Session 13. Biospectroscopy and Oxidative Stress

Lectures

L13.1

Spectroscopic detection of reactive oxygen species and oxidative stress

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Reactive oxygen species (ROS) include both free radicals and non-radical compounds. ESR spectroscopy, especially employing spin traps, is the unique technique allowing for detection, identification and quantification of free radicals *in vitro* and their imaging *in vivo*. However, the sensitivity of this technique is limited and essays based on the use of more sensitive fluorogenic and luminogenic probes have gained enormous popularity. Classic fluorogenic probes such as 2',7'-dichlorodihydrofluorescein diacetate or dihydrorhodamine 123 are broadly used for detection of peroxides, but their specificity is low so often they are regarded as general indicators of oxidative stress than detectors of specific ROS. Dihydroethidine has been routinely used for detection of superoxide, but only one of its oxidation products is specific for this radical so precise identification of the product requires HPLC separation. The common problem with fluorogenic and luminogenic probes, especially when used at high concentrations, is their redox cycling; the probes not only detect but also generate ROS. Recently, a variety of new probes, i. a. boronate and BODIPY based, have been introduced, some of them allowing for a more selective detection of ROS. Probes targeted to mitochondria by a positively charged (usually phosphonium) moiety allow for detection of ROS produced by the main endogenous cellular source of ROS. A promising novel approach consists in the use of ROS-reactive fluorescent proteins. HyPer is a unique genetically engineered yellow fluorescent protein (YFP) for ratiometric measurement of hydrogen peroxide; cpYFP protein has been proposed as a superoxide sensor and pn-Green Fluorescent Protein (GFP) as a sensor of peroxynitrite. Other proteins have been constructed for measurement of other indices of oxidative stress like changes in the redox state of main cellular redox systems. RexYFP is sensitive to changes in the NAD^+/NADH ratio and rxYFP measures changes in the oxidation state of glutathione. Fluorescent proteins can be targeted to various cell compartments allowing for studies of oxidative stress at the subcellular level. However, they have also drawbacks related, i. a., to the effects of other factors on their fluorescence and possible effects of their presence on cellular redox homeostasis.

L13.2

Advanced optical biosensors for new applications in biology and medicine

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The know-how for fluorescence protein-sensing is advancing rapidly owing to the continued introduction of new concepts, new fluorophores, and proteins engineered for sensing-specific analytes. Concerns about the reversibility and selectivity of engineered proteins are being addressed by developing biosensors that are based on the utilization of coenzyme-depleted enzymes. Such biomolecules do not consume the substrate and can exhibit conformational changes upon the binding of the analyte, which can be easily detected as fluorescence change. In addition, concerns about the stability of biosensors can be overcome by using thermostable enzymes isolated from thermophilic microorganisms. Finally, the development of new techniques such as polarization-based sensing, anisotropy-based sensing and lifetime-based sensing, all of which can be accomplished with light-emitting diodes as the light source, is prompting the design of a new class of specific and stable biosensors, as has occurred with blood glucose measurement. These biosensors represent a valid alternative to the conventional clinical chemistry diagnostics. Here, we introduce the recent advances in fluorescence biosensors for monitoring celiac disease, a disease present all over the world.

L13.3

Surface plasmon-coupled emission

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Surface Plasmon-Coupled Emission (SPCE) is a plasmonic phenomenon occurring at metallic surfaces attached to a high refraction index glass prism. It converts near-surface fluorescence into a directional radiation. Induced by the excited molecules traveling surface plasmons are out coupled into the glass prism in a form of a hollow cone with very well defined angle. This angle corresponds to a surface plasmon resonance (SPR) angle at the fluorescence emission wavelength. SPCE offers an ultrasensitive detection from a minimal volume of the sample but preserves fluorescence properties of the excited dyes. We will describe advantages of SPCE radiation and its applications in fluorescence spectroscopy/microscopy.

Oral presentations

O13.1

Multiple assay strategy for revealing molecular mechanism of 5' mRNA cap degradation by Decapping Scavenger enzyme

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The 5’ terminal mRNA cap structure is required for pre-mRNA splicing, export, nucleolytic stability and cap-dependent translation. The abundance and lifetime of mRNA is controlled by two major degradation pathways, one of which entails enzymatic hydrolysis of the cap by Decapping Scavenger. The enzyme functions as a homodimer, and is not expected to exist in a stable monomeric form due to large domain-swapping. In addition to the wild-type protein we expressed and purified two heterodimeric mutants with one of the monomers modified at the binding site for loss of its enzymatic activity. The first mutation, H277N, disables cleavage activity, yet the protomer is capable of binding a ligand. The second mutation, N110A / W175A, results in compromised binding capability. Kinetic studies of the cap degradation by the wild-type protein as well as the mutants gave further insight into the enzymatic process, product release and inhibition. Moreover, the cap-binding mechanism was followed using a broad class of non-susceptible, synthetic, cap analogues. Steady-state fluorescence titration provided the protein-ligand equilibrium association constants. Influence of various chemical modifications within the cap structure gave further insight into the details of the enzyme active site that are not visible form the X-ray structure. Pioneer application of dye-labelled cap analogues enabled a 'reversed' observation concept, focusing on the ligand emission. Fluorescence time-resolved experiments showed changes in labelled cap analog fluorescence life-times upon binding by protein. Furthermore, novel method of circular dichroism measurements in near UV was applied, in which each aromatic amino-acid type exhibited signal change in a different wavelength. Such effect enabled observations of tertiary structure rearrangements. The overall picture was completed with calorimetric measurements. The latter imply energy effect of the electrostatic contacts, especially H-bonds. Comparison of the results with those previously obtained for the initiation factor eIF4E gave initial data for a broader view of the protein-mRNA interactions.

O13.2

L-Arginine can prevent ethanol-induced oxidative stress in the liver

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Background: Alcoholic liver disease is one of the most serious consequences of chronic alcohol abuse [1]. Oxidative stress via generation of reactive oxygen species is suggested to be the major mechanism of alcohol-induced liver injury [2, 3]. **Aims**: This study tested the hypothesis that ethanol-induced oxidative stress in the liver can be prevented by the administration of L-arginine amino acid in the experimental binge drinking model of alcoholism. **Methods**: We investigated the effect of intragastrical administration of L-arginine (500 mg/kg) on oxidative stress and antioxidant status in the liver of male Wistar rats under interrupted alcohol intoxication (8 mg/kg/day (7 days of alcohol intoxication and 7 days of abstinence) during 42 days). **Results**: The results revealed that interrupted alcohol treatment caused a significant increase in the level of lipid peroxidation indicated by increase in the levels of superoxiddismutase (SOD)/glutathioneperoxidase (GSH-Px) activities and decrease in the activity of glutathionereductase (GSH-Red). Activation of lipid peroxidation was accompanied by significant increase in serum gamma-glutamyl-transpeptidase (GGTP) activity and decrease in nitric oxid (NO) concentration. Supplementation of L-arginine significantly lowered the activities of SOD and GSH-Px, decreased the level of lipid peroxidation, enhanced the antioxidant status, normalized the activity of biochemical marker of the liver damage (GGDP) and increased the NO concentration. **Conclusions**: This study demonstrated that ethanol-induced liver damage is associated with oxidative stress and co-administration of L-arginine may attenuate this damage by decreasing oxidative stress in the experimental binge drinking model of alcoholism.

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O13.3

Identification of 2-pyridoxyl-1,3-thiazolidine-4-carboxylic acid, novel metabolite of vitamin B6 and cysteine in human plasma

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Pyridoxal 5'-phosphate (PLP), the active form of vitamin B6 in the human body, is an important co-factor for many enzymatic reactions, including the transsulfuration of homocysteine (Hcy) to cysteine (Cys). The PLP deficiency is the cause of many disorders, such as hypochromic, microcytic or iron-refractory anemia, inflammatory diseases or cancer. PLP is known to react with Cys and Hcy *in vitro*, forming corresponding 1,3-thiazolidine-4-carboxylic acids and 1,3-tetrahydrothiazine-4-carboxylic acids, respectively. We used HPLC-based methods and chemical tests to identify a novel form of vitamin B6 in human plasma. We provide evidence that 2-pyridoxyl-1,3-thiazolidine-4-carboxylic acid (PTCA), an adduct formed in a reaction of PLP with Cys, is present in human plasma. PTCA was separated from other metabolites present in plasma by an HPLC assay that allows simultaneous quantification of PTCA and low-molecular weight thiols, such as Cys and Hcy. The assay is based on pre-column derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) under acidic conditions, affording a stable UV-absorbing derivative that is separated by HPLC and quantified by monitoring at 355 nm, a characteristic UV maximum of thiol-CMQT derivatives. PTCA was identified by its co-elution with an authentic PTCA standard during HPLC, its sensitivity to base hydrolysis, and the recovery of PLP and Cys after base hydrolysis. PTCA was present in human serum at 44.6±15.8 μM, a concentration similar to plasma reduced Cys, but 1000-fold higher than plasma PLP. In conclusion, our results suggest that PTCA is the major form of vitamin B6 in human plasma and provide methodology for examination of the role of PTCA in human health and diease.

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O13.4

Isolation and characterization of cancer cell lines resistant to Photodynamic Therapy

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Photodynamic therapy (PDT) is a medical treatment that utilizes a Photosensitizer (PS) and visible light. PS absorbs light and, in the presence of oxygen, transfers the energy, producing cytotoxic oxygen species that damage cell components and lead to cell death. There are several cellular defense mechanisms against PDT including DNA repair, drug resistance pump, activity of antioxidant detoxifying enzymes and heat shock proteins.
In this study we have grown human cancer cell lines resistant to PDT in order to study their characteristics and resistant markers of PDT. To this aim, eight different cell lines were chosen and exposed for 3 h to 0.6 mM 5-ALA in serum free medium and then irradiated with increasing light dose (635 nm) to achieve survival level in the 4-10% range. From all ALA-PDT tresated cell lines, HeLa, human glioblastoma (U-87) and cervical carcinoma (SKG-IIIa) cell lines showed stable resistant features after receiving about 10 cycles of ALA-PDT and two weeks interval between each cycle. Cell doubling time was considerably longer in U87 ALA-PDT resistant cell line compare to parental cell, but no difference in doubling time of other cell lines was observed. SKG-IIIa showed that needed less courses of PDT than other cell lines to become resistant to the treatment. Besides studying some cell characteristics of resistant cells, activity of base excision repair enzymes has been measured and expression of ABCG2, HO-1, OGG1, APE1 and ABH2 genes has been studied by both qRT-PCR and Western blotting that results of which will be shown.

O13.5

Enzymatic generation of highly fluorescent nucleoside analogues using various forms of purine-nucleoside phosphorylase (reverse reaction)

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Mammalian and bacterial forms of purine-nucleoside phosphorylase (PNP) as well as their variants effectively catalyze syntheses of a variety of highly fluorescent nucleoside analogs, potentially useful as fluorescent probes of the enzyme active site. In particular, ribosides of 2,6-diamino-8-azapurine can be synthesized using a wild-type and the Asn204Asp mutant of the calf PNP, while the *E. coli* PNP can be used to effectively synthesize highly fluorescent adenosine and inosine analogs such as 1,N6-etheno-adenosine and lin-benzo-inosine. In some instances, the non-typical N8- and/or N7-ribosides can be obtained as well. Kinetic parameters of the synthetic and/or phosphorolytic reactions suggest strong interaction between enzymes and substrates. Potential applications for analytical and/or mechanistic studies will be discussed.

Posters

P13.1

Structural and spectroscopic studies of nanosystems based on gemini surfactants as innovative delivery systems in gene therapy

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The process of complex formation between cationic gemini surfactants and nucleic acids is very interesting and important from the point of view of gene therapy. This new method of treatment of genetic diseases based on the application of a transgene introduced into a pathogenic cell and this is the hope for successful treating neurodegenerative disorders or cancer. The most important thing is to find the best vector for gene delivery into infected cell. Perfectly suitable for this purpose are non-viral vectors - delivery system based on surfactants. These compounds have specific properties such as good solubility, low toxicity, biocompatibility and ability to form ordered structures (e.g. hexagonal and regular shape). With the help of spectroscopic and structural studies we try to determine the optimal parameters for such systems [1-3].
In particular, the aim of this study was to determine the possibility to use gemini surfactants as complexing agents for nucleic acids [4, 5].

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P13.2

Lipoic acid, a well-known antioxidant, is able to restore aldehyde dehydrogenase activity blocked by nitroglycerin. Implications for nitrate tolerance

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A link between lipoic acid (LA; 1,2-dithiolane 3-pentanoic acid), its reduced form dihydrolipoic acid (DHLA; 6,8-dimercapto-octanoic acid) and aldehyde dehydrogenase (ALDH) activity is a new hypothesis postulated in the literature of recent years in the context of studies on the enzymatic mechanisms underlying bioactivation of organic nitrates and tolerance to these compounds [1,2].
In the present study we investigated the ability of LA, DHLA and dithiothreitol (DTT): (1) to prevent ALDH inactivation by nitroglycerin (glyceryl trinitrate, GTN) and (2) to restore GTN-inhibited ALDH activity.
The studies were conducted with the use of yeast ALDH. The activity of ALDH was determined in the presence of 1 mM NAD and 8 μM propionaldehyde as a substrate. The enzyme was preincubated in three ways (1) with 0.1 mM GTN in 50 mM sodium phosphate buffer (pH 8) for 20 min; (2) with 0.1 mM GTN for 5 min, then with addition of DHLA/LA (1mM) for further 15 min; (3) with DHLA/LA (1mM) for 5 min, then with addition of 0.1 mM GTN for further 15 min. Catalytic activity of ALDH was determined by the spectrophotometric method based on the test utilizing optical properties of NAD(P)/NAD(P)H/H+. Data are presented as a percentage relative to the control sample containing only ALDH without GTN, LA and DHLA (100%).
The obtained results indicated that ALDH activity in the presence of GTN decreases to 12.1%. When the enzyme was earlier preincubated with GTN and then with LA or DHLA DHLA, ALDH activity was 31.6% and 71.9%, respectively. On the other hand, when ALDH was earlier preincubated with LA/DHLA and then GTN was added the activity was 68.6% and 106.9%, respectively. This means that LA and DHLA were able to restore ALDH activity blocked by GTN. It was also shown that GTN did not inhibit ALDH activity when the enzyme was earlier preincubated with DHLA.

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P13.3

The oxidative influence of iron(III) ions on the polyene antibiotic amphotericin B

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Amphotericin B (AmB) due to its chemotherapeutic properties occupies a special place among the antifungal agents. It has been considered the gold standard in the treatment of systemic fungal infections. The physicochemical mechanism of AmB action is related to the formation of transmembrane channel structures by this antibiotic. The chemical mechanism is associated with destructive effects of the antibiotic on the cell membrane through the oxidative effect. As a result of free radical reactions, differently severe peroxidation of phospholipids occurs in the fungal cell membrane. Since the mechanism of increased permeability of the membrane due to the formation of channels by AmB not fully explain its fungicidal activity (only fungistatic), some authors have shown that the oxidative mechanism is responsible for the killing effect of the antibiotic. Also AmB toxicity against eukaryotic cells may be associated with oxidative processes mentioned above. Due to possessing in the molecule the system of seven conjugated double bonds (chromophore), AmB may be subject to oxidation processes. In the experimental conditions the oxidation of AmB molecule was induced by free iron (iron (III) chloride, pH 2.5) and by heme iron (methemoglobin, pH 7.0). The effects of interaction of iron (III) with AmB was analyzed by electron spectroscopy (absorption and fluorescence) and oscillation (FTIR and Raman). The use of the chelator 1,10 phenanthroline clearly defined oxidative effect of iron (III) on AmB molecule. The observed spectral changes may indicate the selective oxidation of conjugated double bonds in the chromophore of AmB molecule by ferrous ions (III). Presented spectral changes in the spectra of AmB were first observed in our previous studies where the typical oxidant - $KMnO_4$ was used. In the present work, we obtained the same spectroscopic effects, but induced by a significant from a biological point of view oxidant - iron (III) ions. In this case, the observed spectral changes suggest further selective oxidation of the conjugated double bonds in the chromophore of AmB molecule by iron (III) ions.

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P13.4

Fluorescence spectroscopy and lifetime imaging microscopy studies of biological activity of amphotericin b against *Candida albicans*

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Amphotericin B (AmB) is an important antifungal antibiotic widely used in human medicine for over 50 years. It exhibits a broad spectrum of activity and is considered the "gold standard" especially for treatment of deep-seated fungal infections. Despite many years of research, the mechanism of antifungal activity of AmB and it cytotoxicity to the human cells are not fully understood. Many authors have reported that the fungal cell sensitivity to Amphotericin B is determined by the presence of ergosterol in the membrane. Better understanding of mechanism of action of AmB may contribute to the progress in the synthesis of improved formula of the antibiotic.
In this study we used fluorescence microscopy, Fluorescence Lifetime Imaging Microscopy (FLIM) and fluorescence spectroscopy as techniques for imaging of biological activity and studying of molecular organization of Amphotericin B in *Candida albicans* cells. We have analyzed physiological and morphological changes in *Candida albicans* cells in response to exposure to different molecular forms of AmB (monomers and oxidized monomers).
In summary, we present for the first time combination of fluorescence techniques for the studying of molecular organization of AmB in living cells. Additionally, the results presented herein have a highly important aspect related to the possibility of application of fluorescence techniques as extremely sensitive and selective methods of detection of different molecular forms of AmB especially oxidized in living cells.

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P13.5

Intercellular differences in cell cycle progression observed with FUCCI reporter system

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Genotoxic stress causes changes in cell cycle progression and cancer cells exposed to ionizing radiation usually arrest cell cycle in G2 phase. However observation of single living cells using fluorescent microcopy shows that the individual cells sometimes differ in the length of cell cycle phases. The aim of the present study was to compare the cell cycle progression by the individual control and exposed to ionizing radiation HCT116 and HEPA1-6 cancer cells. FUCCI (fluorescent ubiquitination-based cell-cycle indicator) reporter system was used to visualize cell cycle. Both cell lines were transfected with FUCCI reporter genes coding for red (mKO2) and a green (GFP) fluorescent protein fused with cell cycle specific proteins Cdt1 and geminin under the control of their promoters. In transfected cells Cdt1 protein tagged with mKO2 is expressed identifying G1 cells with red fluorescent nuclei. In the S, G2, and M phases geminin tagged with GFP is expressed and cells in these phases are marked with green fluorescent nuclei. HCT 116 cells were observed *in vivo* and images were captured every 1 hour after IR, by time-lapse fluorescence microscope at 37ºC and 5% CO_2. To examine irradiation effects cells were exposed to 4 Gy dose of X-rays immediately before transfer to microscopy.
Observation performed on un-irradiated HEPA1-6 cells showed the presence of cells whose daughter cells were characterized by significantly different times of cell cycle progression. Irradiation caused strong cell cycle inhibition in G2 in HCT116 cells 12 hours after irradiation and showed time-lapsed dynamics in comparison to control cells. The FUCCI system allowed for the detection of asymmetry in behavior of the cells descending from the same mother cell and characterization of cell cycle dynamics in different cell types.

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P13.6

Oxidative modification of protein in pediatric cystic fibrosis patients with bacterial infections

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Introduction: Cystic fibrosis (CF) is a systemic and genetic disease, involving the disorder of secretion of exocrine glands, causing changes in the respiratory and digestive tract. The patient's body produces too sticky mucus that causes abnormalities in the internal organs. Well-characterized genetic cause of this recessive disease is one of 1 500 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) including the most common known as ΔF508. Lungs of patients with CF, due to the presence of viscous mucus already in infancy and early childhood, are colonized by *Staphylococcus aureus* and *Haemophilus influenzae*. These bacteria contribute to the damage of epithelium lining the airways and subsequent colonization with *Pseudomonas aeruginosa* or *Burkholderia cepacia* complex.
Aim of study: This study was aimed at comparison of the usefulness of plasma protein oxidation parameters: carbonyl and sulfhydryl group content; AOPP and AGEs; dityrosine, N'-formylkynureine, kynurenine, tryptophan fluorescence and Amadori products, as well as erythrocyte antioxidant enzyme activities (SOD, CAT and GST) for assessment of oxidative stress in *P. aeruginosa* and *S. aureus* chronically infected pediatric stable CF patients.
Results and conclusions: Elevated glycophore (4.22±0.91 and 4.19±1.04 *vs* control 3.18±0.53 fluorescence units (FU)/mg protein; p 0.05) and carbonyl group levels (1.9±0.64, 1.87±0.45*vs* control 0.94±0.19 nmol/mg protein; $p<0.05$) as well as increased glutathione *S*-transferase activity (2.51±0.88 and 2.57±0.79 U/g Hb *vs* 0.77 ± 0.16 U/g Hb; $p<0.05$) were noted in *Pseudomonas aeruginosa* and *Staphylococcus aureus* infected CF. Kynurenine level (4.91±1.22 *vs* 3.89±0.54 FU/mg protein; $p<0.05$) was elevated only in *Staphylococcus aureus* infected CF. These results confirm oxidative stress in CF and demonstrate the usefulness of the glycophore level and protein carbonyl groups as markers of oxidative modifications of plasma proteins in this disease.

P13.7

The effect of lipoic acid on the activity of glyceraldehyde-3-phosphate dehydrogenase *in vitro*

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme which plays a crucial role in glycolysis, is a protein whose activity is affected largely by oxidative modification. The activity of GAPDH is mainly dependent on the presence of Cys-152 in the active center of the enzyme. Reactive -SH group of cysteine can undergo the processes of S-nitrosylation, S-glutathionylation, S-sulfhydration and oxidation to sulfenic, sulfinic and sulfonic acid. Most of these reactions leads to a loss of the enzymatic activity of GAPDH [1-3].
In this study, we investigated the effect of lipoic acid (LA) on the skeletal muscle–derived glyceraldehyde-3-phosphate dehydrogenase activity in vitro. Lipoic acid, its oxidized form (disulfide) is used as adjunctive therapy in the treatment of diabetes and diabetic complications.
The enzymatic activity of GAPDH was monitored by the increase in NADH concentration (ε_{340} (NADH) = 6.220 $M^{-1}cm^{-1}$). The control mixture contained 50 mM Tris-HCl buffer (pH 7.5) with 5 mM EDTA, 20 mM potassium phosphate, 1mM glyceraldehyde-3-phosphate (GAP), 1 mM NAD^+ and 25 ug/ml GAPDH. The assay mixture contained additionally 1 mM LA.
Preliminary studies showed that lipoic acid inhibited GAPDH activity by more than 50% as compared to controls, suggesting that the LA modifies thiol groups of GAPDH by S-lipoylation. It seems that these results are essential to supplement our knowledge of the regulation of this enzyme's activity in the glycolytic pathway. On the other hand, they show additional biological aspects of lipoic acid used in therapy of many diseases (diabetes, neurodegenerative diseases, diseases of joint) [4].

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P13.8

Evaluation of the impact of coenzyme Q and vitamin A and E on the activity of protein metabolism enzymes of liver of rats intoxicated with sodium fluoride

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The studies have shown that exposure to fluoride causes oxidative stress, which is one of the causes of metabolic disorders in various organs. Thus, recent researches have confirmed that various antioxidant can stop oxidative changes after exposure to fluoride. The aim of this study was to assess the impact of co-administration of CoQ with vitamins A and E on the activity of protein metabolism enzymes and TAS in liver of rats that were exposed to NaF.
The study was conducted on 18 female Wistar rats. The animals were divided into 3 groups with 6 animals in each. Rats in the control group received distilled water. In the I and II study groups animals received 2.5 mg NaF/rat/24 h. Additionally, animals in the group II were given 200 μg CoQ/rat/24 h, 3 mg vitamin E/rat/24 h and 250 IU vitamin A/rat/24 h.
The experiment lasted 5 weeks. Then the animals were anesthetized by intraperitoneal injection of 0.5 ml of 1% heksobarbital/100g of body mass. At necropsy the liver was taken. The activities of aspartate aminotransferase (AST) (EC 2.6.1.1), alanine aminotransferase (ALT) (EC 2.6.1.2) and γ-glutamyltranspeptidase (GGTP) (EC 2.3.2.2) were determined in liver homogenates. Total antioxidative status (TAS) and protein concentration was also determined.
The studies showed a statistically significant decrease in AST activity in the liver of experimental animals. In contrast, ALT and GGT activity was slightly reduced, indicating a fluoride ions interference in the metabolism of proteins. Co-administration of fluoride and antioxidants showed beneficial effects on strengthening of the total antioxidant capacity of the liver, and the activity of the assayed protein metabolism enzymes. The values of these parameters were similar to those in the control group.
In the summary, the results indicate the possibility of preventing changes related to exposure to fluoride by strengthening the antioxidant defense system.

P13.9

Amifostine — investigation of the prodrug transformation process into the biologically active thiol form

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Amifostine ([S-2-(3-aminopropylamino)-ethylphosphorothioate]; WR2721; Ethyol) is one of a few drugs able to protect healthy tissues during radio-and chemotherapy. The drug is administered intravenously or subcutaneously in the inactive form in which the thiol group is phosphorylated. In endothelial cells, amifostine is dephosphorylated in the reaction catalyzed by alkaline phosphatase (ALP) to the active metabolite with a free sulfhydryl group (-SH) ([2-(3-aminopropylamino)-ethanethiol; WR1065). This compound acts mostly as a reactive oxygen species (ROS) scavenger. Amifostine selectively protects normal tissues without reducing the toxic action of radio- and chemotherapy due to low ALP activity in cancer cells.

The aim of the present study was to develop a new *in vitro* method for examination of kinetics of ALP-catalyzed transformation of the inactive drug form into the active form. The method of determination of –SH groups with the Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid (DTNB)) is not reliable under these conditions because DTNB is unstable and decomposes non-enzymatically in alkaline environment. Moreover, the Ellman's method is not useful for determination the active form of WR1065 in biological material due to the presence of other thiols (e.g. glutathione).

The studies were conducted with the use of endnothelial ALP from bovine intestine and amifostine manufactured by LKT laboratories. The studies led to development of the method for monitoring of ALP-catalyzed reaction of WR2721 phosphorylation to WR1065. It consists in a direct measurement of absorbance at a wavelength of 233 nm selectively absorbed by WR1065. Thus, the methods allows for a direct continuous monitoring of an increase in product concentration. This method was used for determination of kinetic parameters (K_m and V_{max}), however, despite that a wide range of amifostine concentrations was used, saturation of the enzyme with the substrate was not achieved. Similar situation was described for WR2721 and ALP from the canine liver.

Monitoring of the reaction of amifostine transformation into its active thiol form is important for further studies aimed to widen its use. WR1065 can be oxidized to a disulfide form or can form mixed disulfides in this way influencing cellular thiol status.

P13.10

Expression of antioxidative enzymes and lipids peroxidation in kidneys of frog exposed to heavy metal ions

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Heavy metal ions (Cd^{2+}, Hg^{2+}, Pb^{2+}) are environmentally persistent toxins. Toxic effect exerted by these metal ions is multidirectional. There are three main reasons for their high toxicity in living organisms: 1) high affinity for thiol-, amino- and carboxyl groups of amino acid residues in proteins (change of the structural and catalytic properties of proteins), 2) the effect on the antioxidant potential of cells — a reduced level of glutathione can ultimately leads to the generation of oxidative stress, 3) they compete with divalent cations of metals such as copper or zinc in binding for active sites of proteins what leads to the inhibition or modification of their activity.

Main goal of the experiment was the determination of malone dialdehyde concentration (one of lipid peroxidation products) and a change of gene expression (glutathione peroxidase, catalase and superoxide dismutase — cytoplasmatic and mitochondrial form) in the kidney of *Xenopus tropicalis*. Sulfurtransferases genes expression were also assayed. The animals had been exposed to lead ions (28 mg/l), cadmium (40 mg/l), and mercury (1.353 mg/l) for 10 days.

The exposition to heavy metal ions caused an increase in amount of mRNA for all sulfurtransferases (γ-cystathionase, 3-mercaptopyruvate sulfurtransferase and rhodanese) with one exception for mercuric ions which caused a decreased expression of 3-mercaptopyruvate sulfurtransferase. Expression of glutathione peroxidase, catalase and cytoplasmic form of superoxide dismutase was also increased. The amount of mRNA for γ-cystathionase and mitochondrial superoxide dismutase remained unchanged. The exposition to heavy metal ions was without any effect on lipid peroxidation under the experimental conditions.

Obtained results suggested that the exposure to heavy metal ions generates oxidative stress in the frog's kidney. That effect was associated with the increased expression of antioxidative enzymes in the kidney.

P13.11

Antihemolytic, antioxidant and MDR reversal activity of *Actinidia arguta* leaf extract

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Polyphenolic compounds exhibit a series of beneficial effects on human health. For this reason, new substances of high biological activity, and without side effects, are sought for. Leaves of mini-kiwi are a rich source of polyphenols, but are not used in health protection mostly due to scanty reports on their biological activity. The results of our earlier studies showed that substances contained in kiwi leaves do not exert any destructive effect on the erythrocyte membrane but, on the contrary, make it stronger and more resistant to changes in the medium tonicity. When in contact with membranes, the substances become imbedded in the hydrophilic region of the membrane, which makes them potential, effective scavenges of free radicals that arise in membrane vicinity. In addition, the changes in physical properties of the erythrocyte membrane inducted by the extract may suggest that the compounds interact strongly not only with membrane lipids but can also modify the membrane proteins.

The aim of the study was to determine the biological activity of a mini-kiwi (*Actinidia arguta*) leaf extract. In particular, the ability of the extract compounds to protect erythrocytes and their membranes against hemolysis and peroxidation induced by various physicochemical agents. In addition, the investigation was to examine the effect of the extract on activity of the protein responsible for multidrug resistance of L5178 mouse lymphoma T cells transfected by MDR gene.

The spectrophotometry and cytometry methods were used in the investigation. The antioxidant activity of the extract was determined based on the concentration of malondialdehyde formed during lipid peroxidation. The extract's antihemolytic activity was found based on the concentration of hemoglobin released as a result of the oxidative damage of erythrocytes. The extract's effect on activity of the MDA protein (glycoprotein P1) was determined on the basis of the accumulation of 123 rhodamine in MDR treated and parental cell lines.

The present investigation showed that the mini-kiwi extract is effective in protecting the erythrocyte membrane against oxidation induced by UVC and UVB radiation. Moreover, the compounds contained in extract protect erythrocytes against hemolysis induced by free radicals. A study on the effect of extract on MDR protein activity in cells of mouse lymphadenoma showed that the extract modifies the protein's activity only slightly.

Substances contained in kiwi leaf extract are effective in protecting erythrocytes and their membranes against the harmful action of free radicals and practically do not influence multidrug resistance of tumor cells.

P13.12

Antioxidant action of cocaine? Studies in the rat kidney after acute administration of cocaine

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Objective: The studies aimed to evaluate the changes in redox balance observed in the rat kidney after a single acute intraperitoneal administration of cocaine at a dose of 15 mg/kg b.w.

Methods: The levels of the following compounds were determined in the rat kidney homogenates:

1) non protein –SH groups (NPSH), which include the thiol tripeptide glutatione being a natural reservoir of reductive power of cells;

2) malondialdehyde (MDA) and reactive oxygen species (ROS) as indicators of possible pro-oxidant action of cocaine;

3) sulfane sulfur, i.e. exceptionally metabolically reactive labile sulfur occurring in 0 or -1 oxidation state covalently bound to another sulfur atom, possessing antioxidant properties (sulfane-sulfur-containing compounds scavenge free radical and increase activity of antioxidant enzymes);

4) hydrogen sulfide, a gaseous transmitter showing antioxidant action that increases glutathione level in cells and activity of antioxidant enzymes;

Results: After acute cocaine administration, the NPSH level in the rat kidney homogenates remained at the control level. The content of reactive oxygen species and malondialdehyde concentration significantly decreased vs. control while sulfane sulfur level statistically significantly rose with hydrogen sulfide level remaining unchanged.

Conclusion: Unexpectedly, the present studies can suggest antioxidant action of cocaine. However, it seems improbable in the light of many reports indicating that cocaine causes damage of many tissues and organs, including renal ischemia [1-3]. The above results demonstrate a large increase in sulfane sulfur level accompanied by the decrease in the levels of ROS and malondialdehyde. Since redox balance should be preserved in the organism, these data suggest shifting of this balance towards reducing compounds and thus development of another pathology, namely, reductive stress.

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P13.13

Factors influencing compact-extended structure equilibrium in oligomers of Aβ1–40 peptide — an ion mobility mass spectrometry study

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Oligomers formed by amyloid beta peptide (Aβ) are widely believed to be the main neurotoxic agent in Alzheimer's disease. Studies discovered a broad variety of oligomeric forms, which display different levels of toxicity. Some of these forms may further assemble into mature fibrils, while other might be off-pathway from conversion to fibrils and assemble into alternative forms. To better understand a relationship between the structure and toxicity of Aβ oligomers, systematic characterization and classification of all possible forms is required, facilitating rational design of the beneficial modifiers of their activity. In previous ion-mobility analysis of Aβ1–40 oligomers, we have detected the coexistence of two alternative structural forms (compact and extended) in a pool of low-order Aβ1–40 oligomers. These forms may represent two pathways of the oligomer evolution, either leading to fibrils or to off-pathway oligomers, potential candidates for the neurotoxic species. Here, we have analyzed the impact of incubation time, the presence of selected metal ions and the effect of a series of point mutations on mutual population of alternative forms. We have shown that a salt bridge D23K28 provides stabilization of the compact form whereas G25 is required for the existence of the extended form. We have found that binding of metal ions also stabilizes the compact form. These results improve our understanding of the possible molecular mechanism of the bifurcation of structural evolution of non-monomeric Aβ species into an off-fibril pathway, ultimately leading to the formation of potentially neurotoxic species.

P13.14

Degradation of vitamin E derivatives modulated by UVB light

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In the past few years, most research on tocopherols has focused on structural variations of vitamin E (α-Toc) with the aim to improve the proapoptotic potency of these agents and simultaneously less susceptible to oxidation than α-Toc. The most studied member of these developed compounds is α-tocopheryl succinate (TS).
In search of synthetic derivatives of vitamin E with strong antitumorigenic potency and more resistant to influence of light, we examined the irradiation decomposition process of two novel esters: di-α-Tocopheryl maleate (TMA) and α-Tocopheryl malonate (TMO), and commercially available α-Tocopheryl succinate (TS). To our knowledge, this study presents the first time the efficacy and mechanism of photodegradation of vitamin E ester derivatives induced by UVB light in a single study.
The photodecomposition of α-Toc and its esters in some organic solvents was monitored by absorption spectroscopy. Upon UV illumination of α-Toc the absorption bands at 223 and 295 nm become less pronounced indicating the consumption of vitamin E. Simultaneously, a new absorption band appears around 250 nm attributed to α-tocopherol quinone. The absorbance bands of studied esters peaking at 279 nm and 285 nm for similar irradiation conditions as for α-Toc, decreased with irradiation time, while in the ranges 240-270 nm and 290-310 nm the absorbance increased. During irradiation the isosbestic points in the absorption spectra at 230 nm, 274 and 290 nm for investigated esters and α-Toc were observed. The spectral and kinetic changes in the other investigated solvents are similar to those obtained in methanol.
The α-Toc absorbance at 295 nm plotted as a function of irradiation time can be well fitted to a single exponential decay with a rate constant of $k = 0.723\ s^{-1}$. For all tested esters the absorption changes at 285 nm can be fitted to a linear function. Our results shown that α-Toc esters degraded through direct absorbance of UVB radiation. The different kinetics of degradation of α-Toc esters indicate that mechanism of oxidation of vitamin E induced by UVB light have been changed as a result of ester-linked moiety attached to the position-6 oxygen atom of the phenolic ring of the chroman head.

P13.15

The impact of propofol on oxidative-antioxidant balance in the experimental model of Parkinson's disease in Wistar rats

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Introduction: Propofol is a medication used in general anesthesia, which has antioxidant properties that's why it appears that propofol may have protective effects in neurodegenerative diseases by influence the oxidation-antioxidant balance.
Aim: Evaluation of selected parameters of the oxidation-antioxidant system in the liver, kidney and blood serum in an experimental model of Parkinson's disease in Wistar rats treated with propofol.
Materials and Methods: 6- hydroxydopamine (6-OHDA) was given to neonatal rats to destroy the dopaminergic central nervous system. The tests were performed on 32 rats divided into 4 groups: 1: control, 2: 6 — OHDA, 3: control of propofol, 4: 6 — OHDA propofol. The rats were decapitated at 45 days of age, organs and blood serum were collected. In blood serum concentrations of malondialdehyde (MDA), lipofuscin (LPS), total antioxidant capacity (TAC) and total oxidative status (TOS) were measured. Homogenates of the liver and kidneys were performed, the enzyme activity of catalase, glutathione peroxidase (GPX), glutathione transferase, glutathione reductase and the concentration of TAC, TOS, MDA were assessed.
Results: The results of liver: in group 4 a statistically significant increase in the activity of glutathione reductase, catalase, and TAC and statistically significant reduction in the concentration of TOS and MDA comparing to group 2 was found. Statistically significant increase in the activity of catalase, the TAC and a significant decrease in the concentration of MDA was observed in group 3 compared to 1. The special correlation between increase in catalase activity and administered propofol was found.
In the kidney group 4 was characterized by the statistically significance increased GPX activity and reduced MDA concentration compared with group 2.
In the blood serum a statistically significant reduction of MDA concentration, LPS and TOS in group 4 comparing with group 3 was observed. A similar trend is also demonstrated in comparing group 2 and 4.
Conclusions: Propofol stimulates the activity of antioxidant enzymes and reduces the level of oxidative stress in individuals with Parkinson's disease.
In the liver catalase plays a main role in oxidative stress reduction, whereas in the kidney glutathione peroxidase.
The administration of propofol individuals with lesions of the central dopaminergic system restores proper balance of oxidative-antioxidative system to a level comparable with healthy individuals.

P13.16

The impact of estrus induction on bactericidal activity of peripheral blood cells in dairy cows

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Research shows that in case of inflammations and tumors [1-3] as well as after physical exercises [4] the increase of blood chemiluminescence (CL) can be observed both in humans and animals. It is worth pointing out that very few studies have been carried out in cow and if so, they mainly concerned the animals with *mastitis* [5].
The aim of the study was the evaluation of the impact of hormonal induction of estrus on activity of blood neutrophils in dairy cows.
The study was carried out on 36 clinically healthy animals of Holstein-Friesian breed. Only the animals over 50 days after the parturition, with no traces of uterus asymmetry in the *per rectum* examination or pathological vaginal discharge in the *per vaginam* examination and *corpus luteum* in the ovary were qualified for the induction program. The health condition of the animals was determined on the basis of palpation and routine haematological examinations.
The estrus was induced with a single intramuscular cloprostenol injection (a synthetic analogue PGF2α) at a dosage of 500 μg/animal. Blood from the external jugular vein was taken from each cow twice, prior to the injection and on the 3rd/4th day after the injection. The animals were observed for 7 days after the injection and the day of estrus occurrence was marked.
Spontaneous (WS) and stimulated by opsonized zymosan (OZ) luminol dependent CL was used to measure neutrophil oxygen metabolism in whole blood. The assessment was performed using a microplate luminometer (Thermo-Labsystems, Finland).The kinetic method was used in the assay measuring CL for 60 min at 38°C. The results were presented as a value of CL integration in relative light units.
The animals were divided into 3 groups: the 1st being the cows in which clinical estrus occurred after the hormone injection and pregnancy was confirmed on the 42nd day following the insemination; the 2nd being the cows in which clinical estrus occurred after the hormone injection and pregnancy was not confirmed and the 3rd the cows in which clinical estrus symptoms did not occur.
On the 3rd/4th day after the hormone injection, WS and OZ CL increase was observed in relation to the CL levels prior the injection. The increase of CL WS was statistically significant in all groups, while the increase of CL OZ was significant only in the 1st and 2nd group.
Studies showed the increase of oxygen-dependent bactericidal activity of blood granulocytes in dairy cows after the induction of the estrus. Therefore, this reaction should be taken into consideration when CL measurements are used for diagnostic purposes.

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P13.17

Fluorescent dyes in studies on the mechanism of antifungal action of *Galleria mellonella* lysozyme against *Candida albicans*

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Fluorescent dyes are particularly advantageous in biological research because they combine very high sensitivity and selectivity in target detection with low toxicity. As such, they have become a method of choice for tracing and localization of proteins, detection of specific molecules in cells and tissues, and staining of cellular organelles. Changes in the potential of cell membranes, the presence of reactive oxygen species in cells, cell death by apoptosis can be also visualized using a fluorescent dye.

Our research focuses on explaining the mechanism of antifungal action of *Galleria mellonella* lysozyme using *Candida albicans*. Lysozyme plays an important role in innate immunity against invading pathogens in animals. This protein is well known antimicrobial polypeptide exhibiting antibacterial and antifungal activities. Antibacterial action of lysozyme is related to enzymatic muramidase activity as well as to non-enzymatic activity resembling a mode of action of cationic defense peptides. However, the mechanism of lysozyme fungistatic and/or fungicidal activity is not clear. Our previous study revealed that purified *G. mellonella* lysozyme, which like the human lysozyme belongs to c-type family of lysozymes, bound to the cell surface of different yeasts and filamentous fungi. Moreover, *G. mellonella* lysozyme inhibited *C. albicans* growth at a relatively low concentration (0.5 μM).

To characterize the effect of lysozyme action on the fungal cells we used a number of methods based on fluorescent dyes and imaging of cells in a laser scanning confocal microscope. Using FITC-labeled *G. mellonella* lysozyme we showed directly that lysozyme binds to the surface of *C. albicans*cells. The metabolic activity of *C. albicans* cells after incubation with 0.5 μM lysozyme was tested by LIVE/DEAD staining using FUN-1 dye. These experiments revealed reduction of metabolic activity of cells treated with *G. mellonella* lysozyme. Furthermore, Calcofluor White staining of the lysozyme-exposed *C. albicans* cells showed no changes in the cell wall in comparison with the control cells. These observations, together with the inability of the lysozyme to degrade standard chitinase substrates, suggested that *G. mellonella* lysozyme could reduce the fungal growth through a non-enzymatic mode of action. Staining of *C. albicans* protoplasts with FITC-conjugated Annexin V and Propidium Iodide after incubation with 0.5 μM lysozyme showed that *G. mellonella* lysozyme can induce apoptosis in *C. albicans* cells. The use of fluorescent dyes in our study allowed to obtain information relevant to explain the mechanism of antifungal action of *G. mellonella* lysozyme.

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P13.18

The influence of methionine on the activity of enzymes and the morphological picture of kidneys of rats intoxicated with sodium fluoride

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When exceeding the toxic dosage, the fluorine can affect the enzyme system and also induces morphological changes in many organs, leading to an impairment of their function. Numerous studies have demonstrated that the disturbances in redox processes play important role in the pathomechanism of the observed changes. The administration of antioxidants may prevent the oxidative stress associated with the exposure to fluoride by strengthening the antioxidant system of the organism.

The aim of this study was to assess the impact of administration of methionine on the activity of certain enzymes of the carbohydrate metabolism and enzymes associated with glutathione as well as to examine the morphology of the kidneys in rats exposed to sodium fluoride.

The study was conducted on 18 female Wistar rats. The animals were divided into 3 groups with 6 animals in each: control group, in which animals received distilled water for drinking, the experimental group I (NaF) and the experimental group II (NaF+Met), in which rats were given 10 mg NaF/kg of body weight /24h. In addition, animals in group II received 2 mg of methionine/per rat/24 h.

The experiment lasted for 35 days. After this time the rats were anesthetized by intraperitoneal administration of 0.5 ml 1% hexobarbital/100 g of rat body weight. For pathological examination, the livers were dissected and fixed in formalin. The activity enzymes: ALD, MDH, LDH, SDH in the kidneys were determinated. Pathomorphological changes in livers were assessed on the basis of preparations made by means of paraffin technique and stained with hematoxylin and eosin.

The study showed changes in the activity of all examined enzymes. The activity of LDH, SDH, ALD in experimental group I decreased in comparison to the control group, while the MDH activity increased. The changes in enzymes' activity were accompanied by morphological changes in kidneys. The administration of methionine counteracted the observed changes in ALD activity as well as morphological changes observed after exposure to fluoride, although the antioxidant was not fully effective.

P13.19

The influence of anthocyanidin and UVB radiation on keratinocytes elasticity and viability

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The ultraviolet B radiation is a common treatment for numerous skin diseases. On the other hand, a chronic exposure to the solar UVB radiation advances the ageing process of the skin, causes oxidative stress and the formation of reactive oxygen species (ROS). It also may lead to the development of melanoma and non-melanoma skin cancer [1]. As a result, a lot of research was made about the influence of photochemopreventive agents on the reduction of the occurrence of skin cancer, especially the botanical antioxidants like delphinidin [2]. However there are no investigations presented regarding the surface morphology and elasticity of irradiated living keratinocytes. There is an increasing evidence that the changes in the mechanical properties of living cells may regulate different processes and the survival of the cells, but also it can be used as an early diagnosis of several diseases [3]. Moreover, the decrease of the cell stiffness for living epidermal keratinocytes treated with sodium lauryl sulphate was presented [4]. Therefore, we examined the effect of UVB radiation and the addition of delphinidin on the mechanical properties of keratinocytes using the atomic force microscopy (AFM) correlated with the fluorescent microscopy and the viability test.

For the presented studies, we used the immortalized human keratinocyte (HaCaT) cell line. We examined the influence of delphinidin on HaCaT cells applying different concentration (5-10 μM; both pretreatment and post-treatment) After 24 h of the incubation, the cells were radiated with a dose of 100 mJ/cm^2. MTT assay was applied to define the number of living cells after the UVB radiation. The AFM measurements of the keratinocytes' stiffness and the visualization of the actin filaments were carried out after 24 h from the UVB radiation. All determined Young's modulus values were normalized to the value obtained for the control measurements.

The ultraviolet B radiation decreased the viability of HaCaT cells and the stiffness of cells measured by the AFM. Likewise, the fluorescence microscopy revealed changes in the organization of actin filaments in the radiated cells. However, the stiffness of irradiated cells were at level of control for pre-treatment with 10 μM delphinidin and post-treatment with 5 μM delphinidin.

These results show that UVB radiation has a significant influence on the viability and mechanical properties of HaCaT cells. The combined AFM/fluorescence microscopy study presents the photochemopreventive effect of delphinidin. This approach can be used to track chemically-induced changes in cell elastic properties under physiological conditions.

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