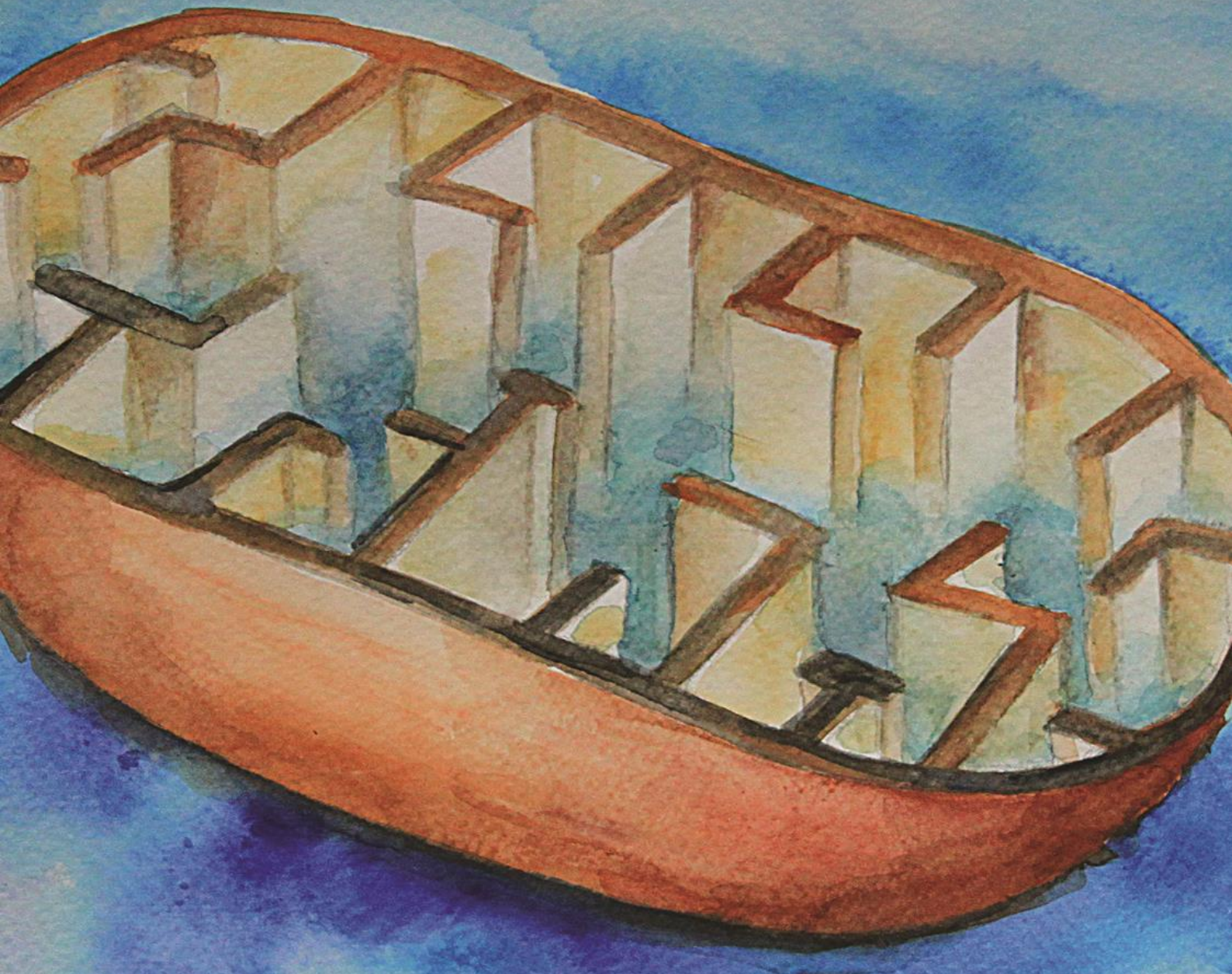
Serbian Society for Mitochondrial and Free Radical Physiology

Fourth Congress

CHALLENGES IN REDOX BIOLOGY



BOOK OF ABSTRACTS

September 28-30. 2018.
Belgrade, Serbia

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Dear Colleagues,

The Fourth International Congress of the Serbian Society for Mitochondrial and Free Radical Physiology is held September 28-30, 2018 at Rectorate Palace of the University of Belgrade, as a part of the celebration of the University of Belgrade's 210th anniversary.

Life is a challenge, and redox biology can help us to understand it. The twenty-first century may be the century of the bloom of redox biology.

The International Congress of the Serbian Society for Mitochondrial and Free Radical Physiology aims to be a meeting place of scientists from around the world, enable exchange of opinions and knowledge and create a pleasant ambience for young scientists who step towards **Challenges in Redox Biology**.

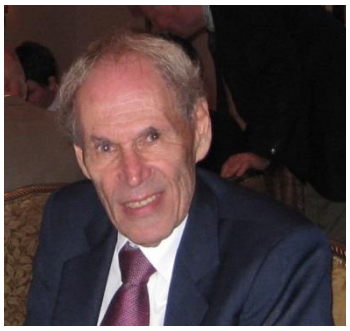
The Serbian Society for Mitochondrial and Free Radical Physiology is grateful to everyone who creates this scientific challenge.

The organizing committee has one more challenge, to present Belgrade and Serbia as good hosts, and to gather again at the biennial meeting of the Society for Free Radical Research Europe, which will be held in 2020 in Belgrade.

Sincerely,

Bato Korac
On behalf of the Organizing Committee

In memoriam: Professor Lester Packer



Professor Lester Packer passed away on July 27th 2018. He was born in New York and earned his PhD in Microbiology and Biochemistry from Yale University. During his post doctoral studies he started working on mitochondria and biological oxidation at the University of Pennsylvania. In the early 1960's he joined as professor of Molecular and Cell Biology the University of California at Berkeley. During 40 years he was the Head of the Packer Lab at UC Berkeley. Professor Lester Packer was a pioneer in the field of Free Radical and Antioxidant Research. He is at the origin the Antioxidant Network Concept discovering the redox cycling of vitamin E by vitamin C. He showed that exogenous lipoic acid is one of the most potent antioxidant known and is able to regenerate vitamins C and E. Professor Lester Packer has attracted in the Packer Lab many researchers from all over the world. He was a mentor of numerous great scientists in the field of Free Radical in Biology and Medicine. In 2000 he became adjunct Professor of Pharmacology and Pharmaceutical Sciences at the University of Southern California and pursued studies on the biological action of antioxidants and their benefits for Human Health.

Professor Packer published more than 700 scientific papers that generated 33,000 citations and more than 100 books on Antioxidants and Health. He was the editor of the series Oxidative Stress and Disease. In 1994 Professor Packer founded the Oxygen Club of California (OCC) and served as President for many years. Afterward he was honorary President of OCC. He was also President of the Society for Free Radical Research International (SFRRI). Professor Packer organized numerous international meetings in America, Europe, Asia with the aim to enhance interactions between researchers interested in Free Radicals and Antioxidants in Biology and Medicine. He always encouraged and supported the young investigators with numerous awards.

In 2007 Professor Packer was honored by the French Republic as Chevalier (knight) of the National Order of Merit. He was promoted as Officer in 2012.

Apart from his work he shared with his wife Anne a passion for the sail.

This eminent scientist was also a great humanist. His exceptional interpersonal skills led him to have many friends worldwide.

The scientific work bequeathed by Professor Packer is immense. His enlightened and avant-gardist spirit has significantly contributed to the knowledge of Redox Biology.

For all those who are concerned by the progress of Science, Professor Lester Packer will remain a reference.

Professor Emeritus Josiane Cillard
President of SFRR-Europe

PLENARY LECTURES

THE INTERACTIONS BETWEEN NITRIC OXIDE AND OXYGEN AT THE MITOCHONDRIA. BIOLOGICAL IMPLICATIONS

Salvador Moncada

Manchester Cancer Research Centre, Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, 555 Wilmslow Road, Manchester M20 4QL, UK

Nitric oxide (NO) inhibits cell respiration reversibly and in competition with oxygen (O_2) through the inhibition of the mitochondrial cytochrome-*c* oxidase (Complex IV). At concentrations lower than those required to inhibit respiration, endogenous NO enhances the reduction of the electron transport chain, thus enabling cells to maintain their O_2 consumption. This facilitates the release of superoxide anion, which initiates the transcriptional activation of NF-κB as an early signal of a stress response. Through free radical formation, long-term inhibition of mitochondrial respiration by NO leads to persistent inhibition of Complex I. This is dependent on the S-nitrosylation of a specific thiol in the active form of this protein. S-nitrosylation of Complex I might indicate the early stages of a pathological process. Inhibition of respiration in cells with glycolytic capacity leads to an increase in mitochondrial membrane potential that acts as a cell defence mechanism and is dependent on the generation of glycolytic ATP. The increase in glycolysis is the result of the activation of the enzyme AMP kinase and the subsequent activation of the enzyme PFKFB3 that generates fructose-2-6-phosphate, which is a powerful allosteric activator of the glycolytic enzyme PFK1. Neurones, which have little or no quantities of PFKFB3 are unable to activate this mechanism when their mitochondria are inhibited or at low O_2 concentrations and therefore die rapidly in apoptosis. The implications of all these findings will be discussed.

ADVENTURES WITH ERGOTHIONEINE

Barry Halliwell
*Chairman, Biomedical Research Council, A*STAR Senior Advisor, Academic Appointments and Research Excellence, National University of Singapore (NUS), Singapore*

Oxygen free radicals and related "reactive oxygen species" (ROS) are fundamental to survival; they help drive evolution yet the damage that they can do ("oxidative damage") is involved in most, if not all, human diseases and in ageing itself. My earliest contribution to this field was to elucidate the pathway used by plants to remove hydrogen peroxide (H_2O_2) (the ascorbate-glutathione cycle). Plants are key to human life; they supply us with oxygen, they provide a variety of nutrients with antioxidant abilities, and diets rich in plants lower the risk of developing many diseases, including diabetes, atherosclerosis, dementias and stroke. Exactly why is uncertain. Despite the key role of oxidative damage, there has been a general lack of effectiveness of supplements of such "classical" antioxidants as ascorbate, vitamin E and β-carotene in decreasing risk or severity of human disease. There are multiple reasons for this, one being that these antioxidants are often ineffective in decreasing levels of oxidative damage in humans. They work better in cell culture and in rodent models (which questions the relevance of some rodent models of human disease, and cell culture studies can generate many artefacts). So how then can we minimize oxidative damage in the human body? Strategies will be discussed. Much of our research now focuses on ergothioneine, a diet-derived antioxidant that is avidly retained by the human body and particularly accumulated at sites of tissue injury, where it may help to diminish tissue damage. We have conducted a detailed study of how ergothioneine behaves when administered to humans or mice. Ergothioneine is made by fungi and some bacteria, although the list of those able to make it grows daily. Data on the relevance of ergothioneine to neurodegenerative diseases, and other conditions will be presented.

ROLE OF GLUTATHIONE IN EPIGENETIC REGULATION

José Luis García-Giménez, Carlos Romá, Federico V. Pallardó
Faculty of Medicine and Dentistry, University of Valencia-CIBERER-INCLIVA, Valencia, Spain

Epigenetics is a powerful regulatory network of mechanisms at the DNA, histone or RNA level that regulate gene expression. We have found that gamma-L-glutamyl-L-cysteinylglycine, glutathione (GSH), a physiological antioxidant and second messenger in cells, is a new post-translational modifier of the histone code that is able to change the stability of the nucleosome and the opening of chromatin. But possibly, the role of GSH in epigenetics goes beyond a mere structural function. A growing number of reports support the hypothesis that there is a link between GSH metabolism and the control of epigenetic mechanisms by controlling the substrate availability, enzymatic activity for DNA methylation, changes in the expression of microRNAs, or through its role in the histone code. We will show the molecular pathways by which GSH, or changes in the oxidative stress balance can control epigenetic events and the mutations in enzymes involved in GSH metabolism. We will give in our presentation different examples. There is a growing number of diseases induced by aberrant epigenetic regulation. It is of paramount importance to elucidate the intricate network between GSH metabolism, oxidative stress and epigenetics. New approaches in this field would contribute to the development of new therapeutic strategies to treat several types of cancer and neurodegenerative and metabolic pathologies.

HEALTH-ENHANCING EXERCISE AND REDOX HOMEOSTASIS: CORRELATION WITH GENOMIC MODIFICATIONS

Daniela Caporossi

Department of Movement, Human and Health Sciences, University of Rome "Foro Italico", Rome, Italy

Physical activity has been demonstrated to be effective in the prevention and treatment of different chronic conditions, including type 2 diabetes (T2D). In particular, several studies highlighted how the beneficial effects of physical activity may be related to the stability of the DNA molecule, such as longer telomeric ends. We analyzed the effect of exercise training on telomere length, spontaneous and H_2O_2-induced DNA damage, as well as the apoptosis level in leukocytes from untrained or trained T2D patients vs. age-matched control subjects (CS) (57-66 years). Moreover, expression analysis of selected genes belonging to DNA repair systems, cell cycle control, antioxidant and defence systems was performed. Subjects that participated in a regular exercise program showed a longer telomere sequence than untrained counterparts. Moreover, *ex vivo* treatment of leukocytes with H_2O_2 highlighted that: (1) oxidative DNA damage induced similar telomere attrition in all groups; (2) in T2D subjects, physical activity seemed to prevent a significant increase of genomic oxidative DNA damage induced by chronic exposure to pro-oxidant stimulus, and (3) decreased the sensitivity of leukocytes to apoptosis. Finally, the gene expression analysis in T2D subjects suggested an adaptive response to prolonged exercise training that improved the response of specific genes.

ENVIRONMENTAL NOISE IS A CARDIOVASCULAR RISK FACTOR – MECHANISTIC INSIGHTS ON OXIDATIVE STRESS AND INFLAMMATORY PATHWAYS FROM STUDIES IN MICE

Andreas Daiber[1], Swenja Kröller-Schön[1], Sebastian Steven[1], Katelyn Frenis[1], Frank P. Schmidt[1], Matthias Oelze[1], Sanela Kalinovic[1], Ksenija Vujacic-Mirski[1], Erwin R. Schmidt[2], Steffen Rapp[2], Thomas Münzel[1]

[1]*Center for Cardiology I, Molecular Cardiology, University Medical Center Mainz, Mainz;* [2]*Institute for Molecular Genetics, Johannes Gutenberg University, Mainz, Germany*

Large epidemiological studies (reviewed in Münzel…, Daiber, J. Am. Coll. Cardiol. 2018; Antioxid. Redox Signal. 2018) point towards a link between the incidence of arterial hypertension, ischemic heart disease, metabolic disease and exposure to noise, supporting its role as an independent cardiovascular risk factor. Recently, the mechanistic parallels in the pathophysiological processes induced by noise and classical cardiovascular risk factors/diseases were discussed in detail (Münzel and Daiber, Antioxid. Redox Signal. 2018). We characterized the underlying molecular mechanisms leading to noise-dependent adverse effects on the vasculature in an animal model of aircraft noise exposure identifying oxidative stress and inflammation as central players in mediating vascular dysfunction (Münzel, Daiber et al., Eur. Heart J. 2017). Peak sound levels of 85 and mean sound level of 72 dB(A) applied for 1, 2 and 4d caused an increase in systolic blood pressure, stress hormones and induced endothelial dysfunction, oxidative stress and inflammation. Control exposures to white noise for 4d did not induce these changes. In a subsequent study, we revealed a crucial role of Nox2 (by using gp91phox$^{-/-}$ mice) for the pathogenesis of noise-induced vascular dysfunction and the associated underlying neuroinflammation and cerebral oxidative stress (Kröller-Schön, Daiber et al., Eur. Heart J. 2018). Noise-induced damage was more pronounced in mice that were exposed to noise during the sleep phase than during the awake phase. Next generation sequencing revealed substantial alterations of gene signaling networks including the circadian clock and FoxO3 (also confirmed by pharmacological FoxO3 activation) pathways. This animal model enables future studies of molecular mechanisms, mitigation strategies and pharmacological interventions to protect from noise-induced vascular damage.

Financial support by the Foundation Heart of Mainz and the Boehringer Ingelheim Foundation „Novel and neglected cardiovascular risk factors: molecular mechanisms and therapeutic implications" is gratefully acknowledged.

ABDOMINAL AORTIC ANEURYSM (AAA): IS THERE A ROLE FOR THE PREVENTION AND THERAPY USING ANTIOXIDANTS?

Joël Pincemail, Jean-Olivier Defraigne, Audrey Courtois, Adelin Albert, Jean-Paul Cheramy-Bien, Natzi Sakalihasan

Department of Cardiovascular Surgery, Surgical Research Center (CREDEC) and Plateforme Nutrition Antioxydante et Santé. University Hospital of Liège, Sart Tilman, 4000 Liège, Belgium

Abdominal aortic aneurysm (AAA) is a degenerative disease that causes mortality in people aged > 65 years. Increased reactive oxygen species (ROS) and oxidative stress seem to play a pivotal role in AAA pathogenesis. Several sources of ROS have been identified in aortic tissues using experimental models: inflammation, increased activity of NAD(P)H oxidase (NOX), over-expression of inducible nitric oxide synthase (iNOS), uncoupled endothelial nitric oxide synthase (eNOS), platelets activation and iron release from hemoglobin. Human studies confirmed that oxidative stress and endothelial dysfunction, an important source of ROS production, were well associated with AAA development. Reducing oxidative stress by antioxidants can therefore be a good strategy for limiting AAA development. There is currently no evidence showing that strategies using classical low molecular weight antioxidants (vitamins C and E, β-carotene) as target for ROS is effective to reduce human AAA progression. However, recent laboratory experiments and epidemiological data have highlighted the positive role of polyphenols and a diet enriched in fruits which contains high amounts of antioxidant polyphenols. By their ability to restore endothelial function and also their capacity to stimulate enzymatic antioxidants through activation of the Keap1/Nrf2/ARE pathway, polyphenols can represent a promising treatment target for reducing human AAA progression. Clinical studies are therefore urgently necessary to confirm the potential beneficial effect of polyphenols in preventing or limiting AAA (Current Drugs Target, 19, 2018).

NEUROVASCULAR COUPLING MEDIATED BY NEURONAL NITRIC OXIDE IN HIPPOCAMPUS MIGHT BE SUSTAINED BY ASCORBATE AND NITRITE UNDER HYPOXIC CONDITIONS

João Laranjinha, Cátia F. Lourenço, Nuno R. Ferreira, Rui M. Barbosa
Center for Neuroscience and Cell Biology and Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

Neurovascular coupling is a highly regulated process that is critical for providing active neurons with bioenergetic substrates. Failure in neurovascular coupling, either during aging and disease (Alzheimer's disease, AD) or following acute hypoxic conditions, compromises brain integrity and functionality. The regulation of neurovascular coupling is under the concerted cooperation of the cells comprising the neurovascular unit. However, the complementary task of identifying modulators of NO activity on neurovascular coupling has remained largely underappreciated. We have come to conjecture that the redox and functional interplay of nitric oxide with ascorbate and nitrite would modulate the functionality of glutamatergic synapses in terms of neurovascular coupling. By using a multimodal approach to probe the dynamics of NO, ascorbate and cerebral blood flow *in vivo* in hippocampus of Wistar and Fisher 344 rats and of a triple transgenic mice model of AD we support that (1) neuronal-derived NO acts as a direct mediator of neurovascular coupling, (2) upon glutamatergic stimulation, volume signaling by NO is an intrinsically controlled mechanism due to increased blood flow, (3) neurovascular coupling is impaired in AD and aging due to vascular dysfunction, (4) under acidic/hypoxic conditions, nitrite is reduced by ascorbate to NO and (5) the redox interaction of nitrite/ascorbate/NO contributes to neurovascular coupling. Given that nitrite increases NO bioavailability and augments cerebral blood flow in hippocampus one may envisage that dietary nitrate via the nitrate:nitrite:NO pathway may help sustaining neurovascular coupling in aging and disease.

Supported by POCI-01-0145-FEDER-029099.

OXINFLAMMATION IN RETT SYNDROME

Giuseppe Valacchi[1,2], Alessandra Pecorelli[2], Carlo Cervellati[3], Joussef Hayek[4]
[1]Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy; [2]Department of Animal Science, NCSU Research Campus, Kannapolis, NC; [3]Department of Biomedical and Specialist Surgical Sciences, University of Ferrara, Ferrara, Italy; [4]Child Neuropsychiatry Unit, University Hospital, Azienda Ospedaliera Universitaria Senese (AOUS), Siena, Italy

Rett syndrome (RTT) is an orphan progressive neurodevelopmental disease affecting almost exclusively females (frequency 1:10,000). RTT clinical expression is typically characterized by loss of purposeful hand movements, severe mental retardation and motor impairment, breathing disorders, ataxia and increased risk of sudden death. Although the main genetic cause, i.e. mutation in the methyl-CpG binding protein 2 gene (MECP2), has been already identified, the molecular and pathogenic mechanisms by which MECP2 deficiency drives pathology in RTT remains not fully understood. A wealth of evidences from our and other laboratories suggests a potential causal relationship between MECP2 dysfunction and systemic redox imbalance, a condition that has been widely found in association with RTT. In turn, a “short-circuit” of redox pathways may contribute to the systemic immune dysfunction expressed as cytokines/chemokines deregulation, a feature clearly emerged from two recent studies on RTT patients. In conclusion the undisclosed deregulation of the immune system in mutual combination with a persistent abnormal redox balance predisposes the individuals affected by the disorder to a chronic and subclinical inflammatory state. These findings open novel windows and targets for future therapy against this devastating and still drug orphan neurodevelopmental disorder.

MITOCHONDRIA, METABOLIC ARREST AND STRATEGIES OF ADAPTATION TO ENVIRONMENTAL STRESS

Kenneth B. Storey
Institute of Biochemistry, Carleton University, Ottawa, Canada

Mitochondria are not just the powerhouses of the cell; these organelles are crucial components of cellular physiology and influence many central metabolic and signaling pathways that support complex multicellular life. Not surprisingly, they play vital roles in biochemical adaptation to support animal survival under extreme environmental conditions - e.g. freezing, anoxia, dehydration, and winter hibernation. One common component of most extreme survival strategies is strong metabolic rate depression (MRD) that requires coordinated global suppression of metabolism as well as reprioritization of most metabolic processes. To facilitate MRD, adaptations of multiple aspects of mitochondrial function are required, including energetics, antioxidant defenses, gene regulation, and enzymatic controls. This talk discusses these and other factors with a focus on new directions in stress-specific controls on mitochondrial functions including gene and transcriptional regulators, novel mitochondria-encoded peptides, microRNA action, epigenetic controls, and new discoveries about the control of Krebs cycle and urea cycle enzymes via posttranslational modifications.

REDOX REGULATION OF PROTEOLYSIS IN METABOLIC DISEASES

Tilman Grune
Molecular Toxicology, German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany

Proteins which are oxidatively modified are degraded by the 20S proteasome in an ATP- and ubiquitin-independent pathway seemingly with the support of the chaperone system. If the proteasomal system is overwhelmed oxidized proteins aggregate, form a hydrophobic yellow-brownish material that is taken up into the lysosomal compartment by macroautophagy. One of the dominant effects of cytosolic, cross-linked proteins is the inhibition of the proteasomal system. The efficiency of proteasomal inhibition seems to be linked to a direct binding of the proteasome and is dependent on the binding efficiency. Importantly, the proteasomal inhibition is accompanied by a modulation of several regulatory pathways. The original concept that these protein aggregates are merely waste products of cellular metabolism has to be overthrown, since mounting evidence demonstrates an active participation of such protein aggregates in several physiological and pathophysiological cellular responses, relevant for aging and diseases. Most of the protein aggregation takes place in postmitotic or non-dividing cells; many of them are tightly related to the metabolic performance of the organism. This includes skeletal muscle, cardiomyocytes and the beta-cells of the pancreas. Interestingly age-related changes of the redox status of these cells are associated to the proteolysis regulation and oxidized protein accumulation. Metabolic stress can also induce proteolysis dysregulation, as well in the proteasomal as in the autophagic-lysosomal system. So, exposure to an excess of lipids, can lead to a dysregulation of the proteasomal system in pancreatic beta-cells this is leading to an accumulation of oxidized proteins. Oxidized, cross-linked proteins in turn are disturbing the insulin release from these cells and, therefore, influencing the metabolism of the whole body. Such conditions of metabolic dysfunction is leading also to a proteolysis malfunction in cardiomyocytes, influencing here the contractile functions of the heart. Further studies to reveal the functional importance of proteolysis modulation and protein aggregate formation in age-associated metabolic disease is required in order to develop effective strategies for therapeutic interventions.

KNOCKOUT OF DAPIT PROTEIN DISRUPTS ATP SYNTHASE OLIGOMERISATION AND HAS A PROFOUND ROLE IN REGULATION OF GLUCOSE HOMEOSTASIS

Petr Pecina[1], Hana Nuskova[1], Jana Kovalcikova[1], Lukas Alan[1], Alena Pecinova[1], Vaclav Zidek[1], Vladimir Landa[1], Vilma Kaplanova[1], Frantisek Kolar[1], Frantisek Papousek[1], David Habart[2], Ludmila Kazdova[2], Marie Rodinova[3], Kristyna Bardova[1], Katerina Tauchmannova[1], Zdenek Drahota[1], Michal Pravenec[1], Josef Houstek[1], Tomas Mracek[1]

[1]Institute of Physiology, Czech Academy of Sciences; [2]Institute of Clinical and Experimental Medicine; [3]1st Faculty of Medicine, Charles University, Prague, Czech Republic

F_oF_1-ATP synthase is the key enzyme of mitochondrial energy provision, responsible for production of most of the cellular ATP. Recently, small 7 kDa proteolipid DAPIT, originally recognised as "diabetes associated protein in insulin sensitive tissues" (also termed Usmg5), has been found to be loosely attached to the enzyme, but its biological role is largely enigmatic. To elucidate the importance of this novel protein we produced zinc-finger rat knockout model of DAPIT deficiency on unique SHR background. DAPIT$^{-/-}$ animals were fully viable and contrary to previous data on cell lines, we observed normal levels of fully assembled ATP synthase, however, it was predominantly present in the monomeric form. Contrary to proposed role of ATP synthase dimers in mitochondrial cristae formation, we observed only minor changes in cristae morphology in heart of DAPIT$^{-/-}$ animals. We observed analogous phenotype of ATP synthase dimers absence and almost normal cristae morphology in HEK293 DAPIT knockdown model, further verifying our animal observations. From the biochemical standpoint, we observed mild isolated ATP synthase deficiency in DAPIT$^{-/-}$ animals. Both ADP phosphorylating and ATP hydrolyzing activities were reduced by circa 10% in studied tissues, i.e. liver and heart. DAPIT$^{-/-}$ animals had 20-30% lower body weight and pronounced decrease in total adiposity (by 40%). Based on indirect calorimetry, DAPIT$^{-/-}$ animals preferred utilisation of glucose to other substrates. This was also replicated at the tissue level, with higher glucose oxidation in DAPIT$^{-/-}$ skeletal muscle. Serum levels of glucose were unchanged in both fed and fasted state, but DAPIT$^{-/-}$ animals were significantly more insulin sensitive with decreased levels of serum insulin as well as area under curve in OGTT test. This is due to the improved peripheral insulin sensitivity, as glucose-stimulated insulin secretion from pancreatic islets was normal in DAPIT$^{-/-}$ animals. High fat diet led to further dissociation of phenotype between control and knockout animals. In conclusion, absence of DAPIT protein leads towards preferential oxidation of glucose, increases insulin sensitivity and decreases total adiposity in rat. In addition, it implicates for the first time that mitochondrial ATP synthase can be directly involved in regulation of glucose homeostasis.

Supported by Czech Science Foundation grant 16-01813S.

OXYGEN, SULFUR, SELENIUM AND LIPID RANCIDITY: HOW GPx4 CONTROLS CELL LIFE AND DEATH

Fulvio Ursini, Matilde Maiorino

Department of Molecular Medicine, University of Padova, Padova, Italy

Ferroptosis (FPT) is a form of regulated cell death (RCD) operated lipid peroxidation (LPO). Since LPO is initiated by lipid alkoxy radicals produced from lipid hydroperoxides- irrespective the mechanism of their formation - by ferrous iron complexes, the sole enzymatic reaction competent for inhibition of LPO is the reduction of lipid hydroperoxides by the selenoenzyme GPx4. No other antioxidant enzyme can substitute for GPx4 for this vital function. A specific signal transduction pathway leading to RCD by FPT is not known, while current knowledge supports the notion, instead, that cell die due to the loss of the homeostatic control of LPO. Different elements must all converge to generate a break-point when a peroxidative chain reaction is initiated and executes cell death: i) formation of lipid hydroperoxides during aerobic metabolism - possibly extended by a lipoxygenase activity; ii) inadequate reduction of these lipid hydroperoxides; iii) availability of ferrous iron from the labile iron pool. Selenium catalysis in GPx4 reaction accounts for the vital role of the element, but a physiological down-regulation of expression or activity accounting for an RCD is not known. GSH also is indispensable to life and its cellular concentration - possible target of a physiological regulation - is controlled by the rate of synthesis, metabolism, extrusion and reuptake of cysteine/cystine. Kinetic and mechanistic evidence indicates that it is the efficiency of reductive phase that rate limits the catalytic cycle. GSH reduces the selenenic and mixed selenodisulfide intermediates and release the protein from the membrane, which completes the catalytic cycle. In agreement with the constraints of FPT as RCD, cells actively metabolizing oxygen in mitochondria are the most sensitive to FPT, which takes place when primed by insufficient lipid hydroperoxide removal due to either low GPx4 activity or low GSH concentration, or both. In conclusion, at present level of knowledge, we propose that it is aerobic life that, while supporting bioenergetics, can also be lethal through FPT, when a threshold is overcome in the homeostatic steady-state between formation and reduction of lipid hydroperoxides. On this light, in aerobic conditions, FPT can be seen as a necessary feed-back regulatory mechanism for the fine tuning of cell proliferation. Notably, the mechanism controlling FPT operates through the redox reactions of three (Se, S, O) chalcogens of the VI group with increased electronegativity.

BIOINORGANIC REDOX SIGNALING AND ITS MEDICAL APPLICATION

Ivana Ivanović-Burmazović
Department of Chemistry and Pharmacy, Friedrich-Alexander University, Erlangen-Nürnberg, Germany

The mission of our research is in the application of inorganic reaction mechanisms, as well as design and syntheses of redox active molecules for elucidation and modulation of complex (patho)physiological processes involved in redox signaling, paving the way for improvements in human health and the conquering of disease. Inorganic chemists dedicated to the physiology and pharmacology research are very rare, but their expertise is indispensable for the future development of the modern life sciences and creation of an extended diversity of bioactive and affordable compounds that can meet increasing challenges in health care. This talk will summarise our exciting results in the field of redox signalling and introduce you to the developments in the emerging field of Medicinal Redox Inorganic Chemistry. In particular you will hear about superoxide-dismutase mimetics, bioinorganic chemistry of H_2S and nitrogen species, as well as about crosstalk between NO and H_2S.

CHOLESTEROL OXIDATION BETWEEN HEALTH AND DISEASE: FOCUS ON THE ANTIVIRAL PROPERTIES OF ENZYMATIC OXYSTEROLS

Giuseppe Poli, Fiorella Biasi, Paola Gamba, Gabriella Leonarduzzi, Andrea Civra, David Lembo

Department of Clinical and Biological Sciences, San Luigi Hospital, University of Torino, 10043 Orbassano (Torino), Italy

The epidemiologically proved correlation between cholesterol and defined chronic diseases, in particular cardiovascular pathology, took hold of the collective imagination, so that cholesterol is generally considered as a dangerous molecule. Indeed, the potential contribution of several cholesterol oxidation products called oxysterols in the pathogenesis and the progression of those human diseases whose hypercholesterolemia is a primary risk factor, such as atherosclerosis, Alzheimer's disease and Inflammatory Bowel Disease, has been unanimously recognized. However, this multitasking sterol is not synonym of disease, being actually involved in a number of biological cell and tissue functions. Quite recently, 25-hydroxycholesterol (25HC), physiologically produced by enzymatic oxidation of cholesterol, was demonstrated to act as inhibitor of a wide spectrum of enveloped viruses. In our laboratory, not only 25HC but also another physiological oxysterol, namely 27-hydroxycholesterol (27HC), was shown able to efficiently counteract not only the replication of enveloped human viruses but also that of non-enveloped ones. Different epithelial cell lines were employed, namely MA104, HeLa, Caco-2. Different strains of human rhinovirus (HRhV) and human rotavirus (HRV) were purchased from ATCC. Plaque reduction assay and yield reduction assay were adopted to test the antiviral properties of various oxysterols. Both 25HC and 27HC blocked the infectivity of several HRhV and HRV strains at 50% inhibitory concentrations in the low micromolar range in the absence of cell toxicity. Of note, the two oxysterols exhibited a strong antiviral effect also when added to cell cultures prior to viral infection. Mechanistic insights will be presented and discussed. The two oxysterols of enzymatic origin, tested in the standard *in vitro* model systems, displayed strong inhibiting but also preventing effects against HRhV and HRV infections, suggesting that not only 25HC but also 27HC could be actively involved in the innate immunity response against viruses. Another healthy effect of "good" cholesterol metabolism.

IDENTIFICATION AND QUANTIFICATIONS OF PROTEIN MODIFICATIONS, AND THEIR BIOLOGICAL CONSEQUENCES

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Proteins are major oxidant targets due to their abundance and their high rate constants for reaction with oxidants generated at sites of inflammation (e.g. $O_2^{\bullet-}$, H_2O_2, HOCl, ONOOH). With reactive oxidants, such as HOCl and ONOOH, both side-chain (mainly at Cys, Met, Trp, Tyr and His) and backbone damage can be detected, whereas with less reactive species, such as $O_2^{\bullet-}$ and H_2O_2 damage is both limited and highly selective. Protein damage within cells is often rapidly repaired, or removed, via catabolism. External to cells, where oxidant formation often occurs, the situation is somewhat different, as extracellular materials are usually poorly protected against damage, and there is limited capacity for repair. Elastin is the most abundant extracellular matrix protein in elastic tissues, including the lungs, skin and arteries, and comprises 30-57 % of the aorta by dry mass. Most elastin synthesis occurs during the early years of life, with limited synthesis in adults, therefore most elastin is as old as the host. It can therefore accumulate high levels of modifications with increasing age and disease. Mature elastin is synthesized from monomeric tropoelastin (TE), with the latter undergoing complex processing to form mature elastic fibers. As considerable evidence supports ONOOH formation in the inflamed artery wall, we that TE would be highly susceptible to modification and structural alteration by ONOOH, with consequences for protein function. This damage to TE may play a role in the development of cardiovascular disease as modified matrix species have been implicated in atherosclerotic lesion development and rupture. We have shown that TE is highly sensitive to ONOOH, with extensive dimerization and fragmentation (detected by SDS-PAGE with silver staining or Western blotting) and nitration of tyrosine (Tyr) residues to give 3-nitroTyr (detected by amino acid analysis and mass spectrometry peptide mass mapping). This damage can be detected with equimolar or greater levels of oxidant, and increases in a dose-dependent manner. Quantification of Tyr loss and 3-nitroTyr formation indicates extensive Tyr modification with up to two modified Tyr per protein molecule, and up to 8% conversion of ONOOH to 3-nitroTyr. These effects were modulated by bicarbonate, a competitive target for ONOOH. 3-nitroTyr formation was detected at 12 of 15 Tyr sites in TE treated with equimolar or higher levels of ONOOH; label-free MS quantification revealed extensive nitration (> 50% modification) at some sites. Four Tyr residues have also been shown to be involved in the formation of inter- and intra-molecular di-tyrosine cross-links, with these characterized using an ^{18}O labelling MS approach. TE treatment with ONOOH lowered both the concentration at which TE self-assembles (coacervates), and increased the rate of this process. Studies on human atherosclerotic lesions using immunohistochemistry showed colocalization of 3-nitroTyr with elastin epitopes, consistent with modifications *in vivo*, and also an association of 3-nitroTyr-containing proteins and elastin with lipid deposits. These data suggest that exposure of TE to ONOOH gives marked chemical, structural and functional changes to TE and alters extracellular matrix assembly. This damage accumulates in human arterial tissue with age, and during the development of atherosclerosis.

REDOX SIGNALING AND EPIGENETICS IN ACUTE PANCREATITIS

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Acute pancreatitis is an inflammatory process of the pancreatic gland that eventually may lead to a severe systemic inflammatory response. A key early event in pancreatic damage is glutathione depletion, which is not associated with glutathione oxidation or protein glutathionylation. Importantly, pairs cystine/cysteine and homocysteine/homocysteine as well as protein cysteinylation and gamma-glutamyl cysteinylation markedly increase in pancreas during acute pancreatitis. Two types of targets of disulfide stress were identified: redox buffers, such as ribonuclease inhibitor or albumin; and redox-signaling thiols that include thioredoxin 1, tyrosine and serine/threonine phosphatases. Redox-sensitive PP2A and tyrosine protein phosphatase activities decreased in pancreatitis and this loss was abrogated by N-acetyl cysteine. There is a cross-talk between disulfide stress and proinflammatory cytokines through serine/threonine protein phosphatases, tyrosine protein phosphatases, and MAPKs that greatly contributes to amplification of the uncontrolled inflammatory cascade. Chromatin remodeling during induction of proinflammatory genes would rely primarily on histone acetylation associated with recruitment of histone acetyltransferase CBP and PP2A should be considered a key modulator of the inflammatory cascade in acute pancreatitis through the ERK/NF-κB pathway and histone acetylation. On the other hand, PGC-1α acts as selective repressor of NF-κB towards Il-6 in pancreas and PGC-1α deficiency, which occurs in obesity, markedly enhanced NF-κB-mediated up-regulation of *Il-6* in pancreas in pancreatitis, leading to severe inflammatory response. Hence, both disulfide stress and PGC-1α deficiency play a key role in redox signaling during acute inflammation in the pancreas.

SQUAMOUS CELL CARCINOMA, OXIDATIVE STRESS AND DIABETES

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Diabetes induces many pathophysiological changes on skin. The most frequent are aging and photoaging. Ultraviolet radiation (UV) is directly related to oxidative stress, skin aging and cancer. Although the effects of UV light on normal skin have been much researched, there is scarcity of data concerning diabetic skin. Diabetes was induced in hairless mice by two doses of streptozotocin injection (20 and 30 mg/kg). Diabetic (D) and normal (N) mice were exposed to UVA and UVB radiation 3 times per week for 18 weeks. The irradiation dose was equal to 0,75 Minimal Erythemal Doses (MED) during the first week and increased by 25% each week until the maximal dose of 3,5 MED. In all normal mice was obtained squamous cell carcinoma while in diabetic skin no one was obtained. Oxidative stress was enhanced in the stratum corneum of N irradiated mice, while the variation of hydrophilic antioxidants were similar. UV light on diabetic skin accelerates aging, enhances dryness and decreases sebum. FT-IR and RAMAN spectra showed considerable changes between diabetic and non diabetic mice skin.

NRF2-REGULATED REDOX SIGNALING IN VASCULAR CELLS: IMPLICATIONS FOR CLINICAL TRANSLATION

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Cells have evolved endogenous defence mechanisms to counteract oxidative stress, and the redox sensitive transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) serves as master regulator of antioxidant defences via the induction of phase II and antioxidant enzymes. Under low oxidative, electrophilic and/or nitrosative stress, Nrf2 is sequestered by its cytosolic binding protein Kelch-like ECH Associated Protein 1 (Keap1) and targeted for proteasomal degradation. GSK-3β phosphorylation may also lead to Nrf2 degradation via an adaptor protein β-TrCP independent of Keap1. Modification of Keap1 cysteine residues leads to nuclear accumulation of Nrf2 following de novo synthesis, and in the nucleus Nrf2 binds to a Maf recognition/antioxidant response element in the promoter of phase II and antioxidant genes, such as NQO1, HO-1, and glutamate cysteine ligase and the cystine-glutamate exchanger xCT involved in glutathione synthesis. Our previous studies in wild type and Nrf2 null murine macrophages identified Nrf2 as a key transcription factor involved in the induction of CD36 and antioxidant stress genes in atherosclerosis. Moreover, Nrf2-regulated defences induced by 4-HNE are reduced in fetal endothelial and smooth muscle cells isolated from gestational diabetic and pre-eclamptic pregnancies. As recently reviewed, accumulating evidence implicates impaired Nrf2 signaling and/or Nrf2 epigenetic regulation in diabetic pathologies such as gestational diabetes. We further demonstrated that adaptation of human primary endothelial cells to physiological oxygen levels (5% O_2) markedly alters the redox phenotype of endothelial cells. Under physiological normoxia, Bach1 is upregulated in endothelial cells adapted in the absence of HIF-1α stabilization. Moreover, elevated Bach1 levels regulated a smaller subset of Nrf2 defense genes than those previously identified under standard atmospheric culture conditions, noting that GSH levels and GCLM and xCT expression were oxygen and Bach1 insensitive and may mediate Nrf2-regulated cellular protection *in vivo*. We recently reported significant differences in nitric oxide and Ca^{2+} signaling in endothelial cells under physiological normoxia, highlighting the importance of investigating redox signaling in vascular and other cell types adapted long-term to physiological normoxia in order to provide translational insights for cardiovascular and regenerative medicine.

THE NRF2/GSTP INTERACTOME AND ITS ROLE IN STRESS-MEDIATED REGULATION OF CELLULAR THIOL METABOLISM AND SIGNALING

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A functional interaction between the detoxification and redox signaling protein glutathione S-transferase P (GSTP) and the electrophile-sensitive transcription factor Nrf2 has been recently proposed (Bartolini et al., Free Rad. Biol. Med. 2015), compatible with effects of GSTP expression and activity on the metabolism and redox of cellular thiols. These effects have been recently characterized in GSTP- and Nrf2-manipulated murine embryonic fibroblasts (MEFs) grown in the presence or absence of sulphur-containing amino acids and treated with Se-compounds with different levels of thiol peroxidase activity. Tumoral and non-tumoral human hepatocytes expressing different levels of GSTP were also investigated. *GSTP* gene manipulation was confirmed to upregulate Nrf2 activity then leading to increased Cys uptake and *de novo* biosynthesis of GSH that was readily secreted together with other cellular thiols in the extracellular medium. These effects were sustained by the presence of cysteine in the cell culture medium, and were abnormally stimulated by Se-compounds. The latter modified the GSH/GSSG ratio and the protein S-glutathionylation pattern in a compound-specific manner, with PhSeZnCl behaving as a potent activator of the GSTP-mediated glutathionylation pathway. These effects of GSTP were compatible with a role of this protein in the altered redox phenotype of GSTP-overexpressing human hepatocellular carcinoma cells. SiRNA inactivation of *Nrf2* gene did not produce major effects on $GSTP^{+/+}$ MEFs, while partially impaired the GSH biosynthesis, secretion and redox response to Se-compounds in $GSTP^{-/-}$ cells. Furthermore, *hGSTP1* gene transfection in $Nrf2^{-/-}$ MEFs was associated with a thiol metabolism and response to Se-compounds similar to the Nrf2-competent counterpart. In conclusion, *GSTP* expression influences both the Nrf2-dependent and independent response of cellular thiols to conditions of electrophilic stress and modified availability of Cys thus representing an important redox regulation hub of normal and tumoral cells.

MODULATION OF THE PROTECTING NRF2-KEAP1 PATHWAY IN SKIN BY UNIQUE ACTIVATORS: CHEMICAL, BIOLOGICAL AND ENVIRONMENTAL

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Objective was to explore activation of the Nrf2-keap1 pathway in skin by unique activators and to elucidate the mechanism involved. Skin, a complex and metabolically active organ possessing numerous roles, covers the entire body, and is a major component of the integumentary system. Skin is exposed to various exogenous and endogenous stress-inducing conditions challenging its homeostasis. One of the most important cellular defense mechanisms by which human skin copes with these numerous stressors is the Nrf2-keap1 defense system. All stressors share the same downstream effect within skin cells: increasing reactive oxygen species (ROS) concentrations leading either to induction of oxidative damage (excessive production) or beneficial effect by activating the protective Nrf2-keap1 pathway (moderate production). Our premise is that the defense mechanisms of skin should always be active and alert. We hypothesized that biological, chemical and environmental stressors are responsible for keeping the skin Nrf2-keap1 pathway partially induced and ready to cope with various insults. Research was conducted using models of human skin in culture and cell culture of skin. GC-MS QTOF was used to analyze skin volatiles, while immunological and cell culture techniques were used for the biochemical tests. Activation of the Nrf2-keap1 pathway was monitored using fluorescence methods, histochemical methods, protein determination and enzymes activity. We showed that skin is a highly responsive tissue. Activation of the Nrf2-keap1 pathway is mediated partially by the presence of skin microbiome via its volatile organic electrophiles. Environmental stressors such as unique UV radiation, osmotic pressure, high temperatures and chemicals such as nano-gold particles can activate this pathway using different mechanisms. Skin's defense mechanism against stress is kept under tight regulation and always in an alert condition due to a variety of biological (skin flora) and environmental (chemicals and non-chemicals) stressors. Moderate exposure to such stress conditions is beneficial for the human body.

TELOMERE LENGTH, TELOMERASE ACTIVITY AND INTERACTION WITH REDOX SYSTEM IN ACUTE MYOCARDIAL INFARCTION AND LUNG CANCER

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Telomeres are specific DNA-protein structures at the chromosomal ends, whose role is to protect the genome from nucleotide degradation, recombination and fusion. When telomeres become critically short cell goes to senescence, oncogenesis or apoptosis. Telomere shortening, physiological or pathological is not irreversible. Enzyme telomerase catalyses the reaction of telomere elongation. Reactive oxygen species could influence cellular senescence by inducing DNA damage, including telomeric parts, and also as signalling molecules in this complex process. Since redox disbalance is a feature characteristic for atherosclerosis and its acute event myocardial infarction, and also deeply involved in cancer pathogenesis, the aim of this study was to estimate telomere-telomerase system status measured in peripheral blood leucocytes in acute myocardial infarction (AMI) and also in lung cancer (LC) patients, regarding their redox status. Our study included 100 AMI patients, 90 LC patients and 100 healthy subjects (CG). We have measured blood telomere length (BTL) and several redox status plasma markers: total oxidant potential (TOP), superoxide anion level ($O_2^{\bullet-}$), total antioxidative capacity (TAC), superoxide dismutase (SOD) activity and paraoxonase 1 (PON1) activity. AMI and LC patients had significantly increased oxidative stress markers and decreased several antioxidative parameters compared to CG. Our results showed significant decrease in BTL in all patients group compared to CG (73%, 64% of control group values in AIM and LC groups, respectively; $P<0.001$). We found significant positive correlation between BTL and PON1 activity ($\rho=+0.212$, $P=0.032$), BTL and TAC ($\rho=+0.240$, $P=0.012$) in AIM patients, and significant negative correlation between BTL and TOP ($\rho=-0.330$, $P<0.01$), and $O_2^{\bullet-}$ ($\rho=-0.444$, $P<0.001$) in LC patients. BTL is differently influenced in acute disease like myocardial infarction and chronic disease such as lung cancer. Both pathologies caused intensive telomere shortening and this process could be guided, at least in part, by deep redox disbalance observed in these two patient groups.

THE INDUCTION OF MACROPHAGE EXTRACELLULAR TRAP FORMATION BY HYPOCHLOROUS ACID (HOCl) AND OTHER INFLAMMATORY MEDIATORS

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The production of extracellular traps (ETs) by neutrophils in response to a range of inflammatory stimuli, is now recognized to play an important role within a range of chronic disease settings, including driving lesion development in atherosclerosis. ETs are released following a novel mode of cell death called ETosis, which results in the release of a mesh of DNA, histones, myeloperoxidase (MPO) and proteolytic enzymes. In innate immunity, these structures trap and kill bacteria, but they can also promote inflammation and contribute to pathological processes. Several inflammatory stimuli, including the MPO-derived oxidant hypochlorous acid (HOCl), can trigger ET release from neutrophils. The aim of this study was to examine whether macrophages can also release ETs on exposure to inflammatory stimuli, because these cells play a critical role in the development of atherosclerosis. We show that exposure of human monocyte-derived macrophages to pathophysiological levels of HOCl results in the dose-dependent extrusion of DNA and histones into the cellular supernatant, consistent with ET formation. Concurrent with, but independent of these findings, macrophage exposure to HOCl also resulted in an immediate and sustained cytosolic accumulation of Ca^{2+}, culminating in the increased production of cytokines and chemokines. Polarisation of the macrophages prior to HOCl exposure revealed a greater propensity for inflammatory M1 macrophages to produce extracellular traps, whereas alternatively-activated M2 macrophages were less susceptible to HOCl insult. M1 macrophages also produced ETs on exposure to phorbol myristate acetate (PMA), interleukin-8 (IL-8) and tumour necrosis factor α (TNFα). Taken together, these data indicate a potential role for macrophages in mediating ET formation, which may be relevant in pathological conditions characterised by chronic inflammation or excessive HOCl formation.

MITOCHONDRIAL Fe-S CLUSTERS IN NEURODEGENERATIVE DISEASES

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Neurodegenerative diseases (NDs) are characterized by the progressive deterioration of the structure and function of the nervous system, ultimately leading to neuronal cell death. It is proposed that the pathological mechanisms associated with the development and progression of these disorders involve the accumulation, and intracellular aggregation of misfolded proteins, as well as a complex interplay between redox active metals, and reactive oxygen, and nitrogen species (ROS and RNS). There is accumulating evidence that mitochondrial iron has a role in the pathophysiology of NDs. More specifically, the iron-sulfur (Fe-S) clusters of the mitochondrial respiratory chain Complex I (NADH:ubiquinone oxidoreductase) have been shown to be the target of ROS formed as the result of neurodegeneration. Our recent low temperature electron paramagnetic resonance (EPR) spectroscopy studies of intact neural tissues isolated from transgenic animal models of amyotrophic lateral sclerosis (ALS), and Alzheimer's disease (AD), indicated that for both NDs, the Complex I Fe-S clusters are exposed *in vivo* to higher amounts of ROS compared to the non-transgenic tissues. This was observed as an increased ratio of oxidized-to-reduced, $[3Fe\text{-}4S]^{1+}$ to $[4Fe\text{-}4S]^{1+}$, cluster content, as well as decreased activity, and uncoupling of heme–iron and copper in the O_2 reduction site of Cytochrome *c* oxidase. It is interesting to point out that although the underlying cellular and molecular mechanisms involved in ALS and AD pathologies are different (including the disease-affected proteins), the mitochondrial Fe-S clusters are affected in both cases. It is not clear however, whether this is a cause or a consequence of the disease. This work should aid in understanding the pathways of ND development and progression, and allow for the effective target-based drug design.

THREE-ACT STORY OF LABILE IRON POOL: ADRENALINE, ASCORBATE, AND AMINO ACIDS

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Labile iron pool is composed of complexes of Fe with small ligands, which affect Fe availability and redox activity (and *vice versa*). The involvement of labile iron pool in a number of pathological conditions has been widely recognized. However, little is known about the speciation and mechanisms. Using three examples, we aimed to illustrate the main aspects of this (patho)physiological entity - coordination, redox activity, and free radicals chemistry. Adrenaline forms stable complexes with Fe^{3+} at physiological pH in 1:1 or 3:1 stoichiometry, depending on the (low or high) concentration ratio. Oxygen atoms on the catechol ring represent the sites of coordinate bond formation within physiologically relevant bidentate 1:1 complex. This prevents adrenaline from binding to receptors, and results in the accumulation of adrenaline that may be activated by stronger Fe ligands, such as chelating drugs. On the other hand, the transient complex of adrenaline and Fe^{2+} shows very low reduction potential and catalysed e^- transfer from Fe^{2+} to O_2 with concomitant formation of adrenaline/Fe^{3+} complex. Ascorbate and Fe^{3+} take part in a two-branch redox system. In the first branch, Fe^{3+} acts as a shuttle for $2e^-$ transfer from ascorbate to O_2, to produce H_2O_2. This reaction represents the basis of anticancer effects of ascorbate that have been observed *in vitro*, spurring the interest for the applicability of mega-doses of ascorbate in cancer treatment. However, in the second branch, an 'intra-complex' $1e^-$ transfer from ascorbate to ferric iron gives rise to Fe^{2+}, which prevents H_2O_2 accumulation via Fenton reaction. Unlike H_2O_2, $HO^\bullet$ has a very short diffusion radius and cannot enter and kill cancer cells. Although extremely reactive, $HO^\bullet$ shows selectivity. For example, amino acids react with $HO^\bullet$ at different rates that show negative correlation with polarity and a strong positive correlation with hydrophobicity and hydrophobic hydration. This speaks in favour of the hypothesis that the movement of $HO^\bullet$ in water via chain-reaction: $HO^\bullet + H_2O \rightarrow H_2O + HO^\bullet$, does not go through hydrogen bonds. Transfer of $HO^\bullet$ from the bulk water to the water molecules in the hydrophobic hydration shell and further to an amino acid might be promoted by the lower density of hydrogen bonds down that route. According to this, damaged proteins with exposed hydrophobic side-chains may act as sacrificial antioxidants.

EMERGING PERSPECTIVES FOR DIABETES TREATMENTS: REDOX MODULATORS AND ENERGY METABOLISM

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Redox homeostasis disturbance, mainly caused by increased superoxide anion radical ($O_2^{\bullet -}$) level and/or decreased nitric oxide ($^{\bullet}NO$) bioavailability, represents an important contributing factor in the (ethio)pathology of diabetes. We examined whether targeting of $^{\bullet}NO$ and/or $O_2^{\bullet -}$ by two redox-modulating compounds, L-arginine (substrate for $^{\bullet}NO$ synthases) and Mn(II) pentaazamacrocyclic mimics of superoxide dismutase (SOD), could improve diabetic state and associated impairment of energy metabolism. The experiments were performed in alloxan-induced diabetes (single alloxan dose, 120 mg/kg) in Mill Hill rats. Several methodological approaches including immunohistochemistry, immunofluorescence and Western blot analysis were used for monitoring the effects of L-arginine (2.25%) and SOD mimics (5 mg/kg/day) on pancreas itself and extrapancreatic tissues (skeletal muscle, white adipose tissue, skin). Multiple beneficial effects of L-arginine and SOD mimics in diabetic rats were observed. First of all, acting directly on diabetic pancreas, L-arginine and SOD mimic induce β-cells regeneration (population of insulin immunopositive β-cells) and glucose sensing (GLUT2 membrane localization). In skeletal muscle, those treatments restore diabetes-induced impairment in mitochondrial energy metabolism (OXPHOS) and glucose transport (GLUT4) by targeting 5'-AMP-activated protein kinase (AMPKα) signaling. Similarly, SOD mimic upregulates phopsho-AMPKα protein in white adipose tissue of diabetic animals. In diabetic skin, L-arginine and SOD mimic stabilize diabetes-induced redox disbalance acting on $^{\bullet}NO$ producing (nitric oxide synthases) and $O_2^{\bullet -}$ /H_2O_2 removing (MnSOD and GPx) systems. The data suggest that targeting of $^{\bullet}NO$ and/or $O_2^{\bullet -}$ by L-arginine and SOD mimic could have beneficial implications for several pathological hallmarks of diabetes: impaired insulin synthesis and sensitivity as well as accompanying metabolic complications and speak in favour of therapeutic potential of these redox-active agents in diabetic conditions.

TARGETING INFLAMMATION IN CANCER TREATMENT

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Inflammation orchestrates host response to tissue damage caused by infection or non-infective stimuli. The connection between inflammation and tumor development in all stages is recognized as very important so the inflammation is attributed to the cancer hallmarks. Inflammation also affected cancer cells sensitivity to immune response as well as applied treatments. Extensive and dynamic crosstalk between inflammatory cells and transformed cells is verified through numerous molecules involved. Targeting of inflammatory mediators or signaling pathways is one of the approaches that attract attention in last decade. Clinical practice revealed that HIV protease inhibitors (HIVPIs), apart from preventing viral replication, are also able to suppress the development or to promote regression of HIV associated neoplasia. Initially it was attributed to the reconstitution of the host immune system while later it became clear that these drugs have antitumor potential realized through direct cytotoxicity, interference with drug transporters and angiogenesis. However, low bioavailability, fast degradation and rapid gaining of resistance presented the common disadvantage for their application. To overcome mentioned deficiencies we modified HIVPIs by the covalent attachment of nitric oxide (NO) moiety. NO modified saqinavir, lopinavir and ritonavir showed their efficacy against numerous tumor cell lines *in vitro* and in few *in vivo* models. In comparison to maternal compounds the experimental drugs were more potent in inhibition of the tumor cell growth. The proapoptotic activity of maternal drugs was converted into strongly cytostatic and differentiating inducing properties. Even though the dissimilarities in their mechanism of action and NO releasing capacity exist, it seems that p70S6 kinase is unique intracellular target of these drugs. The antitumor activity of modified HIVPIs, as well as the relevance of NO for its realization will be elaborated in detail.

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THE ROLE OF IMPAIRED REDOX REGULATION IN RENAL CELL CARCINOMA DEVELOPMENT AND PROGRESSION

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Renal cell carcinoma (RCC) belongs to tumors in which significant changes occur in cellular redox balance. It is believed that, since in cancer cells ROS concentrations are higher than in normal cells due to accelerated metabolism and promotion of tumor development and progression, cancer cells manage to develop resistance to ROS by inducing a new redox balance, further resulting in cellular adaptation and proliferation. Indeed, the activities of antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), are down-regulated in RCC, while the concentration of glutathione (GSH) is up-regulated. This is mostly due to increased activity of γ-glutamylcysteine synthetase, a major GSH replenishing enzyme, whose increased activity is recognized as the strategy used by many tumors to enhance GSH content. The change in redox balance is probably the consequence of deregulated Nrf2 pathway, suggested as the master regulator of RCC cellular redox state. Since nuclear accumulation of Nrf2 modulates the expression of a large number of genes, including familiar antioxidant enzymes, but also detoxification phase II enzymes, such as glutathione transferases (GST), changes in cellular redox balance in RCC might also be attributed to alterations in GST phenotype. It has been confirmed that *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* polymorphisms might be associated with the risk of RCC, with special emphasis on *GSTM1-null* and *GSTP1-variant* genotypes. Moreover, combined *GSTM1-null, GSTT1-active, GSTA1 low activity* and *GSTP1-variant* genotype is considered as the "risk-carrying genotype combination" in RCC. Apart from their catalytic role, GSTs act as modulators of several signaling kinases through protein-protein interactions. The fact that *GSTM1-null* genotype is recognized as an independent prognostic factor, associated with favourable postoperative prognosis in RCC, might be explained by the presence of protein-protein interaction between GSTM1 and apoptosis signal-regulating kinase 1 (ASK1).

MODULATION OF NRF2-KEAP1 SIGNALING IN MALE INFERTILITY

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Infertility is an expanding global medical problem. Particularly worrying is the progressive decline in male fertility seen in recent years. This clearly gives importance to development of new strategies to treat sterility. Redox signaling recently emerged as important for spermatozoa function. Accordingly, we have recently showed substantial beneficial effects of redox modulator - superoxide dismutase (SOD) mimic M40403 on sperm function. Molecular mechanisms underlying the advantageous effects of M40403 indicate nitric oxide (NO) involvement, but remain unexplained; we aimed to reveal it here. Compared to the control, incubation of spermatozoa in Tyrode's medium for 3 h, decreased sperm motility, mitochondrial membrane potential (MMP), NO level, expression of NO synthases (NOSs), and completely abolished nuclear localization of NF-E2-related nuclear factor 2 (Nrf2) which was followed by decrease in L-cysteine γ-ligase (GCL), SODs, catalase and glutathione peroxidase (GPX) expression. Treatment with M40403 decreased level of superoxide anion radical ($O_2^{\bullet -}$), increased level of NO, restored expression of NOSs and MMP, and triggered marked nuclear translocation of Nrf2 followed by up-regulation of GCL, SODs, catalase and GPX and increase in sperm motility. In turn, M40403 + $N^{(\omega)}$-nitro-L-arginine methyl ester (L-NAME) treatment nullified all beneficial effects of M40403. Treatment with L-arginine alone achieved beneficial effects, but to a lesser extent than M40403. Presented results reveal the redox based mechanisms of beneficial effects of M40403 SOD mimic in spermatozoa, suggesting fine-tuning of $O_2^{\bullet -}$/NO ratio and consequent activation of Nrf2 as a crucial pathway. Utilization of a redox modulator M40403, as an inducer of Nrf2 is a promising pharmacological approach for the improvement of sperm fertilizing potential and treatment of infertility.

SHORT PRESENTATIONS

eNOS IS UPREGULATED IN HUMAN INTERNAL THORACIC ARTERY AFTER REMOTE ISCHEMIC PRECONDITIONING

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Remote ischemic preconditioning (RIPC) is an intriguing phenomenon in which short episodes of ischemia-reperfusion in one, remote vascular bed provide a systemic protective phenotype, i.e. protect distant tissues and organs from future ischemia-reperfusion injuries. RIPC has shown beneficial effects in patients with endothelial dysfunction, hypertension, myocardial infarction and better outcomes in patients undergoing vascular surgery or organ transplantation. However, although RIPC was discovered several decades ago, the mechanisms through which it confers whole body protection are unclear. We aimed to reveal the redox signature of RIPC in distant vasculature, with a special focus on the role of the nitric oxide synthases (NOS). To this end, we examined the internal thoracic arteries of patients undergoing urgent coronary artery bypass grafting. After the induction for anaesthesia, half of the patients underwent a RIPC protocol of 3 cycles of 5 minutes of forearm ischemia with 5 minutes of reperfusion by using an inflatable cuff. After harvesting the left internal thoracic artery a sample was taken. Western blotting results showed that RIPC leads to induction of eNOS on the level of protein expression. This was additionally confirmed by immunohistochemistry, where we observed stronger eNOS immunopositivity in the endothelial layer of RIPC patients, along with an especially strong reaction in the smooth muscle cells of the tunica media. We also analysed the protein expression of inducible and neuronal NOS, but found no significant differences between patients undergoing coronary artery bypasses with and without RIPC. These results, for the first time, show that RIPC leads to eNOS upregulation in human arteries, highlighting its role in this protective phenotype.

DEVELOPMENT AND VALIDATION OF AN ANALYTICAL ASSAY FOR THE DETECTION AND QUANTIFICATION OF 3-NITROTYROSINE IN ANIMAL MODELS OF CARDIOVASCULAR DISEASE

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Oxidative stress is a major trigger of endothelial dysfunction and cardiovascular disease. Therefore, accurate determination of reactive oxygen and nitrogen species (ROS and RNS), especially nitric oxide, superoxide and peroxynitrite, is of great importance for the evaluation of disease mechanisms and the potential targets for drug therapy. One of my PhD thesis objectives is to develop an HPLC assay for the specific detection of 3-nitrotyrosine in biological samples of animal models of cardiovascular disease. Nitrated proteins are subjected to total hydrolysis using pronase, a mixture of different proteases. The free 3-nitrotyrosine is then separated by HPLC and quantified by a coulometric (electrochemical) method using the CoulArray system. Concentration-response-curves of 3-nitrotyrosine standards were highly linear. Nitrated bovine serum albumin standards were successfully detected and validated by other methods (ELISA and dot blot analysis using a specific 3-nitrotyrosine antibody). Ongoing studies are dedicated to the quantification of 3-nitrotyrosine in tissue samples of nitrate-tolerant, diabetic, hypertensive and septic mice, also including the comparison of different detection methods. A major draw-back seems to be the separation of the 3-nitrotyrosine peak in complex biological samples, which is currently the main focus of my work. Based on our *ex vivo* data, the CoulArray quantification method for 3-nitrotyrosine seems to have some advantages regarding sensitivity and selectivity. In the future, we hope to be able to establish a reliable automated HPLC assay for the routine quantification of 3-nitrotyrosine in biological samples of cell culture, animal and human origin.

ACTIVITY AND CONCENTRATION OF PARAOXONASE 1 IN BLOOD AND LIPOPROTEIN FRACTIONS IN PATIENTS WITH CHRONIC KIDNEY DISEASE

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Many epidemiological studies have shown that cardiovascular disease represents the most important cause of morbidity and mortality in patients with chronic kidney disease (CKD). Numerous traditional and non-traditional risk factors such as dyslipidemia, hypertension, diabetes mellitus, oxidative stress and inflammation are closely related to impaired renal function. Increased production of free radicals, together with weakening of the endogenous antioxidant defense system contributes to elevated oxidative stress in CKD patients. Enzyme paraoxonase 1 (PON1), located mainly on HDL particles, has anti-oxidative role which is reflected primarily by capability of this enzyme to protect LDL and HDL particles against lipids oxidation. The current study had aim to investigate whether failure of kidney function leads to changes in the concentration and activity of PON1 in blood as well as in different lipoprotein fractions. In 77 renal patients (21 chronic kidney disease (CKD) and 56 end stage renal disease (ESRD) patients on dialysis) and 20 healthy subjects, PON1 activity was measured kinetically in blood and on different HDL subclasses. After isolation lipoproteins using ultracentrifugation, PON1 concentration was measured in HDL, VLDL as well as in blood by ELISA test. Serum paraoxonase ($p<0.01$) and arylesterase activity ($p<0.001$) of PON1 as well as its concentration ($p<0.01$) were significantly lower in CKD and ESRD patients compared to controls. Control subjects had higher arylesterase activity of PON1 on HDL2 (CKD and ESRD patients $p<0.001$) and HDL3 (CKD $p<0.05$; ESRD patients $p<0.001$) subclasses in comparison with the both patients groups. PON1 concentration was significantly higher in HDL compared with VLDL fractions in all examined groups ($p<0.001$). In HDL fractions PON1 concentration was lower in CKD and ESRD patients compared with controls ($p<0.01$ and $p<0.05$, respectively), while determined PON1 concentration in VLDL fractions was similar across all investigated groups. This study showed that diminished anti-oxidative protection of HDL reflected through decreased PON1 activity and concentration, together with impaired HDL maturation and metabolism could be important factors in atherosclerosis development in patients with CKD.

INVESTIGATION OF COX4 ISOFORM PAIR BIOLOGICAL ROLE USING HEK293 CELL-LINE BASED KNOCK-OUT AND KNOCK-IN MODELS

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Oxidative phosphorylation (OXPHOS) is responsible for production of majority of ATP in mammalian organisms. This process is partly regulated by nuclear-encoded subunits of cytochrome *c* oxidase (COX), the terminal enzyme of respiratory chain. One of its regulatory subunits, Cox4, is an early-assembling COX component essential for the formation of catalytically functional enzyme. Moreover, regulated expression of its two isoforms (Cox4i1, Cox4i2) is hypothesized to optimize respiratory chain function according to oxygen supply. However, details of functional alterations between the two variants have not yet been described. We established HEK293 cell line-based model with complete absence of subunit Cox4 (knock-out, KO) employing CRISPR CAS9-10A technology, and characterized its impact on OXPHOS complexes. Knock-out of both isoforms Cox4i1 and Cox4i2 (COX4i1/4i2 KO clones) showed general decrease of COX subunits resulting in total absence of COX holoenzyme, making cells fully reliant on OXPHOS-independent ATP production. COX4i1/4i2 KO were subsequently utilized as a platform for knock-in of COX4i1 or COX4i2 isoform using stable overexpression. Expression of both isoforms complemented the respiratory defect of COX4i1/4i2 KO. The content of COX as well as its ability to incorporate into supercomplexes were comparable in COX4i1 and COX4i2 expressing cells. Respiratory rates of permeabilized cells in OXPHOS (coupled, state 3) and ETC (uncoupled, state 3u) states, as well as COX capacity were not distinguishable between cells expressing either isoform of COX4. However, significant changes were detected in COX oxygen kinetics. The p_{50} parameter (partial pressure of oxygen at half-maximal respiration) was approximately 2-fold increased in COX4i2 versus COX4i1 cells. These findings indicate decreased oxygen affinity of COX4i2-containing enzyme. Interestingly, we observed COX4 isoform dependent modulation of reactive oxygen species (ROS) production - COX4i2 KI clones manifested decreased mitochondrial ROS generation. Using this model, we will further focus on the ability of COX4 isoforms to serve as mitochondrial energy and redox sensors for regulation of ATP production and oxidative stress response during hypoxia.

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RELATION OF OBESITY TO REDOX REGULATION AND METABOLIC REPROGRAMMING IN PREMENOPAUSAL BREAST CANCER

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Obesity is a well-known risk factor for breast cancer in postmenopausal women. However, the role of obesity in premenopausal breast cancer is still controversial. We aimed to reveal complex interactions between metabolic reprogramming and redox regulation in premenopausal breast cancer and their relation to obesity. Protein expression of rate limiting enzymes for mitochondrial (acyl-coenzyme A oxidase 1) and peroxisomal (acyl-coenzyme dehydrogenase) β-oxidation, level of 4-hydroxy-2-nonenal (4-HNE) protein adducts and key peroxide metabolizing components in glutathione (GSH) and thioredoxin (TRX) systems: levels of GSH and TRX, glutathione peroxidase (GSH-Px), glutathione reductase (GR), thioredoxin reductase (TR) and catalase were analyzed in tumor tissue of normal-weight and overweight/obese breast cancer patients and in benign tumor tissue of weight-matched controls. In accordance with increased cancer-associated redox homeostasis threshold, higher protein expression of catalase, GSH-Px and TRX characterize malignant tumors of normal-weight and obese women alike, in comparison to their weight-matched benign controls. Interestingly, highest immunoreactivity for 4-HNE protein adducts and protein expression of fatty acid β-oxidation enzymes were found in cancer tissue of obese women, compared to cancer tissue of normal-weight women. These results indicate that obesity contributes to peroxidative pressure in breast cancer tissue, evident as an increase in 4-HNE protein adducts, by promoting a metabolic shift towards oxidative metabolism.

COMBINATION OF COLD PHYSICAL PLASMA DERIVED OXIDANTS AND CHEMOTHERAPEUTIC AGENTS INDUCE IMMUNOGENIC CELL DEATH VIA UPREGULATION OF SLC22A16 IN MELANOMA CELLS

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Malignant melanoma is an aggressive cancer that develops drug resistance leading to poor prognosis. Efficient delivery of chemotherapeutic drugs to the tumor tissue remains major challenge in treatment regimens. Using murine (B16) and human SK-MEL-28 melanoma cells we investigated traditional cytotoxic agents in combination with cold plasma derived oxidants. We report an additive cytotoxicity of doxorubicin, epirubicin and oxaliplatin with 30s exposure of cold plasma effluent in 2D and 3D cultures. Combination treatment led to an increased DNA damage response by pATM and γ-H2AX foci and micronuclei formation. There was also an enhanced secretion of immunogenic cell death markers ATP and CXCL10 in cell supernatants following combination treatment. SK-MEL 28 and THP1 co-culture experiments led to enhanced THP1 migration and phagocytosis of tumor cells indicative of immunogenic cell death when compared to controls. The observed additive effects in tumor cells was due to enhanced intracellular doxorubicin accumulation via upregulation of organic cationic transporter SLC22A16 by cold plasma derived oxidants. The doxorubicin uptake was reversed by pretreating cells with antioxidants or calcium influx inhibitor BTP2. siRNA mediated knockdown of SLC22A16 led to inhibition of additive toxicity in tumor cells. Taken together we propose pro oxidant therapies may constitute a new and effective anti-cancer treatment modality to counter chemoresistance in cancer.

THE EFFECT OF SIRT3 EXPRESSION ON HUMAN BREAST CANCER CELLS IN NORMOXIA AND HYPEROXIA

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Sirtuin 3 (Sirt3) has a promising role in cancer tumorigenesis and treatment, but there have been controversies about its role as tumor suppressor or tumor promotor in different types of cancer. Changes in its expression are associated with the excessive production of reactive oxygen species (ROS), thus contributing to mitochondrial dysfunction and age-related pathologies. Hyperoxic treatment (i.e. generator of ROS) was shown to support some tumorigenic properties, but finally suppresses growth of certain mammary carcinoma cells. Due to strikingly reduced Sirt3 level in many breast cancer cell lines, we aimed at deciphering the effect of *de novo* Sirt3 expression in normoxic and hyperoxic conditions in the human breast cancer cells. We stably transfected MCF-7 and MDA-MB-231 cells with Flag-tagged Sirt3 plasmid and characterized Sirt3 overexpressing clones in normoxic and hyperoxic conditions using real-time PCR, western blot and confocal analysis. We monitored the expression of genes involved in antioxidant protection, metabolic regulation, angiogenesis and epithelial mesenchymal transition, and the expression of proteins involved in mitochondrial biogenesis, glycolysis, metabolic regulation and antioxidant defense. The growth rate and metabolic activity of the cells were defined by the MTT and CFU tests, whereas the ROS production and mitochondrial function were monitored by FACS analysis. Effects of *de novo* Sirt3 expression in MCF-7 cells were enhanced upon hyperoxic treatment: decreased metabolic activity and cellular growth, reduced expression of pro-angiogenic genes, induced metabolic switch from glycolysis to OXPHOS, increased ROS levels followed by mitochondrial and antioxidant dysfunction. We observed enhanced susceptibility of MCF-7 cells to hyperoxia and decreased cellular growth upon *de novo* Sirt3 expression. However, Sirt3 markedly promoted growth of highly invasive ERα negative MDA-MB-231 cells. This initial finding suggests that Sirt3 may either have a tumor suppressing or tumor promoting role depending on the invasiveness of the cancer cell and that it should be further explored, along with hyperoxia, *in vitro* and particularly *in vivo*, as an adjuvant tumor therapy in breast cancer malignancies.

THE LEVEL OF OXIDATIVE STRESS DETERMINES THE ROLE OF EXTRACELLULAR HMGB1 PROTEIN IN DIABETIC RAT LIVER

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Oxidative stress through changes in antioxidative enzyme activities, glutathione metabolism and lipid peroxidation, leads to cell damage and even cell death. These changes are integrated in the pathogenetic mechanisms of the long-term, specific complications of diabetes, such as neuropathy, retinopathy, cardiomyopathy, nephropathy and hepatopathy. Recent studies have shed light on new redox sensitive endogenous targets which are important regulators of oxidative stress-induced damage. HMGB1 is a nuclear chaperone with an inflammatory function when released in the extracellular space. Extracellular HMGB1, through interaction with TLR4 receptors in its oxidized state, and with RAGE in its reduced state, controls the equilibrium between apoptosis and autophagy. HMGB1 is a redox sensitive protein with a potentially harmful role. We therefore analyzed the changes in HMGB1 regulated signaling pathways by immunoprecipitation and Western blot that can lead to cell death or cell survival in the liver of streptozotocin (STZ)-induced diabetic rats during decreased oxidative stress after melatonin administration, and when HMGB1 release was inhibited by ethyl pyruvate. Inhibition of HMGB1 release decreased both apoptosis and autophagy, and supported the unchanged state in liver cells in STZ-treated rats as compared to the control animals. The decrease in oxidative stress achieved with melatonin decreased HMGB1 driven apoptosis but upregulated HMGB1 regulated protective autophagy, mitophagy in particular as the second level of antioxidative defense which was detected by electron microscopy. It provided a selective advantage, minimizing oxidant insults when primary antioxidant activities are compromised during oxidative stress. This adaptation led to improved cell survival in the liver of STZ-treated rats. These results showed that modulation of the role of HMGB1 in the extracellular space that was achieved by a decrease in oxidative stress is more desirable than complete inhibition of its release because HMGB1 has a protective role against oxidative injuries in diabetic liver.

DMSO PRE-TREATMENT OF MCF-7 BREAST CANCER CELLS FOR EVALUATION OF THE EFFECTS OF GAMMA RAYS AND CARBON IONS

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Ionising radiation causes DNA damage directly through incoherent interaction with DNA molecules, and indirectly as a result of water radiolysis and formation of free radicals that chemically react with DNA molecules. These reactive oxygen species contribute the majority of irradiation-induced DNA damage. Occurrence of indirect DNA damage within irradiated cellular systems can be reduced through the application of agents with free radical scavenging capacity, such as glycerol, cysteamine or dimethyl sulfoxide (DMSO). The objective of this study was to compare the effects of low (γ-rays) and high linear energy transfer (LET) radiations (^{12}C ions) on MCF-7 breast cancer cells. A non-toxic concentration of 100 mM DMSO was used to limit the impact of produced free radicals. MCF-7 cells were exposed to γ-rays, as well as to ^{12}C ions over a dose range of 1 to 8 Gy. For ^{12}C ions, the irradiation geometry was optimised to obtain the highest biological effectiveness of 62 MeV/u ion beam at the sample site. To evaluate radiosensitivity level of cells, clonogenic assays were performed. Phosphorylation of H2AX histone (γH2AX) was used as the DNA double strand brake (DSB) marker, since it is one of the main proteins involved in repair processes. DNA DSBs were quantified immunocytochemically, using γH2AX foci assay. According to the results of clonogenic survival, ^{12}C ions induce stronger cell killing as compared to γ-rays while treatment with DMSO elevates cell survival due to the efficient scavenging of free radicals induced by irradiations. Formation and disappearance of γH2AX foci were evaluated 0.5 and 24 h after irradiations. Analysis of γH2AX shows that γ-rays and ^{12}C ions increase the number of foci 0.5 h after irradiation, while pre-treatment with DMSO reduces the number of γH2AX foci in both experimental cases. At 24 h after irradiation, the effect of DMSO is greater in γ-irradiated samples than in cells exposed to ^{12}C ions meaning that contribution of indirect effects is more pronounced in low LET irradiations, i.e. γ-rays. The obtained results display good free-radical scavenging capacity of DMSO thus enabling comparison of these two radiation types.

CONCOMITANCE OF POLYMORPHISMS IN GLUTATHIONE TRANSFERASE OMEGA GENES IS ASSOCIATED WITH RENAL CELL CARCINOMA RISK AND PROGNOSIS

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In defining novel approaches to the diagnosis and treatment of renal cell carcinoma (RCC), different patient-related factors, including genetic polymorphisms, have been taken into account. Potential significance of polymorphisms in glutathione S-transferase omega (GSTO) class in the onset of RCC was not investigated as yet. We aimed to evaluate the effect of specific *GSTO1* and *GSTO2* gene variants, independently and in interaction with established risk factors (smoking, obesity and hypertension) on the risk and prognosis of clear cell RCC (ccRCC), as the most aggressive RCC subtype. *GSTO1* (rs4925) and *GSTO2* (rs156697 and rs2297235) polymorphisms were determined in 239 ccRCC patients and 350 matched controls. Plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, was determined by ELISA. As a result, combined effect of all three variant genotypes corroborated almost 3-fold risk of RCC development. Additionally, this association was confirmed at haplotypic [variant *GSTO1**A/*GSTO2**G (rs156697)/*GSTO2**G (rs2297235) haplotype] level, suggesting potential role of those variants in propensity to RCC. Regarding the gene-environment interactions, variant *GSTO2**G (rs156697) homozygous smokers are at higher ccRCC risk (p=0.040). Association in terms of oxidative DNA damage was found for *GSTO2* rs2297235 polymorphism and 8-OHdG (p=0.042). In the multivariate Cox regression analysis *GSTO2**G (rs156697) allele was an independent predictor of higher risk for overall mortality among ccRCC patients (p=0.050), compared with the carriers of *GSTO2**A/A genotype. In conclusion, GSTO polymorphisms might be associated with the risk and postoperative prognosis of ccRCC, with special emphasis on *GSTO2*-variant genotype.

POSTERS

P01

N-6/N-3 RATIO IN LIVER PHOSPHOLIPIDS AS A BIOMARKER OF AGING IN FISH OIL WISTAR RATS TREATMENT

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The phospholipids class, fatty acids (FAs) composition content in membranes are basic determinants of the physical properties of membranes. They have been shown to influence a wide variety of membrane dependent functions such as membrane transport, enzyme activity and receptor function. The FAs profile in tissues partly reflects not only the dietary fat intake, but also the efficiency of FAs metabolism in the body. It seems that aging itself is a risk factor and at least in part lead to higher saturation of FAs in tissues (liver) phospholipids. Also n-6/n-3 ratio as a risk factor and biomarker become higher with aging. Experimental model of aging (young Wistar rats-3 months, n=10 and aged Wistar rats-18 months, n=10) in a great extent showed differences in FAs profiles of phospholipide. Treatment was with fish oil, 6 weeks, in both experimental groups. Fatty acids profiles were determined by GC chromatography. Results showed that young rats had decreased n-6/n-3 ratio vs. aged (3.65±0,36 vs. 6.30±1.46) in liver phospholipids while after 6 weeks of fish oil treatment n-6/n-3 ratio also decreased in young (3.65±0.36 vs. 2.40±0.15) and aged (6.30±1.46 vs. 3.35±0.51) in liver phospholipids. We can conclude that n-6/n-3 ratio could be the marker in membrane composition of tissue aging and that fish oil treatment had beneficial role in decreasing this ratio as a risk factor of cardiovascular diseases, inflammatory processes and some degenerative changes in body.

P02

BAX AND 4-HNE EXPRESSIONS IN RATS TREATED WITH INDOMETHACIN PRIOR TO LIVER ISCHEMIC-REPERFUSION INJURY

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Ischemic-reperfusion injury (IRI) is a process where, after the initial ischemic injury, reflow of blood (reperfusion) induces an additional injury. It is known that in pathophysiology of IRI, oxidative stress and inflammation play a major role. They evoke cell apoptosis and necrosis and can be detected through different tissue parameters that are readily detectable on pathohistological slides, while agents affecting these processes modulate IRI. Indomethacin is one such agent, a drug belonging to the NSAIDs group, whose exact effects in the IRI model are not fully investigated. The aim of the present study was to determine the effects of indomethacin on rat liver IRI by quantifying Bax and 4-HNE positive hepatocytes. The study was conducted on 30 adult male Wistar rats evenly distributed into five groups: (1) the negative control group (the animals were not subjected either to incision or IRI), (2) the sham-opened group (subjected to incision, but not IRI), (3) the positive control group (subjected to IRI), (4) the vehicle-treated group (subjected to IRI) and (5) the experimental group (before being subjected to IRI, the animals were given indomethacin in a dose of 5 mg/kg). Following sacrifice, liver tissue was processed routinely and stained with Bax and 4-HNE antibodies. The quantification was performed using ImageJ software where the total number and the number of immunohistochemically positive hepatocytes were counted. The obtained results were compared using ANOVA and Tukey's post hoc test. Groups 3 and 4 had a significantly higher number of positive hepatocytes in the case of both parameters when compared to groups 1 and 2. Indomethacin significantly reduced the number of Bax- and 4-HNE-positive hepatocytes compared to the group that did not receive indomethacin. The statistical analysis revealed that there was no significant correlation between Bax- and 4-HNE-positive hepatocytes in the group that received indomethacin. The results of the present study showed that indomethacin causes a statistically significant decrease in the number of Bax- and 4-HNE-positive hepatocytes in animals subjected to IRI, suggesting its beneficial effect on rat liver subjected to IRI.

P03

CENTRAL FAT DEPOSITION, PREMENOPAUSAL BREAST CANCER AND BREAST ADIPOSE TISSUE REDOX STATE

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It is known that obesity per se may induce systemic oxidative stress. Increased oxidative stress in accumulated adipose tissue is, at least in part, the underlying cause of dysregulation of adipocytokines and development of cardiometabolic disorders and cancer as well. Association between obesity and breast cancer is well documented in postmenopausal women. However, studies among premenopausal women are inconsistent. This study was conducted with an aim to analyze redox status of breast adipose tissue in women with breast cancer with respect to their nutritive status and regional fat distribution. Overall and regional adiposity was compared between premenopausal women with breast cancer, women with the benign breast conditions and healthy women, while expression of antioxidative enzymes in breast adipose tissue was compared between women with breast cancer and women with benign breast diseases. Our results showed higher tendency of normal-weight women with breast cancer toward central fat deposition (represented by higher values of waist circumference, waist-to-hip ratio, waist-to-thigh ratio and waist-to-height ratio). Normal-weight women with breast cancer had also higher expression of catalase, glutathione-peroxidase, copper, zinc- and manganese- superoxide dismutase and thioredoxin comparing to their normal-weight benign counterparts. These findings could point to the link between central fat distribution, oxidative pressure in breast adipose tissue and carcinogenesis.

P04

THE EFFECTS OF CATALASE ACTIVITY IN HYPERTENSIVE RATS ON KIDNEY FUNCTION IN EXPERIMENTAL MODEL OF ISCHEMIC ACUTE KIDNEY INJURY

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Cardiovascular diseases are the leading cause of mortality and morbidity worldwide, with hypertension being a major risk factor. Significant amounts of oxygen free radicals are generated during kidney ischemia/reperfusion injury, and oxidative stress plays an important role in kidney damage after the ischemia. The available evidence supports the role of oxidative stress in ischemia-reperfusion injury and emphasizes the importance of antioxidant mechanisms in renoprotection. Therefore, we investigated whether endogenous catalase plays a role in the maintenance of kidney function after the kidney ischemia in experimental hypertension. An experiment was performed in anesthetized adult six-month-old male spontaneously hypertensive rats (SHR). SHR were randomly selected in six experimental groups: sham operated (SHAM; n=7); AKI (acute kidney injury) control group (AKI; n=9); and four AKI groups with different treatments (losartan-LOS, novokinin-NOV, apokinin-APO and the combination of apokinin and novokinin-COM; n=9). The right kidney was removed and the renal ischemia was performed by clamping the left renal artery for 45 minutes. Mean arterial pressure (MAP) was measured 24h after reperfusion. Plasma creatinine (PCr) was measured on Cobas Integra 400 plus. Catalase activity was determined by spectrophotometry.

	SHAM	AKI	LOS	APO	NOV	COM
MAP mmHg	147±5	101±3***	86±5$^{###}$	111±4$^{#}$	122±3$^{###}$	110±4$^{###}$
P_{Cr} µmol/l	32,7±3,9	242,7±20,1***	99,1±14,5$^{###}$	186,5±26,4	248,2±18,4	147,6±16,5
CAT %	100±5	77±3***	115±17$^{#}$	70±9	78±4	87±3$^{##}$

***p<0,001 vs. SHAM; $^{#}$p<0,05; $^{##}$p<0,01; $^{###}$p<0,001 vs. AKI

The strong correlation between catalase activity and kidney function implies that catalase could have a central role in the defense against oxidative stress and free radicals during the reperfusion period after ischemia in SHR with AKI episode.

P05

PROTEIN-PROTEIN INTERACTION BETWEEN GLYOXALASE II AND SPECIFIC REDOX DEPENDENT PROTEINS THROUGH S-GLUTATHIONYLATION MODIFICATION

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Glyoxalase II (Glo2), the second enzyme of glyoxalase system, is an antioxidant glutathione-dependent enzyme, which catalyzes the hydrolysis of S-D-lactoylglutathione to form D-lactic acid and glutathione (GSH). GSH is the most important thiol reducing agent inside the cell. For its chemistry features GSH plays a crucial role in the cellular redox state and in various cellular processes, including S-glutathionylation, which involves the reversible formation of a mix disulphide-bridge between specific cysteine residues and a molecule of GSH. S-Glutathionylation, can be spontaneous or catalyzed by enzyme, it is involved in the protection of protein thiol groups from irreversible oxidation and play key role in redox regulation by activation/inactivation of different enzyme. During the hydrolysis of glyoxalase II substrate (SLG), in the active site of Glo2 there is unprotonated glutathione molecule (GS-) which can be transferred to protein target. To demonstrate the active involvement of Glo2 in glutathionylation of different proteins, the enzyme and SLG were incubated with different proteins that are known to be glutathionylated, like malate dehydrogenase, actin or cytochrome c purified proteins. *In vitro* studies demonstrated high propensity of Glo2 to aggregate with other proteins through its catalytic site. To better understand the role of Glo2 in the mechanism of S-glutathionylation, *in silico* analysis have been performed. This approach consists in protein-protein docking investigations followed by atomistic Molecular Dynamics (MD) simulations, and it is useful to predict molecular associations between human Glo2 (in the presence and absence of GSH) and proteins investigated. Computational data confirmed the propensity of the enzyme to interact with the studied proteins through its catalytic site and evidenced a high stability of the Glo2-protein systems when GSH is present. These studies revealed that Glo2 allows a rapid and specific protein-SSG formation using SLG substrate, leading to an enzymatic regulation of S-glutathionylation in proteins of different origin and cellular compartmentalization.

P06

LYSINE METHYLATION REGULATES TRANSCRIPTIONAL CONTROL DURING HIBERNATION IN *ICTIDOMYS TRIDECEMLINEATUS*

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During mammalian hibernation, animals survive in hypometabolic states characterized by reduced body temperature, breathing rate and cellular metabolic rate, from euthermic (i.e., summer) levels. Entering these hypometabolic states allows the 13-lined ground squirrel (*I. tridecemlineatus*) to defend against winter-time environmental stresses (e.g., higher energetic costs, lack of food supply, decreased oxygen) by suppressing non-important cellular pathways and protein synthesis. Part of this regulatory shift is under the control of protein post-translational modifications and by introducing a further level of control over transcription, epigenetic controls have shown to be promising to understanding regulation of hypometabolic states in the ground squirrel. This research is an investigation into the role lysine methyltransferase enzymes play in both histone and non-histone protein methylation during the torpor/arousal cycle in skeletal muscle and liver tissues. Changes in lysine methyltransferase (KMT) enzyme expression and activity as well as changes in the methylation state of downstream histone and nonhistone proteins were observed, whereby methylation caused a shift towards cytoprotective roles during torpor, and early arousal periods. The current research furthers the study of an epigenetic fingerprint of hypometabolism, and provides insight into how the chosen tissues are able to survive extended periods of torpor.

P07

A LITTLE PARP OF DNA DAMAGE AND REPAIR DURING HIBERNATION IN THE 13-LINED GROUND SQUIRREL

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One of the causal factors of DNA damage is the accumulation of both endogenous and exogenous oxidative agents. Through differential gene regulation, many organisms have developed specialized adaptations that enable them to survive prolonged exposures to these oxidative stressors. One such model animal is the thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*. During winter, squirrels are able to survive for months in a hypometabolic state of hibernation, during which time they undergo periodic short bouts of arousal. While squirrels cycle through periods of ischemia, they have developed protective mechanisms to avoid ischemic stress and reperfusion injury. Indeed, research has shown that the squirrels do not accumulate significant DNA damage during hibernation. Multiple pathways function in concert to modulate DNA repair mechanisms, including direct repair, base excision repair, nucleotide excision repair, double-stranded break repair and cross-link repair. PARylation levels rose significantly in the liver and brown adipose tissue during hibernation of ground squirrels but showed no change in the skeletal muscle and were reduced in the white adipose tissue. These results were paralleled by the downstream DNA repair proteins responsible for non-homologous end-joining and homologous recombination pathways. This work will better our understanding of the molecular mechanisms that control DNA repair and cell fate.

P08

THE RESPONSE OF COLD-SHOCK RNA-BINDING PROTEINS DURING HIBERNATION IN 13-LINED GROUND SQUIRRELS

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Thirteen-lined ground squirrels use hibernation to adapt to periods of food scarcity and freezing temperatures. During hibernation, metabolic rate drops to just 1-5% of the normal resting rate at 37°C, body temperature decreases to 5-8°C, and breathing and heart rates drop to below 3% of euthermic levels. Incredibly, these animals emerge from hibernation in the spring without any sign of ischemia-reperfusion damage, muscle deterioration, or organ failure, suggesting they can activate select molecular pathways to enhance survival. Formerly thought to be activated upon mild cold-stress alone, cold-shock proteins respond to stressors such as UV and IR irradiation, chemical imbalances, heat shock, hypoxia, and starvation. Cold-inducible RNA-binding proteins CIRP, HuR, and Rbm3 have roles in mRNA stabilization and transport, and in the control of mRNA translation through their binding to translational machinery. They have yet to be studied in mammalian hibernators that undergo severe cold-stress. Western blotting and qPCR were used to quantify the relative protein and mRNA levels of CIRP, Rbm3, HuR, and select translation factors over 6 time points of the torpor-arousal cycle in 13-lined ground squirrel liver and muscle. In both tissues, CIRP protein levels increased during entry into torpor and Rbm3 levels remained low. HuR protein levels increased in muscle but not in liver. Only liver Rbm3 mRNA levels increased suggesting that the activities of CIRP and HuR are regulated by subcellular localization instead of timely gene expression. Cytoplasmic-nuclear distribution was assessed via Western blotting to suggest changes in RNA-binding proteins' activity between control and torpor. More CIRP was present in the cytoplasm during torpor in liver. Translation factor binding partners of these RNA binding proteins were primarily in their active states throughout the torpor-arousal cycle. Together, these results suggest that RNA binding proteins may have an important role in regulating metabolic suppression through translational activation.

P09

THE HIBERNATING SOUTH AMERICAN MARSUPIAL, *DROMICIOPS GLIROIDES*, DISPLAYS TORPOR-SENSITIVE MICRORNA EXPRESSION PATTERNS

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The marsupial, *Dromiciops gliroides*, is the only known hibernator in South America; its capacity for strong metabolic rate depression allows the species to endure seasonal cold and food scarcity. Hibernation is a complex energy-saving strategy, involving metabolic controls that include changes in gene expression that are elicited in part by regulatory control from the differential expression of microRNAs. To better elucidate the role of microRNAs in orchestrating hypometabolism, a modified stem-loop technique and quantitative PCR were used to characterize the relative expression levels of 85 microRNA species in liver and skeletal muscle of control and torpid *D. gliroides*. Thirty-nine microRNAs were differentially regulated during torpor; of these, 35 were downregulated in liver and 11 were differentially expressed in skeletal muscle. Data reveal an important tissue-specific involvement of differential microRNA expression in torpor facilitation and bioinformatic analysis indicated that these microRNAs contribute to the regulation of selected signal transduction pathways (MAPK, PI3K-Akt, mTOR), thermoregulation, and prevention of muscle disuse atrophy.

P10

DEVELOPMENT OF ANALYTICAL ASSAYS FOR THE DETECTION AND QUANTIFICATION OF REACTIVE OXYGEN AND NITROGEN SPECIES IN BIOLOGICAL SAMPLES IN AN ANIMAL MODEL OF ARTERIAL HYPERTENSION WITH EMPHASIS ON SUPEROXIDE AND NITRIC OXIDE - IMPACT ON INFLAMMATION AND VICE VERSA

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My research is embedded within the research consortium “Novel and neglected cardiovascular risk factors” with a focus on characterization of the molecular mechanisms of oxidative stress in an animal model of angiotensin-II-induced arterial hypertension. An additional focus is the establishment of new analytical assays for the detection and quantification of reactive oxygen and nitrogen species (ROS and RNS), primarily superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$) but also the associated reaction products hydrogen peroxide and peroxynitrite in biological samples (isolated enzymes, cells, animal and human tissues). Traditional cardiovascular risk factors are further exacerbated by environmental risk factors including aircraft noise, and thus I am also working on the determination of cardiovascular and cerebral ROS and RNS in various animal models in response to aircraft noise exposure. Aircraft noise is associated with a significant increase in arterial hypertension, coronary artery disease, heart failure, and stroke. The identification of reliable markers of vascular inflammation in animals with arterial hypertension is also a major objective of my project. Disruption of the delicate balance between reactive species and cellular antioxidants by excessive ROS and RNS is one of the most important risk factors for the cardiovascular system. ROS and RNS confer redox regulation of essential cellular functions like differentiation, proliferation, migration, apoptosis and also initiate and catalyze adaptive stress responses. Consequently, therapeutic targeting of the cellular redox state requires detailed molecular insights into redox regulatory pathways and advanced knowledge of the involved ROS and RNS. Thus, my research project will hopefully contribute to our understanding of redox biology and pathophysiology in a clinically relevant scientific field.

P11

ESTABLISHMENT AND CHARACTERIZATION OF NEW ANIMAL MODELS TO STUDY THE ADVERSE CARDIOVASCULAR EFFECTS OF E-CIGARETTE EXPOSURE USING A NOVEL EXPOSURE SYSTEM

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Electronic cigarettes (E-cig) are battery-powered devices that vaporize a liquid solution (propylene glycol, glycerol and nicotine) to generate an aerosol. The aerosol is inhaled by the user to simulate traditional cigarette smoking. Although E-cig vapour contains much lower quantities of the harmful components than traditional cigarette smoke, it is important to analyse possible damaging effects of E-cig now, so the long-term health risk can be predicted. The aim of the project is to observe the influence of E-cig vapour on cardiovascular function in mice. The main experimental parameters are the levels of cardiovascular reactive oxygen species (ROS) and endothelial function, together with protein and gene expression. Mice (C57BL/6) were exposed to 1, 3 and 5 day of E-cig smoke with and without nicotine. We observed impaired endothelial function in response to all of the E-cig vapour exposures of C57BL/6 mice. Results of oxidative stress measurements are inconclusive, as there is a trend towards higher oxidative stress but it is not always significant. Oxidative stress markers 3-NT and 4-HNE were assessed in heart and cortex and show a trend towards higher oxidative stress damage. Plasma inflammation markers MCP-1 and IL-6 show an increase in exposed animals compared to the control mice. We have also observed an effect of E-cig vapour on the $NO^{\cdot}$/cGMP-signalling pathway. Later, we will use a mouse model of hypertension to examine the effect of E-cig vapour on vascular function, as hypertension is a major heart disease risk factor, and it is present in an ever rising number of people.

P12

THE EFFECTS OF FOOD ENRICHED BY FISH ON OXIDATIVE STRESS IN WORKING DOGS

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Recently, there has been an increased interest in novel dietary antioxidants in dog's nutrition with increased daily physical activity, i.e. working dogs. The aim of this study was to investigate potential antioxidant effects of supplementation with a specific diet enriched with fish in working dogs for 3 months. In this study, we examined working dogs from the police gendarmerie (Belgian Shepherd Malinois). There were 10 dogs (5-females and 5-males), age categories of 3 to 7 years, body weight of 30.2±2.2 kg. In dogs, blood was taken before and after treatment with enriched omega-3 fatty acids. B
lood was taken from the *vena cephalica antebrachii*. Erythrocytes and plasma were isolated, stored at a temperature of -80°C and further analyzed. Antioxidant enzymes - glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), as well as concentration of thiobarbituric acid reactive substances (TBARS), were measured before and after the treatment of food enriched with fish for a period of 3 months. Higher activities of GPx (299.68 ±18.96 U/mg vs. 484.27±23.35 U/mg) and CAT (2.08±0.70 U/mg vs. 2.55±1.05 U/mg) were observed at the end of the treatment. There were no significant statistical differences in other parameters of oxidative stress, as comparing the pre and post-treatment. Based on our results, we conclude that diet enriched with fish for 3 months could improve oxidative status in working police dogs, by enhancing the activities of antioxidant enzymes.

P13

EVALUATION OF WALNUTS FATTY ACIDS, TOTAL PHENOLICS OF WALNUT CULTIVARS IN SERBIA

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Walnuts are a traditional food in Serbia potentially an excellent candidate for improving dietary intakes of ω-3 fatty acids, minerals and fiber in Serbia, a Balkan country that does not consume a Mediterranean style diet. There is mounting evidence of the health benefits associated with ω-3 polyunsaturated fatty acids (PUFAs). Dietary intake analysis showed that whole walnuts, as a significant source of n-3 PUFA and minerals in diet and possible alpha-linolenic acid (ALA) source, are consumed by 20% of the Serbian population. Limited fish consumption determines unfavorable fatty acid profile in Serbian population, which was confirmed analyzing dietary intake. It was shown that fish is consumed by 15.6% of Serbian population, 80.3 g/day. ALA deficiency and an unfavorable n6:n3 fatty acid ratio could be the major explanation for the high prevalence of cardiovascular diseases in Serbian and Balkan countries population. Determination of fatty acids and total phenolics content of walnuts cultivars in Serbia. Total lipids were extracted according to method of Folch, separations of the methyl esters were carried out using a gas chromatograph. The method for determining the total phenol content was based on the reaction between the Folin Ciocalteu reagent. Phenolic compounds are quantified based on their absorbance at 734 nm as modified by Dewanto et al. and Wolfe et al. The results show that walnut cultivars from Serbia were like that reported in other countries in terms of their fatty acid content, and the ratio of linolenic (C18:3, ω-3) to linoleic (C18:2, ω-6) for walnuts is the highest among all the tree nuts. Also, our results indicate that the total phenolic content of walnuts varies between 2095 and 2118 mg GAE/100 g of walnut extract. Future work in addressing public health should focus on encouraging the increased intakes of walnuts in Serbia based on their favorable fatty acid, phenolic and mineral content. Dietary intakes of walnuts should be encouraged to meet the recommended intake of ω-3 fatty acids in this country with a low intake of fish.

P14

ISOLATION, IDENTIFICATION AND ANTIOXIDANT ACTIVITY OF PIPERINE FROM BLACK PEPPER (*PIPER NIGRUM* L.)

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Plants play an important role in maintaining human health and improving the quality of human life for thousands of years. Herbal active ingredients have been used in medicine, cosmetics, food and beverages. Black pepper (*Piper nigrum* L., *Piperaceae*) fruits have been widely used in household spices and also in various traditional systems of medicine. Piperine is a major alkaloid of black pepper used as an analgesic, antipyretic, CNS depressant, anti-inflammatory, antitumor and hepatoprotective agent. In this paper piperine was isolated by Soxhlet extraction from the dried, grounded black pepper fruits (30 g) with 96% ethanol (300 ml). The obtained ethanol extract was filtered and evaporated at the rotary evaporator until a syrup consistency was reached (the obtained volume was 10 ml). Ten milliliters of 10% KOH ethanolic solution was added to the evaporated extract, the mixture was left to stand for 1 h and filtered. The obtained alcoholic solution was left to crystallize for 48 h, the crystals were separated and dried in desiccator. The crude product was recrystallized from acetone, at its boiling temperature, during 10 minutes. The acetone was evaporated and the resulting yellow crystals were dried until constant mass. The characterization of the isolated piperine was carried out using UV-VIS, FT-IR, HPLC and UHPLC/MS methods. The antioxidant activity of piperine was investigated spectrophotometrically using DPPH test. The degree of DPPH radical neutralization depends on the piperine concentration and incubation time applied. The best antioxidant activity showed piperine incubated for 40 minutes. Piperine concentrations needed for 50% of initial DPPH radical concentration neutralization (EC_{50} value) were 4.22 mg/ml (after 20 minutes) and 3.66 mg/ml (after 40 minutes incubation with radical). The presented results showed that the isolated piperine from black pepper is a potential natural antioxidant as an alternative to synthetic additives for phytopreparates production.

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P15

SYNTHETIC TUBULYSIN, TUBUGI 1, INDUCED MITOTIC CATASTROPHE IN THE A-375 MELANOMA CELLS

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Tubugi 1 is a synthetic analogue of its parental naturally derived compounds - tubulysins. These metabolites are widely recognized for their outstanding antiproliferative activity, targeting microtubules in the mitotic spindle through the process of depolymerization. The objective of this study was to determine the cytotoxic effect of tubugi 1 on A-375 human melanoma cell line and to elucidate the underlying mechanisms. Results of MTT and CV viability assays indicated a dose-dependent decrease in the percentage of viable cells upon tubugi 1 treatment. Microscopic evaluation of the nuclei of treated cells pointed to the presence of multiple micronucleation - the main imprint of mitotic catastrophe, cell death related to failed mitosis. Further, flow cytometric analyses revealed loss of dividing potential of treated cells, as well as their accumulation in the G2M phase of the cell cycle which is likely linked to mitotic cell death. Additionally, an estimation of the presence of autophagosomes by flow cytometry has shown that autophagy is occurring upon the treatment with this drug, which is considered to be cytoprotective and transient. In parallel, activation of caspases was shown by flow cytometry indicating upcoming apoptosis. The expression of two main proteins relevant for mitotic catastrophe, IkB-α and caspase 2, was strongly increased. Diminished expression of Bcl-2, elevated presence of cleaved caspase 3 along with late time activation of Bax confirmed apoptosis as a finalizing event in tubugi 1 action on A-375 cells. Also, enhanced production of ROS and intracellular NO in treated cells was found by flow cytometry, which might be connected with the execution of apoptotic cell death after the overall cascade of events present during tubugi 1 treatment on A-375. Altogether, above briefly described mode of action of tubugi 1 against human melanoma cells makes this agent valuable and promising in further preclinical development.

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P16

INTERPLAY BETWEEN REACTIVE OXYGEN SPECIES, ANTIOXIDANT SYSTEM AND NO SIGNALING IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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A common characteristic of malignancies is an increase of reactive oxygen species (ROS) and reactive nitrogen species. In such circumstances macromolecules are damaged and the cell structure and function are disrupted. The *BCR/ABL* oncogene, the diagnostic hallmark of chronic myeloid leukemia (CML) at the molecular level, induces the formation of ROS in hematopoietic stem cells that leads to genomic instability. Chemical interaction of nitric oxide (NO) with ROS constitutes the basis for the formation of additional oxidative signaling elements. Although previous reports showed an increased production of ROS in CML, the role of NO-related parameters was not revealed. In order to clarify this, we have examined the markers of oxidative and nitrosative stress in the CML patients. The study includes 22 *de novo* CML patients and 10 healthy subjects. Markers of oxidative and nitrosative stress in plasma and erythrocyte lysates were determined by the colorimetric methods. The levels of nitrotyrosine and inducible NO synthase (iNOS) were determined in granulocytes using immunocytochemistry methods. The activities of superoxide dismutase, catalase and glutathione peroxidase were reduced in the erythrocytes of CML patients. The malondialdehyde and protein carbonyl levels were elevated in plasma of CML patients. The concentration of nitrite in plasma was decreased while nitrotyrosine and iNOS expression were increased in granulocytes of CML patients compared to healthy subjects. The expression of oxidatively modified macromolecules and the activity of antioxidants demonstrated the presence of oxidative stress in CML. Those changes were associated with the high levels of nitrotyrosine as the NO-related biomarker of oxidative protein modification that could contribute to the pathogenesis of CML.

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P17

PROGESTERON EXERTS ANTI-OXIDATIVE FEATURES IN RAT MODEL OF PERMANENT OCCLUSION OF COMMON CAROTID ARTERIES

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Brain injuries, such as permanent or transient ischemia, induce oxidative stress as a consequence of overproduction of highly reactive oxygen species that overwhelms antioxidant defense capacity. This could lead to uncontrolled membrane lipid peroxidation and generation of specific products, 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) along with oxidative damage of DNA and inactivation of proteins, including protein kinase B (AKT) and its downstream effectors. The adequate neuroprotective therapy should minimize the activation of toxicity pathways and increase the activity of endogenous protective mechanisms. Due to anti-apoptotic, anti-inflammatory and membrane stabilizing properties, steroid hormone progesterone (P_4) is imposed as a compelling natural treatment agent in numerous models of neurological diseases. Nonetheless, there is scarcity of data regarding its implication in stance of chronic cerebral hypoperfusion (CCH), observed in patients with Alzheimer's disease, aging and vascular dementia. Current study assessed the extent of AKT phosphorylation by immunoblotting and employed assays for determination of the level of advanced oxidation protein products (AOPP), prooxidant-antioxidant balance (PAB) and MDA/HNE, to examine P_4 anti-oxidative capacity in rat prefrontal cortex during CCH. Adult male Wistar rats were subjected to sham operation and injected with vehicle (commercial flax oil) or to permanent occlusion of common carotid arteries and treated either with vehicle or P_4 (1.7 mg/kg/day) for 7 consecutive days. In current experimental setup, CCH induced the burst of investigated pro-oxidative parameters and decreased AKT phosphorylation, indicating the occurrence of oxidative stress. Following CCH insult and P_4 treatment, the increased lipid peroxidation and generation of MDA/HNE as well elevated levels of AOPP and PAB were downscaled, while phosphorylation of AKT was upregulated. In conclusion, P_4 might counteract the redox imbalance induced by CCH most likely by activating AKT signalling pathway and direct suppression of the emergence of pro-oxidative products.

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P18

ARE PARAMETERS OF OXIDATIVE/NITROSATIVE STRESS AFFECTED BY TRANSIENT ISCHEMIC ATTACK AND DEHYDROEPIANDROSTERONE?

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Transient ischemic attack (TIA) is a brief episode of neurological dysfunction due to a vascular cause, without detectable infarction. Although oxidative/nitrosative stress is well-recognized cause of post-stroke neuronal damage, its role in TIA pathology has received no attention in experimental field. Among various stroke therapeutics dehydroepiandrosterone (DHEA) has become one of the candidates showing conflicting effects from neuroprotective to neurotoxic. Considering the lack of data focusing on DHEA effects on specific ischemic conditions like TIA, the aim of the study was to investigate potential changes in malondialdehyde (MDA), glutathione (GSH) and nitric oxide (NO) levels following TIA and/or DHEA treatment in rat hippocampus, the brain region most vulnerable to ischemic damage. Adult male Wistar rats were treated either with single dose of vehicle (1.2 g/kg dimethyl sulfoxide) or DHEA (20 mg/kg i.p.) 4h following sham operation or 15 min bilateral common carotid artery occlusion with 24h reperfusion time (I/R). One of the end products of lipid peroxidation, MDA, was evaluated by the thiobarbituric acid reaction in whole tissue homogenate while GSH and NO levels were determined in cytosolic fraction by Ellman method and Griess reaction, respectively. According to the results, MDA and GSH levels remain unchanged in all experimental groups. A significant increase of NO level was detected in both vehicle and DHEA treated I/R groups compared to vehicle treated sham operated animals suggesting potential dysregulation of NO production and changes in nitrosative status. Interestingly, DHEA showed no effect either in physiological or ischemic conditions. In conclusion, presented data may point to the lack of DHEA effectiveness along with TIA-induced altered homeostasis governed by NO. Given that actions of NO are multifaceted and its interactions with oxygen or oxygen-related reactive intermediates may yield numerous reactive nitrosative/oxidative species, obtained data may represent valuable guideline for further research on TIA and DHEA effects.

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P19

ANALYSIS OF PATHOGENIC mtDNA MUTATIONS ASSOCIATED WITH LEBER'S HEREDITARY OPTIC NEUROPATHY: OUR EXPERIENCE

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Leber's hereditary optic neuropathy (LHON) shows a non-Mendelian genetic pattern of inheritance via mtDNA. This involves maternal transmission affecting mitochondrial oxidative phosphorylation pathway “OXPHOS” which takes place in inner membrane of mitochondria. LHON primary pathogenic mutations are impacting drastically on complex I (NADH: ubiquinone oxidoreductase), the largest complex of OXPHOS, and subsequently, reduces ATP supply of retina and optic nerve. Besides primary mutations number of secondary mtDNA changes affects phenotype. This study provides screening for primary and secondary pathogenic mutations associated with LHON in 10 Serbian families, using Sanger sequencing of mtDNA. The most frequent LOHN primary mutations in our group are: mt. 3460 G>A in ND1, mt. 11778 G>A in ND4 and mt. 14484 T>C in ND6 gene. Interestingly, frequency of mt. 11778 G>A mutation was significantly higher than other primary changes (11 of 18 patients or 61.1% of all primary mutations). This mutation converts the highly conserved 340th amino acid arginine to histidine in subunit 4 of Complex I of the mitochondrial electron transport chain. The patients positive for mt. 11778 G>A assembled within 7 families with typical matrilineal involvement. In one family this primary mutation was associated with mt. 2755 A>G mutation in 16S rRNA, which is possibly linked to left ventricular non-compaction (LVNC). In other cases different secondary mtDNA mutations associated with LHON were detected. Analysis of both primary and secondary mtDNA mutations is important for precise diagnostics and better characterization of LOHN - associated phenotypes.

P20

ACTIVITIES OF CARDIAC TISSUE MMP-2 AND MMP-9 IN DIABETIC RATS - THE ROLE OF FOLIC ACID

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The aim of this study was to examine the matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9) activity in cardiac tissue of Wistar male rats in which diabetes mellitus (DM) type I was induced by streptozotocin (STZ), as well as effects of folic acid administration. All experimental animals were divided into five groups: C1 - control (physiological saline 1 ml/kg, i.p. one day; n=8), C2 - control with daily physiological saline treatment (1 ml/kg, i.p. for 28 days; n=10), F - folic acid (5 mg/kg in physiological saline, i.p. for 28 days; n=10), DM - diabetes mellitus (STZ 100 mg/kg in physiological saline, i.p., one day; n=8), and DM+F - diabetes mellitus and folic acid group (STZ 100 mg/kg in physiological saline, i.p. one day and folic acid 5 mg/kg in physiological saline, i.p. for 28 days; n=10). After four weeks of experimental period the animals were sacrificed, the serum glycaemia was measured, and animal hearts were isolated and homogenized for MMP-2 and MMP-9 activity determination by SDS-PAGE zymography according to Stetler-Stevenson method. After administration of STZ, DM was developed (glycaemia level over 11.5 mmol/l) in all treated animals, but there was statistically significant decrease of serum glucose level in the group DMF in comparison to DM group ($p=0.006$). Comparing C and F groups, significant difference in relative MMP-2 activity was not found, while in other groups its activity was significantly increased. Folic acid treatment have reduced MMP-2 activity, comparing C2 and F group ($p=0.019$), as well as DM and DMF group ($p<0.001$). MMP-9 activity was not changed in DM group in comparison with all other groups. A statistically significant strong positive correlation between glycaemia and relative activity of MMP-2 was observed ($r=0.918$, $p=0.028$). The obtained results show increased activity of MMP-2 in diabetic rats that may be the consequence of oxidative stress caused by DM. It can also be concluded that the folic acid application has positive effects as it leads to a reduction in glycaemia and MMP-2 activity in diabetic as well as in healthy rats.

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P21

IN VITRO STUDY OF LOPINAVIR-NO NITRIC OXIDE DONATING PROPERTIES IN MOUSE MELANOMA

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The focus of our attention lies on derivative of Lopinavir obtained by linking the nitric oxide (NO) moiety to the original drug. Lopinavir belongs to HIV-protease inhibitors (HIV-PIs), a class of anti-retroviral drugs initially designed to target viral protease. Here we have investigated the nitric oxide donating properties of Lopinavir-NO in two melanoma cell lines: B16 and B16F10. Modified Lopinavir released significant amounts of NO in both cell lines. There was no release of nitrates in medium alone or in conditioned cell culture medium. In parallel, nitrite accumulation in cell supernatants upon the treatment of cells for 48 h showed dose dependent increase. The kinetics of intracellular NO release reveals that the nitric oxide levels raised significantly after 16 h. Lopinavir-NO released from two to six times more intracellular NO (depending on the dosage) compared to control, which implies that Lopinavir-NO is a smart NO donor, acting by targeted NO release inside the cell. Importantly, in the same time frame and dose range, NO releasing potential of two conventional nitric oxide donors: SNAP and SIN was negligible both extra- and intracellularly. Finally, the viability of cells treated with the combination of Lopinavir-NO and NO scavenger carboxy-PTIO was almost completely restored, indicating that NO-release had a major impact on cell viability. The cumulative intracellular production of ROS/RNS was markedly elevated for both compounds in both cell lines. Neutralization of ROS by the antioxidant N-acetylcysteine did not improve the viability of treated cells indicating that ROS/RNS production does not contribute to Lopinavir-NO mode of action. Taken together, the data presented here will help us understand the molecular mechanisms underlying antitumor activity of HIV-PIs. Lopinavir-NO is new, improved HIV-PI and a promising candidate for future clinical studies.

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THE ASSOCIATION BETWEEN *NRF1* AND *SOD2* GENETIC POLYMORPHISMS WITH THE RISK AND OVERALL SURVIVAL IN PATIENTS WITH CLEAR CELL RENAL CELL CARCINOMA

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Renal cell carcinoma (RCC) accounts for 90% of all kidney cancers. Due to poor diagnosis, high resistance to the systemic therapies and the fact that most RCC cases occur sporadically, current research has given rise to investigation of the molecular mechanisms underlying RCC. It is believed that RCC belongs to tumors in which significant changes occur in cellular redox balance. Therefore, we aimed to determine the potential role of nuclear respiratory factor 1 (NRF1) and superoxide dismutase 2 (SOD2) gene variants as determinants of risk in patients with clear cell RCC (ccRCC), as well as to discern whether phenotype changes reflect genotype-associated risk. Furthermore, we evaluated the effect of *NRF1* and *SOD2* gene variants on overall survival (OS) in ccRCC patients. This hospital-based study included 166 ccRCC patients and 348 control subjects. *NRF1* (rs6721961) single nucleotide polymorphism was determined by polymerase chain reaction-confronting two-pair primers, whereas *SOD2* (rs4880) polymorphisms by quantitative PCR (q-PCR). OS was evaluated using *Kaplan-Meier* survival analysis, during mean follow up period of 43 (1-125) months. 8-hydroxy-2'-deoxyguanosine (8-OHdG), as the most widely used fingerprint of radical attack towards DNA, was determined by ELISA method. No significant difference was observed in the distributions of *NRF1* and *SOD2* gene variants between patients and controls (p>0,05). Although *NRF1 variant* (*CA+AA*) genotype did not confer any significant risk towards ccRCC development, carriers of the SOD2 *variant* (*GA+AA*) genotype exhibited 2-fold increased risk (p=0,007). Moreover, the level of 8-OHdG was higher in the carriers of *NRF1* and *SOD2 variant* genotypes, however, without reaching statistical significance (p>0,05). Still, no association between *NRF1* or *SOD2* polymorphism and OS was found (p>0,05). In summary, determination of *SOD2* genotype might serve as a valuable indicator in ccRCC risk assessment.

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THE EFFECT OF LONG-TERM COCOA SUPPLEMENTATION ON ERYTHROCYTE AND HEPATOCYTE SUPEROXIDE DISMUTASE ACTIVITY IN C57BL/6J MICE

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An increasing body of research evidence has recognized cocoa as a “super food” due to its unique chemical composition: mixture of polyphenols, mainly catechins, and methylxanthines theobromine and caffeine. One of the main aging indicators in all living cells is compromised and damaged redox homeostasis. Related to this, present study was designed to examine cocoa powder functionality in terms of its effect on superoxide-dismutase activity in aged mice erythrocytes and hepatocytes. Investigation was conducted on thirty-six male C57BL/6 mice which were assigned to one of three diets equally groups (n 12/group): control (C), 3% cocoa powder (CP) or 0,087% methylxanthines (M) (the same dose as in the second experimental group). Liver and blood samples were collected after six months supplementation with cocoa and methylxanthines at levels equivalent to human daily cocoa powder dose of 7.3 g ~ two tablespoons. Total SOD and manganese SOD (MnSOD) activity, after the inhibition of copper/zinc SOD (CuZnSOD) with 4 mM KCN, were measured and then CuZnSOD activity calculated. Western blot analysis was applied for determination of proteins in mice liver. The obtained results suggest that total SOD activity did not differ in both erythrocytes and hepatocytes in any group of mice after long term intervention with moderate cocoa/methylxanthines quantities. Hepatic CuZnSOD level showed a statistically significant increase in cocoa and methylxanthines groups in comparison with control ($F=12.8$, $p<0.001$), while activity of this enzyme remained unchanged. When it comes to hepatocyte MnSOD level and activity, supplementation did not have any impact. Future investigations should turn light on cocoa biological compounds mechanism and if there is a dose dependent regulation of SOD and other antioxidant enzyme activity.

P24

SENESCENT PERIPHERAL BLOOD MESENCHYMAL STROMAL CELLS INDUCED BY HYDROXYUREA INHIBITS BYSTANDER HUMAN ERYTHROLEUKEMIA HEL CELL PROLIFERATION

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Hydroxyurea (HU), ribonucleotide reductase inhibitor, is an anti-cancer drug used in the treatment of hematologic malignancies. Although, HU as DNA replication stress inducer may provoke a premature senescence-like cell phenotype its repercussion on bystander cell proliferation has not been shown so far. In this study, HU strongly inhibited peripheral blood mesenchymal stromal cells (PBMSC) proliferation by cell cycle arrest in S phase, and accordingly PBMSC acquired senescence-related phenotypic changes. HU-treated PBMSC increased expression of senescence-associated β-galactosidase and p16INK4 with changes in cell morphology, as well as DNA double-strand breaks and genotoxic effects demonstrated by expression of γH2A.X and micronuclei. Furthermore, HU induced PBMSC senescence (HU-S-PBMSC) was mediated by increased reactive oxygen species (ROS) level, as demonstrated by the ROS scavenger N-acetylcysteine and NADPH oxidases inhibitor Apocynin. HU-S-PBMSC effects on bystander cells were determined by use of the JAK2-V617F-positive human erythroleukemia 92.1.7 (HEL) cells. Coculture with HU-S-PBMSC strongly inhibited bystander HEL cell proliferation, which is mediated by both ROS and transforming growth factor-β expression, as a part of senescence-associated secretory phenotype (SASP). Furthermore, the reduction of HEL cell proliferation was accompanied with increased cell adhesion to HU-S-PBMSC. Besides induction of premature senescence, HU educates PBMSC towards an inhibitory phenotype of HEL cell proliferation. As a final point, our study contributes to understanding the role of therapy-induced senescence by HU as a targeted approach that may improve clinical treatment of hematologic malignancies.

P25

ASSOCIATION OF REPRODUCTIVE FACTORS AND ANTIOXIDANT STATUS IN UTERUS OF GYNECOLOGICAL PATIENTS

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Endometrial cancer is one of the sources of morbidity and mortality among women worldwide; however, women with benign gynecological diseases may also experience increased risk of developing malignancy. Antioxidant (AO) status may influence susceptibility to many diseases associated with the deleterious effects of oxidative stress, including gynecological ones. Since we previously showed that AO status is altered in endometrium of women with hyperplasia and adenocarcinoma compared to those with polyp or myoma, in this study we aimed to evaluate the association of reproductive factors and AO enzyme activities of patients diagnosed with endometrial polyp, uterine leiomyoma, endometrial hyperplasia simplex or complex, and endometrial adenocarcinoma. The material consisted of uterine tissue specimens of women admitted to the Department of Gynecology and Obstetrics, Clinical Center of Banja Luka, for gynecological evaluation within routine checkups or for abnormal uterine bleeding (AUB). In prepared samples, the activities of superoxide dismutase, catalase (CAT), glutathione peroxidase, and glutathione reductase (GR) were determined. The Pearson correlation method and multivariate regression analysis were used to test the association of reproductive factors as predictors (parity, abortions, and AUB) with activity of each AO enzyme. Parity fitted the best predictive model for CAT activity, with significant positive association ($\beta=0.267$, $p=0.01$). The AUB gave the best predictive model for the GR activity ($\beta=0.332$, $p=0.002$), with association of 11%. There was also a significant positive correlation between parity/AUB predictor variables ($r=0.28$, $p<0.01$). The reproductive and other factors may be associated, at least partially, with antioxidant capacity and ability to defend against the oxidative damage in different diagnostic categories. The observed correlations between the predictor variables also indicate possible interactions between them in the prediction of antioxidant enzyme activities.

P26

EFFECTS OF DIETARY CADMIUM AND ZINC ON CATALASE ACTIVITY AND PROTEIN THIOL CONTENT IN *OSTRINIA NUBILALIS* (HBN.) LARVAE

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Heavy metal pollution is a global environmental problem posing a risk to living organisms. The influence of heavy metals on insects is of great importance from the aspect of global biological diversity. Especially intriguing is the impact of these metals on the economically important insects, like *Ostrinia nubilalis* (Hbn.), one of the most dangerous pests of corn in many parts of the world. One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress and increased production of reactive oxygen species (ROS). Redox inactive heavy metals, such as cadmium (Cd) and zinc (Zn), are known to induce oxidative stress through indirect mechanisms, by depleting cells' major antioxidants, particularly thiol-containing antioxidants and enzymes. The aim of this study was to explore the effects of Cd and Zn on catalase activity and protein thiol content in *Ostrinia nubilalis* larvae. Neonate larvae were fed with an artificial diet containing Cd and Zn at different concentrations ($CdCl_2$: 0.005, 0.05, 0.1, 0.5 mg/kg; $ZnCl_2$: 10, 50, 100, 200 mg/kg) or without heavy metals (control) under laboratory conditions. The results indicated different mechanisms of action of Cd and Zn at a given concentration, which can be expected since Zn is a biogenic element and Cd is not. The analyzed parameters have undergone significant changes only in the Cd treatments. The two highest concentrations of Cd increased the activity of catalase, while all the analyzed concentrations of Cd decreased the total content of thiol groups indicating their important roles in Cd induced oxidative stress defence.

P27

OXIDATIVE STRESS IN NATURAL DEVELOPING TADPOLES OF *PELOPHYLAX ESCULENTUS* COMPLEX FROG

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Anurans represent an interesting model organism for investigating changes in the oxidative stress during life time, as they have unique life cycle among tetrapods (consisted of eggs, tadpoles, juveniles, subadults and adults) related with terrestrial and aquatic environment. During the early phases of the life cycle, anuran individuals undergo drastic changes in the course of the transition from aquatic to terrestrial life style. Those changes are followed by metabolically demanding processes underlying rapid growth and responses to developmental pressures. All those processes could be responsible for the increased production of reactive oxygen species (ROS) and possible oxidative stress. The aim of this study was to investigate changes in antioxidative system response (SOD, CAT, GSH-Px, GR, GST, GSH and SH groups) and levels of oxidative damage (TBARS - lipid peroxidation) in natural developing *Pelophylax esculentus* complex frogs. Frogs were caught from May to July in 2017 at Special Nature Reserve Deliblatska Peščara. We divided development in four stages with clear differences: feeding and free-swimming tadpoles (covering 25-31 Gosner stage - GS), tadpoles with developed hindlimbs (35-41 GS), tadpoles with both limbs (42-46 GS) and juvenile individuals. Our results revealed that individuals at earlier stages (25-31 GS) faced with higher oxidative damage (higher TBARS and lower SH groups) that was followed by higher GR, GSH-Px and GST in comparison to other stadiums. Increased growth of individuals in this stadium and exposure to environmental factors directly for the first time may result in higher ROS production and as a consequence higher antioxidative system response and oxidative damage.

P28

CuZnSOD AND nNOS LOCALIZATION IN ERYTHROCYTES FROM DUCHENNE-BECKER PATIENTS

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Aside of sarcolemma dystrophin deficiency associated with Duchenne-Becker muscular dystrophy (DMBD), higher oxidative pressure also contributes to muscle fiber degeneration and disease progression. The increased activity of catalase, CuZn superoxide dismutase (CuZnSOD) and glutathione transferase (GST) in order to protect both muscle and non-muscle cells from oxidative damage has been shown, but their cellular localization remains unsolved. Also, the role of neuronal nitric oxide synthase (nNOS) situated near dystrophin in sarcolemma has not been investigated in non-muscle cells. We have previously shown significant differences in the erythrocytes' catalase localization in DMBD patients comparing to control and this study aimed to further examine presence and localization of CuZnSOD and nNOS in erythrocytes of DMBD patients. In the present study immunogold labeling was used to compare CuZnSOD and nNOS localizations in erythrocytes from patients with Duchenne-Becker muscular dystrophy and from age-matched normal boys. Immunogold labeling was performed on ultrathin Araldite sections (70 nm), using antibodies against CuZnSOD and nNOS, examined under the transmission electron microscope and quantified by Image J. Significantly higher amount of CuZnSOD and reduced amount of nNOS is present in erythrocytes of DMBD patients compared to control. Ultrastructural localization of CuZnSOD and nNOS by gold immunocytochemistry showed that the label is mostly localized in erythrocytes' cytoplasm, with sporadic decoration of the cell membrane. In addition, clustering of nNOS-gold complexes was observed in DBMD samples. The lesser presence of nNOS is observed in acquired and inherited neuromuscular disorders including Duchenne muscular dystrophy while the increase of CuZnSOD is noticed in vascular dysfunction in various diseases. In the light of disturbed erythrocyte structure, deficiency of nNOS may lead to and may contribute to reduced blood flow, e.g. tissue ischemia and thus degeneration of muscle fibers in DMBD as well. The timely treatment of muscular dystrophy patients, based on reduction of oxidative damage, could diminish muscle injury, and possibly change progression of the disease.

P29

ANTI/PRO-OXIDANT AND PROAPOPTOTIC ACTIVITIES OF *CENTAURIUM ERYTHREA* EXTRACTS ON COLON CANCER CELLS

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The European centaury (*Centaurium erythrea* Rafn.) is a plant very rich in secondary metabolites, with various biological activities and therapeutic applications in the treatment of different medical problems, especially digestive tract diseases. This study provides data about *C. erythrea* phenolic content and its redox potential in two model systems: *in vitro* evaluated by DPPH method and live model systems, HCT-116 and SW480 colon cancer cells, as well as cytotoxic and proapoptotic activity on these cell lines. Three different extracts from *C. erythrea* (methanol, acetone and ethylacetate) were prepared for spectro-photometrical evaluation the total phenolic content, flavonoid concentrations and antioxidant activity. The phenolic content in extracts ranged between 34.02 to 62.99 mg GA/g. The highest concentration of phenolics and flavonoids was observed in methanol and acetone extracts respectively. The high linear correlation between the values of total phenolic content and antioxidant activity in DPPH method was observed. Among investigated extracts, ethylacetate extract showed significant cytotoxic activity on both cell lines as did acetone extract on SW480 cells (IC_{50} values are approximately 30 μg/ml), without cytotoxic effect on normal human fibroblasts. In accordance with cytotoxic effects, extracts induce apoptosis, where treated cells showed changes in cellular morphology typical for apoptotic cells stained with acridine/orange ethidium bromide, without necrotic effects. The apoptosis is induced due increased protein expression of Fas receptors on cell membrane, increased activities of caspase 8 and 9 in treated cells compared with control. The treatments by *C. erythrea* extracts cause change in redox status of cancer cells: mainly they induce prooxidant activity by increasing $O_2^{\bullet -}$ concentrations and antioxidant by reducing iNOS protein expression and NO level, as well as enhanced antioxidant protection by increasing of GSH level in treated cells. Based on observed results, ability of *C. erythrea* to affects signal molecules in cancer cells and modulate redox status can be used for design of drugs originating from nature, with desirable properties in prevention and treatment of cancer.

P30

EFFECTS OF OLIVE LEAF EXTRACT ON OXIDATIVE STRESS AND KIDNEY FUNCTION IN EXPERIMENTAL FOCAL SEGMENTAL GLOMERULOSIS

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Olive (*Olea europaea* L.) leaf extract (OLE) is rich in phenolic compounds that possess antioxidant properties. Oxidative stress contributes to the progression of chronic kidney disease. In this study we investigated the effects of OLE treatment on oxidative stress and kidney function in spontaneously hypertensive rats (SHR) with experimental focal segmental glomerulosclerosis (FSGS). Adult female SHR were divided in three groups. Control rats (CON) received vehicle, while FSGS and FSGS+OLE group received adriamycin (2 mg/kg body weight *i.v.*) twice in 3-week-interval. After the second injection, FSGS+OLE group received OLE, 80 mg/kg/day, by gavage for 6 weeks. Plasma albumin, albuminuria and renal antioxidant enzymes activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were analyzed. In FSGS group albuminuria was significantly increased (p<0.001) and plasma albumin level was significantly decreased (p<0.05) compared to control. OLE treatment markedly, but not significantly decreased albuminuria compared to FSGS group. Plasma albumin level was slightly, but not significantly increased in FSGS+OLE compared to FSGS group. SOD and GPx activities in the kidney were significantly decreased in FSGS group (p<0.05) compared to control. In FSGS+OLE group SOD activity was similar as in FSGS group and significantly decreased compared to control. OLE treatment increased GPx activity to the level not significantly different from control. Renal CAT activity was significantly increased in FSGS+OLE compared to both FSGS and control group. Chronic OLE treatment improves renal antioxidative defense indicating to a potential role in slowing down the progression of kidney disease in experimental FSGS.

P31

A NOVEL ROLE FOR AN ANTI-OXIDANT TRANSCRIPTION FACTOR NRF2 IN REGULATING THE AGE-RELATED CHANGES IN SKELETAL MUSCLE CLOCK FUNCTION

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The circadian clock is an evolutionary conserved intrinsic timing mechanism that governs most physiological and metabolic processes across species. Disruption of circadian rhythms in humans has been identified as a major risk factor for several age- related diseases characterised by an altered redox control. We have previously published that NRF2-dependent antioxidant defence system is subject to circadian clock control both *in vitro* and *in vivo*. Here we investigated the hypothesis that NRF2, a master regulator of antioxidant defence, is a clock-controlled gene in skeletal muscle, which itself modulates the circadian clock function. Using genetic, pharmacological and real-time imaging approaches, we identified a feedback mechanism between the molecular clock machinery and the NRF2/KEAP1 antioxidant pathway. Pharmacological manipulation of NRF2 exerted robust effects on both the amplitude and phase of circadian clock oscillations. Moreover, single muscle fibre analyses demonstrated cell-autonomous changes in the core clock gene expression in fibres isolated from *Nrf2* KO mice or cells isolated from Keap1 KD mice Interestingly, similar effects on clock gene expression were evident in muscle fibres isolated from old wild-type mice. Loss of *Nrf2* or ageing led to diminished muscle clock gene cycles but activated *de novo* rhythmic transcriptional cycles of genes involved in inflammation and stress resistance. All together, these findings implicate NRF2 as a novel therapeutic target which may be utilised to reset disrupted circadian rhythms seen in several age-related diseases associated with skeletal muscle wasting including COPD and sarcopenia.

P32

THE SERUM LEPTIN CONCENTRATIONS AND THE MARKERS OF OXIDATIVE STRESS AND INFLAMMATION AMONG OVERWEIGHT AND OBESE YOUNG ADULTS

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Obesity can induce systemic oxidative stress through multiple biochemical mechanisms, such as superoxide generation from NADPH oxidases, oxidative phosphorylation, protein kinase C activation. The possible contributors to oxidative stress in obesity include hyperglycemia, elevated lipid levels, vitamin and mineral deficiencies, chronic inflammation, endothelial dysfunction, impaired mitochondrial function. Obesity is described as a state of chronic low-grade inflammation, which is another important source of oxidative stress in obesity. Obesity is associated with elevated plasma leptin levels. Leptin plays an important role in obesity-induced oxidative stress. The hormone leptin activates NADPH oxidase and induces the production of reactive intermediates. The aim of the study is to investigate the association between oxidative stress, total oxidant status, adiponectin and leptin with BMI in young adults. The study group is divided according to their BMI in three groups: normal weight (BMI < 25; N=106), overweight (BMI ≤ 25 to < 30; N=37), obesity (BMI ≥ 30; N=32). We determined clinical parameters, oxidative stress parameters, total oxidant status (TOS), thiobarbituric acid reactive substances (MDA), protein carbonyls, antioxidants, reduced glutathione, glutathione peroxidase, catalase, total antioxidant capacity, systemic inflammatory markers (high-sensitivity C-reactive protein), leptin and adiponectin. Overweight and obese subjects have higher serum levels of MDA, TOS and hs-CRP vs. normal weight subjects. Overweight and obese subjects have lower levels of adiponectin vs. normal weight subjects. Our data indicated that hs-CRP levels were positively correlated with BMI and leptin. Alteration in leptin, hs-CRP and oxidative stress levels were found to be related to overweight and obesity. All this data may play an important role in pathogenesis of chronic disease related to the increase of BMI.

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EFFECT OF ORGANOMODIFIED CLINOPTILOLITE (ZEOLITE) ON REDOX BALANCE OF COLOSTRUM AND BLOOD PLASMA OF PRIMIPAROUS DAIRY COWS

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Periparturient period, defined as last 3 weeks before parturition to first 3 weeks after parturition, is critical for health and subsequent performance of dairy cows. Oxidative stress, i.e. “oxygen paradox” associated with rapid differentiation of secretory parenchyma, mammary gland growth, and the start of colostrum/milk synthesis and secretion in this period, can greatly contribute to various disorders in dairy cattle and consequently newborn calves. We have investigated the effect of orally administered organomodified clinoptilolite (Minazel Plus®, Patent Co., Serbia) (150 g per day, in 1 L of water) on antioxidant status of blood plasma and colostral serum of 20 primiparous dairy cows measured by FRAP and TBARS assay, starting from 20±5 days before the expected calving term up to 7 days after calving. The control group of animals (n=16) has received pure water in the same amount and time as treated animals. Supplementation with clinoptilolite induced an increase of 41% and 14% in FRAP activities of blood plasma on 2^{nd} and 7^{th} day after parturition, respectively. Regarding the colostrum samples, 33% higher antioxidant capacity of colostral sera 24h after calving was shown in zeolite treated animals. Seven days after calving level of measured lipid hydroperoxide-MDA by TBARS was 40% lower in blood plasma of treated group. Interestingly, TBARS analysis of colostrum revealed MDA level in clinoptilolite treated group 120% higher than MDA level in control animals 24 h after calving. Although considered as pro-oxidants, revealed higher level of ROS in colostrum of treated animals can be beneficial to overcome poorly developed intestinal defense mechanisms against bacteria in neonate calves. In line with increased antioxidant capacity measured by FRAP in the same colostrum sample, altogether results indicate positive effect of clinoplilolite on redox status of colostrum. Results allow recommending use of the organomodified clinoptilolite as an agent for preventing development of oxidative stress in dairy cows during adaptation for parturition.

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EFFECT OF *N*-ACETYL-L-CYSTEINE ON PROLIFERATION OF DENTAL TISSUE STEM CELLS: A PROPOSED MECHANISM OF ACTION

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N-acetyl-L-cysteine (NAC) is a powerful antioxidant. NAC is a precursor of L-cysteine that results in elevation of glutathione biosynthesis. It acts directly as a scavenger of free radicals, especially oxygen radicals. NAC stimulates proliferation of various types of mesenchymal stem cells. Obtaining high number of stem cells is of interest for cell based therapies. The aldehyde dehydrogenase (ALDH) group is composed of several enzymes which detoxify aldehydes into carboxylic acids and are involved in self-protection, differentiation and cellular expansion. ALDH has been identified as an important enzyme in the protection of stem cells, and is also widely used as a marker to identify and isolate various types of normal stem cells. In addition, emerging evidence suggests that ALDH1 is not only a marker for stem cells, but may also play important functional roles related to self-protection, differentiation, and expansion. The objective of our study was to determine the possible mechanism of NAC stimulation of dental tissue stem cells (DTSC) proliferation. DTSC proliferation after treatment with different NAC concentration was determined by flow cytometry, using CFSE staining. Higher intensity of CFSE (mean fluorescence intensity) had population of cells with higher population doubling time (PDT). Fluorescent ALDH substrate (BODIPY® - aminoacetaldehyde (BAAA/Aldefluor®) is used to identify ALDH+ cells by flow cytometry. According to the manufacturer's instructions (StemCell Technologies), the Aldefluor kit allows to differ a population with high ALDH vs. low ALDH enzymatic activity (hereafter referred to as $ALDH^+$ and $ALDH^-$). NAC in concentration of 0.1 mM stimulated proliferation of DTSC in sixth passage. NAC also stimulated increasing the percentage of $ALDH^+$ cells in total population of DTSC. It was shown that percentage of $ALDH^+$ cells in total DTSC population was in negative correlation with PDT. We propose that the underlying mechanism by which NAC stimulated the proliferation of DTSC was by increasing the percentage of $ALDH^+$ cells in total DTSC population.

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BOTH SATURATED AND UNSATURATED HIGH FAT DIETS ALTER BLOOD PRESSURE, LIPID AND OXIDATIVE STATUS IN WISTAR RATS

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The incidence of cardiovascular diseases increases rapidly, one of the main factors that favor it is excessive consumption of fat. Reviewing the evidence about saturated and unsaturated fatty acids consumption is incredibly confusing. The associations between dietary fat and risk of cardiovascular diseases remain controversial, for every study showing harmful, there is another one showing beneficial effects. The aim of our study was to investigate influence of saturated (lard) and unsaturated (soybean oil) high fat diets on blood pressure, as well as lipid and oxidative status in normotensive condition. Wistar rats were randomly divided into three groups and fed with a standard diet (STD) or with one of two high-fat diet for 8 weeks. High fat diet groups received commercial food enriched with either lard (LD) or soybean oil (SO) up to 20% of the food mass. Blood pressure was measured at the end of experiment. Lipid profile and protein oxidation (advanced oxidation protein products - AOPP) as a marker of oxidative stress were measured in plasma.

	Diastolic blood pressure (mmHg)	Total cholesterol (mmol/L)	Triglycerides (mmol/L)	LDL-C (mmol/L)	AOPP (µmol/L)
STD	57.25±3.75	1.09±0.06	0.53±0.04	0.24±0.02	33.56±3.00
LD	68.75±3.22*	1.48±0.03***	0.65±0.07	0.41±0.03***	54.31±2.3***
SO	70.75±1.74**	1.24±0.03**	0.64±0.09	0.38±0.02***	45.31±2.21**

($p<0.001$; $p<0.01$; $p<0.05$ vs. STD)

Our results show that both high-fat diets directly favor development of hyperlipidemia. Elevation of blood pressure in both high-fat diet groups was associated with increased oxidative stress, indicating that both saturated and unsaturated high fat uptake carried a cardiovascular risk. This basic research demonstrates importance of balanced diet as a way to prevent cardiovascular disorders due to elevated oxidative stress followed by high blood pressure.

P36

RUTIN SUPPLEMENTATION IMPROVES OXIDATIVE STRESS AND REDUCES SOD ACTIVITY IN SPONTANEOUSLY HYPERTENSIVE RATS

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Flavonoids are polyphenolic compounds that occur in most foods of plant origin (vegetables, fruits, herbs, leaves, etc.). Our previous study showed rutin as one of the most abundant compounds of *Urtica dioica* L. leaf extract that exerts antioxidative and antihypertensive properties in spontaneously hypertensive rats (SHR). Earlier was shown that, in cases of prolonged consumption, rutin and its metabolites were being accumulated in plasma. To evaluate whether rutin alone could be responsible for antioxidative and antihypertensive effects, adult male rats were intragastrically supplemented with 2 mg/kg/day of rutin trihydrate for 28 days (SHR+R group). Control SHR received vehicle (SHRC) by the same procedure. Systolic arterial pressure (SAP) was recorded on Cardiomax III in anesthetized rats. Blood samples were obtained by puncture of the abdominal aorta and centrifuged to separate plasma for the thiobarbituric acid reactive substances assay (TBARS). The remaining erythrocytes were washed in cold saline and prepared for the determination of superoxide dismutase (SOD) and catalase (CAT) activities. Four-week administration of rutin did not change SAP of SHR (SHR+R: 204.42±8.23 vs. SHRC: 203.38±5.76 mmHg). However, in SHR+R group plasma TBARS was significantly reduced compared to value in SHRC group ($p<0.05$), and was positively correlated ($r=0.5730$, $p=0.026$) with the significant reduction of erythrocytes' SOD activity ($p<0.01$). The activity of CAT was also reduced in the erythrocytes of SHR+R group compared to SHRC ($p<0.05$). A significant positive correlation was observed between erythrocyte SOD and CAT activities. These data indicate that decreased oxidative stress could have been the result of the direct antioxidant activity of rutin as a scavenger of reactive oxygen species.

P37

THE EFFECTS OF FISH-BASED AND MILK-BASED DIETS ON LIVER TISSUE ANTIOXIDANT ENZYMES IN FEMALE WISTAR RATS

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Recently, there has been an increased interest in novel dietary antioxidants, including omega-3 fatty acids and bioactive proteins present in milk. The aim of this study is to investigate potential antioxidant effects of four-weeks long fish-based and milk-based diets in female Wistar rats. Four-months old rats were divided in three groups receiving either: control diet, diet enriched with fish meal, or diet enriched with milk. The activities of antioxidant enzymes - glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT) were determined in liver homogenates obtained at the end of treatment period. Statistically significant higher activities of GPx (3.52±0.73 U/mg) and CAT (147.25±15.93 U/mg) were detected in rats fed with fish-based meal, in comparison with both the control (GPx: 1.93±0.11 U/mg, $p<0.001$; CAT: 99.37±10.03 U/mg, ($p<0.05$) and the group fed with milk-based diet (GPx: 1.72±0.52 U/mg, $p<0.001$; CAT: 104.18±37.49 U/mg, ($p<0.05$). There were no significant differences in the activities of SOD between the groups. Furthermore, a significant correlation was found between GPx and CAT activities ($p<0.05$, r=0.795), while SOD positively correlated with both GPx and CAT, but not significantly. In the control group, we observed significant correlation ($p<0.05$) between the GPx and CAT activity as well. Based on our results, we conclude that diet enriched with fish could improve one's oxidative status by enhancing the activities of antioxidant enzymes in liver tissue. On the contrary, we obtained no results suggesting that milk could serve as a source of dietary antioxidants.

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HYPERBARIC OXYGEN PRECONDITIONING DECREASES OXIDATIVE STRESS IN EXPERIMENTAL MODEL OF ISCHEMIC ACUTE KIDNEY INJURY

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Oxygen-derived free radicals play an important role in several models of reperfusion injury. Increased oxidative stress is one of the main problems in the ischemic type of acute kidney injury (AKI). Hyperbaric oxygen preconditioning has been shown to prevent ischemia-reperfusion injury in different tissues. The aim of our study was to compare the effects of hyperbaric oxygen preconditioning on oxidative stress in normotensive and spontaneously hypertensive rats who suffered from kidney ischemia-reperfusion injury. The experiment was performed in anesthetized, adult six-month-old male normotensive Wistar (W) and spontaneously hypertensive rats (SHR). Rats were randomly divided into four experimental groups: Wistar AKI control group (WAKI); SHR AKI control group (SHAKI); Wistar and SHR AKI group with hyperbaric oxygen preconditioning (WHBO and SHHBO). The right kidney was removed and the renal ischemia was performed by clamping the left renal artery for 45 minutes. Treated rats were placed into experimental HBO chambers and exposed to pure oxygen, twice a day (in a 12-hour interval) for two consecutive days (oxygen pressure: 2.026 bar) for 60 minutes. AKI was performed on the next morning. Plasma lipid peroxidation (TBARS), superoxide dismutase (SOD) and catalase (CAT) activity in erythrocytes were measured spectrophotometrically.

	MAP, mmHg	TBARS, nmol/mL	SOD %	CAT %
WAKI	86±2	20.4±2.6	135±10	167±20
WHBO	87±4	6.0±0.4***	238±30**	140±7
SHAKI	102±6	12.9±1.5	100±3	100±7
SHHBO	92±6	8.4±0.9$^{\#\#}$	106±9	130±10$^{\#}$

** p<0.01 and *** p<0.001 vs. WAKI, $^{\#}$ p<0.05 and $^{\#\#}$ p<0.01 vs. SHAKI

Our results suggest that HBO decreases oxidative stress of W and SHR with AKI episode, but they also imply that preexisting hypertension does not affect the beneficial effects of HBO preconditioning.

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WARM ACCLIMATION DURING WINTER DIAPAUSE DIFFERENTLY AFFECTED SOD, CAT AND GST ACTIVITY IN LARVAE OF EUROPEAN CORN BORER *OSTRINIA NUBILALIS* HBN

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As a major corn pest, Lepidopteran species of *Ostrinia nubilalis* has been of great agricultural and economic interest for many decades. However, since larvae of *O. nubilalis* overwinter in diapause, a state of arrested development during which they become freeze-tolerant, they have been an excellent model system for the investigation of relation between diapause and cold hardiness. However, due to ongoing and profound climate changes, European winters became milder, frequently with periods of unusually warm waves and it is a question how those changes will reflect on cold-adapted species. The aim of this study was to explore effects of long term warm acclimation of diapausing larvae of *O. nubilalis,* which are adapted to winter temperatures during evolution. Larvae were sampled during the time course of diapause from two distinct experimental groups of corn stalks – one kept in field conditions (FC), exposed to field subzero temperatures, and second from corn stalks held indoors at high ambient temperatures (14-22°C), warm acclimated (WA). In order to analyze the influence of warm acclimation of diapausing larvae on their antioxidative system, the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) was recorded in the whole body homogenates. Results have showed that the activity of SOD, CAT and GST changed differently, depending both on the ambient temperatures and period of diapause. The activity of SOD proved to be more responsive to changes in ambient temperature during diapause, being higher in FC group, while the activity of CAT and GST had similar trend in both groups and seems to be more under the control of endogenous program of arrested development.

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THE EFFECT OF DHA SUPPLEMENTATION IN MICROGLIA CELLS, A MODEL FOR ACOX1 DEFICIENCY

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The central nervous system (CNS) has been long recognized as an immune-privileged site. But over the last several years, evidence has accrued suggesting that the CNS contains resident immune cells that actively participate in immune surveillance and shape the CNS development and neuronal function under steady states. These resident cells include various types of macrophages, including the most abundant and best studied population, microglia. *Acox1* gene encodes ACOX1 involved in the peroxisomal beta-oxidation of very long chain fatty acids (VLCFAs) and this pathway participate also to the endogenous synthesis of DHA by shortening the C24:6 precursor. ACOX1 enzyme is found in cell structures (organelles) called peroxisomes, which contain a variety of enzymes that break down many different substances. Specifically, it is involved in the first step of a process called the peroxisomal fatty acid beta-oxidation pathway. This process shortens the VLCFA molecules by two carbon atoms at a time until the VLCFAs are converted to octanoyl-CoA and acetyl-CoA, which are transported out of the peroxisomes for reuse by the cell. The aim of this study was to analyse the effect of DHA supplementation in mutant microglia BV2 cells ACOX1-deficient, which leads to dysregulation of lipid metabolism and the development of neuroinflammation in the context of the neurodegenerative disease. BV2 cells (wild type - WT and ACOX1 deficient - D9) were prepared for different analysis before and after supplementation with DHA. For the proliferation and maintenance of biological constants of BV2 cells, synthetic media (DMEM High Glucose) with 10% serum and 1% antibiotics (Penicillin-Streptomycin to avoid cell contaminations) was used. The passage of BV2 cells and the medium change were applied when 85% of confluence is reached. ACOX1 and catalase activities were measured by spectrophotometer using the transparent 96-well plate specific for UV reading. MTT test was used for determination cytotoxicity of different concentrations of DHA (12.5, 25, 50, 100 and 150 µM). Proliferation of BV2 cells with 85% confluence was achieved during 2 days of incubation in medium at 37°C. Catalase activity was higher in ACOX1-deficent BV2 cells compared to the wild-type BV2 cells before supplementation with DHA. Catalase activity increased in ACOX1-deficent BV2 cells after supplementation with DHA. Catalase activity did not show any change in wild-type BV2 cells after supplementation with DHA. Supplementation with each concentration (12.5, 25, 50, 100 and 150 µM) did not show cytotoxicity in ACOX1-deficent BV2 cells. Supplementation with 150 µM DHA showed less viability in wild-type BV2 cells. Microglia BV2 cells ACOX1-deficient showed higher catalase activity compared to wild-type BV2 cells both before and after DHA supplementation.

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COMBINED *NRF2* AND ANTIOXIDATIVE ENZYME GENE POLYMORPHISMS IS ASSOCIATED WITH OVERALL SURVIVAL OF PROSTATE CANCER PATIENTS

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Prostate cancer (PCa) is the second most commonly diagnosed malignance in men. Incidence of the disease rises every year, leading to a substantial public health burden in western countries. In order to facilitate treatment, decision-making and outcome prediction, current research focuses on discovering and integrating potential prognostic factors into risk stratification groups. Available data show that reactive oxygen species might influence the prostate carcinogenesis. Therefore, the aim of our study was to determine whether glutathione peroxidase 1 (*GPX1* rs1050450), superoxide dismutase 2 (*SOD2* rs4880) and nuclear factor erythroid 2-related factor 2 (*NRF2* rs6721961) single nucleotide gene polymorphisms contribute to risk for PCa development and patients' overall survival (OS). This case-control study consisted of 160 patients with prostate cancer and 160 healthy men in the control group. *GPX1* and *SOD2* gene polymorphisms were determined by quantitative polymerase chain reaction (q-PCR) and *NRF2* polymorphism by PCR with confronting two-pair primers (PCR-CTPP). Logistic regression was used to assess the association between polymorphisms and PCa development and *Kaplan-Meier* survival analysis for estimating OS during 41±7 month follow-up. According to our results, there was no association between observed gene polymorphisms and risk of PCa development ($p>0.05$). Furthermore, there was no difference in OS when *GPX1, SOD2* or *NRF2* variant allele carriers were compared to those carrying respective referent genotypes ($p>0.05$). However, *NRF2* polymorphism had an additive effect when combined with *SOD2* and *GPX1* polymorphisms. Patients holding both *NRF2 variant* (*CA+AA*) and *GPX1 referen*t (*CC*) genotypes, or *NRF2 variant* (*CA+AA*) and *SOD2 referent* (*GG*) genotypes had the shortest OS ($p=0.046$ and $p<0.001$, respectively). In conclusion, determination of *SOD2*, *GPX1* and *NRF2* genotypes might contribute to better evaluation of the risk and prognosis of PCa patients.

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THE IMPACT OF OXIDANTS ON AKT/mTOR SIGNALING IN HUMAN ERYTHROLEUKEMIA HEL.92.1.7. CELL LINE

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The *JAK2*V617F mutation represents the most common genetic defect in patients with Philadelphia chromosome negative myeloproliferative neoplasms (Ph^- MPN), that leads to a constitutive activation of Janus kinase - signal transducers and activators of transcription (JAK-STAT) and PI3K/Akt/mTOR pathways, resulting in uncontrolled cell proliferation and excessive production of reactive oxygen species (ROS). The oncogenic PI3K/Akt/mTOR signaling pathway is a potential target for ROS action. In order to examine how Akt and mTOR are regulated by oxidation, we analyzed the effect of oxidants on kinases' phosphorylation and activation in HEL cells, as a representative Ph^- MPN model cell line. In addition, we analyzed cell viability and cell cycle activity after treatment with oxidants and rapamycin. The influence of H_2O_2 and 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH) oxidants and antioxidant N-acetyl cysteine (NAC) on AKT/mTOR signaling pathway, cell cycle and survival were tested by Western blot, flow cytometry and MTT test in HEL cells. The treatment of HEL cells with H_2O_2 caused the time and dose dependent activation of AKT/mTOR signaling, while long-term treatment with AAPH, as the source of continuous production of peroxyl radicals, increased only the activity of mTOR kinase. The reduced viability of HEL cells by H_2O_2 reached statistical significance after pretreatment with mTOR inhibitor rapamycin, wherein the NAC acted cytoprotective. The mTOR inhibition significantly reduced the number of HEL cells in the S phase of cell cycle. The AKT/mTOR signaling was changed under the influence of the type of oxidant as well as time and dose dependent. The mTOR kinase stimulated cell survival and cell cycle progression in oxidative stress conditions.

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CELL MODELS OF INSULIN RESISTANCE: STUDYING THE ROLE OF MITOCHONDRIA

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Impairment of mitochondrial function has been linked to insulin resistance, but the precise role of mitochondria in the development of this state is not yet clearly understood. The goal of this research is to establish two different cell models of insulin resistance, using HuH7 hepatocyte and C2C12 skeletal muscle cell lines, and study the role of mitochondria in the conditions of *in vitro* insulin resistance (IR). In contrast to other published cell models of insulin resistance, induced mostly by palmitate exposure, we exposed cells to chronic insulin treatment. We followed the alterations of insulin sensitivity at the level of insulin signaling, by following the level of phosphorylation of protein kinase B (Akt) by immunoblot. In order to understand if insulin sensitivity changes throughout the process of differentiation in C2C12 cells, and at which stage it is best to start with the insulin treatment, we studied the response to acute and chronic insulin stimulation at different stages of differentiation: from proliferative myoblasts to fully differentiated myotubes. Concentrations of insulin treatments used on muscle and liver cells found in literature are very different, and in most cases a lot higher than the physiological concentrations found *in vivo*, so we also tested the response to insulin stimulation using a wide range of different insulin concentrations for acute and chronic treatments. In these *in vitro* IR models we have measured ROS production by flow cytometry and mitochondrial respiration by high-resolution respirometry. We found no significant increase in ROS production with insulin treatments up to 24 hours long. Cell respiration was differently affected in the two cell lines - chronic insulin treatment causes an increase in respiration in C2C12 cells, while it does not significantly affect respiration in HuH7 cells. Establishing these cell models of insulin resistance in hepatocytes and myocytes opens a possibility of comparative analysis that could clarify potential tissue-specific mechanisms for regulation of insulin sensitivity that include mitochondria.

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THE INFLUENCE OF *SOD2* AND *GPX1* GENETIC POLYMORPHISMS ON THE RISK AND OVERALL SURVIVAL IN PATIENTS WITH END STAGE RENAL DISEASE

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The onset and progression of end stage renal disease (ESRD) has been linked to oxidative stress. The antioxidant enzymes, superoxide dismutase (SOD) and glutathione peroxidase (GPX) form the primary defence system against oxidative stress. Genes coding these enzymes are polymorphic, which ameliorates their antioxidative capacity. We hypothesised that *SOD2* and *GPX1* gene variants can influence the risk, as well as, overall survival among ESRD patients. This hospital-based study included 256 ESRD patients and 374 healthy controls. *SOD2* (rs4880) single nucleotide polymorphism was determined by quantitative PCR (q-PCR) while *GPX1* (rs1050450) polymorphism by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The influence of the genetic polymorphisms on the risk of ESRD development was assessed by logistic regression. Overall survival (OS) rate was estimated by *Kaplan-Meier* survival analysis during mean follow up period of 55.38±33.21 months. Patients homozygous for *SOD2 variant* allele (*Val/Val*) were more frequent among patients than in controls (32% vs. 22%, respectively), as well as, in 2-fold higher risk of ESRD development (p=0.002). Although *GPX1* polymorphisms alone did not show an association with ESRD risk, it had an additive effect when combined with *SOD2* polymorphism. Individuals who carried both *GPX1* and *SOD2 variant* genotypes (*GPX1 Leu/Leu* and *SOD2 Val/Val*) had a 3-fold higher risk of ESRD development (p=0.019). Regarding *Kaplan-Meier* survival analysis, carriers of at least one *variant* allele of *SOD2* gene showed trend towards decrease in OS. Interestingly, in case of *GPX1* polymorphism, patients homozygous for *GPX1 variant, Leu* allele, had a survival advantage (p=0.038). Assessment of *SOD2* and *GPX1* genotypes might serve as a significant indicator of both ESRD risk and patients' survival.

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EVALUATION OF PAB VALUES AND PON1 ACTIVITY IN COLORECTAL CANCER

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Oxidative stress may have a significant role in colorectal carcinogenesis. A direct connection between lipids and oxidative stress status is probably achieved through HDL anti-oxidative protective effects which mainly depend on its undisturbed bound with a paraoxonase1 (PON1) enzyme. PON1 is an aryldialkylphosphatase, which helps in eliminating lipid peroxides and protects HDL and LDL particles from oxidation. Prooxidative-antioxidative balance (PAB) is a valid parameter that provides the most complete information on the redox state of the organism. The aim of our study was to explore the association between general biochemical parameters, especially HDL-cholesterol (HDL-C) and oxidative potential, expressed as PON1 activity and PAB values in colorectal cancer (CRC). 99 CRC patients and 101 healthy controls were included in our study. PON-1 activity was determined toward substrate paraoxone. A modification of Hamidi-Alamdari's method was used for PAB assessment. Lower levels of HDL-C, LDL-C and TC were observed in the CRC group, while elevated PAB values and diminished PON1 activity followed disturbed lipid status. According to patient's gradus stratification, none of the examined parameters differed, but when divided by Astler Coller staging system, statistically significant results existed regarding BMI and LDL-C, with highest BMI values in B stadium ($p<0.010$) and LDL-C in D ($p<0.050$). Significant positive correlations were noticed between PON1 activity and HDL-C ($p<0.050$; $\rho=0.277$); PON1 and LDL-C ($p<0.050$; $\rho=0.244$), as well as between PON1 and TC ($p<0.01$; $\rho=0.350$). PAB was in positive relation to HDL-C in the control group ($p<0.050$; $\rho=0.352$), while this correlation was insignificant in CRC. Univariate logistic regression showed that PAB is a significant independent predictor of CRC (OR=1.068; $p<0.001$; 95% CI (1.035-1.102)), and its influence remained significant after including PON1 and HDL-C values in the regression model. PAB might be considered as an excellent parameter in predicting the development of CRC and estimation of CRC risk. The observed associations between PON1 activities and lipid parameters in CRC could form a ground for future investigations in order to elucidate complex metabolic interactions in cancer-associated dyslipidemia.

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ANTIOXIDANT POTENTIAL OF DIFFERENT HYDROLATE MIXTURES

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Oxidative stress is defined as an imbalance in the production of reactive oxygen species and antioxidative defense of the organism. Hydrolates, aqueous products of hydrodistillation have antioxidative capacity which may be contributed to herbal active compounds such as phenols and flavonoids. The aim of the study was to examine *in vitro* antioxidant capacity of three different hydrolate mixtures with or without rosmarinic acid addition. Serum samples of healthy people were collected from which sera pool was obtained. Parameters of oxidative stress (total oxidative status - TOS, prooxidant-antioxidant balance - PAB) and antioxidant protection (total antioxidant status - TAS, the total content of sulfhydryl groups - SH) were determined in sera pool with or without tert-butyl hydroperoxide (prooxidant) with the addition of three different hydrolate mixtures with or without rosmarinic acid. These parameters were used to calculate oxy scores and TAS/TOS ratios, which represent a quantitative measure of the relation between oxidative stress and antioxidant protection and therefore better reflect the redox balance. The *in vitro* analysis was performed after 2 h and 24 h of incubation. The Kruskal-Wallis test showed statistically significant difference between oxy score 2 h ($p<0.001$), oxy score 24 h ($p<0.001$) and TAS/TOS 2 h ($p<0.001$). The Mann-Whitney U test was used to determine where the exact differences lie. This test showed significant difference between mixtures with and without rosmarinic acid addition, which implies that the rosmarinic acid significantly improves their antioxidant properties. When comparing oxy score 2 h and TAS/TOS 2 h between groups, we noticed that Gerania Immune Herbal Complex (mixture of *Geranium robertianum, Foeniculum vulgare*, *Lavandula angustifolia* and rosmarinic acid) and Rosemary Mental Herbal Complex (mixture of *Rosmarinus officinalis*, *Foeniculum vulgare, Geranium robertianum* and rosmarinic acid) had the largest antioxidant capacity, suggesting these mixtures could be particularly useful for short-term antioxidant protection. We may conclude that different hydrolate mixtures are a good source of antioxidants, and that the addition of rosmarinic acid significantly enhances their antioxidant capacity.

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CYTOTOXICITY OF MONTENEGRIN MERLOT WINES ON HUMAN COLON AND CERVICAL CANCER CELL LINES

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Since ancient times, red wine was frequently used as dietary product all around the world. Its beneficial effects are primarily linked to phenolic compounds that exhibit anti-oxidant, anti-inflammatory and anti-proliferative effects. One of the anti-carcinogenic mechanisms of action of wine phenolics is certainly initiation of reactive oxygen species mediated DNA breakage and consequent cancer cell death. The objective of this study was to evaluate cytotoxic potential of three Merlot wine samples [commercial wine (Comm) and two wines obtained from recognized clones (VCR 1 and VCR 101) of Merlot variety], vintage 2011, on human colon (HCT116) and cervical (HeLa) cancer cell lines. Cytotoxicity of analyzed wines was measured by sulforhodamine B (SRB) assay. Exponentially growing HCT116 and HeLa cells were treated with different volume ratio of examined wines (5, 10 and 20%). After 48 h cells were fixed with trichloroacetic acid, stained with 0.4% SRB and absorbance was measured at 550 nm. The results obtained indicate that all examined wines induced cytotoxic effects in both tested cancer cell lines. HeLa cells demonstrated higher sensitivity to Comm wine for all volume ratios compared to HCT116 cells ($p<0.001$). Regarding HCT116 cell line, VCR 1 and VCR 101 wines had higher cytotoxic effect compared to Comm wine for all volume ratios. Furthermore, Comm and VCR101 wines showed higher cytotoxic effect on HCT116 cells at 5% volume ratio than at 20% ($p<0.001$ and $p<0.01$, respectively). The similar trend was observed for Comm and VCR 1 wines affecting on HeLa cell line ($p<0.05$ and $p<0.001$, respectively). Namely, some polyphenols from red wine at high concentrations could act as growth stimulators rather than inhibitors. This could explain higher cytotoxic effect of lower wine concentration observed in this study. Finally, it can be concluded that Comm wine affected more HeLa cells, while VCR 1 and VCR 101 showed higher cytotoxic effect on HCT116 cells.

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TOXICOGENOMICS ANALYSIS OF PROTECTIVE ROLE OF MAGNESIUM AGAINST CADMIUM-INDUCED OXIDATIVE STRESS

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The antioxidative properties of magnesium (Mg) have been demonstrated, suggesting that Mg deficiency leads to increased levels of oxidative stress markers and weakened antioxidant defense. Moreover, the protective role of magnesium (Mg) against cadmium (Cd) induced oxidative stress, has been shown on animals exposed to this cumulative toxic metal, suggesting the positive effects of Mg on antioxidant enzymes and non-enzymatic antioxidants such as superoxide dismutase (SOD) or glutathione (GSH), respectively, etc. However, the molecular mechanisms of these positive effects are not investigated enough. The aim of this study was to explore the protective role of Mg against Cd-induced oxidative stress, using the toxicogenomics approach. Comparative Toxicogenomics Database (CTD; http://ctd.mdibl.org) tools, My Venn and SetAnalyser, were used to identify the genes common to Cd and Mg involved in the detoxification of reactive oxygen species (ROS), while GeneMania predictive server (http://www.genemania.org) was used to explore the interaction between the identified genes. According to CTD base, Cd affected 18 genes involved in detoxification of ROS, while Mg interacted with 6 such genes. Among these genes, 5 were common for Cd and Mg - SOD1, SOD2, GSTP1, PRDX6, and TXNRD1. The effect of Mg and Cd on all of these genes was contrary - Mg increased the activity of SOD1, while Cd bound to this gene and decreased its activity. Likewise, Mg increased the expression of SOD2, GSTP1, PRDX6 and TXNRD1 mRNA, while Cd decreased the expression of their mRNA. Further GeneMania analysis of 5 common genes, along with 20 related genes, revealed that more than half of them (57.64%) had physical interactions. These results indicate the protective role of Mg against Cd-induced oxidative stress on the gene level and provide a basis for further *in vitro* and *in vivo* investigation of its molecular mechanisms.

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PHOTOSTABILITY OF SELECTED BACTERIOCHLORINS - BACTERIOCHLOROPHYLL *A* AND BACTERIOPHEOPHYTIN *A* IN METHANOL SOLUTIONS

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The interaction of bacteriochlorin derivatives such as bacteriochlorophyll *a* (BChl*a*) and bacteriopheophytin *a* (BPheo*a*) with oxygen under the influence of UV-VIS light (to produce harmful reactive oxygen species) and their photostability, has been a matter of great interest, having in mind that both BChl*a* and BPheo*a* are good UV-VIS absorbers and also well-known photosensitizers. Their photostability against different irradiation treatments could play important role in various fields of research and their applications. The objective of this study is to determine photostability of BChl*a* and BPheo*a* during continual UV-A (350 nm) and UV-B (300 nm) irradiation treatments (with total measured energy flux 12.9 W m^{-2} and 15 W m^{-2}, respectively), in methanol solutions. The changes were studied by absorption UV-VIS spectroscopy (providing kinetic analysis). Continuous UV-A and UV-B irradiation of BChl*a* and BPheo*a* in methanol resulted in their irreversible degradation. Bacteriochlorophyll *a* is significantly reduced by UV-A and UV-B after 1 and 0.5 min, while BPheo*a* after 20 and 10 min of irradiation treatment, respectively. Both, BChl*a* and BPheo*a* degradation induced by UV-A and UV-B irradiation obeys first-order kinetics. Kinetic analysis showed significant differences in the degradation rate constant values (in min^{-1}) for BChl*a* and BPheo*a*: for UV-A irradiation treatments are 0.30202 min^{-1} and 0.00516 min^{-1} and for UV-B 0.40985 min^{-1} and 0.04382 min^{-1}, respectively. Bacteriopheophytin *a* showed significantly higher photostability in comparison to BChl*a* for both irradiation treatments - possibly related to their differences in chemical structures. Bacteriochlorophyll *a* has a central metal Mg, and in the solutions occurs with extended life of its excited states induced by irradiation, which can produce much more reactive oxygen species (through photosensitive reactions) in comparison to BPheo*a*. In turn, formed reactive oxygen species could directly participate in degradation of selected bacteriochlorins. As expected, photostability of chosen bacteriochlorins is also energy dependent.

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THE ROLE OF MITOCHONDRIA IN ANTIMELANOMA ACTION OF ORGANIC ESTER COMPOUND

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Melanoma is a highly aggressive malignant skin tumor with an increasing incidence in the last 30 years and rather poor prognosis. Cyclohexyl analogue of ethylene diamine dipropanoic acid ethyl ester (EE) is an organic compound with proven cytotoxic activity. The aim of our study was to investigate the role of mitochondria in antimelanoma action of EE. Mouse melanoma cell line B16 and 6-8 weeks old female C57Bl/6 mice were used in the experiments. Mitochondrial depolarization, production of superoxide anion and reactive oxygen species (ROS) in B16 cells treated with EE were investigated using flow cytometry. Subcutaneous mouse model of primary melanoma was used to assess expression of apoptosis-related mitochondrial Bcl-2 protein members in tumor tissue with RT-qPCR. EE induced mitochondrial membrane depolarization in B16 cells as early as 0.5h after the beginning of the treatment reaching the depolarization level that corresponds to uncoupler CCCP after 4h. This effect increased with time, reaching maximum after 6h. Increased production of ROS and superoxide anion were detected after 2h, and reached the maximum of production at the same time as the peak of mitochondrial depolarization. In *in vivo* experiments we observed a change in the expression of several Bcl-2 proteins in melanoma tissue of mice receiving EE for 2 weeks, weekend off. There was an increase in the expression of proapoptotic *Bax* that forms Bax/Bak1 oligomers involved in mitochondrial outer membrane permeabilization; an increase in expression of BH3-*only Bad* that inhibits antiapoptotic proteins; and an increase in BH3-*only Bcl2l11* (Bim) that both inhibits antiapoptotic proteins and activates Bax. The expression of mitochondrial proapoptotic *Bak1*, *Bbc3* (Puma) and *Pmaip1* (Noxa) was not changed. In conclusion, EE induces prompt mitochondrial membrane depolarization followed by increased production of ROS in B16 cells. Involvement of mitochondria also includes change in the expression of different Bcl-2 proteins that shifts the proapoptotic/antiapoptotic balance towards the proapoptotic side in subcutaneous mouse melanoma tissue.

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INTERACTIONS OF ENTEROLACTONE AND ENTERODIOL WITH HUMAN SERUM ALBUMIN INCREASE ITS THIOL GROUP REACTIVITY

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Human serum albumin (HSA) is recognized as an important antioxidant in plasma. The interaction of fatty acids (FA) with HSA change the reactivity of the HSA Cys34 thiol group (HSA-SH). Enterolactone (EL) and enterodiol (ED) are metabolites of dietary plant lignans abundant in seeds, whole grains and berries. Influence of EL and ED as well as cooperative and competitive interactions between them and FA on HSA-SH reactivity were investigated. FA-free HSA was prepared by defatting commercial HSA with charcoal treatment and then reducing with dithiothreitol. For preparation of FA-bound HSA samples, solution of FA-free were mixed with appropriate volumes of stearic acid (S), EL or ED solutions and then incubated. HSA-SH reactivity were investigated by the determination of the pseudo first order rate constants (*k*') for the thiol reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) measuring absorbance change at 412 nm. Binding of S increased k' value of thiol group with DTNB from $8.9\pm0.1 \times 10^{-3}$ s^{-1} for FA-free to $11.6\pm0.3 \times 10^{-3}$ s^{-1} for S/HSA complex 1:1 and $15.3\pm0.1 \times 10^{-3}$ s^{-1} for S/HSA complex 4:1. In all assayed HSA-enterolignan complexes EL and ED increased the reactivity of HSA-SH by 9.1-33.1% and this effect is more pronounced at lower molar ratios of S/HSA. The highest effect was found for FA-free HSA-EL complex. S reduced the effect of EL on HSA-SH reactivity. The change of HSA molecule reactivity found in our study could be important for the expression of enterolignans antioxidant effects *in vivo*.

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THE EFFECTS OF NEWLY SYNTHESIZED PLATINUM(IV) COMPLEX AND *PHELINUS LINTEUS* EXTRACT IN CO-TREATMENT ON THE MIGRATORY POTENTIAL AND REDOX STATUS OF COLON CANCER CELL LINES

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The use of natural bioactive substances and chemotherapeutics in co-treatment, through synergistic anticancer activity, contributes to the reduction of the negative effects of chemotherapeutics. Suppressed cells motility is one of the main strategy in cancer therapy. In this study we investigated anti-migratory effects and changes in redox status after co-treatment with Pt(IV) complexes ([PtCl4(dbu-S,S-eddp)] - Pt(IV) complex 1, [PtCl4(dpe-S,S-eddp)] - Pt(IV) complex 2, their corresponding ligands (Ligand 1 and Ligand 2), and cisplatin as positive control) and the methanol extract of *Phellinus linteus* on colorectal cancer (HCT-116, SW-480) cell lines. The main state of study was analysis of protein expression and localization of pro/anti-migratory markers (E-cadherin, β-catenin, N-cadherin and vimentin) by immunofluorescence staining and quantification of fluorescence intensity by ImageJ software, as well as concentration of MMP-9 by colorimetric ELISA assay. Co-treatments, with emphasis on *P. linteus* + Ligand 1, show more effective anti-migratory action compared to individual treatments, with a significant increase in E-cadherin expression and reduction in pro-migratory proteins (nuclear fraction of β-catenin, N-cadherin, vimentin) on HCT-116 and SW-480 cells. The reduction of MMP-9 content in cultured media indicated that co-treatment had significant anti-invasive potential on investigated cells. The co-treatments express cellular specific biological effects on HCT-116 and SW-480 cells. Changes in redox status parameters, in terms of increased $O_2^{\bullet -}$ concentration, can be considered responsible for the anti-migratory/anti-invasive effects of co-treatments especially on HCT-116 cells. Combined treatments of the chemical complex with natural bioactive substances provide promising results on colorectal carcinoma cells. Given the cell-specific responses obtained in this study, the introduction into therapy and the application of controlled doses of certain nutritional supplements lead to the achievement of better antitumor effects in invasive forms of colorectal carcinoma.

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ANTITUMOR EFFECTS OF NEWLY SYNTHESIZED 3-(4-SUBSTITUTED BENZYL)-5 ISOPROPYL-5-PHENILHYDANTOIN DERIVATIVES ON HUMAN BREAST CANCER CELL LINE MDA-MB-231

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Breast cancer is one of the most common cancer types and represents the leading cause of women mortality worldwide. Recently, various hydantoin derivatives have been shown to exert significant antitumor activity on different tumors. We have examined the possible anti-tumor mechanisms of a series of newly synthesized 3-(4-substituted benzyl)-5-isopropyl-5-phenylhydantoin derivatives on human breast cancer cell line MDA-MB-231. The effects of these compounds on proliferation index, cell survival, and migration/invasion capacity were determined. The cells were treated with increasing concentrations of derivatives (0.01 μM up to 100 μM) during 24 h and 72 h, after which the evaluation of proliferation index and nitric oxide production was performed. The concentrations with optimal antiproliferative effects (1 μM and 10 μM) were chosen to conduct the examination of the effects on apoptosis level, migration capacity and cell invasion parameters. After 72 h treatment migration index was determined by Boyden chamber transwell migration assay, while the invasion capacity was examined by measuring the level of MMP-9 and COX-2 gene expression. All tested compounds expressed the strong anti-proliferative activity and induced dose- and time-dependent increase in the level of nitrites. The compounds applied in both used concentrations significantly decrease cell survival, migration capacity and the expression of invasion genes. These data suggest that tested hydantoin derivatives express considerable anti-tumor effects by reducing cell division, inducing apoptosis level and inhibiting breast cancer cell motility and invasion. The results obtained in this study indicate these compounds as molecules with the potential beneficial role in the future development and strategies of new therapeutic strategies against breast tumor cell growth and invasion.

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THE EFFECTS OF ARONIA JUICE ON SERUM OXIDIZED LOW-DENSITY LIPOPROTEIN CHOLESTEROL LEVELS IN ADULTS WITH ONE OR MORE CARDIOVASCULAR RISK FACTORS

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Oxidized low-density lipoprotein cholesterol (ox-LDL) is recognized risk factor of atherosclerosis. Aronia is fruit rich in polyphenols, suggested to have antioxidant properties, but **t**here is limited data regarding the effects of aronia on ox-LDL levels in human subjects. The aim of this study was to investigate the effects of polyphenols contained in aronia juice on ox-LDL in adults at risk of cardiovascular disease. Subjects were included in study based on the presence of one or more established cardiovascular risk factors: i) elevated body mass index (BMI ≥ 25 kg/m^2), and (or) waist circumference (≥ 80 cm for women, ≥ 94 cm for men), ii) blood pressure values above optimal (systolic/diastolic blood pressure (SBP/DBP) ≥ 120/80 mm Hg), iii) elevated fasting glucose levels (≥5.5 mmol/L), iv) altered lipids levels, triglycerides (TAGs>1.7 mmol/L), total-cholesterol (≥ 5.0 mmo/L), LDL-cholesterol (> 2.6 mmol/L) and HDL-cholesterol (men, < 1 mmol/L; women, < 1.2 mmol/L). A total of 34 (14 women, 20 men) adults (41.07±7.99 years) were included in study. Among them, 73.52% had elevated BMI, 71.42% of women and 80% of men had elevated waist circumference. The SBP was above optimal values in 64.70% and DBP in 44.11% of subjects. Altered fasting glucose levels were detected in 35.29%, TAGs in 41.17% and LDL-cholesterol in 94.11% of subjects. Altered HDL-cholesterol levels were detected in 20% of men, and 21.42% of women. Subjects were randomized to drink 100 mL daily for 4 weeks of 1 of the 3 study treatments: (1) aronia juice (AMJ, 1174.11 mg GAE 100 mL^{-1}) (n=12), (2) polyphenol-lacking placebo (PLB) containing same minerals, vitamins and sugars as AMJ (n=12), (3) ¼-AMJ juice obtained mixing AMJ with PLB (1:3) (294.28 mg GAE 100 mL^{-1}) (n=10). Serum ox-LDL was measured at the baseline and at the end of the study using ox-LDL enzyme-linked immune-absorbent assay kit 369 (Cell Biolabs Inc., San Diego, USA). Intergroup comparison was performed using two-way ANOVA with repeated measurements, and simple main effects were assessed using paired t-test. The analyses were performed using SPSS (Ver. 22, Chicago Il.) and *P* values ≤ 0.05 were considered statistically significant. There was no difference between 3 groups regarding ox-LDL at the baseline nor at the end of the study. The only significant change in mean ox-LDL occurred in group drinking AMJ (decrease from 131±24.01 to 110.58±17.75 ng/ml, *P*=0.022) comparing the end of the study with baseline. In conclusion, high amount of polyphenols contained in aronia juice could potentially decrease oxidation of LDL, but further research is needed due to lack of significance in intergroup comparison upon intervention.

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MORPHOLOGICAL HETEROGENEITY OF MITOCHONDRIA IN MILLIPEDE DEFENSIVE GLANDS: AN ULTRASTRUCTURAL STUDY

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The defensive secretions in millipedes have been the subject of growing number of studies, but there is only a handful of papers dealing with morphological and cytological features of the defensive glands (ozadenes) in these arthropods. Hence, we have investigated defensive glands of *Apfelbeckia insculpta* (L. Koch, 1867) (Diplopoda: Callipodida: Schizopetalidae) using light and transmission electron microscopy. Defensive glands of *A. insculpta* consist of a reservoir and an efferent duct which opens through an opening (ozopore). The entire inner surface of the gland is encompassed with cuticular lining (intima). Only the most basal part of the reservoir consists of cells that take part in the synthesis of defensive secretions which are based on phenolic compounds (*p*-cresol and phenol). Ultrastructural features of mitochondria present in cells of the secretory region were in focus of our study. The basal domain of secretory portion of glandular reservoir is characterized by the presence of mitochondrial accumulations in the proximity of basal labyrinth infoldings. These mitochondria are ovoid or bean-shaped with wide intermembrane space and few irregularly arranged cristae. In contrast, mitochondria in cells of the middle domain of secretory region are elongated, with more cristae arranged parallel to one another. Finally, mitochondria present in cells of apical domain can be both ovoid and elongated with long, parallel cristae. Observed mitochondrial heterogeneity probably reflects alterations in metabolic state of cells comprising secretory region of *A. insculpta* defensive glands.

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DID Cd AND Pb MIXTURE CAUSE A STRONGER EFFECT ON OXIDATIVE STRESS PARAMETERS IN RATS COMPARED TO SINGLE CHEMICALS?

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Cadmium (Cd) and lead (Pb) are well known hazardous substances. Having in mind that in real life scenarios we are exposed to mixtures of chemicals rather than the single chemicals, it is important to establish whether these chemicals in mixture produce more pronounced effect than single chemicals. Oxidative stress has been recognized as one of the pivotal mechanisms of these metals toxicity. The aim of this study was to investigate levels of malondialdehyde (MDA), advanced oxidation protein products (AOPP) and total anti-oxidative status (TAS) in plasma of Wistar rats, after an acute oral exposure to Cd, Pb, and their mixture. The experimental groups (each containing 6-8 animals) received 15 mg Cd/kg b.w. (Cd group), 150 mg Pb/kg b.w. (Pb group), and mixture of these two (Cd+Pb group). Control group of animals was untreated. Twenty-four hours after the intoxication animals were sacrificed, blood was collected by cardiac puncture and plasma samples were obtained. The Cd+Pb group had significantly higher levels of MDA ($p<0.05$) and AOPP ($p<0.01$) when compared to groups receiving single dose of metal. Furthermore, mixture treatment induced significant elevation of AOPP if compared to control values. TAS levels were significantly higher in mixture group in comparison to groups treated with single chemical. The obtained data give an insight into the effects of Cd and Pb mixture on oxidative status in plasma and provide evidence of more pronounced disturbances of oxidative status in the presence of both toxic metals.

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MITOCHONDRIA IN PACHYTENE: THE FRAGILE POINT OF MATERNAL SUBCLINICAL HYPOTHYROIDISM AFFECTION

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The stimulative effects of thyroid hormones on mitochondria are realized through both non-genomic and genomic mechanisms, affecting respiration, mitochondrial plasticity and biogenesis. The subclinical form of maternal hypothyroidism in rats induces significant reduction of mitochondria number but also an augmentation of their area in neonatal and early infantile offspring dyctiotene oocytes. This study aimed to investigate if this form of subclinical hypothyroidism affects mitochondrial morphology and distribution in the early prophase of meiosis I oocytes. It was performed on newborn control (C) (n=10) and hypothyroid (SCH) (n=10) female rat pups derived from control (n=6) and propylthiouracil treated pregnant dams (n=6), respectively. Ovaries of all pups were removed and processed for transmission electron microscopy. The morphological features of mitochondria in the early prophase I oocytes until dyctiotene were assessed. No substantial differences were found in leptotene and zygotene oocytes in SCH group comparing to control, except just a few mitochondria characterized with shortened cristae, presence of wide pale area centrally positioned and membrane disruption. Pachytene mitochondria in treated pup oocytes were in great extent with disrupted membrane, shortened cristae and wide pale area centrally positioned while these features were rarely observed in control ones. Our results confirm altered mitochondria morphology found in primordial and primary follicles in case of maternal hypothyroidism, indicating their impaired function and possibly, propensity to programmed cell death. Further investigations may indicate to what extent pachytene, as a meiotic checkpoint, appears to be a milestone possibly predetermining the future of the cell.

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ANTIOXIDANT PROPERTIES OF DIFFERENT COLOURED MAIZE TOWARDS IMPROVED TOLERANCE TO ROS INDUCED SEED DETERIORATION

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Generated by electron transport activities of chloroplast, mitochondria, and plasma membrane or as a side-product of various metabolic pathways localized in different cellular compartments, ROS are unavoidable side-products of normal cell metabolism. ROS production in various cell compartments under normal growth condition is low. However, various environmental stresses disrupt the cellular homeostasis and enhance the level of ROS produced. High temperatures and relative humidity, as adverse environmental factors, lead to seed deterioration, which is expressed as the loss of quality, viability and vigor. The major causes of seed deterioration refer to free radical-mediated lipid peroxidation, enzyme inactivation or protein degradation, disruption of cellular membranes and damage to genetic integrity. In this experiment, oxidative damage of ROS on seed germination and seedlings performances was evaluated on four maize genotypes differing in kernel colour and type (white and yellow semi-flints, red dent and dark red popcorn), that have been subjected to accelerated aging (AA). The same genotypes were evaluated regarding content of total phenolics, flavonoids, anthocyanins, phenolic acids, individual anthocyanins, as well as total antioxidant capacity. For determination of deterioration rate, seeds were exposed to 42°C and 100% RH for different time points (three and six days of AA). Three biological replicates were performed for each treatment, as well as germination assay with 50 seeds in each set of the replicate. Compared to non-stressed seeds (control), aging was evidenced by decreased germination energy (48.9%), total number of seedlings (40.8%) and seedlings growth (70.0% for root length, 44.0% and 10.5% for root and shoot fresh weight, 5.0% for seed rest, respectively, as well as increased number of abnormal seedlings (22.0%). Correlation analysis revealed that higher content of total phenolics and flavonoids, and both higher ABTS and DPPH radical scavenging capacity contributed to lower level of deterioration rate, particularly regarding energy of germination, seedlings root and shoot length and fresh weight, as well as to lower number of abnormal seedlings.

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NUCHAL ORGAN OF THE LARGE BRANCHIOPOD CRUSTACEAN *TRIOPS CANCRIFORMIS* (LAMARCK, 1801) SHOWS A DISTINCT ARRANGEMENT AND ULTRASTRUCTURAL DIFFERENCE OF MITOCHONDRIA

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Nuchal organ is well-defined structure present in most crustaceans. It is generally accepted that this organ has a chemoreceptor function, the role in uptake of substances, or in ion transportation. In the large branchiopod T. cancriformis, the nuchal organ is located dorsally in the head region between the paired compound eyes. It is oval-shaped and usually slightly elevated relative to the surrounding cuticle. In this study, using light microscopy (LM) and the transmission electron microscopy (TEM), the ultrastructure of nuchal organ and localization of its mitochondria were examined. The LM reveals the upper layer of cells (epithelium) arranged in close proximity with one another. They meet thin, naked cuticle in the front of the cuticular dome. A fibrous structure similar to a basal lamina stretches out in the subepidermal space, surrounding the organ. Trans-section of the nuchal organ showed the tight bundle of fibers of the primary sensory cells ('nucleus') in the center, separated by electron-lucent space formed by different kinds of microfilaments embedded in the extracellular material. The cytoplasm of the epithelial cells contains a very few, delicate mitochondria with tiny cristae near the surface of the plasma membrane. In contrast, mitochondria in primary sensory cells are elongated, occupy a great part of the cytoplasm, and closely surround the nucleus. Mitochondrial cristae are numerous and tubule-like. In the basal region cells are sizeable in diameter, with the presence of larger intercellular spaces, and visible arrangements (clusters) of nerve fibers. TEM showed that nerve cells are very rich in large, filamentous mitochondria mainly situated beneath plasma membrane. Ultrastructural features of the nuchal organ of T. cancriformis indicate a high metabolic activity. A web of primary sensory cells, and the nerves extending to this organ, point to the possibility of this organ being some kind of peripheral nervous (sensory) structure.

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BROWN ADIPOCYTES MITOCHONDRIA-PEROXISOMES CROSSTALK THROUGH CATALASE AND MnSOD REDISTRIBUTION IN HYPOTHYROIDISM

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Mitochondria and peroxisomes are highly dynamic organelles which cooperate and cross-talk in the cell. They are metabolically linked through fatty acids, where peroxisomes, as mitochondrial partner organelles, oxidise long and branched fatty acids. It is well established that metabolism in hypothyroidism, lipid metabolism in particular, is changed. As a consequence of that, peroxisomes take over the main role in β-oxidation of fatty acids. Hence, the aim of our study was to examine protein expression, activity and cell localisation of peroxisomal and mitochondrial enzymes − catalase (CAT) and manganese superoxide dismutase (MnSOD), in rat brown adipose tissue. Two month old male Wistar rats were fed with standard pelleted food *ad libitum*. Hypothyroidism was induced with 0.04% methimazole given in drinking water for 7, 15, 21 days respectively; control animals drank tap water. An isolated portion of interscapular brown adipose tissue was examined by: western blot, enzymatic activity assay, immunohistochemistry and immunocytochemistry. Our results show that the treatment increased protein expression of both CAT and MnSOD. Enzymatic activity of CAT was increased during the treatment. In contrast, MnSOD enzymatic activity was slightly increased to the 15th day of the treatment and then significantly decreased. Subcellular localization showed CAT presence in all cell compartments: cytoplasm, nucleus, mitochondria, peroxisomes and around lipid droplets. Also, the number of CAT-immunopositive cells was increased during the treatment. MnSOD was localised in mitochondria and peroxisomes adjacent to lipid droplets. Ultrastructural analysis showed remarkable MnSOD localisation in peroxisomes in experimental groups. The intensity of MnSOD immunopositivity was also increased in experimental groups, especially in the group treated for 15 days. Moreover, we found the harlequin pattern of immunostaining for both enzymes was potentiated in hypothyroidism. The lack of thyroid hormones alters mitochondria-peroxisomes crosstalk through increase in peroxisomes number, catalase expression and catalase activity, but also via catalase and MnSOD organellar redistribution in brown adipocytes.

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THE IMMUNOEXPRESSION OF CuZnSOD, MnSOD AND CATALASE IN TESTES OF HYPOTHYROID RATS

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Although the mammalian testis has for many years been regarded as an organ unresponsive to thyroid hormones, their role in cell proliferation and differentiation during spermatogenesis was recently established. It was shown that thyroid hormones affect both immature and adult testes in sense of their involvement in spermatic cells differentiation and survival through metabolic regulation and that both, hyper- and hypothyroidism are associated with increased oxidative stress in semen. Hence, we addressed the effect of hypothyroidism on CuZnSOD, MnSOD and catalase immunoexpression in rat testes. Two-month-old male Wistar rats were fed with standard pelleted food *ad libitum*. The rats were divided into two groups. Hypothyroidism was induced by 0.04% methimazole in drinking water for 21 days while animals that drank tap water served as control. Isolated testes were examined by immunohistochemistry on 2 μm semi-fine sections using antibodies against CuZnSOD, MnSOD and catalase respectively. The immunopositive reaction for all examined antioxidant enzymes was visible in seminiferous tubule of both, control and hypothyroid groups. CuZnSOD-positive reaction in both groups was observed solely in the spermatids and the hypothyroid group showed stronger CuZnSOD immunoexpression compared to control. In contrast, MnSOD immunopositivity was detected in spermatogonia, spermatocytes and spermatids in both groups. Compared to other analyzed antioxidant enzymes, MnSOD immunoexpression is slightly reduced in the methimazole-treated group compared to control, particularly in spermatogonia. Catalase immunopositivity was visible all through the seminiferous tubules from the basal lamina to the lumen, around and within the cells in both groups. In contrast to control, the hypothyroid group showed more prominent catalase immunopositivity, especially in early spermatogonial stages. It is interesting that spermatocytes nuclei in the hypothyroid group appeared catalase-positive. Our results clearly showed that cell and stage-specific CuZnSOD, MnSOD and catalase immunoexpression in the rat testes were changed in hypothyroidism and may contribute to altered spermatic characteristics.

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A ROLE FOR THYROID RECEPTOR ISOFORMS IN MITOCHONDRIAL BIOENERGETIC CAPACITY OF HUMAN ADIPOSE-DERIVED STEM CELLS

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Thyroid hormone receptors (TRs) α and β are homologous ligand-dependent transcription factors. We recently found that TRα is a predominant form in human adipose derived stem cells (hADSC). Further, we demonstrated that major TRα variants, TRα1 and TRα2 display mainly cytoplasmic distribution and colocalize with mitochondria. In this study, we investigated possible role for TRα receptor isoforms in mitochondrial bioenergetic capacity of hADSC. hADSC were purchased from Invitrogen, and all donors were non-diabetic females. Cells were plated in MesenPRO RS medium and grown to 50% confluency. To knock down (KD) particular TRα isoforms we used custom SMART pool ON-TARGET plus TRα1 or TRα2 siRNA, at 50 nM final concentration for 4 days. ICC was performed on cells cultured in 4-well chamber slides. Cells were fixed with 4% PFA for 20 minutes, rinsed three times with PBS and then permeabilized in 0.1% Triton X-100 dissolved in PBS for 10 minutes. Subsequently, cells were blocked for 30 minutes using 10% goat serum in PBS. Cells were incubated with mixture of fluorophore-conjugated mouse monoclonal antibodies against ATP synthase subunit beta (ATPB, Alexa Fluor 488) and ATPase inhibitory factor 1 (IF1, Alexa Fluor 647), and analyzed using a Leica TCS SP5 II confocal microscope. The colocalization rate and relative fluorescence intensities of ATPB/IF1 were determined at several regions of interest using LAS AF software. Our data revealed significant increase in ATPB and IF1 immunopositivity in both TRα1 KD and TRα2 KD cells compared to control. Elevated levels of ATPB and IF1 after TRα1 or TRα2 knockdown could be a result of compensatory mechanism due to suppression of one the TRα isoforms. This study implicates both TRα isoforms as regulators of hADSCs mitochondrial bioenergetic capacity, and further research is needed to elucidate that role.

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UNRAVELLING THE MYSTERY OF HIBERNATION: IS THE FUTURE COLD?

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The principles of bioenergetics set up here by Professor Djaja over a hundred years ago are still relevant within the field of hibernation which he pioneered. Even back then, it was obvious that endothermia is expensive. More than two thirds of overall metabolic energy are reserved for maintaining body temperature i.e. the production of heat. Extreme environmental conditions, such as very low temperatures and a lack of food, question the very survival of homeotherms. In such times, for many small mammals, the answer to this question is hibernation - leaving homeothermy behind and allowing the body temperature to fall to ambient levels. This is made possible thanks to a fascinating, powerful control of molecular processes which leads to coordinated suppression of virtually all metabolic function. This newly established homeostasis possesses specific adaptations and a capacity which enables hypothermic and hypometabolic cells and tissues to remain undamaged. Unravelling the mystery behind the biochemical and physiological mechanisms that regulate hibernation is important not only for understanding this amazing phenomenon, but also for solving many health-related issues.

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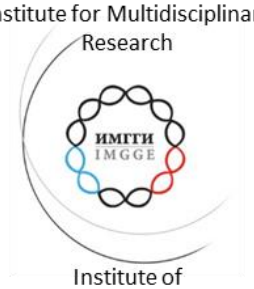

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