Anna Gvozdjáková
Editor

Mitochondrial Medicine

Mitochondrial Metabolism, Diseases, Diagnosis and Therapy

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Anna Gvozdjáková
Comenius University
Medical Faculty
Bratislava
Slovakia

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This Book is Dedicated To The Memory of My Late Husband

Professor Ján Gvozdják, MD, PhD, DSc, FESC Lecturer, WHO Consultant in Cardiomyopathy, Specialist in Cardiology, Internal Medicine, Biochemistry and Mitochondrial Bioenergetics and Expert in the Application of Experimental Research to Clinical Medicine.

Preface

The target groups of this textbook Mitochondrial Medicine are practitioners of medicine, specialists in individual medical branches, pharmacologists, and sports doctors. The updated scientific and clinical information and knowledge provide a broad spectrum for postgradual education of physicians, pharmacologists, and specialists in other life sciences. Students of relevant branches at universities may find in the book a valuable source of information, which might serve as direction indicator in their future professional career.

I am confident that the usefulness of this monograph is warranted also by the international participation of specialists as contributors. The textbook will hopefully provide relevant information for many scientific branches of clinical and experimental medicine and will assist doctors involved in establishing diagnosis and devising the management of mitochondrial diseases.

Professor Rolf Luft can be considered the father of Mitochondrial Medicine as he was the first to carry out a mitochondrial study in man In the years 1959–1962. 1970 is the year of birth of Mitochondrial Medicine in Comenius University in Bratislava, Slovakia. The Pharmacobiochemical Laboratory of the Third Department of Medicine of the Medical Faculty was the birthplace of Mitochondrial Medicine. In cooperation with the Head of the Department, Professor Ján Gvozdják, MD, PhD., DSc, we devoted 36 years of our married life to the problem area of mitochondria. From 1970 up to the present day, the focus of interest of several coworkers and institutions have been metabolic studies of mitochondria in different experimental models (smoke mitochondrial cardiomyopathy, alcoholic mitochondrial cardiomyopathy, ischemia-reperfusion of the isolated heart, diabetic mitochondrial cardiomyopathy, Huntington's disease, Alzheimer's disease, adjuvant arthritis, etc.) and therapeutic intervention in these conditions, particularly the effect of CoQ_{10}. The obtained results elucidated some metabolic processes involved in several diseases and have found their application in clinical medicine.

Mitochondria, small subcellular organelles, are present in all eukaryotic cells. They are considered the generators of energy production in the body and belong to the main sources of reactive oxygen species generation. Coenzyme Q_{10}, the mobile part of the mitochondrial respiratory chain, has a key position in energy production. Evidence on the biological clock of CoQ_{10} and of oxidative phosphorylation of mitochondria has provided insight into the relationship of these processes with the

origin, development and course of many diseases, including cerebral episodes and acute myocardial infarction. CoQ_{10} deficiency, impairment of mitochondrial function and oxidative stress belong to the underlying metabolic causes in the etiopathogenesis of many diseases.

This book presents joint aspects of clinical medicine with metabolic phenomena of mitochondrial function obtained in experimental medicine. Determination of mitochondrial respiration and oxidative phosphorylation does not belong to common diagnostic methods of mitochondrial diseases in patients since several milligrams or even grams of human tissue would be required for the isolation of mitochondria from individual organs or for the preparation of skinned fibers. For this reason we consider metabolic studies of mitochondria in experimental models of mitochondrial derangements to be useful in yielding valuable information also with respect to clinical medicine.

The textbook is focused on four problem areas: mitochondrial physiology, mitochondrial medicine, diagnostic methods in mitochondrial derangements and diseases, and therapeutic interventions aimed at regeneration of impaired mitochondria.

An important supplement of mitochondrial physiology is the information on the biological clock of coenzyme Q_{10} and on the circadian cascade of oxidative phosphorylation, which is presented by Anna Gvozdjáková in Chapter 1.
Zdenka Ďuračková presents an overview on oxidants, antioxidants, and oxidative stress in Chapter 2.

The general overview of chronobiology provided by Franz Halberg, considered worldwide the father of chronobiology, is combined in Chapter 3 with the specialized part on coenzyme Q_{10} and mitochondrial medicine.

In the application of methods of chronobiometric analysis of mitochondrial functions, Miroslav Mikulecký, doctor of medicine and outstanding statistician, presents the mode of statistical evaluation of results with 95% statistical significance in Chapter 4. Chapter 5, presented by Anna Gvozdjáková provides basic information on mitochondrial medicine.

The author of Chapter 6 on mitochondrial cardiology is Ivan Pecháň, medical specialist and biochemist.

The original results of studies of the mitochondrial respiratory chain and coenzyme Q_{10} in endomyocardial biopsies from the transplanted human heart and their relationship to the development of transplant rejection highlight the importance of well-functioning mitochondria with intact ATP production. Chapter 7, provided by Anna Gvozdjáková, is on the impairment, reduced function of the mitochondrial respiratory chain and CoQ_{10} deficiency in direct correlation with the origin and development of human transplanted heart rejection.

In Chapter 8, Jozef Čársky gives an overview of metabolic processes in diabetes. Ram B. Singh and Franz Halberg shaped the idea of the involvement of biological rhythms in the possible relationship between ATP production as well as CoQ_{10} concentration in mitochondria of the heart muscle and brain on the one hand and acute cerebral episodes and myocardial infarction on the other. At our institute, we were intrigued by the idea and on using Halberg's chronobiological method,

semicircadian and circadian rhythms of CoQ_{10} were established in mitochondria of the heart muscle and in the brain in healthy and diabetic rats, along with the mitochondrial circadian cascade of oxidative phosphorylation. The topic of diabetes was thus supplemented by findings on mitochondrial functions of the cardiac muscle in experimentally induced diabetes, the CoQ_{10} clock, and the circadian cascade of oxidative phosphorylation of diabetic mitochondria by Miroslav Mikulecký, Anna Gvozdjáková, Jarmila Kucharská, and Ram B. Singh.

Mitochondrial nephrology is the topic of Chapter 9, written by Katarína Gazdíková and František Gazdík. The survey presents both physiological and pathologically altered functions of the kidney.

The problem area of Chapter 10, by Janka Lipková is energy production in mitochondria of skeletal muscles, oxidative damage, antioxidants, and aspects of these issues relevant in sports.

Chapter 11 by Jozef Rovenský and Karel Pavelka gives a broad survey of rheumatoid arthritis, commenting on diagnostic and therapeutic issues of the disease. The chapter is supplemented by an experimental study of adjuvant arthritis and mitochondria by Katarína Bauerova and Jarmila Kucharská.

Updated knowledge on mitochondrial immunology is presented in Chapter 12 by František Gazdík and Katarína Gazdíková.

Chapter 13 presented by Anna Gvozdjáková is concerned with mitochondrial spermatopathy, providing current information on the role of spermatozoal mitochondria in male infertility.

Current diagnostic methods of mitochondrial defects, concerning particularly metabolic derangements, are presented in Chapter 14 by Anna Gvozdjáková, Jarmila Kucharská, and Anna Hlavatá.

A detailed theoretical survey of nuclear magnetic resonance and its application in metabolic studies can be found in Chapter 15 by Tibor Liptaj.

Chapter 16 falls within the therapeutic part of the monograph. The problem area of coenzyme Q_{10} is brilliantly treated of both from clinical and theoretical aspects. The author of the chapter is the President of the International Coenzyme Q_{10} Association, G. Paolo Littarru with his coworkers.

Supplementation with CoQ_{10} in children with metabolic derangements, in patients with nephropathy, asthma, and diabetes is presented in four individual studies, including original results, in Chapter 17. The first study is by Anna Hlavatá, Jarmila Kucharská, and Anna Gvozdjáková, the second by Anna Gvozdjáková and Jarmila Kucharská, the third by Anna Gvozdjáková, and the fourth by Anna Gvozdjáková, Patrik Palacka, Jarmila Kucharská, and Ján Murín.

Supplementation with CoQ_{10} in experimental models of Alzheimer's disease, Huntington's disease, and adjuvant arthritis is dealt with in three studies with original results, which are included in Chapter 18. The three studies are presented by Jaromír Horecký, Ol'ga Vančová, Jarmila Kucharská, and Anna Gvozdjáková the first study, the second is by Anna Gvozdjáková, and the third by Katarína Bauerová, Jarmila Kucharská, Silvester Poništ, and Anna Gvozdjáková.

A theoretical overview of ω-3 and ω-6 PUFA and their supplementation in clinical and experimental medicine, including original results, are given in Chapter 19 by

Anna Gvozdjáková, Daniel Pella, Jarmila Kucharská, Kuniaki Otsuka, and Ram B. Singh.

The marked improvement recorded in male infertility on supplementation with hydrosoluble CARNI-Q-GEL (carnitine with CoQ_{10}, vitamin E and vitamin C) is considered a significant contribution. The results of carnitine and CoQ_{10} supplementation in male infertility under clinical and experimental conditions are presented by Anna Gvozdjáková in the first study, the second by Anna Gvozdjáková, Jarmila Kucharská, and Pavol Lepieš, and the third presented by Anna Gvozdjáková and Jarmila Kucharská in Chapter 20.

In Chapter 21, Jarmila Kucharská presented an overview highlighting the beneficial effect of vitamin supplementation in mitochondrial derangements.

The survey of new prospective therapeutic methods includes the effect of polarized light on mitochondrial function (experimental studies). Ján Pálinkáš and Alfonz Smola prepared a comprehensive overview of the characteristics and effects of polarized light in Chapter 22. Photographic documentation of healing effects of polarized light on wounds of the diabetic foot and on pressure sores is attached to this chapter.

I am confident that this monograph will contribute to the understanding of the role that mitochondria and CoQ_{10} exert in human mitochondrial medicine. The textbook was prepared with the aim to provide comprehensive information, including new data and aspects, relevant in the field of mitochondrial medicine which may be used to advantage in diagnosis and supplementary therapy (with CoQ_{10}, carnitine, polyunsaturated fatty acids, polarized light) in patients suffering from mitochondrial diseases.

Anna Gvozdjáková

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My sincere thanks go to all contributors of this book, specialists in medicine and research workers, who readily met my request to write individual chapters of the book.

I wish to thank Professor Rastislav Dzúrik, MD, PhD, DSc, who was my first boss and tutor in the field of research in Comenius University, Medical Faculty, Pharmacobiochemical Laboratory in Bratislava, Slovakia. His enthusiasm for research into clinical and experimental medicine inspired my own research zeal.

I would like to thank many of my coworkers, particularly PharmDr Jarmila Kucharská, PhD, for her cooperation of many years, and Maria Kaplánová, Anna Štetková, Valika Ješková and Emil Benko, MSc. for their excellent technical assistance.

I extend my greatest thanks to Dr R K Chopra, President of Tishcon Corp., USA. Under their auspices we performed several clinical and experimental studies. We acknowledge gratefully their donations of different forms of CoQ_{10} (hydrosoluble Q-GEL®, liposoluble, reduced, liposomal), ω-3, ω-6 PUFA, Q-GEL® with α-lipoic acid and CARNI-Q-GEL® with L-carnitine allowing us to carry out several experimental and clinical investigations. I wish to thank Dr Hemmi Bhagavan (Tishcon Corp., USA) for the valuable consultations in mitochondrial studies.

My deep thanks go to my three sons, Peter Gvozdják, architect, MSc., Juraj Gvozdják, MSc, and Ján Gvozdják, MSc for having tolerated their parents' devotion to science, which many times went at the expense of family ease and comfort. Peter (Atelier 2) is to receive my thanks also for his great help in preparing the colour figures in chapters.

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hinted at the reason for the confusion: by sampling at different clock-hours, two groups of mice were found to be characterized by a circadian rhythm with different phases because the experimentals were fed in the morning and the controls fed at night. Opposite effects thus became predictable. (**E**) Abstract illustration of two circadian rhythms in antiphase. Differences in opposite direction or no effect are then anticipated from sampling at different clock-hours. (Copyright Halberg) 56

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in MESOR-normotension (IIC) and monitors transient and/or success of treatment lasting during monitoring (IIIA–C). Illustrative results supporting the need for continued surveillance and for a chronomic data analysis. Benefits are:

- Detection of abnormality during the night when medication is no longer effective, not seen during office visits in the afternoon (I)
- Detection of abnormal circadian pattern of blood pressure (CHAT, "overswinging") associated with a risk of cerebral ischemia and nephropathy larger than other risks (including "hypertension") assessed concomitantly (IIA and B)
- Finding that CHAT carries a very high risk even among MESOR-normotensives who do not need anti-hypertensive medication (IIC)
- Availability of statistical procedures such as a self-starting cumulative sum (CUSUM) applicable to the individual patient to determine whether an intervention such as autogenic training is effective and for how long the intervention remains effective (IIIA)
- N-of-1 designs for the optimization of treatment timing: the same dose of the same medication can further lower the same subject's blood pressure MESOR and circadian amplitude when the timing of daily administration is changed (IIIB and C), as ascertained by as-one-goes (sequential) testing and parameter tests, procedures applicable to the given individual.

I: Stacked from 11 days of around-the-clock monitoring. Office spot-checks cannot detect nocturnal pathology.
IIA: Among risk factors, an excessive circadian blood pressure (BP) amplitude (A) raises the risk of ischemic stroke most.
IIB: Among risk factors, an excessive circadian blood pressure (BP) amplitude (A) raises the risk of nephropathy most.
IIC: An excessive circadian blood pressure (BP) amplitude (A) is a risk factor for ischemic stroke independent from the 24-h mean (MESOR).
IIIA: Individualized assessment (by CUSUM) of a patient's initial response and subsequent failure to respond to autogenic training (AT) (EO, F, 59 years).
IIIB: Individualized blood pressure chronotherapy. Lower circadian double amplitude (2A) and MESOR (M) after switching treatment time from 08:30 (*left*) to 04:30 (*right*).
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On the average, on top, peaks in temperature of the blinded group occur earlier and earlier, but there are uncertainties in such eyeballing. A transient antiphase at 22–23 days after blinding is readily seen. If, around that stage after blinding, 2-timepoint checks are carried out on the 2 groups, opposite results can be obtained on mice with and without eyes and later when they are again in phase, 2-timepoint checks show no difference, a puzzle readily resolved by an objective quantification of the rhythm characteristics. The need for this microscopy in time becomes obvious, notably if an inference is desired as soon as possible with an estimate of uncertainty. (Circadian desynchronization also characterizes congenitally blind ZRD mice.) (Copyright Halberg). 68

Fig. 3.13 *Top*, section I: Desynchronization of circadian rhythm in core temperature of mice after blinding, seen time-macroscopically in IA (much better in Fig. 3.12), here leads, in IB, to time-microscopy with a chronobiologic serial section showing a different time course of the core temperature acrophases, ϕ, with early separation of the two groups by nonoverlapping 95% confidence intervals of ϕ; in IC, to a summary of individual periodograms that form two separate distributions, and in ID to time relations among three variables in a 24-h synchronized (*top*) or free-running (*bottom*) system (of mice, *left*, and of a human, *right*). Section II shows a spontaneous (α) rhythm in circulating corticosterone of mice in antiphase with the slope of an in vitro response rhythm to ACTH, a reactive (β) rhythm of adrenal corticosterone production. The components of the chronome (time structure) are internally coordinated through feedsidewards in a network of rhythms that are more or less spontaneous (α), others primarily reactive (β) or modulatory at a single mapped frequency, such as a circadian (γ), IIC and IID, or at multiply mapped (δ) frequencies, IIE.

The effect of one entity (the actor) upon a second (the reactor), such as the pituitary acting upon the adrenal cortical corticosterone production may be influenced, predictably insofar as rhythmically, by a third entity such as melatonin (the modulator) at the level of the pituitary; the same melatonin also acts directly upon the adrenal. Reproducible sequences of attenuation, no-effect, and amplification, the time-qualified feedsidewards, replace time-unqualified feedbacks and feedforwards (IIC–E). In sections II and III, feedsidewards include the interaction of a modulator (such as ACTH) upon an actor (such as adrenocortical corticosterone production) acting upon DNA labeling in bone (the reactor). The roles played by endocrines can and do change in various feedsidewards that replace time-unqualified feedbacks and feedforwards. Chronomolecular mapping of circadian acrophases has also begun (Fig. 3.21). (Copyright Halberg). 69

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Fig. 3.17 Division of labor in time on a population basis (rat). Extrapolation to individuals is not warranted. (Copyright Halberg) 74

Fig. 3.18 Division of labor in time on a population basis (human). Extrapolation to individuals is not warranted. (Copyright Halberg) 75

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Fig. 22.2 Wound healing after 3-months polarized light effect on diabetic finger 393

Contributors

1. Diana E. **Ayala**, MD, PhD
   Bioengineering and Chronobiology
   Laboratories,
   University of Vigo
   Spain
   rheminda@tsc.uvigo.es

2. PharmDr. Katarína **Bauerová**, PhD
   Slovak Academy of Sciences
   Institute of Experimental Pharmacology
   Bratislava
   Slovakia
   katarina.bauerova@savba.sk

3. Larry A. **Beaty**, BS
   Halberg Chronobiology Center
   University of Minnesota/
   IEEE "Phoenix" Ambulatory Blood
   Pressure Monitor Team Member
   Minneapolis, Minnesota
   USA
   labeaty@bitstream.net

4. Germaine **Cornélissen**, PhD
Professor of Integrative Biology and Physiology
Halberg Chronobiology Center
University of Minnesota
Minneapolis, Minnesota
USA
corne001@umn.edu

5. Jerzy **Czaplicki,** PhD
Université Paul Sabatier
Institut de Pharmacologie et Biologie Structurale
CNRS, Toulouse
France
Jerzy.Czaplicki@ipbs.fr; jhcz@free.fr

6. Professor RNDr. Jozef **Čársky**, PhD
Comenius University, Medical Faculty
Institute of Medical Chemistry, Biochemistry
and Clinical Biochemistry
Bratislava
Slovakia
jozef.carsky@mail.t-com.sk

7. Professor Dipl Ing. Zdenka **Ďuračková**, PhD
Comenius University, Medical Faculty
Institute of Medical Chemistry,
Biochemistry and Clinical Biochemistry
Bratislava
Slovakia
zdenka.durackova@fmed.uniba.sk

8. Associate professor František **Gazdík**, MD, PhD
Slovak Medical University
Department of Immunology and Immunotoxicology
Bratislava
Slovakia
frantisek.gazdik@szu.sk

9. Associate professor Katarína **Gazdíková**, MD, PhD
Slovak Medical University
Department of Clinical and Experimental Pharmacotherapy
Bratislava
Slovakia
katarina.gazdikova@szu.sk

10. Associate professor Anna **Gvozdjáková**, PhD, DSc
Comenius University, Medical Faculty
Pharmacobiochemical Laboratory
Bratislava
Slovakia
Q10@Q10.sk, anna.gvozdjakova@stonline.sk

11. Franz **Halberg**, MD
Professor of Laboratory Medicine and Pathology
Director, Halberg Chronobiology Center
University of Minnesota
Minneapolis, Minnesota
USA
halbe001@umn.edu

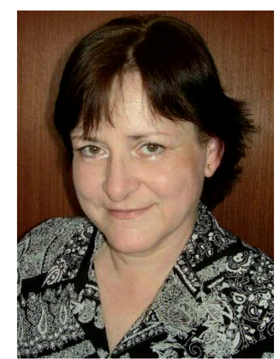
12. Ramon C. **Hermida,** PhD
Director, Bioengineering and Chronobiology Laboratories,
University of Vigo
Spain
rhermida@tsc.uvigo.es

13. Anna **Hlavatá**, MD
Comenius University, Medical Faculty
Second Pediatric Clinic
Bratislava
Slovakia
a.hlavata@gmail.com

14. Associate professor Jaromír **Horecký**, MD, PhD, DSc
Slovak Medical University
Surgical Pathophysiology
and Tissue Engeneering Center
Bratislava
Slovakia
jaromir.horecky@szu.sk

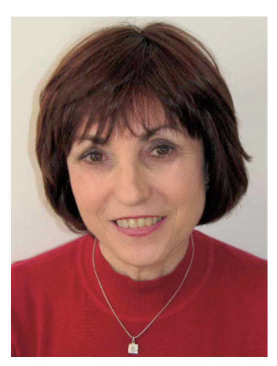
15. George S. **Katinas**, MD
Halberg Chronobiology Center
University of Minnesota
Minneapolis, Minnesota
USA
gkatinas@hotmail.com

16. PharmDr. Jarmila **Kucharská**, PhD
Comenius University, Medical Faculty
Pharmacobiochemical Laboratory
Bratislava
Slovakia
jarmila.kucharska@stonline.sk



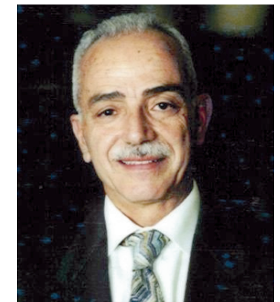
17. Pavol **Lepieš**, MD, PhD
Medical Faculty, Andrology Department
Bratislava
Slovakia
pavol.lepies@faneba.sk

18. Associate professor Janka **Lipková**, MD, PhD
Comenius University, Sport's Faculty
Bratislava
Slovakia
lipkova@fsport.uniba.sk



19. Associate professor Dipl Ing. Tibor **Liptaj**, PhD
Slovak Technical University
Department of Nuclear Magnetic Resonance
Bratislava
Slovakia
liptaj@stu.sk

20. Gian Paolo **Littarru**
Professor of Biochemistry
Director, Institute of Biochemistry
Ancona
Italy
g.littarru@univpm.it
www.icqa.org



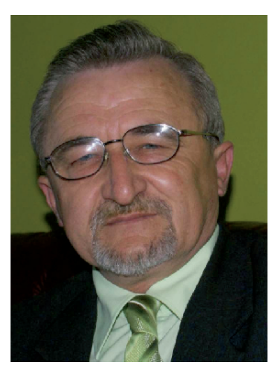
21. Professor Miroslav **Mikulecký**, MD, PhD, DSc
Comenius University, Medical Faculty
First Medical Clinic
Bratislava
Slovakia
statistics@nel.edu

22. Professor Ján **Murín**, MD, PhD
Comenius University, Medical Faculty
First Medical Clinic
Bratislava
Slovakia
murin@faneba.sk

23. Kuniaki **Otsuka**, MD
Department of Medicine
Tokyo Women's Medical University
Medical Center East
Tokyo
Japan
otsukagm@dnh.twmu.ac.jp

24. Patrik **Palacka**, MD
National Cancer Institute
Bratislava
Slovakia
pal_patrik@yahoo.co.uk

25. Ján **Pálinkáš,** MD
Biotherapy
Slovakia
palinkas@biotherapy.sk



26. Professor Karel **Pavelka**, MD, PhD, DSc
Institute of Rheumatology
Prague
Czech Republic

27. Professor Ivan **Pecháň**, MD, PhD, DSc
National Institute of Cardiovascular Diseases
Laboratory of Clinical Biochemistry
Bratislava
Slovakia
pechan@nusch.sk

28. Professor Daniel **Pella**, MD, PhD
PJ Šafárik University, Faculty of Medicine
Preventive and Sport's Medicine Center
Košice
Slovakia
pellad@stonline.sk

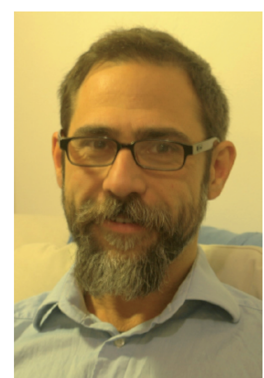
29. PharmDr. Silvester **Poništ**
Slovak Academy of Sciences
Institute of Experimental Pharmacology
Bratislava
Slovakia

30. Professor Jozef **Rovenský**, MD, PhD, DSc, FRCP
National Institute of Rheumatic Diseases
Piešt'any
Slovakia
rovensky.jozef@nurch.sk

31. Ram B **Singh**, MD
Professor of Medicine
Halberg Hospital and Research Institute
Moradabad, UP
India
icn2005@mickyonline.com

32. Professor Ing. Alfonz **Smola**, PhD
FEI Slovak Technical University
Bratislava
Slovakia
alfonz.smola@stuba.sk

33. Luca **Tiano**, MD, PhD
Institute of Biochemistry
Ancona
Italy
luca.tiano@unicam.it



34. Dipl Ing. Ol'ga **Vančová**
Comenius University, Medical Faculty
Pharmacobiochemical Laboratory
Bratislava
Slovakia
olga.vancova@gmail.com

Chapter 1
Mitochondrial Physiology

Anna Gvozdjáková

Abstract Mitochondria, subcellular organelles, serve as centers of genetic information, as central integrators of intermediary metabolism: oxidative phosphorylation, fatty acid oxidation, Krebs cycle, gluconeogenesis, urea cycle, ketogenesis. A key component of the respiratory chain, coenzyme Q_{10}, acts as Q-cycle, as circadian "Q_{10}-CLOCK". Mitochondria are the main energy producers in the body from carbohydrates, fats and proteins, they are one of the main sites of reactive oxygen radical production, they play a crucial role in apoptosis.

Keywords Circadian cascade, energy metabolism, mitochondria, oxidative phosphorylation, "Q_{10}-CLOCK"

Mitochondria are subcellular organelles found in the cytosol of all cells. The quantity of cell mitochondria is different in individual organs. It depends on the function of the cell and the energy demand of the given organ. Thrombocytes contain between two and six mitochondria, most organs contain between 500 and 2,000 mitochondria/cell, however in cardiomyocytes mitochondria make up about 38% of the cell volume, the ovum contains 2,000–20,000 mitochondria/cell and the oocyte possesses close to 100,000 mitochondria. Various shapes and sizes of mitochondria from ring to oval, from small to big can be found in cells.

1.1 History of Mitochondria

In the years 1850–1900, several light-microscopic studies reported the presence of small granules in cells. They were similar in their shape and size to bacteria. In the years 1900–1930, the mitochondrion became well defined. At that time, some functions of mitochondria were suggested: mitochondria serve as centers of genetic information, protein synthesis, lipid synthesis and respiration. In the 1930s, the Krebs cycle (citric acid cycle) and urea cycle were formulated. Electron microscopy allowed the identification of mitochondria in the 1950s. Fatty acid oxidation, respiratory chain and oxidative phosphorylation were

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identified in mitochondria. In the 1960s, complexes of the respiratory chain were characterized.

Coenzyme Q and cytochrome c were identified as mobile compartments of the respiratory chain. In 1964 circular mitochondrial DNA (mtDNA) was discovered. In the 1970s, Mitchell's chemiosmotic hypothesis and mitochondrial "Q-cycle" were accepted. In the 1980s, all genes of mammalian mtDNA and in vitro mitochondrial protein import were identified. First molecular identifications of causes of mitochondrial diseases were documented. The genetic basis of mitochondrial diseases was demonstrated in several conditions: diabetes mellitus, neurodegenerative disorders (Parkinson's disease, Alzheimer's disease) and aging. In the 1990s, the crucial role of mitochondria in apoptosis was established. The important mitochondrial membrane function in the transport of small molecules or ions was proved by discovering mitochondrial contact sites, mitochondrial permeability transition pore [14]. The first publication of *Mitochondrial Medicine* appeared in 1994 [10]. The beneficial effect of coenzyme Q_{10} in the early stage of Parkinson's disease was reported [15]. The mitochondrial "Q_{10}-CLOCK" and circadian cascade of oxidative phosphorylation were detected in rats in 2004 [8, 9].

1.2 Mitochondrial Ultrastructure and Function

The mitochondrion consists of four compartments: *two membranes*, the *intermembrane space* and the *matrix* within the inner membrane (Fig. 1.1). The outer mitochondrial membrane (OMM) separates the cytosol from the intermembrane space. The OMM is responsible for interfacing with the cytosol and its interactions with cytoskeletal elements, which are important for the movement of mitochondria within a cell. This mobility is essential for the distribution of mitochondria during cell division and differentiation [1]. The inner mitochondrial membrane (IMM) separates the intermembrane space from the matrix. The folding of the IMM (*cristae*) serves to increase the surface area of this membrane. Mitochondria are moving along intermediate actin filaments, using kinesin and dynein. In muscles, mitochondria are localized between myofilaments – *interfibrillar mitochondria* (IFM). They are involved in muscle contraction and supply ATP for the actomyosin complex. The function of *subsarcolemmal mitochondria* (SLM) is to maintain the membrane electrical potential [18].

1.2.1 Composition of Mitochondrial Membranes

(a) *Lipid composition* of mitochondrial membranes: phospholipids (phosphatidyl choline – 40%, phosphatidyl ethanolamine – 28.4%, cardiolipin – 22.5%, phosphatidyl inositol – 7%). (b) *Protein composition* of the inner mitochondrial membrane: *external proteins* (loose association with the membrane, soluble in water): succinate dehydrogenase,

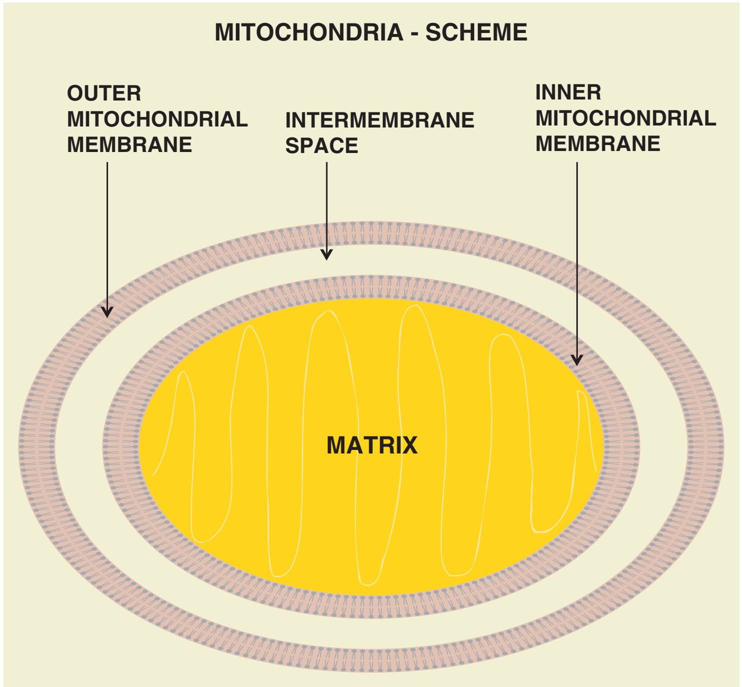

Fig. 1.1 Schema of mitochondria

NADH-dehydrogenase, F_1-ATP-ase, cytochrome c, cytochrome c_1, cytochrome oxidase – subunits 4, 5, 6, 7. *Internal proteins* (insoluble in water): cytochrome b, cytochrome oxidase – subunits 1, 2, 3, iron protein of coenzyme QH_2-cytochrome c reductase, subunits 5, 7, 9 of oligomycin-sensitive ATPase [17].

1.2.2 Functions of Mitochondrial Components

Each of the four mitochondrial components has specific functions.

The *outer mitochondrial membrane* (*OMM*) exerts several functions: protein import from the cytosol to the interior of mitochondria. Smaller molecules can diffuse to the intermembrane space, larger molecules are actively transported into the intermembrane space and matrix [19]. Fusion of the inner and outer mitochondrial membranes establishes contact sites, which are involved in protein import through two complexes: *TOM* (complex of transport outer membrane) and *TIM* (complex of transport inner membrane). Proteins from the matrix are transported across the outer mitochondrial membrane and inner mitochondrial membrane via the *TOM/TIM* complex located at contact sites [4, 20]. Contact sites for multiprotein complexes

are called the *mitochondrial permeability transition pore* (*MPTP*). Activation of the MPTP leads to depolarization and release of cytochrome c into the cytosol, which activates *caspase*-dependent apoptosis. MPTP is an early regulator of apoptosis [3]. Further functions of OMM are ion transfer, oxidation of neuroactive aromatic amines and cardiolipin synthesis.

The *inner mitochondrial membrane* (*IMM*) folds into the matrix to form cristae. Integral membrane proteins represent five complexes of the electron transport respiratory chain (Complexes I–IV), ATP synthase (Complex V- F_1F_0-ATPase), and the adenine nucleotide translocase (ANT). Electron transport through the respiratory chain generates the *electrochemical gradient* ($\Delta\mu H^+$), necessary for ATP production. $\Delta\mu H^+$ is known as *proton-motive force*, which includes the *membrane potential* ($\Delta\psi$) and *proton gradient* (ΔpH) [6].

The *intermembrane space* contains cytochrome c as a mobile electron carrier for the respiratory chain. Release of cytochrome c into the cytosol initiates caspase enzyme activation.

The *matrix* contains the machinery necessary to transcribe and translate the 13 proteins of oxidative phosphorylation. Most of the proteins of oxidative phosphorylation are encoded by the nuclear genome, synthetized in the cytoplasm, and imported into the mitochondria. In the matrix, there are several metabolic pathways, such as pyruvate dehydrogenase complex (PDHC) – oxidation of pyruvate to acetyl CoA, further oxidation of ketone bodies, amino acids, initiation of the urea cycle, fatty acid oxidation, citric acid cycle, process of importing proteins, heme synthesis, suppression of free radical damage through manganese-superoxide dismutase (Mn-SOD).

1.3 Mitochondrial Energy Metabolism

Sources of mitochondrial energy production are carbohydrates, fats and proteins. Carbohydrate metabolism (glycolysis) generates pyruvate, which can cross the mitochondrial membrane. Pyruvate is oxidatively decarboxylated to acetyl-CoA, which enters the Krebs cycle. Another way for acetyl-CoA production is β-oxidation of fatty acids. Acetyl-CoA is further oxidized to CO_2 by the Krebs citric acid cycle [12, 16].

1.3.1 Krebs Cycle

Pyruvate and fatty acids are transported from cytosol into the mitochondria and processes through the Krebs cycle, citric acid cycle. Pyruvate converts into acetyl-CoA, catalyzed by pyruvate dehydrogenase complex (PHDC). Acetyl-CoA condenses with oxaloacetate to form citrate and then isocitrate. After some structural changes, dehydrogenation, decarboxylation and CO_2 loss, α-ketoglutarate is

formed. By further decarboxylation and CO_2 loss, succinyl-CoA is formed. Hydrolysis of succinyl-CoA to succinate releases free energy for the synthesis of guanosine triphosphate (GTP) from guanosine diphosphate (GDP) and inorganic phosphate (Pi). GTP in turn phosphorylates adenosine diphosphate (ADP) to adenosine triphosphate (ATP). The dehydrogenation of succinate is followed by hydratation to form malate and after further dehydrogenation oxaloacetate is formed and the cycle is completed. The resulting high-energy intermediates (NAD, NADH and FAD, $FADH_2$) are utilized in the inner mitochondrial membrane to make ATP. (NAD – nicotinamide adenine dinucleotide, NADH – reduced nicotinamide dinucleotide, FAD – flavin adenine dinucleotide, $FADH_2$ – reduced flavin adenine dinucleotide) (Fig. 1.2).

1.3.2 Fatty Acid Oxidation

Fatty acid oxidation occurs primarily in the mitochondrial matrix, while synthesis of fatty acids is in the cytosol. Fatty acids are important components of biological membranes. The main function of fatty acids is energy production. The metabolism of free fatty acids is complex. Acyl-CoAs, formed in the cytoplasm from fatty acids, cannot cross the inner mitochondrial membrane, cannot enter the mitochondrial matrix. Fatty acids are activated before they enter the mitochondrial matrix. The activation requires ATP and acyl CoA synthase. ATP is hydrolyzed to ADP and inorganic phosphate. End products of beta-oxidation are acetyl CoAs and reducing equivalents. The reducing equivalents are translocated to the mitochondrial respiratory chain and acetyl CoA enters the citric acid cycle. Fatty acids are oxidized to produce water in the respiratory chain and carbon dioxide in the citric acid cycle.

1.3.3 Shuttle Systems

Electrons from cytoplasmic NADH enter mitochondria by shuttle systems. The inner mitochondrial membrane is impermeable for NAD^+ and NADH. Several shuttle systems are located in mitochondria.

1.3.3.1 Carnitine Shuttle

Oxidation of long-chain fatty acids is possible with carnitine, via the carnitine shuttle system. The produced acylcarnitine is translocated through both mitochondrial membranes into the matrix by carnitine-acyl transferase I, II and carnitine-acyl translocase. As one molecule of acylcarnitine is passed into the matrix, one molecule of carnitine is translocated back to the cytosol and acylcarnitine is converted back to acyl CoA. As a result of the carnitine shuttle system, NADH and $FADH_2$ is

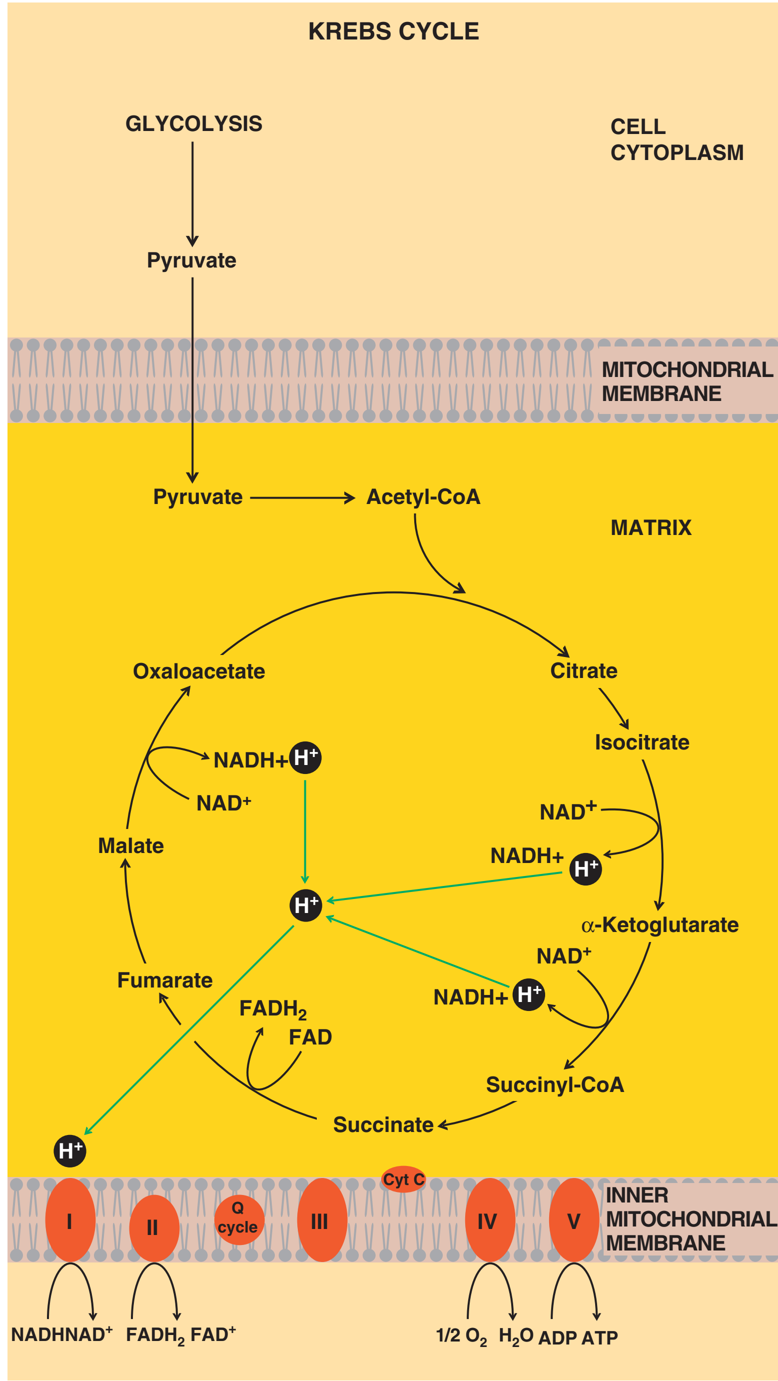

Fig. 1.2 Krebs cycle

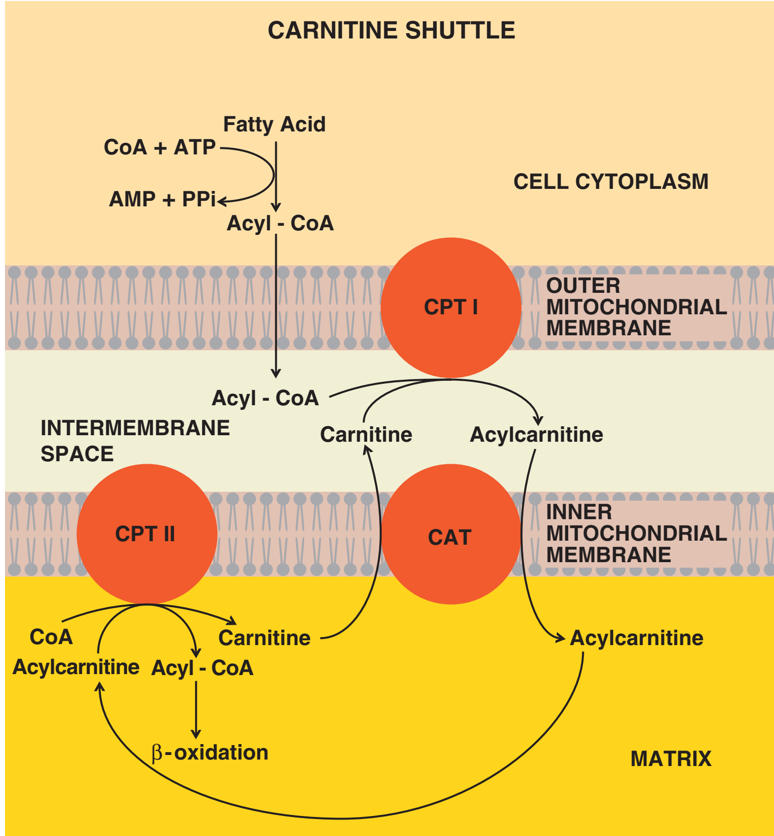

Fig. 1.3 Carnitine shuttle

formed. The acyl CoA can then enter the beta-oxidation pathway. NADH and $FADH_2$ are able to provide protons required for ATP formation by the activity of the Krebs cycle (Fig. 1.3).

1.3.3.2 Glycerophosphate Shuttle

Electrons from NADH formed by glycolysis in the cytoplasm are carried across the mitochondrial membrane by glycerol-3-phosphate. This cytoplasmic reaction is catalyzed by glycerol 3-phosphate dehydrogenase (GPDH). GPDH is reoxidized to dihydroxyacetone phosphate on the outer surface of the inner mitochondrial membrane. Electrons from glycerol 3-phosphate are transferred to FAD of the mitochondrial glycerol dehydrogenase. This enzyme uses FAD as electron acceptor. Dihydroxyacetone phosphate is formed in the oxidation of glycerol 3-phosphate, it

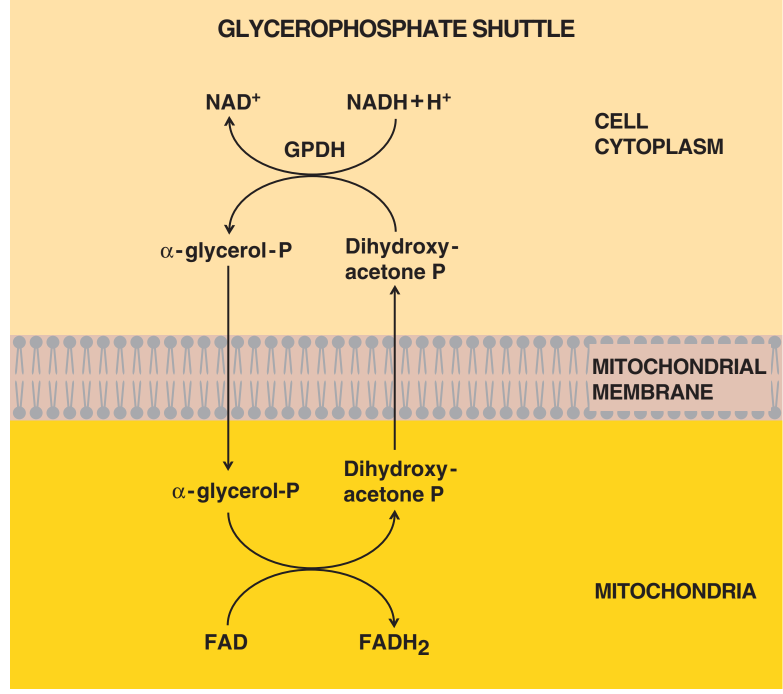

Fig. 1.4 Glycerophosphate shuttle

then diffuses back into the cytosol to complete the shuttle. $FADH_2$ inside the mitochondria transfers its electrons to coenzyme Q, which enters the respiratory chain. Consequently, ATP is formed when cytoplasmic NADH (transported by the glycerol phosphate shuttle) is oxidized by the respiratory chain. This shuttle is irreversible (Fig. 1.4).

1.3.3.3 Malate–Aspartate Shuttle

Electrons from cytoplasmic NADH (in heart and liver) are transferred to the mitochondria by the malate–aspartate shuttle. Electrons are transferred from cytoplasmic NADH to oxaloacetate, malate is produced, it enters the inner mitochondrial membrane and is reoxidized by NAD^+ in the matrix to form NADH. Oxaloacetate is produced, which can not cross the inner mitochondrial membrane, and is then reoxidized by NAD^+ in the matrix to form NADH. Oxaloacetate, which can not cross the inner mitochondrial membrane is formed. By further transamination aspartate is formed, which can be transported to the cytosol. This shuttle is reversible.

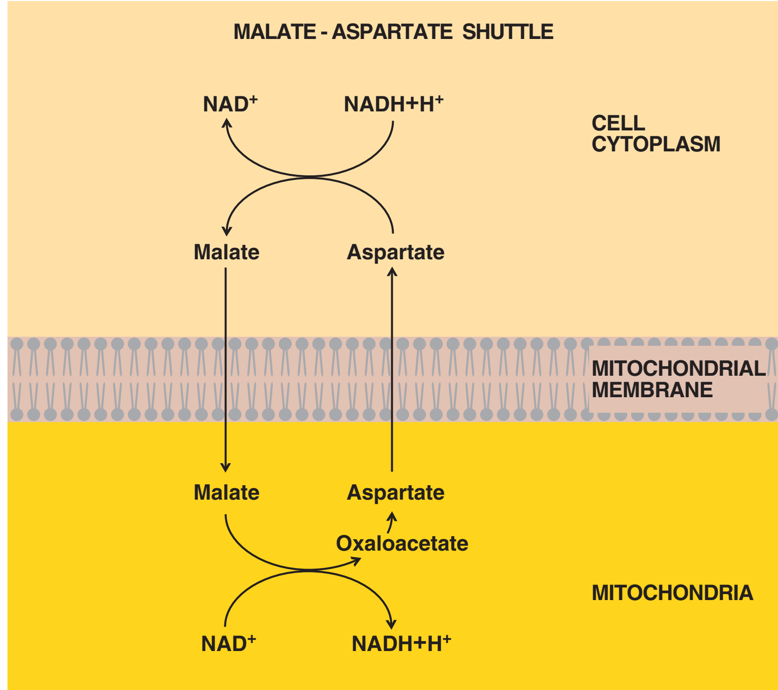

Fig. 1.5 Malate–aspartate shuttle

In addition, NADH can be brought into mitochondria by the malate–aspartate shuttle only when the NADH/NAD^+ ratio is higher in the cytosol than in the matrix of mitochondria (Fig. 1.5).

1.3.4 Ketogenesis

Another way for acetyl CoA metabolism in the liver is its conversion to acetoacetate, which is degraded to acetone. By the reduction of acatoacetate in the mitochondrial matrix 3-hydroxybutyrate is formed. This reaction is dependent on the ratio NADH/ NAD^+ in mitochondria. The condensation of two molecules of acetyl CoA to acetoacetyl CoA occurs in the mitochondria. Acetoacetyl CoA then condenses with another acetyl CoA to form HMG CoA (hydroxy methyl quanosine CoA). HMG CoA is metabolized into acetoacetic acid and acetyl CoA. The acetoacetic acid is reduced into beta-hydroxybutyrate (3-hydroxybutyrate). Ketones can be used in muscle and brain, when glucose is in short supply.

1.3.5 Gluconeogenesis

Gluconeogenesis occurs in the cytosol but depends on the mitochondrial compartments for its initial substrate, oxaloacetate. Gluconeogenesis occurs primarily in the liver and kidney. Initial step: the malate–aspartate shuttle provides reducing equivalents to the mitochondrial compartment via metabolite exchange across the mitochondrial membrane. The key enzyme in gluconeogenesis is PEPCK (phosphoenolpyruvate carboxykinase). The control of gluconeogenesis rests in part with the mitochondrial malate–aspartate shuttle. This shuttle works to transport reducing equivalents into the mitochondria. It is stimulated by the influx of ADP in exchange for ATP. Malate is transported into the mitochondria whereupon it gives up two reducing equivalents and is transformed into oxaloacetate. Oxaloacetate cannot cross the mitochondrial membrane, so it is converted to alpha-ketoglutarate in a coupled reaction that also converts glutamate to aspartate. Aspartate travels out of the mitochondria in exchange for glutamate. In the cytosol, the reactions are reversed. Aspartate is reconverted to glutamate and alpha-ketoglutarate reconverted to oxaloacetate. In turn, oxaloacetate can be reduced to malate or decarboxylated to form phosphoenolpyruvate (Fig. 1.6).

1.3.6 Urea cycle

The urea cycle is initiated in the hepatic mitochondria and finished in the cytosol.

Urea is liberated from arginine via arginase and released into the circulation where-upon it is excreted from the kidneys in the urine. Ornithine, the other product of the arginase reaction, is recycled back to the mitochondrion – again to make citrulline.

1.3.7 Respiratory Chain and Oxidative Phosphorylation

Five protein complexes of the respiratory chain are located in the inner mitochondrial membrane. Complex I: NADH dehydrogenase-ubiquinone oxidase, Complex II: succinate dehydrogenase-ubiquinone oxidoreductase, Complex III: ubiquinone cytochrome c oxidoreductase, Complex IV: cytochrome c oxidase, Complex V: ATP synthase. Mobile components of the respiratory chain are cytochrome c and coenzyme Q_{10}. Coenzyme Q can occur in oxidized form (ubiquinone – Q), reduced form (ubiquinol – QH_2) and radical form ($Q^{\cdot}$), called "Q-CYCLE". The first four complexes receive electrons from the catabolism of carbohydrates, fats, and proteins and generate a proton gradient across the inner mitochondrial membrane. Complex I and Complex II collect these electrons, transfer them to coenzyme Q_{10}, Complex III and Complex IV. Complex I, III and IV utilize the energy in electron

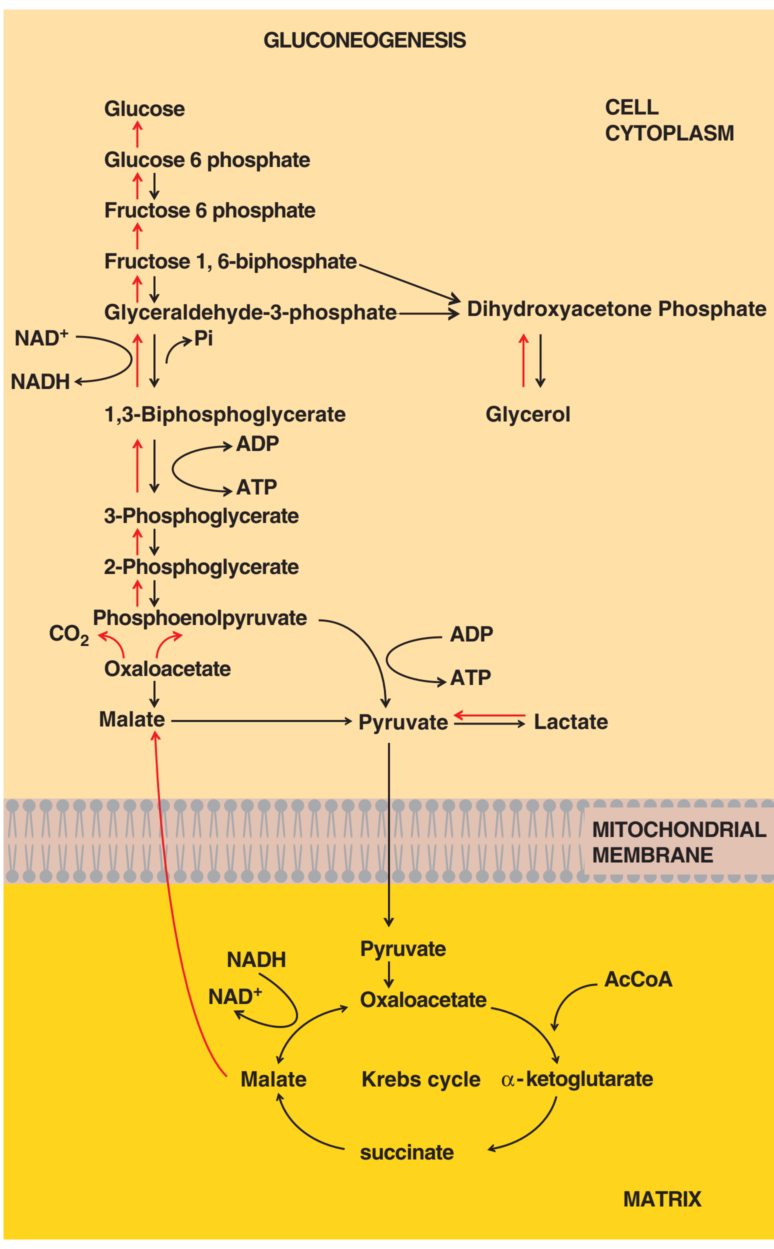

Fig. 1.6 Gluconeogenesis

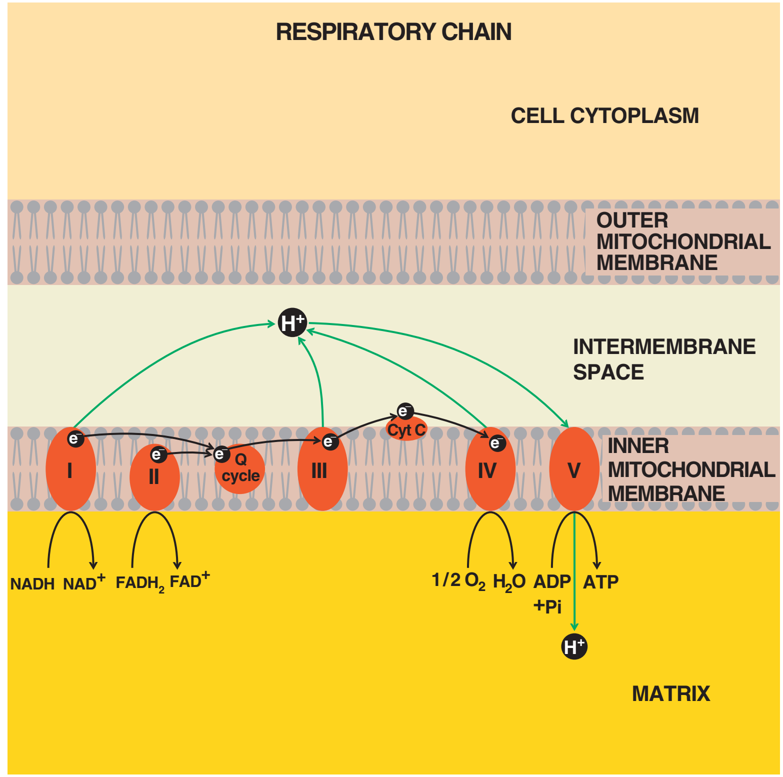

Fig. 1.7 Respiratory chain

transfer to pump protons across the inner mitochondrial membrane, producing a proton gradient, which is used by Complex V for ATP production from ADP and inorganic phosphate. The produced ATP is translocated from the mitochondria into the cytoplasm by adenine nucleotide translocase (ANT) (Fig. 1.7). The inner and outer mitochondrial membranes have numerous contact sites. Both of them contain a large assortment of integral and peripheral proteins as well as numerous phospholipids. The electron transport chain ETC (Complexes I–IV) is localized in the inner mitochondrial membrane, associated with electron transfer components – coenzyme Q and cytochrome c. In the matrix, pyruvate oxidation, β-oxidation of fatty acids, and the TCA cycle pathways are associated. Mitochondrial permeability transition pore (MPTP) includes key components such as the adenine nucleotide translocator (ANT), porin, mitochondrial creatine kinase (CK), hexokinase (HEX) and the inner membrane phospholipid, cardiolipin (CL). The release of apoptogenic peptides, e.g. cyt c, from mitochondria precedes caspase activation, leading to apoptotic cell death. The carnitine shuttle pathway for the mitochondrial import of fatty acids includes carnitine palmitoyl translocase I, II (CPT-I, CPT II) and carnitine translocase (CAT). The generation of reactive oxygen species (ROS) comes from the

mitochondrial electron transport chain (ETC). Mitochondria contain antioxidants as MnSOD and glutathione peroxidase (GPx). The mtDNA is shown with transcripts (mtRNA), which are translated on mitochondrial ribosomes (mtribosome) forming peptide subunits of Complexes I, III, IV, and V.

1.4 Mitochondrial "Q_{10}-CLOCK"

Coenzyme Q_{10} (ubiquinone) as a crucial mobile component of the respiratory chain of the inner mitochondrial membrane acts in three forms in the "Q-CYCLE": CoQ – (ubiquinone – oxidized form), $CoQH_2$ – (ubiquinol – reduced form) and CoQ (ubisemiquinone) – radical form. The dominant human form of coenzyme Q is CoQ_{10}, the dominant rat form is CoQ_9. The central role of CoQ_{10} is electron and proton transfer between Complex I and Complex III, and between Complex II and Complex III. The global functions of CoQ_{10} are in cellular bioenergetics (ATP production), "redox poise" (ratio of reduced to oxidized form), metabolic flux modulation, gene regulation and oxygen radical production. According to the hypothesis of Linnane and Eastwood [11], "CoQ_{10} redox poise" changes determine the key metabolic control function in all subcellular membranes, resulting in the signaling process. Evidence on bioenergetics and antioxidant functions of coenzyme Q_{10} was presented in several papers [2, 11]. CoQ_{10} levels in plasma, tissues and mitochondria of various organs show biological circadian or circasemidian rhythms [7–9].

Heart mitochondrial circasemidian (12 h) and circadian (24 h) and showed statistically significant maximum (PEAKS) only for $CoQ_{10\text{-ox}}$ at 15:27 h and 3:30 h, minimum (NADIRS) for $CoQ_{10\text{-ox}}$ – at 10:00 h and 20:51 h. $CoQ_{9\text{-ox}}$ PEAKS were without statistical significance. Nocturnal activity of rats (22:00–10:00 h) reflected in higher peaks versus diurnal peaks (10:00–22:00 h). It is known that biorhythms in humans are opposite to those in rats (Fig. 1.8, Table 1.1).

In conclusion, heart mitochondrial "Q_{10}-CLOCK" changes may play an important role in the pathogenesis of altered heart function and in mitochondrial diseases of the heart.

1.5 Circadian Cascade of Oxidative Phosphorylation Values

Circadian variations of oxidative phosphorylation showed different times of oxidative phosphorylation parameters at Complex I and Complex II: ADP:O – coefficient of oxidative phosphorylation, OPR – rate of ATP production, S_4 – basal respiration, S_3 – ADP-stimulated respiration, RCI – respiratory control index. These variations exhibited two circadian maxima and two minima of oxidative phosphorylation activity of heart mitochondria of control rats [7–9] (Table 1.2).

Complex I: *Maximum* OXPHOS activity (circasemidian PEAK I – 12 h) is between 9:02 and 14:22 h (lasting 5.20 h) – rat inactivity (circadian PEAK II – 24 h)

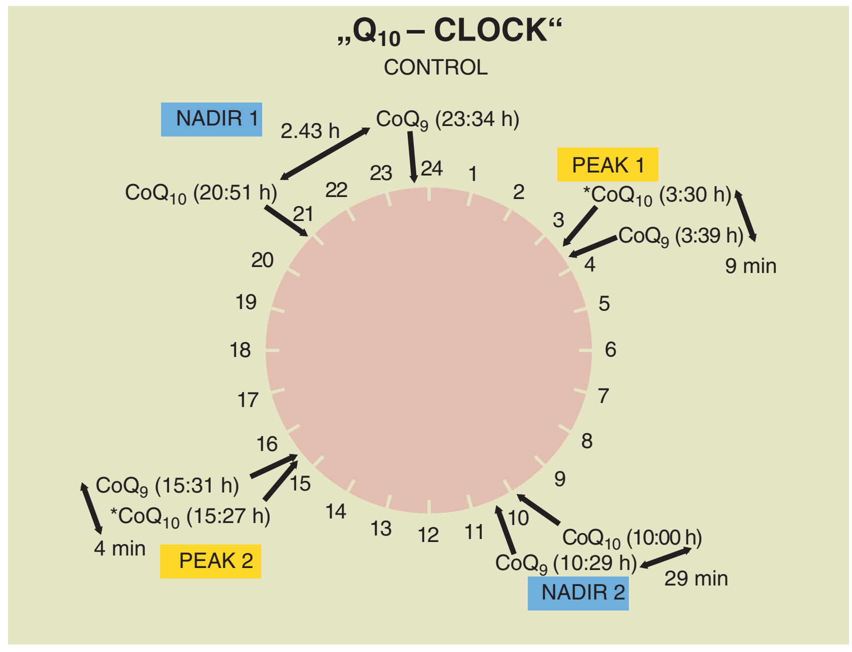

Fig. 1.8 "Q_{10}-CLOCK" in heart mitochondria of control rats

Table 1.1 Circadian variations of coenzyme Q_{10} in mitochondria of control rat heart

Hours	08:00	12:00	16:00	20:00	24:00	04:00
$CoQ_{10\text{-}ox}$ (nmol/mg prot.)	0.327	0.469	0.717	0.390	0.688	0.948
$CoQ_{9\text{-}ox}$ (nmol/mg prot.)	2.290	4.080	4.520	3.047	5.280	7.800

Table 1.2 Circadian variations of oxidative phosphorylation in mitochondria of control rat heart

Hours	08:00	12:00	16:00	20:00	24:00	04:00
Complex I:						
ADP:O (nmol ADP/nAtO)	2.60	2.42	2.19	2.11	2.27	2.31
OPR (nmol ATP/mg prot./min)	442.50	337.90	309.00	239.00	713.80	355.50
$\mathbf{S_3}$ (nAtO/mg prot./min)	180.70	141.90	140.80	112.00	277.10	154.70
$\mathbf{S_4}$ (nAtO/mg prot./min)	38.82	27.74	24.65	25.10	38.65	24.73
RCI (S_3/S_4)	4.68	5.15	5.71	4.44	7.28	6.30
Complex II:						
ADP:O (nmol ADP/nAtO)	1.37	1.31	1.06	1.18	1.48	1.21
OPR (nmol ATP/mg prot./min)	372.90	259.00	247.10	177.10	519.40	302.00
$\mathbf{S_3}$ (nAtO/mg prot./min)	255.00	210.10	236.70	148.20	277.20	251.20
$\mathbf{S_4}$ (nAtO/mg prot./min)	163.50	121.60	149.20	94.00	164.10	153.80
RCI (S_3/S_4)	1.56	1.73	1.57	1.57	1.81	1.65

Table 1.3 Circasemidian (12 h) and circadian (24 h) cascade of oxidative phosphorylation of control rat heart (Complex I)

PEAK I (12 h):	PEAK II (24 h):
S_4 at 9:02 h	S_4 at 23:42 h
ADP:O at 9:16 h	ADP:O at 0:00 h
OPR at 10:47 h	OPR at 0:58 h
S_3 at 11:02 h	S_3 at 1:13 h
RCI at 14:22 h	RCI at 2:14 h

Table 1.4 Circasemidian (12 h) and circadian (24 h) cascade of oxidative phosphorylation of control rat heart (Complex II)

PEAK I (12 h):	PEAK II (24 h):
S_4 at 10:04 h	S_4 at 4:33 h
ADP:O at 10:04 h	ADP:O at 0:11 h
OPR at 10:14 h	OPR at 1:02 h
S_3 at 12:18 h	S_3 at 3:02 h
RCI at 12:51 h	RCI at 6:34 h

between 23:42 and 2:14 h (lasting 2.32 h) – rat activity. *Minimum* of OXPHOS activity NADIR I is between 16:21 and 19:31 h (lasting 3.10 h) and NADIR II between 0:00 and 5:34 h (lasting 5.34 h) (Table 1.3).

Complex II: *Maximum* of OXPHOS activity of circasemidian (PEAK I – 12 h) is between 10:04 and 12:51 h (lasting 2.47 h) – rat inactivity and circadian (PEAK II – 24 h) from 0:11 to 6:23 h (lasting 6.23 h) – rat activity. *Minimum* (NADIR I and II) of OXPHOS activity is between 17:13 and 19:20 h (lasting 2.07 h) and between 0:00 and 5:39 h (lasting 5.39 h) (Table 1.4).

Decreased 12-h heart mitochondrial respiratory chain function and ATP production between 16:29 and 19:31 h and between 4:18 and 7:38 h, as well as "Q_{10}-CLOCK" changes, may be important in the pathogenesis of altered heart function. Understanding of the heart mitochondrial "Q_{10}-CLOCK" and ATP production during circasemidian and circadian rhythms can contribute to the explanation of mechanisms triggering an acute heart attack [8, 9].

1.6 Mitochondria and Molecular Genetics

Human mitochondria have their own double-stranded circular DNA encoding 13 protein components of the four enzyme complexes (i.e. I, II, IV, V) involved in electron transport and OXPHOS. These protein-encoding mtDNA genes are transcribed into specific mRNAs that are translated on mitochondrial-specific ribosomes. The mtDNA also encodes a part of the mitochondrial protein synthesis machinery, including two ribosomal RNAs (rRNA) and 22 transfer RNAs (tRNA) [for review see 14].

1.7 Mitochondria in Apoptosis

Mitochondria play a pivotal role in the process of cell death. Cells die by necrosis when ATP is not sufficient, or they die by the process of apoptosis when sufficient ATP is available. Apoptosis (programed cells death) is a process coordinated by a family of proteases – the caspases, which participate in the molecular control of apoptosis as triggers of cell death and as regulatory elements within this process. Excessive accumulation of Ca^{2+} leads to the formation of reactive oxygen species (ROS) and to opening of the mitochondrial permeability transition pore (MPTP), which depolarizes the mitochondria and leads to mitochondrial swelling. This may also provide a mechanism for the release of cytochrome c from the intermembrane space into the cell cytoplasm. Cytochrome c normally functions as a part of the respiratory chain, but when released into the cytosol it becomes a critical component of the apoptosis execution machinery, where it activates caspases and causes apoptotic cell death. Apoptosis may be triggered by extracellular signals (extrinsic pathway) or by intracellular processes (intrinsic pathway). An increased mitochondrial formation of ROS triggers the intrinsic pathway by opening permeability transmission pores with increased permeability of the outer mitochondrial membrane [5, 13].

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Chapter 2
Oxidants, Antioxidants and Oxidative Stress

Zdenka Ďuračková

Abstract Generation of reactive metabolites (RM) derived from oxygen and nitrogen is a consequence of life in the oxygen atmosphere. In the organism, RM represent a source of oxidative attacks to genetic material, proteins and lipids. RM overproduction, called oxidative stress, can modulate the redox state of the organism. RM interfere with signaling pathways which influence through gene expression cell growth, proliferation and apoptosis, as well as the immune response of the cell. The harmful effect of oxidants can be inhibited to a certain extent by enzymes with antioxidant properties and low-molecular weight endogenous and exogenous antioxidants.

Keywords Antioxidant, oxidant, oxidative stress, polyphenols, signaling

Life in the oxygen atmosphere when organisms started to develop the respiratory chain and other enzymatic systems utilizing oxygen, required the parallel formation of protective systems for elimination of toxic effects of oxygen. Oxygen circulation in nature is associated with photosynthesis in green plants and with reduction of expirated oxygen during the process of ATP synthesis in aerobic cells of heterotrophic organisms. Mechanisms of the final production of ATP have a few "danger sites" when the process of oxygen reduction may become uncontrolled leading to formation of free radicals derived from oxygen. However, oxygen may get out of control also at other sites of metabolic changes [30].

Discussions on the presence of free radicals in biological systems ***started*** 50 years ago [16] and soon thereafter scientists hypothesized that free radicals were produced as by-products of enzymatic reactions [51]. In the early 1960s, superoxide was supposed to bind to multiple enzymes including xanthine oxidase. At the end of the 1960s, superoxide was found to be released from cells to the surrounding environment [81].

The second era of acquiring information on free radical effects in biological systems was triggered by the discovery of the enzyme *superoxide dismutase* (SOD) by McCord and Fridovich [79]. At that time, Slater [122] assumed that hepatotoxicity of tetrachlormethane is a result of free radical reactions and a new direction of thinking started in pathobiochemistry, admitting the presence of free radicals and their participation in processes leading to tissue damage.

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The third era started with the first report on positive effects of free radicals. Mittal and Murad [83] found that superoxide through its metabolites, the hydroxyl radical, stimulated the activation of guanylate cyclase and the formation of the "second messenger" cGMP. A similar effect was reported for hydrogen peroxide [138]. The Nobel Prize winners for medicine, Ignaro and Kadowitz [56] and Moncada and colleagues [105] discovered the regulatory function of nitrogen oxide in the control of smooth muscle relaxation and inhibition of platelet adhesion. Since then an avalanche of new information on positive effects of free radicals (FR) and their reactive metabolites (RM) have been witnessed. The living organism is a well-organized system with not only protective systems against toxicity of FR and RM but also with mechanisms taking advantage of free radicals and their metabolites in regulation of many physiological processes beneficial for life.

2.1 Free Radicals and Reactive Metabolites

Free radicals are atoms, molecules or their fragments with one or more unpaired electrons able of independent existence for a short time. They are either electroneutral or they have an anionic or cationic character. The simplest radical is a hydrogen atom with only one unpaired electron. In addition, we recognize free radicals derived from oxygen, nitrogen or various organic compounds.

Free radicals are mostly very reactive substances which can pair their unpaired electron with an electron taken from other compounds, causing their oxidation. Therefore they are called ***oxidants***. Other very reactive metabolites can be formed from free radicals. They can often be even more reactive and toxic than their maternal molecules. Free radicals derived from oxygen include the superoxide anion, shortly ***superoxide*** ($O_2^{\bullet-}$) and ***hydroxyl radical*** ($HO^\bullet$). Reactive metabolites of oxygen (RMO) include nonradical molecules such as **hydrogen peroxide** (H_2O_2), ***hypochlorous acid*** (HOCl), ***singlet oxygen*** ($^1O_2$) and ***ozone*** (O_3). From nitrogen, both radical and nonradical reactive metabolites (RMN) are derived, such as ***nitric oxide***, shortly ***nitroxide*** ($NO^\bullet$), ***nitrogen dioxide*** ($NOO^\bullet$) and ***peroxynitrite*** ($ONOO^-$). Alcoxyl ($RO^\bullet$) and peroxyl ($ROO^\bullet$) radicals (30) belong to organic radicals. The terms "free radicals" and "reactive metabolites of oxygen" should be strictly distinguished. Due to simplification, the terms "free radicals" or RMO/RMN are often used for both groups of these reactive substances. The expression "reactive" does not always describe the property of radical/metabolite – its reactivity. For example: H_2O_2, $NO^\bullet$ and $O_2^{\bullet-}$ react quickly with only a few substrates, while $HO^\bullet$ can react very rapidly with almost every molecule. Other reactive RMO/RMN, such as $RO^\bullet$, HOCl, $NO_2^\bullet$, $ONOO^-$, react similarly as other intermediates in the organism [50]. Since most "reactive" metabolites contain at least one oxygen atom, classification to metabolites derived from oxygen and from nitrogen is irrelevant. Therefore, it is simpler to use the term "reactive metabolites" (RM) (Fig. 2.1).

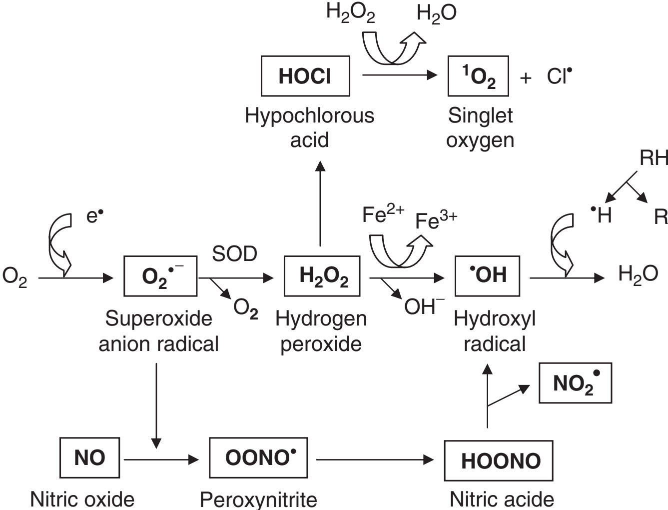

Fig. 2.1 Mutual relations among RM

FR and RM can react very rapidly with biologically important molecules, such as lipids, proteins and nucleic acids, causing their *primary damage*. During these reactions, products can be formed, e.g. aldehydes, which cause *secondary damage* to cells and organs.

The effect of FR in biological systems (positive or negative) depends mostly on *the presence of oxygen*. On the one hand, oxygen is essential for life because its reduction to water is fundamental for the synthesis of the energetically rich compound in the organism, i.e. ATP. On the other hand, oxygen may be a source of production of extremely toxic reactive metabolites. FR and RM can be produced in both the exogenous and endogenous environment of the organism (Table 2.1).

In spite of their negative effects, FR have maintained their positive role in the living nature since the period of life genesis through whole millenniums till the present. There are many processes or systems where FR and RMO play an irreplaceable role in the function of biological phenomena.

Formation and effect of FR in physiological processes must be under control of various protective systems in order to avoid FR activity at the wrong place and damage to the body's own important biomolecules.

Protective systems reduce toxicity of RM. They are (i) mechanisms preventing FR formation (e.g. inhibitors of xanthine oxidase catalyzing formation of superoxide, as allopurinol, chelating agents trapping ions of transmission elements), (ii) systems eliminating already formed FR and RM (so-called antioxidants), and (iii) reparation systems eliminating oxidatively damaged molecules.

Table 2.1 Sources of reactive oxygen species

Endogenous sources of ROS	90, 103, 141
Phagocytes	32, 121
Mitochondrion	116
Peroxisomes	52, 143
Xanthine oxidase	6, 57
Cascade of arachidonic acid	47, 97, 134
Reactions of ions of transition	14
Elements	73
Inflammation	129
Ischemia-reperfusion states	86
Atherogenesis	84, 132
Hemodialysis	100, 144
Intensive exercise	37
Exogenous sources of ROS	89
Cigarette smoke	101, 133
Radiation	53
Chemotherapeutics	
Ozone	
Exhalates	

2.2 Antioxidants

From the biological point of view, ***antioxidants*** are compounds which at low concentration prevent oxidative damage to molecules by oxidants – free radicals and reactive metabolites, while products of the reaction between oxidant and antioxidant should not be toxic and should not branch the radical reaction. With antioxidants, the source of oxidant and the method for detection of antioxidant ability should always be reported [50].

Antioxidants have various structures and according to the size of their antioxidative molecule, they can be classified into high-molecular weight and low-molecular weight compounds. ***High-molecular weight antioxidants*** include, e.g. the enzyme superoxide dismutase (SOD), catalase, glutathione peroxidase or nonenzymatic proteinaceous antioxidants, e.g. transferrin and albumin. ***Low-molecular weight antioxidants*** include, e.g. hydrophilic vitamin C, glutathione, uric acid or lipophilic antioxidants such as vitamin E and coenzyme Q.

Also natural flavonoids (e.g. catechin, quercetin) or other phenolic (e.g. ferulic acid) or polyphenolic compounds (e.g. resveratrol) contribute to the antioxidant capacity of the organism, getting into the organism by food as natural constituents of fruits and vegetables and exerting significant antioxidative ability [11, 21, 30, 130].

The content of individual antioxidants is different in various organs and animal species. For example the human eye lens contains little SOD and a considerable amount of ascorbic acid, while the rat eye lens possesses much SOD and little ascorbate. In the peripheral nerve tissue of the rat, superoxide dismutase has an activity of 90 U/mg nerve tissue, but in mice only 1 U/mg nerve tissue [111].

Plasma antioxidative capacity is different depending on age. With age the concentration of urates increases (25–30%) [99], while in children ascorbic acid is more abundant. In adults, ascorbic acid presents about 15% of the total antioxidative capacity of the plasma. Moreover, also proteinthiols (25%), albumin (25%) and vitamin E (5%) contribute to the antioxidative capacity. In addition to these main antioxidants, also other low-molecular weight substances as well as enzymes with antioxidant properties participate in the antioxidative capacity of the plasma to a lesser extent [98, 120].

A compound is characterized as an effective antioxidant *in vivo* when it meets the following requirements: (i) it must react with biologically effective reactive metabolites of oxygen; (ii) the product of the reaction of prooxidant + oxidant must not be more toxic for the organism than the removed metabolite; (iii) the potential antioxidant must be present in the organism in sufficient concentration; (iv) the half-life of the antioxidant must be long enough to react with the oxidant [50].

Generally, an ***antioxidant in one system and under certain circumstances need not act as antioxidant under other circumstances and in other systems.***

An increasing body of evidence from experimental studies as well as clinical practice concerning their effect suggests that antioxidants need not play always a positive role. This fact has to be kept in mind especially during therapeutic administration of these compounds.

2.2.1 Enzyme and Protein Antioxidants

Enzyme antioxidants have a negligible significance in the extracellular space. In plasma, extracellular superoxide dismutase (EC-SOD) [75] is present only in a small quantity, there is little GSH-dependent peroxidase activity and a questionable quantity of catalase [120]. In the intracellular space, enzyme antioxidants play a significant role.

Superoxide dismutase specifically catalyzes dismutation of the superoxide radical to nonradical molecules O_2 and H_2O_2.

The general mechanism of ***superoxide dismutation*** catalyzed by ***superoxide dismutase*** can be expressed by Equations (2.1) and (2.2). It is characterized by the redox cycle of copper atom Cu(II)/Cu(I)/Cu(II), etc.

$$M^{n+} + O_2^{\bullet -} \rightarrow M^{(n-1)+} + O_2 \tag{2.1}$$

$$M^{(n-1)+} + O_2^{\bullet -} \rightarrow M^{n+}(O_2^{2-}) \xrightarrow{2H^+} M^{n+} + H_2O_2 \tag{2.2}$$

The redox potential is significantly changed by some SOD inhibitors, e.g. CN^- and N_3^- ions. Human and bovine Cu/Zn-SOD is inhibited by the higher concentration of H_2O_2, which oxidatively damages the histidine unit at the active enzyme center [7].

In the human organism, three SOD isoforms are present. They differ in the content of a metal at the active site, in the number of subunits, in the amino acid composition of the apoenzyme, in the sensitivity to inhibitors, etc.

All types of superoxide dismutase catalyze dismutation of the superoxide. In the human organism, there are intracellular superoxide dismutases containing Cu/Zn and Mn metals in their active site. In the extracellular space, there is EC-SOD containing Cu(II) and Zn(II) in its active site. In the organism, these enzymes occur in different concentrations, depending on the site or tissue of their origin [76].

In spite of the fact that SOD is one of the most important intracellular antioxidative systems, its low activity need not always lead to pathological conditions. Antioxidants can partially substitute one another and the lack of one antioxidant can trigger an increased synthesis of another antioxidant. On the other hand, increased enzyme activity need not always be a positive phenomenon, e.g. in Down's syndrome [33, 39, 88, 145].

The majority of aerobic organisms, excluding some bacteria and algae, contain ***catalase***. In animals, catalase is located in all important organs, especially in the liver and erythrocytes. Brain, heart and the skeletal muscles contain only a small amount of catalase. In cells catalase is bound to *peroxisomes*. Mitochondria, chloroplasts and endoplasmic reticulum contain a negligible amount of catalase. The molecular weight of catalase is 240 000 and it is composed of four subunits containing heme with coordinated Fe(III) atom in their active site. Each subunit binds one molecule of NADPH, stabilizing the enzyme molecule.

Catalase catalyzes the decomposition of H_2O_2 to water and oxygen (reaction 2.3). The mechanism of the catalytic reaction is expressed by Equations (2.4) and (2.5).

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad (2.3)$$

$$\text{Cat–Fe(III)} + H_2O_2 \xrightarrow{k_1} \text{intermediate I} \qquad (2.4)$$

$$\text{intermediate I} + H_2O_2 \xrightarrow{k_2} \text{cat–Fe(III)} + H_2O + O_2 \qquad (2.5)$$

The composition of the intermediate is not exactly known but it is supposed to contain Fe(III)–HOOH or Fe(III) = O complex [48].

The enzyme ***glutathione peroxidase*** (***GPx***) occurs in two forms: selenium-dependent and selenium-independent GPx, differing in the number of subunits, in the selenium bond at the active center and in the catalytic mechanism.

Selenium-independent GPx (glutathione-S-transferase, GST) catalyzes detoxication of various xenobiotics. Selenium atom with the oxidative number (II) present in the enzyme molecule does not participate in the catalytic mechanism [55, 71].

Selenium-dependent glutathione-peroxidase (*GPx*) is composed of four subunits, while each subunit contains one selenium atom at the active center bound in the modified amino acid selenocysteine.

All GPx can reduce peroxides by two electrons producing selenols –Se–OH. In the second stage of the catalytic cycle, selenols are reduced by two GSH back to –SeH.

The significance of these selenoenzymes is based on elimination of peroxides – potential substrates for the fenton-type reaction. In addition, selenols react faster than thiols. During redox reactions, they transfer two electrons preventing the formation of superoxide from the oxygen molecule. For the formation of superoxide only one electron is required.

A high GPx activity was detected in the liver, medium activity in the heart, lungs and brain, and low activity in muscles.

Glutathione peroxidase cooperates with the tripeptide glutathione (GSH) present in cells at relatively high (millimole) concentrations. The substrate for GPx reaction is H_2O_2 or an organic peroxide. Glutathione peroxidase decomposes peroxides to water or alcohol and at the same time it oxidizes GSH (reaction 2.6 and 2.7). It is supposed that GSH reduces selenium in GPx and this reduced form of the enzyme catalyzes decomposition of hydrogen peroxide:

$$2\,GSH + H_2O_2 \rightarrow GSSG + 2H_2O \qquad (2.6)$$

$$2GSH + ROOH \rightarrow GSSG + ROH \qquad (2.7)$$

The majority of glutathione present in cells is more frequently in the reduced form (GSH) than in the oxidized form (GSSG). A part of the total glutathione in cells is present in the form of “mixed” glutathione

```
x–glu–cys–gly
        |
        S–S–R
```

where –R can be the cysteine residue, coenzyme A or a protein containing –SH groups [48]. At physiological conditions, glutathione in cells is mostly in its reduced form (GSH). The ratio GSH:GSSG is 100:1 [25]. In cells, the enzyme glutathione reductase (GR) catalyzes GSSG reduction to GSH (2.8). Glutathione reductase can reduce not only GSSG but also “mixed” disulfides (GSSR). The cofactor of GR is NADPH, produced in the pentose cycle by *glucose-6-phosphate dehydrogenase*

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \qquad (2.8)$$

Glutathione peroxidase activity depends on the concentration of GSH, thus its physiological activity requires a sufficient concentration of glutathione.

Transferrin is a protein in blood plasma binding Fe(III) atoms. Its binding capacity presents only 20–30%. Iron ions bound strongly to transferrin do not catalyze lipid peroxidation [2]. Apotransferrin (transferrin without Fe(III) atoms) has a

great ability to bind Fe(III) atoms released from ferritin or other sources. Thus transferrin acts as an antioxidant because it inhibits formation of the $^{\bullet}OH$ radical and lipid peroxidation.

An important protein of iron metabolism is ***ferritin*** present in plasma and cells as a stock protein. Unlike transferrin, ferritin binds Fe(III) by a weak bond, releasing Fe(III) very easily. Such a release of Fe(III) from ferritin requires the presence of the appropriate reductant, e.g. $O_2^{\bullet-}$. Released and reduced Fe(II) atoms can exert their catalytic properties at hydroxyl radical formation. Thus *ferritin cannot be considered an antioxidant*, on the contrary, it can be a source of prooxidatively acting Fe(II) atoms [110].

Ceruloplasmin is a protein transporting Cu(II) atoms. It binds almost all Cu(II) atoms present in plasma. Its antioxidative effect is based on the following conditions:

1. Ceruloplasmin is able to oxidize Fe(II) to Fe(III) atoms (it is called also *ferrooxidase I*) at simultaneous reduction of O_2 to H_2O (reaction 2.9 and 2.10). Fe(III) atoms can bind to transferrin. During oxidation of Fe(II) to Fe(III) by ceruloplasmin, superoxide is not formed as it is during nonenzymatic oxidation of Fe(II) atoms. By changing the oxidative number of iron, ceruloplasmin expels Fe(II) from participation in the Fenton reaction [49]:

$$\text{CP–[Cu(II)]} + 4\text{Fe(II)} \rightarrow \text{CP–[CU(I)]} + 4\text{Fe(III)} \qquad (2.9)$$

$$\text{CP–[Cu(I)]} + O_2 + 4H^+ \rightarrow \text{CP–[CU(II)]} + 2H_2O \qquad (2.10)$$

2. Cu(II) or Cu(I) atoms bound to ceruloplasmin do not produce the hydroxyl radical in the presence of H_2O_2.
3. Ceruloplasmin can react directly with superoxide. However, the mechanism of the reaction is not dismutative as it is in the reaction with superoxide dismutase [4].

Thanks to these properties, ceruloplasmin belongs to the most important extracellular antioxidants in the human body.

Albumin – its antioxidative ability is based on the bond of Cu(II) atoms to the heme, thus preventing oxidative damage of other molecules in the extracellular space. Albumin is named “self-sacrificing” antioxidant. In the case of Cu(II) ions bound to albumin, radical reactions can be accelerated. Cu(II) atom can be oxidized by the hydrogen peroxide to the highly toxic atom Cu(III). At these redox reactions copper from albumin is not liberated and the protein can be damaged at the site where Cu(II) binds to albumin. However in plasma, albumin is in such a high concentration (600 μmol.l^{-1}) and its overall change is so rapid that possible biological consequences of such damage are nonsignificant [48]. Albumin thus significantly contributes to the antioxidative activity of ceruloplasmin in the extracellular space.

One of the important functions of serum albumin is also its protection against *the oxidative damage by hypochlorous acid* (HOCl) produced by *meyloperoxidase* [45] when HOCl binds to albumin thiol groups.

In addition to high-molecular weight enzymatic as well as nonenzymatic antioxidative systems, also ***low-molecular weight antioxidants*** significantly participate in the metabolism of free radicals. According to their solubility, they are classified to hydrophilic and lipophilic antioxidants. They occur in cells as well as in the extracellular space.

2.2.2 Low-Molecular Weight Hydrophilic and Lipophilic Antioxidants

Antioxidative properties of the ***ascorbic acid*** are shown in reaction (2.11), where R can be $O^{\bullet -}$, $HO_2^{\bullet}$, $ROO^{\bullet}$, $RO^{\bullet}$ $GS^{\bullet}$ and $^{\bullet}OH$ (27, 112).

$$\text{Ascorbate} \underset{+e^-}{\overset{-e^-}{\rightleftharpoons}} \text{Ascorbate Free Radical} \underset{+e^-}{\overset{-e^-}{\rightleftharpoons}} \text{Dehydroascorbate} \qquad (2.11)$$

Ascorbic acid is able also to recycle other important antioxidants from their radical forms, e.g. tocopherol and glutathione.

The antioxidative effect of ascorbic acid is strictly confined by the presence of ions or chelatory bound atoms of transition metals. In their presence, ascorbate behaves prooxidatively through the “Fenton type” reaction (Fig. 2.1) [28], In the presence of ascorbic acid, metal ions in so-called “catalytically effective form”, can catalyze the formation of toxic reactive metabolites, such as $^{\bullet}OH$ radical. Moreover, the prooxidative effect of ascorbic acid *in vivo* is associated with autooxidation of ascorbate to dehydroascorbate, thus changing the redox state of cells leading to changed expression of some genes, e.g. for transcription factors (NFkB and AP-1) and signal molecules influencing the regulation of signaling transduction pathways (Fig. 2.2) [27].

Intracellular dehydroascorbate (DHA) can be enzymatically reduced back to ascorbate, producing H_2O_2, which can oxidatively modulate surrounding molecules and thus change also the cell redox state.

These reactions can be ambiguous, depending on the type of cells in which they are carried out. Shin et al. [119] found that ascorbic acid increases neuron differentiation by the induction of genes, a mechanism not known before. The authors found that ascorbate increases the expression of genes encoding for iron-binding proteins, ferritin, transferrin and for glutathione-S-transferases. The given proteins participate in the regulation of the redox balance and may contribute to the

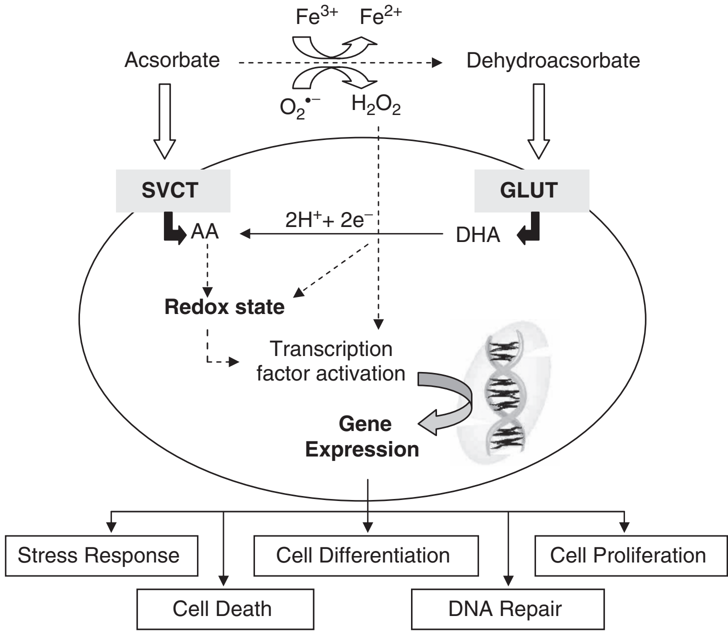

Fig. 2.2 Putative mechanism of ascorbic acid action *in vivo* (Adapted from Duarte and Lunek, 2005). AA – ascorbate, DHA – dehydroascorbate, SVCT – sodium-dependent, vitamin C (ascorbate) transporter, GLUT – glucose transporter

pro-oxidative effect of vitamin C. The authors hypothesized that oxidative stress may play a positive role in inducing neuronal differentiation.

Vitamin E is a term used for a group of compounds composed of the mixture of eight derivatives exerting the activity of α-tocopherol [102] (Fig. 2.3).

The main function of vitamin E is its antioxidative ability. It is able to stop radical chain reactions (e.g. by the reaction with peroxyl radical $LOO^{\bullet}$) or to trap directly oxygen radicals (e.g. hydroxyl radical $HO^{\bullet}$). During these reactions, tocopheryl radical (vitamin $E^{\bullet}$) is formed. This radical can be generated by extraction of hydrogen atom from various places of the chroman nucleus (from the position 5, 7, 9) or more often from the –OH group at the position 6. Tocopheryl radical can be reduced by ascorbate, glutathione or ubiquinol to active α-tocopherol. When vitamin E is located in the lipid part, where the process of lipoperoxidation is carried out, tocopheryl radical can be reduced by lipoperoxides, thus branching the radical reaction [5].

Vitamin E acts synergically with vitamin C. This is possible due to the location of vitamin E in the membrane where its chroman ring with the hydroxyl group is oriented into the hydrophilic part of the membrane, where at the border line of the two phases it can come into contact with ascorbic acid, which can regenerate vita-Min E [93] (Fig. 2.4). However, there are studies refuting cooperation of vitamin E with C *in vivo* [12].

Name	Structure	IU/mg	Comparison to d-α (%)
d-α-tocopherol		1.49	100%
d-β-tocopherol		0.75	50%
d-γ-tocopherol		0.15	10%
d-δ-tocopherol		0.05	3%
d-α-tocotrienol		0.75	50%
d-β-tocotrienol		0.08	5%

Fig. 2.3 Structure and chemical characteristics of α-tocopherol derivatives

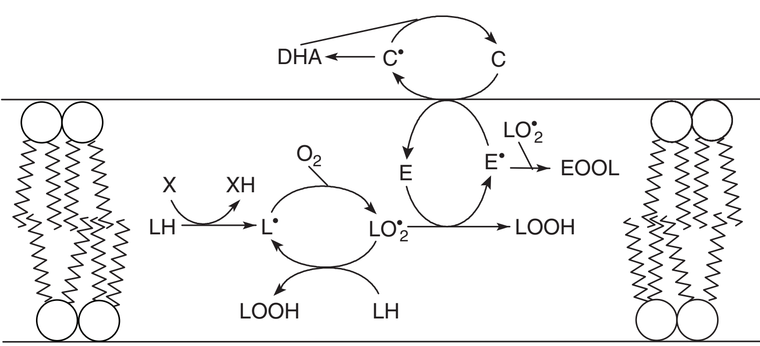

Fig. 2.4 Regeneration of tocopheryl radical by ascorbate E – tocopherol, E• – tocopheryl radical, C – ascorbate, C• – ascorbate radical, DHA – dehydroascorbate, LH – lipid, LOOH – lipoperoxide, X – oxidant, $LO_2^{\bullet}$ – lipoperoxyl radical

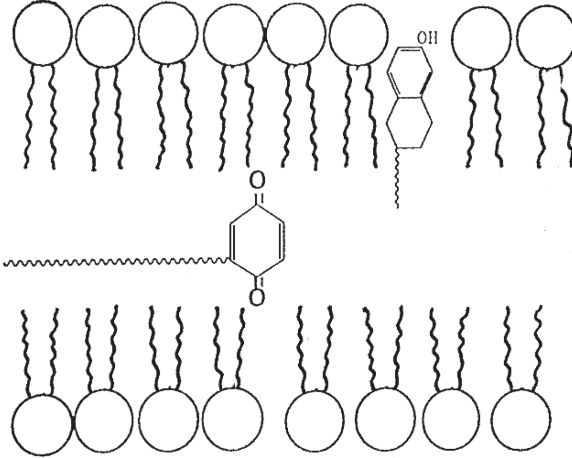

Fig. 2.5 Scheme of vitamin E and coenzyme Q_{10} location in phospholipid bilayer

Physiologically important is also the orientation of the hydrophobic part (fytyl residue) of vitamin E inside the membrane [62], where vitamin E may come into contact with coenzyme Q_{10}, which can also regenerate vitamin E in membranes (Fig. 2.5).

Carotenoids are pigments of plant or microbial origin. At present, around 600 derivatives of carotenoids are known. Of these, 10% possess the activity of provitamin A and can be metabolized to retinol. Only a small number of carotenoids are present in plasma and tissue in sufficient amount. Carotenoids can react with singlet oxygen and return the molecule of the excited oxygen into the basic energetic state. Due to this property, β-carotene belongs to important *in vitro* antioxidants. Antioxidants with this ability are called *quenchers*. Carotenoids can also directly trap free radicals [67, 124]. Of the biologically important natural carotenoids, the most efficient quencher is lycopene [24]. Vitamin A exerts only a negliglible antioxidative ability [127] (Fig. 2.6).

***Coenzyme Q* (*CoQ*)** is a widely used name for 2,3-dimethoxy-5-methyl-6-multiprenyl-benzoquinone. The synonym *ubiquinone* is derived from the English word *ubiquitous*, present everywhere. Coenzyme Q has isoprene units in its side chain, whose number differs in various species (in microorganisms 6, in rats 9, in man 10).

Coenzyme Q was discovered by Crane et al. [17] as a constant component of the mitochondrial succinate dehydrogenase system. In addition, coenzyme Q is associated with the metabolism of free radicals: with their formation, e.g. in mitochondria (prooxidative properties) (Fig. 2.7), as well as with their elimination (antioxidative properties). Coenzyme Q in its reduced form ($CoQH_2$) is a more effective antioxi-

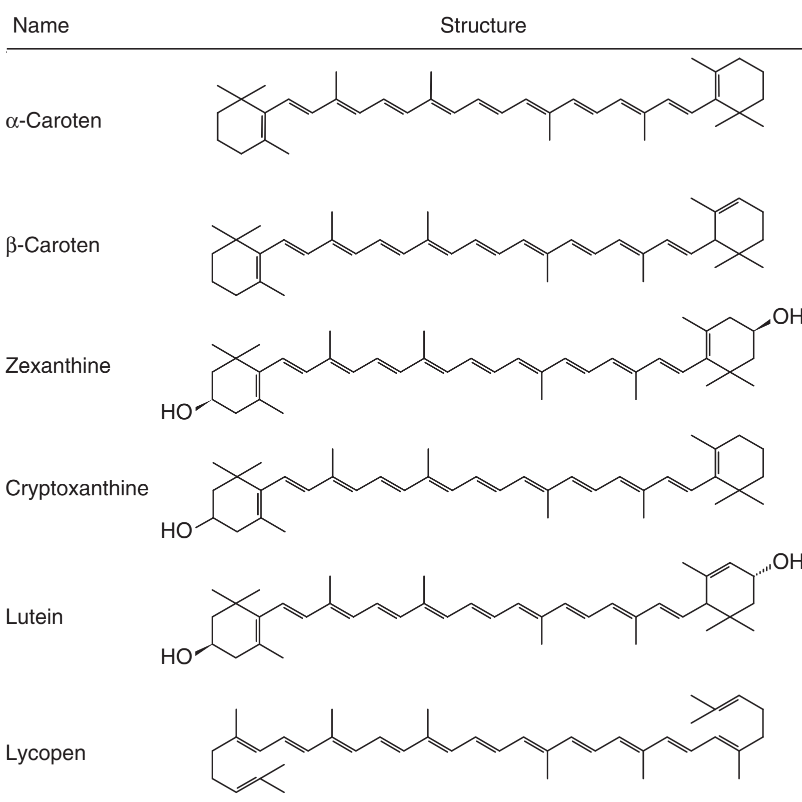

Fig. 2.6 Structures of the most important carotenoids

dant than ubiquinone (CoQ). Coenzyme QH_2 can react directly with superoxide (reaction 2.12), lipid radical (reaction 2.13), as well as with the peroxyl radical (reaction 2.14) [8].

$$CoQH_2 + 2O_2^{\bullet-} \rightarrow H_2O_2 + O_2 + CoQ \tag{2.12}$$

$$CoQH_2 + 2L^{\bullet} \rightarrow 2LH + CoQ \tag{2.13}$$

$$CoQH_2 + 2LOO^{\bullet} \rightarrow 2LOOH + CoQ \tag{2.14}$$

The reactions shown above can also proceed in one stage. The formed semiquinone (reaction 2.15) can be reduced in the respiratory chain in mitochondria (by cytochrome b_{562}), or it can be oxidized to quinone with the potential danger of superoxide formation (Fig. 2.8)

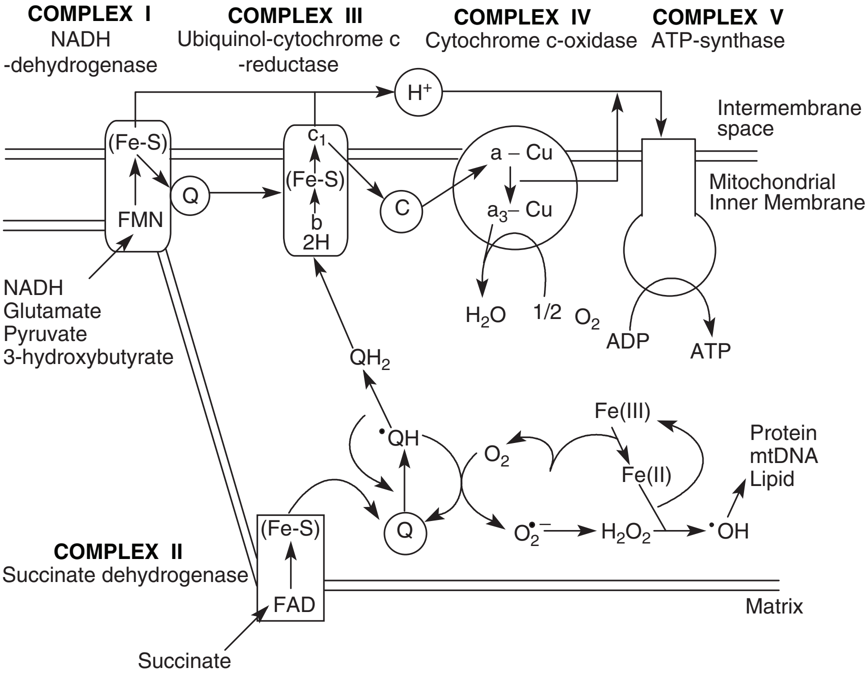

Fig. 2.7 Expected sites of RM formation in mitochondria (modified according to Gabbita et al., 1997 with his kind permission) [38]

Fig. 2.8 Two possible directions of the semiquinone change R (in human mitochondria) = $(-CH_2-CH{=}C(CH_3)-CH_2-)_{10}H$

$$CoQH_2 \; + \; ROO^{\bullet} \; \rightarrow \; CoQ^{\bullet-} \; + \; ROOH \; + \; H^{+} \qquad (2.15)$$

The antioxidative ability of coenzyme Q can be exerted also through regeneration of vitamin E (reaction 2.16) due to the neighboring location of both compounds in the membrane lipid bilayer.

$$CoQH_2 + \alpha\text{-T-O}^\bullet \rightarrow CoQ^{\bullet -} + \alpha\text{-T-OH} + H^+ \qquad (2.16)$$

Although α-tocopherol is a 10 times more effective antioxidant than $CoQH_2$, in case of vitamin E insufficiency in membranes, CoQ becomes an irreplaceable antioxidant. When there is sufficient concentration of α-tocopherol in membranes, $CoQH_2$ has a regenerative function [63].

CoQ is supposed to act as antioxidant also by another mechanism. It was found that coenzyme Q_1H_2 (in the side chain is one isoprene unit) reduces the highly toxic ferryl myoglobin {Mb–Fe(IV) = O}to the less toxic metmyoglobin {Mb–Fe(III)} or to the physiological oxymyoglobin (MbO_2), oxidizing itself to ubiquinone (CoQ). If CoQ_{10} acts also by this mechanism, then the appropriate level of $CoQH_2$, through this feed-back mechanism using heme proteins, could detoxicate H_2O_2 and other hydrogen peroxides which can initiate production of heme oxidants. It is assumed that by this mechanism $CoQH_2$ could protect, e.g. the heart muscle against the harmful effect of oxidants [40, 72].

Bilirubin is a linear tetra pyrole formed *in vivo* by the oxidative splitting of the heme (Fig. 2.9). Bilirubin inhibits oxidation of linolic acid which is bound to albumin, initiated by heme proteins or by peroxyl radical [126]. It quenches $^1O_2$ (26), inhibits hyperoxia (>95% O_2) evoked by lipoperoxidation and production of carbonyl proteins [23]. Bilirubin bound to albumin (similarly as uric acid) reacts slowly with the oxidatively effective hypochlorous acid, and the rate of the reaction increases after conjugation of bilirubin with glucuronic acid. Similarly as vitamin C, bilirubin acts synergically with vitamin E [125].

In healthy adults, bilirubin occurs in plasma only in micromolar concentration and is therefore, without any physiological importance as an antioxidant.

Uric acid was originally considered a catabolic product of degradation of purine metabolites. Uric acid is present in human plasma at high concentration (0.12–0.45 $mmol.l^{-1}$) and is a significant quencher of $^1O_2$ and a trapper of the hydroxyl radical. Uric acid exerts its antioxidative ability by two mechanisms: (i) by direct reaction with some free radicals, and (ii) by the chelatory ability binding ions of transition metals from the environment, eliminating them from their catalytical action in fenton type reactions [30, 41, 95].

At physiological conditions, uric acid stabilizes ascorbate in human serum [118], which is ascribed to its chelatory ability [20]. Uric acid, by formation of

Fig. 2.9 Structure of bilirubin

stable complexes with atoms Fe(III), inhibits oxidation of ascorbate and suppresses peroxidation of lipids. When uric acid chelates atoms Fe(II), it inhibits the Fenton reaction and formation of •OH radical.

Since humans have no *uricase*, allantoin should not be present in plasma under physiological conditions. However, increased concentrations of allantoin were found, e.g. in plasma of patients with rheumatoid arthritis, which is associated with increased oxidative stress [46]. Similarly in patients with Down's syndrome, who are assumed to have increased oxidative stress, allantoin was detected in their plasma, which together with increased levels of uric acid is indicative of the antioxidative role of uric acid in these patients [145].

Glutathione plays a significant role in protection of the organism against oxidative damage for several reasons: (i) It is a cofactor of some enzymes participating in detoxication mechanisms of oxidative stress, e.g. *glutathione peroxidase, glutathione transferase, dehydroascorbate reductase*. (ii) GSH participates in amino acid transfer through the cell membrane. (iii) GSH is a direct trapper of •OH radical and $^1O_2$, it detoxicates H_2O_2 and lipoperoxides during catalytical action of glutathione peroxidase. (iv) Glutathione can reduce the tocopheryl radical directly or indirectly during the reduction of semidehydroascorbate to ascorbate, regenerating these important antioxidants back to their active form (Fig. 2.10). Glutathione plays an important role as an antioxidant in the intracellular space and indirectly affects the antioxidative status also in the extracellular space and membranes, where in cooperation with α-tocopherol it can inhibit lipoperoxidation [3, 77, 78].

Oxidized glutathione is regenerated by *glutathione reductase* (GR) cooperating with NADPH, which is produced in the pentose cycle of glucose degradation. The most important source of GSH is the liver, where 90% of the circulating GSH is

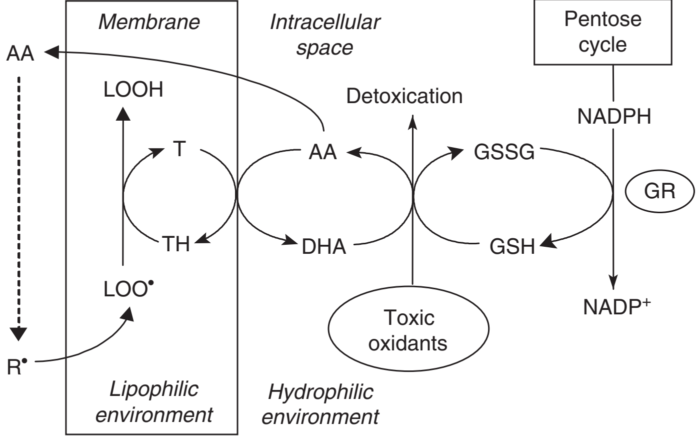

Fig. 2.10 Cooperation of glutathione with vitamin C and E R• – initiator of a radical reaction, LOO• – lipoperoxide, LOOH – hydroperoxide, TH – tocopherol, T• – tocopherol radical, AA – ascorbate, DHA – dehydroascorbate, GSH – reduced glutathione, GSSG – oxidized glutathione, GR – glutathione reductase

synthesized *de novo*. The ratio GSH/GSSG is a suitable marker of oxidative stress in the organism [61, 91] and has the function of "redox buffer". The ratio GSH/GSSG is an indicator of the redox state of the cell [114]. The intracellular concentration of GSH is 500 times higher than its extracellular concentration, thus GSH has an important function in detoxification processes in the cell. Moreover, the correct ratio of GSH/GSSG significantly contributes to the total redox state of the cell. Since many proteins participating in the signaling pathways, such as transcription factors and receptors have thiol groups in the active sites, their function can be influenced by the redox state of the cell. At increased GSSG level, thiol groups of proteins can form mixed disulfides, changing thus their physiological function. In this respect, GSSG appears to act as a nonspecific signaling molecule [134].

2.2.3 Natural Antioxidants, Flavonoids and Polyphenols

Flavonoids are phenolic compounds spread in the plant kingdom. They include more than 4,000 different derivatives and their list constantly increases. Formation of so many derivatives is possible due to the substitution of hydrogen atoms at different sites of the basic structures by hydroxyl, methoxyl and other groups [10]. The basic flavonoid structures include: *flavan-3-ol, flavanone, flavone, flavone-3-ol, anthocyanidin* and *chalcone* [135] (Fig. 2.11).

Flavonoids occur in food either as free monomers (quercetin, catechin) or oligomers (procyanidins) or they are bound to saccharides as glycosides. After ingestion, flavonoids can undergo biotransformation to their metabolites, which can be detected in plasma (Chovanová, unpublished results) reaching a concentration of about 1 μmol.l^{-1} [54].

Consumption of flavonoid-rich food is associated with a lower incidence of coronary heart disease, myocardial infarction [18], cancer [134], neurodegenerative diseases [106], psychic diseases [131], and other chronic diseases [66]. Since in the pathology of these diseases, in addition to other factors, oxidative stress has been assumed to play a role, dietary flavonoids have been suggested to exert health benefits through antioxidant mechanisms. In experiments *in vitro*, flavonoids exert a sig-

Fig. 2.11 Basic flavonoid structures

nificant antioxidative ability, especially due to hydroxyl groups present in the B ring, donating hydrogen atoms to radical reactions [139]. The presence of the double bond at position 2,3 in conjugation with the 4-oxo-group in the C ring and the presence of hydroxyl groups capable of binding transition metal ions, such as iron and copper, contributes to the chelating ability of flavonoids. In addition, flavonoids have a relatively low redox potential enabling them to easily oxidate and reduce free radicals and also to regenerate other antioxidants with a higher redox potential [30, 74].

However, the antioxidative ability of flavonoids cannot be exerted *in vivo* because flavonoid absorption from food is low. The plasma level of flavonoids is an order of magnitude lower than the level of other antioxidants in plasma, such as vitamin C, E and uric acid [15]. Moreover, the half-life of flavonoids in plasma is short because after ingestion they are metabolized to other derivatives.

Low concentrations of flavonoids exert other than antioxidative biological activities *in vivo*. Flavonoids can participate in signaling pathways in cells, thus influencing the fate of a cell [22]. Flavonoids may interact selectively within the MAP kinase signaling pathway which is involved in signaling in neuronal survival, regeneration, development, and death [123] (Fig. 2.12).

The positive effect of flavonoids on the organism is manifest in several directions. In addition to the secondary effect of the antioxidative ability, e.g. through stimulation of antioxidative enzymes [15], they have also a vasodilating effect [34], anti-thrombotic effect [42], anti-inflammatory effect [115] and anti-apoptic effect.

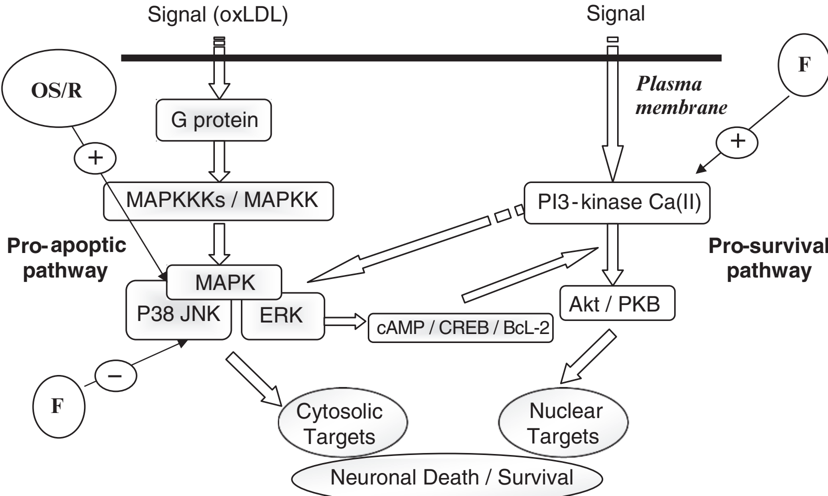

Fig. 2.12 Possible sites of flavonoid intervention into signaling pathways (Modified from Spencer, 2005). MAP kinase – mitogen-activated protein kinase, JNK – c-jun amino-terminal kinase. ERK – extracellular signal-related kinase, PI3 – phosphatidylinositol 3-kinase, Akt/PKB – serine/threonine kinase, CREB – cAMP regulatory-binding protein, Bcl-2 – antiapoptic protein, OS/RS – oxidative /redox stress, F – flavonoids

Flavonoids also possess antimutagenic ability [68] and can inhibit the bond of cancerogenic compounds to DNA.

Results of many epidemiological studies indicate an exceptional place of flavonoids among biomodulating compounds. Well known is the so-called "French paradox". Based on the living style of French people, it could be assumed that their life includes several risk factors for the occurrence of coronary heart disease. However in one region of France, a markedly low incidence of this disease has been detected in comparison with other regions. This fact has been attributed to the consumption of red wine with a high content of flavonoids and polyphenolic compounds [108].

On the other hand, flavonoids exert also their pro-oxidative properties, e.g. in the presence of a high concentration of Cu^{2+} ions (25–100 μmol.l^{-1}) and oxygen [70, 109]. The issue whether pro-oxidative effects of flavonoids can be exerted also *in vivo* has been addressed, yet the answer to this question requires further studies.

2.3 Oxidative Stress

When some of the protective systems of the organism against free radical toxicity fail, the action of free radicals becomes uncontrolled, resulting in damage to molecules, cells and organs, and potentially to the death of the organism [30, 31].

The consequence of the negative effects of FR and RM is called ***oxidative stress***. From today's point of view, *oxidative stress* can be defined as *an imbalance between production and elimination of reactive metabolites of oxygen and nitrogen, in favor of their production, leading to potential damage* [120]. During oxidative

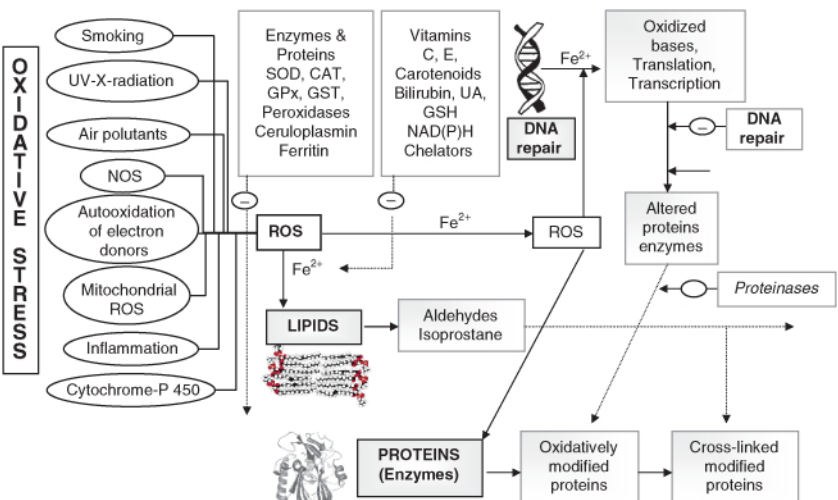

Fig. 2.13 Oxidative stress and its impact on a cell

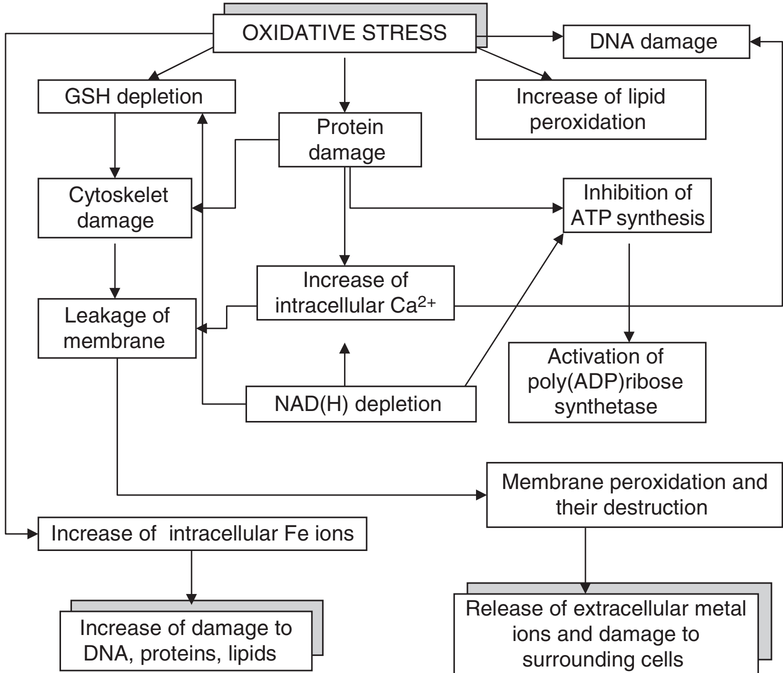

Fig. 2.14 Associations between oxidative stress and damage to the organism

stress, biologically important molecules and cells can be damaged, and this can be significant in the pathogenesis of many diseases. Figure 2.14 shows associations between cell damage and oxidative stress [1]. The term “oxidative stress” has not always been used correctly. It is not correct to use it in a situation when, e.g. increased levels of lipoperoxidation have been detected but the effect of oxidative environment on proteins and nucleic acids is unknown. It should be also noted that the decreased level of one antioxidant need not evoke oxidative stress (Fig. 2.13).

Oxidative stress can result from several conditions (50):

1. Increased production of RM, as in hyperoxia, increased exposure to toxic RM, e.g. $NO_2^{\bullet}$, or promotors of RM formation, e.g. paraquat, or by increased activation of systems producing FR and RM, e.g. activation of neutrophils during inflammation.
2. Diminished levels of antioxidants, e.g. by increased exposure to toxins, which themselves need not be a source of FR but which are eliminated from the organism by glutathione transferase cooperating with GSH, thus leading to its depletion. Futher it may concern insufficient supply of some metal ions in food which are necessary for the function of some protective mechanisms (Zn^{2+}, Se^{4+}, Cu^{2+}, Mg^{2+}), by gene mutation encoding for antioxidative enzymes, e.g. superoxide dismutase, glutathione peroxidase.

FR and RM can damage biologically important molecules either primarily – through the attack of FR or RM on the molecule leading to its damage, or secondarily, when products of FR or RM (e.g. aldehydes) further react with biomolecules (e.g. with proteins and nucleic acids) leading to the formation of conjugates and changing the structure and biological function of lipids, proteins and nucleic acids (Fig. 2.14).

Depending on the given conditions, the intensity and type of oxidative attack, cells and the organism can react differently:

1. *Cells and the organism adapt to* the oxidative stress either completely, e.g. by increased activity of protective antioxidative systems, or partially when they eliminate only one type of the oxidative attack, or the cell builds up its "oxidative immunity" by gradual exposure to a particular oxidant.
2. *Cells can be injured* by oxidative modification of lipids, saccharides, proteins and nucleic acids or by the following nonoxidative reactions as a consequence of the primary oxidative damage (cross-linkage of molecules), change in the level of intracellular ions by the change of integrity of the cell membrane, e.g. Ca^{2+} ions.
3. Damaged molecules in cells can be repaired or replaced by repair systems. In such a case, the *cell can survive* with a partial damage, yet when the repair ability of the organism fails and the cell, especially DNA, is constantly exposed to oxidants, then cell death can be triggered by apoptosis or necrosis.

The term "oxidative stress" is an incorrect term also from the chemical point of view. Oxidation never occurs alone but in association with reduction. Oxidation and reduction are shortly called the redox reaction [134]. Therefore "oxidative stress" should be correctly named "redox stress". For the understanding of the chemical nature of "oxidative stress", the use of terms such as oxidant/prooxidant and antioxidant have their significance. Concerning the redox balance, for the terms "oxidant and antioxidant" there are more precise expressions, such as a *strong oxidant* and a *weak oxidant*. A stronger oxidant (a higher positive redox potential) can extract hydrogen atom or electron from a weaker oxidant (antioxidant), transferring the radical character to the weaker oxidant (antioxidant). According to redox potentials of individual redox pairs, it can be assessed whether a particular antioxidant can regenerate the other one. An antioxidant or a weak oxidant with a lower redox potential can regenerate an antioxidant with a higher potential. According to these indicators, dihydrolipoic acid (DHLA) is the most versatile antioxidant with the lowest oxidative properties. This redox potential indicator strictly follows chemical rules in *in vitro* systems and does not take into account the influence of other cell components and the effect of other antioxidants. The redox balance has thus to be evaluated in the total context of a living organism (Fig. 2.14).

The degree of oxidative stress damage depends on various factors. These include the type of the injured molecule (proteins, lipids, nucleic acids), the mechanism by which the damage is performed (fenton type chemistry, induction by a certain drug or a xenobiotic, activation of enzymes, e.g. NO-synthase, xanthine oxidase) and the type of the oxidative stress.

A strong oxidative stress in the organism can participate in the onset or development of many human diseases [1], e.g. neurodegenerative diseases, Parkinson's and Alzheimer's disease, atherosclerosis, cardiovascular and cancer diseases, autoimmune nephropathy, diabetes mellitus [87], Crohn's disease, rheumatoid arthritis, retinopathy and psychic diseases such as schizophrenia or ADHD [131].

Ischemia-reperfusion states of various organs belong to important and relatively frequent endogenous forms of oxidative stress. ***Ischemia*** is a source of tissue necrosis under various pathological conditions. Symptoms of ischemia appear in several organs, as the heart, intestine, brain, and lungs [104]. In the therapy of these conditions, reperfusion is used, yet it can contribute to tissue damage [94]. It is assumed that during ischemia-reperfusion, free radicals and oxygen reactive metabolites are produced by mechanisms of arachidonic acid [30], by *xanthine dehydrogenase* together with NAD^+ catalyzing oxidation of hypoxanthine and xanthine to uric acid [43], by *activated neutrophils* piling up in reperfused tissue [35], by *catecholamines* released during ischemia-reperfusion states [58], by *mitochondria* producing superoxide generated under certain circumstances in the respiratory chain by reduction of molecular oxygen [30, 36, 140], or by myoglobin in the presence of “catalytical iron” [82, 94].

Ischemia-reperfusion conditions can occur besides the heart also in the brain, gastrointestinal tract, lungs, kidneys and other organs [120].

Oxidative stress appears also in other pathological conditions, e.g. in some viral diseases [44, 117], or during top sport performances [59, 132].

A lower or higher intensity of oxidative stress during life can positively or negatively influence also ***aging of the organism***, depending on the genetic disposition as well as on dietary factors and life style [142].

As already mentioned, oxidative stress is characterized by the imbalance between oxidants and antioxidants. Since oxidants can reduce themselves and antioxidants can oxidize themselves, reactions of oxidants and antioxidants are associated with electron transport. A particular concentration of electrons (redox state) stored in many cellular constituents is characteristic for each cell. ***Redox state*** of cells is maintained within a relatively narrow range, similarly as is pH. Under pathological conditions, the cell redox state alters to lower or higher values. A change of 30 mV in the redox state means an about 10-fold change in the ratio between reductant/oxidant species [114].

The term “redox state” does not represent the state of one redox pair, e.g. GSH/GSSG, $AscH^-/Asc^{\bullet-}$, it rather describes more generally the redox environment of a cell [114] influencing cell signaling systems. Signaling pathways are processes transferring information from extracellular into intracellular space through the cell membrane (“signal transduction”or “cell signaling”) [128]. The signal is transferred to the target by various components of signal transduction, such as receptors, connectors, effectors, second messengers, protein kinases and phosphoproteins [64]. Besides phosphorylative reactions also redox reactions play a regulatory role in these processes [13, 96]. Signal processes lead to enzyme activation, activation of DNA and RNA synthesis, gene expression, induction of muscle contraction, trans-

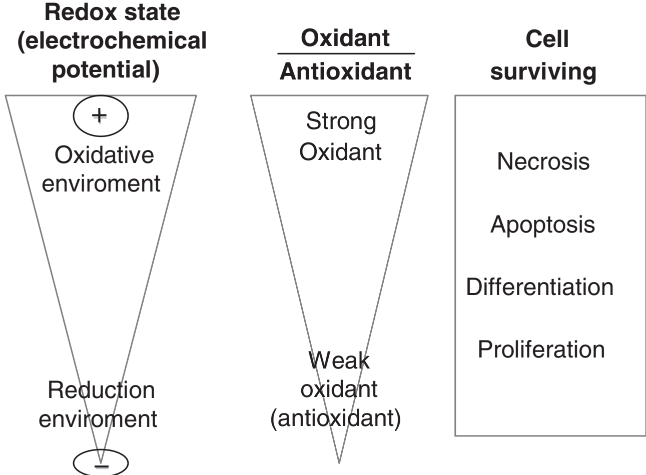

Fig. 2.15 Effect of the redox environment on a cell and its survival

port of nerve signal, the response of the cell to stress conditions such as hypoxia [9], cell proliferation or differentiation and apoptosis [85].

Induction or inhibition of cell proliferation has been suggested to depend on the balance between oxidants and antioxidants in a cell. A more reducing environment of the cell (balance shifted in favor of antioxidants) stimulates proliferation. A balance shifted in favor of oxidants (mildly oxidizing environment) initiates cell differentiation. A further shift towards a more oxidizing environment of the cell leads to apoptosis and the strongest oxidizing environment to necrosis [137] (Fig. 2.15).

The state of redox balance plays an important role, e.g. at the onset and development of cancer diseases. For a cancer cell, a more reducing environment is characteristic. It leads to disturbed balance between cell proliferation and cell death in favor of proliferation. Changes in activities of antioxidative enzymes (SOD, catalase, glutathione peroxidase) as well as in levels of low-molecular weight antioxidants (GSH, ascorbic acid, thioredoxin) are characteristic for a cancer cell leading to an altered redox state of the cell and signaling pathways dependent on the redox state [80]. Reduction of the environment of a nucleus and mitochondria caused by increased level of GSH and thioredoxin inhibits apoptosis and activates the proliferating potential through the redox-dependent nuclear transcription factor [65].

Redox balance affects also DNA repair ability. The human repair enzyme APE (apurinic/apyrimidinic endonuclease) together with Ref-1 (redox effector factor) protein play an important role in the transcription response to oxidative stress (through the regulation of binding activity of transcription factors AP-1, NF-kB, p53, and others) and in reparation of oxidatively damaged nitrogen bases. Increased activity of Ref-1 inhibits apoptosis of cancer cells sensitive to oxidative stress [69].

The oncoprotein Bc1–2 participates in the regulation of apoptosis and blocks the release of cytochrome c, which leads to an increased level of GSH, thus shifting the redox environment of the cell away from apopptosis. This may enhance resistance of the cancer cell, with overexpressed Bc1–2, to oxidative stress–induced apoptosis [29, 92].

Understanding of the mechanisms by which redox-modulators, such as thioredoxin, GSH and Ref-1 maintain intracellular "redox buffer capacity" could contribute to the development of targeted cancer-preventive and therapeutic drugs [134].

RMO modulate the expression of several genes participating in many physiological processes [107]. Interesting is the participation of RMO in influencing longevity, which can manifest differently depending on gender. In many animal species including humans and rats, females usually live longer than males. This fact has been explained by antioxidative effects of estradiol. However, in respect of estradiol concentration, this reason does not seem plausible. Vina et al. [136] showed that the protective effect of estradiol is based on its ability to influence the signaling function through the bond to an estrogen receptor (ER) and activation of the MAP-kinase pathway and the transcription factor NFkB, leading to an increased expression of antioxidative enzymes.

Similar data on adaptation to oxidative stress and increased expression of antioxidative enzymes have been recorded during intensive exercise. During exercise RM are produced at higher concentrations as a consequence of the increased intake of oxygen during exercise [19]. This state evokes an increased activity of antioxidative enzymes through activation of NFkB, followed by activation of genes for Mn-SOD, eNOS, but also iNOS [60], leading to elimination of consequences of oxidants production in mitochondria. The consequences of oxidative stress are manifested only at extreme exercise [113]. Findings on RM signaling function changed our opinion on preventive administration of antioxidants before training. Antioxidants should not be recommended before training as they interfere with muscle cell adaptation and inhibit the generation of antioxidative enzymes. On the contrary, antioxidants may be administered, e.g. before competition representing an extreme exhaustion for the organism with resulting generation of ROS that overwhelm the defensive mechanisms.

2.4 Conclusion

The generation of reactive metabolites (RM) derived from oxygen and nitrogen is a consequence of life in the oxygen atmosphere. In the organism RM represent a relatively constant source of oxidative attacks to genetic material and to important biomolecules such as proteins and lipids, which can partially be modulated by nutritional antioxidants, hormonal or environmental effects. RM overproduction induced by endogenous or exogenous conditions is harmful to the living organism and is called oxidative stress. In addition to the significant action of RM on genetic material through increase of the mutagenic "pool", RM interfer with signaling pathways, which through gene expression influence cell growth and proliferation

or apoptosis, and they affect also the immune response of the cell. These processes are very complicated and their effect depends on the type and concentration of oxidants.

The harmful effect of oxidants can be inhibited to a certain extent by enzymatic antioxidants and low-molecular weight antioxidants. However, their direct antioxidative effect in several diseases, e.g. cancer, is disputable. It is expected that further studies will clarify other biomodulating activities of natural polyphenolic compounds, such as their participation in signaling pathways affecting cell function.

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2.5 Mitochondrial Free Radicals and Antioxidants

Anna Gvozdjáková

Abstract Mitochondrial free oxygen radical production and antioxidant systems are important for living cells of the body. About 2–4% of the oxygen produced via the respiratory chain serves for the production of reactive oxygen species (ROS). Mitochondria are also a source of nitrogen radicals ($NO^{\cdot}$). Since $NO^{\cdot}$ formation requires oxygen, the rate at which it is produced varies with the intramitochondrial oxygen concentration.

Keywords Antioxidants, mitochondria, nitric oxide, reactive oxygen species

Mitochondria are one of the main sources of free radicals production and antioxidant enzymatic systems. In human physiologic metabolism, there is a dynamic balance between the production of free radicals and the antioxidant capacity of the body. Over 95% oxygen consumed by the mitochondria is converted to water at cytochrome oxidase (Complex IV of respiratory chain). Only 2–4% of the oxygen acquires electrons directly, generating reactive oxygen radicals (superoxide radical – O_2^-, peroxide hydrogen – $H_2O_{2,}$ and the very reactive hydroxyl radical – $OH^{\cdot}$), which are normal products of the oxidative process. Radicals may be harmful when produced in increased amounts and when they are not neutralized by antioxidants. These radicals can damage membranes, DNA, and functions of mitochondrial oxidative phosphorylation enzymes. A key component of the mitochondrial respiratory chain is coenzyme Q_{10}, a component with antioxidant properties. Mitochondrial antioxidant enzymes, as manganase superoxide dismutase (MnSOD) and glutathione peroxidase neutralize free radicals. Cytosol antioxidant enzymes are cuprum superoxide dismutase (CuSOD), catalase and glutathione [9].

2.5.1 Mitochondrial Reactive Oxygen Species

The mitochondrial respiratory chain, an integral part of the inner mitochondrial membrane, is created with Complexes I–IV, coenzyme “Q-cycle” and complex V (ATP-synthase). Electrons enter the chain through oxidation of either NADH at Complex I or $FADH_2$ at Complex II. Electrons flow to Complex IV to reduce O_2 to H_2O. Almost all O_2 consumed in mitochondria is reduced to water at Complex IV, a small part of O_2 is reduced to superoxide anion ($O_2^{\bullet-}$). $O_2^{\bullet-}$ can be converted to peroxide hydrogen (H_2O_2) by the mitochondrial matrix enzyme manganese-dependent superoxide dismutase (MnSOD) or by cuprum- and zinc-dependent superoxide dismutase (CuZnSOD) in the intermembrane space. Flow electrons and protons are

translated from the matrix into the intermembrane space. Proton translation from the matrix into the intermembrane space establishes a transmembrane potential ($\Delta\psi$) and electrochemical gradient (ΔpH) across the coupling membrane [8]. The inner membrane is impermeable to protons. Reentrance of protons into the matrix is possible only through Complex V. During aerobic metabolism more than 90% of intracellular ROS are produced at the mitochondrial respiratory chain [4] (Fig. 2.7).

Superoxide formation occurs on the outer mitochondrial membrane, in the matrix and on both sides of the inner mitochondrial membranes. Mitochondrial $O_2^{\bullet-}$ production varies in organs. Complex I appears to be a primary source of $O_2^{\bullet-}$ in the brain under physiological conditions [3]. Complex III appears to be responsible for most of the $O_2^{\bullet-}$ produced in the heart and lung mitochondria. $O_2^{\bullet-}$ production depends also on mitochondrial respiratory chain activity. During state 3, mitochondria are actively respiring, during state 4, mitochondrial respiration is reduced [2].

2.5.2 Mitochondrial Nitric Oxide

Nitric oxide (*NO*) regulates several systems in the organism, e.g. the cardiovascular system, central nervous system, immune system. Mitochondria are important targets of NO and contribute to its several biological functions. Nitric oxide is produced in vivo in the oxidation of L-arginine to L-citruline in a reaction catalyzed by NADPH-dependent enzymes called *nitric oxide synthases* (*NOS*) [1, 6].

$$\text{L-arginine} + O_2 + NADPH_2 \text{--------->} \text{L-citruline} + NADP + H_2O + NO$$

Four isoforms of NOS were discovered. Two of them are constitutive or normally present: neuronal NOS (*nNOS*) and endothelial NOS (*eNOS*). The third one is the inducible form (*iNOS*), typical of macrophages. Each isoenzyme consumes L-arginine, produces equal amounts of NO and L-citruline, and requires Ca^{2+} -calmodulin for its activity. The activities of eNOS and nNOS are regulated tightly by alterations in Ca^{2+} status. Because *iNOS* forms a complex with calmodulin at very low concentrations of Ca^{2+}, its activity is not regulated by Ca^{2+} alterations.

The fourth isoform is ***mitochondrial NOS*** (***mtNOS***) [7, 8]. Mitochondria produce nitric oxide (NO) through Ca^{2+} -sensitive mitochondrial NO synthase (*mtNOS*). The NO produced by mtNOS regulates mitochondrial oxygen consumption and transmembrane potential via reversible reaction with cytochrome c oxidase. Although the O_2-binding site of cytochrome c oxidase is specialized, similarity between the physicochemical properties of NO and O_2 enables NO to bind to cytochrome c oxidase, thus inhibiting the electron flow. NO decreases mitochondrial O_2 consumption, the electrochemical gradient ($\Delta\psi$) and proton gradient (ΔpH), resulting in decreased ATP formation. By decreasing $\Delta\psi$, NO consequently decreases mitochondrial Ca^{2+} uptake. NO produced by *mtNOS* modulates mitochondrial respiration, $\Delta\psi$ and ΔpH, and regulates mitochondrial bioenergetics. *MtNOS* is a crucial biochemical regulator of mitochondrial function.

2.5.3 Mitochondrial Utilization of Nitric Oxide

There are three main reactions that utilize NO in mitochondria.

(a) With $O_2^{\bullet-}$: $NO + O_2^{\bullet-}$--------> $ONOO^-$

The rapid reaction of NO with $O_2^{\,-}$ accounts for about 85% of the rate of mitochondrial NO utilization in aerobic conditions. The simultaneous formation of $O_2^{\,-}$ and nitric oxide produces peroxynitrite ($ONOO^-$), which irreversibly modifies targets within mitochondria and induces oxidative stress. Peroxynitrite is a very strong oxidant and nitrating agent [12].

(b) With ubiquinol: $NO + UQH_2$--------> $UQH + H^+ + NO$

The reaction of NO with ubiquinol produces ubisemiquinone, which in turn operates as a free radical reaction center able to generate $O_2^{\,-}$ by autooxidation in a propagation reaction [10].

(c) With cytochrome oxidase: $NO + Cyt\ a_3^{2+}$-------->$Cyt\ a_3^{3+} + NO$

NO with cytochrome oxidase inhibits the main pathway of oxygen uptake and energy production [5].

2.5.4 Mitochondrial Antioxidants

Mitochondria are prevented from ROS production by several antioxidant systems.

(a) ***Superoxide dismutase:*** Superoxide is enzymatically converted to H_2O_2 by a family of metalloenzymes – SODs. Since $O_2^{\bullet-}$ may either reduce transition metals, which in turn can react with H_2O_2 producing $OH^{\cdot}$, or spontaneously react with $NO^{\cdot}$ to produce peroxynitrite, it is important to maintain the steady-state concentration of $O_2^{\bullet-}$ at the lowest possible level. Thus, although the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2 can also occur spontaneously, the role of SODs is to increase the rate of the reaction to that of a diffusion-controlled process. The mitochondrial matrix contains a specific form of SOD, with manganese in the active site, which eliminates $O_2^{\bullet-}$ formed in the matrix or on the inner side of the inner membrane. The expression of MnSOD is further induced by agents that cause oxidative stress, including radiation and hyperoxia, in a process mediated by the oxidative activation of the nuclear transcription factor NFkB.

Intermembrane space: the steady-state concentration of $O_2^{\bullet-}$ in the intermembrane space is controlled by three different mechanisms. First, this compartment contains a different SOD isoenzyme, which contains copper and zinc – CuZnSOD

and is also found in the cytoplasm of eukaryotic cells. Second, the intermembrane space contains cytochrome c, which can be reduced by $O_2^{\bullet-}$ regenerating oxygen in the process. The reduced cytochrome c can then transfer electrons to the terminal oxidase. Thus, some of the electrons that escaped the respiratory chain producing $O_2^{\bullet-}$ may re-reduce cytochrome c and still contribute to energy production by providing the energy needed to pump H^+ through Complex IV. Finally, the spontaneous dismutation of $O_2^{\bullet-}$ in the intermembrane space is facilitated by the lower pH in this compartment, resulting from the extrusion of H^+ coupled to respiration.

Hydrogen peroxide is the product of $O_2^{\bullet-}$ dismutation and the main precursor of $OH^{\cdot}$. In the presence of reduced transition metals, it is mostly decomposed by the enzyme glutathione peroxidase. In the liver, mitochondria account for about one third of the total glutathione peroxidase activity.

A second glutathione peroxidase associated with the mitochondrial membrane, known as phospholid-hydroperoxide glutathione peroxidase, is specifically involved in reducing lipid peroxides associated with the membrane.

(b) ***Catalase***, a major H_2O_2 detoxifying enzyme found in peroxisomes, is present in heart mitochondria [11], yet it has not been found in mitochondria of other tissues.
(c) In addition to ***cytochrome c***, other electron carriers appear to exert a detoxifying role against ROS.
(d) ***Coenzyme Q*** *(QH – ubiquinol)* has been shown to act as a reducing agent in the elimination of various peroxides in the presence of succinate. Coenzyme Q is a source of $O_2^{\bullet-}$ when it is partially reduced (semiquinone form) and an antioxidant that interferes with the propagation of free radical-mediated chain reactions.

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Chapter 3
Chronobiology, Chronomics and N-of-1 Tests of Timing Coenzyme Q10

Franz Halberg, Germaine Cornélissen, R.B. Singh, Anna Gvozdjáková, Kuniaki Otsuka, Larry Beaty, George Katinas, Ramon Hermida, Diana Ayala, and Jerzy Czaplicki

3.1 Chronobiology

(From Gk *chronos* = time, Gk *bios* = life and Gk *logos* = science) is:

- A computer-aided objective quantification, mapping and investigation of mechanisms underlying biological rhythms, the fundamental mechanisms of life, and broader time structures; its primary raison d'être is to provide throughout science:
- The control information on cycles, broader time structures and their underlying mechanisms replacing imaginary baselines, serving immediately and above all to avoid blunders such as the three comparisons on top of Fig. 3.1, if each were viewed separately. Such blunders are common in an overwhelming majority of current publications ignoring rhythms in the variables investigated or presuming to "eliminate" them by integrating over the period of a known rhythm [23]. Controversy could have arisen if any one of these results (Fig. 3.1A, B or C, *top*) had been published without the realization that two groups being compared differed in the phase of rhythms in the variables investigated (Fig. 3.1 *bottom*). The rhetorical question revolves around how very many actual publications ignoring rhythms by dealing with "baselines" are as wasteful as, e.g., the statistically significant results in A, B or C. Hence, study of Fig. 3.1 is warranted. In themselves, each of the comparisons is statistically significant, albeit they are contradictory effects, opposite in A vs. C (Fig. 3.1, *top*). All are readily resolved by focus upon the characteristics of two detected rhythms that are in antiphase. The need for control data – for characteristics of rhythms in investigations of rhythmic variables – started chronobiology in Minnesota, specifically by
- The designs for the collection and interpretation of serial data along a pertinent timescale, aiming at providing indispensable reference standards that can be time-macroscopic for inspection by the naked eye, e.g., from sampling results on separate groups at intervals of a few hours as plots of mean values at each timepoint

US National Institutes of Health (GM-13981) (FH) and University of Minnesota Supercomputing Institute (GC, FH).

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with their uncertainties, shown in Figs. 3.2 and 3.3. Eventually, such displays are complemented by a microscopic approach providing for any critically rhythmic variable's period, τ (or frequency, f, or $\omega = 2\pi f$), estimates of phase, ϕ and amplitude, A (Fig. 3.4, *top*), and waveform, the latter quantified by the (A, ϕ) pairs of harmonics; this led by multiple regression with least squares to

- A set of inferential statistical methods recommended for the detection and/or parameter estimation of rhythms in unequidistant data, Figs 3.4–3.9, also yielding, as an invaluable dividend, a usually more accurate or more precise average, Fig. 3.5 (*top*). These procedures serve for
- The interpretation of any results and action based on characteristics of rhythms as well as of chaos (such as a correlation dimension [37] or approximate entropy [2] and trends, including, eventually, among others, a timely and time structure-based diagnosis and, if need be, a timely and timed treatment [12, 13]. By no more than changing treatment timing, a high risk of severe vascular disease, Fig. 3.10, section IIIB, can be reduced or the survival rate from cancer

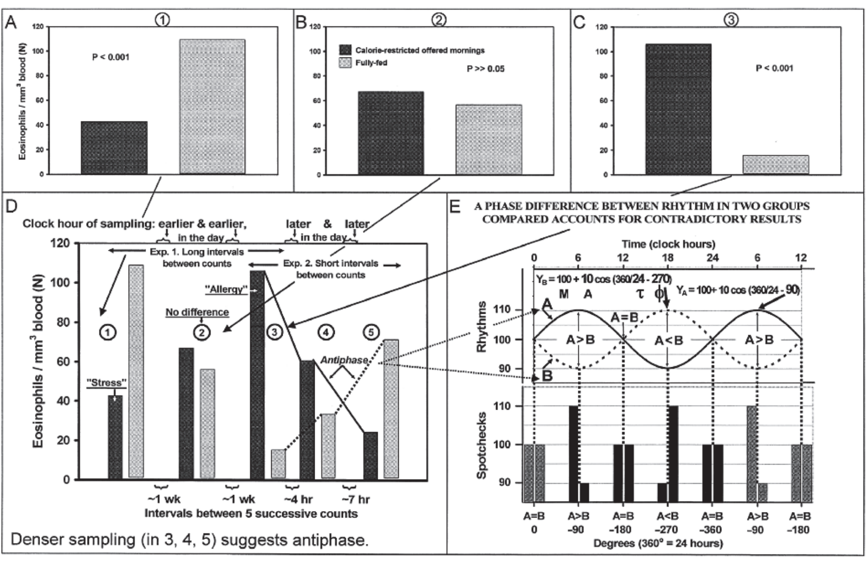


Fig. 3.1 Avoiding blunders: importance of rhythms in assessing intervention effects, illustrated in relation to stress or allergy. (**A**) Eosinophil counts seem to be lowered by fasting (by the associated stress), when a 50% reduction in dietary carbohydrates and fats (with proteins, vitamins, and minerals similar to control group) was fed in the morning to C_3H mice (*dark column*). (In this model, the naturally high incidence of breast cancer is lowered by a diet reduced in calories; the effect upon cancer is not shown.) The result could have been interpreted as an adrenocortical activation, assessed by eosinophil depression, with applications for treating breast cancer and for prolonging life. Steroids that depress eosinophil cell counts and perhaps mitoses could be a mechanism through which caloric restriction (and ovariectomy in the mice on restricted feeding) act in greatly reducing cancer incidence. (**B**) In view of the importance of this finding for the etiology of cancer, results were replicated on a larger group of animals 1 week later. This follow-up study with more animals started at an earlier clock-hour, yielded confusing results, showing no

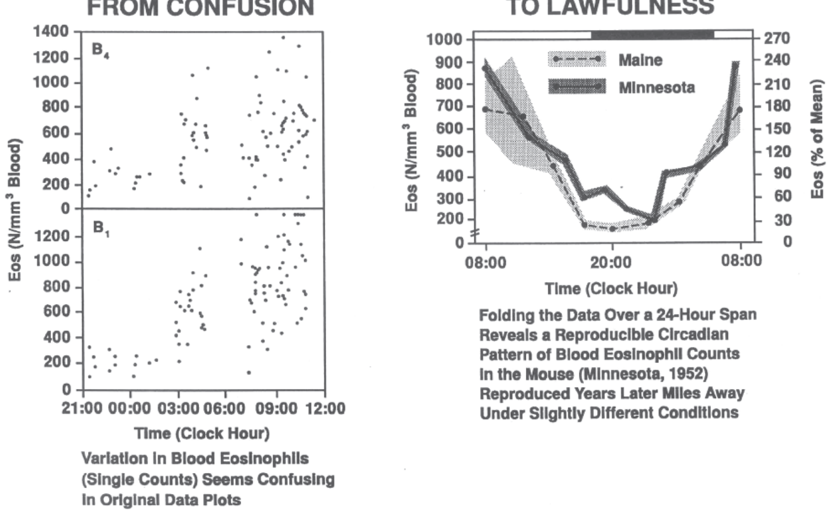


Fig. 3.2 Sampling repeatedly with serial independence as to individuals (each animal sampled only once) shows variability (*left*); new systematic sampling in added studies and averaging blood cell counts reveals pattern to the naked eye. (Copyright Halberg)

radiotherapy of patients with oral cancers can be doubled, Fig. 3.11. Such findings hold the promise of broader applicability.

3.2 Quantification

Any endeavor in experimental and clinical biomedicine benefits from the inferential statistical "isolation" of circadian and other rhythms by desirable hypothesis testing and, irrespective of the rejection of the zero-amplitude assumption, the usually indispensable estimation (with their uncertainties, such as 95% confidence

Fig. 3.1 (continued) statistically significant difference between the two groups of mice. (**C**) After another week, another study, starting at an even earlier clock-hour, yielded results opposite to those in the first experiment. These findings in **C** in themselves could have been interpreted as an allergic response, certainly contrary to the "stress" response in **A**. (**D**) Additional sampling at intervals of a few hours in the third study, in stages called 4 and 5, hinted at the reason for the confusion: by sampling at different clock-hours, two groups of mice were found to be characterized by a circadian rhythm with different phases because the experimentals were fed in the morning and the controls fed at night. Opposite effects thus became predictable. (**E**) Abstract illustration of two circadian rhythms in antiphase. Differences in opposite direction or no effect are then anticipated from sampling at different clock-hours. (Copyright Halberg)

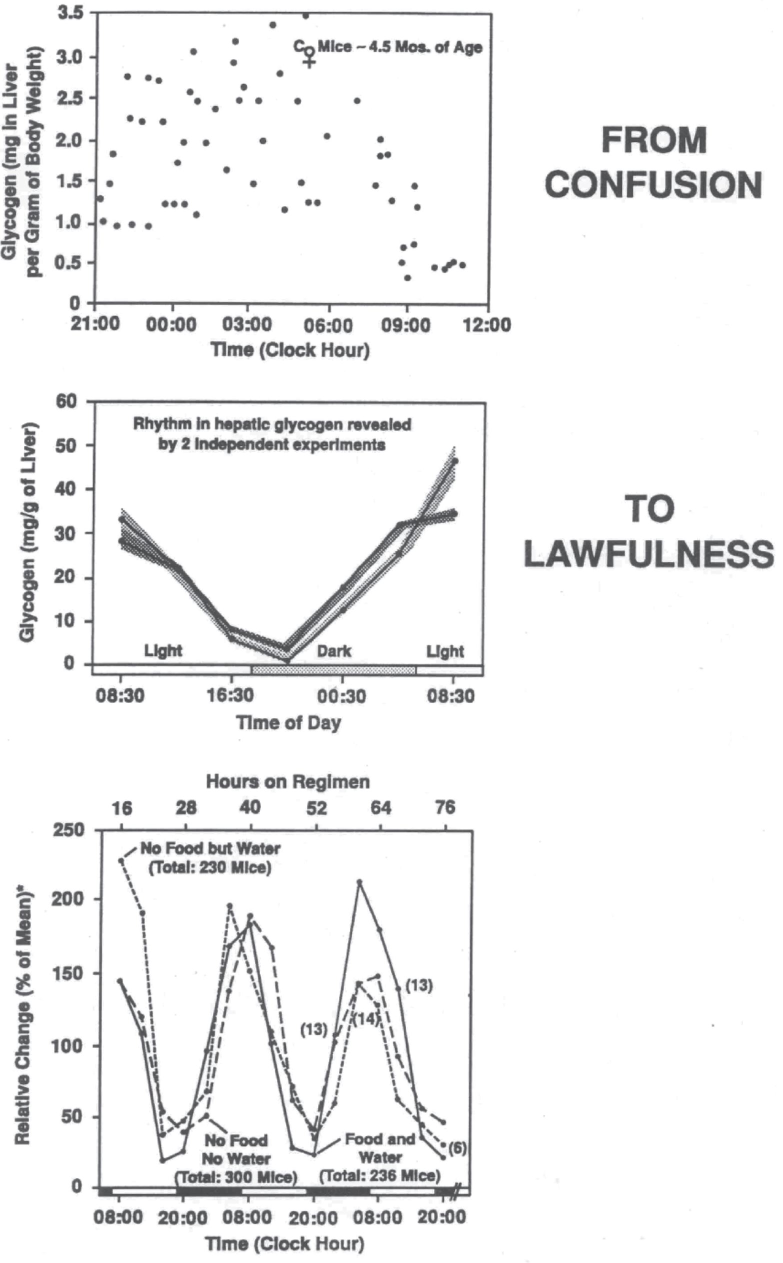
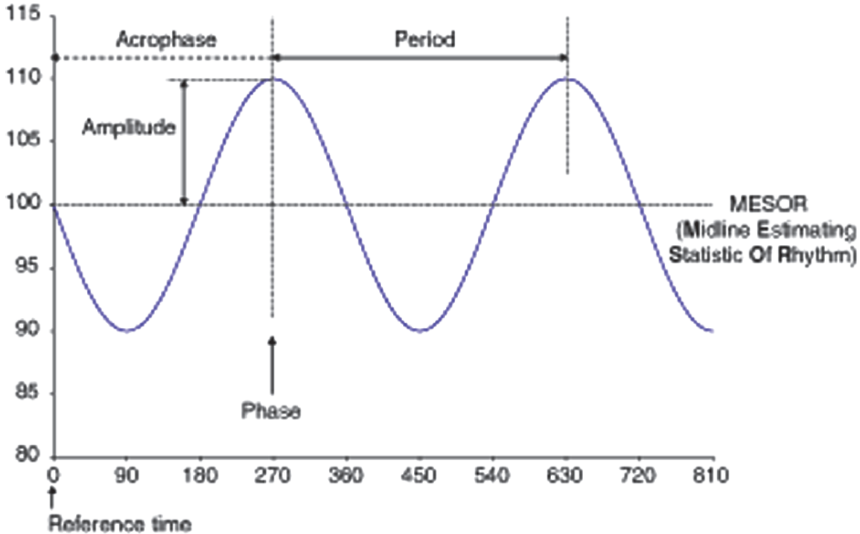


Fig. 3.3 Repeated casual sampling of data on liver glycogen vs. systematic study with averaging. (Copyright Halberg)



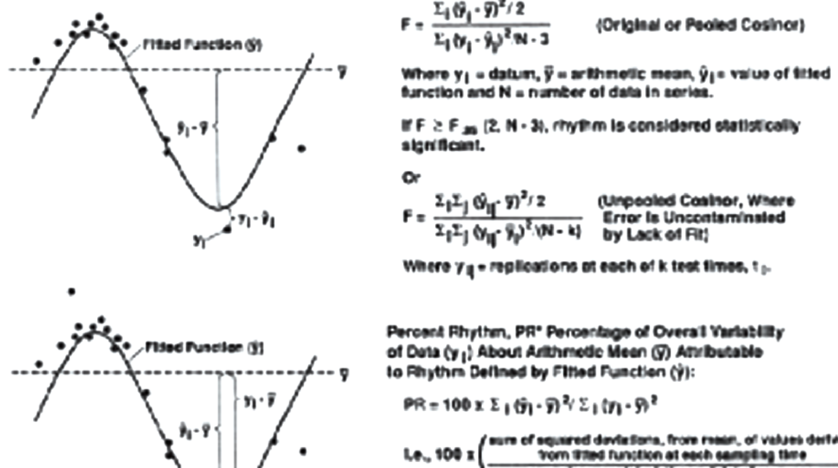
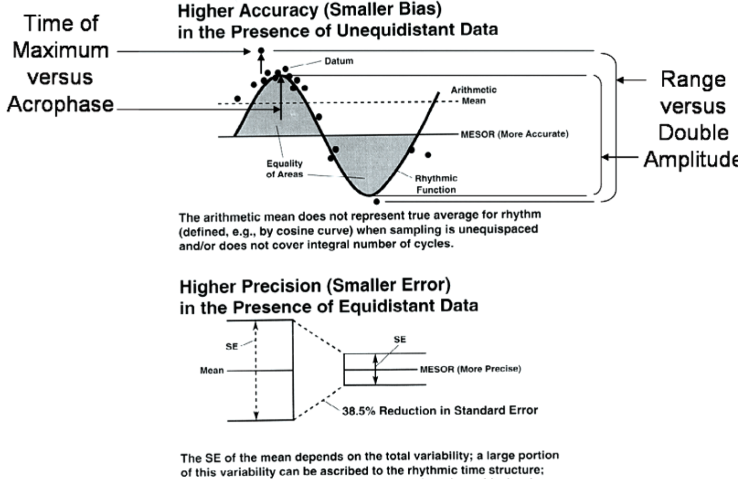
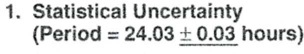


Fig. 3.4 Cycle characteristics on *top* obtained by curve-fitting, the latter at the *bottom* constituting both a hypothesis test (P-value) and a gauge of prominence (% rhythm). (Copyright Halberg)

Advantages of MESOR, Double Amplitude and Acrophase over Arithmetic Mean, Range and Time of Maximum



"CIRCA" IN "CIRCADIAN" CONVEYS TWO CONCEPTS:



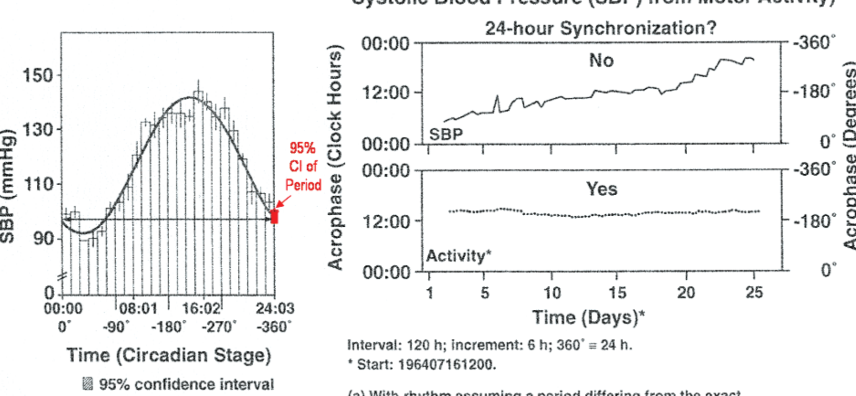


Fig. 3.5 A usually better average than the arithmetic mean is a dividend from curve-fitting, seen on *top*, which can be done on consecutive intervals of the total time series, as shown at the *bottom right*, as a chronobiologic serial section, preferably after a period has been estimated, *bottom left*, with a 95% confidence interval of the period, shown as a box at the right end of the horizontal period line under 1. Statistical Uncertainty, *left*. One major reason for the use of "circa" in "circa-rhythms" includes, among several other considerations, the need for providing inferential statistical uncertainties that qualify the estimate of characteristics such as a period. Another major reason is

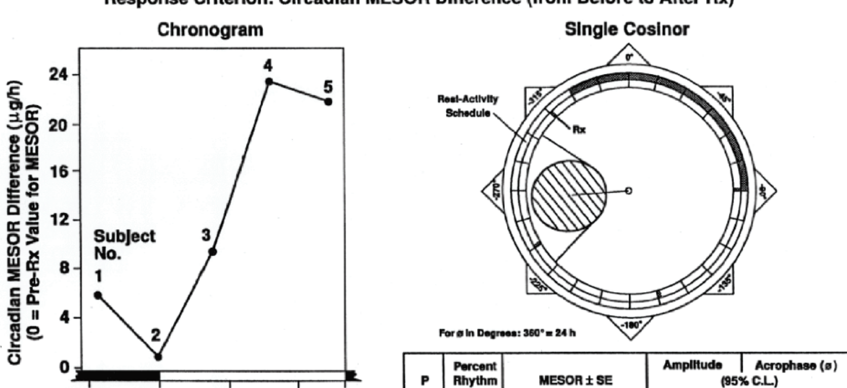


Fig. 3.6 Single cosinor illustrated by a study of time-dependent effects of an ACTH analogue. The approach (Fig. 3.4, *bottom*) on data from a chronobiologic pilot design on only five subjects demonstrates an effect at some circadian stages (validated by the rejection of the zero-amplitude assumption) but not at another circadian time. (Copyright Halberg)

intervals, when possible) of characteristics such as period, τ, and at each τ, an amplitude, A, acrophase, ϕ, and waveform, (A,ϕ) pairs of harmonics. The extended cosinor method based on a linear–nonlinear approach serves this purpose, complemented by chronobiologic serial sections and a combination of gliding and global spectral windows. As a dividend, the MESOR, a **m**idline-**e**stimating **s**tatistic **o**f **r**hythm also obtained by the procedure, is usually superior to the arithmetic mean, Fig. 3.5 [5, 23, 38].

Fig. 3.5 (continued) a pertinent endogenicity, revealed by external and/or internal desynchronization, e.g., of systolic blood pressure (SBP) from sleep–wakefulness (activity) in one and the same person living under a 24-h synchronized hospital routine, assessed by 3 shifts of nurses (*bottom right*). Further support for endogenicity stems from circadian rhythm alteration in mice with clock genes deleted or mutated [10]. (Copyright Halberg)

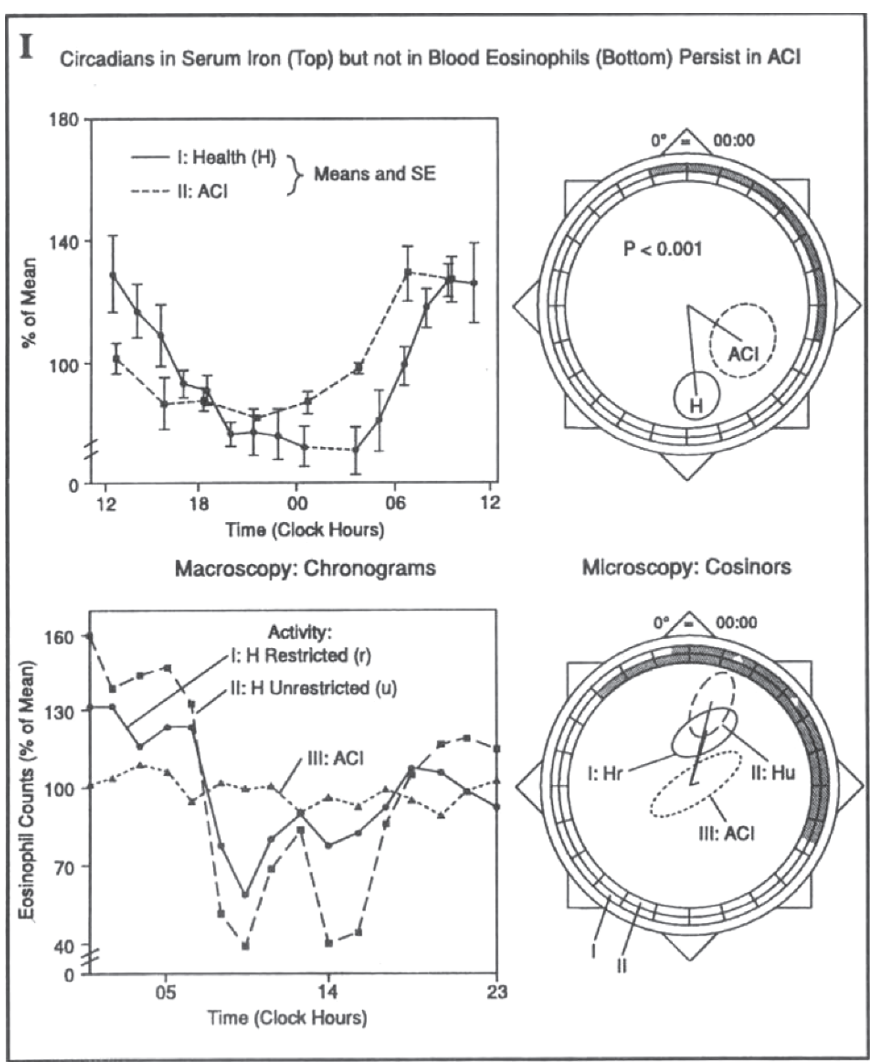
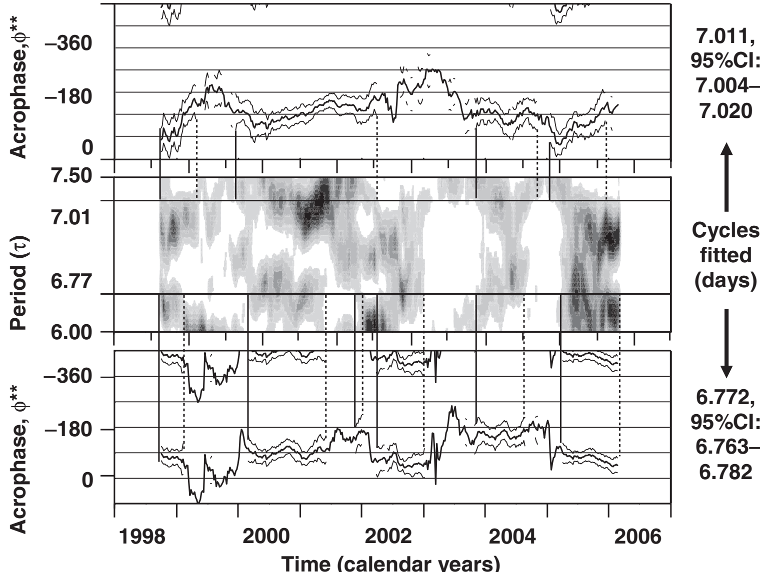


Fig. 3.7 The population-mean cosinor method summarizes abolition of circadian rhythm in counts of blood eosinophil cell (*bottom*) but not in serum iron (*top*) in adrenocortical insufficiency (ACI) vs. health (H). Error ellipse for blood eosinophils in ACI (*bottom right*) overlaps center of graph (pole). Amplification of circadian rhythm by exercise (II vs. I) also apparent. (Copyright Halberg)

A GLIDING SPECTRAL WINDOW (middle) BRACKETED BY CHRONOMIC SERIAL SECTIONS SHOWS CIRCASEPTAN ACROPHASES AT TRIAL PERIODS (τ) OF 7.01 DAYS (top) AND OF 6.77 DAYS (bottom) IN THE HEART RATE OF A MAN*



*** GSK, 72 years of age at start of half-hourly monitoring (N = 134,823). In gliding spectral window, interval = 1 year, increment = 1/24 year; shaded areas show percentage of rhythm (from 0.3 to 3.0 %). Statistical significance shown as 95% confidence intervals (thin partly broken lines) bracketing ϕs (thick line) (top and bottom). Uninterrupted and interrupted vertical lines indicate beginning and end of correspondence between circaseptan components in the phase (top and bottom) and period (middle) domains. 23rd solar cycle began in May 1996, its maximum was in Apr 2000 and the cycle still continued descending after the end of record.**

**** ϕ, in (negative) degrees, with 360° ≡ τ; = 00:00 21 Dec 1997.**

Fig. 3.8 Chronobiologic serial section displaying 95% confidence intervals as thin lines above and below the point estimate of acrophase, missing (in the middle) from a gliding spectral window. (Copyright Halberg)

3.3 Degree of Synchronization in Frequency but not Necessarily in Phase, Intra-Individually Among Different Variables and with the Environment

Circadians include rhythms with periods near 24 h, whether or not they are frequency-synchronized with other cycles or are desynchronized if not free-running. Generally, like the local weather and the broader climate, life also involves the recurrence in *about* (the first *circa)* the same sequences of *about* the same phenomena

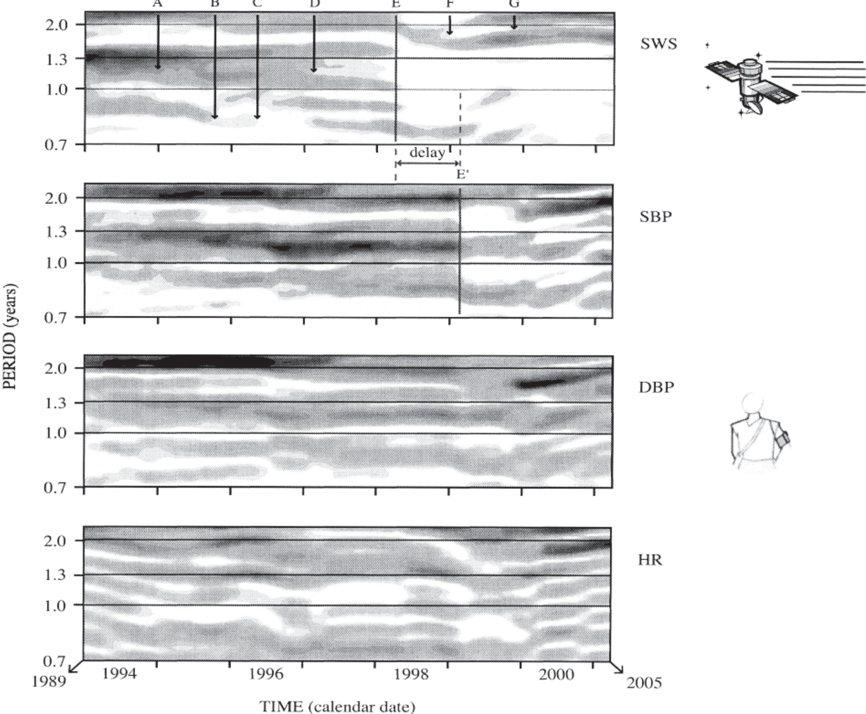


Fig. 3.9 Putative merits of gliding spectral windows. Time courses of the frequency structures of the speed of the solar wind (SWS) (*top*) and of an elderly man's (FH) systolic (S) and diastolic (D) blood pressure (BP) and heart rate (HR) (rows 2–4, respectively), examined by gliding spectral windows. Human SBP selectively resonates with solar wind speed (SWS) (*top* two sections). No obvious resonance, only minor coincidence with DBP or HR (*bottom* 2 sections) of a man (FH), 70 years (y) of age at start of automatic half-hourly around the clock measurements for ~16 y with interruptions (N = 2418 daily averages, total ~55000). Gliding spectra computed with interval = 8 y, resolution low in time but high in frequency, increment = 1 month, trial periods from 2.5 to 0.4 y, with harmonic increment = 0.05. Darker shading corresponds to larger amplitude. When several of these broad bands disappear in the SWS, at E, parts of the bands in SBP also disappear, with a lag (delay) at E′, while other parts persist. These aeolian rhythms in gliding spectra of SWS and SBP change in frequency (smoothly [A] or abruptly [B, C, D], bifurcating [D, F] and rejoining [G], they also change in amplitude (B) (up to disappearing [C, E] and reappearing). During a nearly 16-year span there are no consistent components with a period averaging precisely 1 year in the three physiologic variables, probably an effect of advancing age. While post hoc ergo propter hoc reasoning can never be ruled out, an abrupt change on top in SWS is followed in the second row in SBP by the disappearance of some components, suggesting that as a first demonstration, some of FH's cis- and transyear components were driven by the SW [since they disappeared with a lag of about a transyear following the disappearance (subtraction) of the same components from the SWS spectrum]. The persistence of other spectral features in turn suggests endogenicity, i.e., an evolutionary acquisition of solar transyear oscillations that may reflect solar dynamics for the past billions of years. Aeolian components are presumably built into organisms over billions of years, as persistence without corresponding components in SWS shows, but can be driven in part by the solar wind, as their disappearance after loss of corresponding components in SWS suggests. "Aeolian", derived from Aeolus, ruler of the winds in Greek mythology, who packed the winds up and then let them loose and had them change. (Copyright Halberg)

with *about* (the third *circa)* the same extent, timing and kind of change at intervals that are *about* the same, but extremely rarely all identical. Variability is invariably found during environmental synchronization, but in experimental animals in the laboratory or field, it is usually greater while circa-periodicities persist after organisms (1) are kept under ordinary conditions but deprived by surgery or genetics of the major transducer of the dominant synchronizing environmental illumination cycle, such as the eyes, or (2) are isolated under conditions rendered as constant as possible on earth, at least with respect to environmental light, temperature and societal interactions, and/or (3) are able to self-select the given regimen, e.g., of lighting, rest/activity and/or eating, or (4) are constrained to periodic regimens exceeding the range of synchronizability, whether these are, e.g., shorter than about 20-h or longer than about 28-h "days" (implemented as far as possible by the lighting regimen in the laboratory for certain rodents, e.g., light [L]/dark [D] 10:10 or LD14:14), or whether, with an unacceptable schedule, one administers regressive electric shocks at 12-h intervals until the subject is disoriented in space and time [15, 19]. Further support for a partly endogenous circadian system stems from circadian rhythm alteration in mice with clock genes deleted or mutated [10].

Under all these conditions, the organism exhibits periods described as desynchronized, if not free-running, Fig. 3.12, if they are shown to have been synchronized earlier, are synchronized subsequently or if there are concomitant synchronized controls, or are **a**synchronized, if they differ with statistical significance from those found under synchronization with their environmental near *(circa)* match, such as a societal day or week or a geomagnetic near-week, half-year, near-transyear or far-transyear. Under usual conditions of a regimen of L and D alternating at 12-h intervals in the experimental animal laboratory or for humans in a social routine, the 24-h synchronization of circadians prevails: without generalizing beyond the scope of 232 individuals in one Japanese city (Urausu), each monitored for 7 days, the systolic and diastolic blood pressure and heart rate were desynchronized from 24 h in 8, 7 or 6% respectively, insofar as the 95% confidence interval of the period analyzed by extended cosinor did not overlap the precise 24-h period. Such a circadian desynchronization for all 3 vascular variables was found in only 1 patient out of 232 (0.43%). Interindividual differences in phase, notably under conditions of risk elevation or disease, remain to be determined for each person at a given time.

3.4 Susceptibility Resistance Cycles: A Step Toward Timed Treatment

In the laboratory, whether a stimulation lasted for seconds (like exposure to noise) or was present for longer (like implantation of a carcinogenic substance), its effects can depend to an extremely high degree on statistically predictable circadian stages that account, among others, for the differences between high and low chances of developing a malignant tumor or of convulsions or survival from a toxic dose of a drug [12, 19]. Eventually, by using tumor temperature as a marker rhythm for

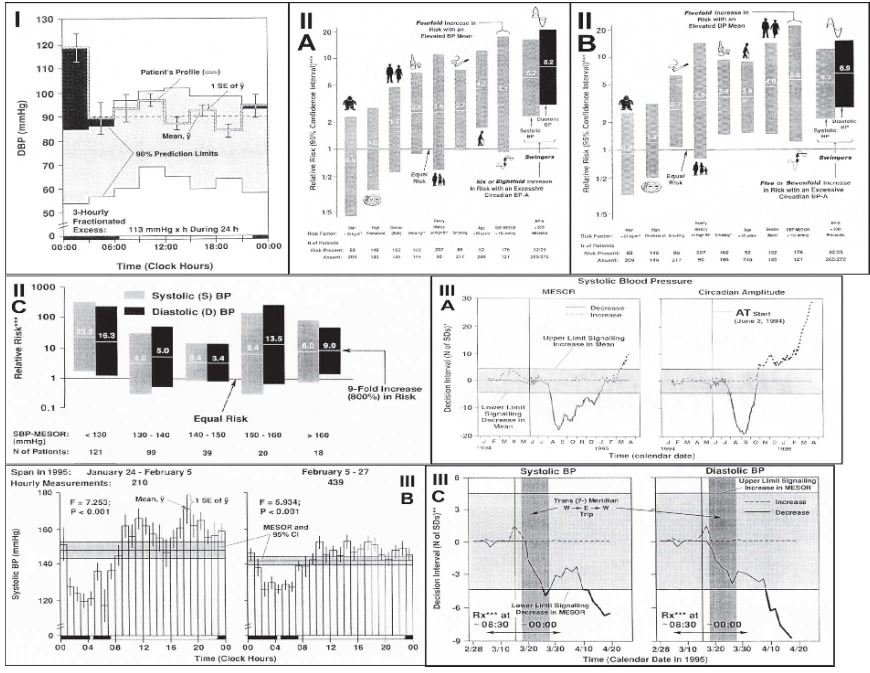


Fig. 3.10 Chronomics detects nocturnal escape from treatment (I), risk of stroke and nephropathy, greater than hypertension (IIA–B), even in MESOR-normotension (IIC) and monitors transient and/or success of treatment lasting during monitoring (IIIA–C). Illustrative results supporting the need for continued surveillance and for a chronomic data analysis. Benefits are:

- Detection of abnormality during the night when medication is no longer effective, not seen during office visits in the afternoon (I)
- Detection of abnormal circadian pattern of blood pressure (CHAT, "overswinging") associated with a risk of cerebral ischemia and nephropathy larger than other risks (including "hypertension") assessed concomitantly (IIA and B)
- Finding that CHAT carries a very high risk even among MESOR-normotensives who do not need anti-hypertensive medication (IIC)
- Availability of statistical procedures such as a self-starting cumulative sum (CUSUM) applicable to the individual patient to determine whether an intervention such as autogenic training is effective and for how long the intervention remains effective (IIIA)
- N-of-1 designs for the optimization of treatment timing: the same dose of the same medication can further lower the same subject's blood pressure MESOR and circadian amplitude when the timing of daily administration is changed (IIIB and C), as ascertained by as-one-goes (sequential) testing and parameter tests, procedures applicable to the given individual.

I: Stacked from 11 days of around-the-clock monitoring. Office spot-checks cannot detect nocturnal pathology.

IIA: Among risk factors, an excessive circadian blood pressure (BP) amplitude (A) raises the risk of ischemic stroke most.

IIB: Among risk factors, an excessive circadian blood pressure (BP) amplitude (A) raises the risk of nephropathy most.

IIC: An excessive circadian blood pressure (BP) amplitude (A) is a risk factor for ischemic stroke independent from the 24-h mean (MESOR).

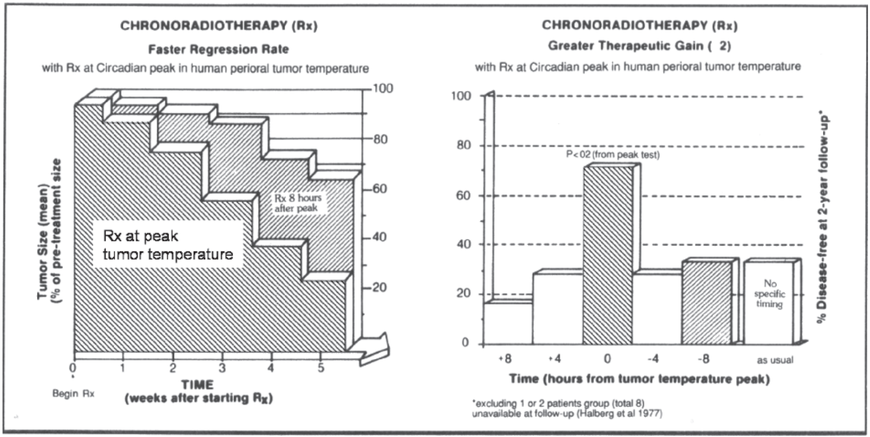


Fig. 3.11 Clinical outcomes show merit of radiation treatment of perioral tumors at circadian peak tumor temperature. (Copyright Halberg)

chronoradiotherapy, Fig. 3.11, 2-year disease-free survival was doubled for patients with cancers in the mouth. Also by using direct as well as indirect tumor markers, chronochemotherapy may become the rule rather than, as currently, the exception for individualized timing [22].

3.5 Marker-Rhythmometry

Automatic, or even manual, blood pressure and heart rate monitoring on a systematic basis, whenever warranted by a changing condition, prevents one from "flying blind." This monitoring constitutes both a diagnostic and a therapeutic marker of risk elevation and overt disease. A chronobiologically detectable circadian overswing of blood pressure constitutes a risk of severe disease greater than hypertension, Fig. 3.10 [9, 13, 17, 24, 36]. This condition, CHAT, short for **c**ircadian **h**yper-**a**mplitude-**t**ension, can be diagnosed and predicts outcomes when day-night ratios

Fig. 3.10 (continued)

IIIA: Individualized assessment (by CUSUM) of a patient's initial response and subsequent failure to respond to autogenic training (AT) (EO, F, 59 years).

IIIB: Individualized blood pressure chronotherapy. Lower circadian double amplitude (2A) and MESOR (M) after switching treatment time from 08:30 (*left*) to 04:30 (*right*).

IIIC: Control chart assesses individualized anti-MESOR-hypertensive chronotherapy. (Copyright Halberg)

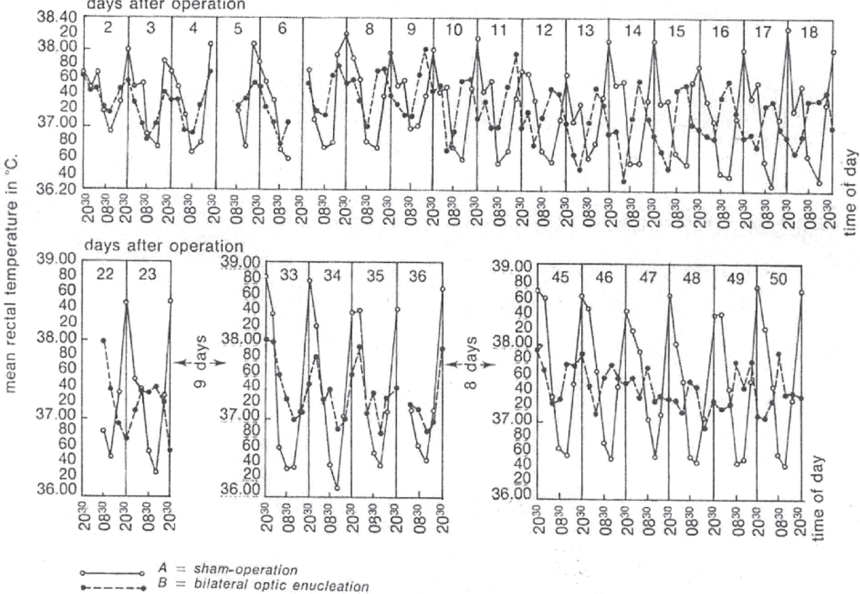


Fig. 3.12 Macroscopic circadian desynchronization in mice after bilateral optic enucleation (dashed line connecting dots) visualizing the need for objective inferential statistical time-microscopic analyses (added in sections IB and IC of Fig. 3.13). While sham-operated controls (solid line connecting open circles) show a daily peak at or close to the vertical 20:30 lines, peak temperatures of blind animals already on day 6 seem to diverge, rising while those of the other group are falling. A graph of the original finding of this separation of the two groups and the decision based thereon to continue measurement every 4 h around-the-clock was interpreted as "paranoia." (At the time, in the precomputer era, the provision of a periodogram on desk calculators took a week, and its checking another week. Indeed, today the approach in Fig. 3.13 (IB) with a moving fit of a 24-h cosine curve is preferred.)

On the average, on top, peaks in temperature of the blinded group occur earlier and earlier, but there are uncertainties in such eyeballing. A transient antiphase at 22–23 days after blinding is readily seen. If, around that stage after blinding, 2-timepoint checks are carried out on the 2 groups, opposite results can be obtained on mice with and without eyes and later when they are again in phase, 2-timepoint checks show no difference, a puzzle readily resolved by an objective quantification of the rhythm characteristics. The need for this microscopy in time becomes obvious, notably if an inference is desired as soon as possible with an estimate of uncertainty. (Circadian desynchronization also characterizes congenitally blind ZRD mice.) (Copyright Halberg)

fail to do so [9, 18]; it can be treated and the elevated risk reduced, along with a reduction in the number of false positive and false negative diagnoses of high blood pressure so that those not in need of treatment are spared the cost, stigma and any side effects and those who need treatment are spared target organ damage and subsequent adverse cardiovascular events, Fig. 3.10.

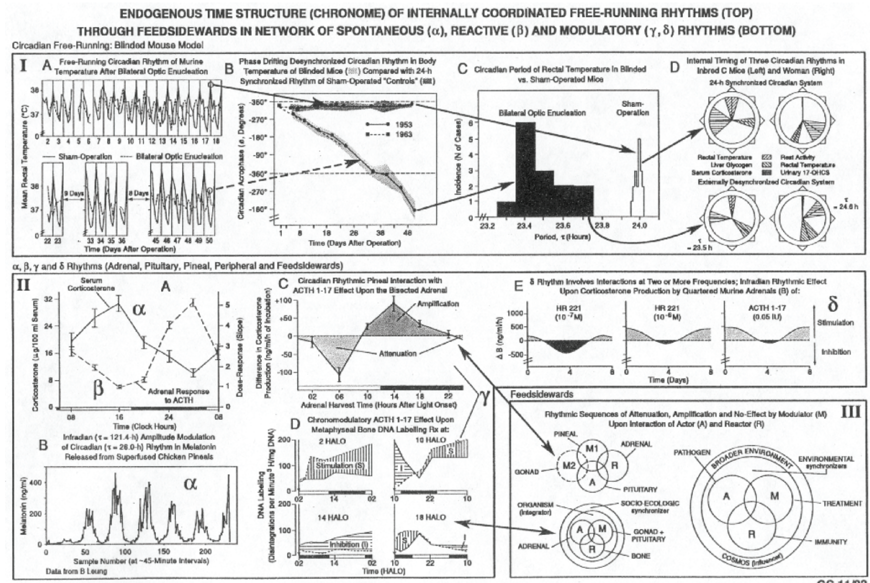


Fig. 3.13 *Top*, section I: Desynchronization of circadian rhythm in core temperature of mice after blinding, seen time-macroscopically in IA (much better in Fig. 3.12), here leads, in IB, to time-microscopy with a chronobiologic serial section showing a different time course of the core temperature acrophases, ϕ, with early separation of the two groups by nonoverlapping 95% confidence intervals of ϕ; in IC, to a summary of individual periodograms that form two separate distributions, and in ID to time relations among three variables in a 24-h synchronized (*top*) or free-running (*bottom*) system (of mice, *left*, and of a human, *right*). Section II shows a spontaneous (α) rhythm in circulating corticosterone of mice in antiphase with the slope of an in vitro response rhythm to ACTH, a reactive (β) rhythm of adrenal corticosterone production. The components of the chronome (time structure) are internally coordinated through feedsidewards in a network of rhythms that are more or less spontaneous (α), others primarily reactive (β) or modulatory at a single mapped frequency, such as a circadian (γ), IIC and IID, or at multiply mapped (δ) frequencies, IIE.

The effect of one entity (the actor) upon a second (the reactor), such as the pituitary acting upon the adrenal cortical corticosterone production may be influenced, predictably insofar as rhythmically, by a third entity such as melatonin (the modulator) at the level of the pituitary; the same melatonin also acts directly upon the adrenal. Reproducible sequences of attenuation, no-effect, and amplification, the time-qualified feedsidewards, replace time-unqualified feedbacks and feedforwards (IIC–E). In sections II and III, feedsidewards include the interaction of a modulator (such as ACTH) upon an actor (such as adrenocortical corticosterone production) acting upon DNA labeling in bone (the reactor). The roles played by endocrines can and do change in various feedsidewards that replace time-unqualified feedbacks and feedforwards. Chronomolecular mapping of circadian acrophases has also begun (Fig. 3.21). (Copyright Halberg)

3.6 Degree of Generality

On earth [12] and in extraterrestrial space [16], circadian rhythms constitute indispensable control information in biological investigation, a consideration of broad scope in experimental and clinical biomedicine [19]. They also constitute the control in integrative and/or molecular studies of circadian timekeeping that are carried out under standardized, if not constant laboratory conditions for a complete and rigorous inference, notably if blunders are to be avoided, Figs. 3.1, 3.12 and 3.13, a point pertinent to scholars of mitochondrial medicine. Within-day changes are reported in the relative mitochondrial volume of pinealocyte perikarya of immature pigs by Lewczuk et al. [29] but only ultradian changes in mitochondrial ultrastructure, only during the respiratory oscillation of *Sacharomyces cerevisiae*, by Lloyd et al. [31].

3.7 Circadian Variation of Ubiquinone or Coenzyme Q10 (CoQ10) in Human Plasma

Reis et al. [39] sampled blood from 16 clinically healthy volunteers during the daytime hours beginning at 09:00 one day and ending at 09:00 of the next day for 13 different determinations, in both April and October, and found a statistically significant circadian rhythm in plasma ubiquinone concentration in one population of Caucasian but not in another small group of African subjects, using a statistical package for fitting multicomponent models [11, 35]. Plasma coQ10 concentrations were also found to be higher in the evening than in the morning (paired $t = 3.566$; $P = 0.001$) in 32 clinically healthy Indian men, 18–61 years of age [7].

3.8 Circadian Systems

Chronobiology developed from the study of about 24-h (circadian) and about-yearly (circannual) rhythmic genetic adaptations to primarily photic environmental cycles, related to electromagnetic radiation, notably in the visible domain, yet it also maps about-weekly, about-monthly and many other cyclic changes in living matter with counterparts in the non-photic environment [4, 12, 28]. Along the 24-h scale in the field and the laboratory, sampling of biological variables usually involves standardized if not, as far as possible, constant conditions, with respect to manageable environmental variables such as illumination, temperature, humidity and sound.

The partly genetic nature of circadian oscillations, apparent by 1950 from differences among inbred strains of mice, Fig. 3.14 [14], was quantified indirectly by free-running after blinding, seen time-macroscopically in Fig. 3.12 and time-microscopically in Fig. 3.13. For human heart rate, heritability was recognized as emergenic, based on studies on twins reared apart, Fig. 3.15 [25, 30, 32–34]. For partly mapped average timing (phases) in circadian systems of populations, Figs. 3.16–3.18,

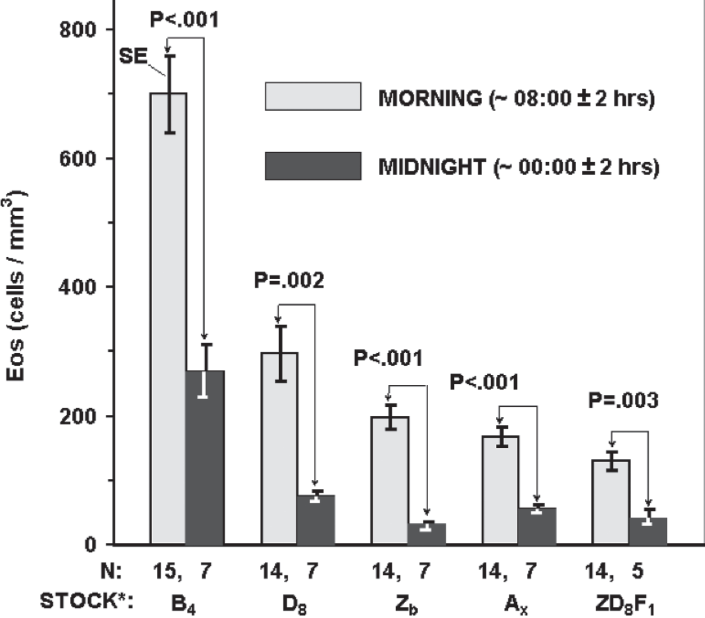


Fig. 3.14 Circadian physiological variation in murine eosinophil counts of four inbred strains and a hybrid (F_1) stock (Adapted from Halberg and Visscher 1950.). Note 1. Large genetic differences, gauged by one-way ANOVA across stocks at 08:00 ($F = 43.1$; $P < 0.001$) and 00:00 ($F = 21.3$; $P < 0.001$) representing differences in genome, and 2. Equally impressive diversity in time, in each stock, gauged by 08:00 vs. 00:00 difference, approximating, by only two timepoints, circadian component of time structure, i.e., chronome ($t = 11.3$; $P < 0.001$ from paired t-test of relative 08:00 vs. 00:00 differences, expressed as percent of mean). The ever-present within-day difference can differ among stocks of mice but more frequent sampling is indispensable for parameter estimation. (Copyright Halberg)

remove-and-replace approaches in humans and rodents lead to adrenocortical, Fig. 3.7, and broader, Fig. 3.19, neuroendocrine, metabolic and other cellular mechanisms, Fig. 3.20, that in turn led to molecular maps, Fig. 3.21. Figure 3.22 visualizes the spectral domains of ultradians and infradians adjacent to circadians. Infradian spectral components, such as circaseptans, Fig. 3.23, or transyears are more prominent early and late in life than in adults and serve to extrapolate to a tree of life [20]. Chronobiology is a figurative microscopy in time, relevant to all bioscience, including but also beyond the disciplines listed on the rim of Fig. 3.24.

3.9 Chronomics

(From Gk *chronos*, time, and Gk *nomos*, rule), a figurative (and partly literal) telescopy in time, Fig. 3.25, investigates aligned time series of organismic and environmental variables, each for separate study in its own right, but also to uncover

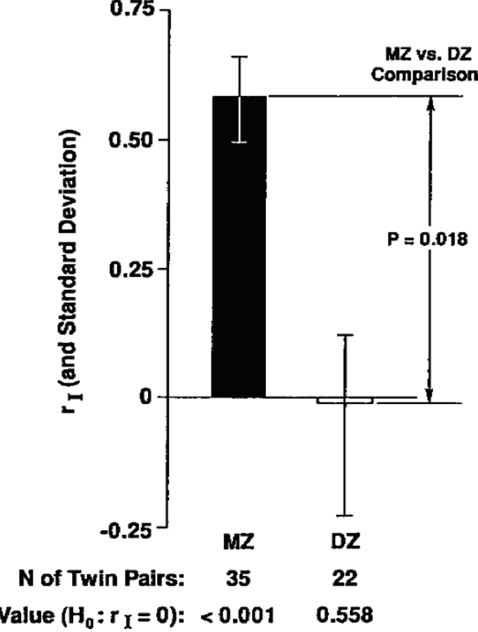


Fig. 3.15 Emergenic heritability of the circadian amplitude of human heart rate, assessed by statistically significant intra-class correlation (r_I) for monozygotic (MZ) but not for dizygotic (DZ) twin pairs reared apart [30, 32–34]. Heart rate was assessed in 24-hour electrocardiograms, amplitude was computed by cosinor; a statistically significant intra-class correlation (r_I) for monozygotic (MZ) but not for dizygotic (DZ) twin pairs reared apart was found. (Copyright Halberg)

(any usually time-varying) interactions among the series. By detecting wobbly non-photic cycles in both the environmental and biological time series, chronomics led to the finding of intra-, inter-, and transdisciplinary congruence among the periods of spectral structures in and around us, congruence defined based on overlying or overlapping in the periods involved, illustrated in Fig. 3.26.

Chronomics developed from the recognition that circadian rhythms are modulated by unseen infradians, apparent in one circadian stage but not in another, Fig. 3.27, and led to the inferential statistical quantification of new biomedical counterparts of known environmental cycles, e.g., of Schwabe's about 10.5-year cycle in relative sunspot numbers and of Hale's sunspot bipolarity cycles, Fig. 3.27, and to the detection of new non-photic cycles in physics as in biomedicine, Fig. 3.25 (*top*), related to non-photics – corpuscular emissions from the sun – and more broadly to helio-geo-atmosphero-magnetics, ultraviolet flux, gravitational and other effects. Chronomics serves biology by using the tools and information from the sciences on the rim of Fig. 3.26 for the study of interactions with the time series from fields including those on the rim of Fig. 3.25; the resulting transdisciplinary information

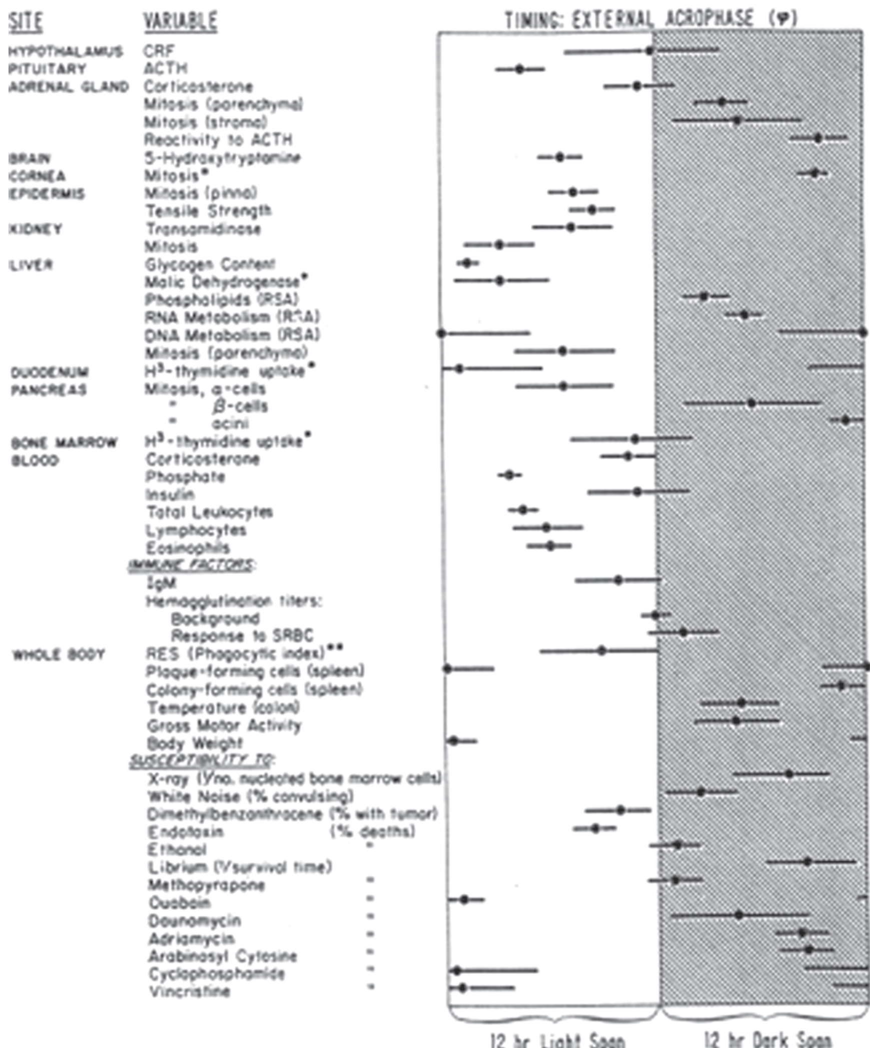


Fig. 3.16 Division of labor in time on a population basis (mouse). Extrapolation to individuals is not warranted. (Copyright Halberg)

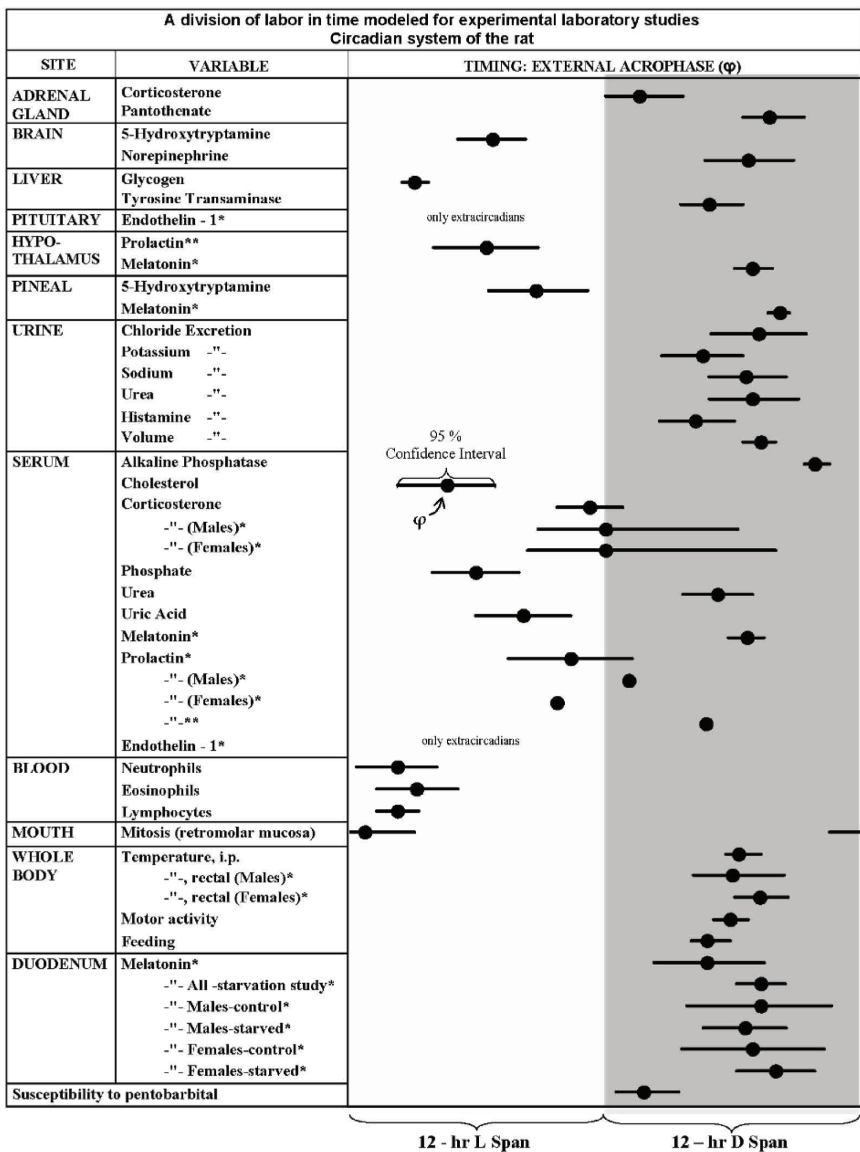
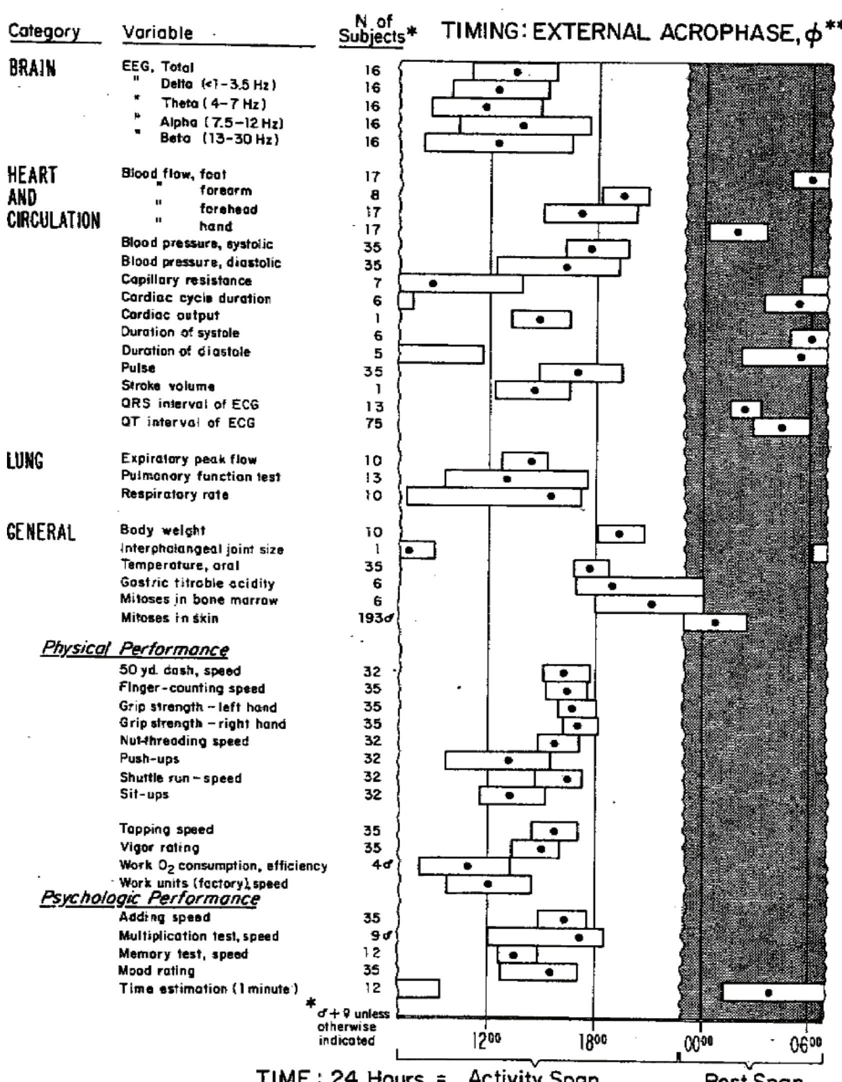


Fig. 3.17 Division of labor in time on a population basis (rat). Extrapolation to individuals is not warranted. (Copyright Halberg)

Human Circadian System : Whole Body and Organs



**Cosinor approximation of high values in circadian rhythm. Dots = acrophases; bars = 95 % confidence limits. Non-overlapping bars indicate statistically significant differences in timing.

Fig. 4. Relative synchronization of several aspects of human physiologic and psychologic performance. Summary of author's and others' published data (Halberg, 1969; Halberg et al., 1977a).

Fig. 3.18 Division of labor in time on a population basis (human). Extrapolation to individuals is not warranted. (Copyright Halberg)

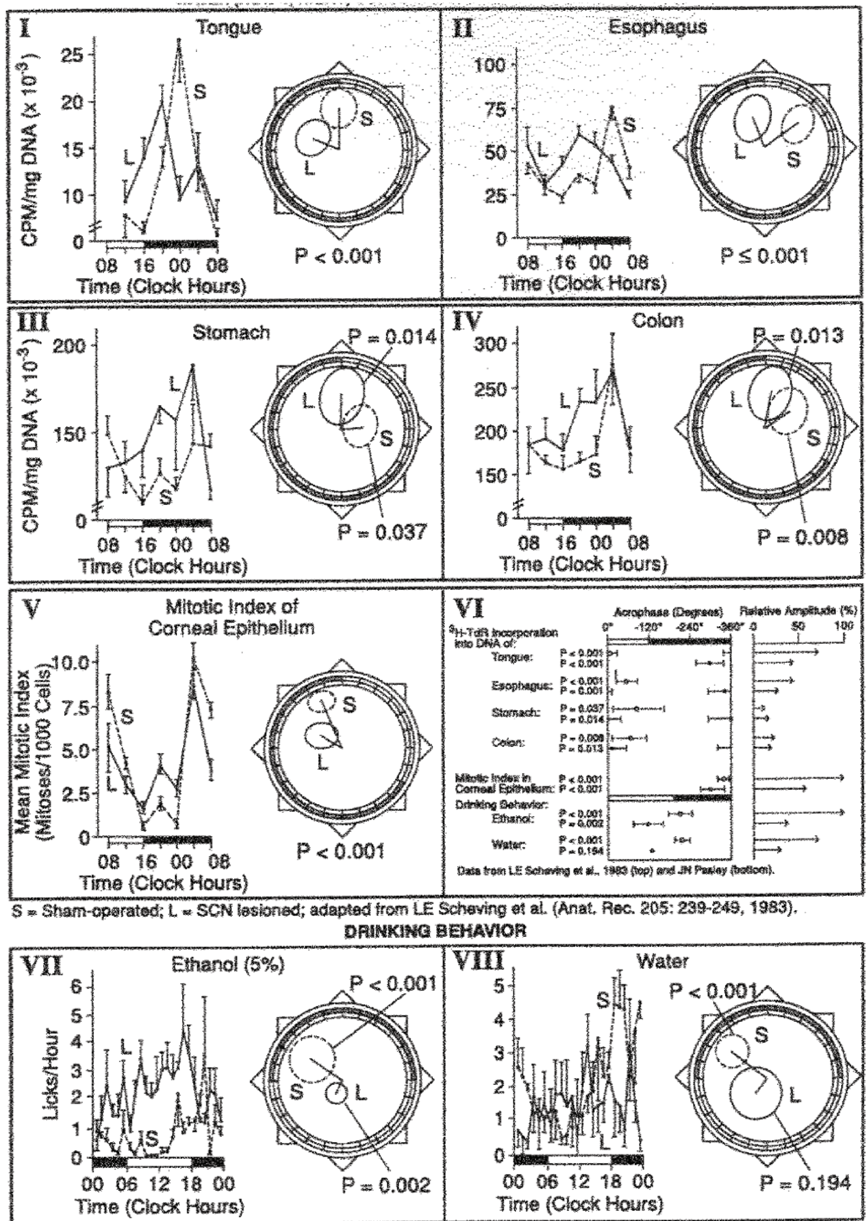


Fig. 3.19 Seven among eight variables examined (counting telemetered circadian rhythm in persistent core temperature, not displayed here) show only changes in circadian amplitude (usually decreased) and phase (usually advanced), with DNA labeling in the stomach showing a numerical increase in circadian amplitude, while the circadian rhythm persists in the presence of histologically validated lesions of the suprachiasmatic nucleus, results pointing to the importance of peripheral mechanisms of circadian systems present in each cell. (Copyright Halberg)

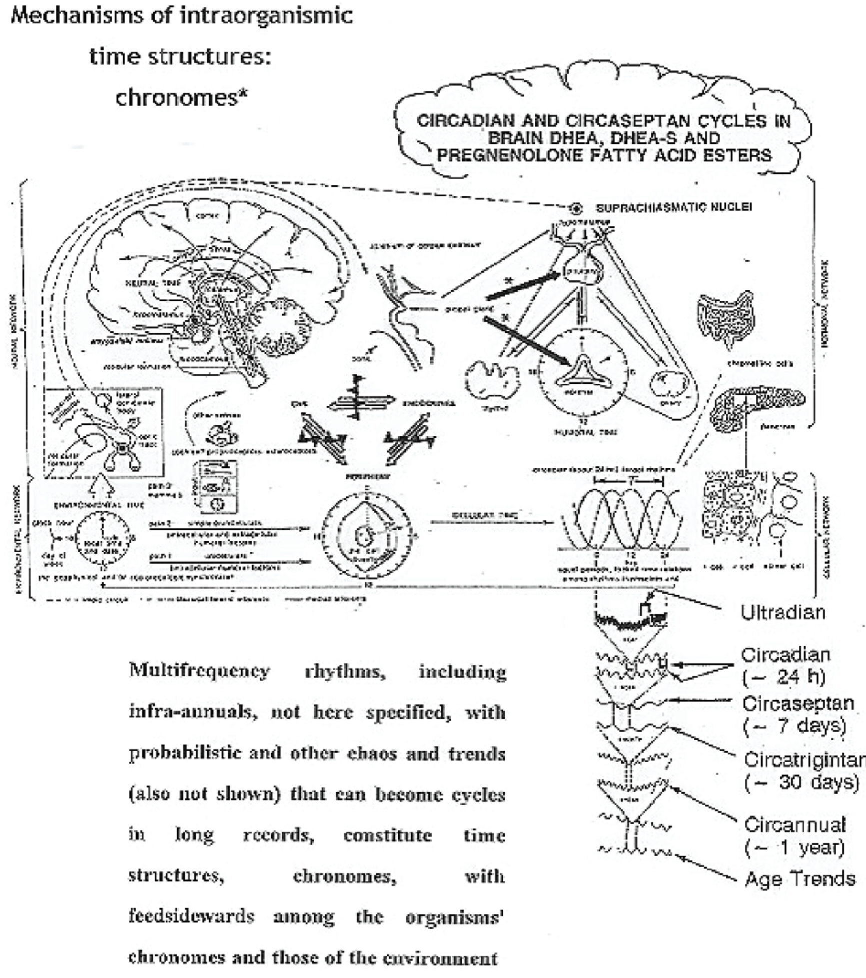


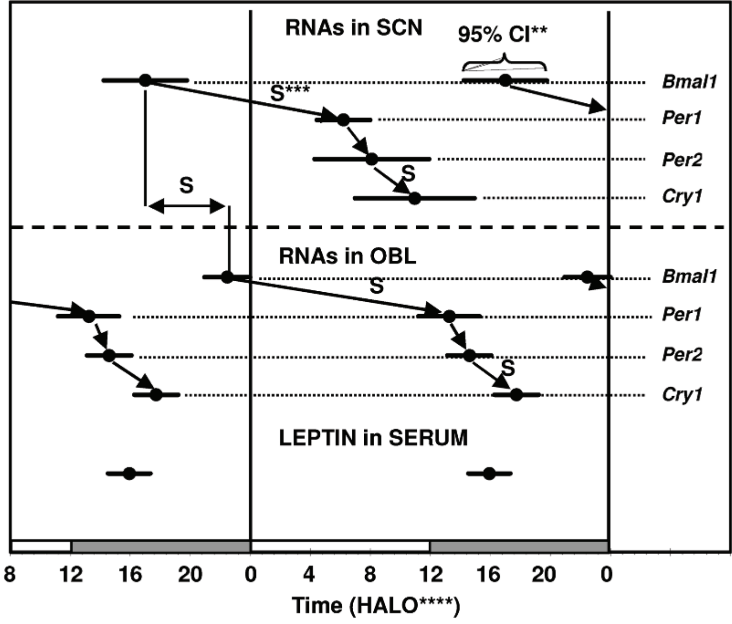
Fig. 3.20 Early scheme of circadian mechanisms with a sketch of a few of many infradian modulations. (Copyright Halberg)

may be useful to physics, e.g., in tracking solar activity by evolution far beyond the earliest recording of auroras.

3.10 Immediate Application

Self-surveillance and self-experimentation of concern to everyone interested in finding out the factors that increase one's risk of stroke from <6% to nearly 100%, before as well as after the presence of high blood pressure and end-organ damage is shown in Fig. 3.27 [6]

TIMING OF CIRCULATING LEPTIN AND CIRCADIAN GENE EXPRESSION IN SUPRACHIASMATIC NUCLEUS (SCN) AND IN OSTEOBLASTS (OBL)* OF RODENTS



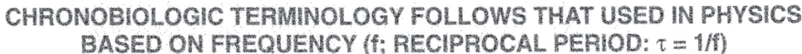
*** Parameter estimations based on relative values expressed as percentage of 24-hour mean from graphs published by S.M.Reppert and D.R.Weaver. Annu. Rev. Physiol. 2001, 63: 647-76 (SCN) and L.Fu et al. Cell 2005, 122: 803-815 (OBL and Leptin).**
**** CI = Confidence interval of acrophase. *** S shows statistically significant phase difference. **** Hours After Light Onset.**

Fig. 3.21 Clock gene expression in suprachiasmatic nuclei leads that in the brain or in osteoblasts. (Copyright Halberg)

and is now possible through a BIOCOS project accumulating an international database of reference values for heart rate and blood pressure (corne001@umn.edu).

3.11 Beneficial Effects of CoQ10 Treatment, Notably on Blood Pressure Variability Assessed with a Chronobiological Study Design

In the experimental laboratory, CoQ_{10} treatment was found to be associated with a statistically significant reduction in toxicity from doxorubicin [27]. In humans, several of us determined, each with an N-of-1 approach, whether coenzyme Q10



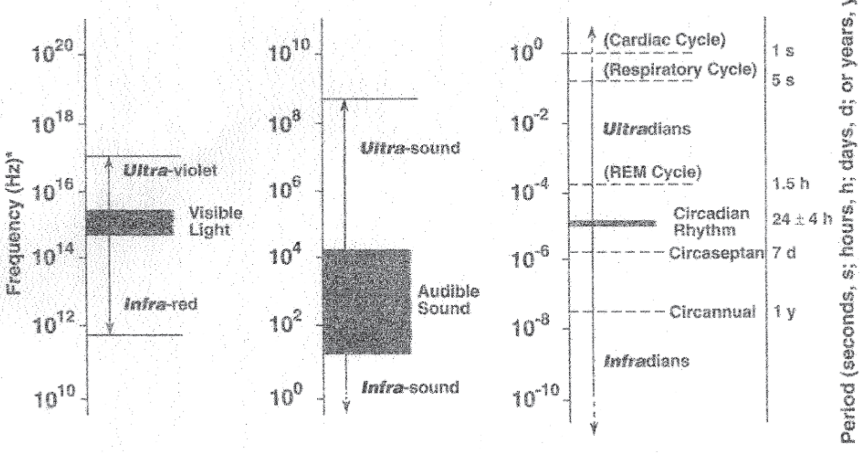


Fig. 3.22 Note that chronobiologic nomenclature is based on frequency, not on their reciprocals, the periods, so that ultradians are periods shorter than 20 h and infradians longer than 28 h

softgels (Q-Gel) supplementation, kindly provided by the Tishcon Corporation (Westbury, NY), affect blood pressure (BP), and if so, what the optimal circadian stage of Q-Gel administration may be. Clinical trials of CoQ10 in the management of high BP reportedly concluded that CoQ10 is associated with a substantial reduction in both systolic (S) and diastolic (D) BP, without the side effects commonly seen with conventional therapy, a proposal by one of us (RBS) [40, 41]. Putative beneficial effects of CoQ10 may not be invariably found on an individualized basis because BP is highly variable and can be influenced by a host of other factors.

It is the more important that a clinically healthy woman (GC, 55 years of age) monitored her BP around the clock at 30-min intervals with only few interruptions for several months prior to the start of Q-Gel administration, using the TM-2430 monitor from A & D (Tokyo, Japan). Starting March 13, 2005, GC took daily doses of Q-Gel (100 mg) during 6 weeks. During week 1, Q-Gel was taken upon awakening, during weeks 2–5, it was taken 3.5, 7, 10.5, and 14 h after awakening, and during week 6, it was taken 17.5 h after awakening or bedtime. The last 6 weeks prior to the start of treatment were used as reference. Data during each of these 12 weeks were analyzed by cosinor to obtain estimates of the MESOR and of the circadian double amplitude (measure of the predictable extent of daily change) and acrophase (measure of the timing of overall high values recurring in each cycle). Student t-tests were used to compare the MESOR and circadian amplitudes of SBP and DBP between the 6 weeks of Q-Gel supplementation and the preceding 6 weeks without treatment. The SBP and DBP MESORs and circadian amplitudes during Q-Gel supplementation were also assigned to the circadian stage of treatment administration

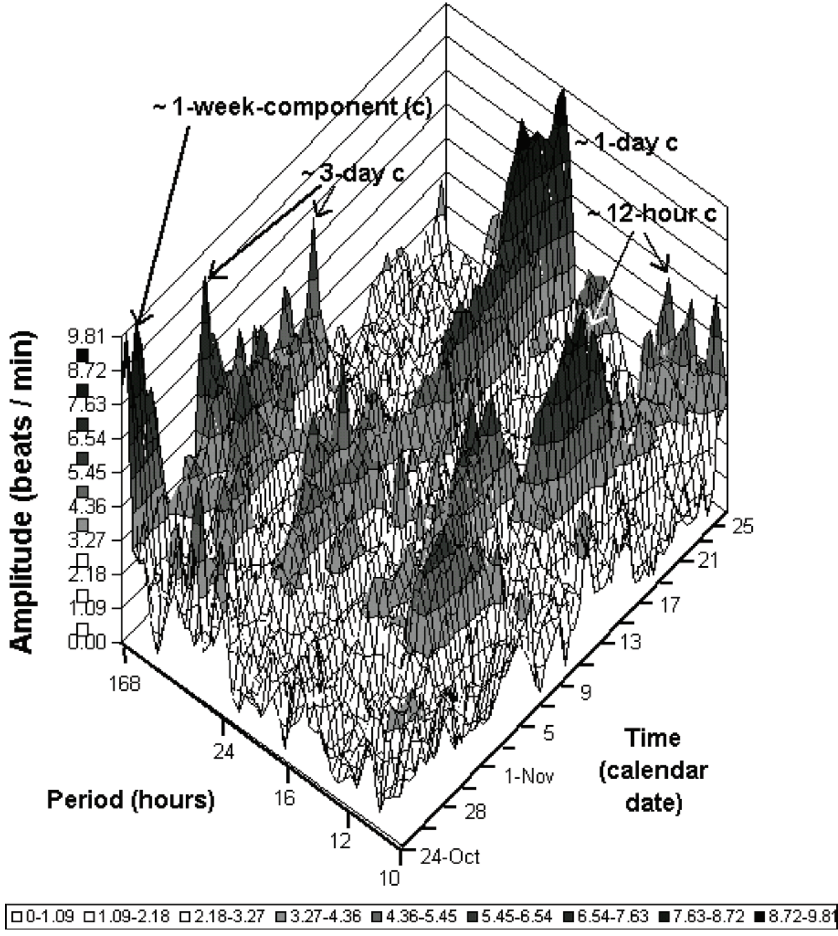


Fig. 3.23 Infradian-to-circasemidian gliding spectral window summarizing half-hourly heart rates of a boy at term monitored during the first 40 days of life: side view of amplitudes. (Copyright Halberg)

to assess any circadian-stage dependence of treatment, using the single cosinor method.

A circadian rhythm in SBP and DBP was invariably demonstrated during each of the 12 weeks of study ($P < 0.001$). As compared to the reference span, Q-Gel was associated with a reduction of the circadian double amplitude of both SBP (from 40.5 to 30.9 mmHg, Student $t = 5.005$, $P < 0.001$) and DBP (from 24.8 to 18.5 mmHg, Student $t = 4.725$, $P < 0.001$) [8]. This effect was circadian stage dependent (SBP: $P = 0.043$; DBP: $P = 0.012$), the largest reduction in circadian amplitude being associated with CoQ_{10} supplementation in the evening (around 14 h after awakening) [8].

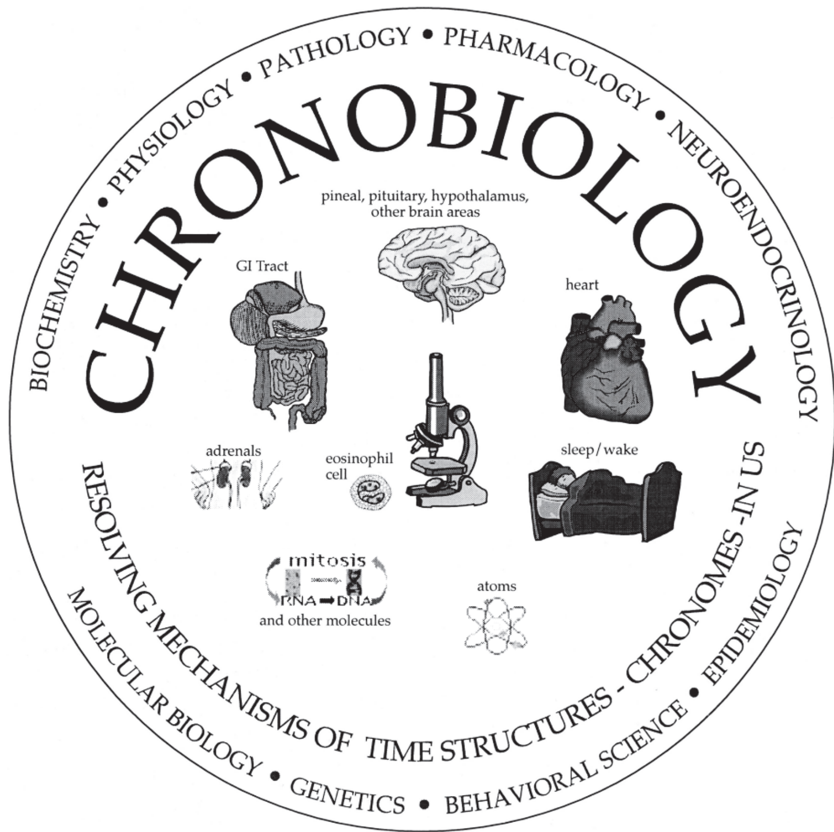


Fig. 3.24 The senior author's endeavors that led him to chronobiology [19], documented by the bibliography on his Web site (*http://www.msi.umn.edu/~halberg/*), constitute a figurative microscopy in time. They started with counts of circulating blood eosinophil cells made with the use of a real microscope initiated in 1948, in developing a bioassay for corticosteroids at Harvard University; by 1950, genetic differences in the extent of within-day changes in count were found, Fig. 3.14, as was a rhythm in abnormal discharges detected by electroencephalography in patients with convulsive disorders [15, 19] and subsequently in rodents. These led eventually to maps of cycles in the metabolism of the cell, the adrenal (Fig. 3.7)–hypothalamic–pituitary–pineal network, Figs. 3.19 and 3.20, and to organismic cycles, including the hours of changing resistance to stimuli such as noise, radiation and drugs, and from there to a budding chrono-physiology, -pathology, -pharmacology and toxicology [12]. These studies were all carried out in an environment rendered as standardized as possible, yet originally only with respect to the availability of food, lighting, temperature, humidity and other routines shielding as far as possible from stimuli in the proximal habitat niche. (Copyright Halberg)

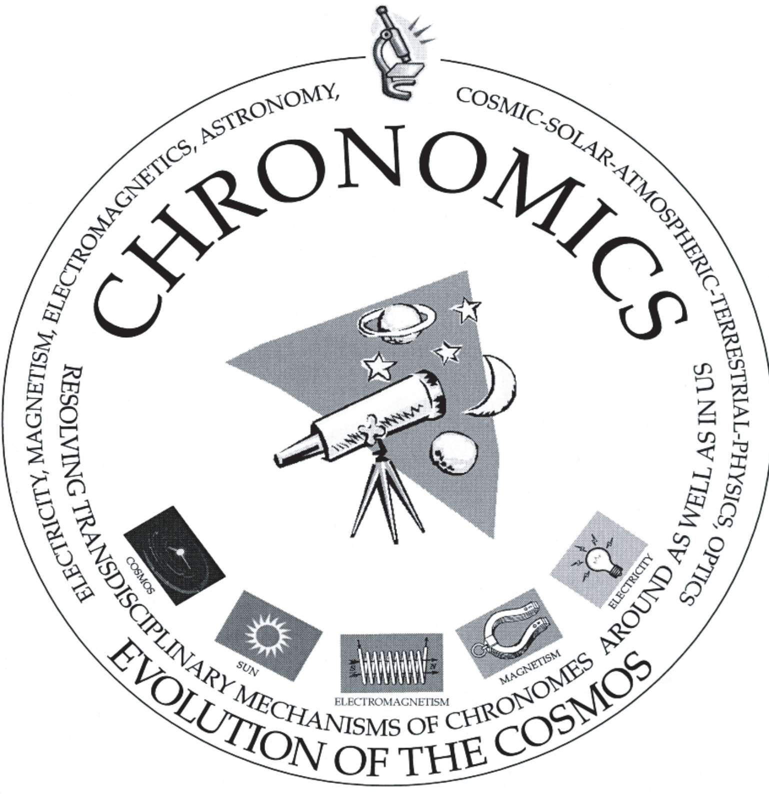


Fig. 3.25 Chronomics, while it discovered near-transyears literally in telescope-monitored solar magnetism is also a figurative, transdisciplinary telescopy of broader scope, examining variables as they change in and around us, with focus on the dynamics of associations, with methods illustrated by the transition from the time-macroscopic displays to the macro-microscopy enabled by modern computers and satellites, Fig. 3.9. (Copyright Halberg)

If and only if the observation in GC can be extended to more subjects, CoQ_{10} supplementation may serve as a nutriceutical intervention to treat CHAT. Notably in the absence of MESOR-hypertension, an active nutriceutical may be preferred to anti-hypertensive medication. Clinical trials should be setup to see whether the results obtained herein are more generally applicable. A chronobiologic design such as the one used herein is advocated so that any individual differences in response can be assessed. For so doing, the chronomically-assessed longitudinal monitoring of BP is critical. Automatic monitors can be obtained with an 80% reduction in price, with analyses, by participating in a project on the BIOsphere and the COSmos (BIOCOS) (by writing to corne001@umn.edu) in exchange for the data. Analyses are usually available free of charge from corne001@umn.edu, and

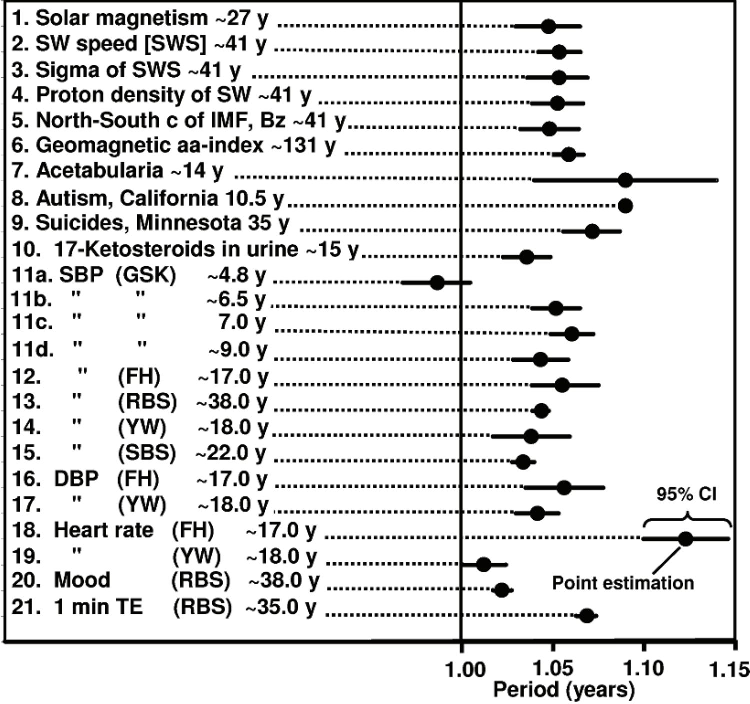


Fig. 3.26 Transdisciplinary congruence among near-transyears in the environment, in a eukaryotic unicell and in human populations and individuals. (Copyright Halberg)

the Phoenix Project (www.phoenix.tc-ieee.org), a group of volunteering members of the Institute of Electrical and Electronics Engineers, which plans to place the software free on the Internet and is working on an inexpensive, cuffless blood pressure monitor, an urgent goal and a challenge for industry.

The purpose of the planned Sphygmochron.org Web site (in the mind of the two senior authors), in keeping with the service provided by BIOCOS (corne001@umn.edu) today, is to constitute a generally accessible Web address that:

1. Would make available concomitantly (Sphygmochron) software for analyzing the time structures of serial blood pressures and heart rates with other related information useful to self-help or otherwise to each person participating directly or via a care provider and

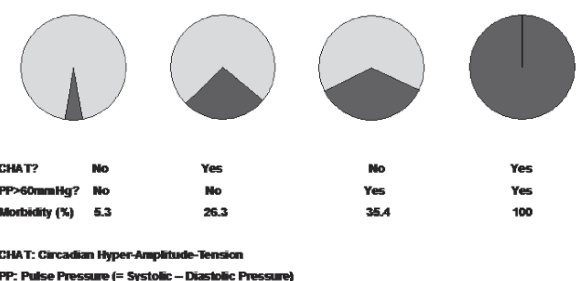


Fig. 3.27 Decreased heart rate variability (DHRV), circadian hyper-amplitude-tension (CHAT) and elevated pulse pressure (EPP) are separate cardiovascular disease risks (cf. Fig. 3.10). CHAT is one of several conditions related to the variability in blood pressure (BP) and/or heart rate (HR) that is associated with an increase in vascular disease risk. The circadian (or preferably circaseptan profile) with too large a pulse pressure (the difference between systolic [S] BP and diastolic [D] BP, i.e., between the heart's contraction or relaxation, or the extent of change in pressure during a cardiac cycle) and a decreased HR variability (gauged by the standard deviation of HR) in relation to a threshold, preferably eventually all in gender- and age-matched peers are two other risk conditions (as is an abnormal circadian timing of BP but not of HR, not shown). Vascular disease risk is elevated in the presence of any one of these risk factors, and is elevated further when more than a single risk factor is present, suggesting that these abnormalities in variability of BP and HR are mostly independent and additive. Abnormalities in the variability of blood pressure and heart rate, impossible to find in a conventional office visit (the latter aiming at the fiction of a "true" blood pressure), can raise cardiovascular disease risk (gauged by the occurrence of a morbid event like a stroke in the next 6 years) from 6% (or even 4% when accounting for EPP, CHAT and DHRV; not shown) to 100%. By comparison to subjects with acceptable blood pressure and heart rate variability, the relative cardiovascular disease risk associated with DHRV, EPP and/or CHAT is greatly and statistically significantly increased. Some of these risks, silent to the person involved and to the care provider, notably the risk of CHAT, can usually be reversed by chronobiologic self-help, also with a non-pharmacologic approach in the absence of MESOR-hypertension. (Copyright Halberg.)

2. Would contribute to a set of advanced basic transdisciplinary and biomedical research databases.

The transdisciplinary base drawing also from government-supported archives of natality, morbidity and mortality, among others such as records of criminality, would aim at a clarification of associations with galactic, solar and terrestrial cycles in human affairs, complementing the ongoing, also government-supported physical environmental monitoring on earth and in space. The medical database would serve to gradually improve the service rendered by the Web site to self-care recipients by a refinement of both the reference standards and the endpoints sought as harbingers in their light.

More specifically, chronomics has been mapping time structure in aligned biological and a variety of physical and social environmental time series, such as

Wolf's relative sunspot numbers, solar wind speed, sigma of speed, proton density and planetary and local geomagnetic and atmospheric indexes, as well as in records of crime and international battles at one extreme and religious motivation at the other. For this purpose, chronomics already uses software to quantify temporal variability in and around humans or other living matter, thus to assess characteristics of any cycles resolved in given time series and their associations. This "microscopy" and "telescopy" in time serves for both a. basic purposes of studying unseen effects of space weather, of corpuscular radiation from space and of other non-photic effects such as heliogeomagnetics broadly, ultraviolet flux, gravitational effects and others; and for b. services available for everyday self-help in health care. A Web site open to researchers as well as any computer-savvy member of the public is the next step. A chronomic analysis of serial measurements can indicate the need for other diagnostics of the human circulation, notably for the self-detection of early vascular variability disorders. It is important for a reliable diagnosis of conditions associated with an elevated risk of cardiovascular disease, just as a biopsy is needed for the diagnosis of cancer.

The Sphygmochron.org Web site is to be implemented by a collaboration between the Halberg Chronobiology Center (University of Minnesota) and the Phoenix Project (Institute of Electrical and Electronics Engineers). These groups are motivated to bring results of research into time-structural analysis of blood pressure and heart rate measurements to the general public in the form of low-cost or no-cost health care products, and to further the underlying research that makes such products possible. Whether or not these plans are realized, the foregoing approach is recommended to governments, foundations and care providers, as a step toward improved preventive (and curative) health care at lesser cost by self-help in dealing, among others, with the current problem of metabolic and premetabolic syndromes.

3.12 Scope of Chronomics Beyond Mitochondrial Medicine

A broad scope of chronomics is facilitated by the development of several novel technologies for monitoring organisms and their environments. These are:

- The availability of portable, personal, long-term ambulatory monitors of biologic variables. Blood pressure, the ECG and EEG, gastric acidity, core temperature and motor activity are cases in point. These and other variables undergo changes that recur spontaneously and as responses.
- The availability of database systems to acquire and analyze volumes of data obtained from personal monitors, Table 3.1.
- The availability of statistical procedures to analyze and model the biologic dynamics and from them to devise optimal dosage time patterns for specific individuals.
- The availability of portable, programmed devices to administer therapy, e.g., by physiologic rate-adjusted cardiac pacemakers, defibrillators or drug pumps.

- A chronomic understanding of the health effect of both photic and non-photic rhythms within everyday physiology. The employment of chronobiologic methods in novel routine medical screening, diagnosis, prognosis, treatment and disease prevention remains a challenge to be met, visualized by opinion leaders in medicine since 1880 [1, 26, 42], leading to a marker rhythm-guided individualized timely diagnosis and timely and timed treatment and the validation of therapy in chronotheranostics [6], Fig. 3.10, in keeping with guidelines from several consensus meetings [3, 13, 21] that can now be partly implemented, Table 3.1, in the detection and treatment of variability disorders in the human circulation as a start. Focus upon day–night differences was helpful, Figs. 3.2 and 3.3, half a century ago, as is still practice with day–night ratios in dealing with blood pressure. But we can do better [9], Table 3.1.

Acknowlegment The support of Raj K. Chopra (Tishcon Corp., Westbury, NY) is gratefully acknowledged.

Table 3.1 Outcomes of chronobiological screens of blood pressure and heart rate*

No of patients (ref)	No at follow-up	Sampling	No measurements: Total (outcomes)	Finding
10	10 (up to 5 years)	5/day daily	Up to 9,125 (only partially analyzed)	Among P. Scarpelli's patients, the 4 who died with malignant hypertension had a larger circadian BP amplitude than the 6 who were still alive (SBP: t = 1.84; P = 0.103; DBP: t = 2.99; P = 0.017)
63 (1)	21 after 28 years	5/day for 2 days	756 (252)	9 of 10 subjects without CHAT are alive while 7 of 11 subjects with CHAT are dead 28 years later (chi-square = 6.390; $P < 0.01$)
56	Concomitant LVMI	q15 min for 24 h	5,376 (5,376)	Classification by Y. Kumagai of patients by LVMI (<100; 100–130; $>130\,g/m^2$) reveals elevation of circadian amplitude at LVMI in 100–130 range whereas MESOR elevation occurs only at LVMI >130
221	221 (time of delivery)	q1 h/48 h in each trimester of pregnancy (336 profiles)	16,128 (16,128)	In addition to an 8 mm Hg difference in mean value between women who will or will not develop complications (gestational hypertension, preeclampsia) already observed during the first trimester of pregnancy, the occurrence of complications is also associated with BP profiles characterized by an elevated circadian BP amplitude. In particular, one case (JK) of CHAT where warning was not heeded, was followed 8 weeks later by severe pre-eclampsia, premature delivery and 26 months of hospitalization of offspring at a cost of about US$1 million
297 (2, 3)	297 after 6 years	q15 min for 48 h	57,024 (57,024)	CHAT or a reduced circadian standard deviation of heart rate, or an excessive pulse pressure (>60 mm Hg) are large risk factors (larger than hypertension) for cerebral ischemic events, nephropathy and coronary artery disease, even when the blood pressure is within acceptable limits
2039 (3)	Concomitant LVMI	Hourly averages for 24 h	48,936 (48,936)	In C.H. Chen's subjects, LVMI is increased in patients with CHAT, a reduced circadian standard deviation of heart rate, or an elevated pulse pressure. The relation between LVMI and the circadian endpoints is nonlinear

(continued)

Table 3.1 (continued)

No of patients (ref)	No at follow-up	Sampling	No measurements: Total (outcomes)	Finding
23 (1)	12 after 7 years	q15 min for 9 days	19,872 (10,368)	10 of 20 patients with no consistent BP abnormality are alive and well; 2 of 3 patients with consistent BP abnormality reported an adverse vascular event (P = 0.015 by Fisher's Exact Test)
80 (4)	Response to treatment administered 2 h before daily BP peak vs. control group treated 3 times a day	q4 h for 24 h before and on treatment	960 (960)	With smaller doses of medications, BP was lowered by R. Zaslavskaya to a larger extent and treatment was accompanied by fewer complications Treatment: propranolol, clonidine, or alpha-methyldopa ($P < 0.05$ for each effect)
18 (5)	18 (12 weeks)	q30 min (≥24 h) on 3 regimens	≥2592 (≥2592)	Treating CHAT may prevent adverse vascular events: As compared to placebo, nifedipine (1 mg b.i.d. at 08 and 20) increases and benidipine (4 mg/day at 08) decreases the circadian amplitude of blood pressure. The resulting increase vs. decrease in the incidence of CHAT on nifedipine vs. benidipine may account for the corresponding difference between the number of stroke events of 7.6 vs. 3.5 and the total number of cardiovascular events of 20.4 vs. 8.8 per 1,000 person-years
Totals:				
2,586	2,533		144,641 (>125,508)	

*SBP and DBP: Systolic and Diastolic Blood Pressure; HR: heart rate; CHAT: Circadian Hyper-Amplitude-Tension, a condition defined by a circadian amplitude exceeding the upper 95% prediction limit of acceptability (in healthy peers matched by gender and age); LVMI: left ventricular mass index. By comparison with several classical studies, the number of measurements in chronobiological work completed thus far is likely to be larger, and confounding by intersubject variability smaller.

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Chapter 4
Methods of Chronobiometric Analysis of Mitochondrial Function

Miroslav Mikulecký

Abstract The methodical hints are given on the basis of the inferential Halberg's cosinor regression for obtaining the optimal information from experimental data organized in the frame of the Halberg's circa(semi)dian design. *First*, the population of experimental animals has to be defined exactly to secure its homogeneity. *Second*, a random choice of separate animals must be kept. *Third*, optimal sampling times have to be settled and realized. *Fourth*, it is recommended to transform the measured data into the Mesor RelatedValues (MRV), to be able to compare mutually the results from various variables. *Fifth*, the best way of expressing the results for consecutive medical considerations and decisions is a graph of the approximating function, including confidence and tolerance corridors. Critically is mentioned the common practice to use standard errors or deviations (representing only 50–68% confidence or tolerance) and p-value, falsely overestimating the impression of an effect. *Sixth*, the same principles should be applied to differences between measurements – a relatively new idea. The evaluation of global effect can be misleading. These modes of presentation are illustrated on coenzymes Q_9 and Q_{10}, as well as on the oxidative phosphorylation cascade for Complex I using data from control and diabetic rats.

Keywords Coenzyme Q, Halberg's circadians, Halberg's cosinor, mitochondria, myocardium, oxidative phosphorylation

4.1 General Design

4.1.1 Definition of the Population of Experimental Animals

Original chronobiometric analysis of Complex I mitochondrial respiratory chain function and coenzyme Q concentrations was used for measured mitochondrial values in rats, male Wistar, 3-month-old. Diabetes was induced by a single injection of streptozotocine (55 mg/kg body weight). It is reasonably supposed that the stock

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of animals available is homogenous [9, 10]. Accordingly, differences between individuals should be only random.

4.1.1.1 Random Sampling

The individual animals for the experiments were selected randomly, as explained at another place of this book. Some known biases, for example that caused by more aggressive behavior of an individual making selection for an experiment more likely, have been avoided with maximal care.

4.1.1.2 Sampling Hours

According to the plan of the experiments, based on the intention to achieve optimal chronobiologic assessment, the following times (hours) for sampling have been planned: 08, 12, 16, 20, 24 and 04 h. All these time intervals had to be covered by duplicate measurements. It turned out, however, that there were many obstacles to fulfil this program. Finally, a substantial reduction of the original intention was necessary due to various disturbing circumstances. Thus, instead of 12 samples in one experiment, only 6–11 were realized.

The real numbers of the measured samples, compared with those planned during the 6 sampling hours, were as follows:

Originally planned sampling: 2, 2, 2, 2, 2, 2
Q_9, Q_{10}: Controls: 1, 1, 1, 1, 1, 1…….Duplicate measurements: 0
Diabetes: 2, 1, 1, 1, 1, 1…… 1 × 2 variables
Difference: 1, 1, 1, 1, 1, 1
Complex I: Controls: 2, 2, 1, 2, 2, 1 …… 4 × 4 variables
Diabetes: 2, 2, 2, 1, 2, 2 …… 5 × 4 variables
Difference: 2, 2, 1, 1, 2, 1
Complex II: Controls: 2, 2, 1, 2, 1, 1 …… 3 × 4 variables
Diabetes: 2, 2, 2, 1, 2, 1 …… 4 × 4 variables
Difference: 2, 2, 1, 1, 1, 1
In duplicate a total of 66 measurements

Abbreviations: Q_9-coenzyme $Q_{9\text{-OX}}$, Q_{10}-coenzyme $Q_{10\text{-OX}}$, Complex I and Complex II – Complexes of heart mitochondrial respiratory chain.

To investigate the possible effect of the defect in sampling, causing differing statistical weights of the measurements performed at various hours, the magnitude of differences between duplicate measurings in 66 couples was evaluated: each couple of two values, obtained for the same variable at the same time, was described as the ratio of the higher value to the lower one, thus yielding always the value over 1 or – in the case of equality of both measurements – equal 1. Of course, only the original measured values, not their overcalculated values or differences were taken into the calculations.

In the *first step*, the normality of the statistical distribution of the whole set of these 66 values were tested according to D'Agostino [1] on the level of statistical significance $\alpha = 0.05$. Normality hypothesis was rejected: the calculated criterion Y was −3.592, while the table values for nonrejecting covered the range between −2.680 and 1.130. Accordingly, nonparametric methods were used for the predictive statistical characterization of the sample [3]: the median is 1.059, with the 95% confidence interval between 1.034 and 1.079. As to the individual values of the ratio, they fluctuate between 1.000 and 1.285. This interval represents the nonparametric one-sided 95.56% tolerance interval with 95% confidence.

It can be therefore concluded that the differences between two contemporary measurements to be expected with 95% confidence for median (3.4 to 7.9%) and for separate measurements (with the extremes of 0 to 28.5%) are not so large that they could influence substantially the results of most circadian and semicircadian model calculations as compared between full double and only partially double sampling.

Another question has to be posed about the randomness of the duplicate measurements: is there a systematic difference between the first and second measurement? The answer can be obtained using the sign (symmetry) test [3]. Of the 66 couples of measurements, three displayed zero difference – both values were equal. Of the remaining 63 couples (=N in the tables on pp 103–107), the first value was higher than the second (denoted as +) in 26, and in the remaining 37 couples the result was opposite – denoted by –. According to the table for the significance $2\alpha = 0.05$ (p 105), the interval of 95% confidence for N = 63 is from 23 to 40. Our values of 26 and 37 are inside this range – there exists therefore no statistically significant systematic tendency for prevalence of positive or negative values. In conclusion, the fluctuation of the two resulting measured values in the couples appears to be random. In other words, no systematic measurement problem has been revealed.

For the final presentation of results, the measured values (MV), expressed for each variable (coenzyme) with the aid of specific biochemical units, were transformed to obtain standardized, mutually comparable outputs. For this purpuse, each measured value was divided by the corresponding calculated mesor value.The latter is the mean value respecting the estimated wave form of the approximation (Mean Estimated Standard Of Rhythm). Accordingly, the unit for expressing the measured value is the mesor. After this transformation, the mesor value will be 1 and all measurements will be given as Mesor Related Values (MRV).

On the other hand, the standardization of differences has to be performed with the aid of dividing each difference by the mesor of corresponding controls or other defined basis of comparison. In other words, the resulting positive or negative Baseline Mesor Related Difference (BMRD) will denote the relative increase or decrease of the baseline value caused by the presence of some factor, e.g. diabetes or treatment. These values, multiplied by 100, will tell the percentage of the increase or decrease due to the influence of the given factor on the baseline, e.g. control values.

The main goal of the present statistical design is to investigate the *circa(semi)dian behavior* of the measured values but also that of their differences by searching for the presence of the 12-h and 24-h rhythm, supposing the zero trend in the data. The practically most important output will be an interval estimate with a given probability: the confidence interval is related to the mean effect and the tolerance one to the effect in an individual. The data stem from [9–11].

4.2 Realization of the Chronobiometric Analysis

The classical Halberg cosinor analysis [2] was used as the basic inferential tool to fulfil the goals described. The computer program was elaborated by Kubáček et al. [12]. In the *first* step of calculations, the measured values (MV) were processed by the cosinor regression to calculate the mesor for each data set, e.g. Q_{10} in controls. In the *second* step, the resulting mesor value and the corresponding measured values of each data set (e.g. Q_{10} in controls) were used to calculate the respective Mesor Related Values (MRV, measured values divided by mesor) for the given set. In the *third* step, the cosinor analysis of MRV for each data set displays the final result for each variable under the given conditions, e.g. Q_{10} in controls or Q_{10} in diabetic rats. These results are the corner stone of the whole work and will be presented for theoretically defined parameters (mesor, amplitudes, acrophases, the latter two for either period length – 12 h and 24 h) in tables and for the real data, measured during a 24-h span, in graphs. The *fourth* step calculates the differences between the measured values (MV), e.g. those in diabetic rats minus those in control rats, if the effect of diabetes versus health has to be studied. The *fifth* step relates these differences to the mesor of the control or other baseline data, to arrive at Baseline Mesor Related Differences (BMRD). Accordingly, the positive values of these differences tell what proportion of the baseline mesor has to be ascribed to increasing influence of the studied factor on the baseline data, while their negative values will speak in favor of its oppossite action. The *sixth*, last step, will subject the obtained BMRD values to cosinor analysis to find out whether the effect of a factor is cycling in a circadian and/or circasemidian fashion. This procedure is based on the premise that all animals stem from one population. If so, then the separate differences can be treated as paired data. Moreover, according to the Lindeberg central limit theorem of the theory of probability, the subtraction of two values can act in favor of the Gaussian distribution of the differences and in this way can improve the conditions for statistical analysis. The effect of an investigated factor will be evaluated versus null in two fashions: globally, using the mesor of differences and its 95% confidence interval, and with respect to separate time intervals using the relation between the corridor of 95% confidence and the mesor straight line. All statistical testing and estimating in these examples is based on the *a priori* chosen significance level of $\alpha = 0.05$.

4.3 The Outcome

4.3.1 *Measured Values Transformed into Mesor Related Values*

4.3.1.1 Analytical Approach: Theoretically Defined Parameters

The control MRV data for Q_{10} measured in myocardial mitochondria were chosen as an example. Instead of the sometimes used standard errors or standard deviations, the 95% confidence intervals were consistently calculated. The significance of Student-t-test values was determined at 1 degree of freedom (six measurements minus five optimized parameters: mesor, two amplitudes, two acrophases). Statistically significant results (with the p-value equal or lower than 0.05) are marked by*. The resulting point and interval estimates for the chronobiometric parameters with corresponding t-values are as follows:

		95% confidence		
	Point estimate	Lower bound	Upper bound	Student t-value
Mesor				
24-h rhythm:	0.9997 = 1.000	0.7427	1.2566	46.8594*
Acrophase (peak time)	01:27	19:13	07:41	3.0495
Amplitude				
12-h rhythm:	0.2226	(−0.1408)	0.5860	7.3780
Acrophase (1st peak time)	03:05	01:35	04:34	24.1736*
Amplitude	0.4643	0.1009	0.8277	15.3894*

The approximation of the data is excellent – the coefficient of determination is 0.9966. This means that 99.66% of the total variance has been explained by the given regression. The adequacy of choosing two period lengths for optimization – 24 and 12 h – not only from the biological but also from the chronobiometrical point of view, can be documented by calculations using separately either period length:

Run only with 24-h rhythm:				
Acrophase (peak time)	01:27	10:46	16:07	1.3016
Amplitude	0.2226	(−0.6324)	1.0776	0.8286
Coefficient of determination:	0.1863			
Run only with 12-h rhythm:				
Acrophase (1st peak)	03:05	01:23	04:47	5.6235*
Amplitude	0.4643	0.0515	0.8771	3.5800*
Coefficient of determination:	0.8103			

Please note that the peaking times of the 24-h rhythm and that of the first peak of 12-h rhythm are situated near to one another, differing only by 98 min.This is manifested by the total approximating function showing two distinct peaks – one (at night) higher and the other (at day) lower, as explained in the next paragraph. Another interesting feature is the narrowing of the confidence intervals for acrophases and amplitudes when both period lengths were optimized.

4.3.1.2 Synthetical Approach: Total Approximating Function

The best way to explain this kind of evaluation is a graphical display of data with the attached prediction of what can be expected at the given probability level for future measurements on another sample withdrawn from the same population. The important, practically useful difference against the parametrical approach is the possibility to predict future measurements directly in the actual situation, not for theoretical parameters. In other words, this evaluation *predicts* what will happen if the observation is repeated. This feature was emphasized by Jules Henry Poincaré. According to him, science *foresees*, and it is this that ensures its usefulness as a rule for acting in *practice*.

This approach is vastly different from the descriptive one, using standard errors or standard deviations and p-values. The latter have been criticized with the proposal to use the replication probability [7]. In contrast with the p-value, the replication probability gives the real probability that the same result will be obtained after repetition of the observation or experiment. It is surprisingly lower in comparison with the impression obtained from the p-value in the eyes of those who do not understand the p-value properly, and – unfortunately – there are many such ignorants [14]. A graphically presented example [13] shows that for the significance level $\alpha = 0.05$ and the p-value, obtained from the data and equal 0.001, the replication probability will be far lower (0.91 or 0.80, the latter when the statistical distribution of sample mean values is taken into account) than the value, sometimes even silently expected by statistical ignorants, i.e. in the given case $1.000 - 0.001 = 0.999$. Another way how to "make" a resulting effect more "sure" is to use standard error or standard deviation. These characteristics are usually understood as "descriptive". They can be interpreted also as inferential ones. In such a case, however, the predicting probability is poor – they delimit only 50–68% confidence (standard error) or tolerance (standard deviation) interval, according to the degrees of freedom between 1 to infinite [5]. The p-value is sometimes erroneously used as the measure of an effect. In fact, it allows only a black-and-white decision, in comparison with the α value [4]. Our way of evaluation is based on estimation rather than on tests [6].

The point and interval estimates of the resulting approximating functions are presented in graphs. C.R. Rao, one of the most famous statisticians of the XXth century, emphasized the importance of graphics, citing the opinion of Ronald Aylmers Fisher, another giant in biometry: "Even graphics is very important ... R.A. Fisher emphasized graphical presentation of data as part of statistical analysis" [8].

Figure 4.1 shows the graph of data from the above-mentioned example and that of prediction based on them. The 95% confidence corridor should include 95% of the total approximating functions (means), while the 95% tolerance corridor has to cover at least 95% of separate measurements. The significant elevation or depression of the mitochondrial Q_{10} level is evaluated directly for separate times as a nonoverlapping of the mesor by 95% confidence corridor in either direction. The excellent fitting of data, mentioned above in connection with the extremely high value of the coefficient of determination, is obvious from the fact that all measurements are exactly covered by the point estimate of the approximating bimodal function.

Figures 4.2a, b illustrate either rhythmic component – that with 24-h and 12-h period length – separately for the time interval extended to 72 h, i.e. 3 consecutive days and nights. Figure 4.2c extends the message of Fig. 4.1. towards the 72-h interval.

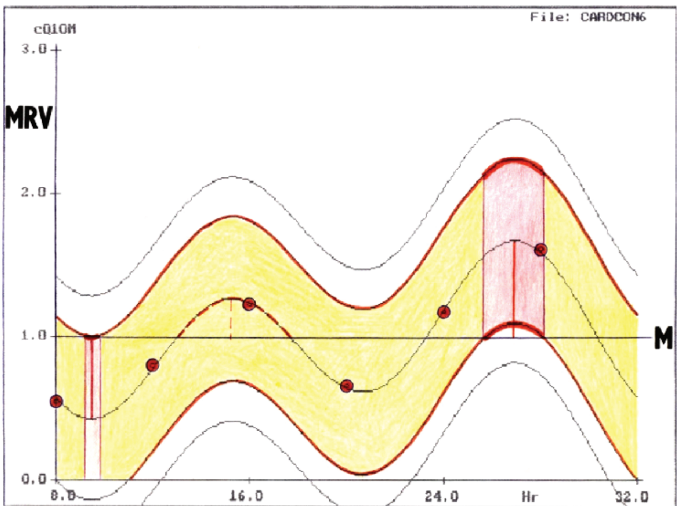

Fig. 4.1 Six measurements (dots), given as the Mesor Related Values (MRV, vertical axis) of the mitochondrial Q_{10} level in control rat myocardium related to the time of day and night. The parts of the 95% confidence corridor (yellow), nonoverlapping the mesor horizontal line (M), are red-shadowed, with the middle times of significant local acme (02:52) or nadir (09:27) marked by the corresponding red straight lines. The broader corridor belongs to the 95% tolerance

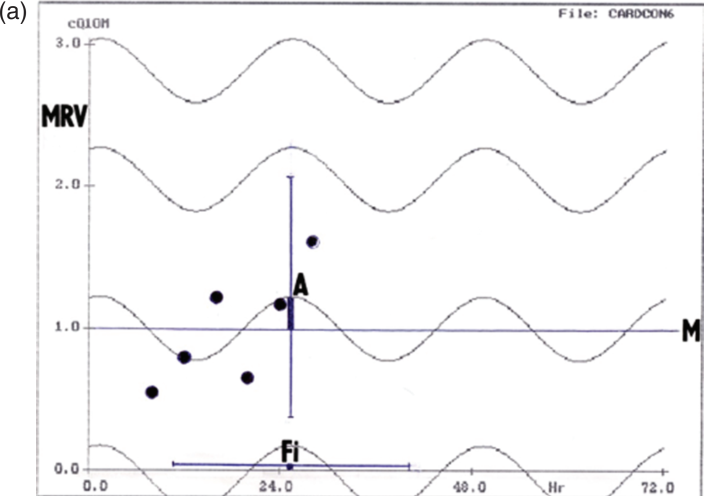

Fig. 4.2 (**a**) The data from Fig. 4.1 optimized using only the 24-h rhythm with the extension of time on the span between 0 (the first midnight) and 72 h (the fourth, last midnight).The point estimate of the amplitude (heavy bar, here the mesor value represents zero amplitude) as well as that of the acrophase time (heavy square) are shown with their 95% confidence intervals at the second peak

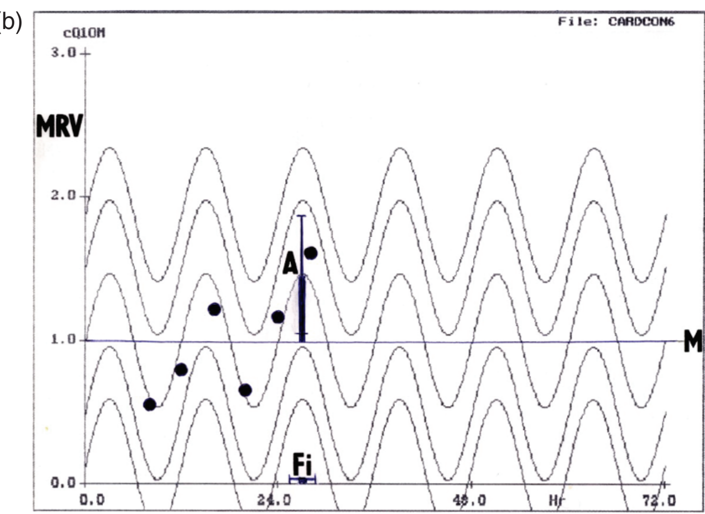

Fig. 4.2 (**b**) Analogy of Fig. 4.2a for the 12-h rhythm used for approximating the data

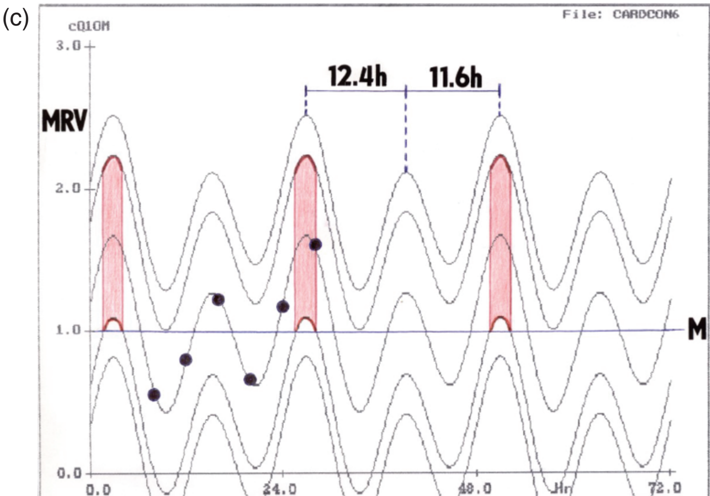

Fig. 4.2 (**c**) Synthetic approximation of the same data,using both the 24- and 12-h rhythm as in Fig. 4.1, yet with extension to the 72-h interval. The time distance from the night peak to that located at day (12.4 h) and that from the day peak to another night peak (11.6 h) are shown

The greatest attention of physiologists is attracted by the sequence of coenzyme peaks appearing at specific day or night times. An example for the Q_9, Q_{10} and Complex I set in controls is shown in Fig. 4.3. The pronounced accumulation of maximal respiratory-energetic activities around midnight is quite clearly visible. It reasonably documents the adequacy of this chronobiometric analysis.

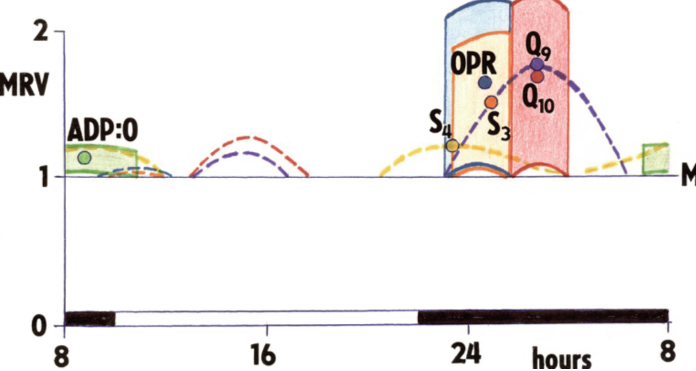

Fig. 4.3 The sequence of peaks for the Mesor Related Values (MRV) of Q_9, Q_{10} and four parameters (ADP:0, S_4, OPR, S_3) of the Complex I in controls. Significant peaking shown as shadowed corridor of the 95% confidence located above the mesor line,with full circles showing the point estimate (mean) of the acme. The point estimates of the approximating functions, located above the mesor, for nonsignificant elevations are shown by dashed lines, with the circles denoting peaks

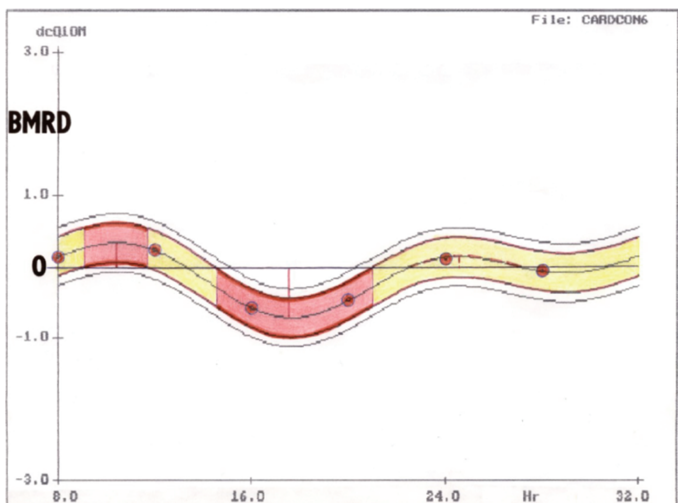

Fig. 4.4 The differences between the Q_{10} levels in diabetic minus control rats, as tranformed to Baseline Mesor Related Differences (BMRD, vertical axis), processed analogically as the Mesor Related Values in Fig. 4.1. The mesor of these differences represents the zero difference (0)

4.3.1.3 Measured Differences Transformed into Baseline Mesor Related Differences

The global difference between the mitochondrial level of Q_{10} in diabetic and control rats is defined by the mesor of the differences (–0.101) with its 95% confidence interval (–0.223 and +0.022). The interval covers the zero value. This speaks in favor of the nonsignificant global decrease under the influence of the presence of diabetes.

Chronobiometrical evaluation of the same data is shown in Fig. 4.4. illustrating the circa(semi)dian fluctuation of the effect of diabetes on the control situation. The presence of the diabetic disturbance appears to be connected with the light but significant elevation of the Q_{10} level in the morning and the more pronounced significant depression in the afternoon and evening.

The comparison of the two conclusions – nonsignificant global effect and significant mutually opposite time limited effects (increase followed by a decrease) demonstrate the usefulness of the chronobiometric approach in evaluating effects and the troubles with a global evaluation, which cannot reflect the partial mutually opposite effects during 24 h.

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Chapter 5
Mitochondrial Medicine

Anna Gvozdjáková

Abstract Mitochondrial medicine represents a complex of clinical, biochemical, pathological and genetic information crucial in diagnosis and treatment. An outline of the development of mitochondrial medicine was for the first time published by Luft in 1994 [22]. Several organizations are focused on mitochondrial medicine, from experimental and clinical research (Mitochondrial Research Society – MRS) to patients application (Mitochondrial Medicine Society – MMS), education and family oriented (United Mitochondrial Diseases Foundation – UMDF), and others. Knowledge concerning mitochondrial DNA (mtDNA) changes in several mitochondrial diseases were published recently [10].

This book presents mitochondrial medicine from the viewpoint of several preclinical studies on chronobiology, aging, Alzheimer's disease, Huntington's disease, diabetes, supplementary therapy with CoQ_{10}, carnitine, alpha-lipoic acid, n-3, n-6 PUFA, and it provides information on clinical application of mitochondrial medicine in cardiology, diabetology, nephrology, immunology, and andrology.

Keywords Aging, mitochondrial disease, mitochondrial medicine

5.1 History of Mitochondrial Medicine

The first mitochondrial disorder was demonstrated by Luft [24] in 1959–1962, and it was termed Luft's disease. The first patient with unusual manifestations, never encountered before, was a 30-year-old woman, suffering since her age of 7 years. The dominant symptoms were profuse perspiration combined with marked fluid intake but with normal urine volume. The daily caloric intake was extremely high (about 3,000 kcal) at a stable body weight of 38 kg and body height of 159 cm. She was asthenic, with progressive weakness. Thyroid function was within the normal range. The main laboratory finding was abnormally increased basal metabolic rate (+180%). Subtotal thyroidectomy with administration of thyroid-depressing drugs was followed by classical myxedema, but the basal metabolic rate was still +100%. From the theoretical point, studies were focused on the patient's skeletal muscle

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mitochondria. In isolated mitochondria of striated muscle uncoupling of oxidative phosphorylation was found. The high level of cytochrome c oxidase, relatively low level of coenzyme Q_{10}, and high content of RNA in muscle homogenate was evidence for mitochondrial protein synthesis. Electron microscopy showed large accumulations of mitochondria, with highly variable size and with paracrystalline inclusions, possibly composed of lipofuscin granules. This was the first demonstration of mitochondrial dysfunction in a human subject [22]. At the beginning of the 1970s, aberrations of the respiratory chain were shown in other mitochondrial disturbances. Spiro et al. [38] identified a respiratory chain deficiency in a disease characterized by dementia, cerebral ataxia and proximal muscular weakness. Defect of the pyruvate-dehydrogenase complex [18] and reduced cytochrome c oxidase in Menken's syndrome were reported [21]. In 1973 a defect of carnitine [11], in carnitine pamitoyl transferase activity, was reported [9]. In 1981 the first complete sequence of mitochondrial DNA (mtDNA) was published [2]. In 1984, evidence was presented supporting the notion that mitochondrial oxidative damage may play an important role in aging [25]. Mutation of mtDNA was first documented in neurodegenerative disorders [42]. More than 120 entities based on alterations in the biochemistry of mitochondria were classified.

5.1.1 Causes of Mitochondrial Diseases

A primary cause of mitochondrial diseases is a defect in nuclear DNA (nDNA) encoding for mitochondrial protein or in mitochondrial DNA (mtDNA). Molecular genetics of human mitochondrial diseases describes mtDNA mutations (mitochondrial protein synthesis, protein-coding genes) and nuclear DNA (nDNA) defects of respiratory chain subunits, mitochondrial motility and importation, mitochondrial transcription or translation. A number of diseases with mutations in the mtDNA have been linked to dysfunction of mitochondrial respiratory chain function and ATP production, of fatty acid oxidation and other metabolic pathways.

Secondary causes of mitochondrial diseases are brought on by factors such as ischemia, reperfusion, cardiovascular diseases, renal failure, pancreatic and hepatic damage, diabetes, infectious agents, gastrointestinal diseases, oncologic diseases, alcohol, smoking, stress, drugs and aging.

5.1.2 Manifestations of Mitochondrial Diseases

(a) *Clinical features*: nervous system: seizures, ataxia, sometimes dementia, generalized seizures, deafness, blindness, short stature. Eyes: ptosis, external ophthalmoplegia, retinis pigmentosa with visual loss. Skeletal muscles: muscle weakness, fatigue, myopathy, exercise intolerance, loss of coordination and balance.

Heart: cardiomyopathy (heart failure, conduction block). Other clinical features involved in mitochondrial diseases are, e.g. gatrointestinal (constipation, acid reflux, vomiting), hepatopathies, liver failure, kidneys – Fanconi's syndrome (loss of essential metabolites in urine), pancreatic diseases – diabetes, sperm defects – mitochondrial "spermatopathy".

(b) *Metabolic features*: mitochondrial disease is associated with low energy production, increased free radical production and lactic acidosis.

5.2 Spectrum of Mitochondrial Diseases

In 2004 several criteria for mitochondrial diseases were published, as Walker criteria, Nijgemen criteria, Nonaka criteria and Wolfson criteria. Mitochondrial diseases were classified by the International Classification of Diseases with clinical modification (ICD-10-CM). This organization assigned codes for 10 groups of mitochondrial disorders – General Mitochondrial Disorders: MELAS, MERRF, Leigh Syndrome, Kearns-Sayre Syndrome, Leber's Hereditary Optic Neuropathy, Mitochondrial Myopathy, and PDH, PCC, PEPCK considered together, and Other Mitochondrial Disorders [28].

On the basis of Scholte's and other reviews, several basic principles in mitochondrial pathology emerged. Some mitochondrial diseases affect only one tissue, most often skeletal muscle, brain, heart, liver, kidneys or endocrine glands. Other organs may be involved secondarily.

The following syndromes are important for the understanding of mitochondrial medicine:

1. KSS – (Kearns-Sayre Syndrome) with ophthalmoplegia, retinal pigmental degeneration, sometimes heart block, ataxia, hyperparathyroidism and short stature. There is a defect in coenzyme Q metabolism in KSS and it becomes manifest before the age of 20 years.
2. MERRF – (myoclonic epilepsy and ragged red fibers syndrome) with intense myoclonus, epilepsy, progressive ataxia, muscle weakness and wasting, deafness, and dementia. Maternal inheritance and reduced activities of Complexes I and IV are established in MERRF.
3. MELAS – (mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes) with episodic vomiting, lactic acidosis, and myopathy with ragged red fibers, sometimes dementia, generalized seizures, deafness, and short stature. Reduced activities in Complexes I and IV are established.
4. CPEO – (chronic progressive external ophthalmoplegia) with signs and symptoms similar to those in KSS but in addition there is retinitis pigmentosa and CNS dysfunction.
5. LHON – (Leber's hereditary optic neuropathy) with optic atrophy and retinal microangiopathy, and in some patients movement disorders, bulbar dysfunction, EEG abnormalities and short stature. Maternal inheritance and reduced activity of Complex I are well established in LHON. The manifestations appear at the age of 12–30 years.

6. Leigh's syndrome with subacute necrotizing encephalomyopathy and early childhood degeneration (with respiratory abnormalities, weak cry, impaired feeding, vision, and hearing, ataxia, weakness, and hypotension). Reduced activities of Complexes I or IV are established in Leigh's syndrome and may be associated in some cases with pyruvate dehydrogenase complex deficiency.
7. Pearson's syndrome, a systemic disorder of oxidative phosphorylation in infants, predominantly affecting the bone marrow and pancreas, with death early in the course of the disease from complications of bone marrow failure and repeated transfusions.
8. Alpers' syndrome or progressive infantile poliodystrophy, with seizures, dementia, spasticity, blindness and liver dysfunction accompanied by specific cerebral degeneration [22, 23].

Mitochondrial diseases can affect many organs, the brain, eyes, myocardium, skeletal muscle, kidney, immune system, pancreas, spermatozoa, etc., Fig. 5.1.

5.3 Mitochodrial Diseases of the Brain

Causes of brain mitochondrial disturbances can be on molecular level (inherited diseases) or on metabolic level, associated with aging.

Down's Syndrome: Down's syndrome (DS) is an inherited disease with onset prior to birth. By the third decade, subjects with DS manifest neuronal pathology resembling Alzheimer's disease, with developing dementia. DS patients have an extra copy of chromosome 21, resulting in an extra gene for cytoplasmic superoxide dismutase (Cu/Zn SOD), with excessive activity of Cu/Zn SOD [12]. In patients with DS, imbalance in activities of individual antioxidant enzymes was documented. Disturbance of antioxidant enzyme balance in patients with DS can be a key to DS pathogenesis [33]. Oxidative imbalance in trisomic cells is age-dependent and depends on the relationship between SOD and (CAT + GPx) rather than on absolute amounts of individual antioxidant enzymes [26]. Mitochondrial dysfunction in DS is likely to begin prior to birth. In cultured cerebral cortex from fetal DS brain, deficiency of a Complex I protein was found [20]. DS-mitochondrial impairment prior to birth increases the risk for its Alzheimer-like degenerative pathology, typically manifested by the age of 30 years. Platelets collected from adult and juvenile DS patients showed reduced activities of mitochondrial enzymes.

5.3.1 Mitochondria in Aging

Aging is a biological complex of progressively decreasing metabolic processes, decreasing energy production and physiological functions of the organism. In aging also ultrastructural mitochondrial changes are involved. Mitochondria become larger and less numerous with vacuolization, crystal rupture and the accumulation

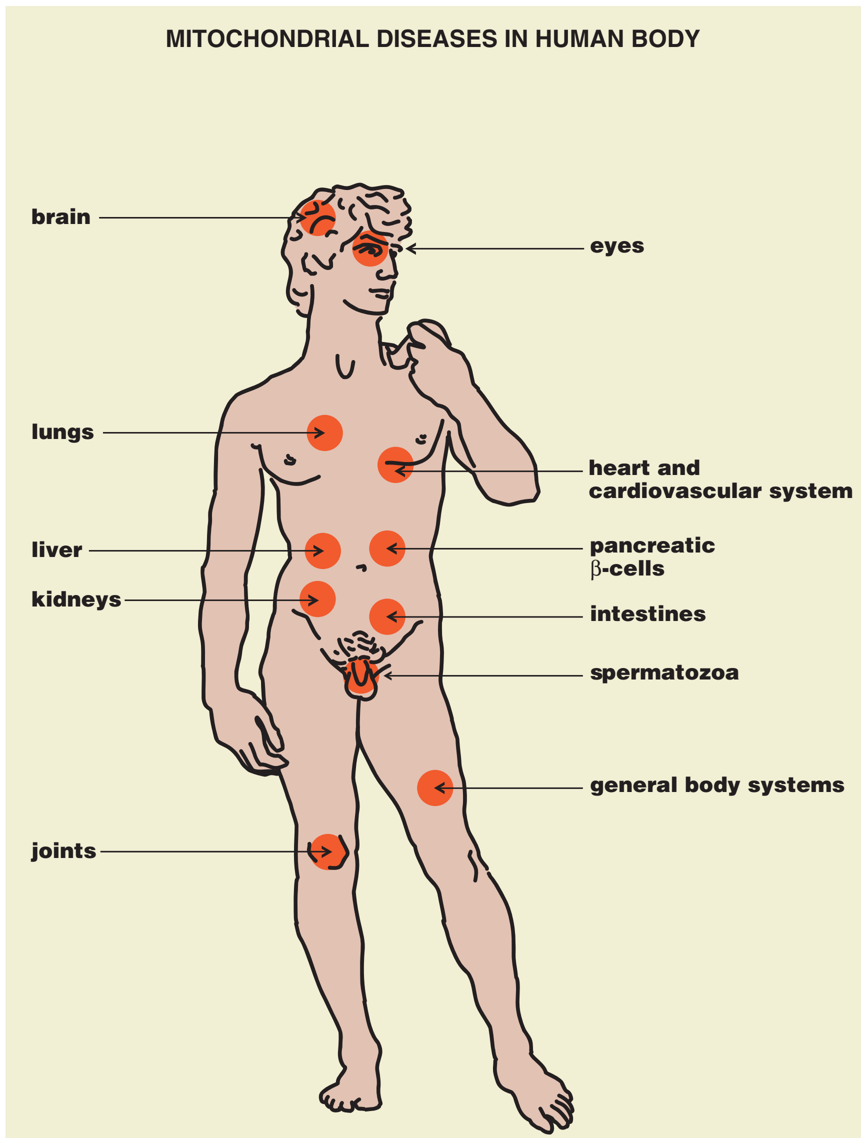

Fig. 5.1 Mitochondrial diseases of human body

of intramitochondrial paracrystalline inclusions. Cytochrome oxidase deficiency in cardiomyocytes of the aged human heart was first demonstrated in 1989 [27]. Histochemical analysis of muscle fibers from elderly subjects has been shown to be associated with very high levels of mutant mtDNA. Free radical accumulation contributes to the progression of changes during aging. Accumulated oxidative damage of mtDNA, proteins and lipids could play a causal role in the aging process. As

biomarker of oxidative damage 8-hydroxy-2 -deoxyguanosine (OH_8dG) is used, which is accumulated with age in mtDNA of the human brain and heart [16]. Age-associated multiple mtDNA deletions in the human heart have been documented [17]. Age-related increase in mtDNA point mutations were observed [43].

Various enzymes participate in the activation and intramitochondrial transport of imported proteins, which takes place in various mitochondrial compartments. The targeting signals of imported proteins have to be cleaved by mitochondrial processing peptidases. Decline or loss of mitochondrial proteolytic enzyme functions probably play an important role in diminished resistance to oxidative stress and in the aging process [4].

5.3.2 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons of the substantia nigra, with reduction of dopamine in the putamen. At 60% depletion of dopaminergic neurons in the substantia nigra and 80% dopamine content in the caudate nucleus, symptoms of PD appear (tremor, abnormal movements, instability, rigidity) [8]. The implication of oxidative stress as a major factor in the pathogenesis of PD is accepted. Abnormally high levels of brain tissue iron of PD patients may serve as an important generator of reactive oxygen species [3].

The etiology and metabolism in Parkinson's disease progression is unknown. In early-onset PD, mutations of various genes were documented, which may damage mitochondrial function and stimulate neurodegeneration. In platelet mitochondria of PD patients, Complex I activity was reduced [15]. Inhibition of Complex I activity may play a pathogenic role in some patients with PD [41].

Mitochondria play a central role in apoptosis – programed cell death. Apoptosis is crucial to multicellular organisms through involvement in elimination of cells during development, removal of cells infected by viruses, and homeostasis in tissues in which production of new cells is balanced by elimination of older cells.

Apoptosis can be activated by both external and internal pathways, which act through activation of cystein-specific proteases (caspases) [32]. The external pathways are activated through ligation of death receptors, such as tumor necrosis factor receptor-1, and the internal pathway works through mitochondria and release of proapoptotic factors, as cytochrome c, which activate the caspase pathways [39]. Treatments targeted at mitochondrial function hold promise to slow down the progression of PD [35]. CoQ_{10} exerts a therapeutic effect in patients with PD [36]. Mitochondrial abnormalities contribute to the initiation and progression of PD.

5.3.3 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by an age-dependent loss of memory and impairment of multiple cognitive functions. AD is

inherited only in 2% of cases, genetic mutations are responsible for causing "familial AD", early-onset AD. In *early-onset* AD, genetic mutations are in genes, concerning beta-amyloid precursor protein (βAPP), which accelerates the disease process, or the proteins preselinin 1 (PS1) or presenilin 2 (PS2) are involved. Presenilins interact with β-catenin to form presenilin complexes. The role of presenilins leading to AD development is not quite clear [13]. Mutations in the human presenilin gene probably play a role in oxidative stress. Increased expression of presenilins increases DNA fragmentation and produces apoptotic changes, which are important consequences of oxidative damage. Oxidative stress plays an important role in AD pathogenesis [37]. All other AD cases are "sporadic", *late-onset* AD. In patients with late-onset AD, pathological changes include amyloid beta plaque production and deposits, NTFs, synaptic damage, and neuronal loss [31]. In patients with late-onset AD, in the absence of genetic mutation, age-related cellular changes control the slower AD progression [30]. The etiology of cellular changes in neurons of late-onset AD is unknown.

AD is associated with the presence of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid beta plaques (Aβ), loss of neuronal subpopulations, mitochondrial oxidative damage, synaptic loss, and proliferation of reactive astrocytes and microglia [34]. Aβ proteins (β-amyloid protein) increase the accumulation of ROS, especially H_2O_2, induce membrane lipid peroxidation [29]. Aβ also interacts with glial cells. Elevation of Ca^{2+} concentration increases the activity of nitric oxide synthase (NOS), resulting in nitric oxide radical (NO) production, which interacts with superoxide radical ($O_2^{\bullet-}$). Elevation of Ca^{2+} concentration and of free radicals contributes to the damage of proteins, lipids and DNA. Aβ proteins may be a trigger in mitochondrial energy dysfunction via increasing ROS production. Impairment of mitochondrial respiratory chain leads to mitochondrial membrane depolarization and mitochondrial permeability transition pore (MPTP) induction. Opening of MPTP could contribute to Ca^{2+} and cytochrome c release from mitochondria. Cytochrome c release supports activation of the caspase cascade and can thus induce cell death (apoptosis). Decline of mitochondrial enzyme activity was reported in AD patients: the pyruvate dehydrogenase complex, the alpha-keto glutarate dehydrogenase complex, and cytochrome c oxidases are affected.

During progression of AD, brain energy metabolism is impaired. Increased production of free radicals induces interruption of brain mitochondrial oxidative phosphorylation and impairs energy homeostasis. Mitochondrial dysfunction in neurodegenerative disorders may contribute to the pathogenesis of Alzheimer's disease [1, 3].

5.3.4 Huntington's Disease

Huntington's disease (HD) is a hereditary neurodegenerative disorder, characterized by degeneration of basal ganglia. Manifestations are chorea, gradual inability to control movements, psychological, cognitive impairment and dementia. The age of

onset is variable but usually in the fourth decade of life, with death within 10–15 years. Strong evidence from studies suggests the involvement of oxidative stress, energy metabolism defects, which can contribute to the altered gene regulation [5, 6]. The mutant gene – huntingtin – was identified [14]. The exact function of huntingtin is not yet known, but mutant protein may interfere with mitochondrial energy production. Mitochondrial dysfunction has been identified as a pathologic factor in HD, impairment of Complexes II and III was found in basal ganglia homogenates from HD patients [14]. An experimental model of Huntington's disease can be developed by 3-nitropropionic acid (3-NP) – specific inhibitor of Complex II. In our study we confirmed that systemic 3-NP administration produced an energy defect in brain mitochondria and increased the activity of creatine kinase system, which could be a compensatory mechanism to decreased ATP production in brain mitochondria of 3-NP treated animals [19]. Pretreatment with coenzyme Q_{10} and vitamin E in the experimental model of HD prevented elevation of creatine kinase activity in cytosol. This positive CoQ_{10} and vitamin E pretreatment effect could contribute to non-mitochondrial activity of CoQ_{10} in trans-plasma membrane electron transport, mediated by cytosolic $NADH/NAD^+$ ratio [19].

5.3.5 Multiple Sclerosis

Multiple sclerosis (MS) is generally considered to be an inflammatory disease with a substantial autoimmune contribution. Impairment of Complex I in chronic plaque zone was associated with oxidative damage to mitochondrial DNA. In 2005, mitochondrial Complex I gene variants were found to be associated with MS.

5.3.6 Amyotrophic Lateral Sclerosis

Mitochondrial dysfunction is a primary feature of amyotrophic lateral sclerosis (ALS). Mitochondrial pathology was observed in an early stage in the degeneration of motor neurons. Muscle mitochondria from ALS patients exhibit impaired mitochondrial electron transport and elevated free radical production. Correlation between mitochondrial pathology in ALS skeletal muscle and abnormal mitochondrial metabolite ratios in the cerebral cortex was shown [40].

5.3.7 Friedrich's Ataxia

Friedrich's ataxia (FA) is an autosomal recessive neurodegenerative disease, characterized by cerebral ataxia and other CNS defects. The most marked pathological changes include loss of large sensory neurons in the dorsal root ganglia and

degeneration of the dorsal columns of the spinal cord. The disease is associated with an expanded trinucleotide repeat in the frataxin gene, mitochondrial protein with iron-sulphur regulation. Mitochondrial oxidative phosphorylation abnormalities, increased free radical generation and iron accumulation in other organs (cardiac and skeletal muscle) are present [7]. Therapy of FA is focused on mitochondrial energy enhancement, antioxidant protection, and iron chelation.

Mitochondrial diseases can affect many other organs, as eyes, myocardium (see Chapter 6, 7), skeletal muscle (see Chapters 10, 11), kidney (see Chapter 9), immune system (see Chapter 12), pancreas (see Chapter 8), spermatozoa (see Chapter 13).

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Chapter 6
Mitochondrial Cardiology

Ivan Pecháň

Abstract The mitochondria belong to the functionally and metabolically most important subcellular organelles, playing a complex multifactorial role in the cell. They are involved in final oxidation of main energy sources with formation of reduced coenzymes, which deliver their electrons to the electron transport chain of the mitochondrial inner membrane. The process of electron transport is realized by some enzymes and other carriers and is associated with the formation of ATP (oxidative phosphorylation pathway). They are also involved in further processes of the cell – they produce the greatest portion of reactive oxygen species, take part in apoptosis and affect many other important cellular functions.

A defect of mitochondrial structure and function is the cause of many diseases or disturbances in the whole human organism. Various functional failures due to individual mitochondrial defects are known. Many are genetically dependent or acquired in connection with different pathological events. Genetically dependent defects are often demonstrated in several organs or tissues. The most afflicted organs are those with very extensive energy metabolism – brain, skeletal muscle and heart. Therefore, encephalopathies and myopathies are very often associated with cardiomyopathies, especially in genetic-dependent mitochondrial defects. Acquired mitochondrial defects concern ischemia-reperfusion injury and other events associated with ischemia or anoxia. In this review some of these mitochondrial defects, especially those associated with cardiomyopathy are discussed.

Keywords Acquired mitochondrial defects, function and metabolism of mitochondria, genetically dependent mitochondrial defects, mitochondrial cardiomyopathies, mitochondrial defects

6.1 Introduction

The heart is a contractile organ which plays the principal role in blood circulation. The main component of this organ are *cardiomyocytes*, contractile cells that constitute approximately 75% of the total volume of the heart [4]. The shape of these

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contractile cells depends on their localization – ventricular cardiomyocytes are greater, long and narrow, atrial are smaller and elliptical. Their number differs with age – in the young organism the heart contains about six billion of these cells, but their number decreases substantially with aging [27].

6.2 Structure of Cardiomyocytes

Cardiomyocytes have cross-striations and are branched, they are surrounded by a membrane called *sarcolemma* and filled with rodlike bundles of contractile elements – myofibrils. Within the myofibrils two principal contractile proteins are located – thick *myosin filaments* and thin *actin filaments, α-tropomyosin, troponin T, I* and *C*, which are direct participants of the contraction–relaxation cycle. Myofibrils contain also a giant protein *titin*. The Z-disc includes the following proteins: *α-actinin, nebulette, myopalladin, filamin* and others. Sarcolemma – unlike the other cells – penetrates into the intracellular space to form tubelike invaginations providing a very close contact with other parts of the cell (*T tubules*) [28].

Cardiomyocytes also contain the common cellular organelles – nucleus, lysosomes, Golgi apparatus. Myocytes have a specific subcellular structure – *the sarcoplasmic reticulum*. This structure is a pendant of endoplasmic reticulum of other cells, but it possesses specific properties and fulfills specific tasks. The sarcoplasmic reticulum consists of a great number of vesicles and tubules to form a longitudinal tubular system. This structure is very rich in Ca^{2+}, which is closely connected with mechanisms of muscular contraction.

Mitochodria have a predominant importance for the function of cardiomyocytes. They represent about 30% of the total volume of cardiomyocytes and supply approximately 90% of cellular energy [7]. These cellular organelles are oval or slightly rounded and represent one of the most important structures of the cell – they are an essential source of ATP as the unique energy donor for cellular function. They have a double-membrane structure, an outer and an inner membrane. The outer membrane is smooth and unfolded, it limits mitochondria against cytoplasmic space and is freely permeable to molecules with molecular weights below 10,000. The inner membrane has many folds directed towards the central part of the mitochondrion – towards the matrix. These membrane invaginations (cristae) substantially increase the surface area of the inner mitochondrial membrane. In the inner layer of this membrane, the electron transport chain and the ATP-synthesizing apparatus are localized. This membrane layer is immediately associated with the mitochondrial matrix, which possesses the enzymes of the citrate and fatty acid oxidation cycles. These metabolic cycles are the main "producers" of reduced equivalents, which are electron donors for the electron transport chain and ATP production in a process known as *oxidative phosphorylation* [16, 17, 27].

6.3 Energy Supply for Heart Function

A typical feature of cardiomyocytes is their high rate of aerobic metabolism. The oxygen demand of the heart is therefore extremely high and it is much higher compared with other functionally important organs, as the liver or kidney. The resting myocardium demands about 10–12 ml oxygen per 100 g of tissue. In contrast to the majority of organs and tissues, under physiological (normoxic) conditions, skeletal muscle and especially the heart obtain their predominant part of energy from fatty acids (60–100%) in comparison with the participation of glucose and lactate (only 0–20%) [19].

Polycarbon-fatty acids are extensively transported by specific transpoters – carnitine – through the mitochondrial membrane as acyl-CoA-derivatives to the mitochondrial matrix and then oxidized via β-oxidation cycle to acetyl-CoA. The produced reduced coenzymes hand their electrons to the electron transport chain of the inner mitochondrial membrane. This transport is closely connected with the ATP-forming system in a process called oxidative phosphorylation, which produces sufficient quantity of ATP molecules necessary for heart function.

6.4 Mitochondrial Pathology of Cardiac Function

Many external and internal factors and events may affect normal metabolism and function of the heart. They can exert their action only temporarily or can induce more pronounced disturbances. The majority of these disturbances are due to the defects of mitochondrial function, associated especially with insufficient energy production. The main causes are usually insufficient oxygen supply due to *ischemia* or other kinds of oxygen delivery to the cells. A typical event of such a situation is the *ischemia-reperfusion* injury of tissue. A result of insufficient oxygen supply is inhibition of the electron transport chain and of the production of ATP. This situation results in the switch of aerobic to anaerobic metabolism. Without oxygen supply fatty acid oxidation is stopped and anaerobic glycolysis becomes the essential ATP producer. The limited production of the so-called "glycolytic ATP" temporarily provides for the function of membrane active transport systems (i.e. Na^+, K^+-transport system). In the reperfusion phase the increased oxygen supply cannot simultaneously renew the previous function of oxidative phosphorylation process because of the defects in enzymes of the electron transport chain and of the ATP-generating system. The low concentrations of *coenzyme* Q_{10} (CoQ_{10}) and the increased production of reactive oxygen species also contribute to the insufficient energy supply of the cell. Owing to the increased permeability of mitochondrial membranes, the formation of proton gradient is limited and cytochrome c release may occur secondarily to the onset of mitochondrial permeability transition pores, which leads to swelling of the matrix space [21, 24]. Therefore the process of full recovery of mitochondrial function is generally not fast and often not completely realized.

6.5 Mitochondrial Cardiomyopathies

Functional and metabolic mitochondrial defects identified in various kinds of diseases and pathophysiological processes have been observed as frequent features in clinical medicine. *Genetically derived* mitochondrial defects represent a substantial part of these pathogenetical expressive diseases, but there are also various clinical events with *acquired mitochondrial defects*. These mitochondrial functional and metabolic defects are manifested predominantly by failure of organs and tissues that have a high energy demand and therefore also a high occurrence of mitochondria. This concerns particularly the central nervous tissue, skeletal muscle and the heart. Therefore deficiencies of mitochondrial function are manifested by typical clinical features – encephalopathies, myopathies and cardiomyopathies.

6.5.1 Genetically Dependent Cardiomyopathies

Mitochondria possess their own genome encoding genes of mitochondrial ribosomal and transfer ribonucleic acids as well as 13 subunits of the electron transport chain [1]. Mitochondrial proteins are predominantly coded by nuclear genes, many of them having essential functional importance. Their mutations concerning mitochondrial (mtDNA) or nuclear DNA (nDNA) evoke some important failures of mitochondrial function seen as *mitochondrial diseases* [11, 38]. Such mutations were identified in more than 30 genes of mtDNA encoding the subunits of electron transport chain and they are responsible for the majority of mitochondrial diseases detected in adult patients. It is interesting that congenital defects in children have been detected only rarely – the majority of them are mutations generated de novo [35]. These mutations are clinically expressed mostly as various myopathies together with associated failure of the heart – cardiomyopathies or with defects of the conducting system of the heart [20]. Dilated and hypertrophic cardiomyopathies are generally due to base substitutions in mtDNA, other nonsense mutations are associated with defects of the conducting system of the heart [39].

Of the genetically dependent mitochondrial diseases, *defects of oxidative phosphorylation* are predominantly seen. Their minimal newborn prevalence is presumed to be about thirteen in 100,000 deliveries. Mutations that evoke defects of oxidative phosphorylation were identified in more than thirty of the total of 37 genes of mtDNA and in more than thirty nuclear genes [34]. The defects of mitochondrial function are related to a number of systems – they can be evoked by defects of enzymes of the electron transport chain directed predominantly to Complexes I–IV, by deficiency of the electron carrier ubiquinone, by oxygen deficiency caused by anemia or ischemia, as well as by increased membrane permeability, resulting in failure of potential of the inner mitochondrial membrane which is necessary for ATP production [13, 14]. These defects of mitochondrial structure and function are often associated with cardiac diseases – hypertrophic and dilated

cardiomyopathy, defects of the conduction system of the heart, ischemic and alcoholic cardiomyopathy, as well as myocarditis.

Deficiencies of protein function of the mitochondrial electron transport chain affect not only to the myocardium, but also other organs and tissues, which are essentially dependent on high energy supply. Such organs – besides the myocardium – are predominantly the brain and skeletal muscle. Thus the most frequent events of this defect – besides cardiomyopathies with heart failure – are mitochondrial encephalopathies and myopathies [8, 18]. In children, cardiopathies are associated with more or less marked encephalopathies or myopathies [15]. Other authors [18] reported that in a group of 301 children and adolescents with encephalopathies and neuromuscular diseases mitochondrial diseases were detected only in one third of them and of these patients only one fifth had simultaneously verified cardiomyopathy. Bhagavan and Chopra [2] reviewed the issue of pediatric cardiomyopathy, which represents a group of rare and heterogenous disorders. About 40% of children with cardiomyopathy receive a heart transplant or die within the first 2 years of life. Feigenbaum et al. [12] are even convinced that analysis of the whole mitochondrial genome is needed for precise diagnosis of any mitochondrial disease, because some less frequent defects could be omitted.

It was also shown that these clinical events can occur not only in connection with defects of the bioenergetic function of mitochondria but the primary defect might be also associated with nonbioenergetic pathways, i.e. with signalization between mitochondria and the cell nucleus or with failure of degrading processes [25].

The predominant damage of oxidative phosphorylation processes in mitochondrial diseases was documented also in experiments on dogs with essential or induced form of cardiomyopathy. In both these cardiomyopathies, defects of heart mitochondrial proteins that participate in processes of oxidative phosphorylation were recorded. These defects concerned predominantly changes of expression of mitochondrial proteins in Complexes I–V [22, 23]. Complex I (NADH-CoQ-reductase) and Complex V (cytochrome-c oxidase) were most often affected. These results are consistent with the observations that the worst prognosis was detected in patients with cytochrome-c oxidase deficiency [18].

Genetic defects concerning processes of *fatty acid oxidation* also fall within the group of typical mitochodrial diseases. Stanley [32] identified nine different kinds of genetic diseases generated by defects in the mitochondrial cycle of fatty acid oxidation. These events include coma, hypoglycemia, various myopathies, as well as cardiomyopathy and liver steatosis. The author concludes that these clinical units are due to inability to use fatty acids as energy fuels and coma could be the consequence of toxic effects of accumulated fatty acids or their metabolites. In a later paper, Stanley and Hale [33] referred already to 16 various defects of mitochondria. One of them – medium-chain acyl-CoA-dehydrogenase – was found to be a relatively frequent defect (1 in 10,000 deliveries) and clinically it featured as sudden heart death of neonates or as Reye's syndrome. Bonnet et al. [3] agreed with these conclusions. They described the same clinical picture in human medicine – with isolated cardiomyopathy, sudden heart death, myopathy or liver disturbances. This defect was found to be not very rare – during one quarter of a century the author detected more than

100 patients with defects of fatty acid oxidation and 24 of them had various kinds of heart arrhythmias.

In connection with defects of fatty acid oxidation, *carnitine* deficiency, another inborn or acquired defect, was observed. This metabolite participates in the transport of fatty acids through mitochondrial membranes and – with participation of three enzyme systems – supplies the mitochondrial matrix as the predominant place of their oxidation with these energetically important metabolites. Studies performed in dogs with diagnosed cardiomyopathy or myocardial dysfunction revealed that these clinical events were due to the deficit of carnitine in cardiomyocytes. Administration of high doses of carnitine augmented its heart concentration and markedly improved heart function. It was concluded that carnitine administration may have a beneficial effect in genetic and acquired failures associated with deficient energy production by recovery of processes of fatty acid oxidation, by more effective elimination of toxic metabolites of acyl-CoA, as well as by renewal of the balance between free CoA and its acyl derivatives. In children two defects were described in which carnitine deficiency blocked fatty acid oxidation due to a recessive defect of the plasmic membrane transporter carnitine. This disease is associated with cardiomyopathy or hypoketotic hypoglycemia as well as with carnitine deficiency due to bonding of carnitine to antibiotics conjugated with pivalate in the form of pivaloylcarnitine. Idiopathic dilated cardiomyopathy induced probably by carnitine deficiency due to malabsorption of this transporter was described.

6.5.2 Acquired Mitochondrial Cardiomyopathies

Mitochondrial cardiomyopathies are not caused only through genetic defects of these organelles, but various kinds of these diseases are acquired.

Chagasic cardiomyopathy belongs to the classic examples of acquired mitochondrial diseases. This defect was particularly analyzed in mice after administration of suspension of Trypanosoma cruzi [37]. Many changes of enzyme activities of the electron transfer chain and of oxidative phosphorylation have been detected in this experimental animal model. It is noteworthy that these changes were not the same through the whole course of infection. During the acute phase of the disease, the most expressive effect was related to the inhibition of Complex I. Complex III was affected during the whole course of infection, while the defect of Complex V was most remarkable in the chronic stage of infection. In the course of this disease, a decreased content of mtDNA and transcripts encoded by mitochondria was detected. Thus the majority of the recorded changes concerned deficiencies of the mitochondrial electron transfer chain. At the same time an essential decrease of mitochondrial ATP production was observed. The expressed inhibition of Complexes I and III, which are the site of reactive oxygen species formation, supports the free radical hypothesis concerning mitochondrial dysfunction in the mitochondrial disease studied.

Increased oxidative stress along with a decrease of antioxidant capacity was demonstrated in 80 patients with Chagasic cardiomyopathy. In this group of patients, markedly increased plasma levels of lipid peroxides were observed. The level of mitochondrial malondialdehyde was increased by 67%, glutathione concentration and the amounts of antioxidative enzymes – glutathione peroxidase and total and Mn-superoxide dismutase – were decreased. These findings support the concept of the essential role of imbalance of oxidative vs. antioxidative processes in the course of mitochondrial dysfunction in this disease [40].

Another example of acquired mitochondrial dysfunction is *Kearns–Sayre syndrome*, which is regarded as a form of mitochondrial diabetes. This syndrome can be observed in adolescents and is characterized by ophthalmoplegia, pigment retinopathy, but is also associated with defects of the heart conduction system, muscular defects and with endocrinopathies, in which diabetes mellitus predominates. Precise molecular-biological analysis revealed marked heteroplasmic deletions of mtDNA [19].

Diabetic cardiomyopathy is a clinical disease, in which – with a high level of probability – the important participation of heart mitochondrial defects is assumed. In a model of diabetic mice, a precise proteonomic analysis of heart proteins was performed and a remarkable number of altered proteins of mitochondrial origin was found [30]. Some changes of mitochondrial structure as well as focal regions with marked injuries were identified. Functionally, decrease of respiration control and decline of glutathione content were detected. The authors suggest an important failure of mitochondrial function and increased oxidative stress in diabetic hearts. This suggestion was supported in a model of transgenic mice with substantially increased expression of Mn-superoxide dismutase localized in mitochondria. Increased expression of this antioxidatively acting enzyme actually improved the respiration of mitochondria and normalized their morphology, and also heart contractility. The suggestion of adverse effects of oxidative stress on the morphology and function of diabetic heart mitochondria was also confirmed in streptozotocine-transgenic mice, which produced metallothionein, a substance with marked antioxidant effect [5]. This animal model was characterized by decreased presence of early heart death due to prevention of diabetic cardiomyopathy. It was suggested that this beneficial reversal could be performed by inhibition of mitochondrial oxidative stress [6].

Among the most frequent acquired mitochondrial defects, failures due to *ischemia-reperfusion injury* can be included. This pathological event is characterized by extensive functional and structural deficiencies of these structures. In spite of the suggestion that the injury of mitochondria is to be regarded as the primary one, there are causal connections among various processes associated with ischemia-reperfusion. Some authors suggest that the mitochondrial permeability transition pores play the principal role in this process. Di Lisa and Bernardi [10] supported this suggestion through experiments with transgenic mice which had deficiency of cyclophilline D. This compound induced opening of these pores by increased sensitivity to divalent calcium ions. In these mice the authors detected a reduction of the heart infarct size. This problem was further analyzed. Administration of carvedilol, a competitive inhibitor of various adrenoreceptors, blocked the

opening of these pores but it simultaneously inhibited the oxidation of mitochondrial thiol groups. The finding confirmed the suggestion about a possible antioxidative effect of this compound.

Thus in ischemia-reperfusion injury the key role in functional disturbances of mitochondria is attributed to oxidative stress as the principal "player" in ischemia-reperfusion. Although mitochondria possess protective mechanisms against toxic effects of oxidative stress (antioxidant systems), they also have several factors that initiate this unfavorable event (production of reactive oxygen species, opening of permeability transition pores, ability to release apoptotic factors). Changes of complexes of the inner mitochondrial membrane and of the function of ion channels evoke imbalance in ion distribution, which have been considered consequences of reperfusion injury [31].

Ischemia-reperfusion injury is one of the principal mechanisms in the *failing heart*. It was shown that defects of the mitochondrial electron transport chain due to disturbances of mtDNA caused a marked increase in the production of reactive oxygen species. The consequence of this event is cardiomyocyte hypertrophy, apoptosis and interstitial fibrosis due to activation of matrix metalloproteinases. The result of these events is myocardial remodelation and failure of heart function [36]. These observations were confirmed through experiments on mutant mice with Mn-superoxidase dismutase deficiency. In these mice progressive heart failure due to specific defects of the mitochondrial electron transfer chain was demonstrated. Besides the specific morphologic changes of mitochondria, increased production of superoxide anion, decreased ATP formation and reduction of heart contractility were observed. Thus restoration of the function of mitochondria is an absolute requirement for the recovery of heart function [9]. An expressive toxic effect of oxidative stress on heart contractility was also confirmed by marked alleviation of heart failure after administration of superoxide dismutase, which removed the present superoxide anion [26]. These findings call attention to the inevitability of new trends in therapeutic strategy of these diseases: extensive use of antioxidatively acting natural antioxidants as well as further drugs with antioxidant capacity [36].

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Chapter 7
Mitochondria of the Human Transplanted Heart

Anna Gvozdjáková

Abstract Mitochondrial oxidative phosphorylation function and coenzyme Q_{10} concentration in endomyocardial biopsies (EMB) of human transplanted heart patients (HTx-pts) were documented. Decreasing mitochondrial energy production and diminished CoQ_{10} concentration in EMB of HTx-pts is in relationship with rejection development of the transplanted heart.

Keywords Coenzyme Q_{10}, EMB, HTx-pts, mitochondrial function, rejection

Heart transplantation is an accepted therapy for patients with end-stage heart failure. Years of patient survival after heart transplantation depend on various factors, such as number of rejections, immunosuppression, production of free radicals, function of antioxidant defense system [8], mitochondrial coenzyme Q_{10} content and oxidative phosphorylation function [2, 3, 7].

Cyclosporin A, which even in very low concentrations damages mitochondrial function, is used as an immunosuppressive drug. It inhibits mitochondrial permeability transition pore (MPTP), which is characterized by progressive permeabilization of the inner mitochondrial membrane, permitting passage to protons, ions, and even small proteins, stimulated by osmotic support [6]. Patients with transplanted hearts (HTx-pts) require continual complex medical care and complex therapy for a lifetime. HTx-pts have to be regularly checked for prevention and therapy of infectious diseases [1]. In spite of effective immunosuppression, acute rejection of the transplanted heart is one of the greatest problems in the first year after heart transplantation. Decreased myocardium mitochondrial bioenergetic processes and diminished coenzyme Q_{10} concentration in the transplanted heart are also involved in the pathobiochemical mechanisms of human transplanted heart rejection [3, 4].

CoQ_{10} is a key part of the mitochondrial respiratory chain connected with oxidative phosphorylation. In patients with heart failure, pronounced deficiency of CoQ_{10} was described [2, 5]. Mitochondrial function of the human transplanted heart is shown in Fig. 7.1 [3, 4].

Mean age of patients was 45 years, range 16–63 years. Twenty eight endomyocardial biopsies (EMB) were divided according to the histologically

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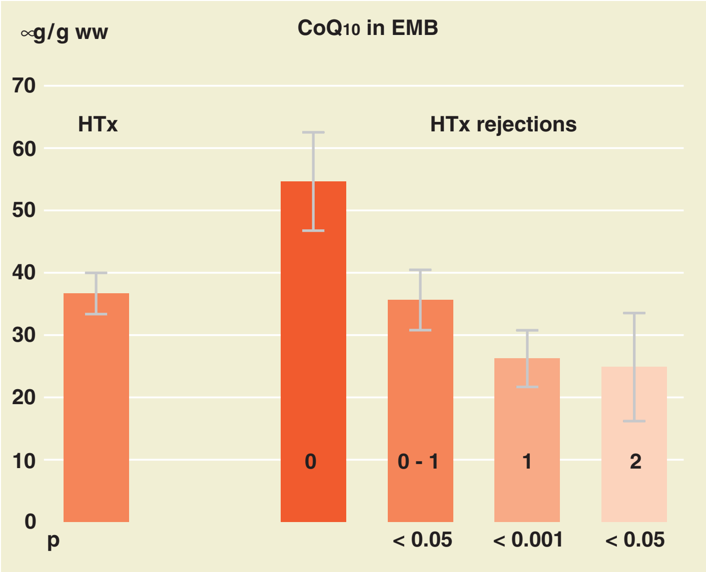

Fig. 7.1 Relationship between coenzyme Q_{10} concentration in EMB and degree of rejection of human transplanted heart

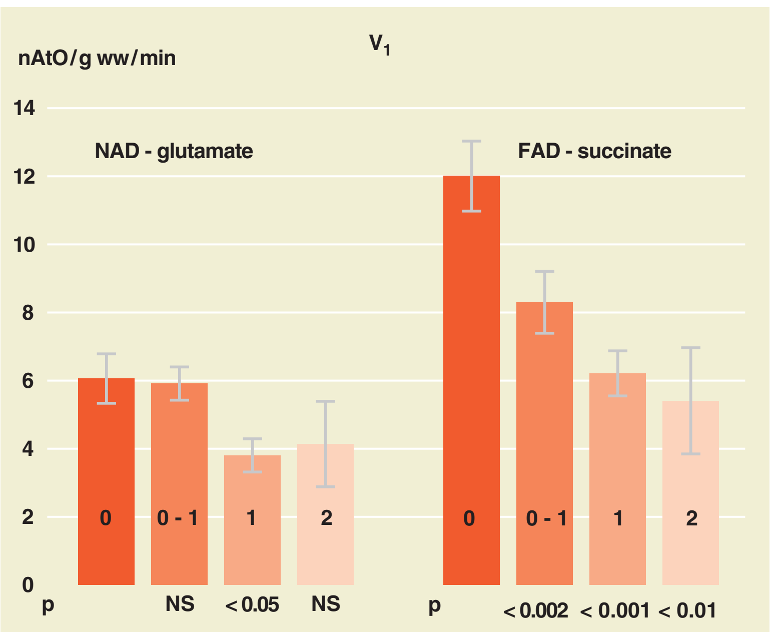

Fig. 7.2 Basal mitochondrial respiration in EMB of HTx-pts in relation to degree of rejection (V_1)

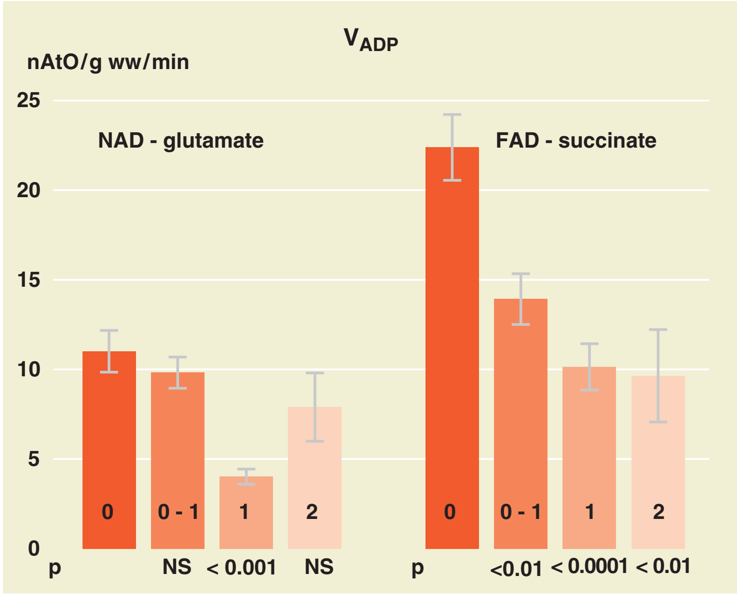

Fig. 7.3 Mitochondrial ATP production in EMB of HTx-pts in relation to degree of rejection (V_{ADP})

confirmed degree of rejection: 0 = without rejection, 0–1 = incipient rejection, 1 = mild rejection, 2 = moderate rejection. Diminished CoQ_{10} concentration in EMB of HTx-pts was in relationship with the degree of rejection of transplanted hearts.

For EMB studies of mitochondrial oxidative phosphorylation function in HTx-pts 2–5 mg heart tissue was used. Skinned fibers for measurements of mitochondrial respiration were prepared by permeabilization of the tissue with saponine [9]. Basal (V_1) mitochondrial respiration and ADP stimulated mitochondrial respiration (V_{ADP}) – energy production in EMB of HTx-pts in relation to the degree of rejection is demonstrated in Figs. 7.2 and 7.3. For estimation of Complex I, NAD-linked glutamate as substrate and for estimation of Complex II, FAD-linked succinate as substrate were used. Damage of mitochondrial respiration and energy production is at the site of Complex II in relationship with rejection development of the transplanted heart (Figs. 7.2 and 7.3).

In conclusion, the decrease of CoQ_{10} concentration in the transplanted heart is in correlation with decreasing mitochondrial energy production and rejection episode development. Decreased CoQ_{10} concentration in heart transplanted patients speaks in favor of supplementary therapy with coenzyme Q_{10}.

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Chapter 8
Mitochondrial Diabetology

Jozef Čársky

Abstract Diabetes mellitus (DM) is a chronic disease caused by disorder of insulin secretion in pancreatic β-cells, and/or its effect in tissues. Insulin acts to lower blood glucose by stimulation of glucose transport to muscle and fat cells and inhibition of its generation in the liver. Insulin promotes storage of the polysaccharide glycogen in the liver and muscles and stimulates glycogen synthesis.

Classification of DM is based on etiopathogenic mechanisms. Type 1 (characterized by β-cell destruction), type 2 (resistance to insulin action), other specific forms (mitochondrial DM included) and pregnancy diabetes.

Chronic hyperglycemia destroys many organs: eyes, kidneys, nervous tissue, heart and blood vessels. These changes are manifested as chronic diabetic complications.

Insulin deficiency and resistance to insulin action lead not only to changes in the metabolism of saccharides but also of proteins and lipids. The main pathobiochemical mechanisms of these changes are: (a) glycation of proteins, lipids, lipoproteins and DNA, (b) oxidative stress induced by hyperglycemia, (c) elevated flux via polyolic metabolic pathway of glucose, (d) changes linked with insulin signal pathway.

Abnormalities in metabolism which are typical for DM influence also functions of mitochondria. Disbalance between oxidative and reductive forms of supporting redox system create conditions for dysfunction of terminal oxidation. Mitochondria are assumed to be the key location for free radical generation because of the higher potential of reduced forms in this system (NADH). There is also a correlation between lower respiratory function of mitochondria and glycation of proteins in Complex II in the respiratory chain.

Keywords Diabetes mellitus, free radicals, glycation, mitochondria, oxidative stress

8.1 Diabetes Mellitus

8.1.1 General Characteristics

Diabetes mellitus is a chronic disease involving a range of metabolic derangements caused by the permanent condition of hyperglycemia (> 6.0 mmol/L

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glucose in plasma). The underlying cause is impairment of secretion or action of insulin, or the combination of both defects. Concentration of glucose in blood is under the control of several factors. One of the most important is the hormonal regulation by insulin, secreted by pancreatic β-cells. Insulin reduces the level of glucose by stimulating its transport particularly into muscle cells and adipocytes and by lowering its production in the liver. In the liver and in muscles, insulin stimulates the production of glycogen, inhibits its degradation, and stimulates glycolysis. In these cells, it stimulates proteosynthesis and inhibits proteolysis. The hormones glucagon, glucocorticoids, catecholamines and growth hormones are most importantly involved in increasing blood glucose concentration. The toxic action of glucose in chronic hyperglycemia causes damage, dysfunction and even failure of organ systems, particularly the eyes, kidneys, nerves, heart, and vessels. This becomes manifest by chronic complications, as retinopathy, nephropathy, peripheral and autonomous neuropathy. Besides these complications, there is increased incidence of atherosclerotic, cardiovascular, peripheral vascular and cerebrovascular episodes, which are the main cause of increased morbidity and mortality rates in diabetic patients. Associated pathological conditions involve a high incidence of hypertension, dislipidemia, gout, and disorders of the periodontium [21, 59].

Insulin insufficiency is an inevitable precondition of hyperglycemia, thus the diabetic state of the organism is considered a status of relative insulin deficiency. However, not always does insulin adequately reduce blood glucose, as seen in some obese diabetics. In these cases, the target cells are resistant to insulin, and insulin resistance becomes an even more serious phenomenon in the pathogenesis of diabetes than is its deficiency. This fact was the basis for the original primary classification of diabetes into insulin-dependent (IDDM) and non-insulin-dependent diabetes (NIDDM). When the pancreatic β-cells release a sufficient amount of insulin, the result is not a diabetic state but rather the development of obesity. In response to this, mitochondrial DNA undergoes relevant changes leading to adaptation to the condition of obesity. This is associated with the development of diabetes since the abnormalities in the mitochondria may induce impaired insulin secretion. Moreover, in muscles and leukocytes, these mitochondrial changes may be associated with insulin resistance. Derangement of insulin secretion and of its action in target tissues becomes manifest in alterations of the metabolism of saccharides, proteins, and lipids, and secondarily also water-electrolyte metabolism is affected. The development of diabetes is enhanced by elevated secretion of antagonistic hormones – somatotropin, glucocorticoids, and adrenalin [4, 32, 61].

A potential defect of insulin secretion can be differentiated from derangements in its action by measuring fasting glycemia – impaired fasting glucose (IFG) and glucose intolerance – impaired glucose tolerance (IGT) on administering a solution containing 75 g glucose. When fasting glucose concentration exceeds mildly the upper limit of the physiological value (6.1–7.0 mmol/L), insulin secretion is impaired, signaling disposition to clinical diabetes. The finding of IGT means derangement of insulin action, associated with its relative deficiency [12, 24, 31].

At present, the number of people suffering from diabetes mellitus reaches practically 180 million worldwide. Prognostic studies expect this number to double within 25 years. The civilization process with changes in lifestyle and environment affecting negatively human health accounts to a great extent for this explosion. The given changes concern not only medicine but also ecology, sociology, and sociopolitical relations. In the majority of countries, diabetes ranks fourth among the most frequent causes of death [5, 67, 68].

8.1.2 Classification of Diabetes

The great heterogeneity of pathophysiological manifestations of this disease makes an unambiguous and objective classification rather difficult. The classification dating from 1979, elaborated by a group of experts (National Diabetes Data Working Group) incited by the National Institute of Health, USA, in cooperation with the WHO, revised in 1985, differentiated five clinical types of diabetes: insulin dependent (IDDM), insulin nondependent (INDDM), gestational diabetes (GDM), malnutrition-related diabetes (MRDM), and other types of diabetes. In 1997, the American Diabetes Association accepted the proposal of a group of experts for a new classification of diabetes based on the etiology of the disease, which no longer contains the terms IDDM and NIDDM, nor does malnutrition diabetes appear as a separate group. The latter decision was based on the finding that malnutrition can lead to different types of diabetes and a direct effect of protein deficiency as the cause of diabetes was considered questionable. Basically, the new classification differentiates type 1 DM, type 2 DM, other specific types, and gestational diabetes (Table 8.1). This classification has become the basis for new diagnostic criteria and border values of glycemia obtained by different tests [1, 26, 70].

8.1.2.1 Diabetes Mellitus Type 1 (Juvenile DM, Labile DM, DM with Predisposition to Ketosis)

In this type of diabetes either the immune system is involved or it is idiopathic. It becomes manifest in childhood or adolescence, but it can appear as late as adulthood. In the diabetic population, the average rate of occurrence of DM type 1 is 5%. On assessing the incidence in individual countries, geographical differences appear. So, e.g. Germany has an incidence of 0.3%, Rumania of 5.2%. The etiopathogenesis of diabetes type 1 is shown in Fig. 8.1.

Immune system-mediated type 1 DM is an autoimmune disease. Its cause lies in the autoimmune destruction of pancreatic β-cells, resulting in deficient insulin production. Destruction of these cells may be the underlying cause of several derangements. In the blood of diabetic patients, antibodies to several antigens have

Table 8.1 Classification of diabetes

Type 1	Destruction of pancreatic β-cells – Conditioned by the immune state – Idiopathic
Type 2	– Predominant insulin resistance – Relative insulin deficiency
Other specific types	– Genetic defects of β-cells (MODY 1–6, MIDD) – Genetic defects of insulin action – Diseases of the pancreas (inflammation, neoplasm, cystic fibrosis, etc.) – Endocrinopathies (acromegaly, Cushing's syndrome, pheochromocytoma, etc.) – Drug and chemically induced (corticoids, diazoxide, thiazides, α- interferon) – Infections (rubella, cytomegalovirus) – Immunologically determined (antibodies to insulin receptors, stiff-man sy.) – Genetic syndromes with occasional appearance of diabetes (m. Down, Turner's syndrome, etc.)
Gestational DM (GDM)	Detection of diabetes or impaired glucose tolerance in pregancy

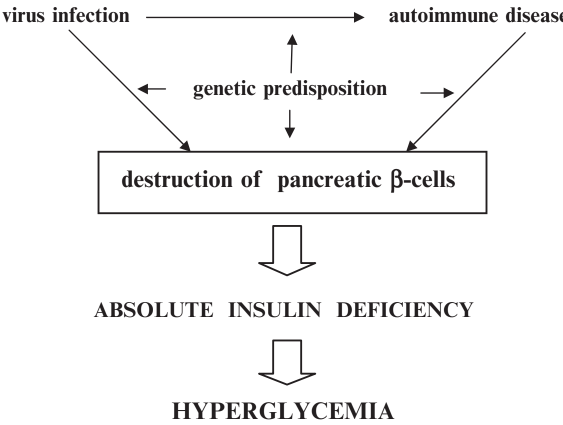

Fig. 8.1 Etiopathogenesis of diabetes mellitus type 1

been found. These include islet cell antibodies (ICA), membrane antibodies (ICSA), insulin autoantibodies (IAA), glutamate decarboxylase antibodies (GAD-AB), antibodies to tyrosine phosphatases IA-1, IA-2, and antibodies to the glucose transporter GLUT-2. Cell-mediated immunity also plays an important role in this type of

diabetes. Studies of the main histocompatibility system revealed genetic predisposition to DM type 1. Its development is associated with the alleles HLA-DR3 and DR4, HLA-DQ8 and DQ2. The allele HLA-DQ6 associated with HLA-DR2, on the other hand, is a factor of insulin resistance. Gene products of the loci HLA-DQ and HLA-DR are molecules responsible for predisposition and resistance. Genes encoding for insulin are presumably also involved in predisposition to type 1 DM. Nevertheless, the majority of subjects with this type of DM do not display a family history of DM, with the exception of but 10 – 15% of cases. In identical twins, both siblings are affected with type 1 DM in about 36% of cases [17, 20, 39, 67].

Idiopathic type 1 DM involves some forms with unknown etiology. The clinical picture exhibits periodic ketoacidosis, yet neither manifestations of autoimmunity to B-cells nor association with the HLA system were identified. The genetic factor plays a very important role in this type of DM [64].

8.1.2.2 Diabetes Mellitus Type 2 (Onset in Adulthood, Stable DM, Resistant to Ketosis)

Diabetes mellitus type 2 involves diabetic states with insulin resistance and relative to predominant insulin deficit, as its secretion is unable to compensate the insulin resistance. The patients are thus dependent on exogenous supply. A slight disposition to ketosis development is characteristic. In many cases insulin therapy is not required since hyperglycemia can be reduced by diet or oral antidiabetics. Despite the strong genetic factor involved, environmental issues play a great part in its development. The most important of these is obesity resulting from too rich nutritional intake and insufficient motor activity. The imbalance between energy intake and consumption increases the concentration of higher carboxylic acids with subsequently decreased utilization of glucose in muscles and adipose tissue. Obesity may then be the cause of insulin resistance, which is inevitably reflected by enhanced insulin secretion. Etiopathogenesis of DM type 2 is represented in Fig. 8.2.

The prevalence of this type of DM is marked by great differences between races and ethnic groups. In the Caucasoid population the rate is 3–6%, in the Japanese 1%, and in some Indian tribes in the USA, Micronesia and Polynesia it reaches 35% [5].

This type of diabetes appears predominantly in adult age and affects mostly women with preceding GDM. The incidence increases with age, yet it may occur also in younger age groups, manifested as permanent hyperglycemia diagnosed before the age of 25 years. For more than 2 years, it can be treated without insulin. It is referred to as Maturity Onset Diabetes of the Young (MODY). Of all the forms of DM, European statistics report an up to 90% incidence of this type. The manifest form of type 2 DM is usually preceded by a period of impaired glucose tolerance of different duration, the so-called metabolic syndrome. This is characterized by varying degrees of insulin resistance, deranged insulin secretion, hyperinsulinemia, hypertriacylglyceridemia, elevated VLDL values, reduced HDL concentrations,

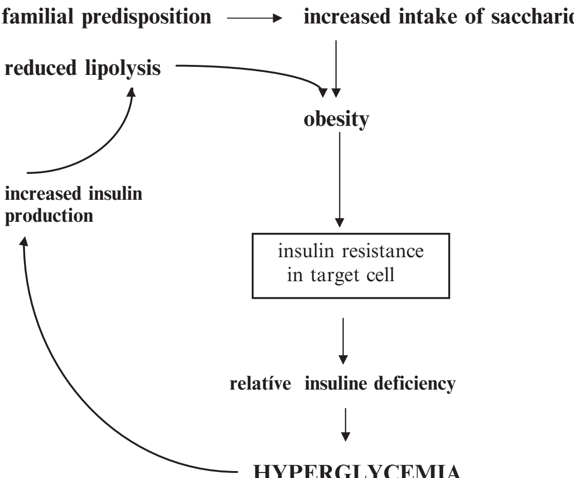

Fig. 8.2 Etiopathogenesis of diabetes mellitus type 2

overweight or even obesity, and hypertension. Hyperinsulinemia and insulin resistance acting together result in impaired glucose tolerance. The clinically manifest type 2 DM is invariably associated with insulin resistance in muscles, adipose tissue, and liver, and there is concomitant defect of insulin secretion of various degrees of severity. The outcome is brought about by mutual combination of these features [33, 49].

According to the WHO criteria published in 1998, a subject with metabolic syndrome has diagnosed diabetes, impaired glucose tolerance, impaired fasting glucose, insulin resistance established by the glucose clamp, and at least two further metabolic abnormalities:

- Blood pressure 140/90 mmHg
- Blood triacylglycerol 1.695 mmol/L and/or HDL cholesterol 0.9 mmol/L in males and 1.0 mmol/L in females
- Central obesity
- Microalbuminuria

In the definition presented by the National Cholesterol Education Program of the USA (NCEP), microalbuminuria and insulin resistance were eliminated and hypertension has been stressed. Generally speaking, the term "metabolic syndrome" applies to clinically assessed disease conditions characterized by resistance, including many subjects with type 2 DM and with prediabetic state of glucose intolerance. Considerations have been voiced indicating that the term "mitochondrial abnormalities" would better characterize the given conditions [1].

8.1.2.3 Other Specific Types of Diabetes

Mitochondrial Diabetes

As established in the 1970s of the 20th century, mitochondria possess their own extranuclear DNA, localized in the matrix of these cellular organelles. Mitochondrial DNA is inherited exclusively from the mother. The ovum is rich in cytoplasm and organelles, while the spermatozoon is virtually without cytoplasm. Rapid mutations are characteristic for mitochondrial DNA. When there is a mixture of mutated and original DNA in the cell, we speak of heteroplasmia, while homoplasmia is a state with only mutated DNA. Mutations of mitochondrial genes are the underlying cause of several degenerative diseases. The association of mitochondrial gene defect and diabetes has been established. In the classification dating from 1999, this form of diabetes was included into the group “Other Specific Types” and the subgroup “Genetic Defects of Pancreatic β-cells”. The following conditions fall under this classification:

Wolfram syndrome – DIDMOAD (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, Deafness). It is caused by mitochondrial DNA defect. Diabetes mellitus is the first condition to appear, and that as early as in childhood or late adolescence. The other manifestations follow later on.

MIDD (Maternally Inherited Diabetes and Deafness) is the point mutation A3243G, substitution of adenine for guanine in the DNA molecule. The clinical manifestations are diabetes and impaired hearing. On investigating a number of pedigrees, it was found to be associated with the MELAS syndrome (Mitochondrial Encephalomyopathies, Lactic Acidosis, Stroke-like Episodes).

In practice, mitochondrial diabetes can manifest itself as type 1 diabetes or type 2, depending on the degree of insulinopenia. With progressive insulinopenia, insulin administration may be indicated within a few years of onset of diabetes [25, 34, 37].

8.1.2.4 Gestational Diabetes Mellitus

Gestational diabetes, with an incidence of 2–5%, appears in pregnancy as impaired glucose tolerance (IGT) or onset of diabetes. Women who had been diagnosed as diabetic before pregnancy do not fall within this group. Predisposition concerns obese women aged over 25 years. Women from some ethnic groups (Hispanic, Afro-American, Southeast Asian) are at a higher risk when diabetes appears in their family history. Early diagnosis makes this type of diabetes manageable. Without treatment, there is a higher probability of type 2 diabetes to become manifest later on. Children of mothers with GDM have a higher predisposition both to obesity and diabetes [46, 66].

8.1.3 Chronic Diabetic Complications

The diabetic status of the organism is determined by the plasma glucose level. Hyperglycemia, induced by absolute or relative insulin deficiency, plays a decisive role in the pathogenesis of chronic diabetic complications. This statement, however, fails to consider the condition of life-important organs, although in the initial phase of the disease their function is mostly normal, as it is in the early phase of experimental diabetes. The tissues of organs of diabetic subjects undergo a time-dependent destruction manifested by complications, which are more pronounced and more frequent in severe long-lasting hyperglycemia. Organs with rich blood supply, as kidneys, retina, and nervous system, are most affected. In the light of these facts, the key issue in the care of diabetic patients is to achieve normoglycemia to prevent the development of destructive changes. This goal can only be accomplished when alternative procedures and methods are found and applied. Even mild hyperglycemia can threaten the state of the organism in sensitive individuals [7, 47, 51, 53].

The occurrence of diabetic complications depends on the age when diabetes became manifest in the patient, on the duration of the disease, on other concomitant disorders, and on treatment received. The complications may concern many organs and their functions (Table 8.2).

In the given problem area, the key issue is to explain the development of capillary destruction and to elucidate the mechanisms involved in the development of chronic diabetic complications. The main pathobiochemical mechanisms of these processes are assumed to be the following [15, 34, 51]:

(a) Glycation of biomolecules (proteins, lipids and lipoproteins, DNA).
(b) Hyperglycemia-induced oxidative stress.
(c) Elevation of the metabolic flux via polyol and hexosamine metabolic pathways and secondary changes associated with this alternative glucose metabolism.
(d) Changes in molecules connected with the insulin-signaling pathway.

Table 8.2 Overview of the most frequently occurring complications

Eyes	Retinopathy (proliferative, nonproliferative), cataract
Kidneys	Nephropathy (glomerulosclerosis)
Nervous system	Neuropathy (peripheral, cranial, peripheral motor and sensory, autonomic, mononeuropathy multiplex)
Skin	Dermopathy (necrobiosis lipoidica, candidiasis)
Diabetic foot	Neurotropic, ischemic
Cardiovascular system	Cardiomyopathies, IHD, ischemic disease of lower extremity vessels, diabetic gangrene, cerebrovascular ischemia
Ligaments, joints	Dupuytren's contracture, Forestier's osteoartrosis
Infections	Necrotizing fascitis, myositis, mucormycoses, candidiases

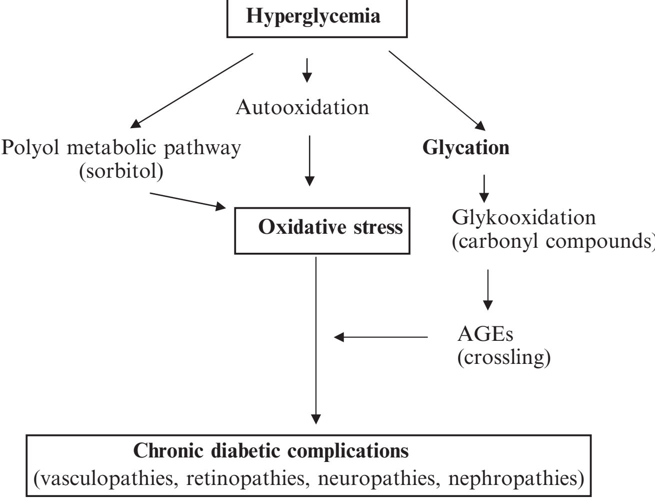

Fig. 8.3 Pathogenesis of chronic diabetic complications

These processes combine with specific conditions of endothelial cells producing nitric oxide (NO) to achieve vasodilation. The interrelationships are schematically represented in Fig. 8.3.

8.1.3.1 Glycation of Proteins and Oxidative Stress in Diabetes Mellitus

Numerous experimental and clinical findings have provided evidence on glycation, a process in which glucose, chemically (nonenzymatically) by covalence bond, modifies the structure and mostly also the biological function of biomacromolecules. Moreover, glucose can directly or indirectly contribute to the generation of free radicals and thus to oxidative damage of proteins, lipids, and DNA, resulting in oxidative stress and the development of chronic diabetic complications. In an environment of chronic hyperglycemia, this effect becomes pronounced and is considered the manifestation of glucose toxicity. In type 1 and type 2 DM the formation of free radicals is evidently enhanced and may be considered one of the risk factors of this disease [2, 8, 27, 65, 74].

In DM, free radical generation is associated not only with the glycation of proteins, lipids, lipoproteins, and DNA but also with glucose autooxidation. This is essentially a physiological process, yet in conditions of hyperglycemia it is potentiated and concerns basically the biological application of Maillard's reaction – "browning reaction". This reaction is an intricate complex of parallel and subsequent nonenzymatic chemical reactions of reducing saccharides (glucose) and their derivatives with amino-groups of biogenic molecules – amino acids, peptides, proteins, lipids, DNA and further biogenic substances. The condensation reaction

proceeds by means of the oxo-group of saccharide and the amino-group of these substances [35, 36, 44].

The process of glycation splits into two directions, which include:

(a) Generation of reactive oxygen species, ROS, (glycooxidation), involved in the development of oxidative stress
(b) Structural modification of substances via the adduct of Schiff base, Amadori product and reactive carbonyls. The Amadori product undergoes oxidation and dehydration changes with the generation of intermediary heterocyclic products of advanced glycation – advanced glycation end-products (AGEs), inducing changes of the physicochemical and functional properties of biomolecules [10, 11, 23], as shown in Fig. 8.4.

The first stage of the glycation process is associated with oxidative changes – oxidation of Amadori product and eventually increased generation of reactive oxygen species, occurring by the catalytic effect of transition metals Fe(II) and Cu(I). The ensuing products are dicarbonyl structures – glucosones, glyoxal and methylglyoxal, which are substantially more reactive than free glucose and react even in very low concentrations with amino-groups of molecules of bioorganic and biomacromolecular substances, resulting in the production of secondary adducts and eventually in the formation of advanced glycation end-products. Free radicals induce structural modification of DNA purine residues by producing 8-hydroxydeoxyguanosine (8-OH-dG), which is used as marker of this oxidative injury. The marker of oxidative injury of proteins is carboxymethyllysine, that of lipids and lipoproteins is malondialdehyde. A general marker of oxidative injury caused by oxidative stress is a diminished ratio of reduced glutathione in cells. A further indicator is the assessment of the overall antioxidative status,

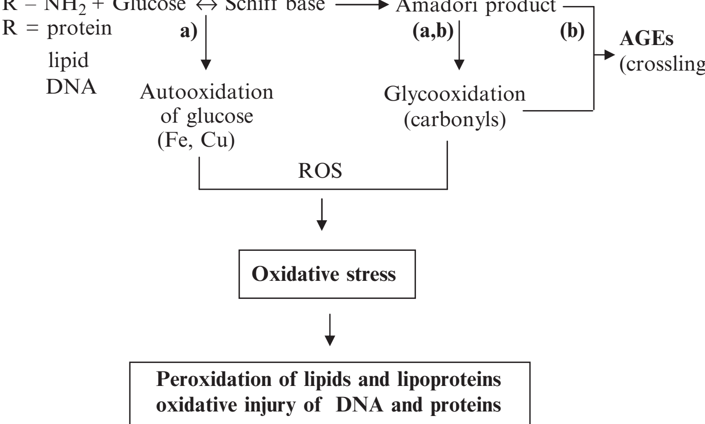

Fig. 8.4 Process of glycation, glycooxidation, and AGE formation

i.e. the antioxidative capacity of serum. Under conditions of diabetes mellitus, however, this parameter may be affected by the reduction capacity of Schiff bases and the Amadori product, developing in the initial phases of protein glycation [18, 54, 56, 57, 72].

The second pathway of glycation results in cross-linking of polypeptide chains of tissue and structural proteins (reduced elasticity and changes in permeability of biological membranes), aggregate formation of crystalline lens (cataract genesis), accumulation of various plasmatic proteins and lipoproteins on the protein constituents of vascular walls (thickening of basement membranes), etc. AGEs are involved in the development of these pathological changes. AGEs are heterogenous heterocyclic structures, products of the Maillard reaction in vivo, which covalently interlink polypeptide chains of identical or different proteins. Based on the mechanism of their origin, they can be considered to be derivatives of saccharides and may be defined as "new chemical entities" or new in vivo fluorophores in proteins with a long turnover half-life, emitting fluorescence at 400–450 nm, with excitation of 330–350 nm [57, 60, 73].

The presence of AGEs in diabetic patients or in subjects of advanced age was proven immunohistochemically by means of antibodies against AGE proteins, and that in the crystalline lens, in the collagen of aortal and renal tissue, in LDL and in other biological material [43]. An example of circulating AGEs is their presence in human serum proteins and in erythrocytes [38].

Prevention of AGE generation lies in inhibition of the effect of free radicals (oxidative stress) by the application of antioxidants. A further approach is inhibition of carbonyl structures, e.g. with the intensively studied effect of aminoguanidine, which scavenges reactive carbonyl compounds in the form of Schiff bases [9, 19]. The novel inhibitors of AGE production include pyridoxamine, penicillamine, derivatives of aminoguanidine – pyridoxylidene aminoguanidine and resorcylidene aminoguanidine, further bioflavonoids, alpha-lipoic acid, beta-carotene, stobadine, etc. Some antioxidants have pro-oxidative properties and may thus exert unfavorable effects [3, 29, 30, 52, 62, 69, 71].

Another pathological action of glycation is inhibition of the catalytic activity of various enzymes. The binding of glucose to free amino-groups located in the active center of these substances results in their reduced ability to interact with the substrate, as observed, e.g. with Na^+, K^+-ATPase in erythrocytes, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and other enzymes. In DM, the diminished enzyme activity along with the decreased concentration of low-molecular antioxidants (glutathione, vitamin C, vitamin E) are substantially involved in creating conditions favorable for the development of oxidative stress [40, 50, 63]. A similar mechanism of impairment is operative also with hemoglobin, when glucose is bound to the allosteric site – the end amino-groups of the beta-chains – assigned for the allosteric effector 2,3-diphosphoglycerate. Due to this structural modification, glycated hemoglobin HbA_{1c} has a higher affinity to oxygen, and thus its transporting ability becomes reduced. In diabetologic practice, determination of HbA_{1c} is used as an objective parameter of metabolic compensa-

tion, expressing the integral value of glycemia for the preceding period of 4–6 weeks [13, 16].

Knowledge leading to the understanding of glycation in vivo conditions has opened new possibilities for an interdisciplinary approach to the study of diabetes and aging of the organism.

Rage Receptors

On investigating the mechanisms of origin and effect of AGEs, specific receptors were detected in the body, which are key elements in materializing the interaction of these products with cells. Several receptor types have been discovered, of which the best investigated is the RAGE receptor, from the group of immunoglobulins. The RAGE receptor is considered to be primarily an intracellular signal transductor, or a proinflammatory peptide with potential significance for inflammatory and autoimmune disorders. Its polymorphism has been demonstrated and its occurrence in type 1 and type 2 DM has been studied, along with its relation to diabetic nephropathy and retinopathy. RAGE exists also in soluble circulating form – s-RAGE, which can exert also its protective role. The formation and binding of AGE to its receptor is assumed to be involved in the development of chronic diabetic micro- and macrovascular complications [58, 75].

The importance of AGE binding to RAGE lies in the removal of glycation modified protein, yet at the same time it acts as a signal for the cell to increase its generation of free radicals and to activate the transcription factor NFkB (Necrosis Factor kappa B). Nitrogen-activated protein kinases (NAP) of the kinase cascade initiate the generation of inflammatory cytokines, procoagulatory, prothrombomodulatory, vasoconstrictively acting endothelin-1, of the vascular cell adhesion molecule VCAM-1, and of other products. This reaction may play an even greater role in the development of atherosclerosis and microangiopathy than do direct consequences of late glycation phases [6].

The investigation of AGEs has still to address the issue of their detailed mechanism of origin and their localization in the organism, as well as to disclose a selective structure which may serve as early marker of the pathogenesis of chronic diabetic complications.

8.1.3.2 Polyol Pathway of Glucose Metabolism

The control of glucose utilization in metabolic processes in the muscles is realized in three degrees:

(a) Supply of glucose to the cell membranes
(b) Transport through the membrane by means of GLUT-4
(c) Intramolecular phosphorylation of glucose to glucose-6-phosphate by hexokinase

In conditions of chronic hyperglycemia, the increased amount of glucose is metabolized by the so-called sorbitol pathway, catalyzed by aldose reductase (AR). This results in accumulation of sorbitol (D-glucitol) and deficiency of NADPH, which besides other functions plays a role in the antioxidative protection of the organism. The oxidation of sorbitol to fructose is accompanied by an enhanced requirement for the oxidized form of coenzyme NAD^+.

The oxidative capacity of the redox system NAD^+/NADH is reduced by this process. The increase in the NADH ratio (not only from the sorbitol pathway but also from glycolysis) creates conditions similar to hypoxia, referred to as ***reductive stress***. The polyol metabolic pathway thus induces an increase in free radical generation (superoxide), particularly in endothelial tissue and peripheral nerves, and advances the relation to the pathogenesis of diabetic retinopathy and neuropathy. In addition, the resulting fructose in the sorbitol pathway intensifies glycation (it is a stronger glycation agent than glucose) and the production of reactive carbonyls – deoxyglucosones. Inhibitors of aldose reductase play an important role in the prevention of pathobiochemical manifestations of the sorbitol pathway in DM. A natural inhibitor is the radical NO, however the elevated superoxide generation proceeding under the given conditions lowers its effect by mutual reaction with the production of peroxynitril anion [45]:

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow O_2 + NO^-$$

Physical exercise exerts a positive effect on metabolic utilization of glucose – on the glucose transporter GLUT-4, on the activity of hexokinase and on glycogensynthase. It increases insulin sensitivity and promotes prevention of type 2 DM development.

The increased potential of NADH production in the polyol metabolic pathway and in glycolysis, participating in the diabetic reduction stress (diabetic pseudohypoxia), results in enhanced formation of diacylglycerol (DAG) which activates protein kinase C (PKC) and its isoformes [28]. It is supposed that this activation is the main factor of endothelium dysfunction and can enhance atherosclerosis in diabetes mellitus. Vitamin E is an inhibitor of PKC, and LY 333531 has a selective inhibitory effect on beta-PKC isoforms. The activation of PKC and its relation to diabetic complications is shown in Fig. 8.5.

Understanding of the molecular mechanisms of pathobiochemical and pathophysiological processes in DM allows to exert influence upon them. A positive effect can be achieved by means of [15, 42]:

- Inhibitors of glycation and of AGE production (aminoguanidine, pyridoxamine, etc.)
- Antibiotics (inhibition of autooxidation and glycooxidation)
- Chelators (binding of metal transition ions catalyzing the generation of free radicals into stable chelate structures)
- Inhibition of aldose reductase (polyol metabolic pathway)
- Inhibitors of phosphokinase PKC

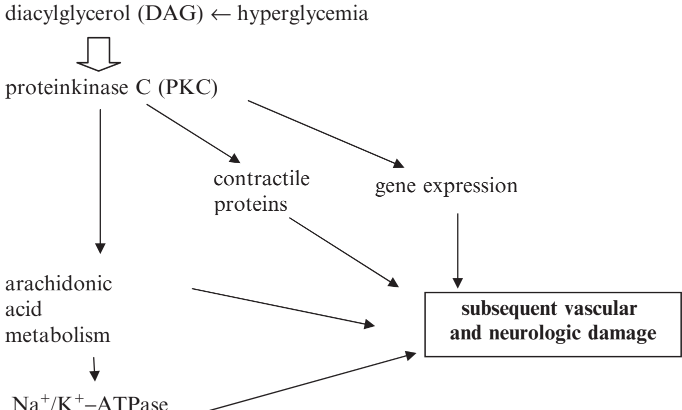

Fig. 8.5 Activation of protein kinase C and its relation to the development of diabetic complications

8.1.3.3 Hexosamine Metabolic Pathway of Glucose

Causes of vascular dysfunction in DM include not only the polyol pathway but also increased activity of fructose-6-phosphate aminotransferase, which catalyzes the transformation of glucose to glucose aminophosphate. This intermediary is able to induce NO formation in endothelial cells.

8.1.3.4 Oxidative Stress and Impairment of Mitochondrial Function

The above-characterized metabolic abnormalities are components of the diabetic status of the organism, affecting also the functions of mitochondria. Their study may result in understanding the development of diabetic complications and the pathogenesis of DM [32, 48, 61].

In metabolic processes in DM, the shift in the balance between the reduced and oxidized form of protective redox systems, NAD^+/NADH, glutathione, etc., creates conditions for an increased supply of reduced coenzyme NADH. This in turn impairs regulation of terminal oxidation in mitochondria. Due to this redox imbalance, mitochondria are assumed to become the key site of superoxide radical generation. Reduced mitochondrial function, brought about by protein glycation and elevated AGE production, has been suggested as the main cause of this development. In conditions of experimental DM, a relationship between diminished mitochondrial respiration and protein glycation was found in rats [55]. Two proteins were identified in Complex III of the respiratory chain in the kidney, where the main production of superoxide takes place both in physiologic and pathologic conditions. Structural modification of these proteins is brought on in the advanced

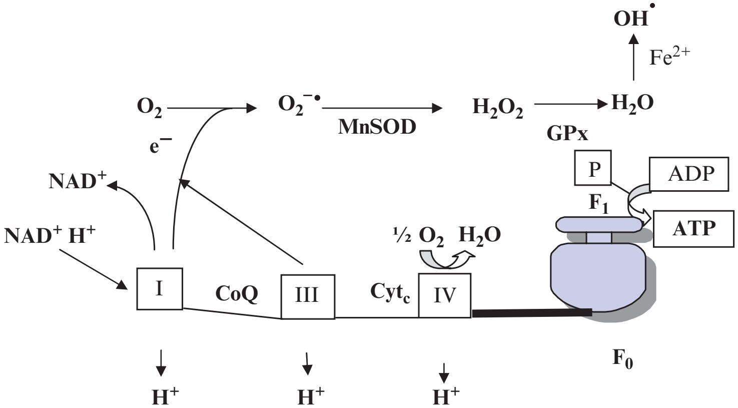

Fig. 8.6 Mechanism of superoxide and hydroxyl radical generation in mitochondria

phase of glycation by hydroimidazolones, whose precursor is methylglycoxal. The glycation-induced abnormality results in impaired mitochondrial bioenergetics (reduced oxidative phosphorylation), increased superoxide generation combined with the development of oxidative stress, and affected β-oxidation of higher carboxyl acids.

Concerning the relationship of mitochondrial function and insulin resistance, interesting findings were observed on using the method of NMR spectroscopy. In a group of nonobese healthy elderly subjects, compared with a group of younger subjects at comparable body mass and adipose tissue values, pronounced insulin resistance was recorded, associated not only with fat accumulation in muscles and liver tissue but also with 40%-reduced oxidation and phosphorylation activity of mitochondria [48]. These findings speak in favor of the hypothesis that reduced mitochondrial function is involved not only in DM but also in aging of the organism.

An experimental DM study showed the existence of a relationship between protein glycation and AGE formation in kidney mitochondria on the one side and pathogenesis of diabetic nephropathy on the other, with suppression of manganese superoxide dismutase (MnSOD) and of Complex I proteins of the respiratory chain, resulting in increased superoxide generation [14]. Such a deranged function of kidney mitochondria can be induced also by extraperitoneal administration of AGE-modified serum albumin [22]. The process of superoxide and hydroxyl radical generation in mitochondria is shown in Fig. 8.6.

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8.2 Mitochondrial Function in Diabetes

Anna Gvozdjáková

Abstract The mitochondrion plays a central role in diabetic beta-cells. Oxidative stress, nitric oxide synthase, oxidative phosphorylation and coenzyme Q damage contribute to the chronic complications of diabetes mellitus.

Keywords Coenzyme Q, diabetes mellitus, mitochondria, nitric oxide, oxidative phosphorylation, oxidative stress

Diabetes represents a collection of diseases characterized by chronic hyperglycemia, which can be accompanied by many other complications, such as cardiomyopathy, myopathy, encephalomyopathy, renal failure, visual failure, stroke, seizures and dementia. Diabetes can result from impaired secretion of insulin by the β-cell, as well as by loss of its action (*insulin resistance*) in peripheral tissues, such as skeletal muscle, fat and liver. It has long been known that increased insulin resistance can lead to decline in function of the pancreatic β-cell [11, 6].

Diabetes results not only in hyperglycemia, but also in elevation of non-esterified fatty acids. Hyperglycemia and excessive non-esterified fatty acids elevation induce mitochondrial ROS production with results in dysfunction of the mitochondria in the β-cell since they are toxic to β-cells. Hyperglycemia also favors an increased expression of inducible nitric oxide synthase (iNOS), which is accompanied with increasing nitric oxide (NO) generation. NO can react with superoxide anion to produce peroxynitrite, which in turn can increase lipid peroxidation, protein nitration, and LDL oxidation, affecting several signal transduction pathways. These metabolic changes are involved in the development of early diabetic injury before the evolution of late complications [1].

The mitochondrion plays a central role in linking metabolism to insulin secretion from the pancreatic β-cell and in this way blood glucose is regulated. Glucose enters the pancreatic β-cell and via cytosolic glycolysis is metabolized to pyruvate, which enters the mitochondrial citric acid cycle. Several mitochondrial mechanisms participate in diabetes.

Mitochondrial ROS production in diabetes. The inner mitochondrial membrane is one of the major sites of ROS production and has a high content of polyunsaturated fatty acids (PUFA). ROS generated in the mitochondrial respiratory chain may react with PUFA causing lipid peroxidation and alterations in the mitochondrial membrane integrity causing irreversible swelling and disruption of mitochondria. Decreased activity of Δ-6 and Δ-5-desaturases provokes changes in composition of PUFA in diabetes [10]. Hyperglycemia causes oxidative stress due to increased mitochondrial superoxide anion production, nonenzymatic glycation

of proteins and glucose autooxidation. Increased superoxide anion production by hyperglycemia could damage mitochondria and mitochondrial DNA (mtDNA). MtDNA depletion was identified in diabetic complications. Excess of superoxide production is accompanied with increased nitric oxide generation in endothelial cells. This mechanism is poorly understood [12].

High glucose concentration leads also to an increase of reducing equivalents such as *NADH and* $FADH_2$ within mitochondria. Their uptake from the cytoplasm occurs by various mitochondrial redox shuttles as well as by increasing uptake of pyruvate. Pyruvate as substrate for mitochondrial oxidative phosphorylation participates in ATP production from ADP and inorganic phosphate. The increased cytosolic ATP/ADP ratio causes closure of the plasma membrane K_{ATP} channels and depolarizes the beta-cell. After depolarization of the plasma membrane, calcium influx into beta-cells leads to secretion of insulin [9]. Mitochondrial dysfunction results in impaired glucose-stimulated insulin secretion [2]. Increased amount of *contact sites* on mitochondria in the diabetic heart can contribute to etiopathogenic mechanisms involved in chronic DM complications [14].

Damaged electron transport of *Q-cycle* can increase superoxide anion production in mitochondria in diabetes [7]. Decreased CoQ_{10} plasma concentration and beneficial effect of CoQ_{10} supplementation in patients with diabetes mellitus (IDDM, NIDDM) was demonstrated [3]. Decreased CoQ_9 and CoQ_{10} found in heart and liver mitochondria of rats with experimental diabetes mellitus participate in mitochondrial dysfunction [8]. Supplementation with CoQ_{10} and n-3-PUFA protect brain and heart mitochondrial function in diabetic rats [4, 13].

Recently it has been documented that the chronome (time structure) of cardiac pathological function undergoes *circadians or circasemidians* in the heart mitochondrial coenzyme Q_{10} and *oxidative phosphorylation*, which may be a clue to the pathogenesis of the diabetic heart [5] (Fig. 8.7).

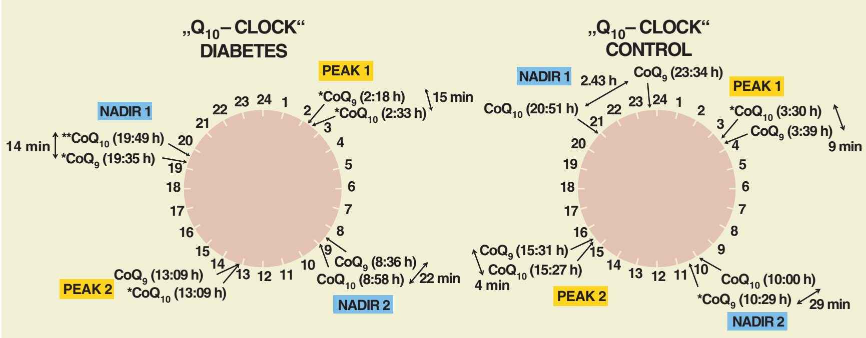

Fig. 8.7 Comparison of heart mitochondrial "Q_{10}-CLOCK" between control and diabetic rats

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8.3 Circa(semi)dian Periodicity of Coenzyme "Q_{10}-CLOCK" and Cascade of Oxidative Phosphorylation in Control and Diabetic Rat Heart Mitochondria

Miroslav Mikulecký, Anna Gvozdjáková, Jarmila Kucharská, and Ram B. Singh

Abstract The synthetic as well as the analytic approach was used for processing the measured data of myocardial mitochondria level of Q coenzymes and of the four parameters of Complex I and II chronobiological cascade of oxidative phosphorylation in control and diabetic rats. The differences were also calculated and processed. The hypothesis tested was based on two expected peaks – one diurnal, the other and higher one nocturnal, mathematically expressed as an interference of 24-h and 12-h rhythms. This pattern was significantly present particularly in the diabetic animals as well as in the differences of diabetes versus controls. A pronounced cycling was observed for Q_{10}(coenzyme Q_{10}) and for ADP:O (coefficient of oxidative phosphorylation). The most consistent effect of diabetes on health – a significant global decrease – was seen with ADP:O in either Complex. These findings can explain some mechanisms of the development of diabetic complications.

Keywords Cascade of oxidative phosphorylation, diabetes, mitochondria, "Q_{10}-CLOCK"

The inferential synthetic model described in Chapter 4 was used for this evaluation. The results, obtained by the cosinor regression, will be presented as graphs in a fashion that makes it possible to allocate the statistically significant elevations or depressions to the corresponding time intervals. It is believed that this form of information is optimal for further considerations and decisions.

Only statistically significant ($\alpha = 0.05$) findings will be displayed and taken into account in this Chapter. Besides both forms of coenzyme Q, also the oxidative phosphorylation (OXPHOS) cascade of Complex I and II will be taken into account. Results obtained in control and diabetic rats will be expressed as Mesor Related Values (MRV) and processed also as their differences (Baseline Mesor Related Differences, BMRD) showing the influence of diabetes as compared to healthy animals.

8.3.1 Mitochondrial Coenzyme Q_9 and Coenzyme Q_{10} in the Myocardium

8.3.1.1 Mesor Related Values (MRV) in Control Animals

Only the Q_{10} myocardial mitochondria level displays statistically significant cycling – "Q_{10}-CLOCK" (Fig. 8.8). The excellent data approximation (almost 100% of the

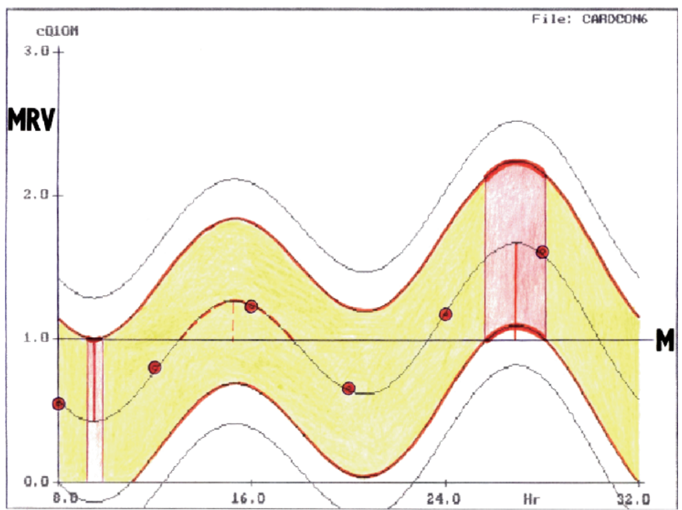

Fig. 8.8 Six measurements (dots), given as the Mesor Related Values (MRV, vertical axis) of the mitochondrial Q_{10} level in control rat myocardium related to the time of day and night. The parts of the 95% confidence corridor (yellow), nonoverlapping the mesor horizontal line (M), are red-shadowed, with the middle times of significant local acme or nadir marked by the corresponding red straight lines. The broader corridor belongs to the 95% tolerance

total variance is explained by the regression) is clearly reflected in the fact that all measurements (Fig. 8.8) are situated on the bimodal curve of the point estimate of the approximating function. There are two significant departures of the 95% confidence corridor from the mesor straight line visible: first, a significant depression occurred in the morning (between 9 and 10 a.m.) with the nadir depth of 0.57 mesor, and second, there was a significant elevation in the night (between 2 and 4 a.m.) with the acme height of almost one (0.68) mesor.

8.3.1.2 Mesor Related Values (MRV) in Diabetic Animals

In diabetic rats, the rhythmicity pattern is rather similar to that in controls but more apparent, being present in either coenzyme Q. Figure 8.9. shows the result for coenzyme Q_9. Between 5 and 9 p.m., a significant depression with the nadir depth of 0.70 mesor happened, followed by significant elevation between 0 and 4 a.m. with the acme height of 0.70 mesor. The chronobiologic pattern is quite similar for Q_{10} (Fig. 8.10). One short period of mild depression (0.24 of mesor) is only added between 8 and 9 a.m. A deep depression (0.87 of mesor) follows between 4 and 10 p.m., and finally a high elevation (0.92 of mesor) starting before midnight and ending before 6 a.m. closes the circadian chronogram. The coefficient of determination testifies also to an excellent fitting of the data (97 and 99%, respectively, of the total variance explained by the regression).

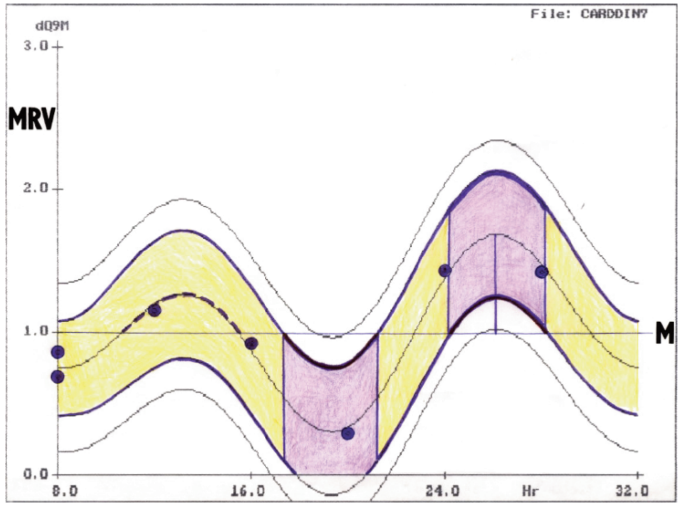

Fig. 8.9 Analogy of Fig. 8.8 for Q_9 mitochondrial level in the myocardium of diabetic rats

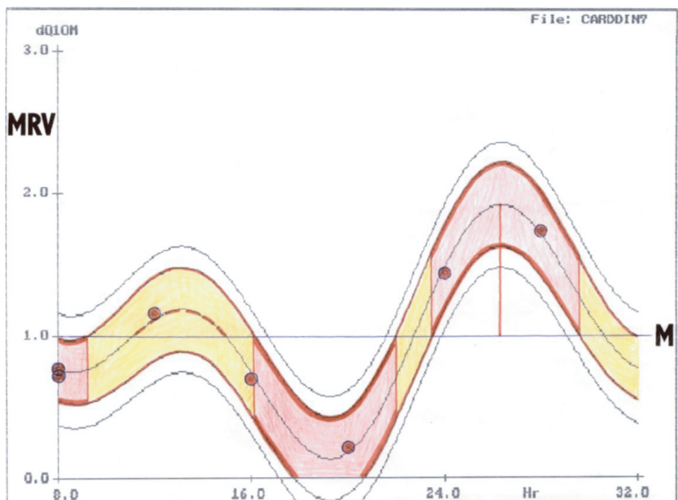

Fig. 8.10 Analogy of Fig. 8.9 for Q_{10}

8.3.1.3 Baseline Mesor Related Differences Between Diabetic Minus Control Measurements

Only the differences for Q_{10} appear as statistically significantly different from zero (Fig. 8.11). Nevertheless, this is true only for the time specified evaluation, showing significant elevation between 9 and 12 a.m. (acme height of 0.36 of the baseline mesor) and significant depression between 2 and 9 p.m. (nadir depth of 0.70).

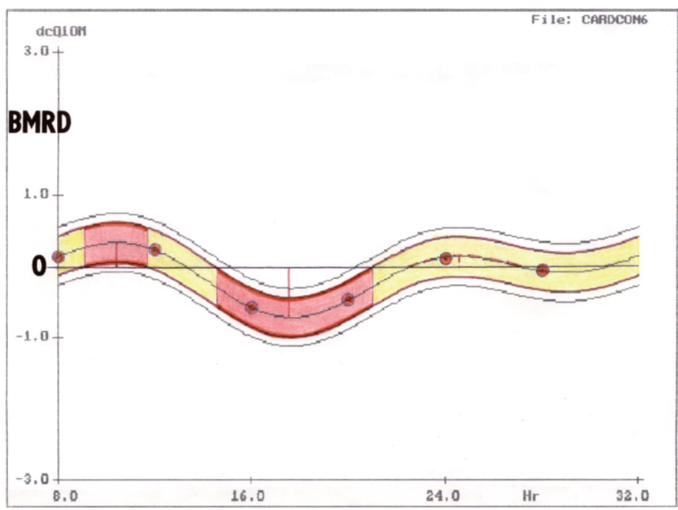

Fig. 8.11 Differences between the Q_{10} levels in diabetic minus control rats, as transformed to Baseline Mesor Related Differences (BMRD, vertical axis), processed analogically as the Mesor Related Values (MRV) in Fig. 8.8

In other words, the diabetic disturbance stimulates slightly the energetic turnover from morning to noon, and decreases it more deeply in the afternoon and early evening. The advantage of the chronobiometric approach is documented by the fact that the global evaluation – through the whole 24-h interval – shows no significant effect: the estimated mesor of the differences is –0.101 with the 95% confidence interval –0.223 and +0.022. This is clear as both the elevation and depression were mutually cancelled. The total variance in the data is fully – 100% – explained by the regression.

8.3.2 Cascade of Oxidative Phosphorylation: Complex I

8.3.2.1 Mesor Related Values (MRV) in Control Animals

Of the four variables (S_4 – basal respiration, OPR – oxidative phosphorylating rate, S_3 – with ADP stimulated respiration, ADP:O – coefficient of oxidative phosphorylation) only three (S_3, OPR, ADP:O) display significant circadian waving. For the parameter S_3, only one significant change – elevation between 11 p.m. and 2 a.m., with the acme height of 0.53 mesor – was identified. A similar pattern is presented by the parameter OPR, only the acme is higher – 0.65 of the mesor. A

quite different result was obtained for the ADP:O: the low elevation (acme of 0.12 of the mesor) lasted from 7 to 11 a.m. The explanation of the variance in the three significant cases is rather fair – between 71% and 78%.

8.3.2.2 Mesor Related Values (MRV) in Diabetic Animals

Here only two parameters – S_4 and ADP:O – do significantly cycle. In either of them, the circasemidian rhythm is prevailing. In the approximated chronogram for $S_{4,}$ the significant morning elevation with the acme height of 0.49 mesor between 6 and 11 a.m. is followed by two depressions, the first one (nadir depth 0.35 of the mesor) being situated between 2 and 5 p.m., and the second (nadir depth 0.25 of the mesor) around 2 a.m. A typically bimodal chronogram was obtained for ADP:O, with the early morning (6 a.m.) – to-noon significant light depression (0.27 of the baseline mesor), followed by two mutually similar significant low elevations - the first (0.08 of the mesor) between 2 and 5 p.m. and the second (0.12 of the mesor) between midnight and 5 a.m.). The variance is explained on 84 and 94%.

8.3.2.3 Baseline Mesor Related Differences Between Diabetic Minus Control Measurements

In spite of the fact that of the eight cases related to the Complex I circa(semi)dian chronograms only five yielded significant cycling, all their four differences displaying significant cyclic behavior. Thus for the difference between both parameter values of S_3, one significant night trough (nadir depth 0.52 of the baseline mesor) starting shortly before 9 p.m. and ending shortly after 6 a.m. was found. The global effect, neglecting the time specified excursions, is the significant depression due to the presence of diabetes: the estimates of the mesor differences are −0.195, with 95% confidence interval between −0.292 and −0.099. A similar result (nadir depth 0.62 of the baseline mesor) with the late evening (starting after 9 p.m.) and night trough (ending at 3 a.m.) was encountered for the variable OPR (Fig. 8.12). The global depression is reflected in the estimates of the mesor of differences: −0.266 with 95% confidence between −0.447 and −0.086. The parameter S_4 for the difference is cycling rather similarly as the same for diabetic rats: elevation (0.53 of the baseline mesor) from 5 a.m. to noon, then a short low elevation (0.22 of the baseline mesor) between 7 and 8 p.m. and a short depression (0.19 mesor) between 1 and 2 a.m. The global effect is here positive, i.e. increase by diabetes: estimates of the mesor of differences are +0.132 with +0.037 and +0.228 as its 95% confidence. Finally, the chronogram of the difference for ADP:O (Fig. 8.13) resembles that for ADP:O values in diabetic animals, but only the depression (0.33 of the baseline mesor) between 6 a.m. and noon is significant.

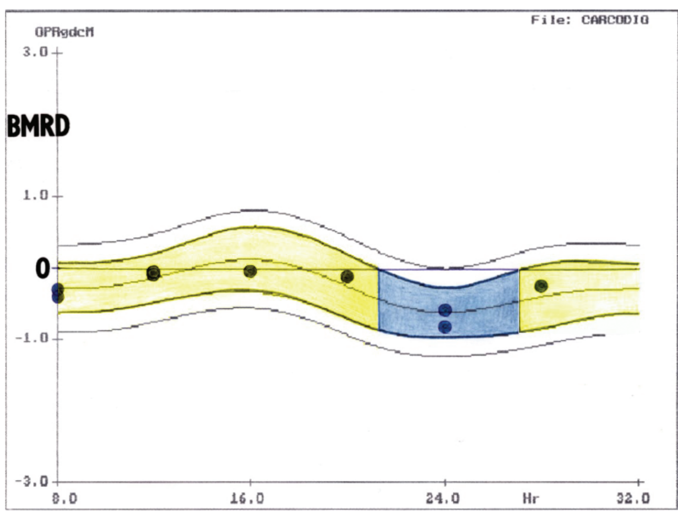

Fig. 8.12 Analogy of Fig. 8.11 for OPR, Complex I

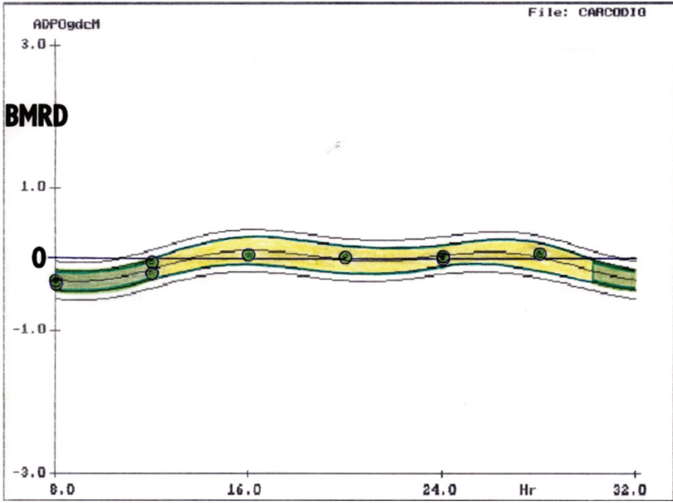

Fig. 8.13 Analogy of Fig.8.12 for ADP:O, Complex I

The whole chronobiologic pattern results in nonsignificant global decreasing effect of diabetes. The approximation of data by the regression function is good – between 77% and 93% of explanation.

8.3.3 Cascade of Oxidative Phosphorylation: Complex II

8.3.3.1 Mesor Related Values (MRV) in Control Animals

Here only one parameter - ADP:O behaves significantly cyclically. The first, morning acme (height 0.12 mesor) lasts from 9 a.m. almost to noon, followed by the afternoon trough (nadir depth 0.20) between 3 and 7 p.m..The second acme (height 0.15 of the mesor) is documented around midnight, from 10 p.m. up to 1 a.m. Of the total variance 87% were explained by the cosinor regression. Some similarity with the pattern seen in controls for the same parameter of Complex I can be traced.

8.3.3.2 Mesor Related Values (MRV) in Diabetic Animals

The results obtained for this combination are different from those described for Complex I. Now only one parameter has significant peaks and troughs, and it is again ADP:O. The first peak (acme height 0.10 of the mesor) has a short duration, starting shortly before 8 a.m. and ending at 9 a.m. It is followed soon by an early afternoon trough (nadir depth 0.10 of the mesor) between 1 and almost 3 p.m. The dominating significant excursion is the peak (acme height 0.23) located between 5 and 10 p.m. Also the second trough is large – with the nadir depth 0.22 of the mesor and duration from midnight to almost 5 a.m. The data are well fitted by this regression – 89% of the total variance are explained by deterministic changes.

8.3.3.3 Baseline Mesor Related Differences Between Diabetic Minus Control Measurements

Three of the four differences display significant time related excursions, only the first one – S_3 does not. The “delta” chronogram, i.e. that for the differences for OPR (Fig. 8.14) is rather similar to the same variable in Complex I: the most pronounced significant deviation from the mesor is the trough (nadir depth 0.57 of the baseline mesor) between 8 p.m. and 5 a.m. Another quite small and short depression (nadir 0.14 of the baseline mesor) happened around noon. The global effect is, of course, the significant depression: −0.194, with 95% confidence −0.265 and −0.122. The result for S_4 differs from that in Complex I clearly – the effect is now opposite, i.e. significant global depression (−0.104.with −0.193 and −0.015 as 95% confidence). There are two troughs here – the first one (nadir depth 0.33 of the baseline mesor) between 3 and 8 p.m., and the second one (nadir depth 0.20 of the baseline mesor) starting shortly before 6 a.m. and ending at 7 a.m. The last parameter – ADP:O (Fig. 8.15), displaying one peak and one trough, shows accordingly no significant global effect. The significant afternoon

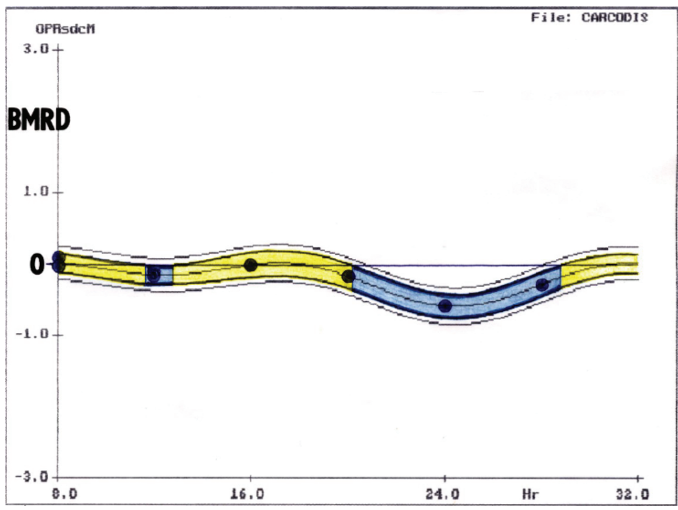

Fig. 8.14 Analogy of Fig. 8.12 for OPR, Complex II

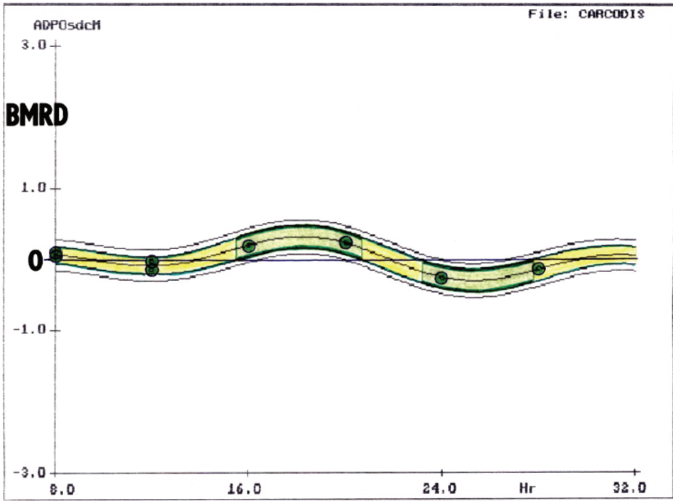

Fig. 8.15 Analogy of Fig. 8.13 for ADP:O, Complex II

and evening peak (acme height 0.33 of the baseline mesor) starts shortly before 4 p.m. and ends shortly before 9 p.m., while the significant night trough (nadir depth 0.30 of the baseline mesor) is obvious between 11 p.m. and 4 a.m. In all three cases, the approximation of data is very good – it explains 90–96% of their total variance.

8.3.3.4 Overview of the Obtained Results

Of two investigated parameters of coenzyme Q in controls, diabetic rats and their differences, covered a total of six tests of which four resulted in significant circadian chronograms. Of four investigated parameters of Complex I in controls, diabetic animals and their differences, involved in a total of twelve tests, nine were significantly cycling. In the same situation with Complex II, only five tests yielded significant results. The highest proportion of significant results – 100% – was obtained for ADP:O parameter, the lowest proportion – 0% – was encountered with S_3 from Complex II. A complete overview of the results is given in Tables 8.3 and 8.4. Ultimately, Fig. 8.16 shows the sequence of the significant and nonsignificant positive peaks for either coenzyme Q and for the four OXPHOS parameters of Complex I. An analogical presentation for Complex II is not shown due to the low number of significant peaks.

Table 8.3 Summary of results obtained by the synthetic procedure

Parameter	Control	Diabetes	Difference	Of the total variance explained
Q_9	0	− +	0	93%, 97%, 72%
Q_{10}	− +	− **−** +	+ −	100%, 99%, 100%
Complex I, S_3	+	0	−	71%, 44%, 89%
Complex I, OPR	+	0	−	71%, 51%, 77%
Complex I, S_4	0	+ **−** −	**+** + −	56%, 84%, 93%
Complex I, ADP:O	+	− + **+**	−	78%, 94%, 88%
Complex II, S_3	0	0	0	72%, 54%, 60%
Complex II, OPR	0	0	− **−**	63%, 38%, 96%
Complex II, S_4	0	0	**−** −	59%, 62%, 90%
Complex II, ADP:O	+ − **+**	+ − **+** −	+ −	97%, 89%, 95%

No significant circa(semi)dian rhythm is marked by 0. The sequence of significant troughs and peaks is illustrated by corresponding symbols − and +. If there are more than one − or +, the lowest − is shown as **−** and the highest + is shown as **+**. The coefficient of determination is given successively for controls, diabetes, and difference in percentages of the total variance explained by the regression

Table 8.4 Summary of results obtained by the analytic procedure
Significant 24-h and 12-h components are marked by +, nonsignificant by 0. Significant global change is designated by + for increase and − for decrease, nonsignificant global change is designated by 0

	Control		Diabetes		Difference		
Parameter	24 h	12 h	24 h	12 h	24 h	12 h	Global change
Q_9	0	0	+	+	0	0	0
Q_{10}	0	+	+	+	+	+	0
Complex I, S_3	+	0	0	0	+	0	−
Complex I, OPR	+	0	0	0	+	0	−
Complex I, S_4	0	0	+	+	+	+	+
Complex I, ADP:O	+	0	+	+	+	+	0
Complex II, S_3	0	0	0	0	0	0	0
Complex II, OPR	0	0	0	0	+	+	−
Complex II, S_4	0	0	0	0	0	+	−
Complex II, ADP:O	+	+	+	+	+	+	0

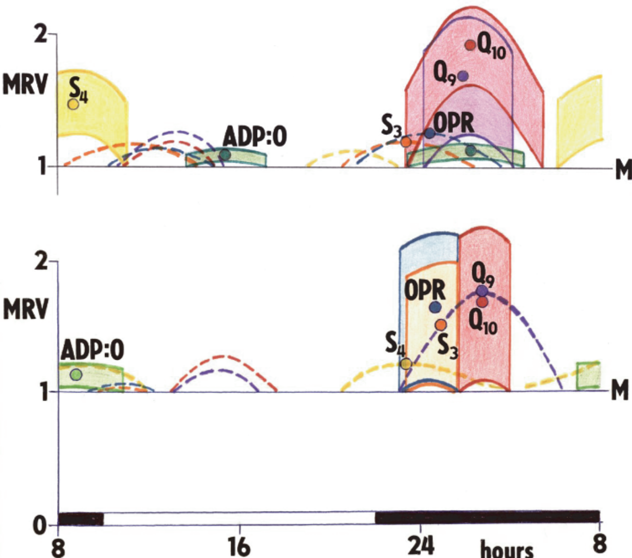

Fig. 8.16 The sequence of peaks for the Mesor Related Values (MRV) of Q_9, Q_{10} and four parameters of Complex I in diabetic (top) and control (bottom) rats. Significant peaking shown as shadowed corridor of the 95% confidence located above the mesor line, with full circles showing the point estimate (mean) of the acme. The point estimates of the approximating functions, located above the mesor, for nonsignificant elevations are shown by dashed lines, with the circles denoting peaks

From the point of view of the analytical approach, the significance of both 24 and 12 h rhythm can be interesting. It was found in diabetes for Q_9 as well as for Q_{10} and in the difference diabetes vs. controls only for Q_{10}. As to the cascade of oxidative phosphorylation, it was the case for Complex I in S_4 as well as ADP:O in the diabetes and the differences, and for Complex II in OPR with the differences, and in ADP:O for all three situations (control, diabetes, difference).

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Chapter 9
Mitochondrial Nephrology

Katarína Gazdíková and František Gazdík

Abstract Mitochondrial defects play an important role in the pathogenesis of tubular syndromes, interstitial nephritis, focal and segmental glomerulosclerosis and diabetic nephropathy. The role of mitochondria in the pathogenesis of nephrotoxicity and kidney carcinogenesis is also discussed.

Keywords Diabetic nephropathy, glomerulosclerosis, interstitial nephritis, mitochondrial nephropathy

9.1 Anatomy and Physiology of the Kidney

The kidneys (ren or nephros) are two essentially regulatory organs. In the adult, they weigh 120–300 g, are 10–12 cm long, 5–6 cm broad, and about 4 cm thick. The kidneys maintain the volume and composition of body fluid by filtration of the blood and selective reabsorption or secretion of filtered solutes. The kidneys are retroperitoneal organs (i.e. located behind the peritoneum), situated on the posterior wall of the abdomen on each side of the vertebral column, at about the level of the twelfth rib. The left kidney is slightly higher in the abdomen than the right, due to the presence of the liver pushing the right kidney down. The kidney is composed of an outer cortex and an inner medulla. The medulla is divided into 8–18 conical regions, called the renal pyramids; the base of each pyramid starts at the corticomedullary border and the apex ends in the renal papilla, which merges to form the renal pelvis and then on to form the ureter. In humans, the renal pelvis is divided into two or three spaces – the major calyces – which in turn divide into further minor calyces. The walls of the calyces, pelvis and ureters are lined with smooth muscle that can contract to force urine towards the bladder by peristalisis.

The kidneys take their blood supply directly from the aorta via the renal arteries; blood is returned to the inferior vena cava via the renal veins. The kidney is unique in that it has two capillary beds arranged in series, the glomerular capillaries, which are under high pressure for filtering, and the peritubular capillaries, situated around the tubule and at low pressure. This permits large volumes of fluid to be filtered and

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reabsorbed. Urine (the filtered product containing waste materials and water) excreted from the kidneys passes down the fibromuscular ureters and collects in the bladder (Fig. 9.1). The bladder muscle (the detrusor muscle) is capable of distending to accept urine without increasing the pressure inside; this means that large volumes can be collected (700–1,000 ml) without high-pressure damage to the renal system.

When urine is passed, the urethral sphincter at the base of the bladder relaxes, the detrusor contracts and urine is voided via the urethra.

The cortex and the medulla are made up of nephrons; these are the functional units of the kidney and each kidney contains about 1.3 million of them.

The nephron is the structural and functional unit of the kidney responsible for ultrafiltration of the blood and reabsorption or excretion of products in the subsequent filtrate. Each nephron is made up of (Fig. 9.1):

- The glomerulus – a filtering unit. As blood is filtered through this sieve-like structure, 125 ml/min of filtrate is formed by the kidneys. This filtration is uncontrolled.
- The proximal convoluted tubule. Controlled absorption of glucose, sodium, and other solutes occurs in this region.
- The loop of Henle. This region is responsible for the concentration and dilution of urine by utilizing a *counter-current multiplying* mechanism. Basically, it is water impermeable but it can pump sodium out, which in turn affects the osmolarity of the surrounding tissues and affects the subsequent movement of water in or out of the water-permeable collecting duct.
- The distal convoluted tubule. This region is responsible, along with the collecting duct, which it joins, for absorbing water back into the body – simple maths will

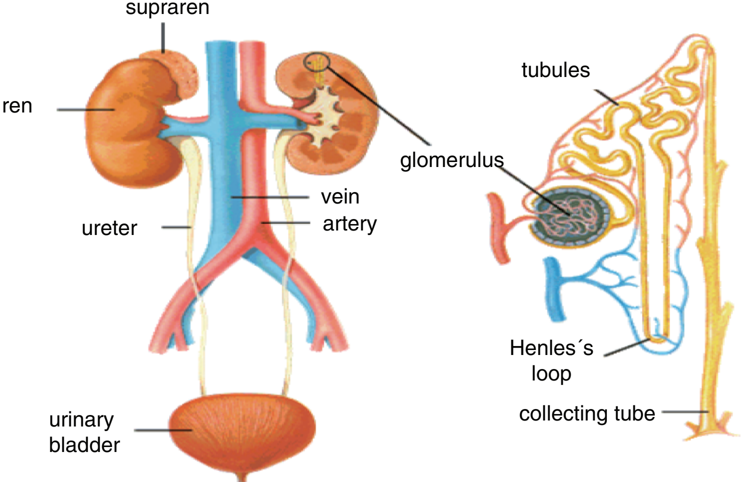

Fig. 9.1 Anatomy of urinary organs. (Redrawn from www.biowebgym.szm.sk)

tell you that the kidney does not produce 125 ml of urine every minute. Virtually 99% of the water is normally reabsorbed, leaving highly concentrated urine to flow into the collecting duct and then into the renal pelvis [1, 2, 41].

9.2 Physiological Function of the Kidney

Urine is formed as a result of a three-phase process: (1) simple filtration, (2) selective and passive reabsorption, (3) excretion.

9.2.1 Filtration

Filtration takes place through the semipermeable walls of the glomerular capillaries, almost impermeable to proteins and large molecules. The filtrate is thus virtually free of protein and contains no cellular elements. The glomerular filtrate is formed by squeezing fluid through the glomerular capillary bed. The driving hydrostatic pressure (head of pressure) is controlled by the afferent and efferent arterioles and provided by arterial pressure. About 20% of renal plasma flow is filtered each minute (125 ml/min). This is the glomerular filtration rate (GFR).

In order to keep the renal blood flow and GFR relatively constant, hydrostatic pressure in the glomerulus has to be kept fairly constant. When there is a change in arterial blood pressure, there is constriction or dilation of the afferent and efferent arterioles, the muscular walled vessels leading to and from each glomerulus. This process is called autoregulation.

Autoregulation of GFR can be achieved by autoregulation of renal blood flow and a feedback mechanism known as “glomerular tubular balance”. When there is a decrease in GFR, there is a resulting decrease in the fluid flow rate within the tubule.

At the loop of Henle, there is greater time for reabsorption of sodium and chloride ions. Thus a decrease in the number of sodium and chloride ions reaching the distal tubule occurs, detected by the macula densa. This in turn decreases the resistance in the afferent arteriole, resulting in an increase in renal blood flow. It also increases the release of renin from the juxtaglomerular apparatus stimulating angiotensin II production and thus causing constriction of the efferent arteriole. These events result in increase of hydrostatic pressure in the glomerular capillary bed and return GFR to normal.

The juxtaglomerular complex consists of macula densa cells, which are special distal tubular epithelial cells. They detect chloride concentration, modified smooth muscle cells and juxtaglomerular cells in the walls of the afferent and efferent arteriole. These cells produce renin. Renin is an enzyme converting the plasma protein angiotensinogen to angiotensin I. Angiotensin converting enzyme (ACE) is formed in small quantities in the lungs, proximal tubule and other tissues. It converts

angiotensin I to angiotensin II, causing vasoconstriction and an increase in blood pressure. Angiotensin II also stimulates the adrenal gland to produce aldosterone, which causes water and sodium retention and together they increase the blood volume. This is a negative feedback system. In other words, the initial stimulus is a fall in blood volume, which leads to a fall in perfusion pressure in the kidneys. When blood volume, renal perfusion and GFR improve, the system feeds back to switch off or turn down the response to the stimulus.

9.2.2 Selective and Passive Reabsorption

The function of the renal tubule is to reabsorb selectively about 99% of the glomerular filtrate. The proximal tubule reabsorbs 60% of all solute, including 100% of glucose and amino acids, 90% of bicarbonate and 80–90% of inorganic phosphate and water. Reabsorption occurs by either active or passive transport. Active transport requires energy to move the solute against an electrochemical or a concentration gradient. It is the main determinant of oxygen consumption by the kidney. Passive transport represents the reabsorption occurring down an electrochemical, pressure or concentration gradient. Most of the solute reabsorption is active, with water being freely permeable and therefore moving by osmosis. In the case of active reabsorption of solute from the tubule, there is a fall in concentration and hence osmotic activity within the tubule. Water then moves because of osmotic forces to the area outside the tubule where the concentration of solutes is higher.

The loop of Henle is the part of the tubule which dips or "loops" from the cortex into the medulla (descending limb) and then returns to the cortex (ascending limb). It is this part of the tubule where urine is concentrated if necessary. This is possible because of the high concentration of solute in the substance or interstitium of the medulla. This high concentration of solutes is maintained by the counter current multiplier. A counter current multiplier system is an arrangement through which the high medullary interstitial concentration of solute is maintained, giving the kidney the ability to concentrate urine. The loop of Henle is the counter current multiplier and the vasa recta is the counter current exchanger, the mechanism described below.

9.2.3 Actions of Different Parts of the Loop of Henle

A The descending loop of Henle is relatively impermeable to solute but permeable to water so that water moves out by osmosis and the fluid in the tubule becomes hypertonic.

B The thin section of the ascending loop of Henle is virtually impermeable to water, but permeable to solute, especially sodium and chloride ions. Thus sodium and chloride ions move out down the concentration gradient, the fluid

within the tubule becomes first isotonic then hypotonic as more ions leave. Urea absorbed into the medullary interstium from the collecting duct diffuses into the ascending limb. This keeps the urea within the interstitium of the medulla and thus it plays also a role in concentrating urine.

C The thick section of the ascending loop of Henle and early distal tubule are virtually impermeable to water. However sodium and chloride ions are actively transported out of the tubule, making the tubular fluid very hypotonic.

The vasa recta are a portion of the peritubular capillary system entering the medulla where the solute concentration in the interstitium is high. They act with the loop of Henle to concentrate the urine by a complex mechanism of counter current exchange. If the vasa recta did not exist, the high concentration of solutes in the medullary interstitium would be washed out.

Solutes diffuse out of the vessels conducting blood towards the cortex and into the vessels descending into the medulla, while water does the opposite, moving from the descending vessels to the ascending vessels. This system allows solutes to recirculate in the medulla and water, in effect, to bypass it.

The final concentration of urine depends upon the amount of antidiuretic hormone (ADH) secreted by the posterior lobe of the pituitary. When ADH is present, the distal tubule and the collecting duct become permeable to water. As the collecting duct passes through the medulla with a high solute concentration in the interstitium, the water moves out of the lumen of the duct and concentrated urine is formed. In the case of absence of ADH, the tubule is minimally permeable to water and large quantities of dilute urine are formed.

There is a close link between the hypothalamus of the brain and the posterior pituitary. There are cells within the hypothalamus, osmoreceptors, which are sensitive to changes in osmotic pressure of the blood. If there is low water intake, there is a rise in osmotic pressure of the blood, and after excess intake of water, the reverse occurs. Nerve impulses from the hypothalamus stimulate the posterior pituitary to produce ADH when the osmotic pressure of the blood rises. As a result, water loss in the kidney is reduced by ADH secreted and water reabsorbed in the collecting duct [3].

The kidneys perform three crucial functions: (a) exocrine – excretion of substances soluble in water; (b) regulation – role in exchange of water, inorganic ions, acid base balance, glucose; (c) endocrine – production of hormones with influence on the kidney and other tissues.

9.2.3.1 Regulation of the Water and Electrolyte Content of the Body

The kidneys allow a person to eat and drink according to his/her habits without changing the composition of the fluid compartments.

Renal blood supply is normally about 20% of the cardiac output. Approximately 99% of the blood flow goes to the cortex and 1% to the medulla. The cortex is the outer part of the kidney containing most of the nephrons. The medulla is the inner part of the

kidney containing specialized nephrons in the juxta-medullary region, in the immediate proximity of the medulla. These nephrons have a greater concentrating ability.

9.2.3.2 Acid/Base Function

The lungs and kidneys work together to produce normal extracellular fluid and arterial pH of 7.35–7.45 (34–46 nmol.l^{-1} H^+ concentration). Carbon dioxide (CO_2), when dissolved in blood is an acid and is excreted by the lungs.

The kidney excretes about 100 mmol hydrogen ions (H^+) daily to prevent the developement of nephrogenic metabolic acidosis (nMAC). Less than 1% is excreted as a H^+. One third of H^+ is excreted bound to anions (titrable acidity) and two thirds bound in ammonium (NH_4^+). In case of kidney function impairment, the ammonium excretion decreases while titrable acidity either does not change or even increases, through increased NH_4^+ excretion by the residual nephrons (main adaptive mechanism to save acid-base homeostasis [77].

9.2.3.3 The Kidney Excretes Fixed Acid and Performs Three Functions to Achieve this

1. Tubular secretion of acid:
   The buffer sodium bicarbonate is filtered by the glomerulus and reabsorbed in the proximal tubule. The sodium is absorbed by a sodium/hydrogen ion pump (Na^+/H^+) exchanging Na^+ for H^+ on the luminal proximal border of the tubular cell. A sodium/potassium pump (Na^+/K^+) forces Na^+ through the cell from tubular fluid in exchange for potassium.
2. Glomerular filtration of buffers combined with H^+:
   The majority of the filtered bicarbonate is reabsorbed (90% in the proximal tubule). The H^+, released as the tubular secretion of acid (above), forms carbonic acid with the bicarbonate (HCO_3^-)

$$H^+ + HCO_3^- <=> H_2CO_3 <=> H_2O + CO_2$$

   Carbonic anhydrase, found in the proximal tubular cells, catalyzes the reaction to carbon dioxide (CO_2) and water (H_2O). The CO_2 diffuses into the cell where it again forms carbonic acid in the presence of carbonic anhydrase. The carbonic acid ionizes to H^+ and HCO_3^-. The H^+ is then pumped out of the cell back to the lumen of the tubule by the Na^+/H^+ pump (see 1 above) and the sodium is returned to the plasma by the Na^+/K^+ pump (see 1 above). Water is absorbed passively.
   Other buffers include inorganic phosphate (HPO_4^-), urate and creatinine ions excreted in urine as acid, in the case of combination with H^+ ions secreted in the distal nephron.

3. Ammonium is produced enzymatically from glutamine and other amino acids, and is secreted in the tubules. Ammonium (NH_3) combines with secreted H^+ ions to form a nondiffusible ammonium ion (NH_4^+) excreted in the urine. Ammonium production is increased by severe metabolic acidosis to as much as 700 mmol. day^{-1}.

Ammoniumgenesis is under the control of pH. Moreover, several hormones participate in its regulation. Angiogensin II, parathormone, glucocorticosteroids, aldosterone, growth hormone and insulin increase ammoniumgenesis and decrease $PGF_{2\alpha}$. The regulation is rather complex and depends on several biochemical pathways.

(a) P-dependent glutaminase – various parts of nephrons contain P-dependent glutaminase with the highest activity and adaptability in proximal tubular cells; glutamine (Gln), resorbed from the primary urine but also taken up from the interstitium, enters mitochondria where it is deaminated by P-dependent glutaminase and the produced glutamate is further deaminated to 2-oxo glutarate by glutamate dehydrogenase (Fig. 9.2). The limiting steps of this pathway appear to be Gln cytoplasmic and mitochondrial transmembrane transport and particularly P-dependent glutaminase activity and expression, all of them stimulated during MAC in proximal tubular cells. Another important limiting step is 2-oxo glutarate concentration. If 2-oxo glutarate is oxidized in he citric acid cycle (lack of ATP), the ammonium production is not limited [88]. In the case

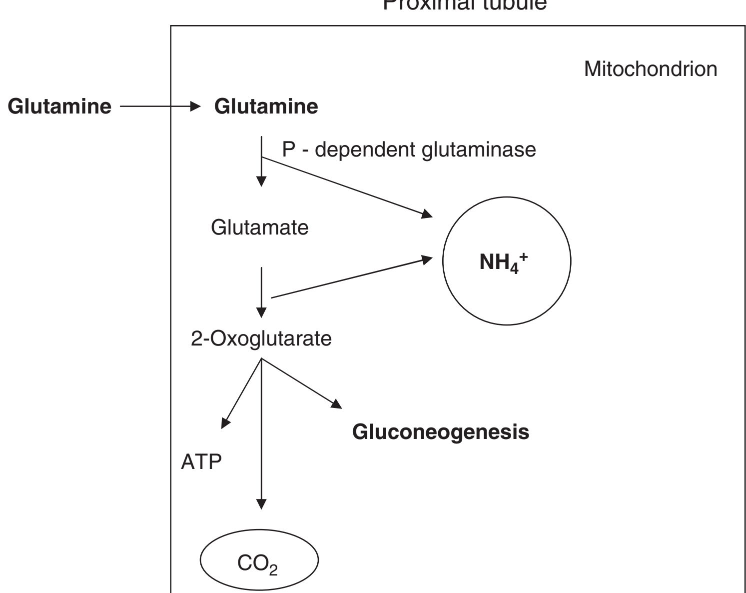

Fig. 9.2 Regulation of ammonium production in the kidney

of sufficient amount of ATP, 2-oxo glutarate enters the gluconeogenesis pathway. Simultaneous expression of P-dependent glutaminase and P-enolpyruvate carboxykinase mRNA was demonstrated [74].

(b) P-independent gutaminase is identical with gamma-glutamyl transferase/transpeptidase, localized at the luminal and basal membrane of proximal tubular cells and participating in the resorption of amino acids in the gamma-glutamyl cycle [43]. The filtered Gln is deaminated to glutamate (Glu) and NH_4^+ is formed intraluminally. Hippurate stimulates the activity of P-independent glutaminase [83]. The originating Glu is either resorbed from the lumen, excreted in urine, or it could be amidated to Gln (kidney glutamine cycle) (Fig. 9.3). From 5% to 48% of ammonium is produced by this pathway [11, 75].

(c) Glutaminase II transaminates Gln to 2-oxo glutaramate deaminated to 2-oxo glutarate by omega-amidase (Fig. 9.4) [77].

(d) Hippurate was shown to be a donor of amino group for the production of ammonium. It is a very interesting pathway, suggested to be a minor pathway of ammonium production [57].

9.2.4 Excretion of Waste Products

Filtration occurs as blood flows through the glomerulus. Some substances not required by the body and some foreign materials (e.g. drugs) may not be cleared by

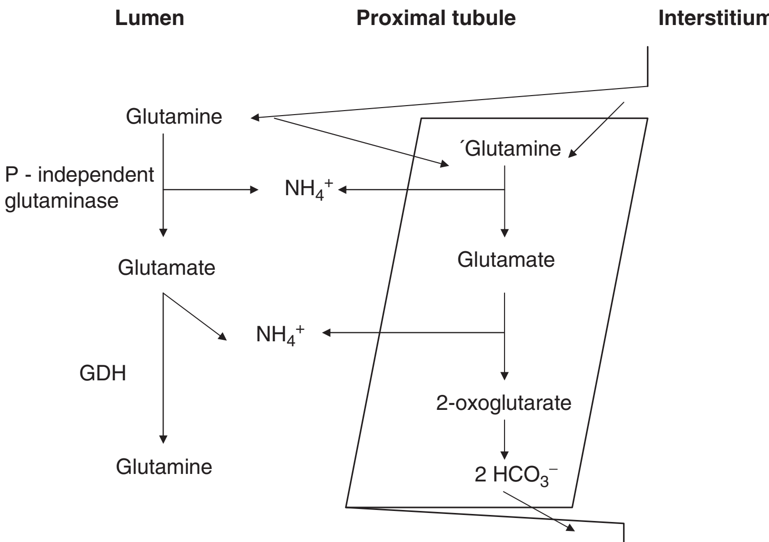

Fig. 9.3 Ammoniumgenesis in proximal tubule

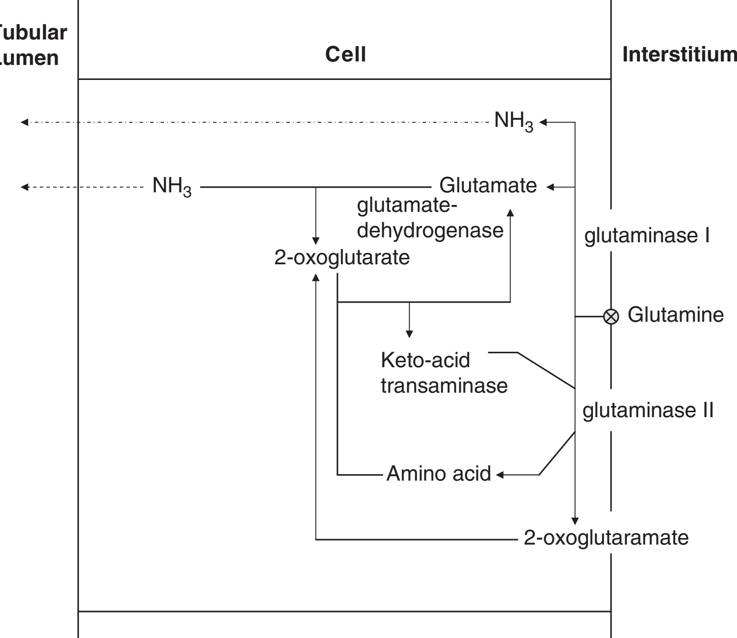

Fig. 9.4 Participation of glutaminase II in ammoniumgenesis

filtration through the glomerulus. Such substances are cleared by secretion into the tubule and excreted from the body in urine.

9.2.5 Hormones and the Kidney

1. Renin increases the production of angiotensin II released when there is a fall in intravascular volume, e.g. hemorrhage and dehydration. This leads to constriction of the efferent arteriole to maintain GFR by increasing the filtration pressure in the glomerulus. The subsequent effects are release of aldosterone from the adrenal cortex, increased release of ADH from the posterior pituitary, inotropic myocardial stimulation and systemic arterial constriction. The opposite occurs when fluid overload occurs.
2. Aldosterone promotes sodium ion and water reabsorption in the distal tubule and collecting duct where Na^+ is exchanged for potassium (K^+) and hydrogen ions by a specific cellular pump. Aldosterone is also released when there is a decrease in serum sodium ion concentration. This situation can occur, for example, when there are large losses of gastric juice. Gastric juice contains significant concentrations of sodium, chloride, hydrogen and potassium ions. Therefore it is

impossible to correct the resulting alkalosis and hypokalemia without first replacing the sodium ions using 0.9% saline solutions.

3. Atrial natriuretic peptide (ANP) is released when atrial pressure is increased, e.g. in heart failure or fluid overload. This situation promotes loss of sodium and chloride ions and water chiefly by increasing GFR.
4. Antidiuretic hormone (ADH) increases the water permeability of the distal tubule and collecting duct, thus increasing the concentration of urine. By contrast, when secretion of ADH is inhibited, it allows dilute urine to be formed. This occurs mainly when plasma sodium concentration falls, as following the intake of large quantities of water. This fall is detected by the osmoreceptors (above). When blood loss or dehydration occurs, the hormones interact to maintain intravascular volume.
5. Erythropoietin (Epo), produced by the kidney in the adult and by the liver in the fetus, increases the number of red blood cells by supporting the survival of erythroid progenitor cells and stimulating their differentiation and proliferation *via* binding to Epo receptor (EpoR). The main signal in the control of Epo production is oxygen; hypoxia stimulates Epo production through activation of Epo gene transcription. Tremendous progress in our understanding of molecular mechanisms of Epo action on erythroid cells and regulation of Epo production has been made by manipulation of cDNAs and genes of Epo and EpoR [72].

9.2.6 Other Substances Produced by the Kidney

1. The most active form of vitamin D, 1,25 dihydroxy vitamin D (calcitriol) is promoting calcium absorption from the gut. The vitamin D endocrine system plays an essential role in calcium homeostasis and bone metabolism, but research during the last two decades has revealed a diverse range of biological actions including induction of cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems. Vitamin D itself is a prohormone metabolically converted to the active metabolite, 1,25-dihydroxy vitamin D [$1,25(OH)_2D$]. This vitamin D hormone activates its cellular receptor (vitamin D receptor or VDR), which alters the transcription rates of target genes responsible for the biological responses. The final step in the activation of vitamin D, conversion of 25-hydroxyvitamin D to $1,25(OH)_2D$ in renal proximal tubules, is now known to involve facilitated uptake and intracellular delivery of the precursor to 1α-hydroxylase. Evidence emerging on using mice lacking the VDR and/or 1α-hydroxylase indicates both $1,25(OH)_2D_3$-dependent and -independent actions of the VDR as well as VDR-dependent and -independent actions of $1,25(OH)_2D_3$. Thus the vitamin D system may involve more than a single receptor and ligand. The presence of 1α-hydroxylase in many target cells indicates autocrine/paracrine functions for $1,25(OH)_2D_3$ in the control of cell proliferation and differentiation.

This local production of 1,25$(OH)_2D_3$ is dependent on circulating precursor levels, providing a potential explanation for the association of vitamin D deficiency with various cancers and autoimmune disease [16].

2. Prostaglandins (PG) are derived from the metabolism of arachidonic acid with the initial step being catalyzed by the enzyme cyclooxygenase (COX). These hormones are produced at a variety of sites within the kidney, including glomerular and vascular endothelium, the medullary and to a lesser degree the cortical collecting tubule cells, and the renomedullary interstitial cells. In general, the tubules primarily synthesize PGE2, while the glomeruli produce both PGE2 and prostacyclin. The renal PG have important local functions but little systemic activity since they are rapidly metabolized in the pulmonary circulation [5].

9.3 Mitochondria and the Kidney

Mitochondria are the subcellular organelles performing many functions in cells. For mitochondrial nephrology two mitochondrial functions are dominant:

Mitochondrial respiratory chain: Oxidative phosphorylation is located on the inner mitochondrial membrane. By oxidation of pyruvic acid, fatty acid, ketoacid, which the kidney intensively eliminates, and amino acid in Krebs'cycle of citric acid, the respiratory chain provides oxidative processes and oxidative phosphorylation with production of adenosinetriphosphate (ATP). Respiration and oxidative phosphorylation is provided by five complexes. Complex I: NADH-ubiquinone oxidoreductase provides transport of electrons from NADH to ubiquinone. Complex II: Succinate dehydrogenase-CoQ oxoreductase transfers H^+ from $FADH_2$ to coenzyme Q. Complex III: Reduced coenzyme Q-cytochrome c reductase tranfers H^+ from coenzyme Q to cytochrome c. Complex IV: Cytochrome c oxidase transfers H^+ from cytochrome to oxide. Complex V: By oxidative phosphorylation, ATP synthase utilizes oxide and phosphorylases ADP to ATP. The complexes utilize some coenzymes, including coenzyme Q_{10} (CoQ_{10}), which is utilized in two complexes [17].

Potential sources of superoxide and peroxide are Complexes I and II, situated near cytochrome b of the respiratory chain (total reduced form of coenzyme Q) [85]. Some authors excluded ubisemiquinone from participation in the production of superoxide anion radical [66]. On the other hand, De Jong et al. [12] marked semiquinone as a site of its production. Cytochrome c peroxidase and Mn SOD perform a crucial role in the detoxication of reactive forms of oxide produced in mitochondria. The outer mitochondrial membrane of rat heart cells dependent on NADH is another site of superoxide production. This is a better source of superoxide than the respiratory chain. Aminooxidases, able to produce peroxides, are located here [17]. Mitochondrial activity is independent of mitochondrial or nuclear genome. It is hormonally regulated. In experimental studies on animals, glucocorticosteroids increased the number and activity of mitochondria [14]. To date it is still unclear how respiratory activity is influenced by hormones or diseases.

A further crucial role of mitochondria in nephrology is *ammonogenesis*. In patients with mitochondrial dysfunction deamination is damaged and in patients suffering from renal tubular acidosis type II [47] the production of ammonium is decreased [7].

9.4 Deficiency of Coenzyme Q_{10} and Kidney Disease

Oxidative stress and decrease of antioxidative capacity participate in the progression and complications of renal diseases. Many data have been collected on oxidation stress in dialysis patients, however a shortage of information is evident in conservatively treated patients. CoQ_{10} is an important antioxidant. It is a vital lipophylic molecule that transfers electrons from mitochondrial respiratory chain Complexes I and II to Complex III. It plays a key role in the synthesis of adenosinetriphosphate and its beneficial effect was demonstrated in several diseases.

In our studies we reported decreased levels of plasma CoQ_{10} and increased level of malondialdehyde in conservatively treated patients with kidney disease. Supplementation with some antioxidants (CoQ_{10}, vitamin C and E) led to increased plasma levels of antioxidants [21–23]. Some authors reported low levels of CoQ_{10} and increased peroxidative damage in uremic (hemodialytic) patients [50, 51, 84]. Lopez et al. [54] decribed the case of an infant with severe Leigh's syndrome, nephrotic syndrome and CoQ_{10} deficiency in muscles and fibroblasts and compound heterozygous mutations in the PDSS2 gene, which encodes a subunit of decaprenyl diphosphate synthase, the first enzyme of the CoQ_{10} biosynthetic pathway. In a 33-month-old male corticosteroid-resistant nephrotic syndrome with progressive encephalomyopathy was diagnosed. In this patient, CoQ_{10} in muscles and fibroblasts was decreased. Oral administration of CoQ_{10} improved the neurologic picture but not the renal dysfunction [70]. In a 9-year-old-girl, mitochondrial cytopathy with steroid-resistant FSGS and dilated cardiomyopathy was reported. Skeletal muscle biopsy showed respiratory chain defect and partial deficiency of CoQ_{10}. In this case, a novel mutation of the mitochondrial tRNA (Tyr) gene was recognized in homoplastic stage in skeletal muscle, blood, and renal tissue [73].

9.5 Experimental Studies of Mitochondrial Nephrology

Haguenauer et al. [31] indentifed and cloned a new member of the mitochondrial carrier family transporting a variety of metabolites across the inner mitochondrial membrane KMCP1 – kidney mitochondrial carrier protein 1, highly homologous to the previously identified protein BMCP1 – brain mitochondrial carrier protein 1. This carrier is expressed predominantly within the kidney cortex in the proximal and distal tubules. KMCP1 is increased during fasting and during the regenerative phase of glycerol-induced renal failure. Both situations are associated with transiently increased expression of superoxide-generating enzymes followed by

increased mitochondrial metabolism and antioxidant defenses. KMCP1 is involved in situations in which mitochondrial metabolism is increased, in particular when the cellular redox balance tends toward a pro-oxidant status. This carier may involve a shift of carbohydrate to lipid metabolism and/or protection from oxidative damage in situations of increased mitochondrial metabolism [31].

In an experimental study, mitochondria of hypertrophied proximal tubular cells were observed to undergo true hypertrophy and no proliferation after unilateral nephrectomy [37].

The kidney is the organ critically affected by prolonged exposure to cadmium. Takaki et al. [81] found that long-term cadmium exposure in rats accelerated accumulation of 4834-bp mitochondrial DNA deletions and impairment of mitochondrial function associated with accumulation of oxidized product.

There are increasing literary data on some new forms of mitochondrial mutations and kidney disease, for example in patients with hypertension or cystinuria.

Hypertension – the mitochondrial genome has been suggested as a site of mutations possibly contributing to susceptibility to hypertension in black Americans who have progressed to end-stage renal disease (ESRD) [90].

Cystinuria – is a relatively common autosomal recessive disorder of the transport of cysteine and dibasic amino acids through the epithelium of the renal tubuli and the interstitial brush border. In a small group of patients, reduction of activities of the respiratory chain complex coded in mitochondria was detected (muscle biopsy). The molecular basis of this disorder is homozygous deletion of 179,311 bp on chromosome 2p16, which encodes the type I cystinuria gene (SLC3A1), the protein phosphatase 2Cβ gene (PP2Cβ), an unidentified gene (KIAA0436), and several expressed sequence tags. The deletion suggests that this unique syndrome is related to a complete absence of the products of these genes, one of which may be essential for the synthesis of mitochondrial encoded proteins. Clinical features were recessive inheritance, cystinuria, lactic acidosis, hypotonia [68].

9.6 Mitochondrial DNA Damage and Kidney Disease

ESRD is associated with enhanced oxidative stress. This disease state provides a unique system for investigating the deleterious effect of exogenous sources of free radicals and reactive oxygen species (ROS) on mitochondrial DNA. Diverse and multiple mitochondrial DNA deletions in skeletal muscles were found in patients with ESRD. These deletions are more prevalent and abundant in ESRD patients compared with the normal population. Accumulation of uremic toxins and an impaired free radical scavenging system may be responsible for the increased oxidative stress in ESRD patients. Such stress may result in oxidative damage and aging-associated mutation of the mitochondrial genome [49].

Increased DNA damage was noted in patients with ESRD. Decrease in the capability of DNA repair in nuclear DNA has been demonstrated in UV-irradiated

lymphocytes of patients with ESRD with or without hemodialysis [20]. Increased incidence of structurally abnormal chromosomes and elevated rates of sister chromatid exchange were also found in patients with end-stage renal disease [9]. Increase of DNA instability may be attributed to accumulation of mutagenic or carcinogenic heterocyclic amines in the dialysate or in plasma of patients with ESRD. These genotoxic substances may induce DNA mutation and promote high mortality or cancer in ESRD patients [69].

As compared with nuclear DNA, human mitochondrial DNA is much more susceptible to damage induced by mutagens or carcinogens as the result of a lack of proofreading and poor DNA repair during mitochondrial DNA replication [91]. The incidence of the 4977 bp mtDNA deletion of hair follicles is not only a biomarker of aging but also an index of the genomic damage in cigarette smokers and may be considered one of the molecular events linked with the occurrence of smoking-associated cancers [52]. Liu et al. demonstrated that ESRD is an add-on factor in promoting mitochondrial DNA mutation in human hair follicles. The predominant 4977 bp deletion of mtDNA may imply the existence of genetic instability in the mitochondrial genome of ESRD patients, and this common deletion may serve as one of the biomarkers for the study of genotoxicity of the uremic milieu in patients with ESRD [53].

9.7 Mitochondrial Disease

Mitochondrial diseases have hetergeneous clinical phenotypes. Organ systems most commonly involved are the central nervous system, skeletal muscles, cardiac conduction system, hematopoietic system, and pancreas, whereas the liver, endocrine glands and the kidney are affected infrequently and usually late in the course of the disease [80].

9.7.1 Mitochondrial Nephropathies

Mitochondrial nephropathies are a group of kidney diseases supported by mitochondrial dysfunction. Familial incidence and maternal inheritance are recorded in this form of diseases. Extrarenal abnormalities suggest a systemic disorder. Renal disease may be the first sign of mitochondrial cytopathy or it may appear together with neurological and neuromuscular manifestations.

Mitochondrial nephropathies can be divided into three groups:

1. Isolated involvement (e.g. Fanconi's syndrome)
2. Multisystemic involvement (e.g. Pearson's syndrome – refractory sideroblastic anemia, neutropenia, thrombocytopenia, proximal tubular dysfunction, hepatocellular and exocrine pancreatic dysfunction)

3. Mitochondrial involvement of another organ or system, which can induce classic form of nephropathy (e.g. diabetes mellitus with mitochondrial dysfunction of beta-pancreatic cells with diabetic nephropathy) [17]

Kidney involvement in mitochondrial encephalopathies, although infrequent and usually late in the course of disease, has been well documented. The most commonly observed abnormality is renal tubular dysfunction, associated with a wide variety of mitochondrial disorders. These include the Kears–Sayre syndrome, mitochondrial myopathy and Pearson's sydrome. Occasionally, patients may first present with renal abnormalities including the de Toni–Franconi–Debre syndrome, renal tubular acidosis, and defects mimicking Bartter's syndrome [18, 26, 36, 56, 87].

MELAS – mitochondrial encephalopathy with lactic acidosis and stroke-like episodes is one of a group of heterogeneous yet clinically distinct syndromes ascribed to a defect in mitochondrial function. In some patients with MELAS renal hypoxic injury and acute renal failure were observed [36]. Lactic acidosis in patients with mitochondrial encephalomyopathies is a consequence of impairment in oxidative metabolism of defective mitochondria. During normal metabolism, pyruvate is transported across the mitochondrial membrane into the mitochondrion to undergo oxidative decarboxylation to acetyl coenzyme A (CoA) by pyruvate

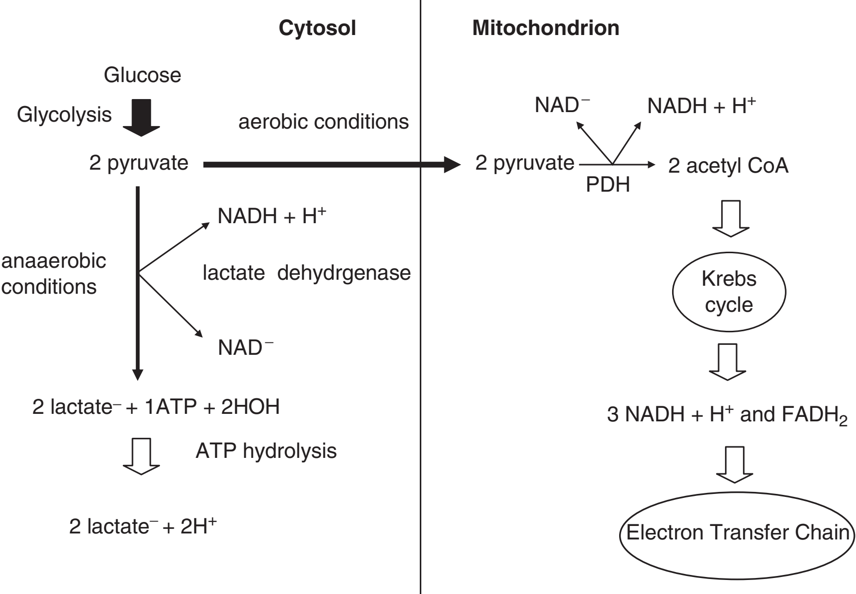

Fig. 9.5 Pathway for lactate production in mitochondrial disease. (Adapted from Hsieh et al. 1996 [36].) If pyruvate ultilization by the mitochondrion is impaired at the level of the electron transfer chain, NADH + H^+, generated by pyruvate dehydrogenase (PDH) and by the Krebs cycle, cannot be oxidized properly to generate ATP. As a result, acetyl-CoA and the NADH//NAD^+ ratio will increase, downregulating PDH. Any increased demand for ATP will drive the formation of lactate, precipitating lactic acidosis

dehydrogenase (Fig. 9.5). Acetyl CoA is shuttled into the Krebs cycle, which then contributes electrons to the electron transfer chain. Defects in Complex I (NADH coenzyme Q reductase), Complex III (reduced coenzyme Q-cytochrome c reductase) and Complex IV (cytochrome c oxidase) of the mitochondrial respiratory chain have been documented in patients with MELAS. Because of the impaired utilization of pyruvate in the Krebs cycle, excess NADH accumulates in the cytosol. Under anaerobic conditions, pyruvate is then reduced to lactate by lactate dehydrogenase and one stoichiometric equivalent of ATP is produced. The hydrolysis of ATP yields one equivalent of hydrogen ion, which must then be buffered by extracellular bicarbonate. Bicarbonate in consumed, lactate accumulates and lactic acidosis develops [36, 46, 78].

To date the following forms of mitochondrial nephropathies are known.

9.7.1.1 Fanconi's Syndrome

The commonest renal manifestation is proximal tubulopathy, resulting in a form of the de Toni–Debré–Fanconi syndrome (Table 9.1).The reason for the predominance of this form of renal disease is that ATP is essential to drive the sodium–potassium-ATPase pump. This generates the electrical gradient across the proximal tubular epithelium and maintains a low concentration of sodium in the cell compared with the outside. This gradient drives all the activities of the proximal tubular cell. All the other absorptive functions – for sugar, phosphate, amino acids, and so on – are handled through cotransporters. The de Toni–Debré–Fanconi syndrome includes urinary losses of amino acids, glucose, proteins, phosphate, uric acid, calcium, bicarbonate, potassium, sodium and water. The proximal tubulopathy is often moderate and several investigators have reported isolated hyperaminoaciduria. Other patients may have acidosis, hypophosphatemic rickets, hypercalciuria, glycosuria and tubular proteinuria. Renal biopsy shows nonspecific abnormalities of the tubular epithelium with dilatation and obstruction by casts, dedifferentiation or atrophy. Giant mitochondria are often observed. Extrarenal symptoms are always present, including myopathy, neurological symptoms, Pearson's syndrome, diabetes mellitus or cardiac problems. Tubular symptoms have been described before the age of two years in the majority of patients and more than 40% of them died during the first year of life [8]. Childern with tubulopathy of unknown origin or progressive renal disease should be investigated for the existence of mitochondrial disease [58].

Table 9.1 Features of Fanconi's syndrome

Renal glycosuria
Nephrogen (generalized) aminoaciduria
Renal calciuria
Renal phosphaturia
Proximal type of renal tubular acidosis (RTA II)

9.7.1.2 Focal and Segmental Glomerulosclerosis (FSGS)

FSGS represents the final common pathway for nephron degeneration in many forms of chronic progressive renal failure. This nephropathy is a most typical renal lesion in adult patients with A3243G mutation [29]. The initial pathologic changes in FSGS are thought to occur in glomerular epithelial cells. Some studies decribed association of FSGS with mitochondrial DNA mutation [92]. Several forms of familial FSGS have been reported, including those involving mutations in glomerular epithelial cytoskeleton components, as e.g. podocin and alpha-actinin 4 or structural components of the slit diaphragm, such as nephrin [6, 42, 44]. The mitochondrial DNA 3243A-G point mutation is regarded as another form of familial FSGS. This mutation has been observed in approximately 0.6–1.5% of patients with type 2 diabetes mellitus and in approximately 16.3/100,000 individuals in the general adult population [24, 55, 93]. The mtDNA 3243A-G point mutation may be the most frequent etiologic mutation in familial FSGS.

The pathogenesis of FSGS lesions with the mitochondrial DNA 3243A-G point mutation exibits some discrepancies. Mochizuki et al. [61] and Doleris et al. [15] reported that vascular smooth muscle cell injury attributable to mitochondrial damage led to arteriolar hyaline lesions, which abolished the autoregulatory mechanism for glomerular pressure; subsequent renal hemodynamic alterations might occur, resulting in FSGS lesions. In contrast, the study by Hotta et al. [35] and Yamagata et al. [92] demonstrated that abnormal mitochondria accumulated in glomerular epithelial cells leading to glomerular epithelial dysfunction, resulting ultimately in FSGS. More than 85% of mitochondrial DNA in analyzed kidney samples exhibited this point mutation.

This mutation progresses to end-stage renal failure and is associated with other clinical features, as short stature, severe headache, hearning loss, diabetes mellitus and hypertrophic cardiomyopathy [13].

In patients with mitochondrial diseases and renal complications abnormal mitochondria of podocytes were found, which may cause glomerular epithelial cell damage leading to glomerulosclerosis [28].

Renal epithelial cell damage attributable to mitochondrial dysfunction in congenital nephrotic syndrome of the Finnish type were decribed by Solin et al. [76]. In this study the authors observed a 30% decrease in mitochondrial DNA contents in patients with nephrotic syndrome compared with normal control samples.

Nephrotic syndrome of the Finnish type can be caused by deficiency of mitochondrial respiratory complexes II + IV [25]. Mitochondria play a major role in apoptosis [40]. Mitochondria-mediated apoptosis has been observed in rat hypertensive nephrosclerosis [94].

9.7.1.3 Tubulointerstitial Nephritis

This group of nephropathies includes patients without findings of Fanconi's syndrome and with histologic findings such as dilatation of Bowman's capsule,

tubular atrophy involving the medullary rays associated with interstitial fibrosis, sclerotis glomeruli and with mitochondrial abnormalities [27, 67, 80]. Mitochondrial DNA A to G mutation was detected in position 5656, possibly the causative factor of the aberrant mitochondria and renal disease [95]. The arteriolar lesions in patients with familial mitochondrial tubulointerstitial nephropathy with progressively impaired renal function may be explained by the suggestion that premature arterio(lo)sclerosis can result from an mtDNA mutation [67]. Tzen et al. described a new A608G mutation of mitochondrial genome in patients with tubulointerstitial nephritis and stroke [86].

The A to G mutation of mitochondrial $tRNA^{Leu(UUR)}$ was decribed by Hirano et al. in a 27-year-old woman with sensorineuronal deafness and renal dysfunction. Renal biopsy revealed many sclerotic glomeruli, advanced tubulointerstitial changes and numerous swollen mitochondria of the tubular cells [34].

9.7.1.4 Diabetic Nephropathy

The mitochondrial DNA 3243A-G point mutation has been observed in approximately 0.6–1.5% of patients with type 2 diabetes mellitus [93]. Guillausseau et al. reported that 28% of patients with type 2 diabetes mellitus and the mtDNA 3243A-G point mutation exibited kidney disease and renal histologic analyses demonstrated FSGS [30]. Mitochondrial diabetes may pre-dispone patients to renal complications, including forms of glomerulonephrithis, such as FSGS and can progress to renal insufficiency [38, 64, 79].

9.8 Diagnosis of Mitochondrial Nephropathies

Since the respiratory chain transfers NADH to oxygen, a disorder of oxidative phosphorylation results in an altered oxidoreduction status in plasma. This feature is a result of functional impairment of the Krebs cycle owing to excess of NADH and lack of NAD, with a secondary rise in blood lactate and an increase in ketone body and lactate/pyruvate molar ratios. This is particularly true in the postabsorptive period, when more NAD is required for adequate metabolism of glycolytic substrates. As a further result of Krebs cycle impairment, ketone body synthesis increases after meals instead of decreasing, owing to the channelling of acetyl-CoA toward ketogenesis. Consequently, the screening for mitochondrial disorders includes the determination of lactate, pyruvate, ketone bodies and their molar ratios in both fasted and fed individuals. However, a normal oxidoreduction status may occasionally be observed, particularly in patients with proximal tubulopathy. In these patients, impaired proximal tubular reabsorption may lower blood lactate and increase urinary organic acid excretion. For this reason, a normal plasma lactate concentration does not rule out a mitochondrial disorder in a patient with proximal tubulopathy. In these cases, the clue to the diagnosis lies in the presence of other unrelated symptoms [27].

The activity of different enzymatic complexes of the respiratory chain is most often evaluated by polarographic and spectrophotometric studies. Polarographic studies consist of the measurement of oxygen consumption by isolated mitochondria or whole cells in the presence of various oxidative substrates (pyruvate, glutamate, succinate, palmitate, etc.). These studies can be performed on peripheral blood mononuclear cells, skin fibroblasts, or a muscle biopsy specimens. Enzyme activities are assessed by spectrophotometric studies on isolated mitochondria or on tissue homogenates. Renal biopsy can be done in cases of nephropathy but other tissues may be more accessible for such spectrophotometric studies – for example skeletal muscle, skin fibroblasts, or peripheral blood mononuclear cells [27].

Histopathological studies may also contribute to establish the diagnosis. On muscle biopsy, the presence of ragged red fibers with irregularly shaped aggregates of abnormal mitochondria and lipid droplets between the fibrils is characteristic of mitochondrial myopathy, but their absence does not rule out the diagnosis. The ragged red fibers may be seen using the modified Gomori trichrome technique. Similarly, the presence of cytochrome *c* oxidase negative fibers is highly suggestive of mitochondrial disorder.

Renal biopsy study on electron microscopy shows abnormalities in mitochondrial number, size and configuration [27]. Mitochondria are increased in number and show prominent variation in size, associated with abnormal arborization and disorientation of mitochondrial cristae, including circular and parallel arrangements. Some mitochondria contain electron dense granular and fibrillar inclusions [80].

Renal biopsy in patients with mitochondrial tubulointerstitial nephropathy show tubular atrophy, moderate interstitial mononuclear infiltrates, interstitial fibrosis, sclerosis of some glomeruli and arteriolosclerosis. Electron microscopy shows various mitochondrial lesions in many distal tubular epithelial cells – an increased thickness of the internal membranes, distended empty core, also seen by light microscopy, dense inclusions enveloped occasionally by irregular or concentric cristae [67].

Large rearrangements of mtDNA are detected by Southern blot analysis and mtDNA point mutations by polymerase chain reaction (PCR) amplification and the sequence of total genomic DNA derived from various tissues. These studies cannot be performed routinely and negative results do not rule out an mtDNA mutation.

9.9 Drug Mitochondrial Nephrotoxicity

Treatment with antiretroviral drugs is associated with varied side effects, many of which are thought to be due to their effects on mitochondria.

Tenofovir disoproxil fumarate administration has been associated with renal dysfunction. The low potential for mitochondrial toxicity involved in the use of tenofovir alone may be accounted for by the fact that its pyrophosphate metabolite inhibits HIV-1 reverse transcriptase at concentrations much lower than those needed to inhibit nuclear DNA polymerases or mitochondrial polymerase γ [4, 39]. Didanosine, on the other hand, has been associated with mitochondrial toxicity at

therapeutic doses [19]. Renal dysfunction can be mediated through mitochondrial nephrotoxicity, influenced by both HIV infection and concurrent tenofovir disoproxil fumarate/didanosine therapy [10]. Murphy et al. described the case of a 49-year-old man with human immunodeficiency virus infection and stable chronic renal insufficiency who developed oliguric renal failure and severe lactic acidosis and died after several regimens of tenofovir added to an antiretroviral regimen that included didanosine. Factors that contributed to this patient's acute renal failure and lactic acidosis included his preexisting renal insufficiency, his use of diuretics and concomitant administartion of tenofovir and didanosine [62].

Tanji et al. described patients treated with adefovir, whose pharmacologic action is to serve as substrate for reverse transcriptase, resulting in premature DNA chain termination. Its potent antiretroviral activity is balanced by potential nephrotoxicity, manifested as acute tubular necrosis and Fanconi's syndrome, involves depletion of mtDNA from proximal tubular epithelium with resultant impairment of cellular oxidative respiration [82].

Cephalosporin antibiotics, cefazolin and cefalotin, decreased the activity of cytochrome c oxidase in isolated mitochondria. This inhibition in mitochondrial electron transport chain by cephalosporins may result in nephrotoxicity [45].

Doxorubicin, an antitumor anthracycle antibiotic, is the drug of choice for the treatment of many solid malignancies and lymphomas. Doxorubicin induced a self-perpetuating nephropathy characterized by early glomerular and late-onset tubular lesions in rats. Lebrecht et al. suggest an important role for quantitative and qualitative mtDAN alterations through the reduction of mtDNA-encoded respiratory chain function and induction of superoxide in doxorubicin-induced renal lesions (Fig. 9.6) [48].

Diclofenac is one of the most frequently used non-steroidal anti-inflammatory drug prescribed to millions of people worldwide for the treatment of osteoarthritis, rheumatoid arthritis and muscle pain. The mitochondrion is clearly a target of diclofenac-induced nephrotoxicity as demonstrated by the oxidative stress and massive DNA fragmentation reported from studies with diclofenac in vivo [33]. A decrease in ATP synthesis was demonstrated in isolated kidney mitochondria with glutamate/malate [60]. Ng et al. concluded that diclofenac inhibited the transport of malate and glutamate into mitochondria via the malate–aspartate shuttle, the most important mitochondrial metabolic transport system in the kidney, liver and heart [65]. The acute renal failure in patients administered diclofenac may result from its bio-accumulation in the kidney.

9.10 Mitochondria and Renal Carcinomas

As early as 1930, Warburg observed that carcinogenesis was associated with an increase in glycolysis and lactate production and with a decrease of oxidative phosphorylation (OXPHOS) in spite of the presence of oxygen [89]. In renal cell carcinomas some characteristic changes occur in carbohydrate metabolism: glycogen and glucose-6-phosphate levels increase, glycolysis is activated, and gluconeogenesis is reduced.

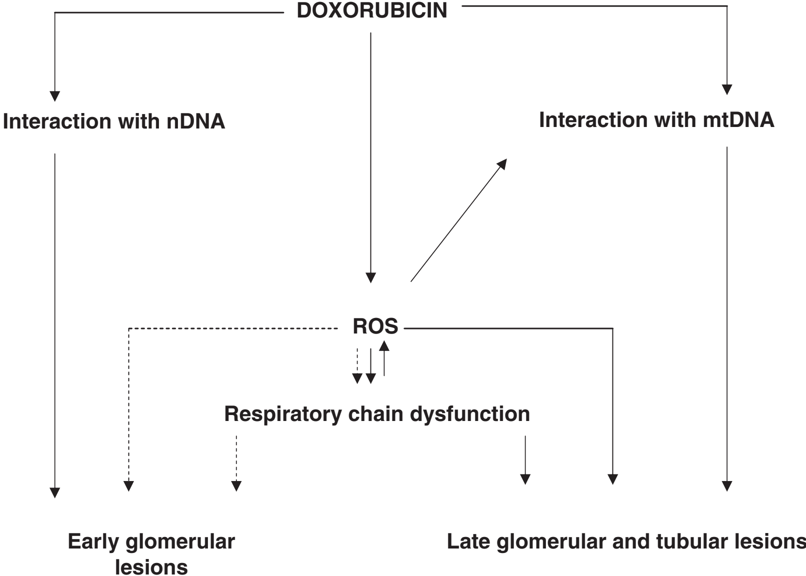

Fig. 9.6 Proposed mechanism of doxorubicin-induced nephropathy. Proposed mechanism of doxorubicin-induced nephropathy with early glomerular and self-perpetuating late-onset lesions in rats

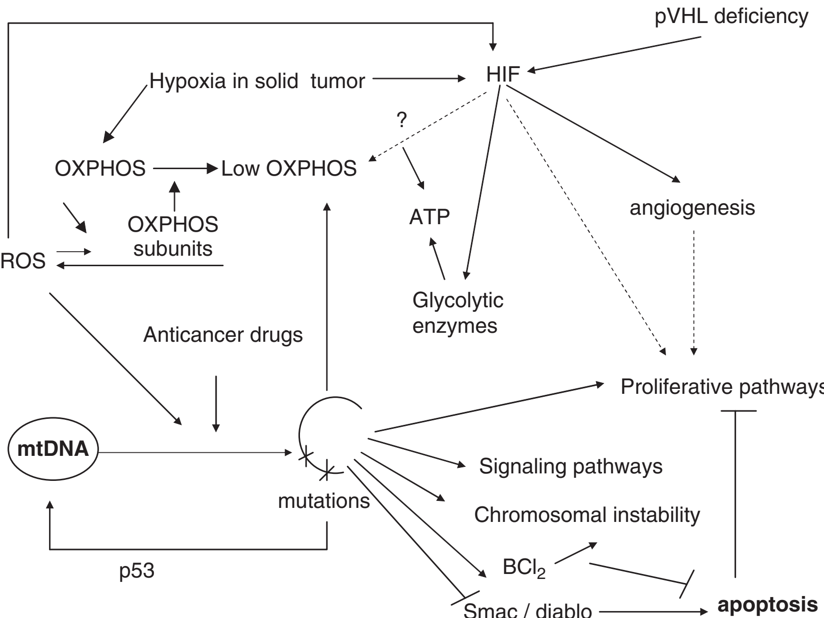

Fig. 9.7 Mitochondrial pathways participating in tumorigenesis of renal cell carcinomas Note: OXPHOS – oxidative phosphorylation, ROS – reactive oxygen species, mtDNA – mitochondrial DNA, ATP – adenosinetriphosphate, VHL – von Hippel-Lindau, BCl_2 – mitochondrial protein

Mitochondria are implicated in two major cellular processes, namely OXPHOS and regulation of mitochondrial apoptosis and they are one of the main sites of reactive oxygen species (ROS) production. The sequencing of mtDNA in end-stage-renal-disease patients and in other renal carcinomas revealed numerous mtDNA mutations [63]. The mutation A3243G usually found in MELAS was seen in pediatric patients with renal cell carcinoma without symptoms of encephalopathy [71].

Different aspects of mitochondrial influence in renal cell carcinomas can be summarized as follows. First, mtDNA mutations and rearrangements decrease OXPHOS, enhance ROS, and deplete aerobic ATP production. The accumulation of ROS increases the risk of mtDNA defects, which are directly implicated in chromosomal instability, in the regulation of apoptosis and in the control of proliferation via signaling pathways, which are still not very well indentified. Second, hypoxia in the tumor, the absence of von Hippel–Lindau lesions in renal cell carcinomas, and/or ROS accumulation may stabilize the hypoxia-inducible transcriptional factor and increase both glycolytic enzymes to produce anaerobic ATP and angiogenesis to improve substrate and oxygen availability to the tumor. The hypoxia-inducible transcriptional factor pathway is also directly implicated in tumorigenesis and eventually more specifically in the evolution of the tumor. Whether or not hypoxia-inducible factor stabilization plays a direct role in OXPHOS complex biosynthesis remains an open question (Fig. 9.7) [32].

Meierhofer elucidated the relationship between tumorigenesis and the mitochondrial energy metabolism in renal neoplasmas. A significant reduction of all mitochondrial enzyme activities, including Complex V, as well as the mitochondrial DNA content, was detected in 34 of 37 renal carcinoma tissue samples as compared with control kidney samples. Low mitochondrial activity seems to represent adaptation to environmental conditions of solid tumors, which have to endure hypoxia during their developement. Low mitochondrial activity leads to lower oxidative stress under hypoxic conditions and might therefore represent an advantage for carcinoma progression. Mitochondrial enzyme activities and mtDNA levels were not statistically different between the convential, papillary and unclassified sarcomatoid type of renal carcinoma, and did not correlate with tumor grade, metastasis, and proliferative activity [59].

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Chapter 10
Mitochondrial Bioenergetics of Skeletal Muscles

Janka Lipková

Abstract Myofibril is the main contractile structure of a muscle; sarcomere is a functional unit comprising thin actin and thick myosin filaments. The process of muscular fiber shortening takes place through insertion of thin actinic filaments in between the thick myosin ones. The whole process is regulated by regulatory proteins troponin and tropomyosin. Energy necessary for muscle contraction is obtained from adenosinetriphosphate (ATP) produced in mitochondria. Energy necessary for ATP resynthesis is obtained by cleavage of phosphocreatine (PCr), carbohydrates, fats and proteins. During the aerobic ATP production, the majority of received oxygen is reduced by hydrogen to water. However a part of oxygen which is not reduced completely produces the so-called free oxygen radicals – univalent oxygen forms escaping from the transport chain. Production of oxygen radicals and their highly reactive derivatives, the so-called *reactive oxygen species*, increases during endurance exercise and may negatively affect the function of muscles and accelerate the process of fatigue.

Exercise can cause imbalance between the levels of oxidants and antioxidants. This state, so-called oxidative stress, causes damage to enzymes, protein receptors, lipid membranes and DNA. On the other hand, exercise positively affects oxidative stress reduction and improves the function of mitochondria. Results of human studies are however frequently inconclusive.

The *antioxidative defense system* depends on the intake of antioxidative vitamins and minerals with the diet (vitamins C, E, β-carotene and selenium), as well as on endogenous production of other substances with antioxidative effects (such as glutathione, coenzyme Q_{10}) and of enzymes (such as superoxide dismutase), whose task it is to suppress free radicals. In most cases, antioxidant supplementation is unnecessary. The question whether supplementation with vitamins and other antioxidants increases sports performance and facilitates regeneration has not yet been explicitly answered.

Keywords Energy, muscle contraction, oxidative damage, physical activity, reactive oxygen species, supplementation

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10.1 Ultrastructure and Function of Skeletal Muscles

The role of muscles is to ensure movement of individual organs and their parts as well as movement of the body as a whole. There are three types of muscular tissues – striated (skeletal muscles), smooth muscles (of internal organs) and heart musculature. Individual types differ from each other by their distribution, internal structure and specific functional properties.

Muscle cells consist of ***myofibrils***, which form the contractile structure of a muscle. The basic functional unit of a myofibril is the ***sarcomere***, a part of myofibril bounded from both ends by the so-called ***Z-lines*** (Fig. 10.1). The center of a sarcomere contains a protein called M-line. There are several proteins, which maintain the architecture of a sarcomere and keep the contractile and regulatory proteins in the correct spatial arrangement for force generation. ***Desmin*** links adjacent Z-discs in a longitudinal and lateral manner. The role of other proteins – ***-actinin, vinculin, talin and dystrophin*** is to anchor the cytoskeleton to sarcolemma by structures called costameres. Actin is joined to the Z-disc by an elastic protein called α-actinin. The role of ***nebulin*** is to regulate the length of thin filaments. The distance between M-line and Z-disc is spanned by ***titin***, which helps to maintain thick filament alignment [7]. The process of sarcomere shortening consists in Z-lines coming nearer to each other. The contractile system of myofibrils consists of contractile and regulatory proteins. Contractile proteins include ***actin*** and ***myosin***; regulatory ones include ***troponin*** and ***tropomyosin*** (Fig. 10.2). Thick filaments include myosin; thin ones include actin, tropomyosin and troponin. A movable link between the head and neck of myosin allows to change the angle between the head and the neck. At contraction, thin actin filaments are telescopically gliding between myosin filaments, while creating links between actin and myosin with the help of so-called ***cross-bridges***. A sarcomere is shortened by actin filaments pushing in (Z-lines are brought nearer to each other) without shortening individual filaments. The bearing structure of actin is created by tropomyosin, which together with troponin regulates the process of actin and myosin push-in, and the creation and disruption of their links. A troponin complex includes three types of troponin, each of which has a different role. ***T-troponin*** binds tropomyosin, ***I-troponin*** prevents creation of an actin-myosin link if necessary, ***C-troponin*** binds calcium. The link between actin and myosin does not exist when the body is at rest since it is prevented by tropomyosin and troponin put in between. The angle formed by the myosin head and neck is 90°. Since the muscle tissue does not work autonomously (its activity is regulated by the nervous system), muscle shortening is initiated by the nervous system. The action potential is distributed over the cell surface and through T-tubules, it gets near to the so-called cisternae – dilated parts of longitudinal tubules of sarcoplasmic reticulum where calcium is stored. Under the influence of action potential, the membrane of cisternae becomes permeable for a short time to pour out calcium into sarcoplasma and to be linked to C-troponin. Higher calcium concentration

produces troponin conformation changes. This allows tropomyosin to divert from its original position and release of actinic links, followed by creation of a link between actin and myosin. A substance is attached to the myosin head that serves as a source of energy – adenosinetriphosphate (ATP). Enzymatic myosin activity (adenosinetriphosphatase) causes ATP breakdown to adenosindiphosphate (ADP) and inorganic phosphate. The angle between the myosin head and the myosin neck changes (from 90° to 45°). However, since myosin is firmly linked with actin, the actinic chain is dragged and the sarcomere is shortened with tilting of the myosin head. The 45° angle is final and further tilt is impossible. Since the shortening within one such cycle is insufficient, any further shortening of the sarcomere needs to disconnect the actin–myosin link with the help of ATP, to raise the myosin head and to repeat the whole process. Shortening continues until the inflow of nervous impulses lasts.

In the vicinity of myofibrils, there are cellular organelles, the so-called ***mitochondria***, frequently called also the cell's power stations, which are also scattered in the sarcomplasma. It is there that the oxidative production of ATP takes place.

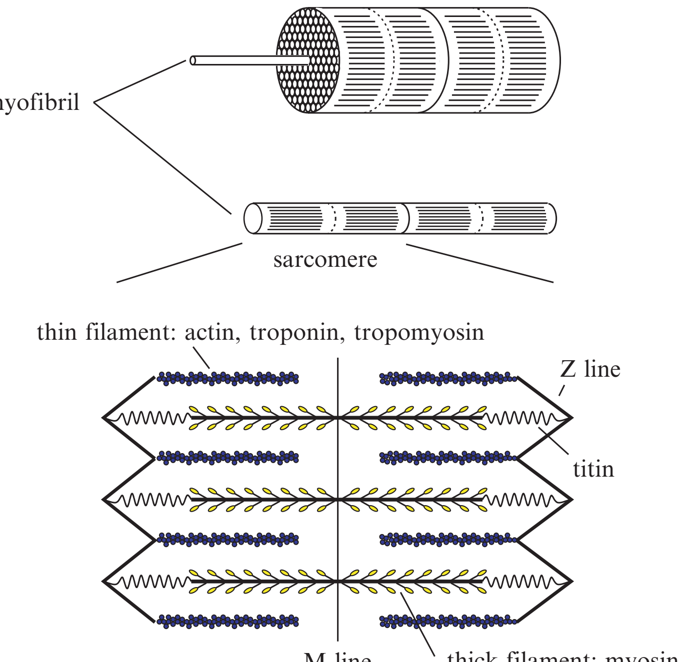

Fig. 10.1 Myofibril – contractile element of skeletal muscle. Sarcomere – the basic functional unit of myofibril, which contains actin and myosin filaments

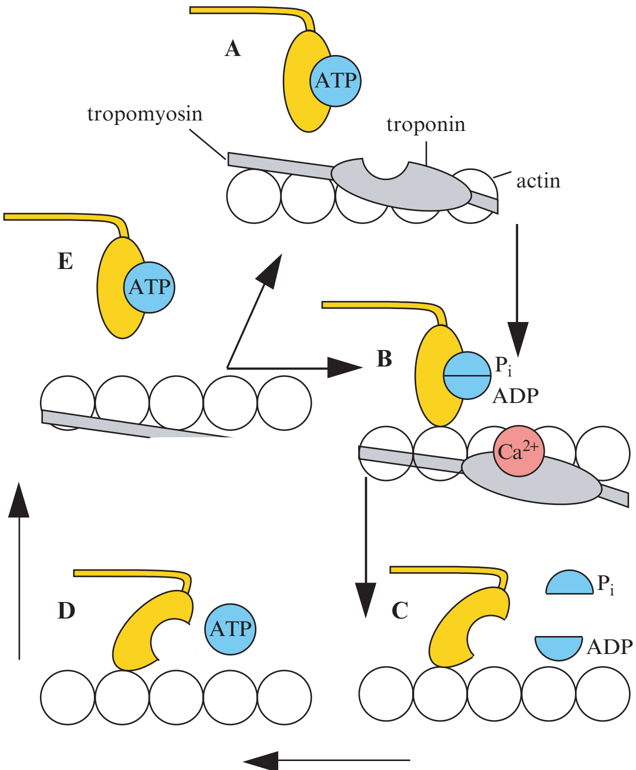

Fig. 10.2 The sequence of events leading to muscle action. (**A**) At rest the active binding sites on the actin molecules are covered by tropomyosin. (**B**) Calcium released from the sarcoplasmic reticulum (following excitation by a nerve impulse) binds to troponin. Troponin pulls the tropomyosin off the active sites – myosin heads attach to the actin. Enzymatic myosin activity (adenosintriphosphatase) causes the ATP breakdown to adenosindiphosphate (ADP) and inorganic phosphate. (**C**) The angle between the myosin head and the myosin neck changes (from 90° to 45°). Actin filaments are pulled towards the centre of the sarcomere. ADP and Pi are released. The energy by ATP hydrolysis powers the process. (**D**) A new ATP molecule binds to myosin head. (**E**) Myosin heads detach from the actin and can attach to another actin unit and the cycle is repeated (→ **B**). When the calcium is actively pumped back to the sarcoplasmic reticulum, muscle action ends (→ **A**)

10.2 Energy Production of Skeletal Muscles

Under the thermodynamic laws, energy can be neither generated nor get lost. However, individual forms of energy can be mutually exchanged while releasing heat. 60–70% of the total energy output of a human body changes to heat. The rest is used for muscular activity and other cellular functions. Energy is obtained from vegetable or animal food. Food contains energy in the form of carbohydrates, fats and proteins. Cleavage of these substances releases energy used by the human body

for various purposes, such as growth and healing of damage, active transport of substances through cellular membranes, and for muscular work. Thus, a part of energy is used by myofibrils for the gliding and pushing of actin and myosin filaments, resulting in muscular activity.

Energy taken from food, however, cannot be used as a direct source of energy for the work of muscles. The only direct, immediate source of energy is ATP. Since ATP reserves are too small, ATP must be constantly renewed. Such renewal takes place through phosphate radical reconnection to ADP with the help of energy that cumulates at such a link. Reactions producing energy for ATP resynthesis can take place under both aerobic and anaerobic conditions.

Anaerobic ATP resynthesis: The source of energy for anaerobic resynthesis includes *carbohydrates and phosphocreatine (PCr).*

Phosphocreatine cleavage is the fastest and promptest manner of obtaining energy for ATP resynthesis. Since it does not produce lactate, this manner is called ***anaerobic alactate*** manner. It is used for energetic coverage of very short and intense activities.

10.2.1 Anaerobic Glycolysis

The final product of anaerobic carbohydrate cleavage (anaerobic glycolysis) is the salt of ***lactic acid – lactate***, therefore this manner of obtaining energy is called ***anaerobic lactate*** manner.

Compared to aerobic glycolysis, anaerobic glycolysis is much faster but energetically less effective (energetic benefit amounts to 2 mol ATP in comparison to 38 mol ATP). A disadvantage is also the production of lactate which acidifies the cell's inner environment, thus deteriorating the conditions of enzyme activity, catalyzing individual reactions.

10.2.2 Aerobic ATP Resynthesis

The third manner of obtaining energy is represented by an ***oxidative system***. It allows cleavage of carbohydrates, fats and in certain circumstances (such as starvation, extreme output), also proteins. Oxidative ATP production takes place in mitochondria. Muscles need a constant supply of energy for continual production of force necessary for long-time activity. In contrast to anaerobic production, the oxidative system has enormous energy producing capacities. This manner of energy obtaining is therefore the primary method of energy production during endurance exercises. In situations where the load intensity is too high and exceeds the body's ability to cover energy demands in an aerobic manner or where the demands for energy appear suddenly, the body is temporarily forced to release the energy in an anaerobic manner. The anaerobic manner of obtaining energy is less effective yet prompter than the aerobic one.

ATP is a universal energy mediator mediating the change of the chemical energy of nutrients to mechanical energy of muscle contraction. It transfers energy from producing to consuming processes.

10.2.3 ATP Production

- Most of the atoms of glucose, carbon, fatty acids, glycerol and amino acids, produced by food carbohydrates, fats and proteins, change to ***acetylcoenzyme A*** (*acetylCoA*).
- Acetylcoenzyme A is oxidized in the Krebs cycle while CO_2 and hydrogen produced by dehydrogenizing reactions are released.
- These reactions are catalyzed by enzymes and the so-called cofactors, including minerals and substances produced by vitamins (nicotinamide adenine dinucleotide – **NAD** and flavinadenine dinucleotide – **FAD**).
- Their reduced forms are produced by hydrogen binding to NAD and FAD.
- NADH and $FADH_2$ transfer electrons to oxygen through an electron transport chain. Energy produced by such a transport of electrons is used for ATP production in ***oxidative phosphorylation*** processes.

ATP production in animal cells proceeds mainly through oxidative phosphorylation. This process takes place on the internal membrane of cellular organelles called mitochondria. Cells contain various numbers of mitochondria, depending on energetic demands of processes taking place in the cells. Therefore energy in the organelles is produced under aerobic conditions. A mitochondrion comprises an outer and inner membrane with an intermembrane space in between. The core of a mitochondrion is called matrix. The folding of the inner membrane produces protuberances called cristae. While the outer membrane is freely permeable for smaller molecules, this is not the case with the inner membrane. This applies to most molecules and ions (including protons). In this way the internal membrane responds to the transport of substances to mitochondria.

During oxidation of various substrates, reduced coenzymes NADH + H^+ or $FADH_2$ are produced by oxidative–reductive reactions in cells. From there electrons are transported through a system of transporters to the final electron acceptor, i.e. oxygen. This system of electron transporters called ***electron transport chain*** is located in mitochondria. It consists of enzymatic complexes that form a part of the mitochondrial membrane and catalyze the transport of electrons from reduced coenzymes to oxygen. The electron transport chain includes also coenzyme Q (ubiquinone) and cytochrome c.

Transport of electrons in the electron transport chain takes place from redox systems with the lowest redox potential (NADH/NAD) to the system with the highest redox potential ($½O_2/H_2O$). Redox (oxidation–reduction) potential is an expression of affinity proportion to electrons.

The process of the so-called terminal oxidation results in a gradient of protons produced on the internal mitochondrial membrane. It represents energy used for ATP production from ADP and inorganic phosphate in the process of oxidative phosphorylation (see Chapter 1).

10.2.4 Proton Gradient Production

The source of energy for ATP resynthesis includes reduced coenzymes produced within oxidation of substrates. Transport of electrons from the system with a low redox potential ($NADH/NAD^+$ and $FAD/FADH_2$) to the system with a high redox potential ($½O_2/H_2O$) is connected with energy release. Within the transport of electrons, transport of protons from the matrix to the intermembrane space takes place in parallel. This way, energy is produced on the inner membrane (***proton-motive force***) in the form of the so-called ***proton gradient***, consisting of electrical and chemical potential. It is therefore called electrochemical gradient. The theory of the electron transport mechanism, proton gradient production and use for ATP production, is called chemiosmotic theory of ATP production and comes from the English biochemist Mitchell.

Within the reduced NADH oxidation, two NADH electrons and one hydrogen ion are transported to an oxygen atom, and one hydrogen cation (H^+) is received from the environment.

$$NADH + H^+ + 1/2O_2 \dashrightarrow NAD^+ + H_2O$$

Simultaneously, energy is released in satisfactory quantities for energetic coverage of synthesis of three ATP molecules in the oxidative phosphorylation process. During oxidation, each molecule of reduced NADH releases energy for synthesis of three ATP molecules. Oxidation of one molecule of reduced $FADH_2$ releases energy enough to produce two ADP molecules.

Electrochemical proton gradient is a form of energy that can be used by mitochondria for ATP synthesis from ADP and inorganic phosphate. This is made possible by the structure of the inner mitochondrial membrane known as F0 and F1. The F0 unit creates a proton canal through which protons can pass from the intermembrane space to the mitochondrion's matrix. The proton canal is connected to the F1 unit where ATP activity takes place. In this unit, ATP synthesis from ADP and inorganic phosphate takes place [1].

10.3 Oxidative Damage and Physical Activity

Positive effects of regular physical activity are well known and include, among others, a reduced risk of various diseases, such as cardiovascular diseases, osteoporosis and diabetes. The mechanism contributing to such an effect also

includes improvement of the antioxidative defence. Although the number and activity of mitochondria are positively influenced by motor activity, excessive physical activity may cause damage to their bioenergetics. Training may cause imbalance between the levels of oxidants and antioxidants, so-called ***oxidative stress***. Oxidative stress may cause damage to enzymes, protein receptors, lipid membranes and DNA.

An excessive load may lead to damage of muscular fibers, while in this process an important role is played by free oxygen radicals produced during aerobic ATP production. In this process, most of the oxygen taken is reduced to water. However, a part of it is not reduced completely and shall produce the so-called ***free oxygen radicals*** – univalent oxygen forms escaped from the ***transport chain***. They may attack lipids of cellular membranes and result in cleavage of lipids, so-called lipid peroxidation.

During intense endurance exercise covered only aerobically, production of free oxygen radicals and their highly reactive derivatives called ***reactive oxygen species (ROS)*** is increasing. It may negatively affect the function of muscles and accelerate the process of fatigue. Strength exercises may damage cellular proteins. Within correction of such damage, macrophages and neutrophils increase, resulting in further release of free radicals that may cause additional damage to cells.

Research done within the last 25 years demonstrated the important role of ROS in physiology and biochemistry of physical exercise, however relevant knowledge is still incomplete. Current knowledge provides a broad base of stimuli for further research into this field. One of the issues to be solved is the question whether oxidative stress is only a by-product of the stress response to intense exercise or whether it plays an active role in immunity changes, muscle damage or overconditioning. Results of further research should provide more information on the issue of fatigue as well as on the role of physical activity in prevention and treatment of various diseases.

The issue of oxidative stress in the context of exercise is, however, a relatively modestly explored field. The fact that exercises are accompanied by increased production of free radicals and by oxidative modification of various molecules is sufficiently supported by evidence. However, mechanisms responsible for the above processes are not yet fully known. In general, increased oxygen uptake by mitochondria is assumed to lead to increased ROS production, although this assumption was not supported by research results [17].

As regards human performance, there is still insufficient direct evidence of its deterioration by ROS. Implications of oxidative modifications caused by training are still unclear, and so is the question whether they cause harmful oxidative damage or whether they form an integral part of redox regulation.

Effects of physical activity on mitochondria and oxidative damage were dealt with by many studies both in animals and humans. Trials with rats [11] showed that modest running on a running belt extended the life of males by 19% and females by 9%, with parallel increase of their performance levels. Modest exercise significantly decreased the oxidative stress development. This was however shown only in young rats. In contrast, Short et al. [13] reported positive effects of 4-month

training on mitochondria in humans, with results similar in all age groups (21–87 years of life). They showed increased activity of mitochondrial enzymes in muscles, mRNK levels of mitochondrial genes and genes of mitochondrial biogenesis. Increased mitochondrial activity of the electron transport chain in muscles of older people followed by increased mitochondrial biogenesis under the influence of exercise was found also by Menshikova [9]. Significant improvement of mitochondrial functions was recorded after 16-week training in obese men and women [8]. Endurance training provoked mitochondrial adaptation resulting in improved oxidation capacity of fatty acids [5]. Adaptation to regular training is the basis of exercise tolerance improvement. Limitation of exercise-related oxidative stress evoked by training can be the result of both lower ROS production and antioxidative systems up-regulation.

In order to limit the increasing level of free radicals, a body has a complicated ***antioxidation defensive system*** at its disposal that depends on both antioxidative vitamins and minerals taken in the diet (vitamins C, E, β-carotene and selenium) and endogenous production of various antioxidative substances (such as glutathione) and ***antioxidative enzymes*** whose task it is to suppress free radicals. Substances with antioxidative effects also include coenzyme Q (CoQ), which in addition to its role in the final ATP production within the electron transport chain participates also in the removal of free oxygen radicals in the antioxidative defensive system. Activities of the antioxidative enzymes SOD (superoxide dismutase), CAT (catalase) and GPX (glutathioneperoxidase) are highest in slow-twich filaments and lowest in fast-twich filaments.

Since enzymatic antioxidative systems represent the first line of defence against ROS, exercises can have inductive effects on these mechanisms of defence. Servais et al. [12] reported on mitochondrial oxygen uptake and H_2O_2 production in intermyofibrillar and subsarcolemmal mitochondria during training in animals. Neither the oxygen uptake nor H_2O_2 production were affected by long-lasting training. In spite of the fact that no changes in oxidative capacity were recorded, the activity of GPX and CAT antioxidative enzymes increased significantly. Although an increased activity of these enzymes in various tissues was shown in trials with animals, results of human studies are often conflicting [10].

Decrease in the number and function of mitochondria is affected by factors of both heredity and environment. Drop of endurance performance results from worsened mitochondrial function. A regular endurance programme of several weeks, however, will increase the oxidative capacity and mitigate the drop of endurance performance. In this way, the effect of endurance exercises on mitochondrial biogenesis may positively influence the reduction of the number of mitochondria caused by aging and thus delay the course of diseases related to age and physical inactivity [4]. Oxidative changes in cells contribute to tissue dysfunction caused by aging and it was ascertained by trials with rats that ROS production increased in both younger and older rats by muscular activity. However, there is no evidence of this production exacerbation with respect to age [16].

The level of antioxidative enzymes is increased by regular exercises also during pregnancy by means of which oxidative stress is reduced, and thus also the

occurrence of preeclampsia. Besides, resistance against prooxidants production caused by higher number of mitochondria is increased and susceptiblity towards lipid peroxidation decreased [19].

Compared to pharmacological means (calcium or aspirin administration), exercising is more beneficial also because there is no risk of negative side effects.

10.4 Antioxidants in Sports Training

Greater physical load can cause damage to the dynamic balance between prooxidants and antioxidants, quicker exhaustion of energy sources and damage of mitochondrial bioenergetics. Increased functional capacity of antioxidative defensive systems is therefore desirable. In athletes subject to demanding training, muscles are damaged by free radicals on the level of cellular membranes and subcellular structures. In comparison to the current nontrained population, higher values of oxidative stress indicators were found in orienteers, basketball players and kayak-paddlers [2]. However, there is also evidence that the antioxidant status can be improved by adaptation response to regular training that may serve as protection against further damage [6].

Antioxidative substances can be received in a natural manner from food; however, their supplementation by pharmacological products is also possible. Certain authors [18] maintain that dietetic supplements may help to block the negative impact of free radicals produced by training. Harmful effects of training, such as DNA damage, may be reduced by supplementation with antioxidants (particularly vitamins C and E) [3]. Improvement of the total antioxidant status as well as reduced oxidative damage of DNA caused by intense training was found in basketball players after 1-month supplementation with α-tocopherol [15].

Nevertheless, the results of research into effects of supplementation with antioxidants are not unambiguous. According to one study, supplementation with daily 200–300 mg of C-vitamin during 3–4 weeks reduced the signs of muscular damage, including post-training pain, while other studies failed to confirm this finding [7]. Most studies dealing with E-vitamin supplementation did not find any reduction of muscular damage. Similarly, no convincing evidence of positive effects was provided by studies dealing with CoQ supplementation effects on metabolism, performance and oxidative damage caused by training. There are even studies that proved negative effects of CoQ on performance and training adaptation [6]. A probable reason why antioxidant supplementation has not shown the expected effect is that a body receiving sufficient amounts of antioxidants from food is able to increase the activity of its own defensive system in response to stress caused by training. Moreover, it was found that physical training itself results in improvement of the defensive antioxidant system. It has been demonstrated by many studies that trained individuals have increased levels of antioxidants and enzymes in both blood and muscles. A lower level of lipid peroxidation, independent of supplementation, was reported as a response to training in well-trained individuals. This, however, is not a rule. Lower values of antioxidant

enzymes – SOD as well as other antioxidant substances (α-tocopherol and β-carotene), in comparison to reference values were found by Gvozdjáková [2] in top athletes, although with relatively big interindividual differences.

In athletes, a diet rich in antioxidants, should be sufficient to suppress free radicals produced by training also without supplementation. Diet supplementation with antioxidants is not harmful in the recommended dosage, yet in most cases it is unnecessary.

The ability of antioxidants to suppress free radicals is probably incontestable. However, the question whether supplementation with vitamins and other antioxidants increases the sports performance and facilitates regeneration has not been unambiguously answered so far. It has been amply studied both in animals and humans, yet the results are still inconsistent. Although the opinion that the sports performance is positively affected by ***supplementation*** with vitamins is quite widespread in the sports public, there is no convincing evidence available, except for cases of deficit correction [6, 18].

Lower levels of oxidants are necessary for the body and its functions. They are part of a functioning immunity system and signals for development of adaptation changes. There is considerable evidence that the adaptation process is stimulated by free radicals. Increasing amounts of free radicals during training may serve as a signal of cellular adaptation to training as it influences the process of gene expression.

Free oxygen radicals belong to nonspecific defence tools. They are located in neutrophilic granulocytes and have bactericidal effects. In normal conditions, their concentration is maintained by reducing enzymes at a low level and their effects are fully developed only with invasion of bacteria [14]. The necessity of oxidants for the body and its functions is also a reason for prudent antioxidant supplementation.

Since there is no clear standpoint regarding their need, athletes are recommended to consume a diet which is a rich source of antioxidants. Supplementation is necessary only in certain substantiated cases, such as sudden rise of the training load, extreme conditions of the outer environment, etc. [6].

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Chapter 11
Rheumatoid Arthritis

Jozef Rovenský and Karel Pavelka

Abstract Definition. Rheumatoid arthritis (RA) is a common severe joint disease that involves all age groups, with maximum occurrence in young people and premenopausal women. There is a multifactorial cause of the disease, which includes genetic predisposition. It is characterized by typical chronic inflammation, initiated, and maintained via autoimmune mechanisms. The course of RA varies considerably. Acute exacerbations follow remissions. In general, the disease progresses and often leads to disability. This disease can shorten one's life span by 10 years.

Clinical manifestations. The pathologic inflammatory process is located in the synovial membrane of joints, bursae, and tendons. Chronic synovitis leads to the erosion of cartilage and marginal bone, followed by destruction of joints. Various extra-articular manifestations developing due to serositis, rheumatic nodules, or vasculitis, can be severe. Approximately two thirds of RA patients have positive rheumatoid factors in their serum.

Therapy. The main targets of treatment of RA are (a) to induce a remission of the disease, (b) if this is not possible, then: suppress the inflammation, decrease the pain, maintain muscle power, maintain function, improve quality of life, and suppress destruction of joints. Treatment of RA combines nonpharmaceutical treatment (rehabilitation, physical therapy), and pharmaceutical therapy (nonsteroidal antirheumatic drugs), disease modifying antirheumatic drugs and glucocorticoids.

Keywords Chronic inflammation, disability, erosion of cartilage, extra-articular manifestation, genetic predisposition, marginal bone, rheumatic nodules, rheumatoid arthritis, rheumatoid factors, severe joint disease, synovial membrane, vasculitis, therapy

Epidemiology. Epidemiologic studies differ according to the classification schemes used for the diagnosis of the disease. Especially older schemes that use expressions like "classical," "definitive," "probable," and "possible," differ from the new Arnett scheme [2] that uses one classification only. The incidence of rheumatoid arthritis (RA), as published in the literature, is 0.1–0.2/1,000 in men, and 0.2–0.4/1,000 in women. Its prevalence varies from 0.5% to 1.0%. It is higher in some ethnic groups

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in the USA, and low in the rural African population [19, 20, 29]. In the Caucasian population in Europe, prevalence is mostly around 1% [15]. Women prevail over men with a 2–4:1 ratio. Some authors reported a decreasing trend of RA incidence over the last 20 years. This fact, however, has to be verified. It seems that RA has decreased, especially in women, and less destructive forms are observed these days. One theory tries to explain the fact of lower incidence in women as a result of taking hormonal contraception. This assumption has not yet been proven.

The ***genetic predisposition*** of this disease has been demonstrated by the fact that in monozygotic twins RA occurrence is four times higher than in dizygotic twins. No single factor of the external environment has been shown to be clearly and consistently related to the development of the disease. RA can start at any age. While in men its incidence increases with age, in women it is higher in the fifth decade of life, maintaining a plateau until the age of 75, to decrease later on.

Etiopathogenesis of RA. The causes of the development of RA have not been revealed yet. It is believed that the disease is triggered by a microorganism in genetically predisposed subjects. The genetic factor is eminent especially in monitoring concordance in monozygotic twins, which is 30% in comparison with 5% in dizygotic twins. It is a polygenically conditioned disease, where the substantial portion of genetic predisposition is within the HLA complex; the portion of HLA genes is estimated to be 40–50%.

RA is associated with the ***HLA-DR4 antigen***. This antigen can be divided into five subtypes: HLA-Dw4, -Dw10, -Dw13, -Dw14, and -Dw15. For the Caucasian population, there is an association of RA with HLA-DR4-Dw4 and HLA-DR4-Dw14 antigens. In the Caucasian population, 70% of patients have HLA-DR4-Dw4 antigens or HLA-DR4-Dw14 antigens. Other groups have different HLA antigens, especially HLA-DR1 [1, 8].

The main biological role of the HLA molecules lies in the presentation of the peptides created from protein antigens of exogenous or endogenous origin. Afterwards, the presented antigens are recognized by T-lymphocytes that develop the immune response together with other cells in a series of interactions. It is not yet known from which antigen the “arthritogenous” peptide comes in RA. The autoimmune process is supposed to be triggered by an infection [8].

Pathological anatomy. In RA, the inflammatory process develops in the tissue of the synovial membrane, which plays a substantial role in the nourishment of a vascular hyaline cartilage in synovial joints. The inflammatory process involves the synovial membrane of articular capsules, recesses in the joint cavity, and bursae. Joint cartilage is gradually damaged because the inflammation impairs both its nourishment and articular cavity drainage. In the location of the synovio-chondral connection, a tissue called ***pannus*** is formed, which expands to the articular cartilage and, starting from the edges, replaces it progressively. Articular capsules, tendons, ligaments, labra, and discs suffer from secondary impairment [15].

The RA picture includes rheumatoid nodules, which occur at sites exposed to pressure, especially elbow joints. These can be affixed to the bone or free and moving. They are almost always associated with the presence of rheumatoid factors. The histological findings are characterized by three layers. In the central part, there

is ***fibrinoid degeneration*** or even ***necrosis***. In the necrotic site, the presence of IgG and IgM immunoglobulins can be detected. The middle zone contains a radical palisade-like rim of histiocytes, and sometimes also multinucleate giant cells, which encircle the central necrotic zone. The outer stratum is made up of lymphocytes and plasmatic cells. Rheumatoid nodules (granulomas) develop in about 25% of patients in their subcutaneous tissue and tendons, and also in their heart and lungs [15].

11.1 Clinical Picture of Rheumatoid Arthritis

RA begins in the winter months twice as often as in the summer. ***RA exacerbation*** is observed twice as frequently in the period from March to April, compared to the summer months. The onset of the disease is mostly *insidious* (50–70%), not so often *acute* (15%), and about 20% of patients experience a *subacute* beginning. Not so common is the emergence of RA via *Still's disease*, developing in *adult age*. Cases in women prevail, just as in people in their third and fourth decades of age. Rheumatoid factors are usually negative and antinuclear antibodies are detected. In all patients, fever is observed and so is exanthema consisting of tiny, salmon-pink-colored maculae. The cervical spine is often affected (resulting in loss of mobility). Pericarditis, pleural exudates, and severe abdominal pain (mesenteric adenitis) can occur. Findings from a retrospective long-term study showed that most patients suffered from a polycyclic type, characterized by exacerbations and remissions. Joint disease may be present, but not necessarily in all cases. Loss of wrist mobility is mostly present; in most severe cases, ***ankylosis*** of this joint is observed.

A palindromic beginning is reminiscent of gout. One joint is usually involved, which swells suddenly and becomes very painful and sometimes red. The entire episode takes several hours. As a rule, it is impossible to differentiate the palindromic type of beginning from other joint affections, which are often time-limited.

Subjective manifestations of RA. Initial ***manifestations*** can be ***articular or systemic***. Of the joint manifestations there is particularly ***joint pain***. It can be of variable intensity and is most pronounced in the morning. It is localized in the joints, and sometimes it shoots into tissues and muscles around the joints. Patients usually feel the pain even when they rest. Another very important manifestation *is* ***morning stiffness***. This is caused by the accumulation of edema in the inflammatory tissue during sleep. Sometimes it precedes the development of articular pain. Usually it lasts longer than an hour, and this differentiates it from osteoarthrosis stiffness, which is of shorter duration (Table 11.1).

Systemic manifestations include overall feelings of discomfort, fatigue, weakness, subfebrilities, weight loss, and sleeping disorders. Patients may show signs of anxiety and depression. Often a psychological trauma is detected in the patient's history, but it is difficult to define its significance in the development of the disease. Systemic manifestations can precede the joint symptoms, they can occur at the same time or begin afterwards.

Table 11.1 Joints involved in rheumatoid arthritis

Joint	Percentage of initially involved joints	Percentage of all involved joints
Metacarpophalangeal	52	87
Wrist	48	82
Proximal interphalangeal	45	63
Metatarsophalangeal	43	48
Shoulder	30	47
Knee	24	56
Ankle	18	53
Elbow	14	21

Objective manifestations of RA. In early RA, most commonly the metacarpophalangeal joints (MCP) are involved, followed by proximal interphalangeal joints (PIP) and radial carpal joints (RC). Large joints are usually affected later than the small joints. Sometimes, e.g. in the elderly, RA starts the other way round – in *shoulder arthrities*. Joint involvement is shown in Table 11.1 (adapted from Harris et al.) [15]. For RA joint involvement, ***symmetrical arthritis*** is typical.

The basic clinical manifestation of joint inflammation is ***joint swelling and palpatory tenderness***. The skin above the joint can be warmer, but erythema is not a part of the RA clinical picture (rather, its presence points towards gout or septic arthritis). A joint is considered to be active when it is swollen or tender when palpated. The presence of pain and swelling is evaluated separately (the number of swollen joints and the number of joints which are tender when palpated). Joint swelling can be intra-articular or periarticular. ***Exudate*** can be detected in the intra-articular localization. ***Joint destruction*** can be determined in a clinical way or via X-ray scans. When destructions are examined clinically, decrease in the range of motion is tested, as is the loss of a fine joint outline, instability, subluxation, joint axis disorder, and the development of deformations [16].

Early RA. There are many definitions of early RA. The time limit usually oscillates around 2 years of its duration (1–4 years) [6].

Other authors use the fact that joint destructions are not present either in the clinical or laboratory picture of this condition as the clinical characteristic of early RA. In this case, early RA has no time limitation and its manifestations are close to those of low activity RA.

From the clinical point of view of early RA, typical fusiform arthrophyma of the interphalangeal joints of the hands is associated with pronounced morning stiffness, ***palpatory tenderness***, and a ***weakened grip*** [38].

The interest in "early RA" has recently increased, especially after Emery and his group [10] published their new epidemiologic studies. They established a network of "Early Clinics" all over England, specialized in early RA.

It has been discovered that:

(a) RA is not a benign disease, it can shorten the life of a patient by as many as 10 years.

(b) Joint erosions develop early, already during the first 2 years of disease development.
(c) While early RA is predominantly a local, articular matter, later the inflammation disseminates further and the disease becomes systemic.
(d) Therapeutic intervention is effective in the initial phase of early RA; after the scar-like, fibrous pannus has developed and deformations occur, the therapeutic possibilities decrease.
(e) RA is a considerably heterogenous disease; some of its forms are benign, either because they are time-limited, or because there are long clinical remissions and less of a tendency to be destructive; however, most forms have a progressive course.
(f) The diagnostic criteria officially adopted by the American College of Rheumatology (ACR) are not ideal for diagnosis of early RA due to their low sensitivity.
(g) The RA course cannot be very well foreseen, but based on the study of large sets of patients, risk factors for the unfavorable course of the disease were selected (Table 11.2).

Progressive disease. In patients polyarthritis persists, their acute phase reactant values are permanently increased, and there are erosions and destructions evident in their X-ray scans. Systemic manifestations can occur. These patients are usually treated with one or more disease modifying drugs (DMARDs) or with steroids. Iatrogenic changes can develop.

Late disease. This term is used for patients with fully developed deformations. They often suffer from some of the complications of this disease, e.g. a cervical spine impairment, tendon ruptures, secondary osteoarthrosis, or secondary osteoporosis. Iatrogenic changes in this stage (e.g. various hepatopathies, osteoporosis) are almost always present. Some patients suffer from the activity of the disease itself, in others the activity "fades away" and they mainly suffer from the secondary effects of the disease (Tables 11.3 and 11.4).

Complete remission, defined as the presence of clinical as well as radiographic remission, is the ultimate goal of treatment of RA. Functional disability in patients with low disease activity is associated with joint inflammation and joint damage. Despite the methodological problems of scoring radiographs, studies show that ***radiographic progression*** is an important outcome measure, and conventional

Table 11.2 Individual prognostic indicators of unfavorable rheumatoid arthritis development

- Persisting, uncontrollable polyarthritis
- Presence of rheumatoid factors, circulating immune complexes
- Presence of anticitrulline antibodies
- Extra-articular manifestations (e.g. vasculitis)
- Systemic manifestations
- Permanently high values of acute phase reactants
- Rapid functional decrease
- Rapidly developing erosions
- The presence of a "common epitope" (DRB1* 0401, 0404, 0405)
- Low education, psychosocial problems

Table 11.3 ACR criteria for remission of rheumatoid arthritis [27]

At least five criteria have to be present during a period of at least 2 months
*Morning stiffness not exceeding 15 min
*No fatigue
*No joint pains
*No palpatory tenderness or pain during passive movements
*No articular or periarticular swelling
*Erythrocyte sedimentation rate not exceeding 30 mm/h in women and 20 mm/h in men

Exceptions that exclude the determination of clinical remission

- Clinical manifestation of active vasculitis
- Pericarditis
- Pleuritis
- Myositis
- Weight loss or fevers that cannot be explained otherwise, just as being associated with RA

Table 11.4 DAS28 evaluation form [12]

Number of swollen joints (0–28) (sw 28)
Number of tender joints (0–28) (t 28)
Erythrocyte sedimentation rate
VAS self-reported by a patient (0–100 mm) patient's general health (GH)

$$DAS28 = 0.56 \times \sqrt{t28} + 0.28 \times \sqrt{sw28} + 0.7 \times \ln(ESR) + 0.014 \times GH$$

sw – swollen joints, t – tender joints, ESR – erythrocyte sedimentation rate,
GH – global score self-reported by a patient
DAS calculation: Available at
http:/www.das-score.nl/www.das-score-nl/DAS28calc.html
http:/www.das-score.nl/www.das-score.nl/DAS28-ne.xls
Disease activity evaluation according to DAS28: remission – DAS28 < 2.6
Low disease activity – DAS28 > 2.8 <3.2
Medium activity – DAS28 > 3.2 <5.1
High activity – DAS28 ≥ 5.1

radiography remains the best available method to assess it. Whether radiographic progression is entirely dependent on the presence of joint inflammation is a matter of debate: some evidence suggests that radiological progression may continue in patients who appear clinically to be in remission [37].

Course of the disease. It can vary considerably. There are several basic types which have modifications or transitory phases [16].

The most frequent type is a ***polycyclic course***. The stages of acute exacerbations and increasing and decreasing disease activity are from time to time replaced with stages of remissions of various lengths. Overall, however, the disease progresses, albeit with varying intensity. This course is manifested in 70% of RA cases. The second type is a ***monocyclic course***, in which at least 1-year-long remissions occur.

It has a relatively good prognosis. It occurs in about 20% of cases. The third type is a ***constantly progressing type***, appearing in about 10% of RA patients. This group includes patients with the course labeled as "malign", with a tendency to experience considerable destruction of joints. They can be divided into two subgroups: (a) those who are responsive to treatment, (b) those who are refractive to disease modifying treatment.

Disease remission. This definition is important from the viewpoint of precise assessment of the course and progression of the disease and the success of the treatment. An ACR and DAS28 (Tables 11.3 and 11.4.) are used for the definition of remission , but unfortunately, these definitions do not include a functional evaluation of the patient [12, 33].

11.2 Impairment of Individual Joints in Rheumatoid Arthritis

Polyarthritis is usually symmetrical and it affects mainly small joints. The impairment of large joints means a more severe course of the disease. The most commonly affected joints are the PIP, MCP, RC, knees, and ankles, but as a matter of fact, any of the joints in the body can be affected [12].

11.2.1 Hands

(a) ***Metacarpophalangeal joints***. A characteristic deformation in RA is the impairment of the MCP joints by subluxation with ulnar deviation. Synovitis of the MCP joints weakens the radial and dorsal structures and relatively prolongs the collateral ligaments. Internal mechanical factors then cause the ulnar deviation of the wrist. The ulnar drift can usually be corrected passively, later it becomes set.
(b) ***Proximal interphalangeal joints***. Synovitis of the PIP joints causes weakening of the support of collateral ligaments, which results in three typical deformations: a "buttonhole" deformation (boutonnière), a "swan neck" deformation, and an "instable PIP finger".

A buttonhole deformation (boutonnière) consists of PIP flexion and DIP hyperextension.
A swan neck deformation consists of MCP flexion, PIP hyperextension, and DIP flexion. It can be mobile, jumping, or fixed.

(c) ***Distal interphalangeal joints***. DIP impairment is not typical for RA, but it probably occurs more frequently than it is believed. It is never isolated or representing an initial impairment. It is recommended to distinguish osteoarthrosis and psoriatic arthritis by differential diagnosis.

11.2.2 Wrist

Symmetrical ***impairment of the wrist*** is typical for RA. In the initial phases, edema can be observed in the area of the processus styloideus ulnae, and flexion is usually restricted. Further weakening of the already weak triangular ligament makes it possible for the wrist to be pushed in the volar direction. The wrist rotates around the strong dorsal ligament and creates a deformation known as supination-subluxation. When intercarpal ligaments are affected, the entire wrist becomes unstable. Less common than instability is the opposite condition: a stiffening of the wrist due to ankylosis.

11.2.3 Elbow

The elbow is frequently affected in RA. It is interesting to note that even if its impairment is considerable, the correct function of the elbow is preserved for a long time. However, if flexion starts to become restricted, it poses a big challenge in the self-servicing abilities of the patient.

When synovitis persists, erosive changes develop first in the humeroulnar joint. If the cartilage between the humerus and ulna is damaged, the head of the radius is pushed into proximal direction, preventing both flexion and extension. After flexion is lost, a loss of extension of the elbow follows. The permanent pressure of the radial head to the *capitulum* generates a typical lateral pain, restriction of supination and crepitus in the pronation and supination. Edema on the medial side of the elbow with joint destruction can provoke an isthmic syndrome of the ulnar nerve. In the elbow area, the bursa of the olecranon is often affected by a rheumatic process, or sometimes by an infection.

11.2.4 Shoulder

Impairment of the shoulder in RA varies. It can be the initial location for RA, e.g. in "old age RA." Most often, destruction of the shoulder joint is the manifestation of a progressing disease. Due to synovitis, erosions and destructions of the humerus head and glenoidal cavity occur. The subacromial bursa is often affected, and can perforate. Similarly, the long tendon of the biceps can crack, and this is manifested by a lump in the biceps when the patient flexes against a resistance. When the tendon of rotators is affected, upper migration of the humeral head occurs. These processes result in severe impairment of shoulder function, especially in abduction, but also in rotation and flexion. The ability of the patient to perform any self-service can be considerably decreased.

11.2.5 Cervical Spine

The cervical spine is often affected in RA, especially the C1–C2 area. The space between the odontoid projection and the curve of the atlas is usually 3 mm or less. If it is more than 3 mm, we term the condition ***atlantoaxial subluxation***, which can reach as much as 10 mm. Atlantoaxial subluxation appears in as many as 30% of serum-positive RA cases. There are lateral, horizontal, and vertical atlantoaxial subluxations. They can be clinically asymptomatic, or they can be manifested by refractory pain in the sense of cervicocranial or cervicobrachial syndrome. Magnetic resonance imaging can be a significant help in diagnosis of cervical myopathy. It can distinguish, e.g. subluxation and destruction of the transversal ligament from the pressure caused by the inflammatory pannus. When the cervical spine is affected, there is always a risk of fatality or the development of quadriparesis. Intubation of these patients in any common surgery can be life-threatening.

The basic types of cervical spine impairment in RA include: atlantoaxial subluxation, narrowing of the intervertebral disc, spondylodiscitis, manifold subluxations, erosions of the intervertebral disc and sclerosis, erosion of the apophyseal joints, and osteoporotic fracture. When impairment of the C1–C2 area is considered, three subareas are discussed. One third is constituted by the protuberance of the odontoid vertebra, the other third by the spinal cord, and the last third is free. If the shift of the C1–C2 exceeds 10 mm, the free part is lost and there is imminent danger of spinal cord damage. Between the transversal ligament of the atlas and the rear part of the odontoid vertebra, there is the right synovial joint. The transverse ligament prevents the movement of the C2 body forward, over to the C1. Persisting synovitis in this joint can impair the ligament and erode the protuberance of the odontoid vertebra. Synovitis can affect the apophyseal joints of the atlantoaxial connection. If it results in loss of bone or change of shape at this site, the odontoid vertebra may penetrate the *foramen magnum* and establish a condition called basilar invagination, thus endangering the cervical spine and *medulla oblongata*. From a clinical viewpoint, these patients can show manifestations of basivertebral insufficiency. However, they are endangered by sudden death after unexpected vomiting or physical trauma. The indication of neurosurgical intervention is unmanageable pain or manifestations of pyramidal system pain.

11.2.6 Hip Joint

Coxitis detection is always a significant finding in RA, because it often occurs in younger patients and usually causes rapid destruction of the joint, which requires total hip endoprosthesis. The first manifestation is usually pain, localized either deep in the joint or predominantly in the groin, emanating to the thigh. Sometimes the patients limp. Restriction in the range of motion appears relatively late. On X-ray

scans, coxitis shows no visible manifestations. An ultrasound scan is beneficial for diagnosis, where the width of the joint capsule bigger than 8 mm or a lateral difference bigger than 1 mm means coxitis. Gradually, diffuse narrowing of the joint space develops, destruction of the joint follows, and often migration to the head and a condition called *acetabulum protrusion* develops. Not so often, when the head is necrotic and collapses (e.g. in patients treated with glucocorticoids), *lateral migration of the head* may develop instead of acetabulum protrusion. Bursa impairment or bursitis should be distinguished from direct joint impairment. The most common bursa involvement is synovitis, bursa effusion of the iliopsoas that can look like a unilateral or bilateral formation in the groin, with or without swelling of the extremity. It usually does not communicate with the hip joint. Manifestations can often be mistaken for a hernia, lymphadenopathy or an aneurysm.

11.2.7 Knee Joint

The knee joint is very often involved in RA, almost in all cases. With not much effusion, the positive sign of this condition is a bulge. When there is more effusion, the patella ballottement is positive. The inflammatory process can be proven via a sonogram or through the analysis of synovial fluid. The skin above the joint is warmer when inflammation is present. When synovitis lasts longer, extension of the knee is restricted. Subjectively, the patient usually feels the pain even when resting. Frequent complication of knee synovitis is the development of a popliteal Baker's cyst. The synovial fluid gets to the popliteal bursa, localized between the heads of the gastrocnemius muscle, through a pump mechanism. There is no backward flow of the fluid. The cyst may migrate along the medial side of the calf as far as the ankle area. There may be a rupture of the cyst and the effusion can extend into the calf. This condition is reminiscent of acute thrombophlebitis, for which patients are often mistakenly treated. Nowadays, the sovereign diagnostic method for the detection and localization of cysts is ultrasound.

Later in the course of the disease, when synovitis persists, destruction of the knee joint develops. With the loss of cartilage, the longitudinal and transversal ligaments weaken, anterioposterior instability develops, with later deformation of the knee joint. *Genu valgum* is more frequently observed than *genu varum*. The development of flexion in the knee joints presents discomfort for the patient.

11.2.8 Joints of the Foot

(a) ***The metatarsophalangeal joints*** are commonly affected, sometimes becoming very painful, because all the body weight lies on them. ***Chronic synovitis of the MTP heads*** causes severe erosive changes, the development of cysts and geodes, and subluxation of the heads. Due to the extension of the synovial

granuloma and degradation of the bone, a callus on the sole develops. Further damage of the tissues results in the penetration of synovial fluid on both the dorsal and plantar surface of the foot and sometimes skin fistula develops. Secondary infection excludes surgical intervention. Walking is immensely painful, if not impossible in these cases.

(b) ***Interphalangeal joints***. On the toes, ***hammer toe deformities*** develop. This is caused by flexion of the interphalangeal joints and extension of the MTP joints. Lateral deviations of both big toes are common (*hallux valgus*). Both a longitudinal and transversal fallen instep will appear. ***Diffuse edema of the foot*** (pitting edema) is due to local venous insufficiency, or obstruction of the lymphatic vessels or veins when the ankle, knee, or hip joint are swollen.

(c) ***Hindfoot joints***. The subtalar and talonavicular joints are often involved in RA. Synovitis of these joints causes pain and stiffness, and sometimes even subtalar dislocation. A spasm of the peroneal muscles induces valgus-like deformation of the foot and peroneal spastic fallen arches. When cartilage is lost and destructions develop, the valgus-like deformation of the foot is further worsened and the longitudinal arch of the foot falls. There are cases with a severe collapse of the whole middle part of the foot. Heel pain is more common in spondylarthritis, but it can occur in RA as well. Mostly it is associated with the development of nodules in the Achilles tendon, and its rupture has been described in the literature [29].

11.3 Extra-articular Involvement in Rheumatoid Arthritis

RA is a systemic disease that can have both significant ***systemic manifestations***, such as increased body temperature, nausea, and weight loss, and it can also affect many organs and systems. From a clinical point of view, the following are the most serious: ***vasculitis, amyloidosis***, *and* ***pulmonary fibrosis*** (Table11.5).

Laboratory manifestations, often present in systemic diseases, are anemia, increased levels of acute phase reactants, thrombocytosis, and increased readings on hepatic tests. Some studies have shown extra-articular impairment more often with the presence of HLA-DR-1 and HLA-DR-4 antigens.

Table 11.5 Extra-articular manifestations of rheumatoid arthritis

- Rheumatoid nodules
- Eye manifestations
- Pulmonary manifestations
- Cardiac manifestations
- Neurological manifestations
- Hematological manifestations
- Hepatic manifestations
- Vasculitis
- Amyloidosis

11.3.1 Rheumatoid Nodules

Rheumatoid nodules are more frequent in patients in whom the presence of rheumatoid factors is positive. In these patients rheumatoid nodules occur in 20% of cases. They normally accompany a more active impairment. Nodules mostly develop in places where higher pressure occurs, i.e. hand joints, sacral prominences, or the foot sole. Rheumatoid nodules are often attached to the periosteum. Histologically, a central necrosis surrounded by fibroblasts is typical. With successful treatment with DMARDs, rheumatoid nodules may retreat. Paradoxically, even at successful treatment with methotraxate (MTX) and a decrease of the general activity of the disease, nodulosis develops, mainly in the finger tendons. Rheumatoid nodules are benign and altogether a cosmetic matter, but when they occur in a place where they hinder the patient mechanically (e.g. on the foot sole), they should be surgically removed.

11.3.2 Eye Involvement

Dry keratoconjunctivitis is the most frequent eye manifestation of RA. It occurs in 10–35% of patients, mostly with a positive rheumatoid factor. When this disease and xerostomy occur at the same time as RA, we talk about secondary Sjörgen's syndrome. Dry keratoconjuctivitis is diagnosed by decreased creation of tears via Schirmer's test, and based on proof of damage to the cornea by dyeing, either with fluorescein or Bengal red.

Episcleritis can be nodular or diffuse. It causes redness in the eye and pain, but only rarely sight disorders. It mostly correlates with the disease activity. This is a more frequent but less severe impairment.

Scleritis is less frequent, but more severe. It often correlates with the presence of vasculitis and long-term, highly active RA. A complicated form of scleritis is necrotizing scleritis. Untreated scleritis may result in ***scleromalacia perforans***.

Other infrequent eye involvements in RA include ***uveitis, episcleral nodulosis***, *or* ***peripheral ulcerating keratitis***.

The eye can also be damaged by certain pharmaceuticals used in the treatment of RA. This is mainly the case of ***secondary cataract*** and ***glaucoma*** after corticoid application, and ***keratopathy*** and ***retinopathy*** after treatment with antimalarial drugs (Table 11.6).

Table 11.6 Eye manifestations of rheumatoid arthritis

- Dry keratoconjuctivitis
- Scleritis
- Scleritis complications
- Toxic manifestations of pharmaceuticals
  – Corticosteroids
  – Antimalarial drugs

11.3.3 Pulmonary Involvement

Pulmonary involvement is relatively frequent in RA, though often not diagnosed, since it can have weak symptoms or be misdeemed for another pulmonary disease (Table 11.7).

Pleuritis is mostly asymptomatic. According to departmental studies, it occurs in as many as 50% of RA cases. Rheumatoid pleural exudates are transudates with a low number of cells, with a prevalence of leukocytes. Glucose concentration is low (less than 1.4 mmol/l). Concentrations of complement in exudates are decreased, rheumatoid factors are positive. In leukocytes, inclusions containing rheumatoid factors can occasionally be shown. Pleuritis may sometimes even precede the development of arthritis in RA, though it is mostly present at a higher overall activity. Chronic exudates may result in fibrosis.

Rheumatoid nodules are usually found in seropositive patients with a higher activity of the disease and with the presence of nodules in another locality. They may differ in dimensions, e.g. smaller than 1 cm, but also 6–8 cm. They are often located peripherally – subpleurally. They can create cavities, evoke the creation of pleural exudates, and bronchopleural fistulas. Parenchymal pulmonary rheumatoid nodules are mostly asymptomatic.

C*aplan's syndrome* is characterized by pulmonary rheumatoid nodulosis and pneumoconiosis in RA patients. Nodules are multiple and occur throughout the entire periphery of the lung field. Caplan's syndrome occurs in RA individuals who were massively exposed to coal dust, but a similar situation occurs with exposure to siliceous and asbestos dust.

Diffuse interstitial pulmonary fibrosis occurs relatively often. The percentage differs depending on the detection methodology applied. A more sensitive method, HRCT (High Resolution Computerized Tomography), has been introduced recently, which allows diagnosis of the initial stages. Another progress is the introduction of biopsy methods. Histological findings vary from alveolitis with a prevalence of lymphocytes, through neutrophilic inflammation, up to pulmonary fibrosis. In the pathogenesis of rheumatoid pulmonary fibrosis certain pro-inflammatory mediators are present, as are certain immunogenetic signs (e.g. HLA-DRB1 or HLA B40), and some RA independent factors, such as smoking and the presence of the alpha-1-antitrypsine phenotype.

Table 11.7 Respiratory manifestations of rheumatoid arthritis

- Cricoarytenoid arthritis
- Pleuritis
- Rheumatoid nodulosis
- Caplan's syndrome
- Diffuse interstitial pulmonary fibrosis
- Bronchiolitis obliterans
- Constrictive bronchiolitis
- Drug toxicity manifestations

Other impairments of the lungs:

- *Bronchiolitis obliterans* is relatively rare; it reacts well to corticosteroid administration.
- *Constrictive bronchiolitis* does not adequately respond to treatment and has a poor prognosis.
- *Toxic drug manifestations in the lungs* are described after gold salts, penicillamine, and methotrexate.

Pulmonary involvement in RA increases the morbidity and mortality of the basic disease. So far, therapy has been empirical.

11.3.4 Cardiac Involvement

Pericarditis is the most frequent disease of the heart in RA (Table 11.8). Analogous to pleuritis, it occurs in seropositive patients with a higher activity of the basic disease.

Myocardial diseases. The myocardium can be affected with nodular, granulomatous lesions, or even multiple diffuse fibrotic changes. Nonspecific myocarditis is mostly asymptomatic, and it only exceptionally influences the size of the heart and its function. Cardiomalacia is slightly more frequent in males with RA than in the population without RA. Impulse conductivity disorders were also described in these cases of myocarditis.

Endocardium diseases. Echocardiographic examinations reveal valvular lesions in as many as 30% of cases, which are, however, hemodynamically irrelevant. The involvement of the radix of the aorta is much rarer than in ankylosing spondylitis and the development of aortal insufficiency is a rarity.

Other heart diseases:

- *Coronary arteritis* occurs sporadically as a part of vasculitis and is treated as such
- *Amyloidosis of the heart* occurs very rarely
- *Vasculitis* of small blood vessels is a serious manifestation of RA and it underlies many clinical manifestations of RA

The most frequent clinical features are skin manifestations; these involve macular or papular urticaria, livedo reticularis, nail bed infarction, and skin ulcers, mostly

Table 11.8 Cardiac manifestations of rheumatoid arthritis

• Pericarditis
• Myocarditis
• Granulomatous valvulitis
• Cardiac valve insufficiency
• Coronary arteritis
• Cardiac amyloidosis

on the legs. In vasa nervorum affection, a peripheral motor neuropathy or distal sensorial neuropathy occurs. From a prognostic point of view, extremely serious is the development of finger tip gangrenes, expansion of the processes into other areas, the development of multiple neuropathies, and general symptoms of the disease, like increased body temperature and loss of weight. These may be signals of the development of systemic rheumatoid vasculitis with arterial involvement also in the mesenteric, coronary, and cerebral areas, and present in general a bad prognosis.

The often pronounced statement that vasculitis occurs more often in patients treated with corticosteroids does not hold since it normally occurs also in patients who have never been treated with glucocorticoids. Chronic administration of glucocorticoids, however, may result in transformation of vasculitis into occlusive vasculopathy.

11.3.5 Hematological Abnormalities

Anemia is a frequent finding in RA and it is an expression of the activity of the disease. The cause of anemia in RA is multifactorial. In principle, it is a matter of so-called *anemia in chronic diseases*. Iron utilization is impaired, which is reflected in a decreased concentration of plasmatic iron and transferrin. Like in other inflammations, concentrations of ferritin and hemosiderin are higher, and there is an abnormal retention of iron via the reticuloendothelial system from the vanishing red blood cells. Further contributing to the development of anemia is a decreased concentration of erythropoietin and a slightly decreased reactivity of bone marrow to erythropoietin. Infective erythropoiesis is also manifested with a premature destruction of red blood cells. The lifespan of erythrocytes is only slightly shortened. Anemia in RA is typically normochromic and normocytic, if it is not complicated by other influences like chronic deficits (e.g. on administration of NSA), wrong nutrition, intercurrent infections, bone marrow suppression, or drug induced hemolytic anemia.

Anemia due to iron deficiency may also occur in RA. This is characterized by low concentrations of the serum iron and by a normal or high iron-binding capacity. Megaloblastic anemia is infrequent. It may occur in coincidence of RA and pernicious anemia, or it may be invoked by drugs (methotrexate, sulfasalazine). Anemia in RA is usually chronic, patients are well adapted to it, and it does not cause them any problems even though the decrease of hemoglobin is considerable. Anemia improves when the overall activity of the underlying disease is successfully suppressed. The drug that can improve the blood count of erythrocytes, without influencing the activity of the underlying process, is erythropoietin. Administration of iron by itself does not usually provide an adequate effect.

Thrombocytosis is a manifestation of RA activity, it correlates with the number of active joints, and it is often present in extra-articular manifestations. The mechanism of thrombocytosis development is not yet clear. Thrombocytosis is not a predisposing factor of thrombotic events.

Thrombocytopenia in RA is rare. It can be a manifestation of drug toxicity (e.g. with sulfasalazine) or Felty's syndrome.

Eosinophilia occurs relatively often. Association with extra-articular RA manifestations has been reported, especially with involvement of the lungs. There are also a number of RA drugs that induce eosinophilia, mainly gold salts.

Lymphadenopathy is relatively common, as its occurrence is estimated to be as high as 30%. It is more common in active forms of RA. Enlarged lymph nodes can be palpated as soft and moving, especially in the axillae, inguen, or epitrochleal area. Histological examination can reveal follicular hyperplasia, sometimes reminiscent of lymphoma. In the macrophages, hemosiderin is often detected, and the presence of iron in lymph nodes is increased.

11.3.6 Felty's Syndrome

Felty's syndrome is defined as a combination of RA with splenomegaly and leukopenia. The syndrome is observed mainly in patients with long-lasting RA that is seropositive, nodular, and deforming. In some patients, however, the articular activity of the disease is low at the time of development of Felty's syndrome. Many patients have positive findings of antinuclear antibodies, ulcerations in the lower extremities and hyperpigmentations.

Successful treatment of the underlying disease may sometimes regulate the cytopenia as well. In an effort to increase the number of granulocytes, corticosteroids were administered (increasing the risk of infection), parenteral testosterone, lithium, and recently, granulocyte-macrophage colony-stimulating factor (GS-CSF). Splenectomy is indicated when there are recurrent severe infections caused by neutropenia, which are refractive to treatment by long-term modifying therapy. The result of a splenectomy is mostly correction of hematologic and bone marrow abnormalities, while the RA activity is usually not influenced.

11.3.7 Hepatic Involvement

Active RA is usually connected with increased values in hepatic tests – transaminases and the isoenzyme of alkaline phosphatase. Increased values in hepatic tests can occur in parallel with thrombocytosis and sedimentation. When the disease activity is decreased, hepatic tests yield results comparable to normal in most cases. At the time of increased hepatic test values, histological examination of the hepatic parenchyma is either completely negative or there are only minimal, nonspecific changes with slight periportal infiltration by mononuclear cells.

The abnormalities of hepatic tests can be caused by certain drugs, especially NSAIDs (e.g. diclofenac, salicylates). It can be quite difficult to differentiate hepatopathy in the framework of an underlying disease from drug-induced hepatopathy,

as after the discontinuation of NSAIDs the values of hepatic tests are usually normal. All the disease modifying drugs can be hepatopathic, yet methotrexate is so to a higher extent (see chapter on Disease Modifying Drugs).

11.3.8 Neurologic Involvement

Peripheral nerve lesions can develop due to compression or vasculitis in the nerve sheaths. The spinal cord can be affected by compression due to atlantoaxial dislocation.

Compressive syndromes develop when an inflamed pannus presses the nerve against a solid structure. The most common compressive (tunnel) syndrome is carpal tunnel syndrome, in which the *n. medianus* is compressed in a narrow channel under the *retinaculum flexorum*. Diagnosis is established in a standard way (via the patient's history and physical examination), and with help of electromyography (EMG). In the case of positive EMG findings, potential surgical intervention should be considered. A not so common tunnel syndrome is ***n. ulnaris* compression** in the area of the elbow joint, and ***tarsal tunnel syndrome*** due to compression of the *n. tibialis*.

Distal sensory neuropathy has a stocking-like appearance. It consists of hyperesthesias and paresthesias with minimum motor weakness and a good prognosis.

Sensory and motor neuropathy has usually a rapid onset and a bad prognosis with fatal outcome within 2 years. It occurs in severe cases of RA with constant activity, mostly of the seropositive type, erosions, generalized vasculitis, nodules, and a high titer of rheumatoid factor.

11.3.9 Amyloidosis

Amyloidosis occurs rather rarely in long-term RA. It can be *primary*, associated with myeloma or similar diseases, or *secondary*, accompanying various other diseases, e.g. chronic inflammatory diseases resembling RA. The most common involvement concerns the kidneys, manifested by proteinuria, or even by nephrotic syndrome. Amyloid can be detected histologically, with help of biopsy of the affected tissue. Treatment of amyloidosis is not very successful and the prognosis of patients is often unfavorable.

11.3.10 Other Conditions Complicating Rheumatoid Arthritis

Septic (infectious) arthritis occurs in RA more frequently than in the general population. As many as 10% of RA patients are reported to have septic joints through the course of their disease. Infections belong to the causes of shortened average life

span of RA patients. Infections can be contracted spontaneously, e.g. in immune-compromised patients (due to corticoid and/or cytostatic treatment), or spreading from ambient locations (e.g. infected tylomas on the soles), or after administration of an intra-articular injection. The classic picture of a septic joint with reddened skin above the joint, hot joint, fever, or shivers induced mainly by pyogenic staphylococcus, is no longer so common today. More frequent is the less dramatic picture of synovitis, often with no systemic manifestations and no reddening, which is induced by other, less virulent organisms. Some of these have not even been considered to be pathogenic until recently (e.g. *Staphylococcus epidermidis*).

Secondary osteoporosis. Secondary osteoporosis (OP) is very common in RA, even in patients who were never treated with corticosteroids; however, it is more frequent in patients who receive hormone therapy. In RA, *periarticular* and *diffuse* osteoporosis may develop. It has been discussed for a long time whether diffuse OP can develop in patients who were never treated with glucocorticoids. Recent papers, e.g. Sambrook [28], have clearly shown on groups of monozygotic twins of whom only one suffered from RA that diffuse OP was present also in the untreated form of RA, though not in all patients. Possible triggering mechanisms of secondary OP are in Table 11.9.

Corticosteroid induced osteoporosis is the most serious problem of long-term treatment with glucocorticoids. It depends mainly on the cumulative dose of glucocorticoids, although some authors stress the significance of a daily dosage of glucocorticoids as well. A safe daily dosage of glucocorticoids was proposed to be about 5–8 mg of prednisone or its equivalent a day [28] – but it was not definitively substantiated. Prevention of the development of OP is in suppression of the underlying disease activity, with corticoids administered in a maximally reduced dose, stimulation of physical activity and prophylactic administration of calcium and vitamin D (Table 11.10). The principles of therapy of glucocorticoid-induced osteoporosis are given in Table 11.11.

Table 11.9 Potential factors triggering generalized osteoporosis in rheumatoid arthritis

- Systemic effect of inflammatory mediators associated with the activity of the disease (IL-1, IL-6, TNF, etc.)
- Changes in the concentrations of circulating hormones
- Changes in calcium metabolism
- Changes in skeleton load
- Effect of RA therapy drugs

Table 11.10 Prevention of glucocorticoid-induced osteoporosis

- Use corticosteroids in the lowest possible dose and for the shortest possible period
- Try to suppress RA activity
- Stimulate physical activity, avoid immobilization
- Do not use sedatives and antihypertensive agents (orthostatic hypotension)
- Eliminate the risk of a fall, especially at home
- Supply calcium in the minimal daily dose 1,000 mg
- Correct potential deficit of vitamin D (800 IUdaily)

Table 11.11 Therapy of glucocorticoid-induced osteoporosis

- The same strategy as in prevention of osteoporosis induced by glucocorticoids, combined with follow-up therapy
- In postmenopausal women: hormone replacement therapy (HRT), estrogens combined with progestagens in women with in situ uterus
- In premenopausal women, men, and postmenopausal women with contraindicated HRT therapy: bisphosphonates
- Exceptionally during strong pain caused by vertebral fractures: calcitonin
- Exceptionally in older cachectic patients: anabolic steroids

Table 11.12 ACR Classification criteria for rheumatoid arthritis diagnosis [1]

Criteria	Definition
*Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 h
*Arthritis of three or more joint areas	At least three joint areas simultaneously with soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
*Arthritis of hand joints	At least one area swollen (as defined above) in a wrist, MCP, or PIP joint
*Symmetric arthritis	
*Rheumatoid nodules	Simultaneous involvement of the same joint areas (as defined in criteria no. 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
*Serum rheumatoid factor	Subcutaneous nodules over bony prominences, extensor surfaces, or in juxtaarticular regions, observed by a physician
	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
*Radiographic changes	Radiographic changes typical of RA on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Note: A patient suffers from RA if at least four criteria are positive, while the criteria nos. 1–4 have to last for at least 6 weeks.

11.4 Classification Criteria for Rheumatoid Arthritis

Recently, diagnosis of RA in clinical studies, epidemiological surveys, and also in routine clinical practice has been done via the criteria suggested by Arnett et al. [2] for the needs of ACR (Table 11.12).

Unlike the previous criteria, these do not use RA categories such as "classical," "definite," or "probable." Sometimes they are criticized for insufficient sensitivity, mainly for incipient early RA. Their specificity is not ideal either, because there is a group of patients who undoubtedly fulfill all the criteria and suffer from other diseases. It has also been discussed that the criteria consider only X-ray scans of hands, although it is known that RA often starts with destructions in the legs.

11.5 Laboratory Findings in Rheumatoid Arthritis

Rheumatoid factors (RF) are determined mostly via the latex fixation test. When this method is used, 70–80% of patients with RA are labeled to be "seropositive." This method is used mainly for determining the rheumatoid factor of the IgM class. ELISA can determine the RF of IgG, IgA, and IgE classes. The positive finding of rheumatoid factor is sometimes mistakenly considered to constitute RA. However, it occurs in at least 1% of the population and its positive presence is detected in other diseases as well, such as sarcoidosis, leprosy, tuberculosis, hepatopathy, pulmonary fibrosis, and the like. The highest titers of RF are detected in Sjögren's syndrome (SS) and macroglobulinemia, which are not accompanied by arthritis. It should also be noted that RFs are often not positive at the beginning of the disease but appear later in its course, and this further weakens the diagnostic value. RA patients with positive findings of RF, who are labeled as seropositive in common rheumatologic practice, have a worse prognosis than "seronegative" patients. They show an overall tendency to erosions and destructions, higher functional impairment, and less frequent remissions.

Anticitrulline antibodies recognize many proteins that contain citrulline, which is modified from posttranslational modification of arginine. IgG and IgM anticitrulline antibodies are produced by B-cells in rheumatoid synovial tissue and synovial fluid. They are produced in early RA as well as during the preclinical phase in some patients and have some significance for diagnosis and prediction of severity of the disease [3].

Blood count. *Acute phase proteins. C-reactive protein (CRP). Serum protein electrophoresis. Immuno-electrophoresis. Circulating immune complexes*. Antinuclear antibodies (ANA). In RA, they are positive in 33–52% of cases. In 70–90% their titers are low. Most frequently (73%), it is a diffuse type of fluorescence. The assumed correlation of ANA occurrence in RA with the severity of extra-articular involvement has not yet been proven.

11.5.1 Biochemical Examination, Urine Examination, Other Specific Examinations

Immunogenetic examination. This is performed only in specialized centers. In 67% of RA patients, the HLA-DR4 antigen is positive (determined by DRB1* 0401 allele), while it is found in 30% of the control population.

Synovial fluid analysis. Synovial fluid is opaque, with a yellow, whitish, or green tinge. Its viscosity is lowered, and the mucine coagulum is of low quality. The content of proteins is high, the overall hemolytic complement, as well as C3 and C4 elements, are decreased (unlike in serum where they have normal values). The number of cells varies from 2,000 to 60,000. Polymorphonuclear leukocytes pronouncedly prevail in the balance. The outcome of the cultivation of synovial fluid in "pure" RA is always sterile.

11.6 Imaging Methods for the Assessment of Rheumatoid Arthritis

11.6.1 X-ray Examination

For RA assessment, *X-ray scanning* is still the basic imaging algorithm. X-ray changes can be divided into early and late ones. Early changes include the proliferation of soft parts and periarticular porosis. Due to the proliferation of synovial fluid, the joint cavity can extend for a short period of time. Initial periarticular porosis becomes global under the impact of therapy through the course of the disease. Early erosions are mostly localized in marginal positions, i.e. in the areas not covered by cartilage (called a bare area), thus they are exposed first to the invasion of the pannus [39]. Late changes include a pronounced narrowing of joint space, which is always symmetrical. Destructions are situated either marginally, or centrally. For advanced stages of RA, subluxations are characteristic, mainly in the hands and feet, and so is bone ankylosis. The disease can become complicated by osteonecrosis, due also to therapy (corticosteroids). Another late manifestation of RA is subchondrally located cysts (geodes, pseudocysts). The current explanations of their etiology are so far inconsistent. They are presumably caused by the expansion of the pannus to bone or, as some authors believe, they are intraoseally situated rheumatoid nodules [19].

Steinbrocker's RA classification according to X-ray changes has four categories. However, they do not sufficiently represent the progression of the disease. Loren's or Sharp's classification suits the purpose better, and together with his explanatory atlas, it is used today in clinical trials.

X-ray picture of the hands and wrist. The basis for RA radiodiagnostics is a good quality skiagram of both hands in anterioposterior and slant projection (according to Norgaarden). After nonspecific changes (see above), erosions develop which are characteristic for the aggressive type of synovitis. Initial erosions are typically localized in predilection areas of the *proc. styloideus ulnae* and also in marginal areas, in a radial way in the MCP joints of the second and third finger, and in the PIP joints of the third finger. Before the first erosions are developed, lamellas often become decalcified. Late changes resemble ulnar deviations and buttonhole or swan neck deformations. The destruction of cartilage in the radioulnar joint leads

to a loosened connection with the dorsal migration of the ulna and radial migration of the wrist. Late manifestations involve ankylosis, which affects mainly the carpal bones.

X-ray picture of the feet. The feet are affected in 80–90% of RA patients. In about 10–15% of patients, changes in the feet precede changes in the hands. That is why it is important always to take X-ray scans of both the hands and feet. First erosions are typically located near the medial and lateral circumference of the *V. metatarsus* heads. In other MTP joints, erosions are in the predilection medial location. Early erosive changes can also be found in the interphalangeal joint and MPT joint of the big toe. Later, *halux valgus* deformation develops. A narrowing of the joint space is symmetric and subluxations that develop in later stages are fibular. Ankylosis affects predominantly the area of tarsi. In the calcaneus, fibroostitis, enthesopathies develop, but at the same time destructions at the insertion of the Achilles tendon and plantar aponeurosis appear. Their occurrence is less common than in seronegative spondylarthritis.

X-ray picture of the knee. The basis of this assessment is in an anterioposterior and lateral projection, while standing, i.e. under full load. Usually both knees are affected. Exudate is a frequent manifestation, localized supralaterally, which causes obscuring and extension of the soft sections and ventral dislocation of the patella. The first erosions are localized at the medial and lateral circumference of the tibial and femoral condyles (bare area). Symmetrical narrowing of the joint space follows, with the development of synovial pseudocysts and deformations. A typical deformation is *genua valga*.

X-ray picture of the hip joints. The hip joints are affected in about 50% of RA patients, and they always represent a significant finding and a considerable worsening of functioning for the patient. The X-ray scan of hip joints is also always done while standing, i.e. under full load. The first erosive changes are usually localized in the bony-and-cartilage rim of the femoral head at the place where it joins the neck of the femur. Synovial cysts develop on the femoral head and on the acetabulum. Narrowing of the joint space is always concentric. In later stages, the femoral head migrates in an axial direction, and the acetabulum protrudes into the lesser pelvis.

11.6.2 Specialized Techniques

Some of the latest techniques were developed with the aim of better surveying the soft parts and detecting erosions as thoroughly as possible.

Magnifying techniques. These work with enlarged scans and therefore should detect the erosive changes sooner than conventional nonmagnifying techniques. The disadvantage of these techniques is a higher exposure to radiation for patients. Magnification can be achieved through either an optical or radiographic method. When the radiographic method is used, special equipment with a microfocal X-ray tube is employed. This method uses less radiation exposure than when an optical

magnification is used. The disadvantage is the difficult positioning of the patient, and a picture of only a small part of the joint in the scan.

Xerography. Enables a more detailed imaging of both the bone edges and soft parts. Because of its high radiation exposure, it is not used anymore.

Scintigraphy. Bone scintigraphy can be beneficial for RA in three aspects: substantiation of the presence of synovitis, determination of its distribution, and possibly its development. Scintrigraphy can prove the presence of hyperemia and inflammation which are not pronounced on a conventional scan.

Magnetic resonance imaging (MRI). The use of MRI in RA diagnostics has undergone a stormy development, and in the future MRI could become the single most important imaging method in this disease. However, many issues should be resolved before this happens (resolution, interpretation, and validation of the examination), and last but not least the price.

MRI shows higher sensitivity in the detection of *early erosive changes*. It can depict the marrow part of the bone, joint cartilage, muscles, tendons, and fats. MRI has become the first choice in the early detection of *osteonecrosis*, frequently found in RA. MRI can depict *the articular cartilage*, distinguish hyaline and fibrous cartilage and its tiny lesions. MRI can depict the tendons and connective tissue, and it is a suitable method for the assessment of the *rotator cuff*.

Currently, validation of this method is being substantiated, when a gadolinium contrast is applied in MRI with the aim to distinguish synovial hyperplasia from the presence of exudate. MRI is the first choice in cervical spine imaging in RA when there is neurological clinical impairment [15, 19].

11.7 Differential Diagnostics in Rheumatoid Arthritis

Differential diagnostics in RA involves a wide spectrum of both methods and rheumatic diseases. The most relevant conditions for differential diagnostics are:

1. Systemic disease of the connective tissue
   (a) Systemic lupus erythematosus
   (b) Polymyositis-dermatomyositis
   (c) Sclerodactylia in scleroderma
2. Crystal induced diseases
   (a) Gout arthritis
   (b) Disease due to deposition of calcium pyrophosphate dihydrate (CaPPD)
3. Spondylarthritis
   (a) Peripheral form of ankylosing spondylitis
   (b) Reactive arthritis
   (c) Psoriatic arthritis
4. Osteoarthrosis (OA)
5. Other diseases

(a) Rheumatic polymyalgia
(b) Acute bacterial arthritis
(c) Arthritis in Lyme boreliosis

11.8 Assessment of Rheumatoid Arthritis and its Treatment

For the modern therapy of RA, several principles are important:

- Therapy should be provided by a multidisciplinary team, for both hospitalized in-patients and clinical out-patients. Besides a rheumatologist, the team should include a physical therapist, an orthopedic surgeon, a social worker, a psychologist, a specialist for employment, and a general practitioner. The whole team is to be managed by the rheumatologist.
- Early diagnosis is very important, as well as the assessment of risk prognostic factors and the activity of the disease.
- Although there are some algorithms of therapy, the treatment plan for a patient should be strictly individual.
- Information given to the patients is very important, as well as their active engagement in treatment and compliance.
- Regular assessment of functionality is very important, because any decrease in the patient's functionality should be considered as an alarming indicator (while other indicators, e.g. acute phase reactant, may at the same time seem to show success of the treatment).
- In specific intervals (1–2 years), a radiological examination should be carried out for detecting X-ray progression. It is known that the disease can continue through the destruction of joints, without any manifestations of synovitis.

11.8.1 Assessment of the Patient's Functionality

The exact assessment of a patient's functionality is the basis for both routine clinical therapy and for the scientific evaluation of different pharmacotherapeutical procedures. In the clinical practice of our country, Steinbrocker's evaluation is often used, which distinguishes four classes of functional impairment (Table 11.13). This classification is, however, too vague, and completely unsuitable for clinical studies.

In principle, a function can be assessed either by ***direct measurement*** (e.g. goniometric measuring or the measurement of time needed for a specific activity, e.g. walking 20 m), or through questionnaires that evaluate the function via a system of questions to the patient. As a high correlation was discovered between the two methods, currently only questionnaires are used since they are not so time

Table 11.13 Functional evaluation of patients by Steinbrocker

A.	The patient is able to do all the activities of daily life
B.	The patient is able to do normal activities but with some discomfort due to pain or restricted mobility
C.	The patient's activities are limited both in everyday life and at work
D.	The patient is confined to bed or a wheelchair and is able to look after himself/herself only a little or not at all

demanding. Globally, the ***Health Assessment Questionnaire (HAQ)*** is used. In several foreign centers, the HAQ fulfillment is a part of every routine medical check and lasts about 5–10 min.

The generic questionnaires assess the function, disability and a negative stress, e.g. McMaster Health Index Questionnaire (MHIQ), Nottingam Health Profile (NHP), Gröningen Activity Restriction Scale (GARS), and others, which are also used in rheumatology.

Specific questionnaires for arthritis typically provide data resulting from the presence of arthritis and joint damage. The most commonly used specific questionnaire is the HAQ/FDI, Health Assessment Questionnaire via the Functional Disability Index and the Arthritis Impact Measurement Scales (A-IMS). It assesses the activities of daily life related to motion, self-service, special hand function, and activities needed for work and leisure time which are of special importance for the independence of the patient. The complex of physiological and psychological interactions, together with the external environment, constitutes the individual's ability to carry out these activities. This ability can be limited only a little, more severe impairment requires changes in these activities. The most severe impairment is manifested by total disability and dependence on others [9, 13].

11.8.2 Rheumatoid Arthritis Activity Assessment

The assessment of RA is the basis for successful treatment of RA and clinical pharmacology of anti-inflammatory drugs. RA is a chronic, inflammatory, very heterogenous disease, and moreover, it has a wave-like course. In some patients, the individual symptoms of the disease are not in balance (no correlation of swelling and pain, laboratory and clinical indicators, and the like). Therefore, more indicators are needed to describe the activity of the disease.

On the one hand, there is a trend to use ever more of these indicators in an effort to judge all the attributes of the disease in a more thorough way (the divergence trend). On the other hand, clinical practice requires the reduction of these indicators to a smaller set, to more simple ones, or a tendency to summarize them (the convergence trend).

The American College of Rheumatology (ACR) and EULAR have both published a list of basal indicators for the activity of the disease and response criteria that should be used in RA clinical studies (Tables 11.14 and 11.15).

11.8.3 NonPharmaceutical Treatment of Rheumatoid Arthritis

The main objectives of the treatment of RA are:

(a) Induce a remission of the disease.
(b) If this is not possible, then: suppress the inflammation, decrease the pain, maintain muscle power, maintain function, improve quality of life, maintain fitness for the ability to work, suppress the destruction of joints seen on X-ray scans.

11.8.4 Pharmaceutical Treatment of Rheumatoid Arthritis

Nonsteroid anti-inflammatory drugs (NSAIDs) are usually prescribed in combination with other drugs during the entire course of an active RA stage. It should be noted that this is a symptomatic treatment which alleviates pain, decreases

Table 11.14 Indicators for rheumatoid arthritis

Disease indicator	ACR	EULAR
Number of tender joints	68	28
Number of swollen joints	66	66
Visual analog scale pain	+	+
Overall patient's evaluation	+	+
Overall physician's evaluation	+	+
Functions	+	+
Acute phase proteins	+	+
X-ray evaluation	+	+

Table 11.15 Response criteria

Improvement in	Paulus (four out of seven)	ACR (first two and next three out of five)
Number of tender joints	20%	20%
Number of swollen joints	20%	20%
Erythrocyte sedimentation rate	20%	20%
Overall evaluation by physician	40%	20%
Overall evaluation by patient	40%	20%
Morning stiffness	20%	–
Pain	–	20%
Disability	–	20%

Note: The non-pharmaceutical therapy of RA is composed of: regimen measures – bed confinement and rehabilitation, physical therapy, prosthetics

stiffness, and thus secondarily improves functionality, but in principle, it does not influence the underlying disease, i.e. it does neither suppress the acute phase reactants nor does it slow down the progression of the disease monitored on X-ray scans. When we apply them, we should consider the potential, sometimes severe side effects. Patients respond differently to the administration of NSAIDs, therefore the strategy is to try to find suitable NSAIDs for individual patients. The decision whether the chosen NSAID is effective or not, whether it should be substituted by another one, is decided after 2–3 weeks of the administration of a full anti-inflammatory dosage [26].

Disease modifying antirheumatic drugs **(DMARDs)**, have become recognized globally for their ability to modify the course of the disease, i.e. they objectively suppress the inflammatory reaction, including suppression of acute phase reactants, and slow down the progression of the disease monitored on X-ray scans. In this way they improve the quality of life of the patients. The above mentioned effect was proven for many of these drugs in numerous controlled studies, of both short- and medium-term (3–24 months). Long-term studies, which would prove the efficacy over a long interval (up to 20 years), are not available and due to ethical reasons it is difficult to imagine that they will be performed.

From 1980 onward, the number of these pharmaceuticals has been growing and their therapeutic possibilities have increased (Table 11.16). It should be highlighted that the onset of action of some of the new drugs is more rapid (usually 3–6 weeks), compared to some of the older drugs (gold salts, penicillamine), and this has influenced the choice of medication and the strategy of the initial treatment.

Table 11.16 Disease modifying antirheumatic drugs (DMARDs)

Globally administered DMARDs
Antimalarials
Gold salts (injections, peroral)
Sulfasalazine
Penicillamine
Methotrexate
Azathioprine
Cyclophosphamide
Cyclosporine
New DMARDs – in the process of registration
Leflunomide
Biological treatment
Etanercept
Infliximab
Adalimumab
Rituximab
In the stage of clinical trials
Inhibition of function and depletion of B-lymphocytes – Humanized mAb to IL-6 receptor – blocking effect on synthesis IL-6
Inhibition of costimulatory molecules on activated T-cells

Strategy of DMARD therapy. In the last few years, there have been essential changes in the strategy of DMARD therapy. The basic reasons for these changes are as follows:

(a) RA is not a benign disease – it can make the life of the patients shorter by as many as 10 years.
(b) Polyarthritis which is not under control results in disability in as many as 50% of the cases after 5 years and in as many as 90% after 10 years.
(c) Erosions and destructive changes occur in the first 2 years.
(d) Most of the DMARDs are not tolerated for longer than 2 years.

Trends in DMARD application. The traditional procedure in this therapy was called a pyramidal process by Smythe. The treatment started with NSAIDs and if the desired effect was not achieved three to 6 months later, DMARDs were applied. Some clinical specialists waited with DMARDs until a definite diagnosis was established, which according to the criteria in those days, meant waiting till erosions developed. Then sequences of individual DMARDs were tested, starting from the least effective to the most effective all the way up to the experimental drugs. This approach was also called "go low, go slow." Even though there was some logic in this approach, it did not suit some of the patients with active disease. In the first 2–3 years, no control of the disease activity was achieved in patients with the aggressive form of synovitis, irreversible morphological changes occurred, and often the side effects of the NSAIDs became manifested as they had to be administered in high doses. Therefore some rheumatologists proposed more aggressive schemes that have since been verified in clinical practice [7]. The procedures are as follows:

1. Early administration of DMARDs. Most centers have recently stopped the practice of several months initial waiting for the response to NSAIDs and they prescribe DMARDs immediately after the diagnosis has been determined.
2. DMARDs are administered consistently and continually, especially in the first years of the disease, i.e. the treatment should be continued also after its effect has been observed.
3. In very active forms, with unfavorable prognostic factors, the physician should not hesitate with the administration of more aggressive DMARDs (e.g. methotrexate) right at the beginning of the disease.
4. Before the effect of the administered DMARDs is declared to be unsatisfactory, an attempt should be made to increase the dosage to the maximum tolerated dose (e.g. with MTX to 20–25 mg, sulfasalazine to 3,000 mg, Azamun to 150 mg daily).
5. Various combinations of DMARDs should be administered in the forms which are resistant to monotherapy and in the early forms with a very aggressive course. When the effect of one DMARD is suboptimal, its administration should not be discontinued and it should not be substituted by another DMARD, but the initial treatment is to be continued and another DMARD should be added.
6. In very active forms with a rapid decrease of functionality and the development of erosions, the procedure of the inverted pyramid should be used, i.e. a com-

bined aggressive approach at the beginning, with a transition to maintenance monotherapy with one DMARD after gaining control over the disease.

7. For the intervals with the highest activity and on occurrence of extra-articular manifestations, bridging effect procedures are recommended. These include pulse steroid therapy (see below), intramuscular administration of steroids (see below), intravenous pulses of, e.g. cyclophosphamide, very high doses of intramuscular methotrexate administered over a limited period. To some extent, the regular application of intra-articular glucocorticoids works in the same way.
8. For the most severe forms, which are called "malign" and which rapidly destruct joint tissues and disable the patients, there are hopes in some of the new experimental ways of treatment, e.g. biological therapy (anti-TNF-monoclonal antibodies – Remicade, Adalimumab) and Enbrel. The Czech Rheumatologic Society published guidelines and proposed biological therapy also for patients with RA after failure of one or two DMARDs [36]. As *ultimum refugium*, some other procedures are being tested, such as bone marrow X-ray exposure or lymph node X-ray exposure.

Combined therapy. About 10 years ago, a few authors proposed a combined treatment of RA with several DMARDs. At that time, they relied on a body of empirical knowledge and some outcomes from uncontrolled studies that were available.
The rationale for the introduction of combined therapy:

(a) If the study of rheumatoid pannus results in the opinion that it is reminiscent of "a non-malign H-lymphoproliferative disease," logic has it that a combined treatment is approached according to the model of oncological therapy.
(b) The objective is to suppress the rheumatoid inflammation completely and for the longest possible time, thus to prevent both the initial and progressive destruction of joints and worsening of joint functionality. Because none of the available DMARD monotherapies has managed to achieve this, attempts to combine two or more DMARDs should be made.
(c) The precise mechanism of action and the location of intervention of individual DMARDs has not been elucidated, but it can be presumed that for different DMARDs they will not be identical. This fact may contribute to their synergic effect.
(d) The combination of drugs with different toxicities could enable their use in smaller doses and achieve higher safety.
(e) It is beneficial to combine a drug with a more rapid onset of its effect (e.g. sulfasalazine, methotrexate), with a drug with a slower onset of its effect (e.g. gold, antimalarials).

In recent years, combined therapy has extended into rheumatologic practice. O'Dell discovered in a questionnaire survey that combinations of various DMARDs are used by 99% of rheumatologists in the USA [24, 25]. Almost 40% of rheumatologists consider the combination to be the right approach as the initial treatment in indicated cases and 33% of rheumatologists use the combinations in more than one third of their RA patients. The recommended combinations are given in Table 11.17.

Table 11.17 DMARD combinations

With proven effect	methotrexate + antimalarials methotrexate + antimalarials + sulfasalazine methotrexate + cyclosporine sulfasalazine + methotrexate sulfasalazine + cyclosporine methotrexate + etanercept methotrexate + infliximab
With probable effect	methotrexate + injected gold sulfasalazine + antimalarials
With unsubstantiated effect	injected gold + antimalarials antimalarials + cyclosporine methotrexate + azathioprine penicillamine + antimalarials

It should be noted that as of yet rigorous proof of the efficacy of any of them has not been provided.

Combined treatment can be provided in different ways. Basic indications are:

- RA refractory to treatment with several DMARD monotherapies
- Early RA with high activity and the presence of unfavorable indicators for prognosis (see above)
- Patients with just a suboptimal curative effect of the monotherapy given
- Patients with just a suboptimal curative effect of the DMARD monotherapy, where it is impossible to increase the dosage for intolerance

11.8.5 Biological Treatment of Rheumatoid Arthritis

The biochemical characteristics of cytokines were established in 1970. The cDNA was cloned for the interferon alpha, beta, and gamma IL-2, TNF-α, and lymphotoxin (LT). It was soon shown that these cytokines are continually expressed in the synovial membrane of RA patients [11,17,22]. Complex relations of the entire cytokine cascade were studied and finally, a fundamental cytokine was identified. If the action of this cytokine – TNF-α – is neutralized, RA activity can be suppressed. The fact that this theory is right has been demonstrated by many preclinical and clinical trials. Available preparations are given in Table 11.18. Etanercept is a soluble receptor for TNF-α. It is a recombinant human TNF-receptor II (p 75), bound to the Fc fragment of IgG1 rhu TNF R:Fc. Etanercerpt (Enbrel) [23] is administered twice a week in the dose of 25 mg s.c., but at present 50 mg is administered once a week, s.c. It is a highly effective medication that rapidly decreases RA activity. It works well in the combined therapeutic regimen in patients in whom MTX achieved only a partial effect. After adding Enbrel to their therapy, their condition substantially improved. The therapy is relatively safe and the infection rate is only slightly higher.

Table 11.18 Present Biological Treatment

Title	Description
Adalimumab	Completely human anti-TNF-α mAb
Etanercept	Fusic protein containing 2 recombinant p75 TNF-α receptors and Fc segment of human IgG1
Infliximab (cA2)	Chimeric murine/human anti-TNF-α mAb

Van der Heijde et al. [34] evaluated the efficacy, including radiographic changes, and safety of etanercept and methotrexate (MTX), used in combination and alone, in patients with RA in whom previous treatment with a disease-modifying antirheumatic drug other than MTX had failed. They observed that etanercept in combination with MTX reduced disease activity, slowed radiographic progression, and improved function more effectively than did either monotherapy over a 2-year period. No increase in toxicity was associated with combined treatment of etanercept and MTX.

Van der Heijde et al. [35] compared patient-reported measures of function, health related quality of life (QoL), and satisfaction with medication in patients with RA treated with methotrexate (MTX), etanercept, or both for up to 1 year. They concluded that combination therapy with etanercept and methotrexate improved function, QoL, and satisfaction with the medication significantly more than monotherapy.

Infliximab (Remicade) is a chimeric monoclonal antibody against anti-TNF-α, which contains 75% of human and 25% of murine protein. It is administered intravenously, via infusion, 3 mg/kg of body weight, usually in intervals of 2, 6, and 10 weeks with another dose following after the eighth week. Within several weeks, 60–70% of patients responded favorably (ACR 20) to this treatment. The drug is also used in combination with MTX. Recent reports have shown very good and pronounced efficacy in slowing X-ray progress. Its safety profile is similar to that of etanercerpt. Maini et al. [21] demonstrated that throughout 102 weeks of therapy, infliximab plus MTX provided significant, clinically relevant improvement in physical function and quality of life, accompanied by inhibition of progressive joint damage and sustained improvement in the signs and symptoms of RA in patients who previously had an incomplete response to MTX alone. Smolen et al. [31] assessed the relationship between inflammation and joint destruction in RA patients who did not respond clinically to treatment. The results showed that even in patients without clinical improvement, treatment with infliximab plus MTX provided significant benefit with regard to the destructive process, suggesting that in such patients these two aspects of disease are dissociated.

D2E7 (Adalimumab) is a fully human anti-TNF-α mAb generated by recombinant DNA techniques that mimic immune selection in human beings. The antibody has a structure indistinguishable from that of normal human IgG1

antibodies. It has a high and specific affinity to TNF-α, neutralizing TNF-α binding to p55 and p75 TNFRs. The estimated half-life was between 6 and 13.7 days.

Adalimumab (Humira) is a fully human monoclonal anti-TNF antibody for treatment of moderate, severe and early RA, psoriatic arthritis and ankylosing spondylitis [4, 5, 14, 30]. Humira has a rapid onset of action (improvement in clinical status occurs as early as in the first week of treatment), a high efficacy in the dose of 40 mg once in 2 weeks subcutaneously and has a sustained effect – more than 7 years [5, 14]. The PREMIER study showed that adalimumab in combination with methotrexate induced remission in 43% of early RA patients after the first year, and in 49% of early RA patients after the second year of treatment. Clinical features were also ameliorated in a great number of patients (ACR20 73/69, ACR50 62/59, ACR70 46/47) and statistically significant inhibition of radiographic progression in early RA in week 52 and in week 104 of adalimumab treatment was reported. A remission occurred only in 25% of patients on monotherapy with methotrexate or adalimumab. However, compared to methotrexate monotherapy, adalimumab treatment inhibited radiographic progression [5].

The new studies DE019, ARMADA, DE005/DE037, STAR, DE010, ReAct allowed to evaluate a long-term (average of 5–7 years) effectiveness of adalimumab treatment in moderate and severe RA, on maintaining the same dose. The clinical status tended to improve during the long-term treatment and the percentage of patients in remission increased from 40% after the second year to 55% after the sixth year of adalimumab treatment [14, 30].

Certainly in introducing this treatment in our countries, in the Czech and Slovak Republic, it should be reserved just for patients with "malign" forms of RA who did not respond to the therapy with several conventional DMARDs, including their combined treatment, but also for very active and progressive early forms of RA. Consensual opinion recommends their application to patients suffering from active RA, in whom the treatment with at least one or two DMARDs has failed, while one of these DMARDs must be MTX [36].

TNF inhibition does not work in all patients. Sometimes adverse effects were observed: serious infections (excluding tuberculosis), tuberculosis, malignancies, demyelination. Total blockade of TNF may result in faster TB activation. Therefore a prompt diagnosis is important, consisting in patient clinical surveillance, lung radiograph checking, proof of TB bacilli in sputum, as well as in positive tuberculin reaction and in positive Quantiferon test – Gold that traces gamma interferon production. Prophylactic treatment should enable inhibition of the TB process and a biologic treatment of RA may be performed. However, it is important to mention a close cooperation between respective specialists, i.e. pneumologists and rheumatologists in diagnosis and treatment of TB in RA, which occurs as an undesired effect of biologic treatment.

Rituximab, currently in combination with MTX, is a new therapeutic option for treatment of patients with RA, particularly patients who have previously not responded to TNF blockers. It constitutes a major advance in the therapeutic armamentarium for patients with RA. Like other biological agents, rituximab does not cure the RA and the disease relapses after varying periods of time after response to treatment, requiring retreatment. Administration of rituximab is 1,000 mg i.v. on

days 1 and 15. How the dose and dosing interval may be adapted during long-term treatment with rituximab has not yet been established [32].

Glucocorticoids in RA treatment underwent a period of initial enthusiasm, followed by almost total rejection, achieving a balanced acceptance. The study by Kirwan et al. [18], which documented the preventive effects of small doses of corticoids on X-ray destruction in early RA, increased the interest in this form of therapy again. Corticosteroids in RA are administered in different ways.

Systemic administration of glucocorticoids. The most common doses are up to 10 mg a day. This procedure is called "low-dose-steroids." This dosage should manage most of the arthritic manifestations, with the exception of extra-articular manifestations, such as vasculitis or serositis, in which higher doses must be used. Glucocorticoids are anti-inflammatory drugs with a very rapid and intensive action. When administered, synovitis recedes quickly, pain alleviates, and morning stiffness decreases, and sometimes even systemic manifestations, such as fatigue and apathy subside. Laboratory parameters can improve, e.g. hemoglobin value. The clinical efficacy in controlled studies has been manifested at the beginning of the disease, but it fades away after a certain period of time. Therefore, small doses of steroids were applied only as a bridging measure till, e.g. DMARDs started to exert their effect.

In the Kirwan study [18], 128 early RA patients were monitored for 2 years. The patients were divided at random into two groups. All of them were treated with NSAIDs and DMARDs. The first half of them received 7.5 mg of prednisolone a day, the second half received a placebo. While after 6 months there were no statistically significant differences in the clinical parameters of the groups, after 2 years the group treated with glucocorticoids had significantly less erosive changes. Similar results were achieved in Wassenberg's study. Glucocorticoids in these studies did not work as symptom modifying drugs but as disease modifying drugs – as the DMARDs. Naturally, these results induced lively discussion whether small doses of corticosteroids should be indicated for all RA patients, as everyone was aware of the not negligible adverse effects of the long-term usage of these medicaments [18].

Corticoid toxicity. The question of long-term treatment with small doses is challenged also due to the possible induction of secondary osteoporosis. Each administration of glucocorticoids longer than 3 months in a dosage higher than 7.5 mg a day should be accompanied by a preventive osteoporotic regimen.

Intravenous pulse therapy. This term denotes the intravenous administration from one to three pulses of soluble glucocorticoids, the most common being methylprednisolone. Three pulses of 1,000 mg each, administered every other day, proved to be the most beneficial, even if some centers administered a smaller dosage of 250–500 mg. Good results for the series of "minipulses" have been reported, when three to five infusions with 100 mg of methylprednisolone were administered.

Local treatment with glucocorticoids. Intra-articular therapy is applied in an effort to manage joint inflammation (synovitis), or some para-articular manifestations, such as tendosynovitis, bursitis, or carpal tunnel syndrome. Their effect is

Table 11.19 Radiopharmaceuticals used for irradiation synoviorthesis

		Joints
Erbium 169	Beta, max. 0.7 mm	Hands (PIP, MCP)
Renium 186	Beta, max. 3.1 mm	Elbow, wrist
Colloidial gold 198	Beta, gamma, max. 2.7 mm	Elbow, ankle Knee, hip joint
Ytrium 91	Beta, max. 8.5 mm	

sometimes excellent and is maintained for several months or even years. There is a dogma (not scientifically verified) that an injection of glucocorticoids applied into one joint should not be repeated more frequently than three times a year.

Synoviorthesis. *Synoviorthesis* is defined as an intra-articular therapeutical application targeted at aggressive suppression of synovial inflammation. Sometimes it is termed *nonblood synovectomy*. It is possible to use both drugs and radioisotopes. Synoviorthesis is the last resort in recurrent joint synovitis that is not responding to repeated injections of corticosteroids. *Chemical synoviorthesis* was done mainly by osmic acid, but this method is currently considered obsolete. *Radiosynoviorthesis* is administration of radioisotopes into the joint cavity. Usually it is performed at a specialized center. An arthrographic check ensures the presence of the medication in the joint. After the radiopharmaceutical is applied, rest in bed is recommended, or immobilization for at least 3 days, so as to prevent the release of the radioisotope into the systemic circulation. The choice of the radionuclide depends on the maximal penetration of beta exposure related to the joint size. When the application was not successful, the next one should be repeated no sooner than in 6 months. The maximum dosage for one joint should not exceed 15 mCi (Table 11.19).

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11.9 Adjuvant Arthritis and Mitochondria

Katarína Bauerová and Jarmila Kucharská

Abstract Adjuvant-induced arthritis (AIA) is an animal model of rheumatoid arthritis (RA) in which joint inflammation is triggered by a single intradermal injection of an immunostimulatory agent (adjuvant). The formulation of commercial immunological adjuvants was the result of intense research in the early 20th century. Adjuvant arthritis research could be divided into three phases. In the first phase, in 1947, Jules Freund introduced a mixture of mineral oils, heat-killed mycobacteria and an emulsifying agent, referred to as complete Freund's adjuvant (CFA). Later mycobacteria were often omitted, in which case the adjuvant was called incomplete Freund's adjuvant (IFA). In the second phase, the experimental data inferred that the mycobacterial component and the oil caused arthritis by separate mechanisms.

At present, each of the rat and mouse models currently used has clinical features that resemble RA in humans. Histological features of all the models of joint disease include synovial hyperplasia with massive infiltration of inflammatory cells, similar to the histological abnormalities in RA patients. The experimental model of AIA belongs to the group of models of erosive arthritis. These rat and mouse models differ between themselves and in comparison to RA with respect to: disease onset, severity of joint inflammation, patterns of joints involved, and various additional clinical and systemic manifestations. Each of these models might provide unique insights into the etiopathogenesis of RA and/or related arthropathies, and also provide a powerful opportunity to probe potential disease mechanisms in RA and to develop new therapies. Reactive forms of oxygen and nitrogen, generated by several mechanisms, are involved in the etiopathogenesis of RA. Free radicals contribute to the damage of tissues in the inflammatory process not only at the site of inflammation but they provoke also systemic damage, such as muscle dystrophies around the damaged joints and myopathies.

Disturbances of mitochondrial bioenergetics mediated by free radicals can participate in the development of myopathies. Thus in arthritic patients, control of inflammation by natural as well as synthetic antioxidants could become a relevant component of antirheumatic prevention and therapy.

Keywords Adjuvant arthritis, animal models, antioxidants, mitochondria, oxidative status, rheumatoid arthritis

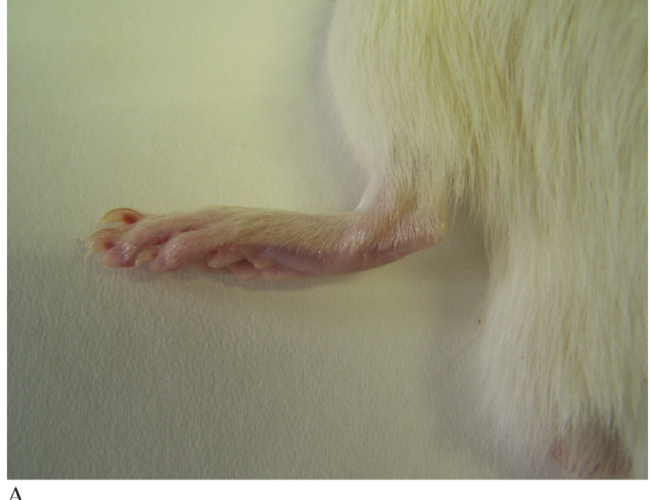
Adjuvant-induced arthritis (AIA) is an animal model of rheumatoid arthritis (RA) in which joint inflammation is triggered by a single intradermal injection of an immunostimulatory agent (adjuvant) [56]. Adjuvant arthritis demonstrates that joint-inflammation can be triggered by nonspecific stimulation of the immune system [25]. This experimental model belongs to the group

of models of erosive arthritis [57]. The different rat and mouse models differ between themselves and in comparison to RA with respect to: disease onset, severity of joint inflammation, patterns of joints involved, and various additional clinical and systemic manifestations. Each of these models might provide unique insights into the etiopathogenesis of RA and/or related arthropathies. Most of the animal models of erosive arthritis share similarities with human RA, but none of them mimics the condition completely. Nevertheless, these models provide a powerful opportunity to probe potential disease mechanisms in RA and to develop new therapies [28].

11.9.1 Pathophysiology of AIA in Comparison to RA

An intradermal injection, at the base of the tail, with heat-killed *Mycobacterium tuberculosis* (Mtb) in incomplete Freund's adjuvant (IFA) results in destructive arthritis within 14 days in susceptible Dark Agouti or Lewis inbred rat strains. AIA can also be induced with cell walls from other bacterial types in IFA, although the arthritogenicity varies [28]. Many experiments are performed with *Mycobacterium butyricum* in incomplete Freund's adjuvant (IFA) [2, 3, 40]. Increased synthesis of tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and IL-6 is detected as early as day four after adjuvant injection. The disease progresses rapidly over several weeks in what appears clinically to be a monophasic process [28]. The chief pathological features of adjuvant arthritis include edema (Fig. 11.1), infiltration into the joint of mononuclear and polymorphonuclear cells, pannus formation, periostitis and erosion of cartilage and bone [58]. Granulocytes and autoreactive CD4+ cells play major roles in the disease. Humoral immune mechanisms do not appear to contribute to the disease process. This unique rat disease model represents a systemic process that involves not only the joints but also the gastrointestinal and genitourinary tracts, the skin and eyes. The development of periostitis, bony ankylosis and most of the extra-articular manifestations are more reminiscent of Reiter's syndrome or of spondyloarthropathies than of RA [13, 57].

In comparison to AIA, RA has a more complicated pathophysiology, influenced by multiple genetic, reproductive, age and environmental factors. Clinically and histologically, AIA has similarities to human RA, which has been suspected to be autoimmune in nature and to be initiated by autoimmune CD4+ T-cells because of the presence of autoantibodies, such as rheumatoid factors (RFs), association with particular haplotypes of the HLA-DR gene (reviewed in [7]), occasional familial clustering with other autoimmune diseases [34, 51], infiltration of CD4+ T-cells in synovial inflammation (synovitis), and successful induction of arthritis in animals by immunization with joint-rich self-constituents, such as type II collagen. It is still unknown, however, whether RA is a joint-specific autoimmune disease or systemic autoimmunity with predominant manifestation of arthritis, or how do CD4+ T-cells contribute to the initiation and progression of RA.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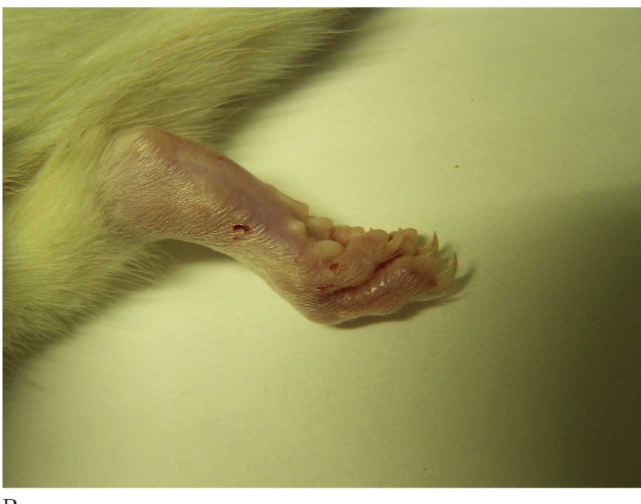
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Fig. 11.1 Comparison of hind paws of healthy Lewis rats (**a**) and Lewis rats with adjuvant arthritis (**b**)

The site of initial joint inflammation in RA is the synovium, which normally secretes the joint fluid for lubrication and nourishment of cartilage. It consists of one or two layers of synovial-lining cells (which are macrophage-like type A and fibroblast-like type B synoviocytes) and underlying loose connective tissue. It is devoid of basement membrane and tight junctions. Macrophage-like synoviocytes, which normally phagocytose debris in the joint fluid, are intrinsically capable of

secreting proinflammatory cytokines, such as IL-1, IL-6 and TNF-α, whereas fibroblast-like synoviocytes, which physiologically form hyaluronic acid, synthesize matrix metalloproteinases (MMPs) and prostaglandin E_2 when stimulated by TNF-α and IL-1. TNF-α, IL-1 and IL-6, which are abundant in the inflamed joint of RA, are largely produced by stimulated synoviocytes. Neutralization of these cytokines or blockade of their receptors is indeed a clinically effective treatment of RA [18, 41]. In addition, synoviocytes actively proliferate in RA, invading and eroding cartilage and bone [19]. One of the key issues in RA research is therefore to elucidate how lymphocytes and synoviocytes, or their products (such as immunoglobulin and cytokines), interact in the triggering and progression of RA.

11.9.2 Adjuvant Arthritis – History and Current State

The formulation of commercial immunological adjuvants was the result of intense research in the early 20th century. Adjuvant arthritis research could be divided into three phases.

In the first phase (1947–1962) the aim was to enhance immunity to tuberculosis, and to achieve high-titered antisera against bacteria. In 1947, Jules Freund introduced a mixture of mineral oils, heat-killed mycobacteria and an emulsifying agent, referred to as complete Freund's adjuvant (CFA) [20, 21]. This concoction proved to be an efficient enhancer of both cell-mediated and humoral immune responses to the antigens it was emulsified with. Repetitive immunizations were sometimes used, yet they were severely detrimental to health since mycobacteria caused formation of persistent foci of inflammation, which were often necrotic (granuloma). Mycobacteria were therefore often omitted, in which case the adjuvant was called incomplete Freund's adjuvant (IFA). Most of the pioneering adjuvant studies were carried out in rabbits and guinea pigs, but once CFA was formulated, it became widely used in many species. In 1955, Lipton and Freund demonstrated in rats that tolerance to central nervous system tissue could effectively be broken by immunization of the tissue together with CFA [21, 22]. Since then, mixtures of adjuvant and autoantigen(s) have been routinely used to induce a wide variety of experimental autoimmune diseases, mainly in rats and mice. Simultaneously with Lipton and Freund, Stoerck reported in 1954 that joint lesions developed in rats after immunization with CFA and rat spleen tissue [48]. Stoerck suspected the spleen tissue to be arthritogenic, but Pearson demonstrated that CFA and not the spleen component was responsible for the development of joint inflammation and established the adjuvant arthritis model in 1956 [45, 46]. A thorough characterization of this CFA-induced arthritis (hereafter referred to as mycobacteria-induced arthritis, MIA) showed that the disease was not joint-specific. It was associated with widespread inflammatory infiltrates and granuloma formation in many organs, for example in the spleen, liver, bone marrow, meninges, skin and eyes. MIA is a severe but self-limiting disease and the affected rats recover within a few months. These early descriptive findings evoked questions about the causative mechanisms. One

hypothesis was that the joint inflammation develops as a consequence of immunity towards microbial antigens spreading to the joints [54].

In the second phase (1963–1996) this idea lost support since it was demonstrated that arthritis could be transferred from diseased to healthy irradiated recipients by cells [55], later shown to be T lymphocytes [33, 49, 50], expressing CD4 [46], and αβTCR (T-cell receptor) [24]. To explain the role of T-cells, several investigators favored another idea, namely that mycobacterial antigens elicited a pathological T-cell immunity that included cross-reactions with joint antigens [52, 53]. This way of breaking tolerance is termed molecular mimicry [42] and it is suggested to be the cause of many autoimmune diseases, including RA [1]. One line of study suggested that the evolutionarily conserved 65 kD heat shock protein (HSP) was the immune target both in MIA and in RA [12, 26]. This protein was however unable to trigger arthritis [5]. While the focus was on HSP, the search for a minimally arthritogenic component of mycobacteria had already in 1977 led to the identification of a peptidoglycan cell wall fragment, which is nonimmunogenic but has adjuvant properties, i.e. muramyl dipeptide (MDP) [31, 32]. It was also reported that mycobacterial components were not even necessary to trigger arthritis, since a synthetic nonimmunogenic adjuvant called avridine could substitute for mycobacteria [11]. Both MDP and avridine were found to induce arthritis when injected together with incomplete Freund's adjuvant (IFA). Furthermore, it was later unexpectedly discovered that IFA (i.e. mineral oil) alone induced arthritis in DA rats [24, 30]. This oil-induced arthritis developed also in germ-free DA rats, demonstrating that microorganisms were not involved in the pathogenesis and that responses to heat shock proteins did not occur in germ-free rats [6]. However, most other rat strains, including the LEW (Lewis) strain, which is highly susceptible to MIA, were resistant to mineral oil-induced arthritis (OIA) [8, 10, 25]. On balance, these data infer that the mycobacterial component and the oil cause arthritis by separate mechanisms. Although it has not yet been documented that mycobacteria can induce arthritis without mineral oil, aqueous suspensions of cell walls from other bacteria, for example streptococci (streptococcal cell-wall-induced arthritis, SCWIA), are arthritogenic [14].

In the present phase, as shown in a review by Holmdahl et al. [25], it is established that "adjuvant arthritis" can be caused by "pure" adjuvants (like mineral oil, pristane, avridine or squalene) without involvement of bacterial cell walls or other components known to bind to immune adaptive or innate receptors. Each of the rat and mouse models currently used has clinical features that resemble RA in humans. Histological features of all the models of joint disease include synovial hyperplasia with massive infiltration of inflammatory cells, similar to the histological abnormalities seen in RA patients [57].

11.9.3 Rat Models of Erosive Erthritis

Several experimentally induced rat and mouse models of autoimmune erosive arthritis are currently widely used to provide insight into etiopathogenetic

mechanisms in RA. They are also used extensively to evaluate potential new therapeutic agents [13, 57, 59]. Rat models of erosive arthritis can be classified into three major groups [57].

The first group is induced by hyperimmunization of genetically susceptible rat strains with antigens such as type II collagen (collagen-induced arthritis) or cartilage oligomeric matrix protein (COMP-induced arthritis) in incomplete Freund's adjuvant (IFA).

The second group is induced by intradermal administration of various oil-based adjuvants, of which heat-killed *Mycobacterium tuberculosis* (Mtb) emulsified in IFA is the most widely studied (Mtb-adjuvant-induced arthritis). Chronic erosive arthritis has also been induced with other oil-based adjuvants, including avridine in IFA (avridine-induced arthritis), pristane (pristane-induced arthritis), and IFA alone (oil-induced arthritis).

The third group of rat models includes various forms of bacterial cell wall peptidoglycan-polysaccharide-induced arthritis. The streptococcal cell wall (SCW) arthritis model is the best characterized of the third group. In mice, collagen-induced arthritis (CIA), pristane-induced arthritis (PIA) and proteoglycan-induced arthritis are the major models. Mice are relatively resistant to classical Mtb-induced adjuvant arthritis and SCW arthritis [28].

In addition to antigen-induced arthritis, such as collagen-induced arthritis (CIA) or adjuvant arthritis, recent efforts with transgenic, gene-knockout, or gene-knock-in technology have established several new models of RA. S. Sakaguchi and N. Sakaguchi reviewed recent findings from several mouse models produced by gene manipulation or established as spontaneous mutants, and on the basis of these findings, they discussed the possible pathophysiology and etiology of RA [47].

11.9.4 Antioxidants, Oxidative Status and Mitochondria in RA

Arthritis in rat induced by intradermal injection of mycobacterial adjuvant is widely used as a model for the evaluation of compounds with anti-inflammatory or antirheumatic activity and is an important methodological tool for studying the mechanisms of RA. Reactive oxygen and nitrogen species contribute significantly to tissue injury in RA [3]. Reactive forms of oxygen and nitrogen generated by several mechanisms are involved in the etiopathogenesis of RA [4, 17]. Shift in the plasma oxidant/antioxidant balance in favor of lipid peroxidation leading to tissue damage was reported in RA patients by many authors [27, 29, 44]. Oxidants can be produced by activated macrophages in the synovial membrane and by activated neutrophils in the synovial cavity. Synovial fluid from the knee joint of rheumatoid patients was found to contain increased levels of diene conjugates and thiobarbituric acid reactive substances, suggesting increased peroxidation of membrane lipids in vivo [23]. Somatic mutations of mitochondrial DNA caused by reactive oxygen and nitrogen substances have been detected in synoviocytes and synovial

tissue in patients with RA [15]. Enhanced production of superoxide anion and peroxynitrite by neutrophils was reported in RA patients [43].

Free radicals contribute to the damage of tissues in the inflammatory process not only at the site of inflammation but they provoke also systemic damage, such as muscle dystrophies around the damaged joints and myopathies. In patients with RA, muscle weakness and atrophy are the most common symptoms [39]. Progressive muscle atrophy and inflammatory myopathy found in patients with RA have been proposed to be mediated by disturbances of myofibrils and mitochondria [16]. Disturbances of mitochondrial bioenergetics mediated by free radicals can participate in the development of myopathies. Mitochondrial function is differently affected by oxidative stress [9]. Leakage of reactive oxygen species may lead to damage of the mitochondrial membrane, proteins, and DNA and to inhibition of oxidative phosphorylation [35, 36, 38]. Miesel et al. [38] found a fivefold increase in mitochondrial reactive oxygen species (ROS) production in blood monocytes from RA patients. Elevated mitochondrial oxidative stress correlated with the increase of tumor necrosis factor in plasma and the authors suggest that it contributes to the pathology of rheumatoid arthritis. In muscle bioptic samples from patients affected by RA, ultrastructural alterations such as separation of myofibrils, pleiomorphic mitochondria and lipofuscin deposition in the subsarcolemmal region were observed [16]. Maneiro et al. [37] found reduced activities of Complex II and III and higher proportion of cells with de-energized mitochondria in human osteoarthritic articular chondrocytes compared with cells from normal cartilage. Thus the control of inflammation in arthritic patients by natural as well as synthetic antioxidants could become a relevant component of antirheumatic prevention and therapy [3].

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Chapter 12
Mitochondrial Immunology

František Gazdík and Katarína Gazdíková

Abstract Oxidative stress seems to be of importance in immunopathogenesis of multiple internal diseases. The significance of mitochondrial metabolism is discussed, particularly in light of its participation in immunopathogenesis of bronchial asthma and selected liver-related diseases, such as primary biliary cirrhosis and chronic hepatitis C. The significance of complementary antioxidative therapy in asthmatics, including coenzyme Q_{10}, is also reviewed. New insight into pathogenetic factors of chronic hepatitis C in relation to oxidative stress is presented.

Keywords Antioxidants, asthma bronchiale, chronic hepatitis C, primary biliary cirrhosis, reactive oxidative species

12.1 Structure and Function of the Immune System

The immune system is a diffuse organ, which weighs about 1,000 g in adult subjects. It is composed of approximately 10^{12} lymphocytes and accessory cells (macrophages, polymorphonuclear leukocytes, 10^{20} antibody (immunoglobulin) molecules and millions of molecules of various effector and regulatory substances, e.g. immunohormones (regulating proliferation, maturation and differentiation of cells of the immune system), complement components, microbicidal and cytotoxic substances, etc. The lymphocytes circulate freely in the blood and lymph or are taken up by primary and secondary lymphoid organs. In any individual, the complex network of immune mechanisms, formed during phylogenetic development, maintains the integrity of the internal environment against changes originating during embryogenesis at certain developmental stages, or against changes caused by environmental factors (viruses, microorganisms, chemical substances, unfavorable physical or psychological effects). The immune system ensures the immunological surveillance, maintenance of individuality and health of the individual, as well as defence against parasites and pathogenic microorganisms (viruses, bacteria, yeast and fungi). The term immunological surveillance refers to the ability of the immune system to detect damaged, worn-out or altered autologous cells and structures and

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to ensure their elimination. Similarly, the immune system is able to recognize tumor cells or cells from a genetically different individual of the same species. The system is responsible for the maintenance of the identity and integrity of the individual. This function is performed by its components, i.e. lymphocytes and antibodies, systematically penetrating tissues of the organism via the blood and lymphatic circulation, where they contact and recognize each other and altered or non-self cells or molecules. The key function of the immune system is therefore the discrimination of "self" from "non-self" (foreign).

The reaction of the immune system (immune response) can be useful for the organism (immunity), or harmful (immunopathological reaction). The specific defence (immunity) against infectious disease is an example of an advantageous immune response, whereas the harmful type of immune response is represented, for example, by various allergic reactions (hypersensitivity). The immune response can be induced not only by foreign antigens, but also by self, antigenically altered cells and molecules that are subsequently recognized as foreign. Even in this latter case the reaction may be useful (immunity against tumors) or harmful (autoimmune disease).

12.1.1 Mechanisms of Natural (Nonspecific) Immunity

The term natural immunity designates the nonspecific resistance of the organism against various microorganisms (viruses, rickettsiae, mycoplasmas, fungi, yeast, protozoa), foreign and tumor cells. Events of this type of resistance are controlled by the genome of any individual, but compared with acquired immunity, the mechanisms of natural immunity operate spontaneously, regardless of previous contact with a particular antigen. Natural immunity acts not only against a single, but against many different antigens. Mechanisms of natural immunity are developmentally older. They may be divided into the cellular and humoral type. The basic mechanism of nonspecific cellular immunity comprises phagocytosis, whereas the humoral branch of nonspecific immunity is represented by the complement system, interferons, chemotactic factors, opsonins, chemokines, cytokines, mediators of the inflammatory reaction, multiple enzymes and their inhibitors and some other factors. The main role of phagocytosis is to take up, ingest, kill or inactivate and degrade foreign particles, including invading pathogenic microorganisms. Phagocytosis is performed by particular types of leukocytes, called phagocytes.

It should be stressed, however, that no immune mechanism can perform its function separately. All mechanisms cooperate mutually and complete each other. The complex of substances, cells and tissues participating in natural immunity potentiates the efficacy of specific immunity and is therefore referred to as immunological amplification systems. On the other hand, products of specific immune reactions, particularly antibodies and some lymphokines, facilitate and enhance the course of many reactions of nonspecific immunity.

12.1.2 Mechanisms of Acquired (Specific) Immunity

All cells are coated with various substances. CD stands for cluster of differentiation and there are more than one hundred and sixty clusters, each of which is a different chemical molecule that coats the surface. B-cells are coated with CD21, CD35, CD40, and CD45 in addition to other non-CD molecules. T-cells have CD2, CD3, CD4, CD28, CD45R, and other non-CD molecules on their surfaces.

Specific immune mechanisms consist of two basic types: mediated by antibodies (humoral immunity) and cell-mediated immunity (cellular immunity). When the antigen activates certain B-lymphocytes, the latter differentiate into plasma cells, which synthesize and secrete antibodies with a binding site identical to that carried by the original B-cells that recognized the antigen. Thus, antibodies are responsible for the humoral type of the specific immune reaction (Fig. 12.1).

T-cells are primed in the thymus, where they undergo two selection processes. The first positive selection process weeds out only T-cells with the correct site of receptors that can recognize the MHC molecules responsible for self-recognition. Then a negative selection process begins whereby T-cells that can recognize MHC molecules complexed with foreign peptides are allowed to pass out of the thymus.

When the antigen stimulates the immunocompetent T-cells, specific antibodies are not formed, but various types of regulatory, cytotoxic or delayed-type hypersensitivity reactions occur. These are mediated particularly by the inducer or helper (CD4+ T-lymphocytes), suppressor and cytotoxic T-lymphocytes (CD8+ T-lymphocytes) and their products – lymphokines and interleukins (IL).

The former two subpopulations (inducer or helper T Ly) play a regulatory role in the induction of specific immune responses. The helper T-cells assist the B-cells to recognize the antigen and are essential for initiation of antibody formation and origin of cytotoxic T-lymphocytes. CD4+ positive T-cells are divided according to the different production of cytokines into two subsets Th1 (IL-2, TNF-alpha, interferon-gamma) and Th2 (IL-4, IL-5, IL-10). The function of the Ts-cell subpopulation

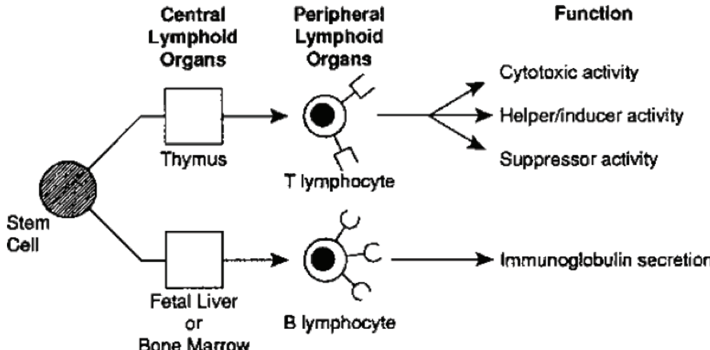

Fig. 12.1 Mechanisms of specific cellular and humoral immunity

is actually performed by cytotoxic T-cells (CD8+ positive T-cells). Cytotoxic T-cells possess a cytotoxic activity, i.e. they damage and kill various target cells which bear on their surface the antigen which originally induced their formation. The target cells for cytotoxic T-lymphocytes include tumor cells, cells transplanted from a genetically nonidentical individual (with different histocompatibility antigens) or autologous cells with a virus-modified surface. The effector and regulatory T-lymphocytes are involved in the mechanisms of specific immunity either directly of via various chemical substances secreted extracellularly. The substances are called lymphokines and have the character of immunohormones.

A particular lymphocyte population – the memory cells – "remember" the antigen after the first encounter. After repeated contact with the same antigen, the memory cells are responsible for more rapid and intensive immune response [12].

12.1.3 Cytokines

Cytokines are chemical mediators released by immune system cells and exert several important functions: they stimulate white blood cell differentiation and division in bone marrow, chemoattraction of phagocytes, lymphocytes, induce fever, produce inflammation/tissue swelling, destroy tumor cells. Cytokines can be divided into three classes: IL-1–18, tumor necrosis factors, and interferons.

12.2 Mitochondria and Immunity

12.2.1 Mitochondria and T-cell Immunity

A functional immune system is dependent on the generation and selection of a lymphocyte repertoire that is sufficiently diverse to respond to innumerable foreign antigens yet has to be adequately self-tolerant to avoid the development of autoimmunity. T-cell homeostasis is maintained by balancing the proliferation and destruction of lymphocytes at multiple steps during the life of an individual. Regulated mitochondria-dependent apoptosis is essential for both the development and subsequent maintenance of the immune system. Firstly, during thymic development, sequential stages of T-cell maturation require strict control of T-cell selection, and secondly apoptosis is essential in controlling the massive expansion of antigen-specific T-cells after activation [16].

Mitochondrial respiration is required to meet the energy requirements of activated and proliferating peripheral lymphocytes. Several mitochondrial proteins have been implicated as regulators of apoptosis in the immune system, required for the prevention of autoimmunity [43]. T-cell activation, proliferation and selection of the cell death pathways depend on the production of reactive oxygen species

(ROS) and ATP synthesis, which are tightly regulated by the mitochondrial transmembrane potential. Mitochondrial hyperpolarization has been proposed as a key mechanism in the pathogenesis of systemic lupus erythematosus and is therefore a target for pharmacological intervention [41].

12.2.2 Immunity and Reactive Oxygen Species (ROS)

The immune system is comprised of innate (natural) and acquired (adaptive) immunity. Acquired immunity is composed of lymphocytes, which are highly active cells that constantly generate ROS as a part of their normal cellular reactivity. Oxidizing pollutants and many viruses can also induce ROS production by normal cells. One mechanism by which the innate branch of the immune system protects humans is phagocytosis and subsequent killing of antigens through an oxidative bactericidal mechanism termed respiratory burst. Phagocytosis of foreign particles by a macrophage or neutrophil activates "NADP" oxidase, resulting in the production of a large amount of superoxide anion (O_2^-) from molecular oxygen. Superoxide anion is then rapidly converted to hydrogen peroxide (H_20_2) by superoxide dismutase. Neutrophils contain myeloperoxidase that converts H_20_2 to the highly potent bactericidal component, hydrochloride ions ($HOCL^-$). Macrophages do not possess myeloperoxidase and depend instead on myeloperoxidase-independent mechanisms to generate other biological oxygen-derived free radicals (OH^-) through Fenton and /or Haber Weiss chemistry. Although ROS are produced as part of the killing mechanism, excessive phagocytic activity can lead to ROS-induced tissue damage. As a defense mechanism, the body produces a number of endogenous antioxidants capable of scavenging the harmful ROS to maintain an optimal oxidant/antioxidant balance and thus to maintain normal cellular function and health. However, under conditions of high oxidative stress, the ability of the antioxidants to eliminate ROS is often overcharged and therefore dietary sources of antioxidants or drugs are required. The most widely used dietary antioxidants include vitamin E, vitamin C, carotenoids, flavonoids, zinc and selenium. The harmful action of ROS does not affect only immune cells but also all cell types. However, immune cells are particularly sensitive to oxidative stress because their plasma membranes contain a high percentage of polyunsaturated fatty acids (PUFA) and they generally produce a considerable amount of ROS [29].

The traditional concept of ROS function is that they indiscriminately destroy cell components. However, exciting research has more recently elucidated the role of these reactive species in signal transduction, gene regulations, and disease etiology [21]. The mitochondrial electron transport system utilizes approximately 85% of the oxygen consumed by the cell to generate ATP, being thus the most important source of ROS. Cytochrome c located between the inner and outer mitochondrial membrane plays a critical role in the apoptotic process. The release of cytochrome c is regulated by pro-apoptotic and anti-apoptotic proteins. Therefore the mitochondrion is likely a key player in immunity and disease, and the localization of carotenoids

in mitochondria is of particular relevance. The presence of these carotenoids in subcellular organelles can protect the immune cells against oxidative injury, and ensure optimal cellular functions, including apoptosis, cell signaling and gene regulation [51].

Immune cells are particularly sensitive to oxidative stress because of their production of ROS, which play a role in their normal function as important signaling molecules in the regulation of various cellular processes [27]. Thus the oxidant-antioxidant balance is critical for immune cell function. Antioxidants maintain the integrity and function of membrane lipids, cellular proteins, and nucleic acid, and the control of signal transduction of gene expression in immune cells. This explains the particular sensitivity of immune cells to changes in their antioxidant status. Moreover, since the immune system cells have a high percentage of PUFA in their plasma membrane, these cells usually contain higher concentrations of antioxidants than other cells [29].

The NK activity, which is very important in the innate immune response to infection and tumors, is carried out through the production of ROS [44]. The proliferation of lymphocytes is a pivotal function of the immune response triggered by antigens or mitogens. In this proliferation, ROS are also involved and proliferation and NK activity are controlled by cytokines, such as IL-1 beta and mainly IL-2 [27]. ROS have more recently been found to function as intra- and intercellular signal molecules and therefore may participate more directly as mediators of the inflammatory response. ROS can act as second messengers and influence gene expression, phosphorylation, DNA synthesis and cellular proliferation [31]. ROS, either directly or via the formation of lipid peroxidation products such as acrolein, 4-hydroxy-2-nonenal and F(2) isprostanes, may play a role in enhancing the inflammation through the activation of stress kinases (**JNK,MAPK**, p38, phosphoinositide 2 (PI-3)-kinase/PI-3K-activated serine-threonine kinase Akt) and redox sensitive transcription factors such as NF-kappaB and AP-1. Oxidative stress and proinflammatory mediators can alter nuclear histone acetylation/deacetylation allowing access for transcription factor DNA binding, leading to enhanced proinflammatory gene expression in various lung cells [46].

Antioxidants possess anti-inflammatory and immunomodulating effects, e.g. supplementation of selenium in asthmatics (selenium is comprised in the enzyme glutathione-peroxidase), as demonstrated in clinical studies and in vitro experiments [14, 15, 19, 23].

12.3 Autoimmunity and Mitochondria

12.3.1 Primary Biliary Cirrhosis

Primary biliary cirrhosis (PBC) is an autoimmune disease, which affects predominantly women and is characterized by antimitochondrial antibodies and specific

Table 12.1 Frequencies of antimitochondrial antibodies in PBC

Antigen	Frequency (%)
PDC-E2	95
BCOADC-E2	53–55
OGDC-E2	39–88
PDC-E1 alfa	41–66
E3BP	95

Notes:

PDC-E2: the E2 subunit of pyruvate dehydrogenase complex

BCOADCE-E2: the E2 subunit of branched-chain 2-oxo acid dehydrogenase complex

OGDC-E2: the E2 subunit of 2-oxo glutarate dehydrogenase complex

PDC-E1 alpha: the E1 subunit of pyruvate dehydrogenase

E3BP: dihydrolipoamide dehydrogenase basing protein

destruction of small bile ducts. Patients with this disease do not only have high titer antibodies to mitochondria, but also liver specific CD4 and CD8 cells directed at the same mitochondrial autoantigens. These mitochondrial autoantigens are all members of the 2-oxo dehydrogenase complex and include the E2 component of pyruvate dehydrogenase as the major autoantigen [22]. Using peripheral blood mononuclear cells (PBMCs), an HLA-A2-restricted CTL epitope of the E2 component of pyruvate dehydrogenase (PDC-E2) was identified [28]. Rates of antimitochondrial antibodies in patients with PBC are shown in Table 12.1.

All cells in the body have mitochondria but biliary cells appear to be subject to specific destruction. Biliary epithelium cells are heterogenous not only in their size but also in the expression of cell adhesion molecules, response to cytokines, growth factors, and so on. This heterogeneity may explain why only the small bile duct epithelial cells are targets of immune-mediated damage in PBC. Only small bile duct biliary endothelial cells express the target molecules PDC-E2 and E3BP.

12.4 Oxidative Stress-related Diseases

12.4.1 Chronic Hepatitis C

Introduction

Infection with hepatitis C virus (HCV) is a global healthcare challenge. Approximately 3% of the population is infected with HCV, representing about 170 million persons worldwide. Although hepatitis C is a systemic disease that can affect most organs, the liver is the primary target for the HCV. The HCV was

first identified in 1989, and was found to be the cause of 80 to 90% of cases of Non-A, Non-B hepatitis [32]. HCV infection is associated with significant morbidity and mortality. Study of the natural evolution of acute HCV infections has shown that approximately 30% of cases result in spontaneous clearance, whereas 70% evolve towards chronic infection. In the past few years, the prevalence of hepatocellular carcinoma has increased in western countries and currently cirrhosis related to HCV is the most common cause of liver transplantation worldwide [5, 10].

Chronic hepatitis C virus infection is treated with pegylated interferon-alfa in combination with ribavirin, however a significant fraction of patients either fail to respond or relapse after cessation of therapy. There is thus an obvious need to develop effective therapeutic strategies to improve the clinical treatment of HCV-associated hepatitis. Advances have already started to reveal a number of viral functions which may provide attractive targets for the development of new and more effective anti-HCV therapies.

12.4.1.1 The Structure of Hepatitis C Virus

HCV is classified as a third genus of the family Flaviviridae and contains a single-stranded RNA genome of positive polarity. The genome consists of a single, approximately 9 kb, open reading frame (ORF) that is enclosed by 5′ and 3′ noncoding (NC) regions. The single ORF of HCV encodes structural proteins (C, E1, E2) at its 5′ end and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) at its 3′ end (Fig. 12.2).

The HCV genome encodes at least four enzymatic activities within the nonstructural (NS) region. NS2 is a metalloprotease responsible for cleavage at the NS2–NS3 junction. The gene product of NS5B is an RNA-dependent RNA polymerase required for viral replication. The 631 residue NS3 protein is a bifunctional enzyme. The N-terminal domain is a serine protease, which cleaves the nonstructural polyprotein into NS3, NS4A, NS4B, NS5A and NS5B proteins. An RNA helicase activity resides in the C-terminal 450 residues of NS3. The helicase removes double-stranded segments of RNA to form a single-stranded molecule, which is required during translation and transcription [54].

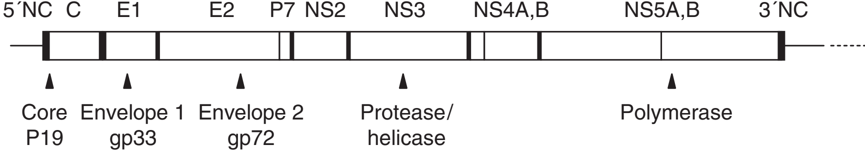

Fig. 12.2 Genomic organization of HCV

12.4.1.2 Oxidative Stress and Chronic Hepatitis C

The mechanisms of liver injury in chronic hepatitis C virus infection are poorly understood. HCV induces a state of hepatic oxidative stress that is more pronounced than that present in many other inflammatory diseases. A possible deregulation of calcium recycling between the endoplasmic reticulum and mitochondrial network has been proposed to provide new insights into the pathogenesis of hepatitis C. The hepatitis C virus core protein plays an important role in this phenomenon. The core protein binds to mitochondria, particularly at the points of contact between mitochondrial outer membrane and endoplasmic reticulum. Its expression causes inhibition of electron transport at Complex I, increased Complex I ROS production, decreased mitochondrial glutathione, and increased mitochondrial permeability transition pore (MPTP) in response to exogenous oxidants and tumor necrosis factor-alpha [30, 42]. On balance, interaction of core protein with mitochondria and subsequent oxidation of the glutathione pool and Complex I inhibition may be an important cause of the oxidative stress seen in chronic hepatitis C (Fig. 12.3). These results provide new insight into the pathogenesis of hepatitis C and supply the rationale for investigation of antioxidant therapy [40]. The resulting oxidized redox state has a

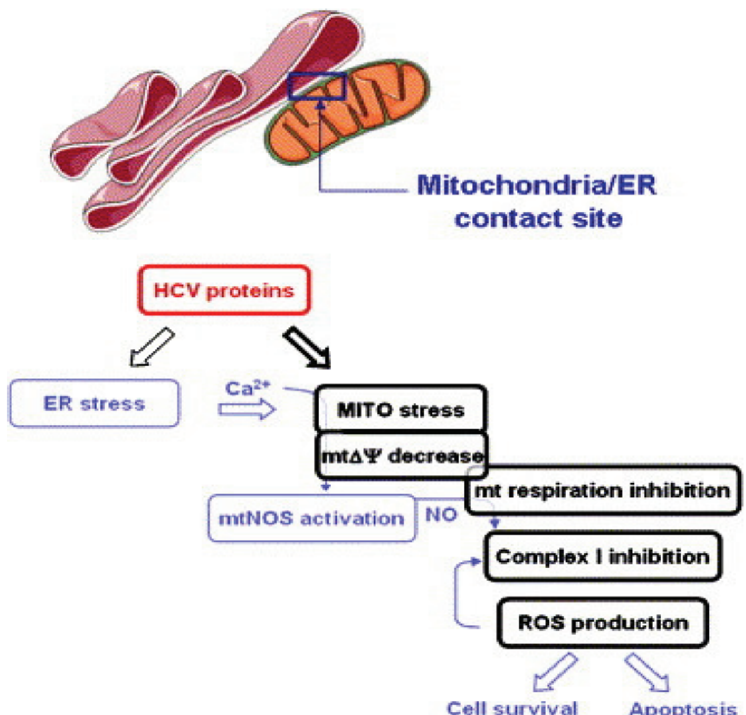

Fig. 12.3 Mitochondrial dysfunction caused by HCV infection

number of potential consequences for liver function. It interferes with the antiviral innate immune responses and potentiates fibrosis and carcinogenesis [52].

HCV infection is frequently associated with the development of hepatocellular carcinomas and non-Hodgkin's B-cell lymphomas. HCV infection causes cellular DNA damage and mutations, which are mediated by nitric oxide (NO). NO often damages mitochondria, leading to induction of double-stranded DNA breaks (DSBs) and accumulation of oxidative DNA damage. The mitochondrial damage and DSBs in HCV infected cells are mediated by both NO and ROS. Of the HCV proteins, core, E1, and NS3 are potent ROS inducers, their expression leading to DNA damage and activation of STAT3 [34, 38].

Endogenous oxidants generated by multiple intracellular pathways are an important class of naturally occurring carcinogens. ROS are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS include a number of species such as superoxide, hydroxyl, and peroxyl radicals and certain nonradicals such as singlet oxygen, and hydrogen peroxide that can be easily converted into radicals. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis. Moreover, a number of recent studies demonstrated that ROS at submicromolar levels act as novel intra and intercellular secondary messengers and thus modulate various aspects of cellular functions including proliferation, apoptosis and gene expression [1]. In humans it is not clear whether ROS production is triggered by the action of HCV per se or by inflammation, which is instrumental in producing ROS in a variety of organs. In a transgenic mouse model for viral hepatocarcinogenesis, ROS overproduction occurred in the absence of any inflammation, suggesting that the HCV core protein per se, which was expressed at levels similar to those in the liver of chronic hepatitis C patients, is sufficient to induce oxidative stress in the mouse liver. This leads importantly to the idea that the presence of HCV itself may induce the production of ROS in the human liver and render hepatocytes susceptible to DNA damage, which on accumulation may result in malignant transformation [38, 39].

12.4.1.3 Mitochondria and Innate Antiviral Immunity

Recent studies have uncovered two signaling pathways that activate the host innate immunity against viral infection. One of the pathways utilized members of the Toll-like receptor (TLR) family to detect viruses that enter the endosome through endocytosis. The TLR pathway induces interferon production through several signaling proteins that ultimately lead to the activation of the transcription factors NF-kappaB, IRF3 (interferon regulatory factor) and IRF7. The other antiviral pathway uses the RNA helicase RIG-I as the receptor for intracellular viral double-stranded RNA. RIG-I activates NF-kappaB and IRFs through the recently identified adaptor protein MAVS (mitochondrial antiviral signaling), a CARD domain containing protein (caspase recruitment domains) that resides in the mitochondrial membrane. MAVS is essential for antiviral innate immunity, but it also serves as a target of hepatitis C virus, which employs a viral protease to cleave MAVS of the mitochondria, thereby allowing HCV to escape the host immune system [33, 35, 49] (Fig. 12.4). MAVS is

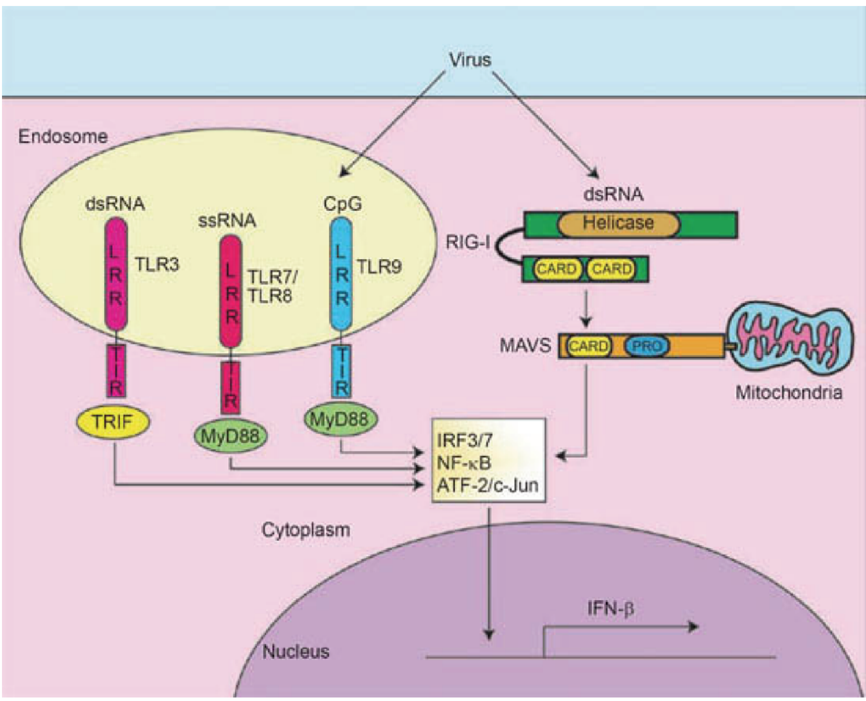

Fig. 12.4 TLR and RIG-I – two antiviral innate immunity pathways

required for the phosphorylation of IRF3 and IkB and functions downstream of RIG-I, an intracellular receptor for viral RNA [50].

These results provide an example of host-pathogen interaction in which the virus evades innate immunity by dislodging a pivotal antiviral protein from mitochondria and suggest that blocking the cleavage of MAVS by NS3/4A may be applied in the prevention and treatment of HCV infections [33]. MAVS is an essential antiviral signaling protein and may be a prime target of viruses that have succeeded in evading the host immune system. MAVS is indeed the proteolytic target of HCV NS3/4A protease. HCV is a pathogen that targets MAVS to evade the host immune system.

12.4.2 Asthma Bronchiale

Introduction

Asthma is a complex disorder characterized by reversible airway obstruction, bronchial hyperresponsiveness and airway inflammation. Key pathological features include infiltration of the airways by activated lymphocytes and eosinophils, damage to and loss of the bronchial epithelium, mast cell degranulation and

collagen deposition in the epithelial subbasement membrane area. There is increasing evidence that oxidative stress and reactive oxygen species (ROS) are involved in inflammatory airway disease, including asthma [3, 48]. The sources of the increased oxidative stress in patients with asthma derive from the increased burden of inhaled oxidants, and from the increased amounts of ROS generated by several inflammatory, immune and structural cells of the airways. Oxidative stress may also reduce glutathione levels and cause inactivation of antioxidant enzymes such as superoxide dismutase, with the consequent increase in apoptosis, shedding of airway epithelial cells and airway remodelling. Bronchoalveolar lavage fluid cells from asthmatic patients show increased production of superoxide anions, compared with cells from normal individuals [24] and this production is increased further after allergen challenge [7]. There is evidence of increased oxidative stress in the circulation [45, 47]. Circulating eosinophils from asthmatic patients produce excessive superoxide after activation [20], and this is increased even further after allergen challenge [11].

12.4.2.1 ROS Production in Asthmatics

Oxygen radical production is increased in asthma and is closely related to its pathogenesis. The mechanism by which oxygen radicals cause asthma pathology is oxidation or nitration of proteins, lipids, or DNA, resulting in dysfunction of these molecules. The physiological antioxidant system, which is equipped to protect the host from detrimental oxidants, is impaired in asthma, possibly due to inflammation. Thus the oxidant/antioxidant imbalance is critical in asthma pathogenesis.

Many inflammatory and structural cells which are activated in asthmatic airways, including eosinophils, macrophages, mast cells, and epithelial cells, produce ROS [3]. Superoxide anions are generated by NADPH oxidase and are converted to hydrogen peroxide by superoxide dismutases (SODs). Hydrogen peroxide is then degraded to water by catalases. Superoxide and hydrogen peroxide may interact in the presence of free iron radical to form highly reactive hydroxyl radical [4]. The lungs of asthmatics are exposed to oxidative stress due to the generation of reactive oxygen and nitrogen species as a consequence of chronic airway inflammation. Increased concentrations of NO, H_20_2 and 8-isoprostane have been measured in exhaled breath and in sputum of asthmatic patients [36]. Oxidants also activate NF-kB, which orchestrates the expression of multiple inflammatory genes that undergo increased expression in asthma, thereby amplifying the inflammatory response [2]. Boldogh and colleagues provided evidence that pollen itself was playing an active role in triggering aspects of the host allergic response and perhaps directly damaging host cells. This active role depends on pollen NADPH oxidase activity, the same enzymatic activity that eosinophils trigger upon exposure to the invading pollen. The endogenous ROS-generating activity of pollen can expose airways cells to ROS prior to the recruitment of inflammatory cells [6].

12.4.2.2 Antioxidants and Asthma Bronchiale

Oxidative stress is described as an imbalance between ROS and antioxidants. The normal production of oxidants is counteracted by several antioxidant mechanisms in the human respiratory tract [8]. Accumulating data indicate that asthma bronchiale is a chronic inflammatory disease. Airway inflammation and its control became the principal focus in asthma pathogenesis and treatment. The mechanisms underlying inflammation have not been fully clarified. Current understanding suggests that the imbalance between increased production of ROS and decreased antioxidative defence seems to be of importance. In this respect, the imbalance could be considered as the important triggering factor as well as the factor for the maintenance of chronic inflammation. The normal production of oxidants is counteracted by several antioxidant mechanisms in the human respiratory tract. The major intracellular antioxidants in the airway are catalase, superoxide dismutase (SOD), and glutathione, which is formed by the selenium–dependent enzyme glutathione peroxidase. Extracellular antioxidants include the dietary antioxidants vitamin C (ascorbic acid) and vitamin E (alpha-tocopherol), uric acid and lactoferrin [8].

The current therapeutic strategy tends to use the combination of preparations with anti-inflammatory effects to reduce manifestations of adverse reactions. Thus the administration of antioxidants seems to be useful in the therapeutic approach and is considered to be complementary to standard antiasthamtic therapy.

Several authors described decreased antioxidative status in asthmatics. Vitamin C and carotene intake was low particularly in males with severe asthma [37]. Carotenoid levels in whole blood, but not in plasma or sputum, were deficient in asthmatics [53]. Decreased selenium status was reported in patients with intrinsic asthma bronchiale [18, 25]. Selenium supplementation showed corticoid sparing effects in asthmatics [15].

Decreased levels of vitamins A, C, E and decreased enzyme activity of SOD were found in asthmatics [9, 26].

Decreased concentrations of CoQ_{10} and alpha-tocopherol both in plasma and whole blood were assessed in patients with asthma, compared with healthy volunteers. The plasma levels of malondialdehyde, a marker of end-stage lipid peroxidation, were elevated over the reference range in asthmatics. These results suggest a possible contribution of CoQ_{10} to antioxidative imbalance in asthmatics [13]. Supplementation with antioxidant combination (CoQ_{10}, E and C vitamins) reduced the corticoid dosage in patients with bronchial asthma [17].

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Chapter 13
Mitochondrial "Spermatopathy"

Anna Gvozdjáková

Abstract Several pathobiochemical mechanisms are involved in male infertility and many of them are still unknown. It has been documented that decreased sperm motility and semen quality, impairment of balance between reactive oxygen and nitrogen species production and reduced total antioxidant capacity, mitochondrial DNA defects, disturbances of sperm mitochondrial function and energy production participate in male infertility. Supplementation with antioxidants as vitamin C, vitamin E, glutathione, carnitine, coenzyme Q_{10} may have beneficial effects in treating male infertility.

Keywords Energy, male infertility, mitochondria, oxidative stress, "spermatopathy"

13.1 Sperm Mitochondrial Function

Sperms contain many mitochondria helically arranged around the mid-piece axoneme. Mitochondria are subcellular organelles of eukaryotic cells. The number of mitochondria in a cell is individual and depends on the cell's requirement for ATP.

Cells with a high requirement for ATP will have a larger number of mitochondria. Oocytes possess up to 100,000 mitochondria, while mature sperm cells have 22–28. The generation of ATP is the key function of each mitochondrion. ATP regulates cell survival, cell dynamics during cellular division and cellular motility. Mitochondria play a key role in the energy production and maitenance of spermatozoa motility [13, 27].

Various pathobiochemical mechanisms are involved in male infertility, as impairment of balance between reactive oxygen species production and reduced total antioxidant capacity [22, 23, 19, 2, 27].

One of the major roles in male infertility concerns decreased sperm motility and sperm mitochondrial ATP production, as well as some sperm mitochondrial DNA defects which make DNA unavailable for amplification [15]. The etiology of sperm DNA damage, much like male infertility, is multifactorial and may be due to intratesticular, post-testicular, or external factors, as protamine (the principal sperm nuclear protein) deficiency, apoptosis, drugs, cigarette smoking, leukocytospermia, high level of ROS production, varicoceles, chemotherapy, and radiotherapy [20].

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Sperm DNA damage is clearly associated with male infertility (and abnormal spermatogenesis), but a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage.

Sperm motility is used as indicator of semen quality and male fertility, since it is decreased in male infertility. Recently increased mitochondrial DNA defects were found to be in correlation with decreased sperm motility [6].

Another cause of infertility may be a disturbance of sperm mitochondrial function and energy production. This sperm mitochondrial damage is termed "*mitochondrial spermatopathy*". Our previous study showed reduced sperm mitochondrial respiration and ATP production in the ejaculate and decreased concentration of coenzyme Q_{10} in oligoasthenozoospermia patients. Coenzyme Q_{10} was not detectable in the ejaculate of azoospermic patients [10]. It is evident that proper mitochondrial oxidative phosphorylation function, an intact mtDNA genome, and decreased ROS production are vital for sperm motility.

13.2 Oxidative Stress and Sperm Function

Oxidative stress has been widely considered a cause of infertility [2, 3, 24, 9, 25]. However, low levels of ROS generation by normal sperm are involved in the physiological control of some sperm functions and are essential for capitation, including hyperactivation and acrosome reaction [1, 12].

Reactive nitrogen oxygen species (NOS) in excess can be toxic for sperm function. The effect of NO. radical depends upon its concentration and interaction with hydrogen peroxide, which is considered to be the most toxic ROS for human spermatozoa [12]. The primary mechanism of nitric oxide is induction of sperm damage on mitochondrial level and DNA synthesis. High production of ROS by large numbers of active leukocytes in semen, as is the case in infections, leads to peroxidation damage and impairment of sperm functions, including motility, longevity and fertilizing ability. High levels of ROS were detected in the semen of practically 25% infertile men but not in the semen of fertile men. ROS induce a chain of events leading to sperm immobilization, axonemes are affected, and limited endogenous repair mechanisms are available to reverse the damage, resulting in ATP depletion [7, 8]. Oxidative stress induces peroxidative damage in the sperm plasma membrane and DNA fragmentation in both the nuclear and mitochondrial genomes [2]. Elevated levels of ROS and negative correalation with sperm motility were demonstrated in semen of men with spinal cord injury [21]. Ejaculated sperms are normally protected from oxidative stress by antioxidative enzymes present in the seminal plasma.

13.3 Antioxidants and Sperm Function

Over the last years, the role of oxidative stress in infertility and especially the therapeutic use of vitamins with antioxidant properties have been widely considered [2, 3, 24, 9, 25].

Several antioxidant systems are present in spermatozoa. These include glutathione peroxidase [4], superoxide dismutase [18] and catalase [14]. Superoxide dismutase depletion has been assumed to be associated with sperm immobility [16] and catalase deficiency with asthenozoospermia [14]. Pro-oxidant and antioxidant substances are present in both seminal plasma and spermatozoa. In semen pathology, the initiating mechanism can be different and may trigger subtly different responses of the antioxidant systems according to the particular circumstances of oxidative stress.

Numerous antioxidants, such as vitamin C, vitamin E, glutathione, coenzyme Q_{10} and carnitine, have shown beneficial effects in treating male infertility [5, 26, 23, 10, 24]. Supplementation with vitamin C, vitamin E and intramuscular glutathione improved sperm quality. Glutathione is not only vital to the sperm as an antioxidant, it is also essential to the formation of glutathione peroxidase – an enzyme inportant in spermatids, which becomes a structural protein in the mid-piece of mature spermatozoa. Deficiencies of glutahione can lead to instability of the mid-piece, resulting in defective motility. A decreased level of reduced glutathione during sperm production causes disruption in membrane integrity of spermatozoa as a consequence of increased oxidative stress. Glutathione peroxidase and glutathione reductase may directly act as antioxidant enzymes involved in the inhibition of sperm lipoperoxidation [24]. The combination of folic acid and zinc supplementation was shown to increase the sperm count. The beneficial effect of carnitine supplementation on male fertility was demonstrated. It has been found that CoQ_{10} biosythesis is markedly active in the testes and a high level of reduced CoQ_{10} (ubiquinol) is present in the semen. The endogenous level of coenzyme Q_{10} showed a significant correlation between sperm count and sperm motility [17]. Reduced CoQ_{10} concentration in seminal plasma and sperm cells of infertile men with idiopathic varicocele was associated with asthenozoospermia. CoQ_{10} concentrations were not detectable in the ejaculate of asthenozoospermic patients [10]. Supplementation with the combination of L-carnitine, hydrosoluble coenzyme Q_{10}, vitamin E and vitamin C probably offers a new way for treating male infertility [11].

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Chapter 14
Methods for Diagnosis of Mitochondrial Diseases

Anna Gvozdjáková, Jarmila Kucharská, and Anna Hlavatá

Abstract Diagnostic methods for mitochondrial diseases are presented: biochemical analysis of blood, muscle biopsy, isolation of mitochondria and preparation of skinned fibers for respiratory chain and oxidative phosphorylation analysis, analysis of activity of respiratory chain enzymatic complexes, histochemistry, electron microscopy, molecular and MRS analysis.

Keywords Diagnosis, methods, mitochondrial diseases

The diagnosis of inherited mitochondrial diseases is difficult. Mitochondrial diseases should be considered in differential diagnosis when there are unexplained features, especially when these occur in combination with *encephalomyopathy*: seizures, developmental delay or regression (including early and late-onset dementia), myoclonus, movement disorders (dystonia, dyskinesias, chorea, etc.), complicated migraine, stroke; *neuropathy*; cardiac conduction defects or *cardiomyopathy; hearing deficits; short stature; disorders of extraocular muscles* (including ptosis, acquired strabismus and ophthalmoplegia); *diabetes; renal tubular disease; visual loss* (retinitis); *lactic acidosis* (which can be mild. More than one organ may be involved in mitochondrial diseases, particularly the functions of tissues with high energy requirement (brain, heart, skeletal muscle, liver, kidneys) are impaired. Affected subjects often show various combinations of signs and symptoms, thus in cases of multiorgan involvement a mitochondrial disorder should be considered.

Clinical manifestations of mitochondrial disease may occur even before birth and up to late adult life. *In the neonate* (up to 1 month), nonspecific findings are seen, as e.g. lethargy, irritability, hyperactivity, failure to feed well, hypothermia or fever, cyanosis, seizures, vomiting, jaundice, diarrhea, abdominal distension. Further manifestations are ketoacidotic coma with recurrent apneas, severe hypotonia, liver enlargement, proximal tubulopathy, severe neonatal sideroblastic anemia, concentric hypertrophic cardiomyopathy, hepatic failure. *In infancy* (1 month to 2 years) the manifestations include failure to thrive, recurrent episodes of acute myoglobinuria, proximal tubulopathy (de Toni Debre Fanconi syndrome), severe trunk and limb dwarfism, early-onset insulin-dependent diabetes mellitus

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(Wolfram syndrome), rapidly progressive encephalomyopathy, subacute necrotizing encephalomyopathy (Leigh's disease). *In childhood* (over 2 years) *and adulthood* the following neuromuscular clinical features may be present: muscle weakness with myalgia and exercise intolerance, progressive sclerosing poliodystrophy (Alper's disease), encephalomyopathy with myoclonus, ataxia, hearing loss, muscle weakness and generalized seizures (MERRF), progressive external ophthalmoplegia (PEO), mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS), Leber's hereditary optic neuroretinopathy (LHON), neurogenic muscle weakness, ataxia, retinitis pigmentosa and variable sensory neuropathy with seizures, mental retardation or dementia (NARP), mitochondrial myopathy and peripheral neuropathy, encephalopathy and gastrointestinal encephalopathy (MNGIE) [17].

Most cases have deficient activity of the oxidative phosphorylation complex or disorder of the pyruvate dehydrogenase complex. In some patients an isolated disorder of a single complex is found, while in other cases a combined disorder with deficient activity of several complexes is present.

14.1 Metabolic Analysis

Standard screening tests include the determination of:

1. Plasma lactate
2. Lactate/pyruvate molar ratio = redox status in the cytoplasm
3. Ketonemia ("paradoxical" elevation in fed individuals)
4. β-hydroxy butyrate/acetoacetate molar ratio = redox status in mitochondria
5. Blood glucose and free fatty acids
6. Urinary organic acid = lactate, ketone bodies, citric acid cycle intermediates.

When standard tests are inconclusive, the following determinations should be made:

(1) Blood determinations in fasted individuals and one hour after food intake: glucose, lactate, pyruvate, ketone bodies and their molar ratios at 15 min, 30 min, 45 min, 60 min, 90 min
(2) Lactate/pyruvate molar ratios in the CFS (only when no elevation of plasma lactate is observed)
(3) Redox status in plasma following exercise

14.1.1 Differential Diagnosis of Lactic Acidemia

Elevated plasmatic lactate is the diagnostic marker of disturbed mitochondrial metabolism and of respiratory chain disorders. Normal lactate values depend on age and laboratory method used. Normal plasmatic lactate value is 0.7–2.1 mmol/l.

Normal plasmatic pyruvate value is 0.034–0.102 mmol/l. Normal plasmatic 3-hydroxy-butyrate value is 0.030–0.300 mmol/l. Determination of the lactate: pyruvate ratio in body fluids is another important parameter. Elevation of this ratio over 25 can be a sign of oxidative phosphorylation disorder. Low values of this ratio are a sign of pyruvate dehydrogenase complex disturbance. Elevation of alanine in plasma and CSF is a sign of mitochondrial inherited metabolic disorders.

Determination of acetoacetate and 3-hydroxy-butyrate is useful for quantifying ketosis in the diagnosis of pyruvate carboxylase deficiency.

14.1.1.1 Blood Sampling Technique for Lactate Determination

The blood sample is taken after fasting and after a meal from an uncuffed vein or artery. False elevation (up to 4–6 mmol/l) is often due to increased muscular activity in a struggling child, in patients on assisted ventilation, or it may be due to seizures.

14.1.2 Lactic Acidemia

Primary lactic acidemia is a common sign in different inherited metabolic disorders. It can be caused by the following group of disorders:

(1) *Mitochondrial disorders*: respiratory chain disorders, PDH (pyruvate dehydrogenase) deficiency, HHH syndrome (hyperornitinemia, hyperammonemia, homocitrullinuria)
(2) *Gluconeogenesis defects*: PCD (pyruvate carboxylase deficiency), PEPCK (phosphenolpyruvate carboxykinase) deficiency, fructose-1,6-biphosphatase deficiency
(3) *Glycogen storage disease*: GSD 1a, 1b, 3
(4) *Organic acidemias*: methylmalonic acidemia, propionic acidemia
(5) *Fatty acid oxidation defects*: CPT I, II, VLCAD, LCHAD, MCAD, SCAD, glutaric aciduria type II
(6) *Other*: biotinidase deficiency, Krebs cycle defects

Secondary lactic acidemia: elevated lactate may be to deficiencies in blood taking techniques and elevated lactate can be a sign of other disorders as well secondary causes. *Elevated lactate* may be due to the following groups of disorders:

- Vascular: shock (hypovolemic, posthemorrhagic)
- Infectious: septicemia, malaria
- Respiratory: hypoxemia, hyperventilation, status asthmaticus
- Neurological: seizures, spasticity, perinatal asphyxia
- Cardiac: cardiomyopathy, heart failure
- Diabetes mellitus: ketoacidosis, biguanidines

Table 14.1 Differential diagnosis of primary lactic academia

Lactate fasting	Lactate after meal	Lactate: pyruvate fasting	3-OH-Butyrate: acetoacetate	Ketones	Blood sugar fasting	Typical for
(N)- ↑↑↑	(Rise)	(N)- ↑↑	(N)- ↑↑	(↑)- ↑↑	N	Respiratory chain disorders
(N)- ↑↑↑	Rise	N	N	N	N	PDH
(N)- ↑↑↑	Fall	(N)- ↑	(N)- ↓	↑↑	(↓)	PCD
(N)- ↑↑↑	Fall	N	N	N-(↑)	↓↓	GSD I
N	(Rise)	N	N	↑-↑↑	↓↓	GSD III
N- ↑	(Fall)	N	N	↓↓	↓↓	Fatty acid oxidation defects
(N)- ↑↑	(Rise)	N- ↑↑	N- ↑↑	↑- ↑↑	↓-↑	OA

- Gastrointestinal: chronic diarrhea, malabsorption, short bowel syndrome
- Renal: chronic renal insufficiency, renal tubular acidosis, urinary tract infection
- Hepatic: acute liver failure, liver cirrhosis, chronic hepatopathy
- Malignancies: leukemia, lymphoma, (Hodgkin's disease, NHL)
- Drugs: biguanidines, salicylates, probiotics, statins, stavudine
- Nutritional: thiamine deficiency
- Intoxications: ethanol, methanol, CO

Of the imaging methods, nuclear magnetic resonance of the brain or NMR spectroscopy is used. CT of the brain is of less diagnostic value in this group of disorders [2, 7, 11].

14.2 Muscle Biopsy

Muscle biopsy can be used for histochemical, electron microscopic, biochemical or genetic analysis.

Histopathology and electron microscopy. Histochemical features often correlate with specific metabolic and genetic changes. Ragged red fibers, presented in biopsy of skeletal muscle, indicate a defect of respiratory Complexes I–IV. Increased lipid within myofibers is a regular feature of Kearns-Sayre syndrome and progressive external ophthalmoplegia (PEO), but not in myoclonic epilepsy with ragged red fibers (MERRF) and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Total deficiency of succinate dehydrogenase indicates a severe defect in Complex II. Total absence of cytochrome-c-oxidase activity in all myofibers correlates with a severe deficiency of Complex IV, or deficiencies in cytochrome c oxidase activity, or deficiency of coenzyme Q_{10} [24]. Electron microscopy may confirm abnormal appearance of mitochondria.

Immuno-histochemistry. Defects in the presence or absence of specific proteins – can rule out other diseases or confirm loss of respiratory chain proteins.

14.2.1 Biochemistry

In most cases, deficient activity of the oxidative phosphorylation complex or disorder of the pyruvate dehydrogenase complex is found. In some patients an isolated disorder of a single complex is observed, while in other cases a combined disorder with deficient activity of several complexes is present. For evaluation of mitochondrial metabolism in the organism, the determination of other parameters is necessary, such as plasmatic alanine, lactate, pyruvate and 3-hydroxy-butyrate, and that repeatedly during the day (before and after meals), alanine in CSF, urinary acylcarnitines profile and elevated urinary excretion of Krebs cycle products (citrate, aconitase, isocitrate, oxoglutarate, succinate, fumarate, malate) and dicarboxylic aciduria [1].

14.3 Mitochondria

In isolated mitochondria or skinned fibers from tissue biopsy, activities of mitochondrial enzymes and respiratory chain function, connected with oxidative phosphorylation, can be measured.

14.3.1 Isolation of Mitochondria

Mitochondria from heart muscle, skeletal muscle, liver and brain can be isolated by means of differential centrifugation [18, 23]. Isolation solution for heart and skeletal muscle mitochondria contains 180 mmol/l KCl, 4 mmol/l EDTA and 0.1% of albumin with addition of Nagarase 2.5 mg/g of the tissue. Sedimented mitochondria are washed twice in isolation solution without albumin. Liver mitochondria are isolated in a solution containing in mmol/l: mannitol 225, saccharose 75 and EDTA 0.2. For isolation of brain mitochondria a solution containing in mmol/l: saccharose 320, K^+ EDTA 1 and Tris-Cl 10 is used [3].

14.3.2 Respiratory Chain Analysis and Oxidative Phosphorylation

Mitochondrial oxidative phosphorylation is measured at 30 °C by means of an Oxygraph (Gilson) using a Clark oxygen electrode [21] in a solution containing in mmol/l: HEPES 10, KH_2PO_4 5, KCl 120, EDTA 0.5 and dextran 2%. The function of Complex I is measured with the substrate glutamate (5 mmol/l) or glutamate + malate (2.5 + 2.5 mmol/l). The function of Complex II is measured with succinate (10 mmol/l) and rotenone is used to inhibit Complex I. Respiration is measured before and after addition of 600 nmol ADP and expressed as state 3 and 4, respectively.

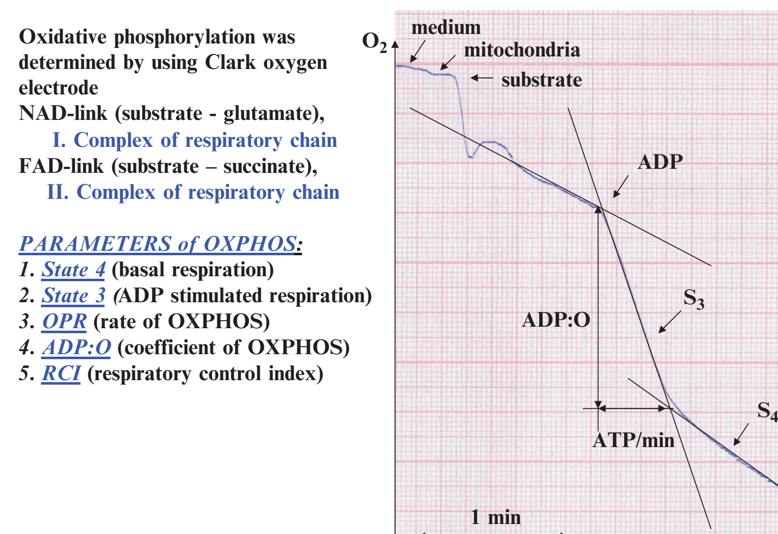

Fig. 14.1 Illustration of oxidative phosphorylation parameters

14.3.3 Mitochondrial Function Parameters

For purity and functional characterization of intact mitochondria the following parameters are used:

RCI – (S_3/S_4) – respiratory control index – indicator of mitochondrial membrane functional integrity; *State 3* – with ADP stimulated mitochondrial oxygen consumption (in the presence of substrate and ADP added); *State 4* – basal mitochondrial respiration (after consumption of the added ADP); *ADP:O* – coefficient of oxidative phosphorylation (indicator of coupling oxidation and phosphorylation); *OPR* – oxidative phosphorylating rate (indicator of ATP production rate) is calculated [5] (Fig. 14.1).

14.3.4 Determination of Coenzyme Q Homologues

Concentrations of coenzyme Q_9 and Q_{10} are determined in isolated mitochondria by HPLC method [15] with some modifications [12]. Mitochondrial suspension is extracted twice by mixture of hexane/ethanol (5/2 v/v), the organic phases are collected, evaporated under nitrogen and the residue dissolved in ethanol is injected on to the column SGX C18 7 µm (Tessek). The mobile phase consists of methanol/acetonitrile/ethanol (6/2/2 v/v/v, Merck). Concentrations of oxidized form of coenzymes Q_9 and Q_{10} are detected spectrophotometrically at 275 nm using external

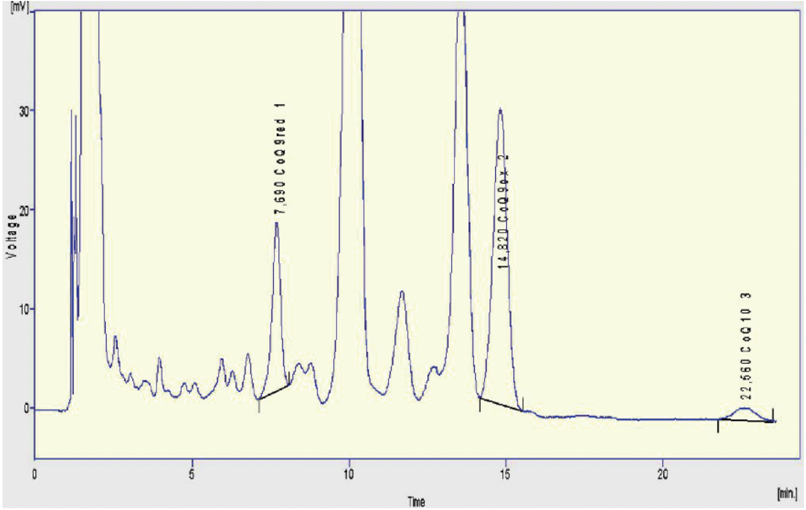

Fig. 14.2 Chromatogram of coenzyme Q

standards (Sigma) and calculated in nmol/mg of proteins. Mitochondrial proteins are determined [16] (Fig. 14.2).

For mitochondrial content of cytochromes b, c_1, c aa_3, spectrophotometric method is used at 605–630 nm [20]. Cytochrome c release from mitochondria is used as apoptotic signal [28].

14.4 Skinned Fibers Preparation and Oxidative Phosphorylation

Endomyocardial biopsy, skeletal muscle biopsy 3–5 mg (or spermatozoa) are used for the preparation of skinned fibres and measurements of mitochondrial respiratory chain function.

14.4.1 Isolation of Saponin-skinned Fibers

Wet tissue is cut into small slices 5–6 mm long. Bundles of fibers are placed into 1 ml of an ice-cold incubation medium containing in mmol/l: 20.0 HEPES, 8.0 $MgCl_2$, 0.5 dithiotreitol, 220.0 saccharose, 5.3 ATP, 15.00 creatine phosphate, 5.00 phosphate, 1.0 g albumin – in 100 ml, pH 7.4. After 20-min exposure to saponin (50 µg) skinned fibers are received; all procedures are carried out at 0–4 °C.

For saponin removal 10.0 mg/1 ml of isolation solution is used [13, 14, 26].

14.4.2 Respiration Measurements

Mitochondrial oxidative phosphorylation in skinned fibers is measured by using Clark oxygen electrode, polarographic method, in a medium consisting of 5.0 mM, KH_2PO_4, 120.0 mM KCL, 0.5 mM EDTA, 2% dextran, pH 7.2. As substrate glutamate, pyruvate, succinate can be used in a concentration of 10.0 mM. Mitochondrial ATP production is stimulated with 500 nmol of ADP [21].

14.4.3 Parameters of Oxidative Phosphorylation in Skinned Fibers

Basal respiration – V_o (as *State 4* in isolated mitochondria of differential centrifugation)

Stimulated respiration with ADP – V_{ADP} (as *State 3* – ATP production in isolated mitochondria)

Ratio of stimulated and basal respiration – V_{ADP}/V_o (as RCI in isolated mitochondria) [8, 9].

14.5 Measurement of Mitochondrial Enzyme Activity

14.5.1 Citrate Synthase

Citrate synthase is a Krebs cycle enzyme located in the mitochondrial matrix. It may be used as an internal control of functional mitochondria [24]. Citrate synthase activity assay is done according to the method of Coore et al. [4].

14.5.2 Analysis of Enzymatic Activity of Respiratory Chain Complexes

***Complex I* assay** (NADH-Ubiquinone Oxidoreductase)

Complex I activity is measured by determining the decrease in NADH absorbance at 340 nm, resulting in reduction of ubiquinone to ubiquinol [19].

***Complex II* assay** (Succinate-Ubiquinone Oxidoreductase)

Complex II activity is measured by the secondary reduction of a dye (6,6-dichlorophenolindophenol) by the ubiquinol formed, monitored at 600 nm [10].

***Complex III* assay** (Ubiquinol Cytochrome C Reductase)

Complex III – enzyme donates electrons from ubiquinol to cytochrome c, leading to reduction of cytochrome c, monitored at 550 nm [19].

***Complex IV* assay** (Cytochrome C Oxidase) is measured by evaluating the oxidation of cytochorme c as a decrease in absorbance at 550 nm [27].

***Complex V* assay** (ATP-synthase)

The enzymeatic activity of mitochondrial ATP-synthase is monitored at 660 nm, according to the method of Taussky and Shorr [25].

14.6 Molecular Tests

The mitochondrion has its own DNA (mtDNA) with a single, circular structure. The mtDNA communicates with nuclear DNA (nDNA). Five complexes of respiratory chain are encoded by nDNA and mtDNA:

Complex I = 41 subunits (mtDNA encoded: 7, nDNA encoded: 34 subunits)
Complex II = 4 subunits (all 4 are encoded by nDNA)
Complex III = 10 subunits (mtDNA encoded: 1, nDNA encoded: 9 subunits)
Complex IV = 13 subunits (mtDNA encoded: 3, nDNA encoded: 10 subunits)
Complex V = 12 subunits (mtDNA encoded: 2, nDNA encoded: 10 subunits).

Disorders due to mutations in mtDNA, disorders due to mutations in nDNA – are included in mitochondrial diseases (encephalomyopathies) [6]. Pathological alterations of mtDNA are involved in three major classes: point mutations, rearrangements and copy number mutations (depletions). *Point mutations* include amino acid substitutions and protein synthesis mutation (mRNA, tRNA). Most of these are maternally inherited, heteroplasmic, but they are associated with a striking variety of clinical phenotypes (LHON, MERRF, MELAS, NARP, Leigh's syndrome, diabetes and deafness). The *second class* of mtDNA diseases are deletions with duplications of the mt genome. They are usually sporadic, heteroplasmic, occurring during early development (KSS, Pearson's syndrome, CPEO, diabetes, deafness). The *third class* of mtDNA diseases are mtDNA depletions due to copy number mutations.

Finally, while detection of mtDNA rearrangement or base substitution confirms the genetic origin of the disease, negativity of these investigations does not rule out an mtDNA mutation nor does it represent a clue that a nuclear mutation is involved.

Recently it was demonstrated that damage to mtDNA and loss of the mitochondrial membrane potential participate in apoptotic cell death [22].

14.7 Magnetic Resonance Spectroscopy

(MRS) of muscle and brain. Phosphorus MRS allows to study muscle and brain energy metabolism in vivo. Inorganic phosphate (Pi), creatine phosphate (PCr), adenosine mono- di- or tri-phosphate (AMP, ADP, ATP) and intracellular pH may be measured.

The Pi/PCr ratio is a most useful parameter and may be monitored at rest, during exercise, and recovery. An increased ratio is found in most patients and MRS is

becoming a useful tool in the diagnosis of mitochondrial diseases as well as in monitoring therapeutical trials (see Chapter 14).

14.8 Family History

History of family members can sometimes indicate inheritance pattern by noting “soft signs” in unaffected relatives. These include deafness, short stature, migraine, headaches and PEO.

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Chapter 15
Nuclear Magnetic Resonance

Tibor Liptaj

Abstract In this chapter a brief and simplified description of the most important features of Nuclear Magnetic Resonance (NMR) is presented. In the first part, the physical principles and definition of basic NMR phenomena like spin, magnetic moment, magnetization, etc. are given. Then the behavior of NMR samples exposed to the effect of external magnetic fields is discussed with explanation of the related terms like spin precession, spin relaxation, free induction decay (fid). The effect of rf pulses, description of the time domain NMR experiments, as well as basic parts of a typical NMR spectrometer/imager are given. In the third part fundamentals of NMR imaging are shortly discussed and illustrated. The basis of NMR spectroscopy, with the definition of related spectral parameters like chemical shift, indirect spin–spin interaction, direct dipolar spin–spin interaction, nuclear Overhauser effect (noe) are presented. In the last part, the spectroscopic biochemical applications of NMR with the emphasis on metabolic studies are shortly discussed.

Keywords Biological application of nuclear magnetic resonance (NMR), electromagnetic irradiation, magnetization, metabolic studies of NMR, NMR spectroscopy, spin relaxation

Mitochondria play a crucial role in energy metabolism and hence influence the whole cell metabolism and many other functions of living organisms. Nuclear Magnetic Resonance (NMR), or simply Magnetic Resonance (MR) as referred to in medicine, is one of the most powerful methods available today for the monitoring and investigation of cellular metabolism. It is therefore useful to discuss in this book the basic principles of NMR and its implementation in biological research and medicine.

15.1 General Characterization

NMR is a physical phenomenon, which is based on the mechanic and magnetic properties of atomic nuclei, which are manifested when nuclei are exposed to the external magnetic field. During the NMR experiment, the orientation of nuclei in a

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magnetic field is changed. Such a change is associated with interaction of nuclei with electromagnetic irradiation from the radiofrequency (*rf*) region. The *rf* radiation is very weak, has low energy, cannot disrupt any chemical bond, and is therefore safe for biological systems. The other favorable feature of this irradiation is that it easily penetrates through biological objects and can thus be employed for imaging inner objects.

In all NMR applications, the NMR active nuclei act as "spies" reporting about the magnetic fields, which they are "feeling". Depending on the experimental arrangement, they carry information about the spatial distribution of the nuclei (methods of NMR imaging) or about their chemical environment (methods of NMR spectroscopy).

NMR is employed in many branches of natural sciences, including biochemistry and medicine. The importance of NMR for modern research and medical practice was publicly acknowledged by the award of three Nobel Prizes for this method in the last two decades [6].

15.2 Physical Principles

NMR is based on three properties of atomic nuclei: ***spin, magnetic moment*** and ***quadrupolar moment***. Two of them, spin and magnetic moment, are essential for NMR. The quadrupolar moment affects NMR properties of some nuclei, but for biologically important nuclei this effect is absent or not important and it will not be discussed below.

15.2.1 Spin

Nuclear spin is an internal property of the atomic nucleus equivalent to the rotation of the nucleus around an axis passing through its center. Due to spin some nuclei can be considered as small microscopic gyroscopes trying to keep their orientation in space. Spin is a vector property, and thus two quantities must be specified for its characterization: its magnitude and its orientation. We designate the spin vector as $\boldsymbol{I}$.

Since atomic nuclei are microscopic objects, the quantum theory must be used for their description. This theory says that the ***magnitude of a spin*** is characterized by the spin quantum number s, which can have the values:

$$s = 0, 1/2, 1, 3/2, ...$$

The value of s depends on the internal structure of the nucleus (number of protons and neutrons) and is therefore specific for each isotope. Some naturally abundant isotopes, like ^{12}C and ^{16}O, have $s = 0$ and are therefore NMR inactive. Isotopes with spin quantum number $s = ½$ possess the most convenient NMR properties for biochemical and medical application. Such isotopes are ^{1}H, ^{31}P and ^{13}C. Among biologically

Table 15.1 NMR properties of some biologically important nuclei

Nucleus	Spin quantum number *s*	Resonance frequency at 1T[MHz]	Natural abundance [%]	Relative sensitivity
^{1}H	1/2	42.55	99.98	1,000
^{2}H	1	6.55	0.016	0.0015
^{12}C	0	–	98.9	–
^{13}C	1/2	10.68	1.1	0.18
^{14}N	1	3.06	99.6	1.0
^{15}N	1/2	4.30	0.37	0.0038
^{16}O	0	–	99.76	–
^{17}O	5/2	0.04	0.011	
^{19}F	1/2	40.04	100.0	830.0
^{23}Na	3/2	11.28	100.0	93.0
^{31}P	1/2	17.23	100.0	66.0
^{35}Cl	3/2	4.17	75.4	3.5

important isotopes with $s \geq 1$ are ^{2}D, ^{23}Na, ^{35}Cl, etc. The list of the biologically most relevant isotopes, along with their NMR properties, is shown in Table 15.1.

Concerning the ***orientation of a spin***, there is a large amount of uncertainty. Normally for determination of orientation of any vector three independent (mutually perpendicular) vector components should be specified. The quantum theory says that only one component of spin vector can be determined with confidence, while the remaining two are completely undetermined with zero averaged value. The orientation of this "well defined component" depends on the state of the spin and can be arbitrary in space. In line with the statistical interpretation of quantum theory we can identify the spin vector $\boldsymbol{I}$ with this *well defined component*. This approach will allow simple (but correct) interpretation of many NMR phenomena (at least for spins with $s = 1/2$).

The existence of a spin is for NMR so fundamental that NMR active nuclei with nonzero *s* are frequently referred to simply as "spins". We will follow this convention and use simply "spins" instead of "NMR active nuclei".

15.2.2 Magnetic Moment

Nuclei with a nonzero spin pose also a nonzero *magnetic moment*, i.e. they behave like tiny microscopic magnets. The magnetic moment is "a handle" by which spins interact with the rest of the world. All NMR interactions are of magnetic origin.

The nuclear magnetic moment vector (we denote it as $\boldsymbol{\mu}$) is parallel and proportional to the spin vector

$$\boldsymbol{\mu} = \gamma . I$$

The proportionality constant γ, called the gyromagnetic ratio, depends on the internal structure of the nucleus and is unique for each isotope. If a spin is placed outside the magnetic field, the orientation of its $\boldsymbol{\mu}$ and $\boldsymbol{I}$ is fixed and has zero orientation energy. If we put the spin into the magnetic field $\boldsymbol{B}$, two things happen: First, the

magnetic field tries to align $\boldsymbol{\mu}$ along itself (exactly as the needle in a compass is aligned to the North). But because of the simultaneous existence of spin, both $\boldsymbol{\mu}$ and $\boldsymbol{I}$ start to precess around $\boldsymbol{B}$ with the frequency proportional to the strength of the applied field and gyromagnetic ratio of the nucleus:

$$\nu = (\gamma / 2\pi).\ B[\text{Hz}].$$

In commonly available magnetic fields (0.1–20 T) this frequency is from the radiofrequency region 1–900 MHz. The important feature of the precession motion is that the relative orientation of $\boldsymbol{\mu}$ and $\boldsymbol{B}$ (precession angle Θ) remains unchanged, while the orientation of $\boldsymbol{\mu}$ with respect to the plane perpendicular to $\boldsymbol{B}$ (azimuthal angle φ) is periodically changing (see Fig. 15.1).

The second consequence of the placement of the spin into a magnetic field is a sudden change of its potential energy, which is determined by the orientation of $\boldsymbol{\mu}$ with respect to the field $\boldsymbol{B}$:

$$\text{E} = -\ \boldsymbol{\mu}.\ B.\ \cos(\Theta).$$

Note that during precession the orientation energy of the spin is not changed as it does not depend on the azimuthal angle φ. Orientations more parallel with $\boldsymbol{B}$ (smaller angle Θ) have lower energy than more antiparallel orientations.

We may summarize that NMR active nuclei can be considered magnetic oscillators, whose frequency is determined by the strength of the applied field. This frequency is in NMR of prime interest as it bears encoded information about chemical or spatial location of the spins.

15.2.3 NMR Sensitivity

As NMR deals with low energies, it is naturally an insensitive method and its sensitivity is always an important issue. NMR sensitivity of a specific nucleus depends strongly on its gyromagnetic ratio and is also proportional to its natural

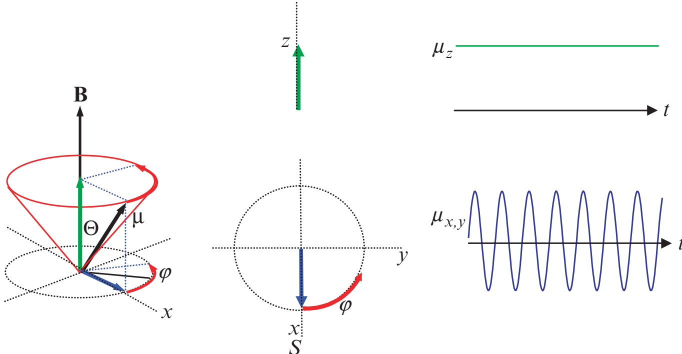


Fig. 15.1 Precession of the nuclear magnetic moment $\boldsymbol{\mu}$ in magnetic field $\boldsymbol{B}$

abundance. The relative sensitivity of biologically most relevant nuclei with respect to the sensitivity of the most sensitive nucleus (^{1}H) is shown in Table 15.1.

Sensitivity of NMR experiments may be increased by accumulation of an acquired signal in repeated experiments (scans) with the square root of the accumulated scans. Sensitivity of some insensitive spins (^{13}C, ^{15}N) can be significantly increased by their indirect observation via the ^{1}H spin. A prerequisite is the existence of appropriate ^{1}H–^{13}C (^{15}N) interactions, which enables the transfer of the signal between the two spins.

15.2.4 Magnetization

Objects investigated by NMR (NMR samples) contain a large amount of spins. The magnetic moments of individual spins are not observable individually but contribute to the resultant macroscopic magnetic moment of the whole sample, denoted as sample *magnetization* **M. M** is the vectorial sum of magnetic moments $\boldsymbol{\mu}_i$ of all active nuclei in a sample.

$$M = \Sigma \mu_i$$

$\boldsymbol{M}$ depends on the orientation distribution of $\boldsymbol{\mu}_I$ in a sample (see Fig.15.2). When the sample is out of a magnetic field, the orientation of individual magnetic moments is fixed and all orientations of $\boldsymbol{\mu}_i$ are equally probable as all orientations have the same (zero) potential energy. As a consequence, the resultant magnetization $\boldsymbol{M}$ is zero. When the sample is then placed into the magnetic field $\boldsymbol{B_0}$, two things happen immediately. First, all spins start to precess around $\boldsymbol{B_0}$. However, because they precess with he same frequency and keep their precession angle (Θ), their relative orientation and hence the resultant magnetization is not changed and remains zero. Second, the state of the sample is suddenly changed from an equilibrium to a nonequilibrium. This is associated with a sudden change in potential energy of each spin from zero to the energy depending on its orientation. The former uniform orientation of spins does no longer correspond to the equilibrium distribution. Since the nonequilibrium state of the sample is not stable, the sample tends to change its state into a new equilibrium by the process called *spin relaxation*. In a new equilibrium, states with lower energy (small angle Θ) should be more populated than states with higher energy ($\Theta \rightarrow 180°$). We will discuss the relaxation process later and now focus on the new equilibrium state.

It is useful to introduce a laboratory reference frame now. Let this frame has its *z*-axis oriented parallel with $\boldsymbol{B_0}$ and the *x*- and *y*-axis in any direction in a perpendicular plane. The angle Θ now describes the deviation from the *z*-axis. If we relate this axis with the polar axis of the Earth, then using geographical terminology, we can say that in the equilibrium state, orientations of spins in the northern hemisphere should be more populated than in the southern hemisphere. The density of population should decrease when going by the meridian from the North Pole

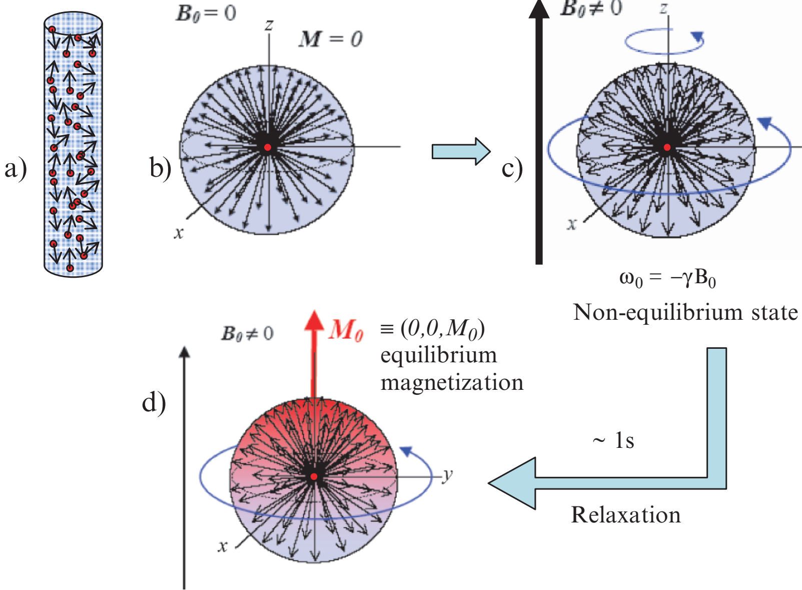


Fig. 15.2
(a) Sample out of magnetic field, magnetic vectors have random orientation.
(b) For vector addition it is convenient to shift them into the same origin; the ends of spin vectors form a sphere with a uniform distribution of their orientation; the resultant magnetization is zero.
(c) In the first moment after insertion of the sample into the magnetic field the spins start to precess, all with the same frequency, around $\boldsymbol{B_0}$; the resultant magnetization remains zero.
(d) After relaxation period the spins still precess around $\boldsymbol{B_0}$ but the population of their orientation on the "north" hemisphere is higher; the resultant (static) M_z magnetization in the direction of $\boldsymbol{B_0}$ is created. The higher population is encoded by red color.

(parallel orientation with $\boldsymbol{B_0}$) to the South Pole (antiparallel orientation), in accord with the Boltzman law. The density of population of spin orientation is constant when passing by any parallel (energy does not depend on the angle φ). We say that the sample is partially polarized by $\boldsymbol{B_0}$. All spins are in constant precession around the z-axis but their relative orientation remains constant. With such distribution of spin vectors the resultant equilibrium magnetization is oriented parallel the with the z-axis. We denote it as the vector $\boldsymbol{M_0}$ with three components,$(M_x\ M_y,\ M_z) \equiv (0,\ 0,\ M_0)$. Despite the fact that all spins are moving, $\boldsymbol{M_0}$ is static and no information about a spin precession frequency can be retrieved from it.

Polarization of a sample and creation of $\boldsymbol{M_0}$ is reached spontaneously and its creation is a starting point of all NMR experiments. From equilibrium magnetization other nonequilibrium forms of oriented sample can be prepared artificially.

Let us now assume nonequilibrium magnetization, which was prepared by simultaneous flipping of all spins around some axis in an x,y plane (see Fig.15.3). The phase of spin vectors in an xy plane (angle φ) is no longer uniform but some preferred value exists. This partial "phase coherence" is manifested by the exist-

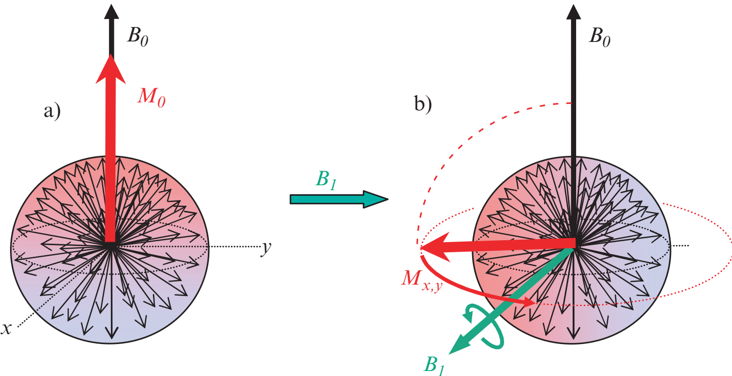


Fig. 15.3 (a) An equilibrium sample is polarized in the direction of $\boldsymbol{B_0}$ (b) $\boldsymbol{B_1}$ on resonance rotates each vector around itself. *Polarization* of the sample is converted into the *phase coherence* in the xy plane. This coherence is manifested by the existence of M_{xy}. Each individual spin vector continues to precess around $\boldsymbol{B_0}$. So does M_{xy}

ence of the nonzero M_{xy} component of magnetization. It is important to note that because all spins are in precession motion with the same frequency, M_{xy} also precesses in line with all of them. In contrast to $\boldsymbol{M_0}$ (or any M_z component) the M_{xy} component of magnetization is an easily measurable dynamic property of the sample. It carries the information about the precession frequency of the spins. It is the property that provides the measurable NMR signal.

Measurable M_{xy} is a nonequilibrium state of the sample and will therefore cease after some time by sample relaxation. M_{xy} can be destroyed also by the effect of spatial $\boldsymbol{B_0}$ inhomogenities. Such inhomogenities lead to differences in precession frequencies of spins, which in turn destroy the phase coherence in the xy-plane. The rate of the decay of M_{xy} is proportional to the product $\gamma \Delta B_0$, where ΔB_0 is the amplitude of field inhomogenities and γ is the gyromagnetic ratio of the given spins.

15.2.5 Effect of Electromagnetic Irradiation

Irradiation used in NMR is from a radiofrequency (*rf*) region and is therefore applied to the sample via an *rf*-coil, which acts as a transmitter antenna. Such irradiation has a high degree of coherence. It means that besides the same frequency, the emitted photons have also a highly coordinated phase. The important feature of coherent irradiation is that its effect on all spins in the sample is coordinated (has the same phase) and does not destroy any organized state of the sample but is able to transfer one organized state (e.g. polarized sample) into another organized state of sample (e.g. a phase coherence). On the macroscopic scale, coherent irradiation can be associated with an oscillating macroscopic electric and magnetic field. For NMR only its magnetic component (labeled as $\boldsymbol{B_1}$) needs to be considered. The placement of the *rf* coil is such that vector $\boldsymbol{B_1}$ oscillates in the xy plane, perpendicular to $\boldsymbol{B_0}$. We say that the *rf* coil produces linearly polarized irradiation. This can be

considered a superposition of the two circularly polarized components, both rotating in the *xy* plane, but with opposite sense. However, only a component rotating in the same direction as the spins can have some effect on them. We will identify $\boldsymbol{B_1}$ with this component, while we neglect the effect of the other component.

$\boldsymbol{B_1}$ has a negligible effect on spins unless it is "on resonance" with them, i.e. to be effective it must have the same or very similar frequency as the spins. To visualize its effect it is convenient to look at spins from a frame of reference which rotates around $\boldsymbol{B_0}$ with the same frequency as the spins. From such a frame the spins would seem to be still, i.e. they could be considered to be placed in a zero field $\boldsymbol{B_0}$. The field $\boldsymbol{B_1}$ applied on resonance would look like a static field and it would be the only field acting on spins. Its effect would therefore be analogical to the effect of $\boldsymbol{B_0}$ in the laboratory frame: after switching on $\boldsymbol{B_1}$, the spins would start to precess around $\boldsymbol{B_1}$ with the frequency

$$\nu_1 = (\gamma / 2\pi).\ B_1\ [Hz].$$

An important feature is that during this precession the *z*-component (and one component from the *x,y* plane) of magnetization is periodically changed. This corresponds to the periodical exchange of energy (resonance) between spins and field $\boldsymbol{B_1}$.

Usually $\boldsymbol{B_1}$ is applied only for a short time (in *a pulse*) to rotate spins by a desired flip angle. Besides the flip angle, the resulting effect of the pulse depends also on the phase of $\boldsymbol{B_1}$ (its orientation in *xy*). Pulses with flip angles 90° and 180° applied from axis *x, y*, −*x* or −*y* are the most frequent ones. The 90° pulses are used as excitation pulses, which from equilibrium $\boldsymbol{M_0}$ magnetization create measurable M_{xy} magnetization. More generally, 90° pulses are used for transformation of one organized state of the sample into another. Transfer of magnetization among interacting spins can also be accomplished by 90° pulses.

The 180° pulses are used as inversion and refocusing. They invert some components of magnetization but do not cause any transfer of magnetization.

The demands on the performance of pulses may be different. On some occasions, a uniform effect is desired in the whole spectral window (nonselective pulses), on other occasions, a selective effect is needed in some part of the frequency window. In general, nonselective (hard) pulses covering a broad spectral window have a short duration (range of μs) and large $\boldsymbol{B_1}$ amplitude, while selective (soft) pulses have a smaller $\boldsymbol{B_1}$ amplitude and longer pulse duration (tens of ms). Ideal pulses should have a uniform effect in the desired spectral window and no effect outside this window. The spectral characteristic of the simplest rectangular pulses ($\boldsymbol{B_1}$ constant during pulse) is far from ideal. To improve the spectral characteristic of pulses, a whole battery of pulses was proposed in which the amplitude (and phase) of $\boldsymbol{B_1}$ is suitably tailored to achieve the desired improvement. Such pulses have found extensive use in both NMR spectroscopy and imaging.

15.2.6 Spin Relaxation

Spin relaxation is a process of establishing thermodynamic equilibrium in an NMR sample. This process is spontaneous and cannot be replaced by any artificial

manipulation of spins. It is the only way how magnetization of a sample can be created and it is therefore of fundamental importance for NMR.

Systems studied by IR or UV spectroscopy (bond vibrations, electrons) can reach equilibrium very quickly (10^{-12}–10^{-8} s) by spontaneous emission of excess energy. For spins such a type of relaxation is negligible and the only way how to reach an equilibrium is the exchange of energy among spins or between spins and other suitable objects in the sample (called lattice). This energy exchange is mediated by internal magnetic fields, always present in a sample (fields coming from magnetic moments of neighbor spins, fields rising up by movement of electrons and other charged particles in a sample, etc.). The mechanism how these random fields affect spins and participate in their relaxation is analogical to the mechanism of action of $\boldsymbol{B_1}$ and $\boldsymbol{B_0}$ fields. A substantial difference between random fields and $\boldsymbol{B_0}$ and $\boldsymbol{B_1}$ is that while the latter act coherently in time and space on the whole sample, the action of random fields can be considered the action of many local weak pulses distributed in time and space randomly.

The relaxation of M_z and M_{xy} is principally different. The reason is that M_z relaxation is affected only by on-resonance components of random fields (analogues of $\boldsymbol{B_1}$), while in M_{xy} relaxation also zero-frequency components of random fields (analogues of nonhomogenous $\boldsymbol{B_0}$) are involved. M_z and M_{xy} have also different equilibrium values (M_0 and 0 respectively).

Phenomenologically relaxation of M_z and M_{xy} can be described by simple differential equations, stating that the rate of relaxation is proportional to the deviation from equilibrium.

$$dM_Z/dt = -(M_Z - M_0)/T_1 \qquad dM_{xy}/dt = -(M_{xy} - 0)/T_2$$

T_1 (spin-lattice or longitudinal relaxation time) and T_2 (spin–spin or transversal relaxation time) are time constants, which characterize the rate of relaxation of M_z and M_{xy} respectively. Depending on the state of the sample, T_1 and T_2 may have values of several milliseconds up to several tens of a second. For small molecules in nonviscous samples both T_1 and T_2 are from the region of ~1 s. Solid or rigid samples may have very long T_1 ~ 10 s but very short T_2 ~ < 1 ms. It should be stressed that these spin relaxation time ranges are for NMR very fortunate. On one side, the relaxation times are sufficiently long to give enough time for complex manipulation of spins (complex NMR experiments) and to provide well-resolved spectra, and simultaneously they are short enough to allow rapid accumulation of signal (to cope with low NMR sensitivity).

15.2.7 NMR Experiments

At present, NMR experiments are performed almost exclusively in a *time domain*. That means that during the course of the NMR experiment spins are manipulated and evolving with time according to a predefined scheme (pulse sequence) and eventually a response of spins as a function of time is acquired. Each NMR

experiment can be divided into several periods. The number of periods depends on "dimensionality" of the NMR experiment, which is directly related to the dimensionality of spectra (or images) which it produces. The simplest one-dimensional (1D) NMR experiments consist of only two periods: *preparation* and *detection* period. These experiments produce conventional spectra, in which peaks are plotted as the function of one frequency coordinate. Experiments with higher dimensionality (2D NMR, 3D NMR, etc.) have an additional *evolution* (and possibly a *mixing*) period for each additional dimension. A multidimensional experiment is realized as a series of 1D experiments in which the duration of evolution periods is systematically incremented. 2D NMR, 3D NMR, etc. experiments provide spectra in which each peak is associated with 2, 3, or more frequency coordinates.

To increase the sensitivity of NMR, the experiment (the whole series of experiments in case of 2D, 3D, … NMR) may be repeated and the response systematically accumulated. At the end the final result (spectrum or image) is computed from the accumulated response.

15.2.7.1 Preparation Period

Each NMR experiment starts with a preparation period. During its first part the sample is let to relax to allow creation of the starting state of the sample (M_z). To get a maximum future signal, spins should relax ~ 5 times the longest T_1 relaxation time in a sample. In practice (to achieve faster accumulation rate) shorter preparation periods are frequently used (~ 1- 3T_1). Then it should be kept in mind that spins with long T_1 may be partially saturated and their signal diminished (T_1 weighted spectra or images are thus obtained). In the second part of the preparation period the sample is excited by a proper excitation block of a pulse sequence. In the simplest case, excitation consists of just a single pulse, which transforms (at least partially) M_z into M_{xy} magnetization. In more complex excitation schemes, the formally excited magnetization is further modified (by T_1 or T_2 relaxation) or transferred to another spin (for example from ^1H to ^{13}C to increase the sensitivity of the experiment).

15.2.7.2 Detection Period and Fourier Transformation

M_{xy} prepared in a preparation period (1D NMR) or present after the last evolution (and mixing) period (*2D*, *3D* NMR) is measured in a detection period. During this period it *freely* precesses in an *xy* plane of the NMR probe and *induces* a signal in the receiver coil. The signal *decays* with time because of T_2 relaxation. The detected signal is therefore referred to as "*Free Induction Decay*", or shortly as "*fid*". If all measured spins in a sample were in exactly the same field, *fid* would contain just one frequency component. In such a case, spectroscopic information (frequency, amplitude, etc.) would be "visible" directly in the *fid*. However,

because of different chemical environment (NMR spectroscopy) or different location in a variable magnetic field (NMR imaging), spins may feel different field. As a consequence, a *fid* may contain many different frequency components and can be a very complex function of time. From such a fid spectroscopic information can be obtained by employing the mathematical transformation called *Fourier transformation* (see below).

15.2.7.3 Evolution Period

In a *2D* NMR experiment, magnetization created at the end of a preparation period is allowed to evolve in an evolution period. The duration of an evolution period is systematically incremented in a series of experiments. It is natural that any periodical modification of the signal during the evolution period must be manifested as some modification of the final detected signal with the same periodicity. The behavior of spins during the evolution period is thus encoded in the detected signal, and in principle, it can be determined.

In 3D, 4D… NMR experiments, additional evolution period/s is/are inserted into the pulse sequence and cause additional modification of the detected signal. Similarly as in a 2D NMR experiment, the behavior of spins during each evolution period is thus encoded in the detected signal and in principle, it can be determined. The dimensionality of the experiment (number of evolution periods) is limited because of T_2 relaxation and prolongation of the experimental time.

15.2.7.4 Mixing Period

An important feature of many pulse sequences is transfer of the signal between spins. This transfer is possible only if there is an interaction between spins and is accomplished in a specially designed block of pulse sequence. The actual design of this block depends on the details of interaction between spins (its further discussion is beyond the scope of this text). In some multidimensional NMR experiments, this block is inserted after the evolution period and is called the *mixing period*.

15.2.7.5 Fourier Transformation

All primary spectroscopic or imaging information about the sample encoded in a time domain fid can be made visible in the form of a spectrum or an image by the mathematic transformation known as *Fourier transformation* (*FT*), which relates unambiguously both forms of signal presentation. In the case of a multidimensional experiment, a separate signal (1D fid) is detected for every duration of each evolution period. Such a series forms one *nD fid*, which is the function of *n* time coordinates (duration of *n-1* evolution periods and the time running during the detection period). By application of *n*-dimensional *FT* (*nD -FT*), a corresponding *nD* spectrum is obtained.

Each signal in the obtained *n*D spectrum has *n* coordinates. One coordinate determines the precession frequency of a signal during detection period, the other (*n*-1) the precession frequencies during the (*n*-1) evolution periods. Each peak thus bears with it the history of its evolution during the experiment. If for example, there was a transfer of signal during 2D NMR experiment from spin A to spin B, then the peak corresponding to the transferred part of signal will have one coordinate belonging to spin A (frequency during evolution) and the other to spin B (frequency during detection). The existence of such a peak is an unambiguous proof of the coupling between spins A and B, which can provide very useful structural information. In imaging experiments, there is no signal transfer. Spins evolve under different conditions during the evolution and detection period. For example, the experiment can be set so that the precession frequency of spins in a selected plane during evolution period depends on their *y* coordinate and during detection period on their *x* coordinate. The 2D experiment thus unambiguously determines both coordinates (localization in selected plane) of spins.

The depth of the evolution of the spins is proportional to the product of the driving force of evolution and the time of evolution. Therefore there are two ways how to achieve the same depth of evolution of spins during the incremental evolution period. The first, as described above, is by keeping the driving force (conditions during evolution) constant and incrementing the evolution time. The second is achieved by incrementing the strength of the driving force during a constant evolution time. The second method is frequently used in imaging experiments, where instead of prolongation of the evolution period, the strength of the field gradient (and thus the magnetic field as a function of coordinates) is incremented. In imaging experiments, which rely on the use of linear gradients of the magnetic field, it is therefore convenient to record the fid not as a function of time but as a function of coordinates which are directly proportional to the depth of the evolution, the "*k*" coordinates. And because with linear gradients of the magnetic field the *k* coordinates are proportional to the space (*x,y,z*) coordinates, the FT of such a fid yields the result (an image) which is the function of the space coordinates.

15.2.8 NMR Instruments

Construction of the NMR instrument (spectrometer or imager) is determined by the character of irradiation used. The essential parts of NMR instruments are therefore very similar to the radio transmitter (transmitter channels through which irradiation is applied to the sample) and radio receiver (receiver channel of spectrometer for the measurement of fid). The quality of the *rf* part is very high (digital synthesis, linear amplifiers, high stability and precision, etc.). The performance of transmitter and receiver channels is controlled by the acquisition processor according the predefined experiment pulse scheme. The response of the sample is in the final step digitalized and stored in the memory of the acquisition computer.

As all NMR experiments are performed in a magnetic field, the dominant part of each NMR instrument is a strong magnet, a generator of field $\boldsymbol{B_0}$. Today superconducting magnets are used almost exclusively. The magnet has a cylindrical ring-like shape. In the central open part there is a working place, in which the probe is inserted. The essential part of the probe is an *rf* coil, which is used as a transmitter antenna through which irradiation is applied to the sample. The same coil is used as a receiver antenna for the measurement of the sample response in the last phase of NMR experiments.

The "supervisor" of the whole NMR experiment is the host computer. It is used to set up, to start and to monitor NMR experiments. After the data acquisition also data processing (Fourier transformation, spectrum or image manipulation, etc.) and data storing and distribution (via Internet) is performed. The scheme of NMR spectrometer is shown in Fig.15.4.

So far we have supposed that all measured spins are immersed in the same magnetic field and have the same precession frequency. However such a situation would imply very limited practical applications. Actually, in practice we would like to discriminate the spins in different chemical or space locations. Fortunately, nature has taken care to differentiate spins in different chemical locations and discrimination of the space location of spins can be achieved by simple modification of the applied magnetic field. In the following sections we will shortly discuss the two basic kinds of NMR applications based on this spin discrimination, NMR imaging and NMR spectroscopy.

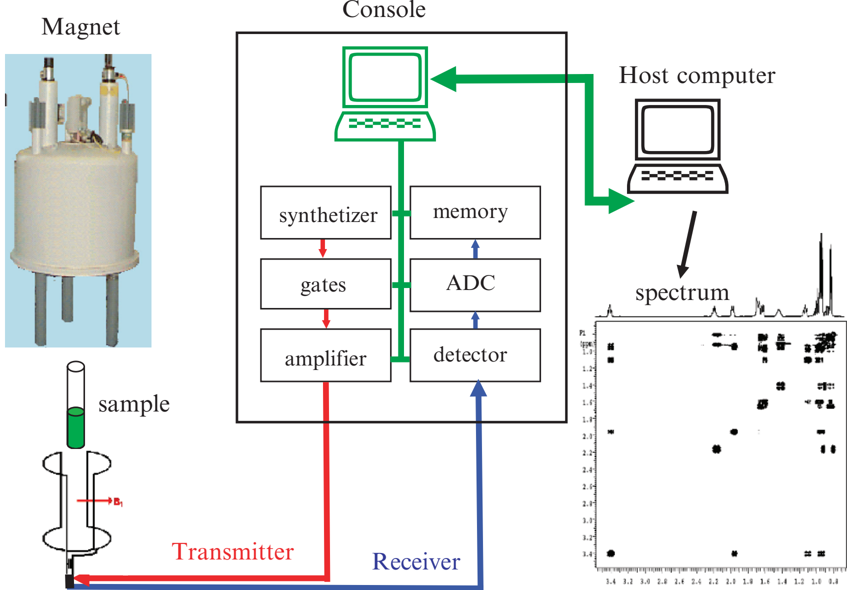


Fig. 15.4 Basic scheme of an NMR spectrometer

15.3 NMR Imaging

In NMR imaging we wish to highlight the space distribution of spins. Any dependence on the chemical environment of spins is in this case undesirable and detrimental. For an ideal image we would need a substance which has only one chemical type of spins and is distributed in the whole object. Fortunately, in biological systems, water with two equivalent hydrogen nuclei is a substance which almost perfectly meets these requirements. Water ^{1}H spins in biological systems are in very large excess to all other hydrogen spins and have convenient relaxation properties. As a consequence, the signal from all other hydrogens is relatively small and can be neglected (the only exception is lipid signal in fatty tissue).

If we measured the signal of water in a constant magnetic field, we would not be able to determine its spatial distribution, as the frequency of the signal from all parts of the sample would be the same. However, if we measured it in a variable magnetic field with the linear gradient in a certain direction (in the direction of the axis *x,y* or *z*), then the spatial coordinate in the gradient direction would be encoded in the precession frequency of spins. This is illustrated in Fig. 15.5.

By repetition of the experiment with proper application of gradients of the magnetic field (see Fig. 15.6) and subsequent processing of the series of acquired data, the inner image of the measured object can be obtained. The image is usually made in black–white fashion, while the lightness of pixels corresponds to the water concentration in the given location. High quality images of soft tissues can be obtained. Besides the water concentration, the contrast of the image can be to a

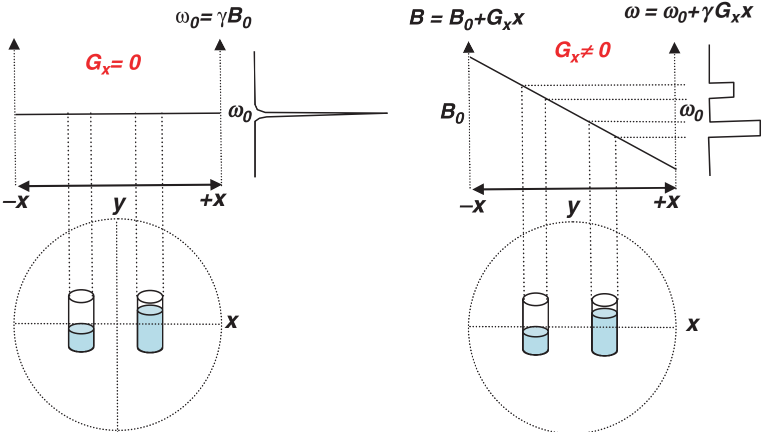


Fig. 15.5 Principles of encoding the space coordinates. (a) Two samples of water are in the same magnetic field and their position cannot be distinguished because both have the same resonance frequency. (b) The same samples but in a variable magnetic field. The left tube is in a higher field and thus the water in it has a higher resonance frequency than the water in the right tube. Notice that the intensity of the signal is higher for the tube with the larger content of water

certain degree controlled by the properties of water like T_1 and T_2 relaxation times, water diffusion coefficients, water flow, etc. (Fig. 15.6). By proper design of the imaging experiment, special information about the studied object, for example blood flow (NMR angiography) or location of the centers of specific activities in the brain (functional imaging) can be obtained. Some applications are illustrated in Fig.15.7.

It is an important feature that water properties, which influence the image contrast, depend to a certain degree on the physiological state of the tissues. NMR images thus not only describe the inner anatomy of the object but may have also high diagnostic value.

In relation to the functions of mitochondria, NMR imaging methods can be utilized for the investigation and mapping of such pathological states as ischemia and edema. Imaging methods are, e.g. able to visualize the size and position of cerebral ischemic lesions. The early stages of ischemia are characterized by intracellular water accumulation and cell swelling. This is accompanied by a decrease of water diffusion coefficients but not by a change of water T_2 relaxation time. Thus to observe early stages of cerebral ischemia (up to 5 h), diffusion weighted images should be acquired. This is in contrast with later stages of ischemia, when the accumulation of extracellular water in the interstitial space causes an increase in the T_2 relaxation time, which becomes visible also in T_2-weighted images.

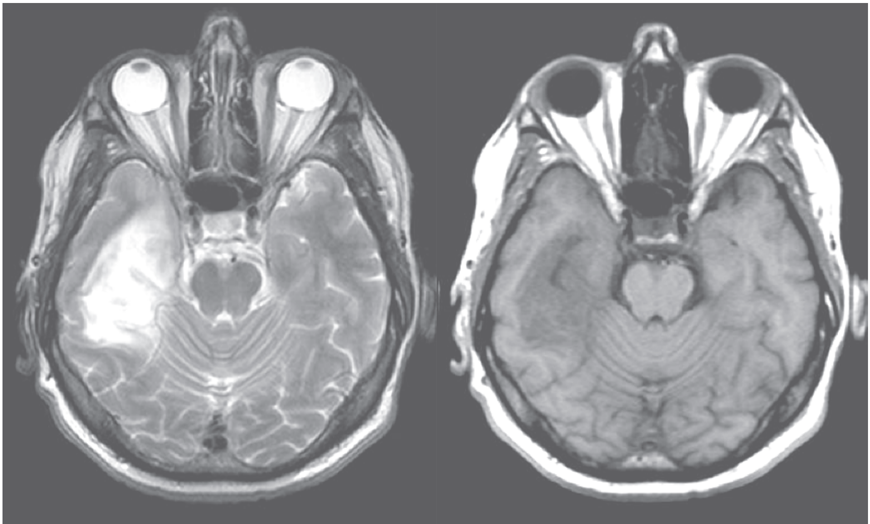


Fig. 15.6 T_2 (left) and T_1 (right) – weighted images of the brain of a patient. The water in the tumor (left side of the brain) has longer T_2 and T_1 than the water in the normal tissue and this is reflected in the contrast of the two images

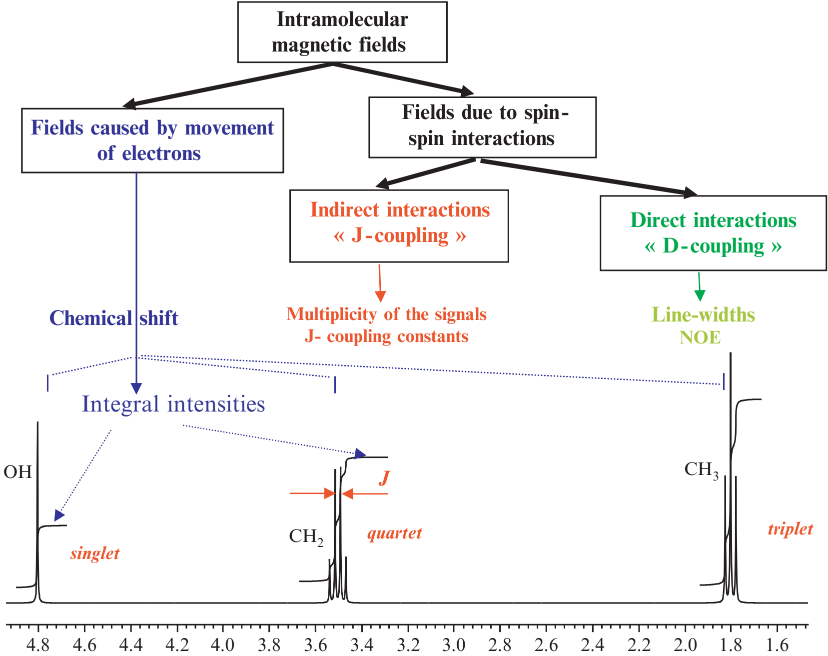


Fig. 15.7 Formation of high-resolution NMR spectrum. The signal dispersion in the spectrum is caused by the effect of the electrons induced by field $\boldsymbol{B_0}$, *J*- interaction causes the multiplet structure of the signals and it is usually observed if the two spins are located on the neighbor bonds. The effect of the direct spin–spin interactions is in the standard spectrum visible only as a contribution to the width of the spectral lines. However D-coupling is behind the important NOE, which is observed in special spectra and bears valuable information about the pairs of spatially close spins

15.4 NMR Spectroscopy

The spectroscopic applications of NMR are based on the fact that spins of the same type (^{1}H, ^{13}C), when placed in chemically different positions in a sample, feel different magnetic field. It is an effect of internal magnetic fields always present in a sample. These fields have different values in different chemical positions and are added to the external field $\boldsymbol{B_0}$. As a consequence, spins in a sample have different resonance frequencies. It is the goal of spectroscopy to investigate the spectrum of these frequencies and provide structural and dynamic information about the sample studied.

All internal interactions of spins depend on the orientation of the molecule or crystal in the external field $\boldsymbol{B_0}$. In a liquid sample, the orientation of molecules is rapidly changing, which causes averaging of internal interactions with the consequence of a dramatic simplification of NMR spectra. On the contrary, spectra from rigid structures are very complex, composed of very broad, nonresolved lines. In conditions of standard NMR experiments, these lines are buried in the base line and

not observable. Therefore in heterogeneous systems, like biological systems, only small mobile molecules dissolved in liquid parts of systems (cytosol, body fluids) are observable, provided they are in sufficient concentration.

The most important types of internal magnetic fields are magnetic fields arising from surrounding electrons and magnetic fields of neighbor spins. They are responsible for the phenomena called *chemical shift* and *spin–spin interaction*. The effect of internal fields manifested in a typical high-resolution NMR spectrum is illustrated in Fig. 15.7.

15.4.1 Chemical Shift

The chemical shift is of fundamental importance for chemical application of NMR. It is caused by internal magnetic fields $\boldsymbol{B}_{int}$, created by the effect of the external field $\boldsymbol{B}_0$ on electrons and is manifested as a shift of the resonance frequency of spins. The amplitude of $\boldsymbol{B}_{int}$ depends on the state of electrons in the molecule (crystal) and is therefore different in each chemically different fragment. As the effect is proportional to the strength of $\boldsymbol{B}_0$, the dispersion of signals and spectral resolution become better at higher fields. It is therefore favorable to run spectroscopic experiments at higher fields.

Chemical shifts are tabulated in a δ scale, which is independent of the field:

$$\delta_i = (\nu_i - \nu_{ref}) / \nu_{ref},$$

where ν_{ref} is the resonance frequency of the reference compound. In ^1H and ^{13}C NMR tetramethylsilan (TMS) is used as reference compound. ^{31}P NMR chemical shifts are referenced to the external 85% H_3PO_4 (sealed in a capillary) or signal of phosphocreatine (in biological application). Chemical shifts of spins in different structural fragments are well mapped and there are numerous data bases (printed or electronic) from which the information about the relevant chemical shift can be obtained.

The chemical shift separates NMR signals into groups. From the position of the signal in a spectrum (from its chemical shift), the structural type of the fragment can be estimated. The number of signals in a spectrum therefore determines the number of chemically different groups in the molecule (sample). The intensity of signals is proportional to the number of equivalent spins associated with each signal. This is important structural information on the type of structural fragments from which the sample is composed and on their relative amount.

15.4.2 Spin–Spin Interactions

Spin –spin interaction is a mutual effect of magnetic moments of a pair of spins. There are two ways how this interaction is transmitted: either directly through space or it is indirectly mediated by electrons. The two types of spin interactions

have different effects on NMR spectra but both are of crucial importance for NMR. Unlike chemical shifts, spin –spin interactions do not depend on the strength of $\boldsymbol{B_0}$ but solely on the properties of interacting spins. The strength of spin–spin interactions is expressed as the coupling constant in frequency units (in Hz). The coupling constant for indirect interaction is denoted as ^nJ, where n indicates the number of bonds intervening the two interacting spins. The direct coupling is denoted as D.

15.4.2.1 Indirect Spin Interaction

In the indirect interaction, spins (by their magnetic moment) first polarize electrons in their vicinity. Polarization is then transmitted via bond electrons to the neighbor spins, where it is manifested as an additional magnetic field. The transfer depends on the number, type and orientation of the bonds which separate the two interacting spins. In general, the strength of this interaction is diminishing with the number of intervening bonds. Interactions between spins separated by one, two or three bonds ($^1J(^1H, ^{13}C)$ ~ 100- 200 Hz; $^nJ(^1H, ^1H)$,$^nJ(^1H, ^{13}C)$ ~ −20 − + 20 Hz, n = 2,3) are important. Interactions through four or more bonds are usually negligible ($^nJ(^1H, ^1H)$,$^nJ(^1H, ^{13}C)$ ~ 0 Hz, n = 4,5,..).

In conditions of liquid state NMR experiments, indirect spin interactions cause the multiplet structure of NMR signals. The strength of spin interaction can be determined by multiplet analysis. The spin interaction can be artificially removed (averaged to zero) by additional irradiation of the partner spin. This procedure is called spin decoupling.

One of the most important outcomes of the existence of indirect spin interaction is that it allows to identify the pair of spins on the neighbor bonds.

15.4.2.2 Direct Spin Interaction

In direct spin interaction the magnetic moment of one spin directly (through space) interacts with the magnetic moment of a neighbor spin. The strength of this interaction is significantly higher than the strength of indirect interaction (up to D ~ 30,000 Hz versus J ~ 1-200 Hz) but it falls rapidly with the separation of spins (negligible after 0.5 nm) and is strongly dependent on the orientation of spins with respect to $\boldsymbol{B_0}$. In liquid state conditions, the direct interaction is rapidly changing. In isotropic liquids its mean value is zero. As a consequence, the effect of direct coupling is only marginally manifested in liquid state spectra (it affects the width of spectral lines). Despite its zeroing, this interaction is of primary importance for NMR as it provides the main NMR relaxation mechanisms in liquid and solid state.

The important practical outcome of direct spin interaction, closely related to spin relaxation, is a phenomenon called *nuclear Overhauser effect* (NOE), which allows identification of spins located closely in space. NOE is defined as the relative change of intensity of the signal of one spin if its partner (the neighbor spin) is

disturbed from its equilibrium. It is important that NOE is observed only if the two spins are in strong direct interaction, i.e. close in space. NOE is not observed in standard spectra but can be detected in specially designed experiments.

Both indirect and direct spin interactions can act as a vehicle for transfer of the signal between coupled spins. It has a close analogy with coupled mechanical oscillators, which can also exchange energy. As mentioned earlier, such transfer of the signal is a frequently occurring part of various methods of 2D (or 3D) NMR. These experiments allow very convenient identification of the pair of spins involved in signal transfer. In these experiments indirect spin interactions are exploited to transfer the signal between spins (and thus to identify these spins, frequently ^{1}H – ^{1}H or ^{1}H -^{13}C), which are located in the neighbor fragments. This enables to combine fragments identified on the basis of their characteristic chemical shifts into bigger blocks and possibly to determine the whole skeleton of the molecule. On the contrary, direct interactions are exploited for the identification of pairs of spins (almost exclusively ^{1}H – ^{1}H) which are close through space. This information enables to determine the configuration of the molecule and 3D structure of molecules in a solution.

The ability of NMR to identify structural fragments on the basis of their characteristic chemical shifts is similar to other molecular spectroscopic methods. However its ability to combine these fragments into bigger blocks is a unique feature of NMR. This feature makes NMR the leading method in structure determination of organic molecules in a solution.

15.5 Biological Applications of NMR Spectroscopy

Spectroscopic applications of NMR in the field of biochemistry and medicine are very broad and diverse.

One important group of application is the determination of 3D solution structure of biologically important molecules: natural products, saccharides, peptides, proteins, fragments of DNA, etc. These applications require the preparation of a sample consisting of the solution of a single compound (water) in sufficient amount and quality (in the case of bigger biomacromolecules possibly enriched in ^{15}N and ^{13}C, (^{2}H) isotopes) and employ a whole battery of multidimensional NMR experiments with subsequent intensive data processing and interpretation using specialized software. Efficient protocols for high sample throughput have been developed and are in wide use (especially for proteins).

The second important group of applications deals with the study of interactions of biomolecules. This group can be divided into several subgroups depending on the complexity of the substrate and the target molecules. A very efficient example of NMR use is its application in SAR (structure affinity relationship) studies. The ^{15}N enriched protein with known structure and assignment of all signals is mixed with the group of substrates. From the effect on the characteristic region of the protein spectrum induced in the presence of specific substrate(s), the location of active center(s)

and the structure of the complex can be determined. This approach is very efficient when the target is the design of substrates with high affinity (potential drugs).

Another group of applications consists of NMR metabolic studies. In many respects this is a very diverse group. The samples investigated are of different complexity and in different forms. Standard and special in vivo instrumentations are employed. For example, ^{1}H NMR analysis of samples of body fluids (urine, blood plasma,…) can be considered to be typical organic chemistry application of NMR done on a classical instrument and using a standard organic chemistry protocol. On the other side, a localized in vivo NMR spectroscopy from humans or experimental animals is performed on instruments with wide-bore horizontal magnets and "tricks" adopted from imaging techniques. Between these two extremes, there is a variety of sample types (tissue extracts, homogenates, perfused cell cultures, tissues or organs) and experimental arrangements (standard NMR probes, bioreactors in NMR tube, suffice coils, etc.). The aims of the studies may also be different. In many metabolic studies the content of specific metabolites is of main interest, while in other studies the kinetic characteristics of some metabolic changes are investigated.

The structural applications of NMR have been in wide use and they have proved to be very useful tools for investigation of solution structures and interactions of biologically important molecules. However due to the special subject of this book, we will not discuss them in greater detail (the reader may refer to the numerous books on this topic) but we will focus on the last mentioned group, i.e. NMR metabolic studies.

15.5.1 NMR Metabolic Studies

A typical sample in NMR metabolic studies is "a soup" consisting of a mixture of several constituents. The identity of the mixture components, the metabolites, is usually known and the main goal is the determination of their relative concentration. Several "bio" nuclei are suitable for this purpose but ^{1}H and ^{31}P are by far the most frequently exploited. Another nucleus which is of great interest in metabolic studies is ^{13}C. This concerns especially experiments with ^{13}C-enriched substrates, which provide very valuable information.

15.5.1.1 ^{1}H NMR Spectroscopy

Depending on the sample type (brain, muscle, body fluid, etc.) and form (intact in vivo, extracts, etc.), relatively few, up to several dozens of metabolites can be observed in ^{1}H NMR spectra. For each type of sample a characteristic spectrum of metabolites is expected, however depending on the sample form (and quality of NMR instrument) only part of them can be resolved in the spectrum. For example, in perchloric acid extracts of brain tissue up to 20 metabolites can be observed, while in in vivo spectra obtained on standard imaging scanners only 3–5 of them (NAA, PCr–Cr, choline, lactate) are observable. The list of common metabolites

found in ^{1}H NMR spectra of tissues together with indication of spectral localization of individual signals is shown in Table 15.2.

On working with extracts of biological material, body fluids, tissue homogenates or biological systems (tissues, organs, cells cultures, etc.) perfused in an NMR tube, the classical methods for "NMR in a sample tube" including multipulse or multidimensional experiments can be used. Clearly, the best quality spectra are observed in pure solutions (extracts, urine sample). Sample heterogeneity deteriorates the quality of spectra. In case of homogenates, special experimental arrangement, i.e. spinning of the sample at the "magic angle" (54.74° with respect to $\boldsymbol{B_0}$) allows to reduce this adverse effect.

To get in vivo ^{1}H spectra from humans or experimental animals is one of the most challenging tasks in NMR. There are many difficult problems (selection of volume of interest (VOI), water suppression, nonhomogeneity of $\boldsymbol{B_0}$ and $\boldsymbol{B_1}$), which must be solved in a limited experimental time in order to get usable spectra. Many different strategies and techniques were employed to achieve this task. For their review we refer the reader to the excellent book [3]. The importance of the information available from such spectra is indeed very high, thus greatest effort has been invested to improve the quality of the spectra. The result is that the recently published in vivo ^{1}H spectra of the rat brain are of a quality approaching that of "tube" NMR spectra.

In the metabolite ^{1}H NMR spectrum, the diagnostic information is encoded as the deviation of the relative signal intensities of the individual metabolite from normal levels. The absolute metabolite concentration can be easily estimated by means of the external quantitative standard in "NMR tube" experiments. However the use of the external standard in "whole-body" experiments is limited, therefore absolute concentrations are estimated from the comparison with the signal of the internal

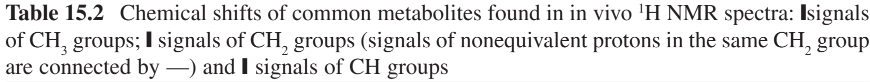
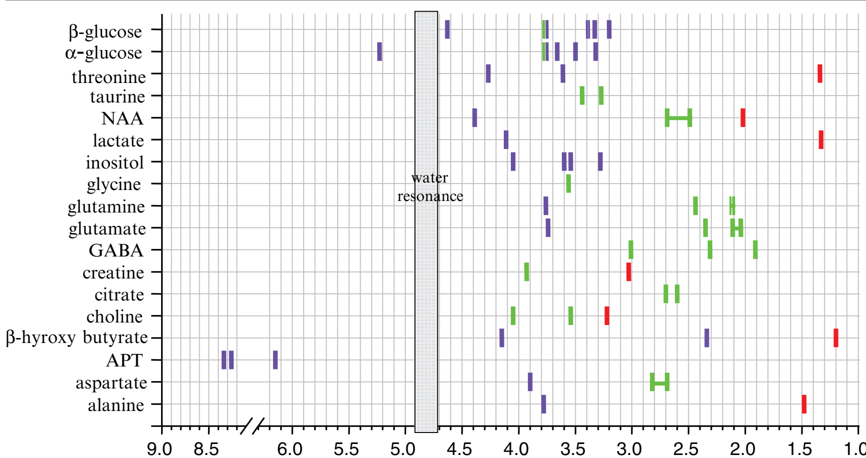


Table 15.2 Chemical shifts of common metabolites found in in vivo ^{1}H NMR spectra: [red bar] signals of CH_3 groups; [green bar] signals of CH_2 groups (signals of nonequivalent protons in the same CH_2 group are connected by —) and [purple bar] signals of CH groups



compound, whose concentration is believed to be well defined. It can be for example the signal of water measured under conditions without water suppression.

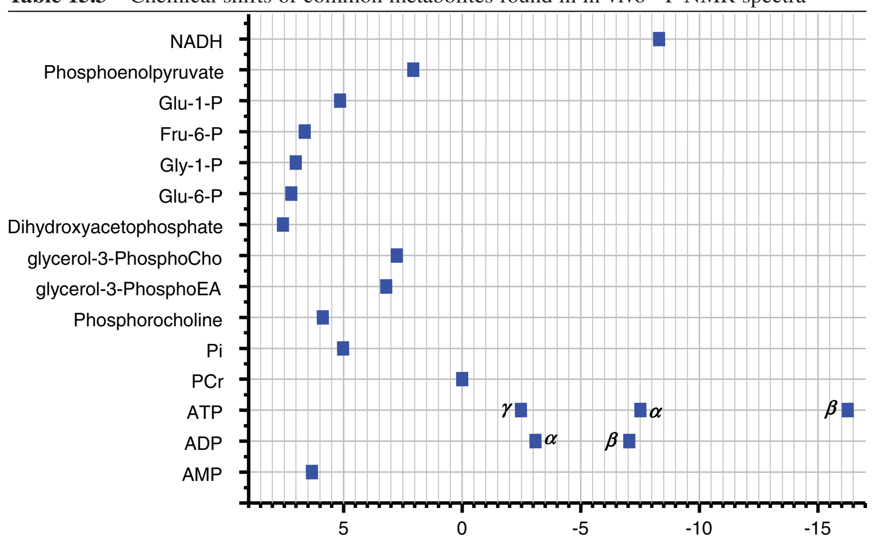
If the NMR metabolite map of the system studied is well known, then it is of advantage to do a full automatic analysis of the samples using appropriate software. This can make the analysis very efficient and reliable and suitable for high sample throughput.

15.5.1.2 ^{31}P NMR Spectroscopy

^{31}P nucleus is frequently used in the NMR metabolite studies. The reasons are several. First, ^{31}P NMR is capable of monitoring the metabolites which play a crucial role in the energy metabolism of cells. Secondly, the NMR sensitivity is relatively good (about 7% of the sensitivity of ^{1}H NMR spectroscopy), and finally, there is no phosphorus compound equivalent to water in ^{1}H NMR, which would dominate the whole spectrum and would require signal suppression.

^{31}P spectroscopy is capable of measuring the levels of the following metabolites: adenosinetriphosphate ((ATP); a doublet/triplet/doublet corresponding to the – α/β/γ phosphate of ATP), phosphocreatine – (PCr), inorganic orthophosphate (Pi), sugar phosphates associated with high-energy phosphate metabolism, water-soluble phosphomonoesters ((PME), including phosphocholine (PC) and phosphorylethanolamine (PE)), phosphodiesters ((PDE), including glycerol-phosphocholine (GPC) and glycerolethanolamine (GPE)), NADH, and others. The ^{31}P chemical shifts of these compounds are shown in Table 15.3. Other important biological parameters, like intracellular pH, free magnesium, and ADP concentrations, can be deduced from ^{31}P spectra indirectly.

Table 15.3 Chemical shifts of common metabolites found in in vivo ^{31}P NMR spectra



Concerning the experimental arrangement for "whole-body" in vivo studies, surface coils are frequently used for the VOI selection. Of the gradient localization techniques, the same methods as used in in vivo ^{1}H NMR can be applied. However, the ISIS method, which is the least sensitive to the relatively fast T_2 relaxation of ^{31}P signals, is the method of choice.

Information about the energetic status of the tissue investigated can be obtained from different ^{31}P NMR spectral parameters. Integral intensities of individual signals in standard phosphorous spectra (as determined by proper analysis of the spectrum) are proportional to the concentration of the related metabolites. Their concentration ratios in normal tissue should remain constant because of homeostasis. Any deviation from normal values indicates a pathological state.

The exact position of some phosphorous resonances is very sensitive to the pH value (Pi, γ and β ATP) or Mg^{2+} concentration (γ and β ATP), which can be utilized for their indirect determination. The prerequisite is knowledge of the relevant dissociation constants [5].

In tissues with highly variable energy demands (brain, muscle, heart) an important role in energy metabolism is played by the creatine–kinase system. ^{31}P NMR is capable not only of evaluation of the equilibrium concentration of the involved reactants (PCr, ATP, Pi) but is also able to determine the kinetic parameter of creatine–kinase reaction. These kinetic parameters have been shown to be much more sensitive indicators of pathologic changes in energy metabolism than static concentration parameters.

15.5.1.3 ^{13}C NMR Spectroscopy

^{13}C NMR in natural samples is not a convenient method for metabolic studies because of its low inherent sensitivity caused by low natural abundance of the isotope ^{13}C. In fact only signals from concentrated species (glycogen and mobile lipids) can be detected in natural samples. The low signal intensity from endogenous compounds can be converted to advantage if ^{13}C-enriched precursors are used in metabolic studies. The use of appropriately ^{13}C-enriched substrates like [1-^{13}C], [6-^{13}C] or uniformly enriched glucose, [2-^{13}C]- acetate, [3-^{13}C]-pyruvate can provide unique information about metabolic pathways like the tricarboxylic acid cycle, glycolysis, gluconeogenesis, and the pentose phosphate metabolic pathway. Using appropriate mathematical models, the fluxes through the TCA cycle and some other paths (GABA shunt, pentose shunt, etc.) can be evaluated. So far most of the ^{13}C NMR metabolic studies have been performed on in situ systems in NMR tube experiments. However recently in vivo ^{13}C studies on human brain were reported [4, 2].

All NMR metabolic methods which allow determination of metabolites affected by mitochondrial function can be in principle exploited in investigating mitochondrial functions and disorders. A number of papers addressing many mitochondrial functions or disorders have appeared in the literature. The reader can find references to most of them in the recent review by Arias-Mendosa [1].

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Chapter 16
Clinical Aspects of Coenzyme Q_{10} in Relationship with Its Bioenergetic and Antioxidant Properties

Gian Paolo Littarru and Luca Tiano

Abstract For a number of years, coenzyme Q (CoQ_{10} in humans), was known for its key role in mitochondrial bioenergetics; later studies demonstrated its presence in other subcellular fractions and in plasma, and also extensively investigated its antioxidant role. This chapter discusses the relationship between the acknowledged bioenergetic role of CoQ_{10} and some clinical effects. The antioxidant properties of CoQ_{10} are then analyzed especially for their consequences on protection of circulating human low-density lipoproteins and prevention of atherogenesis. The relationship between CoQ_{10} and statins is also discussed in the light of possible involvement of CoQ_{10} deficiency in the issue of statin side effects. New aspects of the antioxidant involvement of coenzyme Q are also discussed together with their relevance in cardiovascular disease. Data are reported on the efficacy of CoQ_{10} in ameliorating endothelial dysfunction in patients affected by ischemic heart disease. Many of the effects of CoQ_{10}, which were classically ascribed to its bioenergetic properties, are now considered as the result of its biochemical interaction with nitric oxide (NO), NO synthase and reactive oxygen species capable of inactivating NO. Clinical studies are reported highlighting the effect of CoQ_{10} on extracellular SOD, which is deeply involved in endothelial dysfunction.

Previous studies have shown decreased levels of CoQ_{10} in the seminal plasma and sperm cells of infertile men with different kinds of asthenospermia. Research has been extended to supplementation with CoQ_{10} of infertile men affected by idiopathic asthenozoospermia. CoQ_{10} levels increased significantly in seminal plasma and sperm cells after 6 months of treatment with concomitant improvement of sperm cell motility.

Keywords Antioxidation, cardiovascular diseases, Coenzyme Q_{10}, male infertility, mitochondrial bioenergetics

16.1 Introduction

The aim of this chapter is to review some basic aspects of the biochemical roles of coenzyme Q and some of its medical implications, namely the ones where I have been specifically involved.

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The discovery of this molecule came about within an intensive program of research, developed by Professor D. E. Green, at the University of Wisconsin, to find out how mitochondria work. This happened in 1957 and it soon became evident that Coenzyme Q was essential to mitochondrial ATP formation. Several years later Professor P. Mitchell was awarded the Nobel Prize, for his studies centered on the vital role of Coenzyme Q in oxidative phosphorylation.

The insight of Professor Karl Folkers, who since those early years re-isolated Coenzyme Q_{10} (CoQ_{10}), the human Coenzyme Q, from human heart, and elucidated its structure, led him to foresee the clinical importance of CoQ_{10}. At the same time Professor Yuichi Yamamura, a pioneer of CoQ_{10} research in cardiology, developed the first clinical trial of CoQ_{10} in patients with cardiovascular disease.

Those intuitions opened a new, variegated, biomedical research field, whose acquisitions expanded together with the basic biochemistry of Coenzyme Q.

It soon became evident that some of the in vitro and in vivo properties of Coenzyme Q were referable to its antioxidant action. Today we have a broader picture of ubiquinone properties, suggesting that some CoQ_{10} effects might be related also to its capability of modulating mitochondrial pore opening, the function of uncoupling proteins [10] and gene expression [23].

In the following sections I will specifically discuss whether some of the medical properties of CoQ_{10} can be ascribed to its bioenergetic or antioxidant properties.

16.2 CoQ_{10} and Mitochondrial Bioenergetics

The essential role of CoQ_{10} in mitochondrial bioenergetics was postulated many years ago [22]. According to this early view, the quinone was considered as a substrate in excess concentration over the prosthetic groups in the lipoprotein complexes of the respiratory chain. The kinetic analyses of Kroger and Klingenberg [28], showed that steady state respiration in submitochondrial particles could be represented as a simple combination of two enzymes, the first one responsible for the reduction of coenzyme Q and the second one causing oxidation of ubiquinol. Experiments of direct titrations of CoQ-depleted mitochondria reconstituted with different CoQ supplements disclosed a Km of NADH oxidation close to a CoQ concentration of 4–10 mM in the lipid bilayer, whereas the Km for succinate oxidation was one order of magnitude lower [17]. As found by Lenaz, Km for CoQ_{10} of NADH oxidation in bovine heart mitochondria is within the range of its concentration in the membrane [17]. The physiological concentration therefore is not saturating and even a small increase in the CoQ_{10} concentration of mitochondrial membranes could lead to an increased respiratory rate. The control exerted by CoQ concentration over mitochondrial respiration could be of particular interest in situations of decreased CoQ levels and/or increased Km values for the quinone in some pathological states. In fact this observation could represent the biochemical mechanism by which exogenous coenzyme Q_{10} ameliorates the bioenergetic impairment in a some mitochondrial myopathies and in cardiomyopathy [43, 46].

Modern views indicate that respiratory complexes may have a supramolecular organization, i.e. stable super complexes [20]. The advantage of this super complex organization would be a more efficient electron transfer by channelling of the redox intermediates. Preliminary data suggest that alteration of the protein/phospholipid ratio and lipid peroxidation disaggregates the supercomplex organization with possible pathophysiological implications. Lenaz postulates [21] that in ageing, and in ischemic diseases, reactive oxygen species (ROS) produced by the mitochondrial respiratory chain induce a progressive peroxidation of mitochondrial phospholipids. This could lead to a dissociation of Complex I–III aggregates and subsequent loss of facilitated electron channelling. Also according to this model, an increased concentration of coenzyme Q may, at least partially, counteract this deleterious effect of supercomplex disaggregation.

16.3 Antioxidant Properties of Coenzyme Q_{10}

Besides the well recognized vital role of ubiquinone in energy transduction and oxidative phosphorylation there is considerable evidence that Coenzyme Q functions as a lipid soluble antioxidant in biological membranes. This evidence has been produced by numerous experimental models, both in vivo and in vitro. These studies have been conducted on artificial membranes, isolated subcellular organelles, cultured cells, isolated perfused organs and clinical models [9]. The first observations were made by Mellors and Tappel in 1966 [39]. These authors demonstrated the antioxidant effect of Coenzyme Q on emulsions of arachidonic acid subjected to peroxidative insult, and on isolated mitochondrial lipids. In the following years Takeshige et al. [62] explored the antioxidant capacity of Coenzyme Q by a new approach: submitochondrial particles were depleted of their Coenzyme Q content, by chemical extraction, which made their endogenous lipids more susceptible to lipid peroxidation. This increased vulnerability could be progressively lessened by reincorporating Coenzyme Q into the submitochondrial particles. Similar results were obtained after extracting mitochondrial Coenzyme Q by pentane and inducing lipid peroxidation by adriamycin: also in this case the increase in peroxidability could be minimized if Coenzyme Q had previously been reincorporated into the mitochondria [60]. Battino et al. [6] pointed out a very evident protective effect of Coenzyme Q on biological membranes: when Coenzyme Q had previously been extracted, exposure of mitochondrial membranes to the oxidative insult brought by γ ray source led to a remarkable loss of their polyunsaturated fatty acids. The same authors also tested the protective effect of CoQ_{10} when exogenously added to cell cultures: survival of these cells to an oxidative insult was doubled if CoQ_{10} was present in the medium.

The antiperoxidative effect of Coenzyme Q has also been checked in lyposomes exposed to radical insult [31], and in micels of unsaturated, fatty acids subjected to thermal autoxidation [34]. In isolated mitochondria, production of reactive oxygen species and free radicals is higher when the extent of reduction of the initial components of the respiratory chain is increased, by means of special inhibitors which

block the electron flux towards the cytochromes. In this condition the luminol amplified chemiluminescence, elicited by the reactive oxygen species, is higher, and can be lowered by supplementing the mitochondria with CoQ_{10} [14]. Several years ago we set up an experimental model in which a homogenate of activated polymorphonuclear leucocytes was tested as a biological source of singlet oxygen and other reactive oxygen species, detectable by a chemiluminescence technique: the addition of 1 μM CoQ_{10} was able to greatly reduce the chemiluminescent phenomenon [33]. I would like to briefly comment on the mechanisms by which Coenzyme Q, mainly in its reduced form, is currently believed to exert its antioxidant effect. Lars Ernster, a pioneer of the studies on the location and the function of Coenzyme Q in mitochondrial membranes, reviewed these mechanisms [15]. Experimental data strongly indicate that the intervention of Coenzyme Q, in its reduced form, can be considered within the antioxidant preventive mechanisms. $CoQH_2$ would reduce the perferryl species ($Fe^{+++} - O_2^{\bullet-}$), thus preventing the radical attack that this species would initiate on fatty acids. By this mechanism QH_2 would practically prevent the formation of alkyl radicals ($L^{\bullet}$) and peroxyI ($LOO^{\bullet}$).

Ubiquinol may also act by slowing down the chain propagation reaction, according to the general mechanism of hydrogen donation to the radicals.

Another mechanism by which ubiquinol exerts its antioxidant properties is the one explored by Packer, Kagan et al. [25]. These authors demonstrated that reduced Coenzyme Q regenerates α-tocopherol, the active form of Vitamin E, by reducing the a-tocopheryl radical. In these reactions the ubisemiquinone radical is formed, whose presence in the mitochondrial respiratory chain has been known for the last 20 years. Ubisemiquinone is stabilized through the binding to special Q proteins. The presence of SOD and catalase would keep under control $O_2^{\bullet-}$ and the H_2O_2 arising from the autoxidation of the ubisemiquinone; however it is unlikely that autoxidation of the ubisemiquinone species generates $O_2^{\bullet-}$ in the phospholipid environment. Ernster stresses the fact that Coenzyme Q is the only lipid soluble antioxidant that animal cells can biosynthesize “de novo” and for which appropriate enzymatic mechanisms exist to regenerate the reduced form. We have demonstrated that reduced Coenzyme Q exerts its antioxidant properties also by inactivating ferrymyoglobin, a species capable of triggering the oxidative attack at muscular and cardiac level [42]. Also through experiments developed with Mordente and Santini [41] we showed that both short side chain ubiquinones and CoQ_{10} can protect several enzymes from oxidative attack, both in solution and when membrane-bound. Antioxidant effects are always more pronounced when Coenzyme Q is used in its reduced form. In certain cases also oxidized Coenzyme Q affords some degree of protection [40, 41].

16.4 Structure and Function of Plasma Lipoproteins

Fats are by definition insoluble in water, yet a lipid interchange exists between different organs, for many metabolic reasons. This interchange, of course, occurs through the blood stream, so that special ways of transport are utilized by our

organism. The "free fatty acids", which represent an important metabolic fuel for the muscle and the heart, are transported by albumin; most lipids are transported in the form of lipoproteins. Different classes of lipoproteins can be classified, and separated, on the basis of different relative amounts of lipids and proteins. Density decreases with increasing percentages of lipids, so that LDL (low density lipoproteins) and VLDL (very low density lipoproteins) have relatively higher content of lipid material, when compared with HDL (high density lipoproteins). LDL, which will be taken into consideration in these pages as the principal atherogenic lipoproteins have a spheroidal structure, where the inner hydrophobic core is made of cholesterol and cholesterol esters, with a minor percentage of triglycerides. The surface layer is made of phospholipids, which are amphypathic, i.e. have a chemical structure which on the one hand can interact with the aqueous environment of plasma, and on the other has a high affinity for lipids. Proteins also stay on the surface, and greatly contribute in stabilizing the LDL in the water environment. Apo B 100 is the protein of LDL, owing its importance to the fact that some sites of its structure are recognized by the LDL receptors. Liver cells assemble and discharge into circulation VLDL particles, which contain endogenously synthesized triglycerides, phospholipids, cholesterol and Apo B 100. As triglycerides, which are the principal lipid fraction carried by VLDL, are removed by lipoprotein lipase at muscle and adipose tissue level, VLDL are progressively transformed into IDL (intermediate density lipoproteins) and finally become LDL, which are poor in triglycerides but very rich in cholesterol. The presence, on the plasma membranes of many types of cells, of high affinity receptors for LDL, which recognize the Apo B 100, allows delivery of cholesterol biosynthesized in the liver to extrahepatic cells, which use it for various purposes. Moreover, since 60–70% of the receptors are located in the liver, this is also a mechanism by which cholesterol from the periphery is returned to the liver. HDL are also essential for the overall cholesterol homeostasis. These lipoproteins are also synthesized in the liver, and initially contain mainly phospholipids. Through the activity of LCAT (lecithin cholesterol acyl transferase) for which Apo AI of HDL is an essential cofactor, HDL effectively remove cholesterol from extrahepatic tissues, then deliver it to VLDL, which will eventually become LDL. LDL are removed by the Apo B 100 receptor in the liver, so that the "reverse cholesterol transport" is completed.

16.5 Lipoprotein Oxidation

Since their isolation was made possible it soon became evident that lipoproteins, especially LDL, are easily oxidized, and LDL oxidation products are cytotoxic. It was also pointed out that metals, especially copper, play an important role in the "in vitro" oxidation of LDL. As LDL are rich in polyunsaturated fatty acids, such as linoleic and arachidonic acid, their oxidation leads to the formation of remarkable amounts of conjugated dienes and malondialdehyde [16, 52]. The role of polyunsaturated fatty acids on the oxidative modifications of LDL was unequivocally demonstrated by Parthasarathy [49]. This author showed that dietary supplementation with oleic acid, which is monounsaturated, significantly lowers peroxidability

of LDL. Substantial modifications of apolipoproteins also take place, in the course of LDL oxidation. These modifications are primarily due to reaction of lipid oxidation products with certain amino acid residues. Some ε-aminogroups of lysine residues, for instance, undergo neutralization of their positive charge following this reaction, so that the apoprotein is no longer recognizable by the high affinity classical LDL receptors, mainly located in the liver. Lipid oxidation products also deeply modify the α-helical regions of the protein, leading to its fragmentation. LDL contain well defined amounts of antioxidants, and their oxidative modification is the result of a delicate balance between oxidative attack and efficacy of the antioxidant mechanisms. It was commonly assumed, until recently, that ascorbic acid and a-tocopherol were the first antioxidants to intervene and to be consumed while exerting their antioxidant action, followed by lycopene and by β-carotene. Reduced Coenzyme Q_{10} is oxidized before Vitamin E, and the concentration of hydroperoxides of phospholipids and of cholesterol begins to increase once available reduced Coenzyme Q_{10} has been consumed, when Vitamin E is still present at concentrations very similar to the initial ones [61].

It is currently believed that high levels of LDL, as well as smoking and hypertension, are primary risk factors, among those contributing to cardiovascular disease. Biochemical mechanisms responsible for the atherogenicity of LDL have been extensively addressed, and experimental evidence bas been produced indicating that oxidatively modified LDL become atherogenic. It was found that endothelial cells are involved in the oxidative attack against LDL [8], as described above. Oxidative attack on LDL deeply affects the apoprotein moiety as well. As a consequence of these changes LDL are no longer "recognized" by the normal receptors, and are taken up more readily by the scavenger receptors of macrophages. LDL leave the blood stream, penetrate the endothelial cell lining and reach the subendothelial space, where they undergo oxidative attack by the endothelial cells. Oxidatively modified LDL are capable of triggering further events, including platelet activation, and exert a chemotactic attraction on circulating monocytes, which migrate to the subendothelial space, where they become macrophages. These cells have only low levels of the classical LDL receptor, nonetheless they are able to take up more rapidly oxidatively modified LDL, and this uptake involves a different receptor, called the "scavenger receptor". As discussed above, oxidatively modified LDL are easily recognized by the scavenger receptors. These events lead to an accumulation of lipids, mainly cholesterol and cholesterol esters, in the macrophages, which will become lipid-laden foam cells. Foam cells may be considered the essence of the atheromatous lesions. A crucial point of the sequence of events we just described is the oxidative modification of LDL in the subendothelial space, and many research efforts are directed towards elucidation of this process. It is likely that LDL, even when minimally modified by the oxidative attack, exert a potent chemotactic attraction on monocytes; furthermore, oxidatively modified LDL are also capable of activating platelets. The presence of clefts on the atheromatous plaque might favor the activation of platelets by the oxidatively modified LDL, so contributing to the thrombotic phenomenon [52]. The role of the endothelium and of its interaction with LDL in determining the sequence of events leading

to the development of atherosclerosis is also connected with the dynamics of the release and of nitric oxide (NO). The enzyme nitric oxide synthase present in the endothelium, using L-arginine as substrate, catalyzes the synthesis of this important vasodilating factor. It was initially named EDRF (endothelium-derived relaxing factor) but soon its identity with nitric oxide became evident, hence the EDNO denomination (endothelium-derived nitric oxide). EDNO inhibits platelet aggregation and induces vasodilatation through stimulation of guanylate cyclase. It is quite evident that release of nitric oxide by the endothelium constitutes an important mechanism in the regulation of vascular tone, including coronary tone regulation. Furthermore, the alteration of coronary responsiveness to a vasodilatory stimulus, as the one represented by acethylcholine, is commonly interpreted as an early symptom of endothelial dysfunction and impairment of the EDNO system preceding the angiografically evident atherosclerosis. A research line primarily stemming from the observation that the vasodilatory mechanism is deficient in the hypercholesterolemic animal has led to the conclusion that oxidatively modified LDL inhibit the mechanism of NO dependent vasodilatation. It is no clear yet whether this inhibitory effect of oxidized LDL is mainly due to inhibition of nitric oxide release or to its inactivation. On the basis of these data it is quite evident that a relationship exists between plasma levels of LDL, their peroxidability, the efficiency of vascular dilative response and the extent of atherosclerotic lesions [52]. Peroxidability of LDL is often expressed in terms of the lag time necessary to induce the rapid phase of peroxidation when LDL are exposed in vitro to a certain oxidative stress: LDL become more susceptible to lipid peroxidation as this time shortens. In this respect a correlation has been found between peroxidability and the extent of coronary atherosclerosis [51]. Several epidemiological surveys are currently conducted in order to investigate the relationship between alimentary habits, dietary availability of antioxidants and incidence of cardiovascular disease in the populations of North and Central Europe compared to those living in the Mediterranean area. We hypothesize that plasma and LDL content of Coenzyme Q_{10} may also play a role in this issue.

16.6 CoQ_{10} and Prevention of Atherogenesis

Based on the above information it appears that lipid oxidation is critical in the development of atherosclerosis; CoQ_{10} supplementation might therefore prevent atherogenesis by counteracting lipoprotein oxidation in the vessel wall. Two different research groups verified this hypothesis. Stocker et al. [70] published the results of a study conducted on the apo lipoprotein E-knockout mice (ApoE-/-). These mice were fed with a diet containing 1.2% (w/w) fat and 0.15% (w/w) cholesterol supplemented with CoQ_{10}, at the concentration of 1% or 0.5%. A group of animals received CoQ_{10} and vitamin E at the same time. After 24 weeks atherosclerosis was assessed by morphometry at the aortic root, arch, descending thoracic and abdominal aorta. Dietary supplementation with CoQ_{10} and/or vitamin E, either alone or

together, led to an increase in both plasma and aortic content of both antioxidants. Compared to controls, plasma from CoQ_{10} or CoQ_{10} plus vitamin E, but not vitamin E-supplemented animals, was more resistant to ex vivo lipid peroxidation induced by peroxyl radicals. In co-supplemented mice, the tissue concentration of lipid hydoperoxides was decreased. Above all, CoQ_{10} 1% and CoQ_{10} 0.5% plus vitamin E significantly decreased the extent of atherosclerosis at all sites examined. CoQ_{10} (0.5%) alone significantly decreased atherosclerosis at the aortic root and arch, but not the descending thoracic aorta, whereas vitamin E (0.2%) alone was antiatherogenic in the aortic root only.

R.B. Singh [57] also reported the results of a similar study conducted in a model of an experimental atherosclerosis in the rabbit. In a randomized, single blind controlled trial, the effect of the administration of CoQ_{10} (3 mg/kg/day) and placebo were compared over 24 weeks on development of atherosclerosis. Both groups of rabbits received a transfatty acid (TFA) rich diet 5–8 g/day for 36 weeks after which the rabbits were examined. Rabbits' diet was oxidised with vitamin C plus iron. Treatment with CoQ_{10} administered after 12 weeks of feeding with the TFA rich diet was associated with a significant decline in TBARs, conjugated dienes and an increase in plasma levels of vitamin E in the CoQ group compared to the placebo group. These changes are clearly indicators of oxidative stress. The treatment with coenzyme Q led to significantly smaller artery plaque sizes. Aortic cholesterol and aortic triglycerides were significantly lower on the treated group whereas aortic vitamin E was significantly higher. The total extent of atherosclerotic involvement of coronary arteries was assessed by the Coronary Atherosclerosis Index. The frequencies in atherosclerotic plaque in different arteries were therefore calculated. Aortic plaque frequency, aortic plaque ulceration, coronary plaque ulceration, aortic plaque thrombosis, coronary plaque thrombosis or hemorrhage were significantly lower in the CoQ group than in the placebo group indicating that CoQ_{10} treatment can improve the quality of the atherosclerotic plaque.

16.7 CoQ_{10} and Inhibitors of HMG–CoA Reductase

HMG–CoA reductase inhibitors, known as statins, represent a potent therapy in the anticholesterol strategy. Cholesterol lowering therapy plays an essential, well recognised role in the secondary prophylaxys of coronary heart disease, as shown for instance by the Scandinavian Simvastatin Survival Study [4S] [63]. As CoQ_{10} and cholesterol share the same biosynthetic pathway it is reasonable to hypothesize that statin therapy may also lead to decreased levels of CoQ_{10}. In fact studies by Folkers et al. reported decreased levels of coenzyme Q, during statin administration, both in rats and in humans [18, 69]. In 1993 we reported the results of the first double blind study showing that both simvastatin and pravastatin produce a decrease in CoQ_{10} plasma levels similar to that of cholesterol [21]. Other studies confirmed those data [5, 11, 12, 24, 26, 45, 50]; a dose-related decrease of serum CoQ_{10} during treatment with statins was reported by Mortensen et al. and that paper also represents a review

of the previous studies [44]. On the whole statins are powerful, effective drugs, usually safe, with an apparently low frequency of side effects but which in some cases have been found to be very serious. In August 2001, a clear relationship between the use of cerivastatin (Baycol) and rabdomiolysis with consequent death of some patients was established. Side effects of statins include muscle weakness, pain, and elevated plasma levels of CK and/or transaminases and these symptoms could, at least in part, be related to a certain degree of CoQ_{10} deficiency. Several years ago we published the results of a study [35] where we monitored CoQ_{10} content and peroxidizability of LDL isolated from patients treated with statins. The decrease of CoQ_{10} was usually parallel to that of cholesterol, and susceptibility of LDL to peroxidation did not change. Only in one case, where CoQ_{10}/cholesterol ratio became lower, did those lipoproteins become more peroxidizable. We can reasonably hypothesize that, in some conditions, where other CoQ_{10}-impoverishing causes exist; treatment with HMG–CoA reductase inhibitors may seriously impair levels of CoQ_{10} in plasma, and possibly in tissues. A physiological decline in CoQ_{10} has for instance been implicated in aging [27, 58], which would make the elderly more susceptible to statin-induced CoQ_{10} depletion. Folkers et al. reported, in a limited number of patients who had been on CoQ_{10} therapy for congestive heart failure, a sudden deterioration of their cardiac function when statins were added to the therapy [18]. This worsening was overcome by increasing their daily dosage of CoQ_{10}. Besides the plasma CoQ_{10} lowering effects, animal studies have also demonstrated tissue depletion in the course of statin treatment [36, 56, 69]. A decrease in tissue coenzyme Q_{10} during statin therapy may have adverse effects on cellular ATP production, as has been proven in dogs and guinea pigs [13, 56]. In particular it was shown [56] that simvastatin treatment leads to a significant decrease in the myocardial level of CoQ_{10} and to an impairment of oxidative phosphorylation in the ischemic dog hearts, and that in guinea pigs [13] lovastatin is able to decrease the cardiac levels of CoQ_{10} and to hamper oxidative phosphorylation, only in the aged animals.

Based on the role of CoQ_{10} in mitochondrial bioenergetics a reduction in the amount of CoQ_{10} in the body may result in hampered mitochondrial function. Some studies have indirectly addressed this issue. In a study by De Pinieux et al. [12] patients receiving statin therapy showed a significantly lowered serum CoQ_{10} concentration and a higher blood lactate/pyruvate ratio (a widely used test for mitochondrial dysfunction), compared to patients not treated with statins. There are only a few studies on the effect of statin treatment, in humans on muscle CoQ_{10} concentrations. In an early study by Laaksonen et al. [30] decreases in serum ubiquinone concentrations in the course of simvastatin treatment did not result in CoQ10 reduced levels in muscle tissue. Those patients took 20 mg simvastatin per day for 4 weeks. In a recent publication the same authors [48] reported the results of a study designed to assess the effect of high dose statin treatment on cholesterol and ubiquinone metabolism and mitochondrial function in human skeletal muscle. Forty-eight hypercholesterolemic patients were randomly assigned to receive 80 mg/day of simvastatin, atorvastatin or placebo for 8 weeks. Plasma and muscle biopsy samples were obtained at baseline and at the end of follow-up. Muscle ubiquinone

concentration was reduced significantly in the simvastatin group. Respiratory chain enzyme activities were assessed in six patients taking simvastatin who underwent markedly reduced muscle CoQ_{10} levels and in matched subjects selected from the atorvastatin and placebo groups. Citrate synthase was also decreased in mitochondria from those six patients, so it is possible that a reduction in mitochondrial number explains the decrease in both muscle ubiquinone and mitochondrial enzyme activity.

CoQ_{10} deficiency may manifest in humans as systolic and diastolic left ventricular dysfunction with symptoms of fatigue and exertional dyspnea. Although statin therapy has been shown to have benefits, the long-term response in ischemic heart disease may have been blunted due to the CoQ_{10} depleting effect. Besides the results reported in the above mentioned paper by Folkers et al. [18] further evidence of this was also shown in diabetic patients, receiving HMG–CoA reductase inhibitors, whose cardiothoracic ratio decreased upon CoQ_{10} treatment [45]. Indications of possible CoQ_{10} deficiency in myopathy in the course of lovastatin treatment had appeared as early as 1989 [68].

It is also possible that the side effects result from a certain deficiency of CoQ_{10}, which is usually well tolerated, together with some other mechanisms of toxicity. Dosage of statins may also be important: in fact it was found that a 12-week lipid-lowering treatment with a daily dosage of 20 mg simvastatin did not negatively alter left ventricular function during exercise [11].

From a practical point of view, characterizing the CoQ_{10} status in patients undergoing statin therapy and treating them with CoQ_{10} could make the use of statins even more effective and safe.

16.8 Protective Effects on DNA Oxidation

Several years ago we also investigated the role of coenzyme Q_{10} in the prevention of DNA oxidative damage. A first experiment was conducted on freshly isolated human blood lymphocytes, pre-incubated in vitro with liposomes loaded with ubiquinone-10 or ubiquinol-10 [66]. Exposure of control lymphocytes to 100 μM H_2O_2 resulted in a detectable increase in DNA strand breaks, measured by the comet assay technique. DNA strand breaks occurred after a significant decrease in the cellular content of ubiquinone-10. Incubation of cells with ubiquinol-10 enriched liposomes increased the total cellular CoQ_{10} from 14.9 ± 1.8 to 61.0 ± 5.5 pmol/10^6 cells. The proportion of CoQ_{10} present in the reduced form also increased. Exposure of ubiquinol-10 supplemented lymphocytes to 100 μM H_2O_2 also resulted in a rapid decrease in cellular content of ubiquinol-10. Despite this loss, however, a relatively large proportion of the cell's CoQ_{10} remained in the reduced, antioxidant-active form. Enrichment with ubiquinol-10 protected lymphocytes from H_2O_2-induced DNA strand breaks. We hypothesized that in such a system ubiquinol-10 acts as an early target for H_2O_2 derived antioxidants: this finding is similar to the situation in LDL, where ubiquinol-10 is the first lipid soluble antioxidant to be consumed in the early stages of lipid peroxidation [61]. Lymphocytes incubated

with liposomes enriched with the oxidized form of CoQ_{10} (ubiquinone-10) increased their content of total CoQ_{10}, but only about 8% was present in the reduced form. Surprisingly however, these ubiquinone-10 enriched cells were also more resistant to H_2O_2 induced DNA damage and loss in viability when compared with control cells.

In a subsequent study, in vivo supplementation with CoQ_{10} was shown to enhance the recovery of human lymphocytes from oxidative DNA damage [67]. The oral intake of 100 or 300 mg/day of CoQ_{10} for 2 consecutive weeks increased the endogenous CoQ_{10} cellular content by 45% and 144% respectively. DNA of CoQ_{10} enriched lymphocytes resulted less damaged by the exposure to oxygen and the extent of DNA strand breaks formation inversely related to the concentration of CoQ_{10} in plasma and cells. The activity of DNA repair enzymes was also assessed, by incubating lymphocyte extracts, containing DNA repair enzymes, with a substrate consisting of oxidized purine basis previously exposed to Ro-19–8022: upon irradiation with a visible light source Ro-19–8022 produces singlet oxygen. DNA repair enzymes introduce breaks at sites of oxidized purines and enzyme activity is reflected by the number of DNA breaks. CoQ_{10} supplementation enhanced DNA repair activity, which was markedly higher in cellular extracts from CoQ_{10}-enriched lymphocytes, 148 ± 25 arbitrary units (au.) versus 55 ± 15 au. and 94 ± 21 au., $P < 0.05$, respectively. The effects of CoQ_{10} might be related to its protective effect against oxidation and a stimulation of the activity of repair enzymes. Changes in the redox state of the transcriptional factors have been proposed as a mechanism regulating gene expression. In a recent study [23] we showed that in a human cell line incubated in vitro with CoQ_{10} there was an increased expression of several hundred genes, involved in cell signalling, metabolism and transport.

We are currently evaluating the results of a clinical trial conducted in patients affected by Down syndrome treated with CoQ_{10} The aim of this study is to measure the extent of DNA damage, and the effect of CoQ_{10} administration, by means of a new, optimized, single cell gel electrophoresis technique [64].

16.9 CoQ_{10} and Ischemic Heart Disease: Bioenergetic Effect or Improvement of Endothelial Function?

We also recently investigated whether in patients with stable moderate congestive heart failure (CHF), oral CoQ_{10} supplementation could ameliorate endothelial dysfunction and functional impairment of the cardiac muscle [7]. Endothelial dysfunction was evaluated by assessment of the brachial artery vasomotor function (flow-mediated dilation, FMD). FMD was measured after release of a sphygmomanometer inflated at 240 mmHg for 4.5 min at the wrist. The sheer stress resulting from readmission of blood flow activates endothelial NO. synthase and the release of nitric oxide induces a vasodilatation measurable by an ultrasound technique. In the same patients maximal oxygen uptake (peak VO_2) was assessed by cardiopulmonary exercise test and myocardial contractility by the sensitive dobutamine stress echocardiography test. Oral CoQ_{10} supplementation significantly

improved the endothelium-dependent relaxation of the brachial artery, left ventricle contractility and peak VO_2.

The result of this study could be interpreted in the light of the bioenergetic, as well as the antioxidant properties of coenzyme Q. We found a significant improvement in left ventricle contractility in dysfunctional segments located in non-infarcted areas served by stenotic arteries, where hibernation and/or chronic stunning is likely to occur. The amelioration of contractile function after CoQ_{10} suggests that chronic post-ischemic stunned cells improve or normalize their metabolism and function. Animal studies had suggested that CoQ_{10} protects stunned myocardium in an open-chest swine model [2]. Myocardial stunning is clearly related to ischemia-reperfusion damage. ATP is depleted during ischemia and experimental evidence indicates increased oxidative stress in stunned myocardium. Ex vivo work in a rabbit heart model of ischemia and reperfusion showed a relative maintenance of tissue stores of ATP, a relative preservation of ATP-generating capacity of mitochondria and a relative absence of calcium overload in CoQ_{10}-pretreated rabbits [47]. We might reasonably hypothesize that in our clinical study administration of 300 mg of CoQ_{10} per day was capable of increasing, even slightly, myocardial ATP content, therefore improving electron transport rate and ATP production. Although the increase in tissue CoQ_{10} content upon oral administration is generally lower then the remarkable elevations in plasma, since the mitochondrial membrane concentration of CoQ_{10} is probably close to the Km value for the NADH oxidation systems, even a small increase could generate an increase in respiratory rate [17], especially in a myocardium with a certain degree of CoQ_{10} deficiency. In a study conducted by Sohal and coworkers [29], CoQ supplementation resulted in an increase in total CoQ content in the heart and the skeletal muscle homogenates. After 4 weeks of supplementation, total CoQ content showed a significant increase in homogenates of heart and skeletal muscle (23% and 45%, respectively; $p < 0.05$). In a more recent paper the same group showed that supplementation of mice with CoQ_{10} significantly increased CoQ_9 and CoQ_{10} content in homogenates and mitochondria of liver, heart, kidney, skeletal muscle and brain [59]. This issue was also addressed by Rosenfeldt et al. in rats and in human myocardial tissue [54]. Hearts isolated from senescent (35 months) rats were tested in vitro in a model where they were subjected to rapid electrical pacing. After pacing, the senescent hearts compared to young, showed reduced recovery of prestress work performance. CoQ_{10} pretreatment of rats from which the senescent hearts were isolated improved the heart recovery of the aged myocardial muscle. In another experiment [54] the same authors showed that trabeculae isolated, during heart surgery, from older patients (>70 years of age) showed reduced recovery of developed force after simulated ischemia compared to younger counterparts (<70 years). CoQ_{10} content was decreased in the myocardium from aged patients. A few years later the study was extended to test the effect of CoQ_{10} therapy before cardiac surgery on mitochondrial function [55]. Patients undergoing elective cardiac surgery were randomized to receive oral CoQ_{10} (300 mg/die) or placebo for 2 weeks preoperatively. Trabeculae from right atrial appendages were excised and mitochondria isolated and studied.

Patients treated with CoQ_{10} had increased ubiquinone levels in isolated mitochondria; mitochondrial respiration (ADP/O ratio) was more efficient and malondialdehyde content was lower in the specimen from CoQ_{10} treated patients. Myocardial tolerance to oxidative stress was also improved.

Other literature indicates that the administration of CoQ_{10} increase cellular ATP production. In a study conducted by Marriage et al., 12 patients with proven defects of oxidative phosphorylation were treated with a combination of CoQ_{10} (5 mg/kg body weight), carnitine, vitamin B complex, vitamin C and vitamin K [38]. At baseline all 12 patients had decreased ATP synthesis in lymphocytes, with all substrates tested, when compared to control subjects. Overall there was a 34% reduction in ATP synthesis when no substrate was added; after 12 months of treatment the ATP synthesis reflecting the oxidation of endogenous substrate was only reduced by 12% relative to mean control values. In order to identify the potential active ingredient(s) in the cofactor mixture, the authors analysed the ability of the individual compounds to influence ATP synthesis rates using lymphocytes from control subjects. Only CoQ_{10} supplementation of control lymphocytes could lead to an increase in ATP synthesis. Interestingly also a marked increase in non mitochondrial ATP synthesis was observed, which is compatible with additional functions of CoQ_{10} as a component of extra mitochondrial redox reduction. Some patients in this study showed an increase in energy levels and improvement in exercise tolerance.

16.10 CoQ_{10} an Extracellular SOD

Extracellular superoxide dismutase (ecSOD) is a particular SOD which plays an important role in protecting the endothelium from oxidative stress; it can be assayed also in plasma, where it is released from the extracellular matrix by means of a functional test. Vascular ecSOD activity is substantially reduced in patients with coronary artery disease. In a recent study conducted in our laboratory [65]. Thirty-eight patients affected by ischemic heart disease were randomized into two groups: one group received CoQ_{10} orally at a dose of 300 mg/day, for 1 month, while the other group received placebo. ecSOD, endothelium dependent relaxation as well as peak VO_2 increases in the CoQ_{10} treated group were statistically higher versus the variations in the placebo group. In particular improvements elicited by CoQ_{10} supplementation were remarkable in subjects presenting low initial endothelium bound ecSOD and thus more prone to oxidative stress. Improvements in the ED relaxation and endothelium-bound ecSOD activity might be related to CoQ_{10} capability of enhancing endothelial functionality by counteracting Nitric Oxide (NO) oxidation. Enhancement of peak VO_2 is likely due to the bioenergetic effect of CoQ_{10} also on the basis of the observed improvement in O_2 pulse. More NO available could also lead to increased ecSOD gene-expression [19], which could in turn contribute to preserve NO from inactivation by O_2.

16.11 Implications of Coenzyme Q_{10} in Male Infertility

A growing body of evidence indicates that damage inflicted to spermatozoa by reactive oxygen species (ROS) plays a key pathogenetic role, implicating oxidative stress as a mediator of sperm dysfunction in the aetiology of male infertility. To counteract the potential hazard effects of oxidative stress, spermatozoa and seminal plasma are endowed with several protective antioxidant systems. Some studies have shown that infertile men have an impaired seminal plasma nonenzymatic antioxidant capacity, suggesting that decreased total antioxidant capacity may exert a pathogenetic role in male infertility. The first determination of endogenous CoQ_{10} levels in seminal fluid was performed by our group [37]. Several years later we showed [1] a significant correlation between the reduced form of coenzyme Q_{10} and sperm count in seminal plasma and an inverse correlation between ubiquinol and hydroperoxide levels, both in seminal plasma and seminal fluid. We also found an inverse correlation between ubiquinol/ubiquinone ratio and the percentage of abnormal sperm cells. Moreover a lower ubiquinol/ubiquinone ratio was shown in sperm cells from idiopathic asthenozoospermic patients (IDA) and in seminal plasma from IDA and varicocele-associated asthenozoospermic patients compared to controls. The above-mentioned studies constitute a rationale which eventually led us to treat infertile subjects with exogenous CoQ_{10}. The effect of CoQ_{10} on sperm motility in vitro had previously been reported by Lewin and Lavon [32].

In an attempt to elucidate a potential therapeutic role, we administered CoQ_{10} to a group of idiopathic asthenozoospermic infertile patients [4]. Twenty-two patients (mean age: 31 years, range: 25–39 years) affected by idiopathic asthenozoospermia, were enrolled in the study. All subjects presented a clinical history of primary infertility of at least 3 years. No female related factor was apparently involved in sterility. Eligible patients had sperm count $>20 \times 10^6$ /ml, sperm motility (forward motility, classes a and b, according to WHO 1999 criteria) <50% at two distinct sperm analyses and normal sperm morphology >30%.

The enrolled patients were administered CoQ_{10} 200 mg/day divided into two doses, for 6 months. Semen analysis, including computer-assisted sperm analysis and motility (C.A.S.A.), CoQ_{10} and phosphatidylcholine assays, were performed at baseline and after 6 months of therapy. A semen analysis was further performed after 6 months from interruption of therapy (wash-out). The results showed an increase of CoQ_{10} levels in seminal plasma after treatment, the mean value rising significantly from 42.0 ± 5.1 at baseline to 127.1 ± 1.9 ng/ml after 6 months of exogenous CoQ_{10} administration ($p < 0.005$). A significant increase of CoQ_{10} content was also detected in sperm cells (from 3.1 ± 0.4 to 6.5 ± 0.3 ng/10^6 cells; $p < 0.05$). As far as semen features are concerned, a significant difference was found in forward (class a + b) motility of sperm cells after 6 months of CoQ_{10} dietary implementation.

A significant increase of curvilinear velocity VCL (from 26.31 ± 1.50 to 46.43 ± 2.28 µm/sec, $p < 0.05$), and straight progressive velocity VSL (from 15.20 ± 1.30

to 20.40 ± 2.17 µm/sec, $p < 0.05$) was found after treatment. No significant differences were found in sperm cells concentration and morphology. Interestingly, although a direct correlation was not found (data not shown), a positive dependence (using the Cramer's index of association) was evident among the relative variations, baseline and after treatment, of seminal plasma or intracellular CoQ_{10} content and of C.A.S.A. (VCL and VSL) kinetic parameters (Cramer's V = 0.4637; 0.3818; 0.3467; 0.5148, respectively). Sperm forward motility was significant reduced after 6 months of wash-out (from 16.34 ± 3.43 to 9.50 ± 2.28%, $p < 0.001$), while no significant differences were found in sperm cells concentration and morphology. The wives of 3 out of 22 patients (13.6%) achieved spontaneous pregnancy within 3 months from the discontinuation of therapy (2.4% pregnancy rate per cycle). The data of the present study show a significant improvement of kinetic features of sperm cells after 6 months of administration of CoQ_{10}, both on the basis of manual and computer-assisted evaluation. Furthermore, these results constitute the first demonstration that exogenous administration of CoQ_{10} increases its levels in seminal plasma and in spermatozoa.

The increment was relevant, especially in seminal plasma where posttreatment levels were three times higher than basal ones. Similar increases of CoQ_{10} concentration (two–three times higher than baseline value) are commonly found in blood plasma after chronic administration of the quinone. As CoQ_{10} is a highly lipophylic molecule, we could reasonably hypothesize that it can diffuse through the phospholipid bilayer of cellular membranes, but we presently do not know whether transport from blood plasma to testicular and accessory male genital glands is a passive one or involves an active mechanism. Statistical analysis did not reveal any significant functional relationship among the therapy-induced variations of CoQ_{10} and kinetic parameters of spermatozoa, probably due to the low number of samples. Nevertheless, the good degree of association among these variables, according to Cramer's V index of association, supports the hypothesis of a pathogenetic role of CoQ_{10} in asthenozoospermia according to previously reported data [3]. The improvement of spontaneous pregnancy rate also suggests a benefit of this therapeutic approach.

16.12 Conclusions

The studies reported in this chapter are not intended to extensively review the biomedical implications of CoQ_{10}. The main purpose was to highlight some old and new studies which give insight into the mechanisms responsible for the biomedical effects of CoQ_{10}. It is possible that the bioenergetic effects of CoQ_{10} are closely linked to its antioxidant properties and its newly discovered role in gene expression might also be related to the redox activity of coenzyme Q. Future research will further investigate many of the unsolved issues.

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Chapter 17
Coenzyme Q_{10} Supplementation in Clinical Medicine

Anna Gvozdjáková

Introduction

Coenzyme Q_{10} supplementary therapy in patients is based on its antioxidant and re-energization effects. CoQ_{10} has been successfully used in clinical medicine as a supplementary therapy in several diseases: in patients with mitochondrial neuromuscular diseases, neurodegenerative diseases (Parkinson's, dementias), cardiovascular diseases (congestive heart failure, cardiomyopathy), statins therapy, chronic fatique syndrom, nephropathy, cancer – breast treatment, chemotheraphy amelioration, diabetes mellitus, bronchial asthma, infertile men, sports medicine, stress and ageing. Bioavailability, dosage and duration of CoQ_{10} treatment are important factors for human tissue uptake and beneficial effect of administered CoQ_{10}.

17.1 Coenzyme Q_{10} Supplementation in Children with Metabolic Diseases

Anna Hlavatá, Jarmila Kucharská, and Anna Gvozdjáková

Abstract Supplementary coenzyme Q_{10} therapy in children with metabolic diseases participates in improvement of clinical and metabolic parameters.

Keywords Children with metabolic diseases, coenzyme Q_{10}, placenta, supplementary therapy

The physiological development of a fetus is dependent mainly on genetic disposition and physiologic function of the placenta. Metabolic disorders in the placenta, increased free oxygen radical production, decreased antioxidant function, as well as mitochondrial metabolic disorders are involved in the development of diseases in children. Environmental conditions (in industrial regions) decreased CoQ_{10} and vitamin E concentrations in human placentas (43 placentas) in comparison with placentas from

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nonindustrial regions (40 placentas). Disturbances of the antioxidant status in the human placenta can affect the genesis and contribute to the rejection of fetus development during pregnancy and the development of metabolic disorders in children.

Based on decreased CoQ_{10} plasma and blood concentrations in 44 children with metabolic disorders, hydrosoluble CoQ_{10} (30–90 mg CoQ_{10} daily) with vitamin E and vitamin C were supplemented to children with myopathies, encephalomyopathies, other neuromuscular diseases, and failure to thrive without gastrointestinal cause. The age of the children ranged from 1 to 17 years. Lactate dehydrogenase, creatine kinase and peroxidation of lipids decreased to normal values, pathological dynamic tests and bicycle ergometry improved. During more than 1 year of supplementary therapy, clinical manifestations of the diseases did not appear. The therapy was well tolerated, no adverse effects occurred. The best results were in children with myopathy. Based on our results, supplementary therapy with hydrosoluble CoQ_{10}, vitamin E and vitamin C was recommended to children with metabolic diseases [1]. (See Chapter 5.)

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17.2 Coenzyme Q_{10} Supplementation in Preclinical Study and in Patients with Nephropathies

Anna Gvozdjáková and Jarmila Kucharská

Abstract Improvement of kidney mitochondrial respiratory chain function and ATP production after hydrosoluble coenzyme Q_{10} (CoQ_{10}) supplementation in a preclinical study substantiated the possibility of CoQ_{10} therapy in patients with kidney diseases. Coenzyme Q_{10} supplementary therapy was actually found to improve kidney function in patients with nephropathy.

Keywords Nephropathy, coenzyme Q_{10}, mitochondria, kidney

Oxidative stress and decreased function of defense antioxidant systems participate in the origin and development of nephropathies as well as in their complications. They are most frequently associated with cardiovascular diseases, diabetes mellitus and dyslipidemias. In mitochondrial nephropathies, decreased CoQ_{10} levels were found. Deficit of CoQ_{10} was shown in dialysed patients [7]. Deficiency of CoQ_{10} and oxidative stress belong to the main factors of kidney mitochondrial dysfunction.

17.2.1 Effect of Hydrosoluble Coenzyme Q_{10} (Q®-GEL) on Kidney Mitochondrial Function in Aged Rats (Preclinical Study)

In a preclinical study, stimulation of kidney mitochondrial function with hydrosoluble CoQ_{10} was shown in aged rats [4]. In a 4-week experiment, 19–22-month-old male rats received hydrosoluble CoQ_{10} by gavage in the daily dose of 200 mg CoQ_{10}/kg body weight. Four weeks of hydrosoluble coenzyme Q_{10} supplementation to aged rats stimulated their kidney mitochondrial respiratory chain function and ATP production at Complex I and II. Partial results from this preclinical study opened a way for hydrosoluble coenzyme Q_{10} supplementation in patients with kidney diseases (Table 17.1).

17.2.2 Clinical Study

Deficit of CoQ_{10} and increased lipid peroxidation were reported in patients with kidney diseases [1, 3, 6]. Fifteen patients with primary nephropathies were involved in the study: 10 patients with chronic tubulointerstitial nephritis, 5 with renal

Table 17.1 Effect of hydrosoluble coenzyme Q_{10} (Q-GEL) on mitochondrial function in aged rats

Groups	Control (mean ± SEM)	Q_{10} (mean ± SEM)	Statistics
Kidney mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	1.495 ± 0.040	1.987 ± 0.216	$p < 0.075^{ms}$
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.245 ± 0.007	0.314 ± 0.050	NS
Complex I.			
S_3 (nAtO/mg prot./min)	36.72 ± 6.13	57.91 ± 5.49	$p < 0.0422$*
S_4 (nAtO/mg prot./min)	15.87 ± 1.93	24.02 ± 3.78	$p < 0.0662^{ms}$
OPR (nmol ATP/mg prot./min)	73.80 ± 15.30	127.60 ± 6.70	$p < 0.0268$*
Complex II.			
S_3 (nAtO/mg prot./min)	155.90 ± 21.90	237.90 ± 4.70	$p < 0.0044$**
S_4 (nAtO/mg prot./min)	123.20 ± 12.30	110.00 ± 11.90	NS
OPR (nmol ATP/mg prot./min)	201.80± 3.70	252.10 ± 11.20	NS

Statistical significance: ms – marginally significant; *significant, **very significant

tubular acidosis. Mean age of the patients was 61 years. Nine patients were treated also for cardiovascular diseases, three patients were on statin therapy.

Effect of supplementary antioxidant therapy during 4 weeks and 3 months: The patients received 100 mg vitamin E, 100 mg vitamin C a 30 mg riboflavin daily during 4 weeks. During the following 8 weeks, hydrosoluble CoQ_{10} was added in the daily dose of 240 mg, divided into 3 daily doses with meals.

After 12 weeks of supplementary antioxidant therapy and 8 weeks of antioxidant therapy with CoQ_{10}, decreased functional excretion of alpha-amino nitrogen and increased ammonia elimination in urine was observed. Parameters of kidney function and spectrum lipids did not significantly change during the study.

Baseline blood and plasma CoQ_{10} concentrations were low, only at 25% of reference levels, lipid peroxidation was significantly increased in comparison with healthy subjects. After 8 weeks of CoQ_{10} supplementation the plasma and blood level increased 18 times and 16 times, respectively.

Hydrosoluble CoQ_{10} (Q-GEL) supplementation resulted in improved kidney function (increased ammonia elimination in urine, significant increase of potassium level in serum, slightly in urine, and decrease of fraction excretion of α-amino nitrogen) [1–3, 5, 6]. Supplementary therapy with CoQ_{10} was well tolerated, no adverse effects occurred. Substitution with CoQ_{10} may represent effective supplementary therapy in addition to the therapy targeted at basic factors of nephropathy progression.(Mitochondrial Nephrology, see Chapter 9.)

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17.3 Coenzyme Q_{10} Supplementation in Patients with Bronchial Asthma

Anna Gvozdjáková

Abstract Bronchial asthma is a chronic inflammatory disease of the respiratory system. It is estimated that there are about 150 million asthmatics in the world, and the mortality rate is approximately 180,000 deaths a year. The inflammatory process in asthma leads to serious disturbances in the dynamic balance of oxidant–antioxidant capacity of the lungs. Oxidative stress due to increased production of reactive oxygen species (ROS) and/or decreased antioxidant fuction contribute to the inflammatory process of bronchial asthma [5]. Long-term administration of corticosteroids has been shown to result in mitochondrial dysfunction and oxidative damage of mitochondrial and nuclear DNA. Decreased plasma and blood CoQ_{10} and alpha-tocopherol levels were reported in patients with corticosteroid-dependent bronchial asthma [1]. An open, crossover, randomized clinical study was performed with 41 bronchial asthma patients, suffering from persistent mild to moderate asthma, clinically stabilized. The patients were divided into two groups. One group was receiving standard antiasthmatic therapy. The second group was in addition given supplementary antioxidant therapy, in the following daily doses: hydrosoluble CoQ_{10} (120 mg CoQ_{10}), 400 mg vitamin E, 250 mg vitamin C. The groups were crossed over at 16 weeks for a total duration of 32 weeks.

The primary benefit of antioxidant supplementation with hydrosoluble CoQ_{10}, vitamin E and vitamin C, was in the reduction of corticosteroid dosage, resulting in lowering the adverse effects of corticosteroid therapy [2– 4]. (Mitochondrial Immunology – see Chapter 12.)

Keywords Bronchial asthma, corticosteroids, hydrosoluble coenzyme Q_{10}, oxidative stress

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17.4 Coenzyme Q_{10} and α-Lipoic Acid Effect in Patients with Diabetic Cardiomyopathy

Anna Gvozdjáková, Patrik Palacka, Jarmila Kucharská, and Ján Murín

Abstract Chronic supplementary simultaneous treatment with coenzyme Q_{10} (CoQ_{10}), alpha-lipoic acid (ALA) and alpha-tocopherol in patients with diabetic cardiomyopathy significantly decreased HbA_1c levels and oxidative stress, it also improved antioxidant protective effect, echocardiographic parameters and myocardium function, without side effects. On the basis of this study CoQ_{10} and ALA treatment could be recommended in patients with diabetic cardiomyopathy as a supplementary therapy.

Keywords Diabetic patients, coenzyme Q_{10}, alpha-lipoic acid, cardiomyopathy

Diabetic cardiomyopathy (DCMP) is a chronic complication of diabetes mellitus (DM). Oxidative stress, decreasing antioxidant activity and energy production in heart are participating in its pathogenesis. Antioxidant effects of coenzyme Q_{10} (CoQ_{10}), (a key component of mitochondrial respiratory chain) and alpha-lipoic acid (α-lipoic acid – ALA) were proved previously in several studies.

17.4.1 Alpha-Lipoic Acid (α-Lipoic Acid, ALA)

ALA was isolated from bovine livers in 1951, also called the thioctic acid. ALA is fatty acid with 8 carbon and two sulfur atoms. It chemically name is 1, 2-dithiolane-3-pentanoic acid. ALA exists in two optical isomers R-(+). Mixture of both optical isomers is called racemic ALA.

17.4.1.1 α-Lipoic Acid Functions

Its function as antioxidant: scavenges ROS such as superoxide radical, hydroxyl radical, peroxyl radical, singlet oxygen and hydrochlorous acid, can remove heavy metals by chelation, regenerates other antioxidants like glutathione, vitamin C, coenzyme Q_{10} and indirectly vitamin E, protects membranes [1, 4]. Reduced form ALA – dihydrolipoate may exert pro-oxidant actions through reduction of iron. Lipoate function is also as a redox regulator of proteins such as myoglobin, prolactin, thioredoxin and NF-kappa B transcription factor [5]. ALA is a part of the pyruvate dehydrogenase complex, located in mitochondrial matrix. It acts as a cofactor of

several enzymatic reactions involved in the pyruvate decarboxylation, the citric acid cycle, and the degradation and biosynthesis of various amino acid.

ALA is water-soluble and is well absorbed from the diet through the stomach and intestines. Its concentrations is various, was found in all muscles and internal organs. Lipoic acid was found in foods, as kidney, heart and liver meats as well as in brocolli, spinach and potatoes [1, 4]. ALA with low molecular weight is absorbed from the diet and crosses the blood-brain barriers. ALA is taken up and reduced in cells and tissues to dihydrolipoic acid, which is also exported to the extracellular space.

17.4.1.2 α-Lipoic Acid Supplementation

ALA administration has been shown to be beneficial in a oxidative stress, ischemia-reperfusion injury, diabetes, cataract formation, neurodegeneration [5]. In patients with diabetes mellitus are depleted levels ALA and neuropathies in diabetes can resulted from this deficit. Beneficial effect of ALA was proved in studies, called ALADIN I, II, III (Alpha-Lipoic Acid in Diabetic Neuropathy), improved cardiac functions were demonstrated [6–8]. In review of MEDLINE publication, from 1996–2005, author offer hope that α-lipoic acid may be a safe and effective therapeutic option for the treatment of patients with peripheral diabetic neuropathy [2].

17.4.2 Simultaneous Effect of CoQ_{10} and ALA in Diabetic Patients

In our original study was evaluated a chronic simultaneous effect of CoQ_{10}, ALA and vitamin E on selected echocardiographic parameters, oxidative stress and antioxidants in plasma of patients with DCMP [3].

Patients and methods: 19 patients with DCMP were included in the study: (12 men, average age 61.6 ± 9.0 and 7 women 63.3 ± 9.0 years) with DM 2.type and DCMP, overweight (BMI 29.4 ± 6.3), average blood sugar values 7.62 ± 1.06 mmol/l, less-controlled hypertension (systolic blood pressure 140.5 ± 6.5 torr and diastolic blood pressure 85.5 ± 14.1 torr) and cardiothoratic index values 0.46 ± 0.05, treated by insulin (n = 10) and/or perorally antidiabetic drugs (n = 12).

Supplementary therapy was given in two daily doses (60 mg hydrosoluble CoQ_{10}, 100 mg ALA and 200 mg vitamin E) during 3 months. All parameters were statistically evaluated by a pair Student's t-test (before start of study and after 3 months of supplementary therapy): glycaemia, glycosylated haemoglobin (HbA_1c), CoQ_{10}, vitamin E levels, lipids peroxidation (malondialdehyde – MDA) and selected echocardiographic parameters of left ventricular (LV) systolic and diastolic function: EF – ejection fraction, LVESD and LVEDD – endsystolic and

Table 17.2 Simultaneous effect of CoQ_{10} and ALA in diabetic patients

Parameter	Before study	Q_{10}+ALA (3 months)	Statistic
Plasma:			
CoQ_{10} (μmol/l)	0.572 ± 0.05	1.204 ± 0.092	$p < 0.0001$
α-tocopherol (μmol/l)	23.94 ± 2.10	30.91 ± 0.243	$p < 0.0003$
MDA (μmol/l)	5.23 ± 0.21	4.51 ± 0.19	$p < 0.0086$
Echocardiographic parameters:			
EF (%)	56.74 ± 2.05	59.21 ± 1.88	$p < 0.0004$
LVEDD (mm)	56.21 ± 0.87	54.90 ± 0.81	$p < 0.0001$
LVESD (mm)	39.74 ± 1.01	38.47 ± 1.07	$p < 0.0001$
IVS (mm)	12.55 ± 0.30	12.00 ± 0.26	$p < 0.0001$
PW (mm)	11.66 ± 0.29	11.16 ± 0.28	$p < 0.0001$
E/A (ms)	0.66 ± 0.01	0.67 ± 0.02	$p < 0.0007$
DT (ms)	291.80 ± 5.42	284.74 ± 5.36	$p < 0.0001$
HbA_1c (%)	8.778 ± 0.440	8.133 ± 0.414	$p < 0.0005$

enddiastolic LV diameters, IVS – intraventricular septum thickness, PW – LV posterior wall thickness, DT – deceleration time and E/A – early velocity/velocity during atrial systole ratio (Table17.2).

Chronic supplementary simultaneous treatment with CoQ_{10}, ALA and alpha-tocopherol in patients with diabetic cardiomyopathy significantly decreased HbA_1c levels and oxidative stress, it also improved antioxidant protective effect, echocardiographic parameters and myocardium function, without side effects. On the basis of this study CoQ_{10} and ALA treatment could be recommended in patients with diabetic cardiomyopathy as a supplementary therapy. (Mitochondrial diabetology see in Chapter 8).

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Chapter 18
Coenzyme Q_{10} Supplementation in Experimental Medicine

Anna Gvozdjáková

Introduction

Since discovery of coenzyme Q (CoQ) by Dr. Crane in year 1957 many studies with CoQ_{10} in experimental medicine has been published. A great interest has been taken in the past years in studying CoQ_{10} dietary supplementation in relation to its tissues and mitochondria concentration of various organs, as brain, heart, kidney, liver and skeletal muscle.

Beneficial effect of CoQ_{10} supplementation was proved in various experimental models of damaged mitochondrial function. This chapter contribute to the knowledge of CoQ_{10} treatment in three experimental models of damaged mitochondria (Alzheimer's disease – Chapter 18.1, Huntington's disease – Chapter 18.2 and Adjuvant arthritis – Chapter 18.3).

18.1 Coenzyme Q_{10} Supplementation in Mitochondrial Alzheimer's Disease (Experimental Model)

Jaromír Horecký, Ol'ga Vančová, Jarmila Kucharská, and Anna Gvozdjáková

Abstract Original minimally invasive surgical procedure for three-vascular occlusion (3-VO) model of Alzheimer's vascular dementia (AD) in aged rats was developed. The main pathological changes observed following 50-min brain ischemia were characterized by decreased parameters of oxidative phosphorylation in brain and heart mitochondria. Four weeks supplementation of liposoluble CoQ_{10} after cerebral ischemia/reperfusion (I/R) injury, regenerated AD brain and myocardium mitochondrial oxidative phosphorylation function. These results indicate, that CoQ_{10} supplementation may stimulate regeneration of the damaged brain and heart mitochondria in patients with Alzheimer's disease.

Keywords Alzheimer's disease model of 3-VO, brain and heart mitochondria, brain I/R injury, coenzyme Q_{10}

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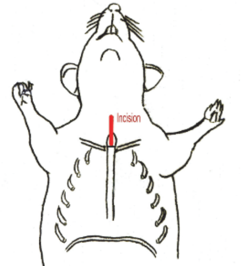
Alzheimer's disease (AD) is an age and brain ischemia-related progressive neurodegenerative disorder characterized by loss of memory, low energy metabolism, oxidative stress and impairment of multiple cognitive functions. Brain amyloid beta proteins (Aβ proteins) can be a trigger in mitochondrial energy impairment via stimulation of excessive ROS production. Mitochondrial dysfunction in neurodegenerative disorder may contribute to the pathogenesis of AD [1, 2]. We supposed, that CoQ_{10} supplementation could improve the function of mitochondrial respiratory chain and oxidative phosphorylation. Our experimental studies supported this assumption.

Experiments were done in 16-months aged Wistar rats. Acute ischemia/reperfusion injury was accomplished by our original surgical procedure for three-vessel occlusion (3-VO) as a vascular model of Alzheimer's dementia and neurodegeneration (Fig. 18.1). Minimally invasive transmanubrial approach was used for occlusion of both, the brachiocephalic trunk (including right common carotid and vertebral artery) as well as left common carotid artery [4]. After 50 min ischemia animals received liposoluble CoQ_{10} by gavage in daily dose 200 mg/kg body weight for 30 days. Brain and heart mitochondria were isolated for coenzyme Q and respiratory chain analyses in the groups as follows: 1. *C* – control group without treatment, 2. *IR* – ischemia/reperfusion group 3. $IR + Q_{10}$ – IR group treated with CoQ_{10} (Table 18.1).

IR induced a significant decrease of brain and heart mitochondrial energy production due to acute oxidative stress. Supplementation of IR group with liposomal CoQ_{10} for 1 month stimulated the activities of Complexes I and II some extent in brain mitochondria of aged rats. Rate of ATP production in Complexes I and II showed a significant increase in myocardial mitochondria in the IR + Q_{10} group. These results show that CoQ_{10} supplementation can stimulate mitochondrial funtion and energy production in both the brain and heart mitochondria in animal models of AD, and therefore these findings are of potential significance in the treatment of AD patients [3, 5].

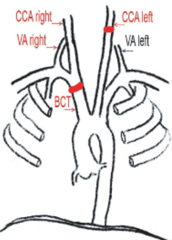
picture A

Incision of the skin an sternal manubrium



picture B

Occlusion of left CCA and brachiocephalic trunk



Abbreviations: VA - vertebral artery, CCA - common carotid artery, BCT - brachiocephalic trunk

Fig. 18.1 3-VO model of rat brain ischemia

Table 18.1 Effect of liposomal coenzyme Q_{10} (Li-Q-Sorb) on mitochondrial function in experimental model of Alzheimer's disease

Groups	Control	IR	IR + Q_{10}
Brain mitochondria			
$CoQ_{9\text{-OX}}$ (nmol/mg prot.)	0.695 ± 0.034	0.709 ± 0.065	0.954 ± 0.075^{+}
$CoQ_{10\text{-OX}}$ (nmol/mg prot.)	0.344 ± 0.026	0.308 ± 0.035	0.334 ± 0.016
Complex I			
S_3 (nAtO/mg prot./min)	62.80 ± 2.53	49.93 ± 5.76	67.21 ± 5.37
S_4 (nAtO/mg prot./min)	19.23 ± 1.75	10.68 ± 1.45**	20.35 ± 1,96^{++}
OPR (nmol ATP/mg prot./min)	201.47 ± 8.42	153.55 ± 17.58*	214.27 ± 20.66^{+}
Complex II			
S_3 (nAtO/mg prot./min)	81.04 ± 3.90	66.24 ± 4.14*	77.93 ± 4.34
S_4 (nAtO/mg prot./min)	46.80 ± 2.36	35.92 ± 2.91*	48.02 ± 3.95^{+}
OPR (nmol ATP/mg prot./min)	142.93 ± 5.97	115.14 ± 8.51*	137.61 ± 10.69
Myocardial mitochondria			
$CoQ_{9\text{-OX}}$ (nmol/mg prot.)	6.756 ± 0.364	6.480 ± 0.410	5.334 ± 0.596
$CoQ_{10\text{-OX}}$ (nmol/mg prot.)	0.940 ± 0.078	0.888 ± 0.041	1.004 ± 0.122
Complex I			
S_3 (nAtO/mg prot./min)	115.87 ± 4.73	78.41 ± 5.44**	87.05 ± 2.31
S_4 (nAtO/mg prot./min)	29.08 ± 1.10	19.74 ± 2.10**	20.02 ± 0.90
OPR (nmol ATP/mg prot./min)	386.96 ± 13.41	260.70 ± 17.35**	317.73 ± 18.22*
Complex II			
S_3 (nAtO/mg prot./min)	174.08 ± 2.64	124.77 ± 7.52**	151.66 ± 4.01^{++}
S_4 (nAtO/mg prot./min)	82.38 ± 1.25	58.98 ± 4.61**	77.42 ± 1.71^{++}
OPR (nmol ATP/mg prot./min)	347.37 ± 11.89	236.32 ±14.03**	299.74 ± 7.92^{++}

Abbreviations: statistically evaluation, Student's t-test
Statistical significance IR vs. control: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$
Statistical significance IR + Q_{10} vs. IR: + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$

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18.2 Coenzyme Q_{10} Supplementation in Mitochondrial Huntington's Disease (Experimental Model)

Anna Gvozdjáková

Abstract Huntington's disease is an autosomal, dominantly inherited neurodegenerative disorder, characterized by degeneration of the basal ganglia and chorea, gradually inability to control movements, intellectual impairment and selective neuronal loss.

Experimental model of HD can be developed by 3-nitropropionic acid (3-NP), which irreversible damages Complex II of mitochondrial respiratory chain (Fig. 18.2). Pretreatment with CoQ_{10} and vitamin E prevented increasing of cytosol creatine kinase activity. Positive CoQ_{10} and vitamin E effect could be in nonmitochondrial level, but in trans-plasma membrane electron transport [1].

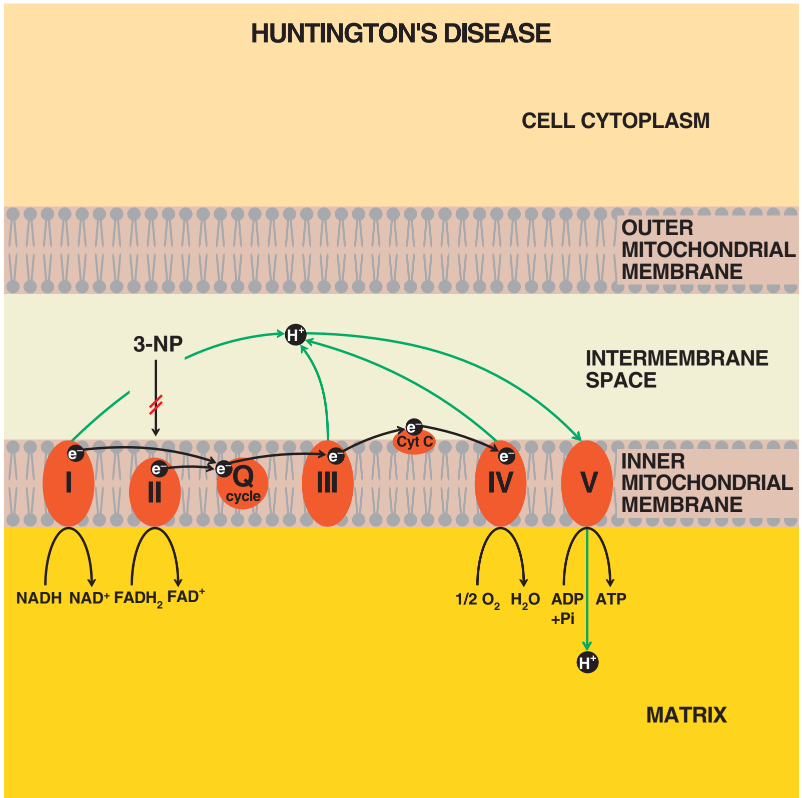

Fig. 18.2 Experimental model of mitochondrial Huntington's disease

Table 18.2 Effect of hydrosoluble coenzyme Q_{10} (Q-GEL) on mitochondrial function in experimental model of Huntington's disease

Groups	Control	3-NP	Q_{10} + 3-NP
Brain mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	0.701 ± 0.016	0.248 ± 0.011**	0.397 ± 0.019*,###
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.331 ± 0.030	0.327 ± 0.014	0.335 ± 0.028
Complex I			
S_3 (nAtO/mg prot./min)	55.30 ± 6.65	41.43 ± 1.67	34.54 ± 2.88*
S_4 (nAtO/mg prot./min)	25.45 ± 3.33	26.13 ± 1.53	20.02 ± 2.00#
OPR (nmol ATP/mg prot./min)	103.73 ± 7.03	87.36 ± 5.29	63.67 ± 5.55**,#
Complex II			
S_3 (nAtO/mg prot./min)	127.75 ± 14.43	36.54 ± 3.97***	39.51 ± 3.80**
S_4 (nAtO/mg prot./min)	108.98 ± 11.72	33.99 ± 4.16***	28.40 ± 6.30***
OPR (nmol ATP/mg prot./min)	119.70 ± 16.21	26.62 ± 14.41**	32.27 ± 5.23**
Complex IV			
Cytochrome oxidase (nAtO/mg prot./min)	1148.0 ± 54.7	1128.6 ± 68.3	1280.0 ± 54.8

Abbreviations: *p < 0.05 vs. c; *p < 0.01 vs. c; **p < 0.001 vs. c;***p < 0.05 vs. HD#

In Huntington disease model of aged rats, liposomal CoQ_{10} supplementation did not restart brain mitochondrial respiratory chain function at Complex II. Complex I and Complex II of brain mitochondria were irreversibly damaged. After 1 month liposomal CoQ_{10} supplementation in experimental model of Huntington disease, liposomal CoQ_{10} supplementation extremely increased ATP and rate of ATP production at Complex I and Complex II in myocardium mitochondria (our unpublished results). These results support possible supplementation of liposomal CoQ_{10} in patients with HD, with regeneration of myocardium damaged mitochondria in HD (see Table 18.2).

Keywords Coenzyme Q_{10}, experimental model of Huntington's disease, mitochondria

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18.3 Coenzyme Q_{10} Supplementation in Adjuvant Arthritis (Experimental Model)

Katarína Bauerová, Jarmila Kucharská, Silvester Poništ, and Anna Gvozdjáková

Abstract Liposomal coenzyme Q_{10} (CoQ_{10}) supplementation was used in a rat experimental model of adjuvant arthritis (AA). After daily administration of 20 mg or 200 mg CoQ_{10}/kg body weight by gavage during 28 days, liposomal CoQ_{10} significantly improved changes of body mass and reduced hind paw volume (HPV) by 23–29%, and prevented CoQ imbalance in skeletal muscle mitochondria. The significantly improved myocardium mitochondrial function indicated that CoQ_{10} supplementation may protect cardiac function also in patients with rheumatoid arthritis.

Keywords Adjuvant arthritis, coenzyme Q_{10}, mitochondrial oxidative phosphorylation, myocardium, skeletal muscle

Adjuvant arthritis (AA) is an animal model of rheumatoid arthritis (RA). Oxidative stress, involved in the etiopathogenesis of RA, may participate in disturbances of mitochondrial function contributing to decreased efficiency and atrophy of skeletal muscle and to damage of the heart muscle. Previously a correlation between stimulation of mitochondrial function in skeletal muscle and coenzyme Q_9 concentration was found in AA rats. The changes were reversed by compounds with antirheumatic properties [3]. Several AA experiments tested the effect of liposomal coenzyme Q_{10} (Li-Q-Sorb) administered to rats by gavage in daily doses of 20 mg/kg body weight during 28 days. CoQ_{10} supplementation differently modulated the function of heart and of skeletal muscle mitochondria [1, 2, 4]. Coenzyme Q imbalance was preferentially improved in skeletal muscle mitochondria of rats treated with CoQ_{10}. In heart muscle mitochondria stimulated oxidative phosphorylation in AA rats returned practically to control values.

In the present study we treated AA male Lewis rats with liposomal form of CoQ_{10} (Li-Q-Sorb) by gavage in the daily dose of 20 or 200 mg/kg body weight during 28 days. AA was induced in Lewis rats intradermally by *Mycobacterium butyricum* in incomplete Freud's adjuvant – *AA group* (see Chapter 11, Fig. 11.1). The *control group* consisted of healthy animals. Hind paw volume (HPV) and changes in body weight (CBW) were monitored. In isolated skeletal muscle and myocardium mitochondria CoQ was measured and parameters of oxidative phosphorylation were determined. Student's t-test was used for statistical evaluation: control vs. AA, and AA vs. AA + Q_{10}.

Liposomal CoQ_{10} significantly improved changes of body weight and reduced hind paw volume by 23–29% compared to untreated AA rats and prevented CoQ

Table 18.3 Effect of liposomal coenzyme Q_{10} (Li-Q-Sorb) on clinical parameters and mitochondrial function in rats with adjuvant arthritis

Groups	Control	AA	AA + Q_{10}	AA + Q_{10}
			(20 mgQ_{10})	(200 mgQ_{10})
Markers of AA (clinical parameters)				
CBW (g)	67.3 ± 19.5	−7.7 ± 8.4***	21.7 ± 8.6^{+++}	17.4 ± 7.51^{+++}
HPV (%)	17.7 ± 4.4	86.9 ± 21.4***	68.5 ± 6.82	61.59 ± 12.61^{ms}
Skeletal muscle mitochondria – respiratory chain				
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	1.300 ± 0.132	2.000 ± 0.295*	1.110 ± 0.108^{+}	1.060 ± 0.110^{++}
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.086 ± 0.012	0.143 ± 0.017*	0.101 ± 0.009^{+}	0.116 ± 0.016
Complex I				
S_3 (nAtO/mg prot./min)	64.76 ± 4.85	51.60 ± 4.22^{ms}	30.96 ± 1.93^{+++}	60.30 ± 8.25
S_4 (nAtO/mg prot./min)	17.31 ± 1.22	16.24 ± 0.78	14.02 ± 1.17	17.30 ± 1.93
OPR (nmol ATP/mg prot./min)	218.76 ± 16.58	174.26 ± 17.06^{ms}	99.47 ± 7.66^{++}	194.00 ± 44.30
Complex II				
S_3 (nAtO/mg prot./min)	85.05 ± 8.53	77.72 ± 4.45^{ms}	43.07 ± 3.01^{+++}	61.80 ± 8.65^{ms+}
S_4 (nAtO/mg prot./min)	58.49 ± 5.88	53.18 ± 4.28	33.30 ± 3.11^{+}	43.60 ± 5.54
OPR (nmol ATP/mg prot./min)	167.95 ± 17.91	168.47 ± 13.06	78.93 ± 3.204^{+++}	114.0 ± 18.2^{ms+}
Myocardial mitochondria – respiratory chain				
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	3.300 ± 0.180	3.050 ± 0.097	4.410 ± 0.174^{+++}	3.680 ± 0.193^{+}
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.335 ± 0.020	0.348 ± 0.012	0.471 ± 0.048^{+}	0.408 ± 0.036
Complex I				
S_3 (nAtO/mg prot./min)	67.96 ± 7.47	60.44 ± 2.10	67.16 ± 2.96^{ms}	64.10 ± 1.03
S_4 (nAtO/mg prot./min)	19.59 ± 2.05	19.75 ± 1.20	22.84 ± 0.79^{+}	27.40 ± 1.23**
OPR (nmol ATP/mg prot./min)	237.28 ± 28.06	193.64 ± 8.81	244.00 ± 12.6^{++}	235.60 ± 12.10^{+}
Complex II				
S_3 (nAtO/mg prot./min)	164.40 ± 4.83	108.57 ± 11.14	126.06 ± 9.22	130.50 ± 4.22***
S_4 (nAtO/mg prot./min)	86.98 ± 1.59	64.50 ± 5.77	70.82 ± 3.44	66.60 ± 4.13**
OPR (nmol ATP/mg prot./min)	356.62 ± 13.34	214.97 ± 8.34**	307.65 ± 12.63^{+++}	255.7 ± 1.17***

Statistical significance: ms – marginally significant; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ *vs. C*, $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ *vs. AA*

imbalance in skeletal muscle mitochondria. Arthritic skeletal muscle mitochondria were regenerated by liposomal CoQ_{10} in higher daily doses of 200 mg/kg body weight, while lower daily doses were insufficient for regeneration of skeletal muscle mitochondrial function [5]. Both daily doses of CoQ_{10} protected myocardium mitochondria in arthritic rats (Table 18.3). Significantly improved myocardium mitochondrial function indicated that CoQ_{10} supplementation may protect cardiac function in patients with rheumatoid arthritis (see Section 11.9, Fig. 11.1)

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Chapter 19
Omega-3-PUFA, Omega-6-PUFA and Mitochondria

Anna Gvozdjáková, Daniel Pella, Jarmila Kucharská, Kuniaki Otsuka, and Ram B. Singh

Abstract Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) belong to the essential fatty acids because the body cannot synthesize them and they must be obtained from the diet. That is why the role of the diet in prevention of mitochondrial damage and pathogenesis of hypertension, coronary artery disease, type 2 diabetes and atherosclerosis has been intensively studied during the last decades. Beneficial effects of *n*-3-PUFA are probably mediated by their antiarrhythmic, lipid lowering, antithrombotic and anti-inflammatory properties. Long-term supplementation of diabetic patients with *n*-3-PUFA could positively influence the mitochondrial energy metabolism of their brain, myocardium and pancreas and preserve theses organs from some later complications of diabetes mellitus. Essential *n*-6-PUFA is only linoleic acid, which is metabolized to arachidonic acid.

Keywords Cardiovascular diseases, diabetes, *n*-3-PUFA, *n*-6-PUFA, mitochondria

19.1 Introduction to ω-3-PUFA and ω-6-PUFA

Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) belong to the essential fatty acids because the body cannot synthesize them and they must be obtained from the diet. In practice, the chemical structure of PUFA molecules is designated as ω- or *n*-. The position of the double bond is indicated by the symbol delta (Δ). For example: Δ^9 – refers to a double bond between carbon atoms 9 and 10 from the carboxyl group. The *unsaturated fatty acids* are created by monounsaturates and polyunsaturates. *Monounsaturated fatty acid (MUFA) is oleic acid* – ω-9 (or *n*-9) MUFA with a single double bond located between the 9th and 10th carbon atoms from the methyl end. It can be synthesized by all mammals, including humans (Fig. 19.1).

19.1.1 Polyunsaturated Fatty Acids (PUFAs)

PUFAs are hydrocarbon chains with a methyl group at one end (called the omega end) and a carboxyl group at the other. PUFAs are with two or more double bonds

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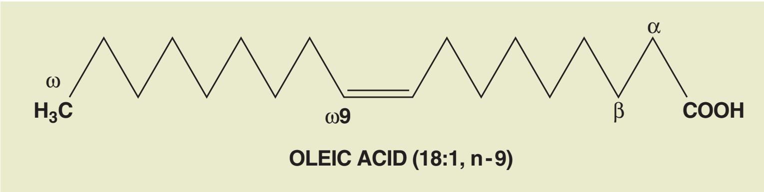

Fig. 19.1 Chemical structure of oleic acid

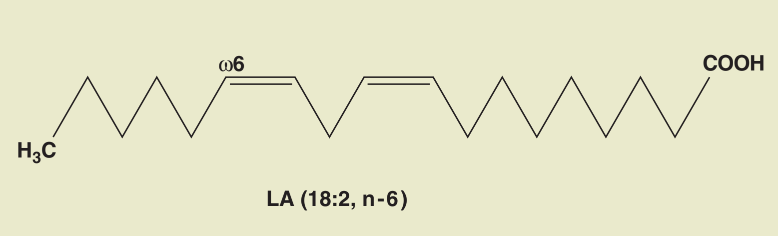

Fig. 19.2 Chemical structures of LA

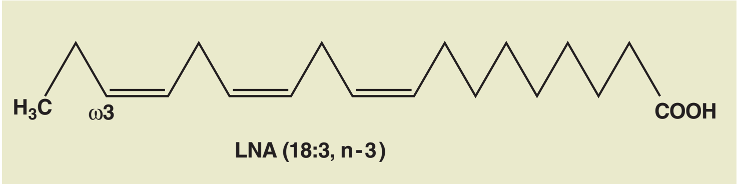

Fig. 19.3 Chemical structure of LNA

[15, 34]. The chemical structures of linoleic acid (LA) and α-linolenic acid (LNA) are shown Figs. 19.2 and 19.3.

19.1.2 Metabolism n-3- and n-6-PUFA

N-3-PUFA is represented by α-linolenic acid (LNA) and *n*-6-PUFA by linoleic acid (LA). Both LNA and LA are metabolized to longer-chain PUFAs, largely in the liver. LNA is converted to eicosapentanoic acid (EPA), and then to docosahexanoic acid (DHA), while LA is the metabolic precursor of arachidonic acid (AA).

Omega-3-PUFAs have a double bond at the third carbon (*n*-3). Alpha-linolenic acid is a so-called *short-chain* omega-3 fatty acid. It is sometimes written as C18:3 omega-3 or C18:3*n*-3 (18 carbons, 3 double bonds with the first at the *n*-3 position).

Long chain omega-3-PUFA includes eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). EPA is C20:5 omega-3, and DHA is C22:6 omega-3.

"Omega-6" refers to the first double bond that occurs at the sixth carbon from the omega (methyl) end. It is also designated as C18:2 omega-6 or C18:2*n*-6 (18 carbons, 2 double bonds with the first at the *n*-6 position) [15]. Elongation and desaturation of *n*-6-PUFA – linoleic acid (LA) produces *n*-6-PUFA – gamma-linolenic acid (γ-LNA), dihomo-gamma-linolenic acid (DHLNA) and arachidonic acid (AA).

AA and EPA are precursors of eicosanoids (prostaglandins, prostacyclins, thromboxanes and leucotrienes. Eicosanoids derived from *n*-3-PUFA – prostaglandins I_3 and E_3, thromboxane A_3 (TXA_3), leucotriene B_5 (LTB_5) – are generally antagonists with anti-inflammatory and anti-aggregatory effects. Eikosanoids derived from *n*-6 AA – prostaglandins I_2 and E_2, thromboxane A_2 (TXA_2), leukotriene B_4 (LTB_4) – are generally agonists with pro-inflammatory and pro-aggregatory effects [36, 37] (Fig. 19.4).

Several supposed mechanisms are involved in the effect of *n*-3-PUFA. It participates in membrane fluidity and phospholipid composition, in regulation of gene expression, transport of sodium, potassium and calcium ions through ion channels and in mitochondrial oxidative phosphorylation.

19.1.3 N-3-PUFA Effect

The effect of *n*-3-PUFA from fish oil is prevention of cardiovascular disorders, cardioprotection, decrease of total cardiovascular mortality and lower incidence of sudden cardiac death [31, 16]. *N*-3-PUFA improves the prognosis of patients not only after AMI but also with heart failure and dysrhythmias. Administration of *n*-3-PUFA seems promising in the primary prevention of atherosclerosis in the group of young individuals at risk.

Epidemiological research associates increased consumption of dietary alpha-linolenic acid with decreased risk of coronary artery calcification and hypertension [6]. *N*-3-PUFA may lower heart rate, reduce inflammation, reduce thrombosis, and inhibit atherosclerosis. Fish in the diet might reduce total stroke risk [3, 25]. *N*-3-PUFA has anti-inflammatory effects, they seem to reduce the use of anti-inflammatory drugs and corticosteroids in patients with rheumatoid arthritis, in patients with asthma and Crohns' disease, and they modestly reduce the risk of developing cataracts. Increased intake of food high in *n*-3-PUFA and decreased intake of *n*-6-PUFA is associated with higher bone mineral density. Fish oil seems to reduce proteinuria and to slow the rate of loss of renal function in patients with IgA nephropathy. Fish supplements might be useful as an adjunctive measure to prevent impaired glucose tolerance and type 2 diabetes, and to prevent progression of type 2 diabetes. For prevention of cancer – fish and fish oil might reduce the overall cancer risk. *N*-3-PUFA exerts protective effects against some common types of cancer, notably those of breast, colon, and prostate [3, 34].

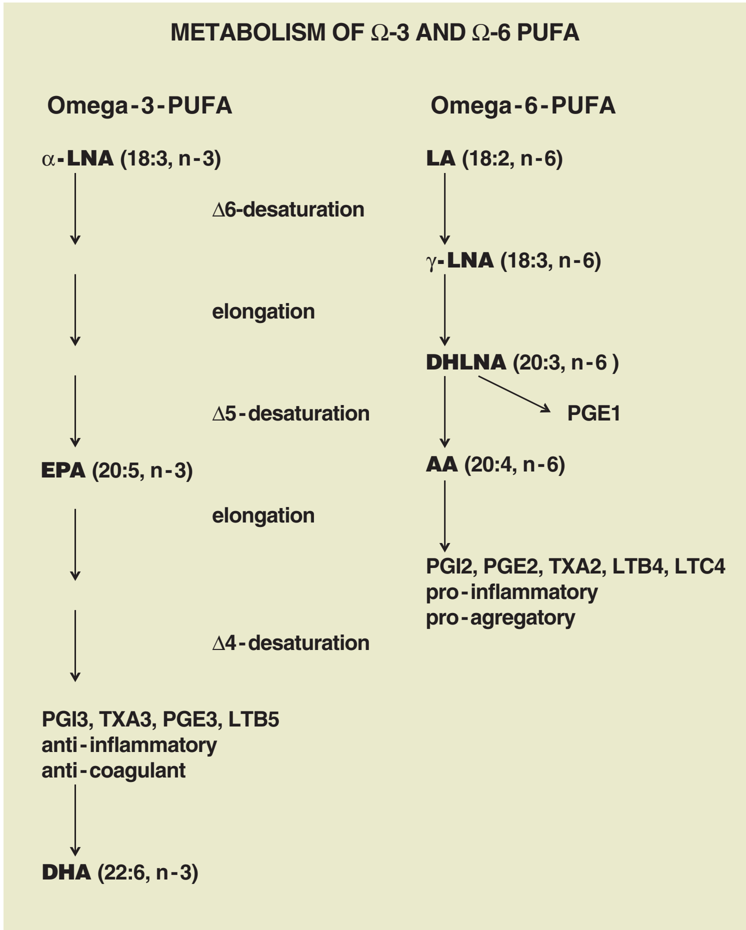

Fig. 19.4 Metabolism of *n*-3- and *n*-6-PUFA
Abbreviations α-LNA (α-linolenic acid), EPA (eicosapentanoic acid), DHA (docosahexanoic acid), LA (linoleic acid), γ-LNA (gamma-linolenic acid), DHLNA (dihomo-gamma-linolenic acid), AA (arachidonic acid).

Conjugated linoleic acid (CLA) is *n*-6-PUFA with 18 carbons, two double bonds and one single bond – between the double bonds. The natural source of CLA is LA. Nine different isomers of CLA have been reported as minor components of food, but only two of them possess biological activity (*cis-9/trans-11* and *trans-10/cis-12*), [32].

Chemical structure of CLA: $CH_3–(CH_2)_5–CH{=}CH–CH{=}CH–(CH_2)_7–COOH$

CLA is naturally present in dairy products (cow's milk, milk products) and meat from ruminants as a consequence of biohydrogenation in the rumen. *Butyrivibrio fibrisolvens*, a ruminant microorganism, is responsible for the production cis-9/trans-11 isomer that is synthetized as a result of the biohydrogenation of linoleic acid [19]. Biological effects of CLA are antiatherogenic [21], hypolipidemic [27, 43], anticancerogenic [4].

CLA decreases abdominal fat and reduces body weight while increasing lean body mass. CLA with L-carnitine activate carnitine palmitoyl transferase activity in liver and kidney mitochondria, a marker of body fat reduction [12].

19.1.4 Fatty Acid Sources

The most important source of *n*-3-PUFA is fish oil. The amount of *n*-3-PUFA varies in different fish species. Other sources of *n*-3-PUFA are vegetables.

Alpha-linolenic acid (LNA) is sometimes called *plant or vegetable omega-3*. Vegetable oils such as soy and canola are the primary sources, and nuts contain also alpha-linolenic acid. Alpha-linolenic acid can be found in high amounts in linseed, walnuts, rapeseed oil, in lesser amounts in hazelnuts and almonds. The body can convert small amounts of alpha-linolenic acid to *eicosapentanoic acid* (*EPA)*, and minute amounts to *docosahexanoic acid* (*DHA*). EPA and DHA constitute a very small part of PUFA in the diet. EPA and DHA are also called *marine omega-3s* because they are found in fish: mackerel, herring, salmon, tuna, trout and halibut. EPA and DHA supplements are sometimes called fish oils. *Linoleic acid* is the predominant dietary *n-6 FA*, comes from linoleic acid in meat, grains, and the seeds of most plants (corn, sunflower, soybean and cotton). Linoleic acid is metabolized to *arachidonic acid (AA)*. AA is converted to inflammatory prostagandins and leukotrienes, which have been linked to cardiovascular and other diseases.

19.2 Cardioprotective Properties of *n*-3-PUFA

Cardioprotective properties of *n*-3-PUFA have been documented in epidemiological, clinical and experimental studies. Especially based on the results of the GISSI Prevenzione Trial and the Diet and Reinfarction Trial (DART), several effects of *n*-3-PUFA have been demonstrated, e.g. antiarrhythmic, anti-inflammatory, antithrombotic and lipid-lowering [2, 10].

19.2.1 Antiarrhytmic Properties of n-3-PUFA

Several studies in healthy volunteers and CAD patients focused on monitoring the relationship between administration of *n*-3-PUFA and heart rate variability (HRV). Low HRV is linked to higher cardiovascular morbidity and mortality and is related to higher risk of sudden death, mostly in AMI patients [41].

In a study of 55 patients after AMI, significant positive correlation was found between the levels of DHA and EPA in platelets and HRV [5]. With increased level of *n*-3-PUFA in platelets, progressive lowering of arachidonic acid accompanied by increased heart rate variability occurred ($p < 0.00001$).

Another study demonstrated a statistically significant decrease of heart rate ($p < 0.0001$), faster heart rate lowering after physical activity ($p < 0.01$) and increase of HRV in patients after AMI with reduced ejection fraction under 40% treated with *n*-3-PUFA (585 mg DHA and 225 mg EPA) when compared to a placebo group [26].

Antiarrhythmic properties of *n*-3-PUFA are assumed to be related to electric stabilization of monocytes via their effect on membrane ion channels, mostly sodium, calcium and potassium, and interactions of *n*-3-PUFA with the phospholipid bilayer of cell membranes. Interestingly, the strengthening of phospholipid membranes with *n*-3-PUFA in experiment is not linked with any antiarrhythmic effect [42]. Redundant removal of *n*-3-PUFA from these membranes using bovine albumine causes no improvement in arrhythmogenic tension [18]. Conversely, infusion of free *n*-3-PUFA into the blood was accompanied by significant antiarrhythmic effect, hence not the esterified but free *n*-3-PUFA is responsible for the antiarrhythmic effect. *N*-3-PUFA in the SN2 position in the membrane phospholipid bilayer has the ability to be released under ischemic influence or ventricular tachycardia, thus exerting an immediate cardioprotective effect.

N-3-PUFA binds to the proteins of sodium channels with effects similar to certain antiarrhythmic agents (e.g. mexiletine) [18]. Extended administration of mexiletine leads to up-regulation of sodium channels, while administration of *n*-3-PUFA does not lead to any negative side effects and shows a better safety profile compared to mexiletine. This is based on the fact that mexiletine increases the synthesis of mRNA coding for α subunit of sodium channels and *n*-3-PUFA increases the hyper-polarization of ischemic cell membranes (which are partly depolarized) and extend the refractive period, therefore a higher intensity of stimulus is needed to generate action potential. *N*-3-PUFA also has the ability to inhibit intracellular influx of calcium ions via L-channels, which is of great importance in ischemia-induced calcium overload. In comparison to calcium channel blockers, *n*-3-PUFA has no negative effect on myocardial contractibility [13].

19.2.2 Antithrombotic Properties of n-3-PUFA

Multiple effects of *n*-3-PUFA on the function of thrombocytes, including coagulation, lowering of thrombocytes and increase of fibrinolytic activity were documented in experimental studies [14]. A detailed scheme of antithrombotic properties of *n*-3-PUFA is shown in Table 19.1.

19.2.3 Anti-inflammatory Properties of n-3-PUFA

The entire complex of anti-inflammatory properties of *n*-3-PUFA can be ascribed to reduced synthesis of arachidonic acid and linked inhibition of cyclooxygenase

Table 19.1 Influence of *n*-3-PUFA on function of thrombocytes and blood coagulation [14]

Factor	Function in atherogenesis	Effect of n-3-PUFA
Arachidonic acid	Precursor of thromboxan and leucotriens	Lowering
Thromboxan A2	Thrombocytes aggregation, vasoconstriction	Lowering
Fibrinogen	Blood coagulation increase	Lowering
Thrombocytes activation factor	Thrombocytes activation	Lowering
tPAI-1	Blood coagulation increase	Lowering
TDGF	Chemoattractant and mitogen of macrophages smooth muscle cells	Lowering

Abbreviations: tPAI-1 – tissue plasminogen activator 1 inhibitor, TDGF – thrombocyte derived growth factor, TPA – tissue plasminogen activator

Table 19.2 Influence of *n*-3-PUFA and *n*-6-PUFA on systemic inflammatory markers [33]

Markers	Before treatment	After treatment	(p)
Group ALA (n = 50)			
CRP (mg/l)	1.24 (0.72–3.70)	0.93 (0.56–1.80)	0.0008
SAA (mg/l)	3.24 (2.30–5.30)	2.39 (1.70–3.90)	0.0001
IL-6 (pg/ml)	2.18 (1.35–3.90)	1.70 (1.30–2.80)	0.01
Group LA (n = 26)			
CRP (mg/l)	1.54 (0.62–3.10)	1.25 (0.64–1.70)	NS
SAA (mg/l)	3.52 (2.10–4.90)	3.34 (2.15–4.40)	NS
IL-6 (pg/ml)	1.77 (1.30–2.70)	2.20 (1.10–2.70)	NS

Abbreviations: ALA – α-linolenic acid (*n*-3-PUFA), LA – linoleic acid (*n*-6-PUFA), CRP – C-reactive protein, SAA – serum amyloid A, IL-6 – interleukin 6

and lipooxygenase pathways of the inflammatory cascade. Anti-inflammatory properties of *n*-3-PUFA may play an important role in modification of subclinical vascular inflammation, which is generally accepted to be involved in the pathogenesis of atherosclerosis, diabetes mellitus, hypertension, metabolic syndrome and chronic cardiac failure. Interestingly, an experimental study of anti-inflammatory effects of *n*-3-PUFA in patients with dyslipoproteinemia has shown that supplementation with high doses of *n*-3-PUFA (8.1 g α-linolenic acid) leads to significant lowering of C- reactive protein, serum amyloid A and interleukin 6 and is linked with the change of *n*-6-PUFA to *n*-3-PUFA ratio from 13.2:1 to 1.3:1 (Table 19.2) [33]. Conversely, in the placebo group without *n*-3-PUFA added to nutrition, there was no lowering effect on inflammatory markers. The anti-inflammatory effect of *n*-3-PUFA may be responsible for deceleration of atherogenesis and a negative ratio of *n*-6-PUFA–*n*-3-PUFA for its acceleration [28, 29].

19.2.4 Lipid-Lowering Properties of n-3-PUFA

The influence of *n*-3-PUFA on serum lipid levels, mainly its effect on triglycerides, is mostly linked with elevation of HDL-cholesterol in experimental studies [30]. In one study, 27 patients on statin therapy for 3 months followed by the addition of *n*-3-PUFA (3 g/day) for the next 3 months were investigated. A statistically significant influence on triglycerides and HDL-cholesterol levels was documented (Table 19.3). The positive lipid-lowering effect was significant only when higher doses (2–4 g/day) of *n*-3-PUFA were used. Interestingly, other positive effects of *n*-3-PUFA were present already at lower doses (1 g/day).

19.2.5 N-3-PUFA and Evidence-Based Medicine

Fish oil is well known from the history for prevention of rickets and other diseases. At present we witness the renaissance of fish oil and its application in the prevention of cardiovascular disorders. In the last decade several analyses of the effects of *n*-3-PUFA from fish oil and its cardioprotective effects were published. One of the latest papers comes from Harper and Jacobson, Table 19.4 [14].

Table 19.3 Influence of additional therapy of *n*-3-PUFA added to statin therapy on lipid levels [30]

Parameter	Before treatment (n = 27)	After 6 weeks with statin treatment	After 3 months with statin and *n*-3-PUFA treatment (3 g/day)	(p)
Cholesterol total	6.48 ± 1.34	5.12 ± 0.86	5.04 ± 0.85	NS
LDL cholesterol	4.21 ± 0.89	3.04 ± 0.72	3.08 ± 0.76	NS
HDL cholesterol	0.92 ± 0.22	1.01 ± 0.29	1.21 ± 0.20	$p < 0.05$
TG	2.96 ± 0.99	2.34 ± 1.01	1.64 ± 0.81	$p < 0.01$

Table 19.4 Randomized controlled trials of *n*-3-PUFA (EPA, DHA) published to date

Study	Kind/doses *n*-3	Controls/Kind/doses	n	Average period Monitoring (months)	Total Mortality (RR, 95% CI)
GISSI-1	EPA + DHA (1:2) 1.85 g/d	Placebo or vitamin E	11,234	42	0.79 (0.66–0.93)
Singh-India	EPA+DHA (1:1) 1.80 g/d	100 mg al. hydroxide	360	12	ND
Nilsen-N	EPA+DHA (1:2) 3.4 g/d	Corn oil 4 g/d	300	18	1.0 (0.45–2.20)
V Schacky-D	EPA+DHA	Vegetable oil	223	24	0.5 (0.0–5.5)
Leng-UK	EPA 0.27 g/d	Sunflower oil	120	24	1.0 (0.21–4.80)
Sacks-USA	EPA+DHA	Olive oil	59	28	0.3 (0.01–7.10)
Burr-UK	EPA 2.4 g/week	Diet without fish	2,033	24	0.73 (0.56–0.93)

From all of the above studies, the most significant results came from the GISSI Prevenzione Trial, Italy, and the British DART study [2, 10]. In the GISSI Prevenzione study, lower doses of *n*-3-PUFA (high purification *n*-3-PUFA – 46% EPA, 38% DHA – 1 g/day) compared to the DART study were used. There was a statistically significant decrease of total cardiovascular mortality (about 21%) ($p = 0.0064$). One of the most important outcomes of the study was the significant lowering of sudden cardiac death (decrease of 44%, p = 0. 0006). With these results there is no doubt about classifying fish oil (drug OMACOR) among drugs with evidence-based cardioprotective properties. Comparison of Omacor to the other drugs used in prevention of sudden death is shown in Table 19.5 [31].

There is growing evidence that *n*-3-PUFA in the form of fish oil or capsules containing purified *n*-3-PUFA will have its place in the newest guidelines of AHA (American Heart Association) for ischemic coronary heart disease. Using 1 gram of EPA + DHA a day (2–4 g in patients with hypertriglyceridemia) as a cardiovascular preventive dose seems reasonable and evidence-based [23].

Table 19.5 Comparison of different therapeutic modalities with respect to sudden cardiac death [31]

Drug	Patient numbers	Relative risk of sudden death (95% CI)
High purification *n*-3-PUFA	11,323	0.56 (0.40–0.79)
ACE-I after IM	15,104	0.80 (0.70–0.92)
Aldosterone blockers	1,663	0.71 (0.54–0.95)
Flekainid, enkainid	1,455	3.60 (1.70–8.50)
Beta-blockers after IM	24,298	0.77 (0.70–0.84)
Amiodaron	6,500	0.71 (0.59–0.85)
D-sotalol	3,121	1.77 (1.15–2.74)
Calcium blockers	20,342	1.04 (0.95–1.14)

19.3 Effect of *n*-3-PUFA in Experimental Medicine

19.3.1 Effect of n-3-PUFA on Mitochondrial Function

The beneficial effect of *n*-3-PUFA on mitochondrial function in rats was documented. Male Wistar rats, aged 11–18 months, weight 250–300 g, received *n*-3-PUFA during 90 days, in the daily dose per kg body weight: 40 mg EPA + 27 mg DHA + 6, 7 mg hydrosoluble CoQ_{10} (Table 19.6) [11].

The beneficial long-term effect of *n*-3-PUFA with low hydrosoluble CoQ_{10} concentration was documented in mitochondria of the brain, myocardium and pancreas. In pancreatic mitochondria, CoQ_{10} oxidized and CoQ_{10} reduced forms were extremely significantly stimulated. One of the metabolic ways of *n*-3-PUFA effects can include improvement of mitochondrial respiration and energy production of various organs [11].

Table 19.6 Effect of *n*-3-PUFA supplementation on mitochondrial function

Groups	Control	*n*-3-PUFA	Statistics
Brain mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	0.768 ± 0.104	0.972 ± 0.088	
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.368 ± 0.030	↑ 0.521 ± 0.040	$p < 0.05$
Myocardial mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	3.240 ± 0.279	↑ 5.600 ± 0.392	$p < 0.0006$
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.370 ± 0.020	↑ 0.960 ± 0.093	$p < 0.0002$
Complex I			
S_3 (nAtO/mg prot./min)	148.74 ± 4.570	↑217.53 ± 24.74	$p < 0.031$
S_4 (nAtO/mg prot./min)	23.37 ± 2.329	↑ 41.44 ± 5.57	$p < 0.0254$
OPR (nmol ATP/mg prot./min)	369.4 7± 15.49	↑577.24 ± 69.45	$p < 0.0153$
ADP:O (nmol ADP/nAtO)	2.480 ± 0.098	2.464 ± 0.026	
RCI (S_3/S_4)	6.678 ± 0.569	5.437 ± 0.287	
Complex II			
S_3 (nAtO/mg prot./min)	226.36 ± 9.640	↑376.52 ± 44.37	$p < 0.0052$
S_4 (nAtO/mg prot./min)	121.76 ± 5.09	↑186.96 ± 35.15	$p < 0.0603$ ms
OPR (nmol ATP/mg prot./min)	291.95± 18.85	↑463.52 ± 56.77	$p < 0.0124$
ADP:O (nmol ADP/nAtO)	1.273 ± 0.045	1.228 ± 0.029	
RCI (S_3/S_4)	1.818 ± 0.088	1.819 ± 0.054	
Pancreas mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	0.239 ± 0.038	↑ 0.520 ± 0.077	$p < 0.058$ ms
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.058 ± 0.020	↑ 14.70 ± 1.910	$p < 0.0001$
$CoQ_{9\text{-}RED}$(nmol/mg prot.)	0.0	0.167 ± 0.054	
$CoQ_{10\text{-}RED}$(nmol/mg prot.)	0.0	↑ 6.460 ± 0.868	$p < 0.0002$

19.3.2 Effect of n-3-PUFA on Diabetic Mitochondrial Function

Several factors contribute to the CVD and diabetes development of mitochondrial damage – genetic predisposition, environmental, dietary and lifestyle factors (such as physical inactivity, unhealthy and unbalanced nutritional consumption of excess calories, simple refined carbohydrates with a high glycemic index and load of high saturated fat (SF), high transfatty acids (TFA), high *n*-6 fatty acids and lack of monounsaturated fatty acids (MUFA) and *n*-3 fatty acids in the diet) can cause mitochondrial damage, contributing to the escalating rates of obesity and mortality due to CVD. About one fifth of the adult population in developing countries and one fourth in industrialized countries are estimated to have CVD and diabetes [8, 17, 38, 39]. Dietary intake of total fat amounts to 25–45% in developing countries. Most of the dietary fatty acids are derived from meat, oil and dairy products, resulting in marked increase in saturated and *n*-6-PUFA but relatively modest increase of MUFA and long-chain PUFA, particularly *n*-3-PUFA. Refinement of vegetable oils has been a major cause for increased consumption of *n*-6-PUFA and hydrogenation of these oils caused greater intake of transfatty acids (TFA). These

factors may account for mitochondrial damage in endothelial cells, cardiomyocytes, smooth muscle, neurons, beta-cells, and liver, leading to increased prevalence of CVD, diabetes, cancers, and neurodegenerative diseases in most countries in the world [7, 8, 17, 24, 39].

19.3.2.1 Mitochondria and Diabetes

Diabetes represents a collection of diseases characterized by chronic hyperglycemia and is one of the leading causes of cardiovascular disease, stroke and renal failure. Diabetes can result from impaired secretion of insulin by the beta-cells, as well as by loss of its action in peripheral tissues (skeletal muscle, fat, liver). Diabetes or insulin deficiency results not only in hyperglycemia but also in elevated non-esterified fatty acid (NEFA) levels. Excessive exposure to NEFA is regarded as a potentially important diabetogenic complication by impairing insulin secretion from pancreatic beta-cells. Hyperglycemia-induced myocardial apoptosis is mediated, at least in part, by activation of cytochrome c-activated caspase-3 pathway, which can be triggered by ROS derived from high level of glucose [21].

19.3.2.2 Effect of *n*-3-PUFA on Diabetic Mitochondrial Function

Hyperglycemia induces mitochondrial ROS production by the mitochondrial respiratory chain of pancreatic β-cells. Increased mitochondrial ROS production during hyperglycemia impairs glucose-stimulated insulin secretion by pancreatic beta-cells. Mitochondrial diabetes can be accompanied by other disorders, such as cardiomyopathy, myopathy, visual failure (retinis pigmentosa, optic athrophy), stroke, dementia [9]. The brain is one of the targets of complications of chronic diabetes mellitus. These complications affecting the brain are manifested as structural, neurophysiological and neuropsychological alterations [1]. The etiopathogenic mechanisms involved are not fully understood. Impaired metabolism of glucose and fatty acids, decreased NAD^+/NADH ratio [35], oxidative stress, changes in membrane fatty acid composition, down-regulation of Na^+/K^+-ATPase [22], and decreased mitochondrial Ca^{2+} uptake have been reported in DM. Impairment of mitochondrial energy production can also contribute to organ damage. Changes in the fluidity of brain and heart mitochondrial membranes in experimental DM were reported [44].

Deficient Q_9 and Q_{10} concentrations of heart and liver diabetic mitochondria and enhanced malondialdehyde production can contribute to the disturbances of mitochondrial energy metabolism in diabetic rats [20]. These changes may be associated with disturbances of lipid metabolism in diabetic rats [26]. Diabetes or insulin deficiency also elevate non-esterified fatty acid (NEFA) levels. Excessive exposure to NEFA is regarded as a potentially important diabetic complication by impairing insulin secretion from pancreatic beta-cells [21]. Supplementation with antioxidants or PUFA could decrease pancreatic beta-cell mitochondrial oxygen radical production and may have a therapeutic effect.

Table 19.7 Effect of *n*-3-PUFA supplementation on diabetic mitochondria

Groups	DM (90 days)	DM + *n*-3-PUFA (90 days)	Statistics
Brain mitochondria			
$CoQ_{9\text{-OX}}$ (nmol/mg prot.)	0.671 ± 0.045	0.762 ± 0.110	
$CoQ_{10\text{-OX}}$ (nmol/mg prot.)	0.494 ± 0.202	0.577 ± 0.090	
Complex I.			
S_3 (nAtO/mg prot./min)	48.490 ± 4.750	↑64.076 ± 4.137	$p < 0.0384$
S_4 (nAtO/mg prot./min)	26.25 ± 2.94	29.78 ± 3.71	
OPR (nmol ATP/mg prot./min)	96.896 ± 9.74	↑139.06 ± 9.07	$p < 0.0133$
Myocardial mitochondria			
$CoQ_{9\text{-OX}}$ (nmol/mg prot.)	4.400 ± 0.218	2.800 ± 0.224	$p < 0.0030$
$CoQ_{10\text{-OX}}$ (nmol/mg prot.)	0.582 ± 0.041	↑1.100 ± 0.169	$p < 0.0177$
Pancreas mitochondria			
$CoQ_{9\text{-OX}}$ (nmol/mg prot.)	0.896 ± 0.039	0.884 ± 0.244	
$CoQ_{10\text{-OX}}$ (nmol/mg prot.)	0.188 ± 0.036	↑ 7.348 ± 2.377	$p < 0.0001$
$CoQ_{9\text{-RED}}$(nmol/mg prot.)	0.0	0.0	
$CoQ_{10\text{-RED}}$(nmol/mg prot.)	0.0	↑ 3.11 ± 1.41	$p < 0.0001$

Beneficial chronic effect of *n*-3-PUFA in diabetic aged rats was proved. [11, 40]. Rats received *n*-3-PUFA during 90 days, in the daily dose/kg body weight: 40 mg EPA + 27 mg DHA + 6, 7 mg hydrosoluble CoQ_{10} (DM + *n*-3-PUFA). Diabetes (DM) was induced by a single dose of streptozotocin (STZ, 55 mg/kg i.v.) (Table 19.7).

In chronic diabetes, brain mitochondrial OXPHOS function was protected by *n*-3-PUFA with low CoQ_{10}. Treatment with CoQ_{10} and *n*-3-PUFA may be protective against cardiovascular or neurological complications in diabetes. Long-term supplementation of diabetic patients with *n*-3-PUFA and Q_{10} could positively influence the mitochondrial energy metabolism of the brain, myocardium and pancreas and prevent some later complications of DM affecting these organs [11, 40].

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Chapter 20
Carnitine

Anna Gvozdjáková

Abstract Carnitine is a necessary component for fatty acid oxidation, plays an important role in many physiological metabolic reactions of the human body. Deficiency of carnitine in diseases, in vegetarians diet and its supplementation is described.

Keywords Carnitine, mitochondria, deficit and supplementation of carnitine

Carnitine is a naturally occuring compound in the body, belongs to a group like vitamin nutrients. It is distributed in 95% in the myocardium and skeletal muscle, and about 4% in the other organs and 1% in the extracellular space. The concentration of carnitine in skeletal muscle is about 70% higher than in plasma. From the subcellular particles – the highest carnitine concentration was found in the mitochondria, dominantly in liver and kidney mitochondria.

Carnitine sources for human body are *external* – from foods (as meats – sheep, mutton, lamb, beef, pork, cow's milk and breast milk) and *internal* – carnitine is produced in the kidneys and liver of the body. Carnitine biosynthesis in the body is in a series of metabolic reactions, which require two amino acids (L-methionine and L-lysine) and vitamin C, vitamin B6, niacin, and iron. Carnitine is a 3-hydroxy-4-N-trimethyl amino butyric acid. Various *types of carnitine* are known: L-carnitine, L-carnitine fumarate, L-carnitine tartarate, acetyl-L-carnitine, propionyl-L-carnitine and aminocarnitines.

20.1 The Physiological Role of Carnitine

Carnitine is peresent in the biological materials as *free carnitine* (in relatively high concentrations) and as *acylcarnitines* (metabolic products of reactions utilizing acyl CoA catalyzed by carnitine acyltransferases). The acyl groups in the acylcarnitines range from short-chain (acetyl) to long-chain (such as palmitoyl).

In 1955 was demonstrated that in liver was formed acetylcarnitine from acetyl CoA and carnitine, in liver was proved stimulation fatty acid oxidation with

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carnitine. A great amount of mitochondrial research activity from years 1960 was in many laboratories after proving that acylcarnitines were substrates for long-chain fatty acid oxidation by mitochondria.

Carnitine was involved in several physiological roles:

- L-carnitine acts as antioxidant (as free radicals scavenger)
- L-carnitine is essential for the transfer of long-chain fatty acid from the cytoplasm into the mitochondria
- L-carnitine acts as scavenger system for acyl groups
- L-carnitine remove of lactic acid from blood and tissues
- L-carnitine is involved in the formation and utilization of ketone bodies
- L-carnitine is involved in the branched-chain amino acid metabolism
- L-carnitine increase ammonia conversion into urea, which is excreted in the urine
- L-carnitine is involved in membrane stabilization

Heart and skeletal muscle obtain most of their energy demands by fatty acids oxidation, which occurs primarily in the mitochondrial matrix. Carnitine is an essential factor for the transport long-chain fatty acids (acyl CoA) from the cytosol to the mitochondrion. Without carnitine, the oxidation of long-chain fatty acids, cannot proceed. Acyl CoA can cross the mitochondrial membrane only after transfer of the acyl group to carnitine, than acylcarnitine and CoA are produced. The basic, reversible reaction is:

$$\text{Acyl CoA} + \text{Carnitine} \leftrightarrow \text{Acylcarnitine} + \text{CoA}$$

Transport acylcarnitine into mitochondrial matrix requires enzymes activity: carnitine palmitoyltransferase I and II (CPT I and CPT II – exclusive mitochondrial enzymes), and the carnitine translocase. Under physiological conditions, long-chain acylcarnitines are formed mostly in the intermembrane space and imported into matrix, whereas short-chain acylcarnitines are formed within the matrix and exported into the cytosol [1, 2].

20.2 Deficit of Carnitine

Deficiencies of carnitine in humans were first noted in 1973 year. Reasons of carnitine deficit in human body are various. Primary deficiency is result of damaged transport mechanism of carnitine in plasma membrane, carnitine is eliminated by urine. Damaged transport of long-chain acylcarnitines into matrix, inhibits of beta-oxidation fatty acids. Reduced carnitine content was found in patients with kidney disease, particularly those undergoing dialysis, may lose up to 80% of their serum carnitine per day. Insufficiency of carnitine can develop progression of cardiomyopathy, myopathy (low carnitine in skeletal muscle) or encephalomyopathy.

Other reasons of carnitine deficiency are associated with genetic metabolic disturbances (defects of beta-oxidation fatty acid, defects in respiratory chain function), or acquired deficiency (hemodialysis, or valporic acid effect in epilepsy therapy).

20.3 Deficit of Carnitine and Vegetarians

Although in a healthy people deficiency of carnitine is rare, in pure vegetarians lysine and methionine are lacking, deficit of carnitine is in their diet. It is well established in both, in children and adults vegetarians, which have lower carnitine concentration in their plasma. Deficit carnitine is in people with strict macrobiotic diets, which participate in loss weight, but also in weakness developing, several nutritional deficiencies and initiation of diseases development [4, 6].

20.4 Supplementation of Carnitine

Benefit carnitine supplementation is in physiology conditions (e.g. in trained athletes) and in pathological conditions, in diseases. In patients with neuromuscular diseases carnitine supplementation is able to improve ankle and knee function and handgrip strength. Patients improved after carnitine supplementation. In patients with hypertension, with anemia, carnitine supplementation (in daily dose 1 g after 3 months) improved myocardial fatty acids utilization. Supplementation with carnitine improves quality of life of chronically hemodialysed patients. In patients with HIV, human immunodeficiency virus beneficial effect of carnitine was after treatment 6 g daily for 6 months [3]. Synergy effect of L-carnitine fumarate (in daily dose 880 mg with 60 mg hydrosoluble CoQ_{10}) decrease men infertility during 3–12 months, offer a new way for improved men fertility [5].

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20.5 Carnitine and Coenzyme Q_{10} Supplementation in Male Infertility

Anna Gvozdjáková, Jarmila Kucharská, and Pavol Lepieš

Abstract Supplementary therapy with the combined effect of hydrosoluble coenzyme Q_{10} and L-carnitine fumarate offers a new way for treating male infertility.

Keywords Carnitine, coenzyme Q_{10}, male infertility, sperm mitochondria

Several pathological mechanisms are involved in male infertility, as oxidative stress, decreased antioxidant function, low level of coenzyme Q_{10} and carnitine, impaired sperm mitochondrial function [8]. Mitochondria of spermatozoa play an important role in energy production, sperm motility and semen quality. Beneficial effects of supplementary therapy with coenzyme Q or carnitine were documented (see Chapter 13).

In our *pre-clinical study* we showed that CoQ_{10} supplementation stimulated sperm mitochondrial oxidative phosphorylation in aged rats (19–22 months old). The animals received daily 200 mg hydrosoluble CoQ_{10} by gavage during 4 weeks. Supplementation with CoQ_{10} significantly increased CoQ_{10} sperm mitochondrial concentration, slightly elevated basal respiration (S_4), ATP production (S_3), as well as the rate of ATP production (OPR) in Complex I and Complex II of the mitochondrial respiratory chain [7] (Table 20.1).

Several clinical studies reported reduction of coenzyme Q_{10} levels in seminal plasma and sperm cells in male infertility [7, 6, 3]. Supplementary antioxidant and energy therapy is widely used in male infertility. Supplementation with carnitine or

Table 20.1 Effect of hydrosoluble CoQ_{10} on testicular mitochondrial function (pre-clinical study)

Groups	Control	CoQ_{10}	Statistics
Testicular mitochondria			
$CoQ_{9\text{-OX}}$ (nmol/mg prot.)	1.260 ± 0.100	1.31 ± 0.10	
$CoQ_{10\text{-OX}}$ (nmol/mg prot.)	0.203 ± 0.039	0.324 ± 0.024	$p < 0.0244$
Complex I			
S_3 (nAtO/mg prot./min)	12.63 ± 0.52	24.30 ± 4.65	$p < 0.067$ ms
S_4 (nAtO/mg prot./min)	5.57 ± 0.20	12.07 ± 3.43	
OPR (nmol ATP/mg prot./min)	23.47 ± 0.17	44.40 ± 7.94	$p < 0.0578$ ms
Complex II			
S_3 (nAtO/mg prot./min)	71.50 ± 6.00	119.80 ± 22.40	
S_4 (nAtO/mg prot./min)	38.83 ± 6.16	54.10 ± 11.80	
OPR (nmol ATP/mg prot./min)	80.70 ± 4.80	134.70 ± 25.90	

Abbreviations: ms – marginally significant

CoQ_{10} has been used for improving male fertility. Numerous antioxidants, such as vitamin C, vitamin E, glutathione, coenzyme $Q_{10,}$ and carnitine, were shown to exert a beneficial effect in treating male infertility [1, 13, 11, 6, 12, 9, 10].

The combination of L-carnitine and CoQ_{10} was found to be more effective than administration of either substance alone in protecting bacteria against oxygen-induced toxicity and mycotoxins [2], on long-chain fatty acid metabolism [5], against ischemia and reperfusion on the working rat heart [4]. In a clinical study, we applied this combination of supplementary therapy in infertile men.

20.5.1 Clinical Medicine

Coenzyme Q_{10} and coenzyme Q_{10} with L-carnitine fumarate were used as supplementary therapy in infertile men.

Coenzyme Q_{10} therapy: In the clinical study we included 75 infertile men aged 26–39 years, with more than 5 million sperms/ml ejaculate. The patients were supplemented with hydrosoluble coenzyme Q_{10} (60–120 mg daily), vitamin E (100–400 mg daily), vitamin C (100–500 mg daily), beta-carotene (10 mg daily). The supplementation with antioxidants lasted from 3 to 12 months. The therapy resulted in improvement of male fertility, sperm count and motility and decrease of plasma lipid peroxidation, as shown in Table 20.2 [6].

Combined supplementation with *L-carnitine and CoQ_{10}* (CARNI-Q-GEL FORTE). Over the years 1997–2006 we compared the effect of classical and antioxidant supplementary therapy with CoQ_{10} alone or in the combination of hydrosoluble CoQ_{10} with L-carnitine in 159 infertile men. The criteria for male infertility were: age – 26–39 years, >5 million sperms/ml ejaculate without leukocyte. Duration of therapy: from 3 to 9 months (Table 20.1).

Supplementary therapy of infertile men with L-carnitine and hydrosoluble CoQ_{10} offers a new way for improving male fertility.

Recommendation: 880 mg L-Carnitine fumarate with 60 mg hydrosoluble CoQ_{10}, vitamin E, and vitamin C, in two daily doses, during 3–9 months. The fertility of men treated with the given dosage improved in 80.43% and pregnancy in women was recorded in 78.26%. These results are original. *Mitochondrial "spermatopathy" see Chapter 13.*

Table 20.2 Supplementary therapy with coenzyme Q_{10} and with the combination of coenzyme Q_{10} and carnitine in male infertility

Group	Number of pts	Improvement of male fertility (%)	Pregnancy in women (%)
(1) Classical therapy	90	10 (11.11%)	6 (6.66%)
(2) Q_{10}	67	27 (40.30%)	27 (38.80%)
(3) Carnitine + Q_{10}	92	74 (80.43%)	72 (78.26%)

(Q_{10} – hydrosoluble CoQ_{10}, Carnitine + Q_{10} – Carnitine with hydrosoluble CoQ_{10}, vitamin E and vitamin C)

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20.6 Simultaneous Effect of L-Carnitine and ω-6-PUFA Supplementation in Human Obesity and in Experimental Medicine

Anna Gvozdjáková and Jarmila Kucharská

Abstract On the basis of the demonstrated synergistic effect of conjugated linoleic acid and L-carnitine on body fat reduction in pre-clinical studies, the combined treatment was applied in clinical studies, in obese people. Long-time supplementation with L-Carnitine, conjugated linoleic acid and Green Tea Extract (L-Carnitine Teavigo Clarinol) significantly decreased cholesterol, triacylglycerols and oxidative stress in obese people. Supplementation with L-Carnitine Teavigo Clarinol can prevent obesity with enhanced energy metabolism and potential cardiovascular and other complications of obesity.

Keywords: Carnitine palmitoytransferase, coenzyme Q, conjugated linoleic acid, L-carnitine, mitochondria

Obesity is a risk factor for cardiovascular diseases, atherosclerosis, type 2 diabetes and certain types of cancer. According to the WHO report, obesity occurs in 250 million people in the world. *Conjugated linoleic acid* (CLA), a ω-6 polyunsaturated fatty acid, has been shown to affect body fat reduction and promote lean body mass. *Carnitine* is an essential cofactor of long-chain fatty acid transport from the cytosol across the mitochondrial membrane into the matrix. Mitochondrial carnitine palmitoytransferase (CPT) catalyses the transformation of long-chain fatty acid-CoA esters to acylcarnitine for transport across the mitochondrial membrane (see Chapter 1, Fig.1.3). CPT activity can be used as a marker of body fat reduction.

20.6.1 Pre-Clinical Study

In isolated rat liver and kidney mitochondria, coenzyme Q concentration, respiratory chain function and ATP production, as well as CPT (carnitine palmitoyl transferase) activity were studied after supplementation with CLA (conjugated linoleic acid) and simultaneous CLA and L-carnitine supplementation. Three-month-old rats were divided into four groups: Control (*C*), conjugated linoleic acid (*CLA* – 91 mg/rat/day), L-Carnitine fumarate (*CAR* – 68.25 mg/rat/day), *CLA* + *CAR* – received L-carnitine fumarate (68.25 mg/rat/day) and CLA (91 mg/rat/day) during 2 weeks, or four times higher daily doses of carnitine and CLA during 4 weeks.

The synergistic effect of CLA + L – Carnitine was observed in stimulation of CPT activity in the corpus adiposum nuchae (Figs. 20.1, 20.2). CLA and CAR

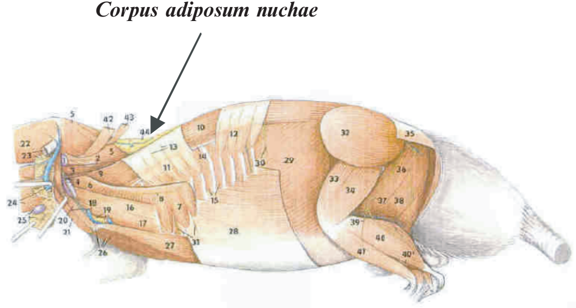

Fig. 20.1 Corpus adiposum nuchae

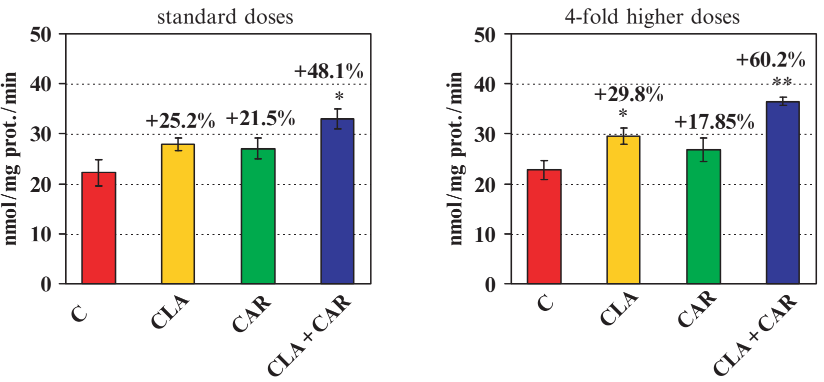

Fig. 20.2 CPT activity in corpus adiposum nuchae after CLA and L-Carnitine supplementation

significantly increased CoQ production in heart and kidney mitochondria. Strong correlations were found between liver mitochondrial CPT activities and coenzyme Q concentration as well as Complex II function (Fig. 20.3). These results indicate that supplementation with CLA and carnitine could prevent obesity and decrease the risk of cardiovascular diseases with enhanced energy and fat metabolism [1, 2, 3, 4, 5].

20.6.2 Clinical Study

Six-month supplementation of L-Carnitine (Teavigo) Clarinol Softgels and placebo softsules were used in 2 groups of obese people (total number 48), aged between 25 and 60 years.

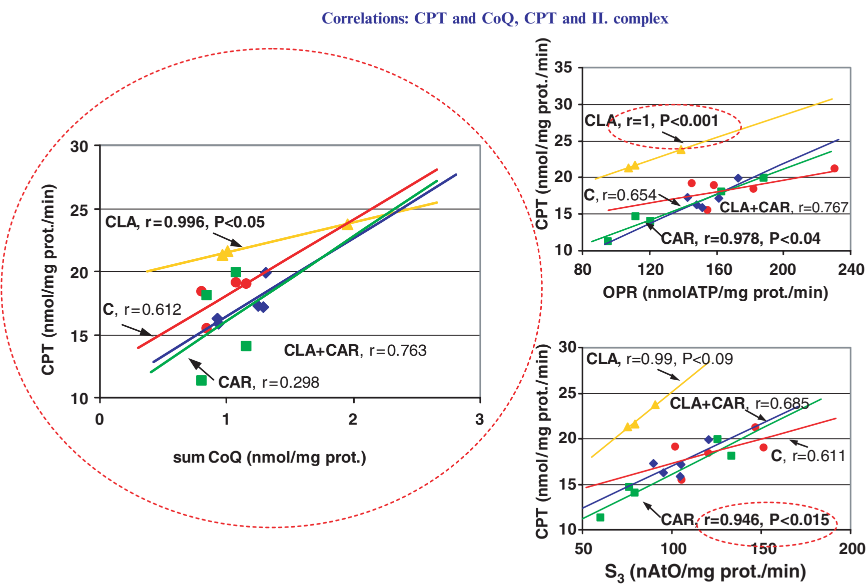

Fig. 20.3 Strong correlations of CPT and CoQ and Complex II (S3, OPR) of liver mitochondria

Group A: obese 12 men and 12 women (placebo)
Group B: obese 12 men and 12 women (anti-obesity therapy)

In Group B the daily dose of L-Carnitine fumarate – 1,000 mg, CLA-3 600 mg, green tea extract 300 mg was divided into three daily doses with each meal. Duration of the study: 6 months, evaluation of all parameters every 2 months.

After 6 months of supplementary therapy with L-Carnitine (Teavigo) Clarinol, group B exhibited significantly decreased BMI (body mass index), waist girth, lipid peroxidation (MDA) – in men, cholesterol, triacylglycerols. No side effects were observed. In Group A (placebo) no significant changes were found [6].

In conclusion, long-time supplementation with L-Carnitine, CLA and green tea extract decreased cholesterol, triacylglycerols and oxidative stress in obese people. This supplementation can contribute to the prevention of obesity and of complications connected with obesity. The effect of this supplementary anti-obesity therapy was higher in combination with increased physical activity [6].

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Chapter 21
Vitamins in Mitochondrial Function

Jarmila Kucharská

Abstract Vitamins are essential in metabolic reactions in the body as catalysts in enzymatic reactions or as coenzymes carrying chemical groups between enzymes. Many vitamins function in enzyme complexes participating in mitochondrial respiration and energy production or they are required for synthesis of mitochondrial respiratory chain components. Biosynthesis of the component of mitochondrial respiratory chain, coenzyme Q, is dependent on vitamins B_2, B_6, B_{12}, folic acid, pantothenic acid, niacinamide and vitamin C. Vitamins B_1, B_2, B_6, niacin, biotin, folic acid and pantothenic acid are important for metabolic pathways in mitochondrial respiration and energy production. Vitamins C, E, niacin and folic acid belong to effective scavengers of free radicals, prevent mitochondrial oxidants formation and mitochondrial aging. Some mitochondrial diseases are linked to vitamin deficiencies and can be improved by vitamin supplementation.

Keywords Metabolism, mitochondria, vitamins

Vitamins are biomolecules that function in essential metabolic reactions within the body. Many vitamins act as catalysts in enzymatic reactions or as coenzymes carrying chemical groups between enzymes and they play an essential role in mitochondrial function.

Vitamins cannot be synthesized in mammalian cells and therefore must be obtained through food intake or in form of dietary supplements. Only few vitamins can be obtained by other means: for example, microorganisms in the intestine produce vitamin K and biotin and one form of vitamin D is synthesized in the skin with the help of sunlight. Some vitamins are produced in the body from provitamins, for example vitamin A from beta-carotene [28, 43, 46].

Deficiencies of vitamins are classified as either primary or secondary. A primary deficiency occurs when they are not obtained in sufficient amount from food. A secondary deficiency may be due to underlying disorders that prevent or decrease the absorption of the vitamin, or it may be caused by some lifestyle factors as smoking, excessive alcohol consumption or drug interactions. Well-known vitamin deficiencies involve thiamine (beriberi), niacin (pellagra), vitamin C (scurvy) and vitamin D (rickets). In developed countries serious deficiencies are

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rare but even minor deficiencies have the potential to cause permanent damage. Few metabolic pathways are dependent on only one vitamin, and thus severe malnutrition can cause multiple deficiencies that result in some disturbances including damage of mitochondrial function. For example: biosynthesis of coenzyme Q as a part of mitochondrial respiratory chain is dependent on vitamins B_2, B_6, B_{12}, folic acid, panthothenic acid, niacinamide and vitamin C [14]. Thiamin, riboflavin, niacin and pantothenic acid have a direct effect on mitochondrial aerobic respiration and energy production [7].

Several vitamin deficiencies, such as biotin or pantothenic acid, increase mitochondrial oxidant formation and accelerate mitochondrial aging [43]. Deficiencies of vitamins are dependent on body stores. Vitamin A, D and B_{12} are stored in significant amount in the body, mainly in the liver, and in an adult they may be deficient for long periods before developing deficiency symptoms. Vitamin B_3 is not stored in the body in significant amounts, stores may last a couple of weeks [46].

The likelihood of consuming too much of any vitamin from food is improbable. Supplementation with large doses of some vitamins can cause side effects such nausea, diarrhea and vomiting. Recovery may be often achieved by reducing the dosage [46].

Vitamins are generally divided into two groups according to solubility in water or in fat:

- water soluble – eight B vitamins and vitamin C
- fat soluble – vitamins A, D, E, K

Function of vitamins and consequences of their deficit will be presented also in relation to mitochondrial function and mitochondrial diseases (Fig. 21.1).

21.1 Water-soluble Vitamins

21.1.1 Thiamin (Vitamin B_1)

Thiamin occurs in the human body as free thiamin or its active phosporylated form, thiamin pyrophosphate (TPP), which is a cofactor in three mitochondrial enzyme complexes central to mitochondrial energy production: pyruvate, α-ketoglutarate and branched-chain ketoacid dehydrogenases [7]. Vitamin B_1 is vital to normal functioning of the nervous system and metabolism (Fig. 21.2).

Deficiency of vitamin B_1 was among the first discovered and was identified as the dietary factor missing in polished rice and responsible for disturbances of the nervous and cardiovascular system, diminished sensation and weakness in the legs and arms, referred to as beriberi [28]. Thiamin deficiency increases the concentration of lactic acid in blood, one of the general metabolic symptoms in mitochondrial diseases. Inborn errors of metabolism, genetic mtDNA mutations and neurodegenerative diseases have been linked to thiamin deficiency. Increased formation of reactive

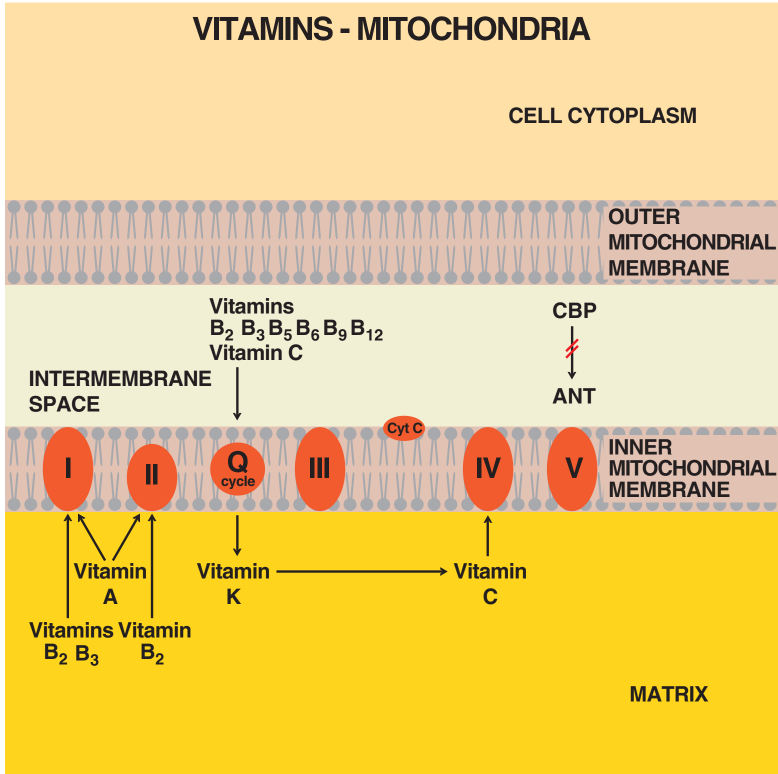

Fig. 21.1 Action of vitamins in mitochondrial respiratory chain

- Riboflavin (Vitamin B_2) is a component of Complexes I and II;
- Niacin (Vitamin B_3) is used for synthesis of coenzymes NADH and NADPH;
- Vitamin A affects activities of Complex I and II;
- Vitamins B_2, B_3, B_6, B_9, B_{12} and Vitamin C are involved in coenzyme Q (CoQ) biosynthesis;
- Carotenoid brakdown products (CBP) impair mitochondrial function targeting adenine nucleotide translocase (ANT);
- Vitamins K and C mediate electron transport from NADH to CoQ or Complex IV.

Fig. 21.2 Thiamin structure

oxygen species in mitochondrial respiratory chain in neurodegenerative diseases, such as Parkinson's and Alzheimer's, can be prevented by thiamin supplementation. [7, 33]. Thiamin deficiency can occur in alcoholics and has been suggested to be involved in colon cancer [5]. Symptoms of deficiency include fatigue, depression, decreased mental functioning, muscle cramps, nausea, heart enlargement.

Thiamin is found in wheat germ, whole-grain products, brown and raw rice, brewer's yeast, beef kidney and liver [43].

The recommended dietary intake ranges 1.0–1.5 mg/day for healthy adults [28, 43, 46].

21.1.2 Riboflavin (Vitamin B_2)

Riboflavin is an integral component of the coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Belonging to the prosthetic group of enzymes termed flavoproteins, it participates in redox reactions in numerous metabolic pathways of carbohydrates, fats and proteins (Fig. 21.3).

Riboflavin plays an important role in mitochondrial energy production. Mitochondria take up riboflavin by the riboflavin transporter and form FMN and FAD. FAD is required for the function of five mitochondrial acyl-CoA dehydrogenases. FAD may be effluxed from the mitochondria by FAD carrier into the cytosol [7]. Riboflavin supplementation has been reported to be effective in some patients with Complex I deficiency [36]. Improvement of muscular tone and exercise capacity were noted suggesting riboflavin as a promising additive to treatment of myopathies [29].

Riboflavin was discovered shortly after thiamin and in distinction from it as heat-stable substance. It is easily destroyed upon exposure to visible light. This can lead to riboflavin deficiencies in newborns treated for hyperbilirubinemia by phototherapy. Age-related formation of cataracts caused by oxidative damage of lens protein was found to be decreased by riboflavin intake [18]. FAD-dependent enzymes glutathione reductase and xanthine oxidase play a role in protecting the organism from reactive oxygen species [7, 28].

Fig. 21.3 Riboflavin structure

A typical symptom of deficiency is inflammation of the lip and tongue, fatigue, depression, anemia. Physically very active people and alcoholics may have increased riboflavin requirements [28, 43, 46].

Major sources of riboflavin are eggs, lean meat, milk, broccoli and enriched bread and cereal products. A small amount of riboflavin is in most plants.

The recommended dietary intake ranges 1.5–2.0 mg/day for healthy adults.

21.1.3 Niacin (Vitamin B_3)

Niacin (nicotinic acid and its amide form nicotinamide) is used in the body to form coenzymes, nicotinamide adenine dinucleotide (NAD^+/NADH) and nicotinamide adenine dinucleotide phosphate ($NADP^+$/NADPH). More than 500 enzymes require niacin coenzymes. All energy metabolic pathways including mitochondrial oxidative phosphorylation, citric acid cycle, β-oxidation of fatty acids and cytosolic glycolysis, are dependent on these enzymes. Entering the cells, nicotinamide is mostly immediately metabolized to NAD^+, which functions as electron carrier to form ATP by mitochondrial respiration or glycolysis, and $NADP^+$, which functions as a hydrogen donor in reductive biosynthetic reactions [7, 28] (Fig. 21.4).

The major role of NADH is to transfer electrons from metabolite intermediates to mitochondrial respiratory chain. Complex I accepts electrons from NADH and passes them to ubiquinone. The rationale for nicotinamide treatment in oxidative phosphorylation disorders is to increase the cellular NADH and NAD^+ concentrations and thereby enhance the substrate availability to Complex I. Antioxidant properties of niacin are mediated by increased cellular NADPH supply, which is able to maintain glutathione in reduced state [29].

Niacin was discovered in the early 1900s during an investigation of pellagra, late stage of severe niacin deficiency. The manifestations of pellagra were commonly referred to as four D's: dermatitis, dementia, diarrhea and death [28]. Most proteins contain tryptophan, which is a precursor of nicotinic acid. Niacin deficiency is thus rare except where the diet is deficient in tryptophan [7].

Niacin is found in yeast, beef liver, fish, meat, cereals, legumes, seeds, milk, coffee and tea. The recommended daily intake ranges 14–16 mg/day.

N COOH N $CONH_2$

Fig. 21.4 Nicotinic acid and nicotinamide structures

21.1.4 Pantothenic Acid (Vitamin B_5)

Pantothenic acid known as vitamin B_5 is essential in numerous chemical reactions in the body. Pantothenic acid is present in the cell in two active forms, coenzyme A (CoA) and acyl-carrier proteins (Fig. 21.5).

Coenzyme A is a molecule essential for 4% of known enzymatic reactions that generate energy from fat, carbohydrates and proteins and in mitochondrial energy production and cellular function. CoA is also required for the first step in biosynthesis of cholesterol and ubiquinone, a part of mitochondrial respiratory chain [15]. As a component of the acyl group carrier protein, CoA is essential for mitochondrial pyruvate dehydrogenase and α-ketoglutarate dehydrogenase of the tricarboxylic acid cycle (TCA), the β-fatty-acid oxidation pathway and leucine metabolism [3, 7]. In a mouse model of muscular dystrophy, supplementation of pantothenic acid improved muscular response suggesting impaired mitochondrial CoA transporter [13]. Pantothenate supplementation prevented the collapse of mitochondrial membrane potential, restored ATP synthesis and activity of antioxidant enzymes in the liver, resulting assumably from increased mitochondrial CoA and reduced glutathione levels [47]. Pantothenate treatment could be useful in liver failure.

Pantothenic acid deficiency in humans is very rare and has been observed only in cases of severe malnutrition. Symptoms include excessive fatigue, sleep disturbances, loss of appetite, nausea or dermatitis [28, 43].

Pantothenic acid is found in all plant and animal cells. Rich sources are in the liver, kidney, yeast, egg yolk, broccoli, fish, chicken, milk, legumes, mushrooms [7, 28, 43].

The recommended daily intake of pantothenic acid is 5 mg.

$$HOCH_2-C(CH_3)_2-CH(OH)-CO-NH-CH_2CH_2COOH$$

Fig. 21.5 Pantothenic acid structure

21.1.5 Pyridoxal, Pyridoxamine, Pyridoxine (Vitamin B_6)

Vitamin B_6 occurs in the form of pyridoxal, pyridoxamine and pyridoxine, which are converted to the biologically active form – pyridoxal phosphate (Fig. 21.6).

Pyridoxal phosphate (PLP) plays a vital role in the function of approximately 100 enzymes that catalyze essential metabolic reactions in the human body. Mitochondrial function is more dependent on PLP than the function of other subcellular organelles. PLP functions as a coenzyme for transaminases, which participate in the catabolism of amino acids, as well as in glycogenolysis as a cofactor for

Fig. 21.6 Pyridoxal phosphate structure

glycogen phosphorylase. Transaminases participate also in the malate–aspartate shuttle that enables mitochondria to oxidize NADH formed by glycolysis [8]. Vitamin B_6 is also involved in decarboxylation reactions in the mitochondrial matrix responsible for the synthesis of heme, a component of hemoglobin. In these reactions B_6 deficiency could increase the formation of reactive oxygen substances because of decreased heme/cytochrome synthesis. For instance, a deficiency of mitochondrial Complex IV could impair respiration and accelerate mitochondrial aging processes [1].

Vitamin B dependency was first recognized in 1954 in children with epileptic seizures beginning early in life [2].

Deficiencies of vitamin B_6 are rare, an increased risk of deficiency is in advanced age, severe malnutrition, excessive alcohol intake, in dialysed patients and patients treated with some drugs (isoniazid, cycloserine, hydralazine, penicillamine).

Symptoms of B_6 deficiency include weakness, mental confusion, irritability, nervousness, inability to sleep, hyperactivity, anemia, skin lesions, kidney stones.

Vitamin B_6 is found in vegetables, whole grain cereals, nuts and muscle meats.

The recommended daily intake of vitamin B_6 is 1.4–2.0 mg.

21.1.6 Biotin (Vitamin B_7 or Vitamin H)

Biotin is the cofactor of enzymes involved in carboxylation reactions. Carboxylases catalyze essential metabolic reactions mostly in lipid metabolism. For example acetyl-CoA carboxylase catalyzes the binding of bicarbonate to acetyl-CoA to form malonyl-CoA required for synthesis of fatty acids. Pyruvate carboxylase is a critical enzyme in gluconeogenesis, the formation of glucose from sources other than carbohydrates, such as amino acids and fats [7, 27] (Fig. 21.7).

Biotin is also a cofactor of mitochondrial carboxylases, which are important in energy production and cellular function. Mitochondrial pyruvate carboxylase catalyzes the first step in gluconeogenesis. Mitochondrial propionyl-CoA carboxylase participates in the last step of β-oxidation pathway, formation of methylmalonyl-CoA and succinyl-CoA, part of the TCA cycle [7, 28].

Fig. 21.7 Biotin structure

Mitochondrial methylcrotonyl-CoA carboxylase catalyzes an essential step in the metabolism of leucine. Deficit of biotin causes accumulation of methylcrotonyl-CoA in the mitochondria and depletes glycine and succinyl-CoA. This prevents heme/cytochrome synthesis and results in formation of reactive oxygen substances in mitochondria [1].

Manifestations of deficiency may include hair loss, dermatitis, anemia, muscle pain, depression, lethargy, impaired immune system. Biotin deficiency has been linked to some inborn errors of metabolism, sudden infant death syndrome, propionic aciduria, and the development of diabetes. Supplementation with biotin decreased glucose level in type II diabetics [7, 28, 43].

Biotin is found in many kinds of food, though in lower amounts than other B vitamins. Rich sources of biotin are egg yolk, liver, yeast, whole wheat bread, cheese [7, 28].

The recommended daily intake of biotin is 30 μg.

21.1.7 Folic Acid (Vitamin B_9)

The function of folate coenzymes in the body is transfer of one-carbon units, important in reactions linked to metabolism of nucleic acids and amino acids. Folate plays a vital role in DNA synthesis from its precursors. Methylation of sites within DNA may be important in cancer prevention [8, 28] (Fig. 21.8).

Folate was originally identified in yeast and liver as a nutrient reducing anemia during pregnancy. Fortification of grain products (flour, bread, cereals) with folic acid started in the USA in 1998 and was associated with a decrease in neural tube defects. Adequate folate status may also prevent the occurrence of other types of birth defects, including heart defects and limb malformation [1, 17, 18]. Folate deficiency has been associated with some types of cancer (cervix, colon, rectum, lung, brain, pancreas, breast), with pediatric leukemia and neuroblastoma.

Folate is essential for metabolic pathways involving cell growth, replication and cell survival. Thirty to fifty percent of cellular folates are located in the mitochon-

Fig. 21.8 Folic acid structure

dria where they are involved in glycine metabolism, important for mitochondrial respiration and energy production. Folate deficiency and increased homocysteine levels induced DNA damage and apoptosis in rat hippocampal neurons, caused mitochondrial dysfunction and oxidative stress [8, 26]. Folic acid can act as a direct antioxidant and scavenger of free radicals [20]. In hypertensive patients supplementation with folic acid decreased oxidative stress biomarkers [39] and prevented mitochondrial damage induced by methanol [41].

Folic acid is found in green leafy vegetables, citrus fruit juices, legumes and fortified cereals. The recommended daily intake of folic acid is 400 µg.

21.1.8 Cobalamin (Vitamin B_{12})

Cobalamin is the largest and most complex of all the vitamins, containing cobalt ion and is synthesized by microorganisms. It occurs in three forms as adenosylcobalamin, methylcobalamin and hydroxycobalamin (Fig. 21.9).

Absorption of vitamin B_{12} from food requires normal function of the stomach, pancreas and small intestine. The active form requires hydrolysis by gastric acid and enzymes. The vitamin is then bound to intrinsic factor, a protein secreted by specialized cells in the stomach, and carried to the small intestine where it is absorbed and then transported to the liver. The most common cause of vitamin B_{12} deficiency is pernicious anemia that develops in autoimmune inflammation of the stomach and lack of intrinsic factor. Antipernicious anemia effect of cobalamin has been recognized in the treatment of patients with fresh liver and liver extracts.

The liver can store vitamin B_{12} up to 6 years, therefore deficiencies are rare. Strict vegetarians need to take cobalamin regularly as a supplement [8, 28]. The form of cobalamin used in most supplements is cyanocobalamin which is converted to adenosyl- and methylcobalamin.

Methylcobalamin is required for synthesis of the amino acid methionine from homocysteine. Methionine is required for the synthesis of S-adenosylmethionine, a methyl group donor used in methylation of DNA, which may be important in cancer prevention. Cobalamin deficiency inhibits methionine synthase, thus leading to an accumulation of homocysteine, which has been associated with increased risk of cardiovascular diseases [8, 28].

Fig. 21.9 Cyanocobalamin structure

Accumulation of methylmalonic acid in cobalamin deficiency can result in neurological complications and has been associated with decreased mitochondrial respiration and energy production [30].

Symptoms of deficiency include nausea, loss of appetite, sore mouth, diarrhea, depression, anemia.

Vitamin B_{12} is found in fish and meat, particularly liver. The recommended daily intake of vitamin B_{12} is 2.4 µg.

21.1.9 Ascorbic Acid (Vitamin C)

Ascorbic acid is present as ascorbate in most body fluids. Ascorbate participates in a number of hydroxylation reactions involved in synthesis of collagen, an important component of blood vessels, tendons, ligaments and bone (Fig. 21.10).

Several other metabolic reactions require ascorbate as a cofactor: synthesis of norepinephrine, a neurotransmitter important for brain function; synthesis of carnitine, essential for the transport of fat into mitochondria for energy conversion; synthesis of bile acids. It plays a role in the immune system in the production

Fig. 21.10 Ascorbic acid structure

of antibodies and as a promoter of interferon, a compound acting against cancerogenesis [28].

The strong reducing potential makes ascorbate an efficient free radical scavenger of several types of free radicals, protecting proteins, lipids, carbohydrates and nucleic acids from oxidative injury. Although ascorbate is present in the aqueous phase, it can protect membranes and lipoproteins from oxidative damage by effective scavenging of lipid peroxidation-initiating radicals at the water–lipid interface [14]. Regeneration of α-tocopherol from α-tocopheroxyl radicals is an important protective function of ascorbate from lipoperoxidation in membranes and mitochondria [21, 32]. Recent findings in human cell culture showed that oxidized form of vitamin C, dehydroascorbic acid, entered mitochondria via facilitative glucose transporter 1, accumulated mitochondrially as ascorbic acid and protected mitochondria from oxidative injury [34].

Severe vitamin C deficiency has been known for many centuries as scurvy, characterized by bleeding, hair and tooth loss, joint pain and swelling. The disease can be prevented by a daily dose of 10 mg of vitamin C. Symptoms of deficiency include prolonged healing of wounds, frequent infections, swollen or painful joints, nosebleeds, anemia.

Vitamin C is found in different fruits (orange, grapefruit, lemon, strawberries, black currants, rose hips) and vegetables (tomatoes, broccoli, cabbage, potatoes) [28].

The recommended daily intake of vitamin C is upward from 60 mg. Many studies indicate that doses about 100 mg may reduce risk of cardiovascular diseases and cancer.

21.2 Fat-soluble Vitamins

21.2.1 Vitamin A (Retinoids)

Vitamin A occurs in three biologically active forms – retinol, retinal and retinoic acid – referred to as retinoids. There are two categories of vitamin A, depending on the food source. Vitamin A found in animal food (liver, milk) is called preformed vitamin A. It is absorbed in the form of retinol and can be made into retinal and

retinoic acid in the body. Vitamin A found in fruits and vegetables called provitamin A is represented by carotenoids. Most common are beta- and alpha-carotene and cryptoxanthin. Beta-carotene is most efficiently made into retinol in the body [9, 31]. Carotenoids are better absorbed when ingested as a part of fatty meal, cooked and minced (Fig. 21.11).

Vitamin A plays an important role in vision. It is required in the production of rhodopsin, the visual pigment used in low light levels. Vitamin A is important for the correct functioning of epithelial cells, synthesis of glycoprotein, in maintaining immune system functions, formation of red blood cells and production of human growth hormone. Vitamin A and carotenoids also act in the body as antioxidants, which may reduce the risk of some diseases associated with increased formation of free radicals [9, 46].

Vitamin A deficiency is common in developing countries but rarely seen in developed countries. One of the earliest signs of vitamin A deficiency is night blindness followed by decreased visual acuity. Vitamin A deficiency also diminishes the ability to fight infections. Excess alcohol consumption can deplete vitamin A, and a stressed liver may be more susceptible to vitamin A toxicity. People consuming excess of alcohol should seek medical advice before taking vitamin A supplements [46].

Vitamin A deficiency was found to affect mitochondrial function [12]. Decreased activity of Complex I and II in rat heart mitochondria which returned to normal after refeeding indicates a beneficial effect of retinol in some heart mitochondrial diseases.

Vitamin A accumulates in the liver and therefore overdose can be harmful or fatal. Toxicity is manifested as bone pain, hepatosplenomegaly, nausea and diarrhea. Excess vitamin A has been suspected to contribute to osteoporosis and increase in birth defects when consumed in early pregnancy. Higher levels of carotenoids achieved by increased consumption of fruits and vegetables are not toxic. Interestingly, high-dose beta-carotene supplementation was associated with an increase in lung cancer in male and female smokers in clinical studies. In non-smokers, opposite effects were found [42, 44]. The mechanism of side effects of high-dose beta-carotene supplementation has been explained recently by increased generation of carotenoid breakdown products (CBP) including very reactive aldehydes and epoxides, accompanied with prooxidant reactions. Accumulation of CPB impairs mitochondrial function. Beta-carotene supplementation should be accompanied with monitoring of its plasma level to avoid

H_3C CH_3 CH_3 CH_3 OH CH_3

Fig. 21.11 Retinol structure

critically high levels. A complex system of antioxidants may contribute to the decreased role of CPB [37].

Vitamin A is found naturally in food that comes from animals, such as whole eggs, milk and liver. Sources of provitamin A carotenoids are carrots, spinach, tomatoes, broccoli, apricots, peaches.

The recommended daily vitamin A intake is for adults about 3,000 IU, not exceeding 5,000 IU [9].

21.2.2 Vitamin D

Vitamin D is a fat-soluble vitamin essential for calcium metabolism. Vitamin D occurs in two main forms: D_2 (ergocalciferol) and D_3 (cholecalciferol) (Fig. 21.12).

Vitamin D_2 is derived from fungal and plant sources and is not produced by the human body. Vitamin D_3 is derived from animal sources and is made in the skin from 7-dehydrocholesterol exposed to sunlight, specially with UVB ultraviolet light, wavelengths 290–315 nm.

Vitamin D is a prohormone with several active metabolites that act as hormones. Vitamin D is biologically inactive, and must be metabolized to its biologically active forms. After it is consumed in the diet or synthesized in the skin, it enters the circulation and is transported to the liver where it is hydroxylated to form 25-hydroxycholecalciferol. In the kidney and other tissues, the dihydroxylated metabolite, the main biologically active hormone 1,25-dihydrocholecalciferol (calcitriol), is then formed and released into the circulation. Bound to a carrier protein, it is transported to various organs. Vitamin D affects many organ systems, but mainly it increases calcium and phosphorus absorption from the intestine and promotes normal bone formation and mineralization (with the assistance of parathyroid hormone and calcitonin). Vitamin D affects the immune system by promoting immunosuppression and anti-tumor activity [10, 28, 46].

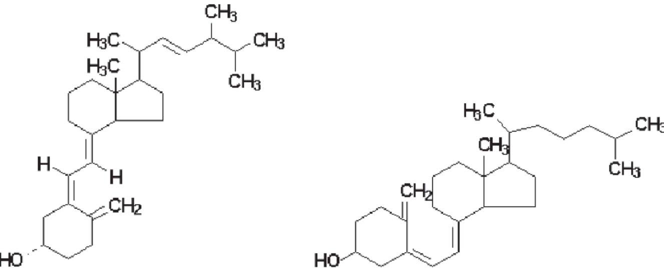

Fig. 21.12 Ergocalciferol and Cholecalciferol structures

Symptoms of vitamin D deficiency include bone pain and muscle weakness. Severe deficiency in children results in failure of bone mineralization and rickets. Deficiency is associated with increased risk of osteoporosis, arthritis, and cancer [28, 43].

Overdose symptoms include anorexia, nausea, vomiting, high blood pressure, premature hardening of arteries, kidney damage.

Vitamin D is found in some fatty fish (mackerel, salmon, sardines), fish liver oils, eggs. Mushrooms are one of the few natural sources for vegans [46].

The recommended daily vitamin D intake for adults is 200 IU, for over 50-year-old people 400 IU.

21.2.3 Vitamin E

Vitamin E is a generic form for eight compounds with high antioxidant activities. Four tocopherols (α-, β-, γ-, δ-) and four tocotrienols (also α-, β-, γ-, δ-) differ in the number and position of methyl groups on the chromane ring, tocotrienols have three double bonds in the side chain. Each form has its own biological acticity, alpha-tocopherol appears to have the greatest nutritional significance in humans [4] (Fig. 21.13).

The major role of α-tocopherol as antioxidant is to protect polyunsaturated fatty acids in membranes and lipoproteins from peroxidation by free radicals. Free radicals are formed in the body during normal metabolism or in the state of increased formation of radicals in diseases or by environmental factors, e.g. cigarette smoke and pollutants. Acting as a scavenger of free oxygen and lipid peroxyl radicals, α-tocopherol is oxidized to tocopheroxyl radical. Active α-tocopherol can be regenerated by ascorbic acid or ubiquinol [21, 38].

Mitochondria contain 35–40% of the total α-tocopherol in the cell and its importance for mitochondrial integrity has been indicated. Despite the location of most α-tocopherol in the outer mitochondrial membrane where it is susceptible to oxidative damage, tocopheroxyl radical can be recycled by mitochondrial electron transport chain [27]. Reduced blood α-tocopherol/cholesterol ratio has been found in patients with impaired function of Complex I [25]. Vitamin E treatment has been used in oxidative phosphorylation disorders [16]. Beneficial effects of coenzyme Q and

Fig. 21.13 Alpha-tocopherol structure

vitamin E on brain energy metabolism have been found in the animal model of Huntington's disease induced by Complex II blockade [22].

The functions of other tocopherols and tocotrienols in humans are not sufficiently explained but recent data indicate their biological activity. For example γ-tocopherol seems to be a more potent antioxidant in vitro with higher activity against free nitrogen radicals, which are involved in the pathology of neurodegenerative diseases and associated with mitochondrial dysfunction [6, 24]. In spite of the fact that γ-tocopherol represents about 70% of tocopherols in the daily diet, blood levels are approximately ten times lower than those of α-tocopherol [19]. This is due to the action of α-tocopherol transfer protein in the liver, which preferentially incorporates α-tocopherol into lipoproteins circulated in the blood; α-tocopherol is thus delivered to different tissues in the body. Tocotrienols possess anticancer and cholesterol-lowering properties that are not exhibited by tocopherols [35]. α-Tocotrienol may be neuroprotective by antioxidant-independent as well as antioxidant-dependent mechanisms [23].

Vitamin E deficiency has been observed in severe malnutrition, genetic defects affecting the α-tocopherol transfer protein and fat malabsorption syndromes.

Severe vitamin E deficiency results in neurological symptoms, including impaired balance and coordination, injury to the sensory nerves, muscle weakness and damage to the retina of the eye. In infants with very low birth weight, vitamin E deficiency can develop neurological symptoms rapidly [26, 46].

Increased dietary intake of vitamin E may prevent or delay coronary heart disease, cancer, development of cataracts, age related macular degeneration and neurodegenerative diseases as Alzheimer's and Parkinson's [46].

Major sources of α-tocopherol are vegetable oils (olive, soybean, sunflower, corn), nuts, whole grains and green leafy vegetables. All eight forms of vitamin E (tocopherols and tocotrienols α, β, γ, δ) occur naturally in foods in varying amounts. For example γ-tocopherol occurs in higher amounts than α-tocopherol in soybean and corn oils, higher amounts of α-tocopherol are in sunflower oil.

The recommended daily vitamin E intake advised for adults is 15 mg. In prevention and treatment of diseases the doses are increased [28, 46].

21.2.4 Vitamin K

Vitamin K is a fat soluble vitamin essential for the functioning of several proteins involved in blood clotting ("K" is derived from the German word "Koagulation").

$$CH_2CH{=}C(CH_3){-}(CH_2CH_2CH(CH_3)CH_2)_3{-}H \qquad (CH_2CH{=}C(CH_3){-}CH_2)_n{-}H \qquad CH_3$$

Fig. 21.14 Phylloquinone, menaquinone and menadione structures

There are two naturally occurring forms of vitamin K: K_1 (phylloquinone) synthesized in plants and K_2 (menaquinone) produced by intestinal bacteria (the forms of K_2 differ in number of 5-carbon units in the side chain). K_3 (menadione) is a water-soluble synthetic form of vitamin and must be alkylated to menaquinone-4 to be biologically active [28, 40] (Fig. 21.14).

The most important function of K vitamins is the maintenance of normal levels of blood clotting proteins, factors II (prothrombin), VII, IX, X, and precursor proteins C and S, which provide control and balance in the coagulation cascade. Coagulation factors are synthesized in the liver, therefore severe liver disease results in lower blood levels of vitamin K-dependent clotting factors and there is an increased risk of uncontrolled bleeding [28].

Vitamin K has been used in the treatment of patients with defects of oxidative phosphorylation because it is assumed to mediate electron transport from NADH to electron acceptors such as coenzyme Q or cytochrome c [29]. Menadione plus ascorbate improved cellular phosphate metabolism in patients with Complex III deficiency [11] and improved clinical and biochemical parameters in a Complex I deficient patient [45].

Although vitamin K is a fat-soluble vitamin, the body stores very little of it and the stores are rapidly depleted without regular dietary intake. Deficiency of vitamin K_2, which is synthesized by intestinal bacteria, can be caused by long-term antibiotic treatment.

Symptoms of deficiency include bleeding manifested as nosebleeds, blood in the urine, stool, heavy menstrual bleeding.

Phylloquinone (K_1) is the major dietary form of vitamin K. It occurs in green leafy vegetables (broccoli, spinach, lettuce, parsley) and some vegetable oils (soybean, olive).

The optimal dietary intake of vitamin K is not yet known, recommended is at least one cup of dark green leafy vegetables daily [28].

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Chapter 22
Polarized Light

Ján Pálinkáš and Alfonz Smola

Abstract The chapter presents polarized light and its effects on the human body. The definition of light is given. Basic features of light from the wave and corpuscular point of view are considered. The effects of particular lights on the human body and their connection to light energy are described. The effects of basic colors of light – red, green and blue on the human body are discussed. The significance and advantages of phototherapy are analyzed. A photographic supplement concerning treatment of some pathological conditions with phototherapy is included.

Keywords Color of light, light energy, phototherapy, polarized light, wavelength

22.1 The History of Healing by Light

Healing by light has as long a history as that of mankind itself. The first recorded use of light energy for disease healing purposes dates back to 2500 B.C. in Egypt and about 1400 B.C in China. Yet the real expansion happened only in cultures of historical Greece and Rome in the form of sun-baths. The original method, from the historical point of view, was ***heliotherapy*** (healing with sun rays, sunlight). In the Middle Ages, the interest faded somewhat, but with the Renaissance period heliotherapy started to be recommended and practiced again. A huge expansion started in the first half of the 19th century and healing with light was definitely established at the end of the 19th century. Its significant contribution to medicine was confirmed at the beginning of the 20th century by the award of the Nobel Prize in 1903 to the Danish scientist N.L. Finsen /1860–1904/ for healing skin diseases, mainly skin tuberculosis/lupus vulgaris/ by using light stimulation. Nevertheless, for quite a long time light healing remained limited to the simple effect of light or the application of light in the infrared part of the light spectrum. Nowadays application of the infrared spectrum belongs to the thermotherapy branch – transformation of energy of the infrared part to heat energy. The actual development of polarized light application started after implementation of laser therapy in the 1960s of the 20th century.

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At that time the term polarized light started to be used as *physically modified light* and polarized light sources were introduced in different branches of medicine. In the 1980s, clinical tests and theoretical works in the light branch led to the creation of *biolight* – simple and cheap sources of polarized light [4, 7, 12].

The last developmental step in the application of polarized light was materialized by biostimulating equipment, devices whose light sources are LED (*light-emitting diode)*, discovered by the laser industry recently. LED is a semiconductor electronical part, emitting tight-spectrum light when crossed by electricity in the forward direction. The light effect is the result of radiant recombination of electrons and is a form of electroluminescence. The color of the emitted light depends on the chemical composition of the semiconducting material used. The first usable LED diode was produced in 1962 by the Canadian scientist Nick Holonyak. In the 21st century, systems of polarized light with LED as light sources appeared. Sources emitting the same wavelength with just a small diffusion are used. Mostly the wavelength of 626–637 nm with diffusion ±18 nm is used. These are highly effective and economical light sources with low consumption.

Phototherapy – therapy with polarized light: it is a biologico-physical method stimulating the activity of living organisms in a naturally way [1]. ***Polarization of light***: it is achieved by processes modifying the oscillation of the light wave in space at one level.

Light sources during polarization: Monochromatic source – laser
Polychromatic source – classical biolights
So-called Q – monochromatic sources – LED sources

Light definition: Light has two principles. One of them is that the light is mainly electromagnetic field waving /Maxwell/, the other concerns its partial character, it is partial– corpuscular /Planck/. Combining both of them, we can say from our point of view, that light is a flow of photocells – the basic elements of light wave that are able to evoke photochemical reactions in living organisms.

22.2 Classification of Light Wave Components

J.C. Maxwell proved that electrical, magnetic and optical events were essentially the same. This confirmed the existence of other electromagnetic waves. These waves are not light visible by our eyes. We know today that the world is literally flooded by elctromagnetic waves from space.

The spectrum of electromagnetic waves can be classified according to wavelength into several categories [11].

Infrared spectrum *(300–760 nm)* – (infra = below) radiation of wavelengths longer than those of visible light, not perceived by human eyes. A part of the optical spectrum with wavelength neighboring on one side with visible spectrum – that is the red wavelength and on the other side connected by the ionizing zone to the area of microwaves and radiowaves. The natural source of such emission is the Sun.

Artificial sources are all solid figures whose temperatures are higher than absolute zero. Humans perceive such emission by heat-sensitive cells on the body surface. Large wavelength and small energy of photons evoke, after perception in tissues, the heat effect (used mainly in thermohealing) with a large penetration effect. It also has a mild analgetic and spasmolytic effect. It penetrates hard environment.

Ultraviolet spectrum *(380–10 nm)* – (ultra = beyond) has a shorter length than visible light and cannot be perceived by human sight. Its sources are objects heated very high (stars, electric arc, etc.). UV is absorbed by the atmosphere and also by simple windowglass. Its small wavelength and high energy of photons result in its low ability to penetrate the human skin, therefore all UV radiation is absorbed in the skin and not getting further to the tissues. Its effects are chemical (whitening), it destroys microorganisms (antibacterial effects used during sterilization) and it also has physiological effects (vitamin D generation, etc.). It has also ionizing effects and in case of excessive exposure, it can have negative effects on the human body (higher risk of skin cancer).

Visible or white light *(760–380 nm)* – It is that part of the light spectrum which the human eye is sensitive to. The whole color range is included in the white light spectrum. It is created by six colors***: red, orange, yellow, green, blue and purple***. In the past, the complete visible spectrum was used for therapeutic purposes (so-called polychromatic light). Modern devices use filtered sources of monochromatic light. Today most of them use the wavelength of white light, mainly the spectrum of red wavelength.

Wavelength – the share of light speed c (c = 299,792 km/s) and its frequency (vibrational number). It is expressed either in nanometers (1 nm = 10^{-9} m) or micrometers, microns (1 mm = 10^{-6} m). It defines the place of light wave in the spectrum and has an influence on light dispersion and its absorption in the tissue. It characterizes the state of emission in the spectrum of electromagnetic radiation. It influences the rate between impinging and reflected energy, as well as light refraction and dispersion. It influences the energy of photons and radiation absorption.

The wavelength defines the level of absorption of polarized light and the interaction with particular tissue levels. Different substances like water, blood (mainly hemoglobin) and melanin – skin pigment – display different degrees of absorption. On studying these parameters, it was discovered that the percentage of light energy absorption by hemoglobin and water is the lowest at the wavelength 600–900 nm, and in this interval also the level of absorption of light energy by melanin decreases. This interval is called – laser window – and it plays an important role in phototherapy. In this spectrum interval the light can penetrate deeper into tissue structures. At a wavelength around UV, the photochemical effect is dominant, in the infrared area it is the thermical effect. In the visible part of the spectrum these effects combine.

Energy of photons as the smallest parts of light waves depends indirectly on the wavelength. Photons with lower wavelength have more energy than those with longer wavelength. So, e.g. photons of UV radiation are richer in energy (stiffer) than photons of visible light. Photons of blue light have more energy than those of red light.

Energy density. We define it as a physical value, giving the amount of light energy (in Watts), impinging the surface unit (in square centimeters).

The energy density has its role in absorption, penetration through tissue, and in the subsequent biological response. Experimental studies showed that a light output of about 50 mW/cm^2 is sufficient for phototherapy purposes.

Penetration depth of light energy is its ability to spread in tissues. It depends on technical parameters of the devices used, skin cleanness and the characteristic features of polarized tissue, its density, chromatosis, amount of fat, etc.

According to the devices used, the penetration depth in the case of biolight is given in mm, in the case of laser in cm, in LED sources in cm.

Light absorption (energy absorption) by the tissue is the final and the most important process without which there would not be any photochemical reaction (chemical change caused by light absorption). The absorbed light energy provides the activation energy for chemical reactions (mostly in cell processes), involved in the body's reaction and response. It affects particularly cell membranes, strengthens the cell metabolism (substance transformation) and so it significantly influences the process of regeneration and healing. It also lowers pain perception and suppresses the inflammation process in tissues [11].

22.3 Biological Effect of Polarized Light on Living Organisms

When the skin is touched by polarized light, a small amount of light rays is reverberated (about 4%) and the rest penetrates into the tissue, where absorption and dispersion take place.

Dispersion and reverberation of some of the ingredients of tissue influence the spreading of light as well as its absorption in the tissue.

Effect of polarized light. Polarized light starts and supports control processes in cells and tissues and contributes to slowing down disease processes in the cells.

(a) ***Biostimulation effect*** concerns cell stimulation by light energy in the course of cell division in different tissues, optimalization of substance transformation and increase of the overall cell energy level or modification of this level to normal.

At the same time, biostimulation speeds up the regeneration of blood and lymphatic vessels and thus the vitality of the tissue. The final effect is revitalization and healing of the damaged tissue. Polarized light influences positively mainly newly built tissue, i.e. after being stimulated by light energy, scarred muscles reach far better tensile strength.

(b) ***Analgetic–antipain effect***. Pain – an effect associated with damaged tissue, is the symptom of many disorders and illnesses. Pain is perceived by sensors reacting to the permeability of the cell membrane. The pain information is brought by nerve fibers to the spinal cord and to the brain. The analgetic effect of polarized light lies in the local effect supported by mucous stimulation and

providing quickening of lymph drainage from the damaged area, stimulation of generation and release of substances – endogenic opiates – opinoids, thus helping to provide the requested analgetic effect, further there is slowing down the degeneration of nerve fibers, and decreasing nerve excitability by direct influence on teleneurons.

(c) ***Anti-inflammatory effect***. Inflammation is the protective reaction in the tissue to damage that can have different causes. The damaged cells release particular substances (histamine, serotonin and many others), local repletion is increasing as well as the permeability of blood vessels, and thus a higher amount of liquids is getting to the intercellular area and edema develops.

In this complex protection system, polarized light plays an important role with its therapeutic effects. Polarized light affects the whole range of inflammation symptoms: pain, heat, redness, swelling, and loss of function. Compared to current classical physiotherapeutic applications, phototherapy has the advantage that there is no thermic (heat) effect. With monochromatic polarized light of red color we can irradiate inflammations from the very beginning. The influence of polarized light on inflammatory changes lies in stimulation of humoral protection and activation of the immune protection of the body. The effect of polarized light on the organism is a complex process resulting in biostimulating, analgetic, and anti-inflammatory effects. The processes of the complex effects of polarized light proceed simultaneously.

22.4 Construction of a Polarization Device

(a) Source of light
(b) Polarizer – where the transformation of diffuse light, either monochromatic or polychromatic, to polarized light takes place.

Percentage of polarization defines the amount of emitted light energy of diffuse light transformed by the polarizer to polarized light. In biolight, as polarizers specially cut mirrors are used, where the angle to the source of diffuse light is called the polarization angle. In biolights, the so-called Brewster's mirror is used and the light reflection is in 56.97 degree angle – polarization angle. In lasers as polarizers, polarization crystals are used. In systems of polarization where LED is the light source, polarization foils are used [11].

22.4.1 Coherent and Incoherent Light

Incoherent light: the produced light after modification by the polarizer (reverberation or transition) is stage shifted – neither time nor space is synchronized. As to

the application site, it impinges with constant intensity and the light is of low consumption. Incoherent light is emitted from biolamps and from LED systems.

Coherent light: is time and space synchronized, it is of high energy concentration. It can be used in laser therapy.

22.4.2 Biolight

The main problem for polarization is in the mirror and its execution itself where the highest values of polarization are in the central part of the mirror and in direction of the edges polarization values become lower.

As light source bulbs are used – polychromatic source, which is emitting different wavelengths and each wavelength characterizes a particular color scale. Each of the colors has specific effects on tissues – we speak of schizophrenia of polychromatic light. For example the wavelengths of red and blue induce different biological responses of the tissue.

The problem of light source in the lamp is related to the technical inability of applying so-called pulse light regimens, known from laser and LED systems, in light therapy.

LED systems. As polarizer, a special silicon foil is used. Diffuse light is polarized by transition. In this system, polarization occurs equally via the whole area of the polarizer, represented by the polarization foil.

22.5 The Area of Utilization of Phototherapy

Research of the effects of polarization – either in laboratory or clinical conditions, has provided knowledge for the application of polarization in humans. From the beginning it was used in dermatology, orthopedics, rehabilitation and sports medicine, as well as cosmetics. Nowadays phototherapy is used practically in all areas of medicine, as e.g. in surgery, gynecology, pediatrics and general medicine [2, 3, 5, 9].

22.5.1 The Meaning of Colors

The healing energy of colors has been used for centuries. The old Egyptians used it in cosmetics as well as in medicine. Later on these methods were forgotten. With the start of modern medicine, it was discovered that each color has a particular wavelength, determining its penetration into tissues and its effect on the human body. It was also discovered that blind people can perceive colors. Light waves are reflected from a particular object into our eyes. Everything we see is reflected light. Low frequency light waves reach the brain via the eye nerve as red color, the

blue color is the result of high frequency light waves. With the influence of molecular structure and the coloring of each object the light rays are mixed, they absorb and reflect with different speed and intensity. Dark objects absorb more light waves and thus reflect less light into our eyes. This absorption creates an illusion of deeper and darker color. Lighter objects reflect more light and thus evoke an illusion of higher contrast and intensity. If daylight falls on a particular color of the object, all the light rays are absorbed in an appropriate way. So the green object catches all the rays, separates the green color frequency and then it is sent to our eyes. The object is perceived as green.

When the light is refracted (reflected or absorbed) to different wavelengths, different colors are created. In the white light spectrum the whole range is involved. The classical proof is the well-known natural phenomenon – the rainbow. This can be shown in the laboratory by refraction of the white light on a grid. This demonstrates the mentioned color spectrum white color refracts. It is created by six colors: ***red, orange, yellow, green, blue and purple***. The visible spectrum is between 380 nm and 780 nm and the colors there have the galaxy amount of tones and variations. Some of the colors can easily influence the higher frequency of the brain because they have high light energy. Other colors can affect the system and the energy of our body (which works slowly) as their frequency is lower. To understand it better, we have to perceive our body as an energy system. Everything alive is created by vibrations. These vibrations are the result of electron and proton movement of each atom in each molecule of every substance in the universe. Vibrations are present in objects, living creatures as well as in the surrounding atmosphere.

All organs, tissues and systems in our body consist of similar vibrating atoms. When such a rhythm is broken (e.g. by disease), the body needs a suitable stimulus to help it recover the original balance. Such stimulus is the light energy of colors *(vibration healing means)* affecting the human body and helping to stabilize the physical and mental state. If the right amount of energy is concentrated in the affected area, we can positively influence the balance there. After reestablishing the balance, the body itself can (with its immune system) manage to get rid of the negative effects blocking the healthy functioning of the organism.

Red color: 620–630 nm. Red color is the representative of so-called warm colors. The energy of this color has the greatest ability of biostimulation. It speeds up the blood circulation as well as other body liquids and is therefore used in cases with problems of blood circulation. Red acts as an energizer. Red color is the first to appear in the visible spectrum. It has the longest wavelength (the shortest frequency) from all the color rays. Red thus means the color of life, the color of the sun, fire, love, happiness, but also of anger and malice, it means power, energy and life strength. Red stimulates when there is lack of energy. It supports the production of erythrocytes, stimulates the heart, breathing, as well as secretion, widens the vessels and supports physiologic congestion. Red light involves the "growth rays" and is therefore used in injuries and wounds (while the wound festers, the blue colour is also used for disinfection). The energy of red is effective in inflammations,

skin diseases, burns, low blood pressure, chronic cough, asthma, throat affections, conditions of the urocyst, and in problems of digestion, sterility, frigidity [3, 5, 6].

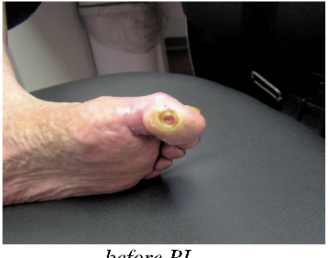
Unsuitable use of red color. It is not recommended to apply red light in fever states and acute infection, since activation and faster spread of the infection may result. Neither is it suitable to apply red light in the area of the thyroid gland with hyperfunction. Scars after thyroid gland surgery are not recommended for treatment sooner than after 6 months, particularly not without consulting the doctor.

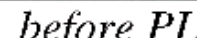
Blue color: 400–490 nm. The light of this color has calming effects – blue is considered to be a cold color. It slows down the pulse frequency, helps the overloaded vessels to get into normal state, acts as an antiseptic, kills pain and cools. For children it is one of the best healing colors. It has chemically acting rays that can be called absorptive, "breaking". Blue provides calmness and strengthens life power, calms down nervousness and the feeling of fear.

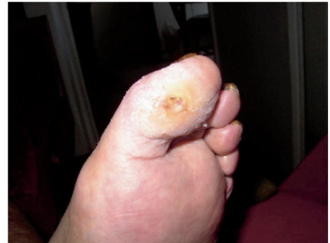
Thearpeutic use of blue color. All high temperature states require blue light for healing and curing. It affects positively festering processes, pain and congestion, bleeding and sleeplessness. Blue also affects the hormonal system of men and women. It is used in painful states of women during menopause. It has great effects in lowering blood pressure and acts as a spasmolytic. It efficiently helps in children's diseases, asthma, varicella, jaundice and rheumatism. Blue is very efficient in combination with red. Blue light can also help with hyperfunction of the thyroid gland, neck pain, fever, high blood pressure, hyperactivity, inflammation, burns, skin infections, eczemas, stomach ulcers, colic, back pain, hemorrhoids, eye infections and sore jaw bones. It positively influences vessel narrowing and supports the functions of the adrenal gland.

Unsuitable use of blue color: muscle cramps, paralysis, problems with congestions.

Green color: 520–560 nm. Green is the most frequently occurring color in nature. It is considered to be neutral. The energy of green light keeps the balance and harmony and is important for the nervous system and the general health state. It strengthens the nerves, provides inner calmness and relaxation. It has alleviating effects mainly in inflammatory and painful conditions. Green affects positively problems with nerves, gout, bronchitis, and whooping cough. It promotes the formation of osseous tissue, acts favorably on the skin in seborrhea and acne. Green

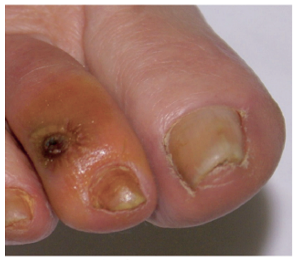






after PL

Fig. 22.1 Wound healing after 3 months polarized light effect on diabetic big toe [10]



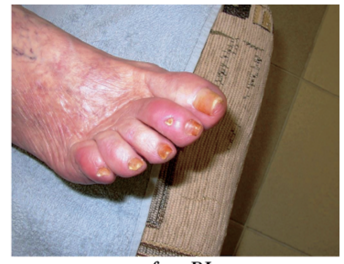


Fig. 22.2 Wound healing after 3 months polarized light effect on diabetic finger [10]

color reduces heart pain, compensates high blood pressure, exhaustion effects and relieves fatigue. It is used in cases with ulcers, cysts and eye problems. Sleeping disorders can be positively affected with green color.

The significance of phototherapy. It is a noninvasive method by which optimal energy rates can be brought about on the level of cell membranes and this state can initiate the regeneration of biochemical reactions of the cell. The application of polarized light is an energy optimizer of the cell membrane both in therapy and prevention, the latter mainly in patients at risk. The effect of polarization on ATP production and free radical reduction is known.

Advantages of using phototherapy: noninvasive, wide range of indications with high efficiency, no side effects, minimum of contraindications, possibility to combine phototherapy with classical and other therapeutic methods, contact-free and sterile application, availability of phototherapy for everyone (no matter at what age), possibility to use it at home – comfortable, fast and cheap.

22.6 Possible Complications During Treatment with Polarized Light

During therapy, several accompanying phenomena may appear, yet they do not necessarily require interruption of treatment. Most of these complications are only temporary and after a break of 24 h without complications, shortened application to the affected site and change in the regimen from pulse to continual can eliminate the undesired effects. When there is bleeding during healing of wounds – a phenomenon that can occur mainly in the case of open wounds (e.g. open ulcer), the direction of the light has to be changed. Bleeding does not present a reason for stopping the treatment, only the application process and the position have to be changed. Such complications can be eliminated by not applying the light right to the middle of the affected area but rather by circular motions (counter-clockwise) on the edge of the defect. This mode is recommended because healing advances

from the edges to the middle and thus this application is the most suitable one. Fresh postsurgery wounds – when polarized light is used, the principles of application in scar healing not complicated by infection should be respected. Treatment is better started after the stitches had been removed, the wound is closed and bleeding is not likely to set in. The healing process by polarized light will not be broken by the removal of stitches. Increased painfulness is a possible complication occurring in about 15% of treated patients, mainly in the case of chronic diseases of the locomotion apparatus (e.g. arthrosis or rheumatic joint changes) and joint disability within a systemic disease.

The problems are not of such a range and intensity that we should interrupt treatment. After 24 h they usually disappear and the required analgetic effect sets in. Despite these problems, the healing process is already taking place in the treated part. In light systems with continual and pulse regimen, we recommend to start first with the continual regimen, while the pulse regimen is suitable after 7–10 applications. Increased itching – in tetter healing mainly around the ear, sometimes after one of the main symptoms of the illness, i.e. itching, has ceased, it reappears after some time. The reason is one of the features of polarized light, namely its drying effect. Therapy with polarized light directed into the ear has to be supported by using common oils for ear treatment (e.g. castor oil).

Photosensitivity – application of certain substances activated by a particular wavelength (mainly UV sources) can evoke unpleasant reactions, mainly abnormal cutaneous responses. In these cases the use of polarized light should be stopped as long as the photosensitizing medicine is administered.

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22.7 Polarized Light and CoQ_{10} Effect in Mitochondria (Pre-Clinical Study)

Anna Gvozdjáková, Jarmila Kucharská, and Ján Pálinkáš

Abstract Simultaneous effect of red polarized light and liposomal solution of CoQ_{10} (LiQ-Sorb) supplementation re-energize mitochondrial ATP production via oxidative phosphorylation in brain, heart and skeletal muscle and affect coupling of oxidation and phosphorylation and regeneration of mitochondrial membrane integrity. We assume that liposomal CoQ_{10} with the effect of polarized light could be used as supplementary therapy in diseases with mitochondrial dysfunction.

Keywords Coenzyme Q_{10}, mitochondria, polarized light

Polarized light (PL) therapy belongs to the phototherapeutics or light therapy methods. Light therapy has been used in infrared and UV rays for a long time. Over the last years, biological effects of a single color from the visible spectrum have been described. Red color from visible light (610–760 nm) delivering energy from light photons, increases cell and mitochondrial metabolism. Different in vivo 2-week effects of PL on brain mitochondrial function were documented recently. While NAD-linked respiratory chain function (state 3, rate of ATP production) was significantly stimulated, FAD-linked respiratory chain function was inhibited (state 3, state 4, rate of ATP production). PL decreased the concentration of coenzyme Q_9 and CoQ_{10} in brain mitochondria [3, 4]. A further study confirmed our assumption, that simultaneous supplementation with CoQ_{10} and PL effect could improve also FAD-linked mitochondrial respiratory chain function.

22.7.1 Mitochondrial Mechanisms of Polarized Light Therapy

Several investigators demonstrated increased ATP synthesis by irradiation, mostly on the basis of in vitro studies. Karu in 1988 was the first to propose the hypothesis of photon therapy mechanism at cellular and mitochondrial level: after primary reactions in mitochondria (absorption of light monochromatic visible and near infrared radiation causes changes in redox properties of respiratory chain components and acceleration of electron transfer), the cascade of secondary reactions of eukaryotic cells follows, involving the cytoplasm, nucleus, cell membrane, DNA and RNA synthesis and cellular adhesion [6].

Two types of photoreceptors of the inner mitochondrial segment were described: rod inner segment (RIS) and cone inner segment (CIS). Mitochondrial CIS photoreceptor produces more ATP than RIS. CIS photoreceptor increases the surface membrane area of cristae and the number of mitochondria at a higher rate than does RIS [5].

Table 22.1 Effect of liposomal coenzyme Q_{10} (Li-Q-Sorb) and polarized light on mitochondrial function in rats

Groups	Control (mean ± sem)	Q + PL (mean ± sem)	PL (mean ± sem)
Brain mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	0.235 ± 0.029	0.429 ± 0.031***	0.396 ± 0.031**
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.117 ± 0.014	0.231 ± 0.023***	0.169 ± 0.016
Complex I			
S_3 (nAtO/mg prot./min)	41.02 ± 1.02	47.56 ± 1.69**	46.84 ± 1.33**
S_4 (nAtO/mg prot./min)	11.37 ± 1.15	12.49 ± 0.51	12.36 ± 0.53
OPR (nmol ATP/mg prot./min)	107.14 ± 6.38	13512 ± 6.25**	135.88 ± 5.10***
Complex II			
S_3 (nAtO/mg prot./min)	64.30 ± 1.78	62.79 ± 4.05	72.52 ± 2.42ms
S_4 (nAtO/mg prot./min)	30.39 ± 2.29	24.05 ± 1.91ms	52.68 ± 2.03***
OPR (nmol ATP/mg prot./min)	106.34 ± 5.62	121.34 ± 7.92	112.89 ± 3.86
Myocardium mitochondria			
$CoQ_{9\text{-}OX}$ (nmol/mg prot.)	2.478 ± 0.245	2.337 ± 0.331	3.798 ± 0.414**
$CoQ_{10\text{-}OX}$ (nmol/mg prot.)	0.264 ± 0.021	0.196 ± 0.021ms	0.289 ± 0.043
Complex I			
S_3 (nAtO/mg prot./min)	91.17 ± 5.30	141.18 ± 9.02***	99.73 ± 3.99
S_4 (nAtO/mg prot./min)	13.55 ± 0.88	26.33 ± 1.55***	22.79 ± 1.57***
OPR (nmol ATP/mg prot./min)	254.00 ± 22.31	444.91 ± 20.05***	308.76 ± 15.97ms
Complex II			
S_3 (nAtO/mg prot./min)	131.29 ± 9.01	182.36 ± 11.28***	122.15 ± 5.20
S_4 (nAtO/mg prot./min)	60.90 ± 6.22	69.75 ± 4.25	78.04 ± 3.03**
OPR (nmol ATP/mg prot./min)	198.25 ± 9.08	332.18 ± 15.95***	222.04 ± 1265

Abbreviations: statistical evaluation, Student's t-test, * $p < 0.01$– very significant, $p < 0.001$ – extremely significant, ms–marginally significant

Review [1] provides evidence that red light increases cellular activity. Irradiation with light between 660–680 nm increases electron transport in purified cytochrome oxidase to increase in turn mitochondrial respiration and ATP production in isolated mitochondria. Near infrared light provokes the mitochondrial signal for acceleration of wound and retinal healing.

In our study we compared the simultaneous effect of liposomal CoQ_{10} and polarized light on 3-month-old rats in vivo during 4 weeks, in the daily dose of 40 mg CoQ_{10}/kg body weight and two times 10 min PL effect. Each group consisted of 8–10 animals. The results were statistically evaluated in comparison with control animals. Means and SEM (±) of values are given in Table 22.1. (For review of polarized light see Chapter 22.)

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