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ABSTRACTS

CEREBRAL CELL DAMAGE AFTER HYPOXIC-ISCHEMIC INJURY IN PERINATAL ASPHYXIA

Alvarez A. (1), Alvarez F.J. (2), Goñi de Cerio F. (1), Gastiasoro E. (2), Caballero A. (3), Valls i Soler A. (2), Solaetxe M. (4) and Hilario E. (1)

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Asphyxia represents one of the major causes of morbility in preterm neonates. In this work, we have used an experimental model of perinatal disease in preterm fetal lambs (Alvarez et al, 2002), in which umbilical blood flow was partially occluded during sixty minutes. After 180 minutes, lambs were sacrificed and brains fixed by perfusion.

The severity, extension and distribution of damage were carried out in cerebral cortex, basal nuclei, hypothalamus, thalamus, hippocampus, amygdaloid body, mesencephalon, pons, cerebellum and white matter. Samples were studied by light and electron microscopy.

In the mesencephalon, pons and intracerebellar nuclei of hypoxic-ischemic groups, scattered great-size damaged cells were observed. In these cells cytoplasm has a poor definition, acquiring a homogeneous and eosinophylic appearance. In the ultrastructural study, these cells showed karyolysis, with nucleoli preserved but with loss of nuclear envelope. In any case, areas of necrosis, hemorrhage or increase in cellularity were observed. In summary, our results suggest a selective cell damage in perinatal asphyxia.

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Alvarez F.J., E. Gastiasoro, M.C. Rey-Santano, A. Arnaiz, J.L. Larrabe, M.A. Gómez, E. Hilario, A. Alvarez, A. Valls-i-Soler. (L. Cabero and J.M. Carrera, ed) The perinatal medicine in the New Milenium, Monduzzi Editore, Bologna 2002, pp. 1085-1089.

EFFECT OF SELENIUM ON THE GROWTH OF *Enterococcus faecium* M-74 IN THE PRESENCE OF SELECTED MUTAGENS *in vitro*

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Selenium (Se), is an essential trace element for animals and human. Several inorganic and organic selenium compounds showed the chemopreventive properties against cancer (Ip *et* Ganther 1992; Lu *et al*. 1995). Probiotics have been defined as "microbial all preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host" (Salminen *et al*. 1999).

In this study we evaluated the growth of the probiotic cells of *Enterococcus faecium* M-74 and *E. faecium* M-74 enriched by selenium in the presence of selected mutagens: 4-nitroquinoline N-oxide (4NQO), methyl methane sulfonate (MMS), nitrovin (NIT), N-methyl-N´-nitro-N-nitrosoguanidine (MNNG) and sodium salt of 5-nitro-2-furylacrylic acid (5-NFA) using the concentration-time-kill-curve method.

The cell growth of both *E. faecium* M-74 and *E. faecium* M-74 enriched by selenium, respectively in the presence of selected mutagens was different. For selenium enriched *E. faecium* M-74 the MICs of all tested mutagens where increased two times. Se-enriched *E. faecium* M-74 grew better in comparison with *E. faecium* M-74 without selenium in the presence of 4 NQO, NIT, MNNG, 5-NFA at concentrations close to the MIC. Generally, significant differences were observed between the $\log_{10}$ CFU per milliliter counts at 24 h for killing curves for mutagens using Se-enriched *E. faecium* M-74 *versus* those for mutagens using *E. faecium* M-74 without selenium.

We have demonstrated that selenium enhances ability of *E. faecium* M-74 to grow in the presence of selected mutagens at concentrations inhibiting growth of *E. faecium* M-74 without selenium.

Supported by grant VTP/178/2000 from the Ministry of Education, Slovak Republic.

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CELL BIOLOGY EDUCATION ON THE INTERNET

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We briefly discuss the role of Internet support for education in cell biology. We have selected websites which are freely available and seem to be useful for learning and teaching. The best websites are found at university or society addresses. Support via the Internet increases democracy in the educational process and makes use of electronic text easier and more economical. We hope that not only recent databases of scientific publications but also artificial intelligence research will improve and assist cell biology learning in the future.

ASSESSMENT OF STAT 1 PHOSPHORYLATION INDUCED BY INTERFERONS IN HUMAN MALIGNANT MELANOMA

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To examine the inducibility of STAT 1 activation by interferons (INFs) in human malignant melanoma cells. STAT 1 levels, basal levels of STAT 1 phospho-forms (i.e. PY 701 and PS 727) as well as IFN-induced STAT 1 phosphorylation were examined by Western blots in 40 primary cell cultures derived from melanoma patients with clinical stage II/III and in 21 established malignant melanoma cell lines.

Using immunoprecipitation and Western blots performed on lysates made of melanoma cells derived from patients and employing specific anti-STAT 1 PS 727/PY 701 immunoprobes, we show that STAT 1 activation response induced by IFN-a/gamma is significantly impaired. Approximately three quarters of patients were lacking phosphorylation at S 727. STAT 1 PY 701 was not inducible by IFN-a in 67.5% and by IFN-gamma in 30% of samples. The phosphorylation at S 727 was induced by IFN-a in 14.3 % (3/21) and after IFN-gamma in 33.3 % (7/21) of established melanoma cell lines. Significantly higher percentage of STAT 1 phosphorylation at Y 701 was observed (76.2% after IFN-a and 90.5% after IFN-gamma, respectively). One melanoma cell line showed no activation at either S 727 or Y 701 induced by any IFN. Phosphorylation signals at both aminoacid residues were recorded in only two melanoma cell lines. Nine melanoma cell lines (42.8 %) showed IFN-induced phosphorylation at Y 701 only. Supported by grant no. NC/7139-3 from the Internal Grant Agency of the Czech Ministry of Health and grant 301/03/0370 from the Grant Agency of the Czech Republic.

The presented data demonstrate that considerable number of melanoma cell lines and primary melanoma cell cultures derived from lymph node metastases were lacking IFN-a and/or IFN-gamma-induced STAT 1 phosphorylation on serine 727 (S 727) and/or tyrosine 701 (Y 701) residues. These results indicate that malignant melanoma associates with the functional impairment of STAT 1 protein.

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REACTIVE OXYGEN INTERMEDIATES-INDEPENDENT ACTIVATION OF TRANSCRIPTION FACTOR NF-kappaB BY TUMOR NECROSIS FACTOR-a

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HL-60 cell line is a useful model for studying regulation of proliferation, differentiation and apopotosis. These processes can be regulated by activation of the transcription factor NF-kB . The activity of NF-kB is controlled by a family of IkB inhibitory proteins that sequester the transcription factor in the cytosol. Tumor necrosis

factor-a (TNF-a) has been found to induce specific IkBa phosphorylation and subsequent degradation, which leads to NF-kB activation. This process is believed to depend on reactive oxygen intermediates (ROI) (Christman J.W, 2000, Janssen-Heininger, Y. M., 2000). We investigated a possible role of ROI in NF-kB activation by TNF-a.

Using a factorial design of experiments, we combined TNF-a, with various concentrations of antioxidant N-acetyl-L-cysteine (NAC), and studied expression of IkBa by Western blotting.

We found reduced IkBa levels after TNF-a treatment. However, NAC had no effect on TNF-a -induced IkBa degradation.

These results suggest that TNF-a induced activation of NF-kB in HL60 cells is ROI-independent.

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DNA BINDING POTENTIAL AND TRANSACTIVATION ACTIVITY OF P73a, P73b, P73g AND P73d AND THEIR ABILITY TO INDUCE APOPTOSIS

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The p53 is the well-known tumour suppressor protein that is involved in control of the cell cycle, apoptosis and senescence. The p73 protein was discovered in 1997. The TP73 gene maps to the 1p36.2-3 chromosome region in which deletions frequently occur in neuroblastoma, colon cancer, melanoma and breast cancer. The p53 and p73 proteins share significant homology both at the genomic and protein levels. Different splicing of TP73 gene is responsible for formation of many p73 C- and N-terminal isoforms. These p73 splice variants differ in their transcriptional activity on p53-responsive promoters depending on the C-terminal structure. N-terminal truncated variants are incapable of transcriptional activation and serve as antagonists of TA family members.
Using radioactive EMSA and cotransfections experiments we compared the in vitro and ex vivo binding activity of p73 isoforms to the different p53 responsive elements. Apoptosis was determined using detection of released nucleosomes.

From our data we can speculate that each isoform has its own regulatory function. We also found that the transactivation ability of these proteins is under strong regulation which, moreover, is cell-dependent. The protein p53 together with p73 isoforms, which combine different C-terminal and N-terminal splicing variants, constitute a complex regulatory network that can accurately reflect the cells requests.

This work was supported by the grant 7131-3 from the IGA MZ CR and grant IAA4004110 from the IGA AV CR.

NITRIC OXIDE SYNTHASE ISOFORMS II AND III EXPRESSION DURING PORCINE OOCYTE GROWTH

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The enzyme nitric oxide synthase (NOS) synthesizes nitric oxide during the conversion of L-arginine to L-citruline. Three isoforms of NOS have been identified: NOS I (neuronal NOS, nNOS), NOS II (inducible NOS, iNOS) and NOS III (endothelial NOS, eNOS) (Moncada et al., 1993). The free radical nitric oxide is known to mediate a wide variety of physiologic functions, including neurotransmission, immune cell cytotoxicity, regulation of the vascular tone (Lowenstein et al., 1994) and has recently been implicated in events associated with a variety of female reproductive functions including ovulation, estradiol synthesis, follicle survival and oocyte meiotic maturation (Jablonka-Shariff et Olson, 1998; 2000; Van Voorhis et al., 1994; Chun et al., 1995). The present investigation was undertaken to verify if nitric oxide synthase isoforms II and III (inducible and endothelial nitric oxide synthase) are present in porcine oocytes during the growth.

The oocytes were isolated from follicles smaller than 2 mm in diameter. Aspirated growing oocytes were ranged according to their size to three groups (80 - 89 μm, 90 - 99 μm, 100 - 110 μm). Oocyte proteins were separated by SDS-PAGE and the NOS isoforms were detected by Western blotting analysis.

The presence of isoforms II and III was demonstrated in growing oocytes of all categories.

These findings may indicate that both isoforms have already been synthesized at the beginning of oocyte growth.

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THE OXIDATIVE STRESS IN LEUCOCYTES FROM YOUNG MICE SHOWING PREMATURE AGEING IS DECREASED BY A DIET SUPPLEMENTED WITH BISCUITS ENRICHED IN ANTIOXIDANTS

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With ageing there is an increase of oxidative stress due to an imbalance between the oxidant production and the antioxidant defences in favour of the former. Since immune cell functions are specially linked to reactive oxygen species (ROS) generation, the oxidant/antioxidant balance is essential for these functions. We have previously studied a model of prematurely ageing mice based on the different behavioral response in a simple T-maze test. Mice classified as PAM (prematurely ageing mice) show an early decline of several nervous and immune functions in comparison to control mice or NPAM (non-prematurely ageing mice) of the same chronological age. These dysfunctions found in PAM are accompanied by a shorter life span. In the present work we have studied several parameters of oxidative stress (different levels of oxidants/inflammatory compounds and of antioxidant defences) in leucocytes from young PAM as well as the effect on these parameters of a diet supplemented with biscuits enriched in antioxidants.

Young female ICR (CD1) mice classified as NPAM (which completed exploration of the first arm of a T maze in 10 s or less) and PAM (which required over 10 s) at 4 months of age were used. PAM and NPAM groups were divided into a treated group, which received a diet supplemented with 20% (w/w) of biscuits enriched in antioxidants (vitamin C, vitamin E, b-carotene, zinc and selenium) for 5 weeks, and a control group, which was fed a standard diet. After this time, peritoneal leucocytes were obtained and in these cells the following parameters were analyzed. As oxidant/proinflammatory compounds: extracellular superoxide anion levels and oxidized glutathione (GSSG) measured by spectrophotometry as well as release of nitric oxide (NO), tumoral necrosis factor (TNFa) and prostaglandine E2 (PGE2), which were determined using commercial colorimetric and ELISA kits. As antioxidant defences: reduced glutathione (GSH), as well as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR) activities, which were determined by spectrophotometry.

We have found that leucocytes from PAM show an increased oxidative stress in comparison to those from NPAM. Thus, oxidation/inflammation parameters such as extracellular superoxide anion, GSSG, GSSG/GSH, TNFa and PGE2, are higher in cells from PAM than in those from NPAM. Conversely, the antioxidant defences, such as SOD, CAT, GR activities as well as the GSH levels, are lower in leucocytes from PAM than in those from NPAM. The ingestion of a diet supplemented with biscuits enriched in antioxidants decreases the oxidative stress of leucocytes from PAM, increasing their antioxidant defences and reducing their oxidant/inflammatory compounds.

In conclusion, it appears, that since leucocytes are a good marker of the oxidative situation of the other cells, the PAM suffer a high oxidative stress, although these animals are chronologicaly young. Moreover, the ingestion for 5 weeks of a diet enriched in antioxidants decreases that oxidative stress. The present findings confirm the important role of antioxidants for health preservation and resulting functional longevity.

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DOWNREGULATION OF HER-2 BY HERCEPTIN DECREASES TRAIL-INDUCED APOPTOSIS IN BREAST CANCER CELLS

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Her-2/neu overexpression and subsequent increase in Akt kinase and MAPK activity is considered to be involved in resistance or reduced sensitivity of cancer cells to apoptosis (Harari et Yarden 2000). The Her-2-targeted therapeutic agent, Herceptin, has been developed to abrogate proneoplastic signaling downstream Her-2 receptor (Carter 1992). We investigated whether combined treatment of Herceptin and TRAIL (tumor-necrosis factor related apoptosis-inducing ligand) could enhance the specific killing of breast cancer cells that overexpress Her-2 receptor.

BT474 cells (a Her-2-overexpressing breast adenocarcinoma cell line) were incubated in the presence of Herceptin followed by cell death induction using his-TRAIL. Apoptotic cells exhibiting sub-G1 DNA content were determined using flow cytometry. The activity of Her-2 downstream kinases, Akt kinase and MAPK, was assessed by immunobloting. Cell surface expression of Her-2, TRAIL-R1, and TRAIL-R2 was by measuring of binding specific antibodies.

Herceptin treatment of BT474 cells resulted in significant inhibition of Akt kinase and MAPK activity as expected. However, Herceptin surprisingly decreased TRAIL-induced cell death in time- and concentration-dependent manner. Furthermore, Herceptin administration caused a reduction in both apoptosis-inducing receptors for TRAIL, TRAIL-R1 and TRAIL-R2.

Although Her-2-induced Akt kinase and MAPK activity has been reported to block TRAIL-triggered apoptosis (Cuello 2001, Chen 2001), our results demonstrate that the activity of Her-2 downstream molecules contributes not only to the high level of TRAIL-R1 and TRAIL-R2 but also to susceptibility to TRAIL-mediated apoptosis in BT474 cells.

This work was supported by IGA MH CR grant NC7133-3/2002 and GA CR grant 301/03/0545.

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THE CULTURED PRIMARY HEPATOCYTE AND ITS APPLICATION IN TOXICOLOGY

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The liver is the main organ involved in the metabolism of xenobiotic (foreign) compounds. The responsible enzymatic systems are the cytochromes P450 (mixed function oxidases or phase I reactions) and enzymes coupling larger water soluble groups to the substrate (phase II reactions). Especially during phase I reactions, highly reactive metabolites can be formed capable of interacting with DNA and causing mutations. On the other hand reactive xenobiotics may be detoxified. Therefore, the primary parenchymal liver cell (hepatocyte) appears to be the optimal and most reliable *in vitro* system for the determination of mutagenicity/genotoxicity. Since however, primary hepatocytes are proliferatively quiescent, a culture system had to be developed allowing for proliferation enabling the determination of induced changes at the chromosomal level. This paper summarizes the special features of primary hepatocytes, the findings on *in vitro* proliferation and the application of hepatocyte cultures for *in vitro* and *ex vivo/in vitro* toxicological testing.

DNA ISOLATION FROM PAC AND BAC CLONES OF ESCHERICHIA COLI AND SUBSEQUENT VISUALIZATION OF B-GLOBIN GENE CLUSTER BY FISH TECHNIQUES

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The BAC (Bacterial Artificial Chromosome) system is used for mapping and analysis of complex genome structure. BACs are DNA vectors based on bacterial F_1 fertility factor. Propagated as single-copy plasmids in

Escherichia coli, it is convenient tool for rapid analysis of selected genomic structures owing to its high clonning efficiency (>300 kb), easy manipulation and high structural stability in host. Other system for DNA clonning is based on bacteriophage P1 Artificial Chromosomes (PACs), which have a maximum clonning capacity of 100 kb.

An example of mentioned clonning systems application is investigation of transgenic properties of a 100 kb DNA fragment containing cluster of human b-globin genes inserted in Bacterial Artificial Chromosome (Kaufman et al., 1999). Initially, 180 kb-PAC insert was found to form random nicks upon in vitro manipulation (Kaufman et al., 1999) and therefore, HindIII partial digest was performed and fragments of 90-110 kb were isolated and cloned into the HindIII site of the 7.4 kb vector pBeloBAC11 (Kaufman et al., 1999). b-globin positive BAC clones were further investigated by Southern analysis, using specific probes for H4, e, Gg, Ag, b and 3´HS-1. The BAC inserts were isolated from its vector and digested with *Not*I, and then it was purified by pulse field gel electrophoresis (PFGE). Finally, the BAC insert was electroporated into host genome (Kaufman et al., 1999).

In our laboratory b-globin positive BAC clone in *E.coli* (a generous gift from dr. W.G. Wood, Institute of Molecular Medicine, Oxford) were propagated and DNA was isolated, purified and fluorescently labelled by nick-translation, in order to use it for Fluorescence in situ hybridisation (FISH) technique. Our experiments were focused on nuclear architecture of b-globin gene cluster composed of 5 functional genes (ε, Gγ, Aγ, δ, and β) arrayed on chromosome 11. These genes are developmentally expressed and transcription of these loci is regulated by DNAse hypersensitive site known as the locus control region (LCR). Studied genes are expressed during development of erythroid cells. The ε gene is active in embryonic yolk sac. Between 6 and 10 weeks of gestation, foetal liver starts to be the site of erythropoiesis and Gγ, Aγ globin genes are expressed. After the birth the site of erythropoiesis is changed to bone marrow and β-globin gene is activated (summary Ristaldi et al., 2001).

In our experiments, we used as a model system, differentiation of human erythroleukemia cell line K-562 by interleukin-3 (IL-3). It was found that b-globin gene is not expressed in progenitor (non-differentiated) K-562 cells, however is activated after treatment of K-562 cell by IL-3. In our experiments we are optimising detection of b-globin cluster (11p15.5) in control and IL-3 treated K-562 cells and in addition, we found relocation of chromosome 11 and its centromeric regions closer to the nuclear periphery during K-562 cell differentiation induced by IL-3.

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HAEMOCYTE MORPHOLOGY DURING THE ONTOGENESIS OF EGYPTIAN COTTON LEAFWORM *Spodoptera littoralis* (Boisd.) AND CHANGES ON THE DIFFERENTIAL COUNT CAUSED BY METYRAPONE

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Morpholgy of hemocytes during the development of *Spodoptera littoralis* (Boisd) (Lepidoptera, Noctuidae) and effects of metyrapone on the differentional count were studied. Four types of haemocytes were found in the haemolymph of *S. littoralis* – plasmatocytes, granulocytes, spherulocytes and oenocytoides. The proportion of individual haemocytes was different through the development. The count of plasmatocytes was higher. Treatment by metyrapone induced developmental changes accompanied by variation in the count of granulocytes. This observation is in correlation with previous findings (Gelbič and Němec 2001) that metyrapon can induce dose dependent higher stimulation of metabolism in the last larval instar of the used species resulted in precocious larval-pupal transformations. Body weight of treated larvae was higher, length of last larval instar was shorter for three days in comparison with control animals. Metyrapon probably induce faster process of

ageing. This hypothesis can be confirmed in this study by the significant decrease in granulocytes which simulated the trend observed during ageing of the last larval instar.

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PATTERNS OF STEM CELLS IN DIFFERENT TAXA OF FREE-LIVING PLATYHELMINTHES: A COMPARATIVE APPROACH

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Platyhelminthes are excellent models for the study of stem cell biology and regeneration capacity. Platyhelminthes are triploblastic, bilaterally symmetrical, unsegmented worms with an acoelomate organization. The most peculiar cell type in Platyhelminthes is a small, round undifferentiated cell called neoblast. The neoblasts are supposed to be the only dividing cells and to be responsible for the renewal of all cell types during development, growth, and regeneration, a unique situation in the animal kingdom. Here, the pool of neoblasts in S-phase is described and compared in an assortment of free-living Platyhelminthes. In order to further characterize these cells, we have applied the marker 5-Bromo-2`-deoxy-uridine (BrdU) for immunocytochemical detection of neoblasts in S-phase. We found a high variation in the distribution of S-phase neoblasts in different free-living platyhelminths ("Turbellaria"). S-phase neoblasts are distributed in two bands along the lateral side of Macrostomum sp. (Rhabditophora), Convoluta pulchra (Acoela) and of a member of the family Otoplanidae (Proseriata). Other Platyhelminthes exhibit S-phase cells all over the body e.g. Convolutriloba longifissura (Acoela) or show a uniform distribution except for the area of the brain and a small free area in the anterior tip (e.g. Nemertoderma sp., Nemertodermatida) and additionally in the tail like Minona sp. (Proseriata). Microstomum lineare (Rhabditophora) reveals S-phase cells in horizontal bands along the body lying parallel to the paratomizing division zone. We assume that the high variability of the distribution of S-phase neoblasts in Platyhelminthes is related to (1) the mode of reproduction (asexual versus sexual reproduction) and (2) the arrangement of brain and nerve cords especially in sexually reproducing animals.

THE NUCLEOLUS: FUNCTIONAL ORGANIZATION AND ASSEMBLY

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The nucleolus is a large nuclear domain generated by the act of building ribosomes. It illustrates the compartmentation of the nuclear functions, since it is in the nucleolus that transcription of the ribosomal genes, maturation and processing of the 47S ribosomal RNAs (rRNAs) into 18S, 5.8S and 28S rRNA, and almost complete assembly of the 40S and 60S ribosome subunits take place. The shape, size and organization of the nucleoli vary with their activity. Nuleolar activity is a cell cycle dependent-process. In electron microscopy, the nucleolus exhibits three main components: fibrillar centers (FCs), a dense fibrillar component (DFC) and a granular component (GC), corresponding to different steps of ribosome biogenesis. The steady state between transcription, processing and export of ribosomal subunits engenders this organization. Conversely, inactivation or blockage of one of these processes modifies the organization of the nucleolus and ultimately induces nucleolar disassembly. The nucleolus is also a plurifunctional domain, a key partner of chromatin architecture in the nucleus and it plays a crucial role in several cellular functions in addition to ribosome production.

The nucleolus is assembled at the end of mitosis, is active during interphase, and disassembled in prophase. The nucleolar transcription and processing machineries are inherited from parental to daughter cells through mitosis. The polymerase I (pol I) transcription machinery is repressed during mitosis although assembled with ribosomal genes. Repression of pol I transcription is achieved at the end of prophase and is maintained during mitosis through phosphorylation of transcription factors by the cyclin-dependent kinase (CDK) 1. The nucleolar processing machineries relocalize from the nucleolus towards the periphery of all chromosomes until telophase and this chromosome association depends on CDK1 activity. As a consequence of natural inhibition of CDK1

activity, pol I transcription is restored in telophase. The processing machineries are recruited to the sites of rDNA transcription after a temporary transit in foci known as prenucleolar bodies.

In conclusion, the behavior of the nucleolus illustrates the fact that the dynamics of nuclear organization are integrated in a network of interactions and controls that is largely dependent on the coordination of cell cycle controls.

CEREBRAL BLOOD FLOW AND APOPTOSIS IN PERINATAL ASPHYXIA

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Hypoxic-ischemic injury gives rise to brain damage in preterm neonates. We set forth the utilisation of an experimental model of perinatal disease in premature lambs, that allows us to study early damage of hypoxic-ischemic injury in the brain. Umbilical flow was partially occluded during 60 minutes. Regional cerebral blood flow was determined by four coloured microspheres infused through the left subclavian artery (Alvarez et al, 2002). After 0, 60 and 180 minutes, lambs were sacrificed and brains fixed by perfusion and divided in 9 selected cerebral areas. In situ apoptotic cell labeling was examined on the paraffin sections using the TUNEL (Gravieli et al, 1992). Regional cerebral blood flow was increased in internal areas, whereas in cortical and cerebellar areas blood flow values were similar to baseline. Moreover, the number of apoptotic cells was increased at 180 min. post-injury in cortical and cerebellar areas. In summary, our results show a direct correlation between the lesser degree of cerebral blood flow and the highest level of apoptotic figures.

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MORPHOLOGICAL AND MOLECULAR CHANGES INDUCED IN THE CALLUS CULTURE OF PEANUT (*Arachis hypogaea* l.) AFTER g-IRRADIATION OF ^{137}Cs

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Calli cultures of higher plants cultivated for a long-time *in vitro* conditions are characterized by higher resistance to γ-irradiation. The causes of this resistance are both genetic and epigenetic induced factors including cultivation conditions and exogenous growth regulators.

The γ-irradiation of ^{137}Cs with the doses D=1; 5; 10; 100 and 500Gy applied on the partially synchronized peanut calli cultures showed that small doses D∈<1; 5> Gy had a slight stimulation effect on the growth processes. Inhibition of the growth began for the dose D≥100Gy. Dose LD_{50} reached 250 ±50Gy. Necrose was not detected but cells of calli cultures changed their color. Stimulating doses D∈<1; 10 Gy> of γ-irradiation decreased morphological polymorphism of cells and nuclei. Gene expression was changed in the first minutes after irradiation. The spectrum of proteins and polypeptides synthesized *de novo* changed depending on the doses used. Protein Hsp60 was registered only for doses D=100 and 500Gy. Proteins connected with the apoptosis pathway (p21; p27; and p53) were synthesized in two waves, the first appeared between 24-48 hours after irradiation and the second appeared in the stationary growth phase.

Changes in the content of GTP-binding proteins as well as auxin and cytokinin binding proteins are indicated by their participation in post-radiation repair mechanisms. The role of r*ad* genes is discussed.

GENE EXPRESSION AND MORPHOLOGICAL CHANGES INDUCED DURING EARLY PHASES OF *Drosera rotundifolia* L. SOMATIC EMBRYOGENESIS

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Embryogenesis is one of plant life cycle essential period. Its background includes complex of molecular, biochemical and physiological processes to regulate a large amount of genes. Results of RNA-DNA hybridization showed that roughly 14 000-20 000 diverse genes are expressed at the mRNA-level (Goldberg et al. 1989). Induction of somatic embryogenesis, compared to natural zygotic one as a top level of plant life cycles, is more complicated. The differentiated cells of plant explants have to change un der effect of exogenous growth regulators their specialization on the active proliferating status. This cells than give the background of initiation of somatic pro-embryonic cells. Approximately after sixth division is received globular stage of somatic embryo with strong polarized cells reach on the actin and tubulin (Dedičová et al. 2000). Some glycoproteins as endoglucanases, chitinases and xylanases are very closely connected with identification and position of somatic embryo cells (Carpita et al. 1996). Genes coding these enzymes rang among auxin-responsible genes and their expression is under control of auxin exo and endogenous level. Trigger mechanisms leads *via* ARE-sequence situated in the upstream region of their promoters. Extracellular matrix shares the compact status of somatic embryos. Proteins participating on the extracellular matrix rank among the hydroxy - proline-reach one (Chapman et al. 2000; Bobák et al. 2000).

The aim of our our work was the study of dynamic morphological and biochemical changes induced in the leaves of *Drosera rotundifolia* during early phases of somatic embryogenesis.

Histological analyses of transversal semi-thing sections of *D. rotundifolia* leaves showed that after 17-th days of cultivation on the MS medium (Murashige, Skoog 1962) supplemented with 1.10^{-8} M NAA appeared dedifferentiated mesophyll cells giving bases to globular structure of somatic embryo. Globular embryo-like structures were induced with tangential and radial dividing dedifferentiated mesophyl cells. Globular embryo-like structures contained from 4-6 isodiametric cells with conspicuous reduced chloroplasts. They had large nucleus and very dense cytoplasm without vacuolar compartment. Scanning electron microscopy endorsed chloroplasts absence and dense cytoplasm. Full differentiated somatic embryo globular structures were received after 24-th days cultivation on MS medium containing auxin.

Protein analyses of extracts from the leaves of *D. rotundifolia* containing somatic embryo-like structures in the different status of re-differentiation (10; 17 and 24 days of cultivation on MS medium) showed qualitative and quantitative changes between low molecular proteins. Many of them created a group of auxin-binding proteins (Mr~11; 22; 24 and 25 kDa). Significant differences compared to untreated control leaves were detected after 17 days of cultivation (4-6 cell's somatic embryo-like structure). De novo synthesized proteins with Mr~12; 14; 15; 17 and 27kDa were appeared. The last two can be with high probability connected with the endogenous cytokinin level.(cytokinin-bindings proteins - Hlinková et al. 1998). After 24 days cultivation quantitative content of proteins with the low molecular masses decreased in the contrary to high molecular proteins. Biochemical analyses showed that changes in the proteins patterns were (high molecular part of protein spectra) induced changes in the amount of peroxidases (Mr~120-60 kDa), glucanase (Mr~25kDa) and lipoxigenase (Mr~12.5kDa). Quantitative changes in the content of p52 protein can be affected amount of large subunits of RUBISCO (protein is coded chloroplast genom – destructions of chloroplasts in the mesophyl cells participating on the conversion of the cells creating embryo-like structure) and β-actin subunits.

Generally, we can conclude that glycoproteins (glucanases, xylanases) and peroxidases play importat role by formation of globular stage of embryo-like structures.

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STUDY OF NATIVE PINE MEGAGAMETOPHYTE CELLS BY NEW LOW VACUUM SCANNING ELECTRON MICROSCOPE VEGA TS 5136

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The seeds of the family Pinaceae are largely composed of thin seed coat surrounding a massive haploid megagametophyte. The megagametophyte is a tissue containing storage substances mainly in the form of lipid and proteins (De Carli et al. 1987).

Spherosomes (lipid bodies, fat bodies), appeared as particles with high level of brightness in non-fixed megagametophytes of European black pine (Pinus nigra Arn.) studied by environmental scanning electron microscopy (ESEM) with the use of a backscattered electron (BSE) detector (Hřib et al.2001).

In a further study, spherosomes were seen as particles with a size up to 1μm in diameter. Apart from them, bright small spherical grains (1 to 2.5 μm) were observed. In these preparations it was not possible to decide whether they were located inside the ovoid protein bodies (particles 4 to 7.5. μm in diameter) or on their surface. When the same preparation site was studied after dehydration and metal shadowing, these grains were globoid in appearance and the majority of them were located inside the protein bodies. Their occasional presence outside protein bodies was caused by their expulsion from the bodies. With a decrease in voltage (30, 20 and 10 kV), the brightness of globoids was reduced but the appearance of spherosomes remained unchanged (Hřib et al. 2002).

In present study, cross sections through the megagametophyte about 0.1 - 0.3 mm thick, were prepared from uncoated and extirpated embryo of European black pine and Scots pine (*Pinus sylvestris* L.) dry seeds. The spherosomes and globoids of protein bodies in these sections were studied by means of a low vacuum SEM Vega TS 5136 (TESCAN Ltd., Brno) equipped with BSE detector and an original low vacuum secondary Tescan detector (LVSTD), patented by Tescan. This detector allows secondary electron imaging under low vacuum conditions. In addition the energy dispersive X- ray (EDX) microanalysis system was used under low vacuum conditions.

LVSTD detector allows to study native surface architecture of dissected cells. The results obtained by means the BSE detector, approved our previous finding showing globoid protein bodies and spherosomes as particles with high level of brightness. In both, globoid protein bodies (diameter up to 2.5 μm) and spherosomes (up to 1 μm), the basic element composition were investigated by means of EDX.

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THERAPEUTIC LASER EFFECTS ON MODEL CELL SYSTEMS

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Therapeutic lasers generate non-invasive, low-power radiation at a power output of up to 500 mW. Although they have been used with varying degree of success for treatment of various pathological conditions in a number of medical specialties, it has not been clearly identified which cell structures or molecules function as primary targets responsible for triggering the cascade of events eventually manifested as proliferative cell activity, enhancement of immune responses, suppression of inflammatory reactions, vasodilatation or analgesic effects. Recent studies have provided fragmentary data on the stimulatory effects of non-invasive laser light on DNA synthesis (Loevschall and Arenholt-Bindslev 1994), collagen and pro-collagen production (Balboni et al. 1986, Abergel et al. 1987), an enhanced rate of cell proliferation (Bednarska et al. 1998, Web et al. 1998) and changes in cell motility and their overall behaviour (Noble et al. 1992). The effect of low-power lasers on mitochondrial

respiratory activity, electric potentials of cell membranes and their subsequent selective permeability for sodium, potassium and calcium ions, and increased activity of certain enzymes, such as cytochrome oxidase or adenosine triphosphatase (Hrazdira and Mornstein 2001). In numerous clinical studies, the therapy of mucosal and skin lesions by infrared laser light has been reported (Kert and Rose 1989). However, there are also data on harmful or destructive effects of low-power lasers. Irradiation with a He-Ne laser resulted in degeneration of bovine oocytes during meiosis (Ocana-Quero et al. 1997). Exposure of HeLa cells to infrared laser light produced a temporary degradation of the microtubular network (Šidlová et al. 2001) or stress fibres (Korbašová and Janisch 2001).

In all studies performed in our laboratory a BTL-10 semiconductor laser (Beautyline, Ltd., Prague, CR) emitting an 830 nm convergent beam was used in either a continuous or a pulsed mode (5000 Hz). The energy density ranged from 2 to 99 J/cm^2 and power output from 72 to 360 mW.

Changes in cell mobility after effects of laser irradiation we studied in *Tetrahymena thermophila*, and *Euglena gracilis* cultures exposed to a red light laser pointer operating at 650 nm, 0.75 J/cm^2, and 5 mW in a continuous mode for 30 sec.

The model systems used to study the effects of non-invasive lasers included monolayer HeLa cell cultures, and protozoan cells of *Paramecium caudatum, Tetrahymena thermophila* and *Euglena gracilis.* Cell responses were studied in the cytoplasmic microtubules of paramecia, the microfilaments, microtubules and intermediary microfilaments of HeLa cells, the motility of *Tetrahymena* and *Euglena* cells and in cell proliferation of HeLa cells. Microtubules were detected by indirect immunofluorescence using TU-01 and SwAM FITC antibodies, intermediary filaments were visualized by Vi-01 and SwAM FITC and microfilaments were stained with rhodamine-phalloidin and observed in a fluorescence microscope or in ultrathin sections by electron microscopy. The proliferation activity of cells was assessed using the MTT assay based on the ability of mitochondrial dehydrogenases in living cells to reduce soluble tetrazolium salts to a blue formazan product. In protozoan cells, changes in velocity were evaluated by comparison of video records of cell trajectories in irradiated and control cells; the VirtualDub 1.4b software product was used.

After the laser irradiation of HeLa cells in a continuous mode at an energy density of 12 J/cm^2, power of 17 mW and irradiation exposure of 21 sec, no changes in microtubules were found. When higher energy densities of 36, 48 and 360 J/cm^2 were used, the fragmentation of cytoplasmic microtubules was observed in most of the irradiated cells and this damage to the microtubular net was reversible. At 1 h after irradiation, the fragmented microtubules were no more revealed, probably due to their repolymerisation (Škorpíková et al. 2000, Réblová and Janisch 2001).

In paramecium cells, changes in the number and distribution of cytoplasmic microtubules were assessed. Irradiation by low-power laser resulted in a partial depolymerisation of microtubules. The reaction of the microtubular system to laser irradiation was not uniform; while cytoplasmic microtubules were depolymerised, the other microtubules remained unaffected (Koutná and Janisch 2001).

In irradiated HeLa cells, partial depolymerisation of intermediate filaments took place. High-energy exposure, in particular, resulted in a disappearance of intermediate filaments and an appearance of clear areas free from any fibres. These changes were probably caused by disruption of bonds amongst the fibres and their associated proteins. However, at the highest doses applied, cells in which intermediate filaments showed minimal impairment still could be found. When cytoskeletal components were compared, intermediate filaments were more resistant to laser irradiation than the cytoplasmic network of microtubules and microfilaments in the previous study by Škorpíková et al. (2001).

The actin cytoskeleton of HeLa cells responded to irradiation by partial depolymerisation of both cytoplasmic microfilaments and stress fibres: fine actin granules were present in high numbers and, when higher doses of irradiation were used, larger granules or their clusters appeared. The granules were mostly located at cell peripheries. Another effect of high-dose irradiation was a partial detachment of cells from the glass surface (Korbašová and Janisch 2001).

In ultrathin section, the microfilaments distributed throughout the cytoplasm in a mesh-like manner were, in some areas, arranged in bundles 25 to 50 nm thick. In cortex regions, microfilaments ran parallel to each other and to the surface and occurred at a higher density, thus forming bundles up to 100 nm in thickness. After irradiation, the distribution of actin microfilaments immediately after treatment was very irregular. Microfilaments, which ran parallel to the cell surface, increased in number and the thickness of their bundles was up to 0.3 μm. The microfilament network in the cytoplasm, particularly in its central part, was markedly thinner. Although thicker bundles were more frequent in this thin network than those found in non-irradiated cells, these bundles were only 30 nm in diameter (Janisch et al. 2002a,b).

HeLa cells irradiated by a 830 nm semiconductor BTL-10 laser in a continuous or pulsed mode at an energy density ranging from 2 to 99 J/cm^2 showed, regardless of the energy density used, a significant increase in proliferation at 72 and 96 h but not at 24 and 48 h. The stimulation of proliferation was related to the mode of irradiation. The cells irradiated in the pulsed mode (5 000 Hz) showed a higher proliferation activity than the cells treated by continuous laser light (Koutná et al. 2003).

Euglena gracilis and *Tetrahymena thermophila* cells responded to laser irradiation by an increase in their mobility. The increase in cell velocity was significant, compared with control cells, at both wavelengths used, i.e., 650 nm and 830 nm ($P<0.001$). In euglena cells, cell velocities, expressed as medians, increased by 10.3% and 14.1 % after exposure to 650nm and 830nm laser irradiation, respectively, as compared with control cells. In tetrahymena, irradiated cells responded to 650nm and 830 nm wavelengths by a 25.1 % and 26.3 % increase in velocity, respectively (Koutná and Janisch 2003).

It can be concluded that exposure to laser light of a very low energy (5 to 360 mW) results in temporary changes in the cytoskeletal system of cells and the stimulation of proliferative activity of cell cultures and mobility of individual cells. The results reported here were obtained in *in vitro* systems that enable us to determine and control experimental conditions with great precision. However, these can never fully substitute the environment of a multicellular organism whose structural and functional complexity provides a much large scope of interactions between living structures and laser light.

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STIMULATION OF LEUCOCYTE FUNCTIONS OF AGED MICE BY A 5 WEEK INGESTION OF A DIET SUPPLEMENTED WITH BISCUITS ENRICHED IN ANTIOXIDANTS

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Ageing is associated with a decline of many physiological functions, including those of the immune system. This impairment of the immune system exerts a great influence on the increasing morbidity and mortality observed in ageing. Since ageing is linked to oxidative stress, it is assumed that supplementation of the diet with

antioxidants may be useful to prevent pathological ageing and to increase longevity. In view of the above, the aim of the present work was to know if the ingestion of a diet enriched in antioxidants during a short period of time could improve several immune cell functions which are depresed in old mice.

ICR (CD1) mice (18 months old) received a diet supplemented with 20% (w/w) of biscuits enriched with antioxidants (vitamin C, vitamin E, b-carotene, zinc and selenium) for 5 weeks, or standard diet (controls). After this time the peritoneal suspensions (containing the main immune system cell types, i.e.: macrophages, lymphocytes and natural killer (NK) cells) were obtained and the following functions were studied. In macrophages, the different steps of the phagocytic process (adherence to tissues, mobility to the infectious focus or chemotaxis, phagocytosis capacity, both number of particles ingested by 100 macrophages or phagocytosis index (P.I.) and the percentage of ingesting macrophages or phagocyting efficiency (P.E.), and the digestion capacity determined by the measurement of intracellular production of free radicals and reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide) were assessed. In lymphocytes, the proliferative response to ConA, a T lymphocyte mitogen, and the release of interleuquine 2 (IL-2) were determined. The NK activity against cells from a murine lymphoma was also analyzed.

The results show that the ingestion for a short period of time such as 5 weeks, of a diet supplemented with biscuits enriched in antioxidants stimulates all the leucocyte functions studied, which were depresed in these old animals.

Since a well preserved function of the immune system is an excellent marker of health and longevity, the improvement of macrophage, lymphocyte and NK functions after ingestion of the diet used in the present study suggests that this antioxidant supplementation may be useful for the preservation of health and functional longevity in ageing populations.

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THE EFFECT OF NITRIC OXIDE SYNTHASE (NOS) INHIBITION ON MEIOTIC MATURATION AND PARTHENOGENETIC ACTIVATION OF OOCYTES IN VITRO

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Nitric oxide (NO) is one of the free radicals that are implicated in a variety of intracellular signaling mechanisms. NO is synthetized from l-arginine by three different nitric oxide synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The aim of this work was to study the effects of two NOS inhibitors, Nω-nitro-L-arginine methyl ester (L-NAME) and NG- monomethyl-L-arginine (L-NMMA), on meiotic maturation and parthenogenetic activation of oocytes in vitro. Both inhibitors have inactive conformers (D-NAME and D-NMMA), used as negative controls.

The oocytes were isolated by manual puncture of porcine ovaries (follicles bigger than 2 mm and smaller than 5 mm). The oocytes in germinal vesicle (GV) stage were cultured in vitro in modified M199 medium containing various concentrations of the following chemicals: L-NAME, D-NAME, L-NMMA, D-NMMA and SNAP (S-nitroso-N-acetyl-L-penicilamin) as NO donor, for parthenogenetic activation, at 39 oC in humidified atmosphere with 5% CO2 in air for 48 or 72 h. At the end of culture, the oocytes were denuded and fixed in methanol : acetic acid (3:1) and stained. All oocytes were examined with an inverted microscope and classifield as being at the following stages: 1) metaphase II (MII) healthy oocytes with a polar body, 2) activated oocytes with pronucleus 3) atypical oocytes with degenerative changes or atypical morphology and 4) oocytes at other stages of meiosis.

The addition of L-NAME or L-NMMA to the culture medium had a significant impact on meiotic development and parthenogenetic activation of oocytes. Compared with control oocytes, the oocytes treatment with either L-NAME or L-NMMA had a significant effect on their meiotic maturation or activation. This was reflected in the vast reduction in the percentage of oocytes at MII stage and activated oocytes and a large increase in the percentage of atypical oocytes compared with the control group of oocytes.

In conclusion, we have provided evidence that NOS - derived NO is a modulator meiotic maturation and parthenogenetic activation of oocytes in vitro.

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AFFLICTION OF SPERMATOGENESIS IN THE HYPODACTYLOUS RATS

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Rat hypodactyly leads in homozygotes (Hd/Hd) to male sterility. Present study was aimed to verify wheather germ cell death during differentiation of seminiferous epithelium is a dominant feature of this disorder.

Testes of adolescent and maturing Hd/Hd and +/Hd rats were processed for light and electron microscopy. The evaluation of apoptosis was performed by TUNEL Assay using In situ cell death detection kit POD (Roche).

In the course of the third week (W-3) seminiferous epithelium was arranged into 2-4 layers composed of two generations of germ cells: spermatogonia and primary spermatocytes. The onset of spermatogenesis was associated with germ cell degeneration, in the central region of some tubules apoptotic spermatocytes were detected, however significant difference between the Hd/Hd and +/Hd testes was not confirmed. At the end of W-4 all meiotic stages of primary spermatocytes and single spermatids were observed. Some tubules of Hd/Hd testes displayed an irregular distribution of germ cells and their adluminal compartment consisted mainly of ramifying processes of Sertoli cells. At W-5 late spermatids belonged to the regular findings in +/Hd testes. In Hd/Hd rats structural differences of seminiferous tubules were found: decrease in the number of differentiating germ cells, loosening and vacuolization of epithelium and well developed spermatogenetic areas. Single TUNEL positive spermatogonia and scattered apoptotic cells in the adluminal compartment were detected in Hd/Hd and +/Hd males as well.

Examination did not confirmed marked germ cell degeneration in the course of the onset of spermatogenesis in Hd/Hd males in comparison with +/Hd ones.

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UNWINDING OF RIBOSOMAL GENE AND ALTERATION IN UBF EXPRESSION AND PHOSPHORYLATION DURING G0/G1 TRANSITION OF HUMAN LYMPHOCYTES.

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Mitogenic transformation of quiescent T lymphocytes into lymphoblast-like proliferating cells stimulated with phytohemagglutinin (PHA) is accompanied by the strong promotion of ribosomal gene transcription, and translation and phosphorylation of an enhancer of rRNA synthesis - nucleolar upstream binding factor UBF (Cabart 1998, Kalousek 2000). Ochs and Smetana 1989 demonstrated increasing number of RNA-polI positive domains in nucleoli of PHA stimulated cells. These processes, as well as DNA replication reached maximal rate in the time interval from 40 to 64 h and a question of direct connection between increasing amount of rDNA sequences containing UBF binding domains and synthesis of UBF molecules arose. In this study we investigate the synthesis and phosphorylation of UBF protein and antiUBF immunofluorescence patterns of fibrilar centers (FC) in nucleoli of PHA stimulated lymphocytes and lymphocytes arrested in the first G_1 phase.

Replication of DNA was monitored using BrdU proliferating ELISA. Translation of UBF protein was measured by 35S metabolic labelling and PHA induced phosphorylation by immunodetection of phosphorylated UBF fraction with antiphosphoserine antibodies. Distribution of UBF positive domains in lymphocytic nucleoli was monitored by antiUBF immunofluorescence microscopy. G_1 or G_1/S arrest was induced by supplementation of PHA containing growth media (10 ㎎/ml) with 1 mM sodium n-butyrate or 2.5 mM thymidine.

In PHA treated lymphocytes the rate of translation of UBF protein as well as DNA replication activity and number of UBF immunoreactive FC increased in time reaching maximum at about 48 h after PHA addition. In lymphocytes stimulated with PHA and arrested in G_1 phase the neosynthesis of UBF and redistribution of FC were independent of G_1 arrest and in early stage of mitogenic transformation ribosomal gene unwinding preceded UBF synthesis. Data also confirm our previous revelation of underphosphorylated UBF pool in quiescent lymphocytes and its phosphorylation in early stage of transformation.

Despite of the fact that in stimulated lymphocytes the synthesis of UBF culminates simultaneously with activity of DNA synthesis and with accomplishment of FC redistribution, UBF synthesis is independent of DNA replication and of ribosomal gene unwinding in early stage of transformation. Unwinding of fibrilar centers can likely increase their synthetic activity exposing their contents of underphosphorylated UBF molecules to protein kinases. Despite of the possibility that neosynthesized UBF molecules may contribute through interaction with

rDNA to the stability of transcriptionally permissible rDNA structure, the unwinding of ribosomal genes in early stage of mitogenic transformation is likely independent of UBF neosynthesis.

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MOLECULAR CLONING, EXPRESSION AND GENE TARGETING OF THE MOUSE GALECTIN-4 GENE

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Galectins, soluble-type lectins, have been found to participate in biological processes such as immune response, development, signal transduction or metastasis. Galectin-4 is a prototype molecule of monomer divalent galectin members, which include highly homologous galectin-6 and less homologous galectin-8, -9 and -12. Little is known about function of these galectins in vivo. They have been found to be up/down-regulated in particular types of cancer but their role during early embryogenesis, in cell adhesion or cell activation has not been precisely elucidated yet. Galectin-4 was localized in the alimentary tract of several mammalian species. In mouse, Northern blot data indicate specific expression of galectin-4 and/or -6 in stomach, small intestine and colon (Gitt et al. 1998). Our aims are to clone and map the mouse galectin-4 (-6) gene and to analyze precisely its tissue and cell-specific expression. For an examination of the role of both galectins in vivo, we develop genetically modified mouse models using transgenic and gene-ablation approaches.

Molecular cloning of the mouse galectin-4 gene We designed several pairs of primers for mouse galectin-4 gene using galectin-4 complete cDNA sequence that we have already cloned, sequenced and submitted to the GenBank (AY044870). Using PCR approach we amplified, purified and sequenced several DNA fragments, which were subcloned into the plasmid vector pCR3.1 (Stratagene). Analysis of these sequences revealed that we obtained four gene fragments spanning the galectin-4 region of 7.5 kb with 10 exons and three fragments containing a part of the galectin-6 gene locus. To isolate DNA fragment encompassing galectin-4 gene region, we performed screening of the λFIXII phage 129/Sv genomic library (Stratagene) using hybridization with a 981 bps galectin-4 cDNA probe. From 500,000 tested plaques, one galectin-4 positive clone was found and purified. Further analysis using PCR amplification with phage DNA and galectin-4 specific primer set supported the presence of the galectin-4 genomic locus. Study of tissue distribution of the galectin-4 (-6) We isolated total RNA from several mouse tissues and cell lines and use the samples as a template for RT-PCR with primers specific for galectin-4 (-6). Transgenic and gene-deficient mice models We constructed the transgenic and gene knock-out vectors and introduced them into ES cells. We are currently testing selected ES cell clones and are working on the generation of transgenic and gene deficient mice.

Using PCR amplification, we were able to obtain a set of galectin-4 (-6) gene fragments. These fragments were subcloned into plasmid DNA vector and further sequenced and the complete sequence map of the galectin-4 gene locus was prepared. Screening of the λFIXII 129/Sv genomic library resulted in an isolation of the positive phage clone encompassing desired galectin-4 sequence. After a partial mapping of the galectin-4 DNA insert using restriction endonucleases, the particular galectin-4 gene fragments are being prepared for the cloning into pBS KSII vector. Using RT-PCR with a set of specific galectin-4 (-6) primers we detect the galectin-4 (-6) mRNA in several mouse tissues. Our results indicate galectin-4 (-6) expression in the small intestine, colon, liver, kidney, spleen and heart and also in cultured cells of mouse T cell lymphoma BW-5147. The transgenic vectors pGale4 TG and phis-Gale4 TG were prepared by cloning of the galectin-4 cDNA and 6xHis tag-modified cDNA into the pCI-Neo mammalian expression vector (Promega) carrying the CMV promotor. These vectors are planned to be used for constructing transgenic mice over-expressing the galectin-4 protein. The gene targeting vector pGale4 K-O, constructed by cloning the galectin-4 gene fragments into pPNP-1 vector carrying the loxP-NeoR-loxP cassette, was introduced into ES cells by electroporation and selected G-418 resistant cell clones are currently being tested for the presence of the mutated galectin-4 allele.

We assembled the complete composite of the 7.5 kb galectin-4 gene locus using sequencing of the several PCR-amplified fragments. We also isolated phage clone carrying the mouse genomic galectin-4 sequence of 129/Sv mouse strain origin, and in addition, some DNA fragments, which were analyzed as putative parts of the galectin-6 gene. We have detected galectin-4 (-6) mRNA expression in several mouse tissues and in the nonmetastatic BW-5147 T-cell lymphoma. Our data indicate that this molecules are more broadly distributed

than it has been described and suggest that biological function(s) of galectin-4 (-6) may not be limited to the alimentary tract. To study the significance of galectin-4 altered expression in vivo, we are working on the generation of mutated mouse strains using transgenic and gene-ablation approaches.

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CHEMICALLY INDUCED PLASTOME REGRESSION IN CLOSELY RELATED FLAGELLATES – GREEN *Euglena gracilis* AND COLOURLESS *Astasia longa*

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The chloroplast system of the photosynthetic flagellate *Euglena gracilis* is sensitive to various agents – bleaching (review, Krajčovič et al. 2001). The colourless *Astasia longa* looks like a bleached mutant of *Euglena*. Complete sequences of both plastomes re-opened the question of a possible transformation of *E. gracilis* into *A. longa* through induced bleaching of *Euglena* (Hallick et al. 1993, Gockel and Hachtel 2000). To answer it we have studied the effects of two antibacterial drugs with different modes of action – inhibition of protein synthesis and DNA-gyrase, streptomycin and ofloxacin, respectively (bleaching agents in *Euglena*), on *E. gracilis* and *A. longa* growth and PCR detection of selected plastid genes.

Organisms: *Euglena gracilis*, Pringsheim strain Z; *Astasia longa*, strain 1204-17a; "Samlung von Algenkulturen" Gottingen, Germany; antibacterial drugs: ofloxacin (Sigma) MW 364.1; streptomycin sulphate (Sigma) MW 1457.4; PCR conditions: 94°C – 5 min., 32 x (94°C – 1 min., 55°C – 45 sec., 72°C – 2 min.), 72°C – 4 min.

E. gracilis plastome encoded genes – photosynthetic: *rbc*L, *psa*B, *psb*B, *psb*C; non-photosynthetic: *rpl*16, *ori*, *atp*B, *atp*I, all were detected by PCR in streptomycin treated cultures with only a slight time dependent reduction in signal intensity (if any), even after two weeks. Regarding the second half of those genes – photosynthetic *pet*G and non-photosynthetic: *rpl*16, *rpl*12, *tuf*A, *rpo*A, *rpo*C1, *orf*516 the time dependent reduction in signal intensity was much more profound. In ofloxacin treated cells after one and two weeks treatment, no PCR products were detected with those primers. In *A. longa*, PCR products were detected for both *Astasia*-specific plastome localised genes (*rbc*L and *rpl*16) in both streptomycin and ofloxacin treated cells. However, there was a significant time dependent reduction in signal intensity. With primers for nuclear encoded genes of *E. gracilis* (non-photosynthetic actin and α1-tubulin, photosynthetic *rbc*S, *cab*, *pet*J, *psb*O) and the mitochondria encoded *cox*I gene, PCR products were detected in both control and treated *E. gracilis*, but no significant time dependent reduction in signal intensity was observed. Although *Euglena* and *Astasia* share a common ancestor the evolution of a stable and functional *A. longa* plastome was most probably different from the process of induced bleaching in *Euglena*.

PCR studies with primers for selected genes revealed differences during drug treatment with respect to the organism (*E. gracilis versus A. longa*), drug (streptomycin *versus* ofloxacin), subcellular localisation of the genes (nucleus, plastid, mitochondria), but not the function of the genes (photosynthetic *versus* non-photosynthetic).

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DETECTION OF CHLOROPLAST DNA DAMAGE AND FREE RADICAL SCAVENGING ACTIVITY OF FUNGAL GLUCAN DERIVATIVES

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Fungal glucans are known as biological response-modifiers, and exert an amazing range of immunopharmacological activities. We have investigated two types of glucans: b–D glucan from yeast *Saccharomyces cerevisiae* and b-glucan-chitin complex from the mycelium of filamentous fungus *Aspergillus niger*. Since these glucans are insoluble in water, their water-soluble derivatives, carboxymethyl-chitin-glucan (CM-CG), sulfoethyl-glucan (SE-G) and carboxymethyl-glucan (CM-G) were prepared (Šandula et al. 1999). The aim of this study was to assess the antioxidative activity and antimutagenic effect of these glucan derivatives against genotoxicity of ofloxacin and acridine orange (AO) in *Euglena gracilis* assay.

The antioxidative activity was determined on a photochemiluminometer, Photochem, Jena, absorption spectra were run on a Biochrom 4060 spectrophotometer, Pharmacia, Upsala and antimutagenicity test was valued on *Euglena gracilis* Z strain (Križková et al. 2001).

The luminol-dependent photochemical method using trolox as a standard showed that CM-CG, SE-G and CM-G possess high antioxidative properties. CM-CG exhibited the highest antioxidative activity (2.15±0.14 nmol exhibits the same activity as 1 nmol of trolox), followed by SE-G (2.99±0.15 nmol) and CM-G (4.59±0.14 nmol). These glucans were confirmed to exhibit different, statistically significant activity in reducing damage of chloroplast DNA of *Euglena gracilis* induced by ofloxacin and acridine orange (AO). Our findings suggest that the antimutagenic effect of CM-CG, SE-G and CM-G against ofloxacin is based on their antioxidative capability to scavenge reactive oxygen radicals ($p<0.001$). As far as AO is concerned, the reduction of the chloroplast DNA lesion could be a result of the absorptive capacity of the glucans ($p<0.001$).

We found out that the water-soluble fungal glucan derivatives, obtained from biotechnologically important fungal strains, possess very high antioxidative activity as well as impressive antimutagenic effects exerted through different mode of action.

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THE EFFECT OF EXOGENOUS NITRIC OXIDE ON PRIMARY RAT HEPATOCYTE CULTURE

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The endogenously produced gas nitric oxide (NO) has been reported to influence wide spectrum of cellular functions. The net effect of NO on cell viability or cell death varies greatly according to different situations and experimental settings. In the present study we tried to evaluate the effect of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) on the viability and functional parameters of primary rat hepatocytes as a pilot study for further experiments.

Isolated hepatocytes were seeded on collagen-coated and coverslip-equipped Petri dishes and were allowed to attach for 3h in complete culture medium. After that, cells were treated with SNAP (0, 200, 400 or 800 μM) and incubated for appropriate time intervals. Morphological assessment of hepatocyte viability, apoptosis and/or necrosis as well as quantification of these changes was performed in routine H-E staining. NO levels, expressed as nitrite concentration, urea biosynthesis and alanine-aminotransferase activity in culture supernatants were analysed.

This study revealed the concentration dependent increase of NO soon after the SNAP addition. During continuation of incubation, nitrite levels gradually decreased. The quantitative morphological evaluation showed improved hepatocyte viability that was reflected by increased percentage of viable cells compared to the proportion of necrotic and apoptotic cells. Neither ALT leakage to medium nor urea biosynthesis seemed to be influenced by NO.

In our experimental setting, NO donor slightly improved rat hepatocyte viability but it did not abolish spontaneous cell death in culture completely. Also exogenously added NO did not significantly affect functional parameters. The beneficial effect of NO on primary rat hepatocyte viability in our culture system will be examined in further studies dealing with different kinds of cell injury.

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DUAL ROLE OF b-CATENIN IN COLORECTAL CANCER: STUDY OF INTERACTIONS AND TYROSINE PHOSPHORYLATION

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b-catenin is a protein known to play a crucial role in both cell-cell adhesion and signal transduction. Its aberrant activities are thought to participate in the development of colorectal cancer. Previously, we have found b-catenin present at high concentrations in different colorectal carcinoma cell lines. The presented work was focused on intracellular translocation of b-catenin as well as on its dual function in the mentioned cells.

The interaction of b-catenin with different members of the focal adhesion protein clusters were examined in HT29 and LS174T cells. Besides E-cadherin, b-catenin was also bound to the MUC-1 and p85 subunits of PI-3 kinase. Binding to MUC-1 appeared to increase significantly in the cells treated with 1.8 mM $CaCl_2$ for 1 hour. b-catenin was detected in cytoplasmic, nuclear and membrane fractions of HT29, SW480 and SW620 cells. The amount of the nuclear portion of b-catenin remained unchanged for 48h, whereas that of the cytoplasmic portion changed probably due to its time-dependent degradation. The stability and abundance of b-catenin are not affected by sodium butyrate. Unlike E-cadherin, b-catenin became markedly tyrosine-phosphorylated. Interestingly, this kind of phosphorylation occured in the nuclear b-catenin and did not occur in its cytoplasmic form.

Our work should help answer some questions regarding a possible role of tyrosine phosphorylation on the function of b-catenin as a mediator of cell-cell interactions and transcription co-factor.

CORRELATION BETWEEN CASPASE 3 ACTIVATION AND TUNEL – POSITIVE CELLS DURING ENAMEL KNOT SILENCING

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Odontogenesis involves complementary cellular events as cell proliferation, migration, differentiation and death, which overlap in time and are controlled by epithelio-mesenchymal interactions. Apoptosis seems to play a crucial role in morphogenesis involved in final tooth shape and position arrangement. The enamel knot has been described as a transitory structure with a specific arrangement of the cells and presumable signaling functions in tooth development. Apoptosis is understood to be a general mechanism whereby the enamel knots are terminated. Cystein proteases called caspases are an important part of apoptotic cell machinery, the central one is the caspase 3.

TUNEL test – terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (Roche Diagnostics, Germany) followed by POD/DAB (peroxidase/diaminobenzidine) modification was used to localize apoptotic cells appearing in embryonic development of dental primordia. Polyclonal rabbit anti-caspase 3 antibody (Transduction Laboratories, France) was applied and streptavidin-avidin detection system used for light microscopy evaluation of active caspase 3 distribution. Field vole (*Microtus agrestis*) embryos ED 13.5 – 17.5 (formalin fixed + cryoprotected) were employed.

TUNEL positive (apoptotic) cells were clearly demonstrated in the primary enamel knot and also in the area of secondary enamel knots. Active caspase 3 was shown in restricted areas corresponding with temporospatial distribution of TUNEL positive cells. These data suggest that caspase 3 is activated during apoptotic elimination of cells in enamel knots. Other steps in apoptotic signaling pathways activated during tooth development represent major aims for our future research.

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GROWING PORCINE OOCYTES HEAT SHOCK RESPONSE

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The heat shock response is a molecular reaction to stressful, but sublethal, temperature, and is characteristic of all organisms. When these organisms or cells are subjected to high temperatures, they rapidly redirect gene expression to maximize synthesis of a distinct group of proteins. These heat shock proteins are beneficial to cells, helping them adapt to the inducing temperature or survive exposure to higher, otherwise lethal, temperatures (Plesofsky-Vig 1996).

The aim of this study was to analyze the expression of heat shock proteins 70 (the inducible form of the HSP 70 family) in growing porcine oocytes after heat shock.

The growing porcine oocytes 80 - 99 µm and 100 - 115 µm in diameter were exposed to heat shock (43˚C) during 1, 4 and 6 hours. The proteins from oocytes were separated by 10% SDS-PAGE electrophoresis and transferred to nitrocellulose membrane. Immunoblotting was performed using a mouse anti-HSP 70 monoclonal antibody (StressGen Biotech. Corp.). Then the membrane was incubated with anti-mouse secondary antibody conjugated to peroxidase and developed using ECL Western blotting analysis system (Amersham Pharmacia Biotech., UK).

The level of HSP 70 in oocytes 80–99 µm in diameter was maximal after one hour of acute heat shock, after four hours was observed decrease the expression of these proteins. After six hours was observed a mild increase expression of HSP 70. An increase in the amount of HSP 70 in oocytes 100–115 µm in diameter was detected from the first up to the fourth hour of heat shock and then the synthesis of HSP 70 gradually decreased.

After heat shock, the level of free HSP 70 goes down when HSP 70 binds to denatured proteins. The depletion of the pool of free HSP 70 triggers the activation of genes encoding heat shock proteins and the synthesis of HSP 70 mRNA and then HSP 70 increases rapidly. These observations suggest that growing oocytes 80 - 99 µm in diameter have an earlier initiation of heat shock response than growing oocytes 100–115 µm with respect to their higher proteosynthesis activity.

This work was supported by the Czech University of Agriculture in Prague (Grant No 236/10/46801/10).

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ORGANIZATION OF REPLICATION SITES IN HeLa CELLS

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Replication of mammalian genome occurs at distinct replication sites (RSs). Light microscopy (LM) mapping of RSs provides several patterns. Small fluorescent foci dispersed in nucleoplasm are observed during early S-phase while larger ones localized in perinuclear and intranucleoar chromatin are found in the late S-phase. The LM and electron microscopy (EM) was used to provide a high-resolution description of the organization of RSs in human HeLa cell line in this study.

HeLa cells were synchronized with a double 2´-deoxythymidine block and released into normal medium for various periods of time. RSs were labeled with BrdU or biotin-16-dUTP in early, mid and late S-phase. Cells were processed for LM and EM immunocytochemistry and analyzed.

In agreement with previously published results, small fluorescent foci were found in early S-phase and larger and less numerous in mid and late S-phase. In contrast to LM results, EM has revealed similarly sized RSs during the whole S-phase of the maximum size 240 nm. Importantly, the average size of RSs was independent of the length of pulse labelling. The number of RSs was similar in early and mid S-phase (about 1100) and decreased toward the end of S-phase.

Our EM data have revealed surprising homogeneity in the size of RSs during S-phase. We named these RSs replication units, as their size was insensitive to the prolongation of incorporation of replication marker. In addition, we have shown that the number of replication units is invariable during S-phase progression.

CHANGES IN THE HUMAN MYOCARDIUM AFTER CARDIAC SURGERY INTERVENTION

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Cardiomyocytes of patients with coronary insufficiency display some qualitative and quantitative changes distinguishing them from healthy myocardium. It is known that oxygen deficiency evokes a prompt unfavorable effect on their structure. The aim of this study was to examine functional morphology of cardiomyocytes after cardioplegic period and demonstrate a localization of Nitric Oxide Synthase (NOS) and apoptosis.

Sixteen patients, indicated to for aortocoronary bypass, were investigated. Needle biopsies from left ventricular wall (the first immediately before aortic clamping, the second af-ter aortic clamping removal) were taken and processed for electron microscopy. Semithin sec-tions were used for immunohistochemical detection of NOS (nitric oxide producing enzyme), and M30 CytoDEATH antibody for evaluation of apoptosis.

Cardiomyocytes displayed enlarged mitochondria with lesser density of matrix and ir-regularly arranged cristae with local disintegrations of both enveloping membranes. A higher prevalence of glycogen granules, lipid droplets and tertiary lysosomes belonged to typical signs. Relatively delicate alterations of intercalated discs were present. Immunofluorescent detection of NOS confirmed its localization in the endothelium and in the sarcoplasm as well. On the contrary apoptosis was confirmed rarely.

Patients undergoing cardioplegia manifested postoperative complications as necessity of defibrillation, hypotension and arrhytmias. Direct affecting of Na, K-ATP-ase of the sar-colemma by oxygen deficit was referred. But also a direct effect of produced NO, supported by findings of NOS, can be accepted, too. Expected stimulated apoptosis was not confirmed.

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STORAGE EFFECT - NEW TOOL FOR RECOVERY AND ENHANCEMENT OF GROWTH OF INDUSTRIALY STORED SEEDS

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Table. **Frequency of aberrant metaphases** during storage 50 % water content of seeds of *Vicia faba* L. treated by 0.6 mM MH

Days/C or MH		32 hrs	48 hrs	56 hrs	72 hrs	96 hrs
0 days	Cont.	1.73 ± 1.32	1.50 ± 0.50	1.00 ± 0.00	4.60 ± 1.60	1.66 ± 0.57
0 days	MH	73.33 ± 6.67	61.50 ± 3.35	68.04 ± 8.98	68.85 ± 4.13	78.83 ± 3.23
14 days	Cont.	3.26 ± 0.10	4.41 ± 0.33	5.69 ± 1.04	1.60 ± 0.40	2.57 ± 1.60
14 days	MH	29.29 ± 4.80	30.40 ± 11.50	35.87 ± 7.01	37.72 ± 14.60	33.65 ± 21.90
28 days	Cont.	2.53 ± 1.22	3.11 ± 0.23	4.50 ± 0.86	3.22 ± 1.02	1.12 ± 0.23
28 days	MH	6.81± 2.27	7.40 ± 2.25	13.25 ± 5.43	17.41± 8.88	10.44 ± 2.14

The experimental storage of plant seeds is the only tool we know which allows the enhancement of the repair capacity of injured cells in a simple physiological way. Imbibition of seeds in the first hours of germination followed by process of re-drying down to 50% water content will cause arrest of the meristematics cells in the G_1 phase of mitotic cycle. Consequently this condition artificially prolongs the period between the damage caused by different kinds of xenobiotics and/or environmental stresses and the onset of DNA replication from hours to days. The condition described is favourable for pre-replication recovery from the induced DNA damage. This assumption was confirmed in seeds of *Hordeum vulgare* and *Vicia faba* L. for chromatid aberrations (CAs), single-strand breaks (SSBs), M1 survival, M1 seed setting and the frequency of M2 chlorophyll mutants.

The above described storage effect is a method well known from the intensive research which has been carried out already for almost thirty years (1). This method offers a simple and effective method of enhancement of DNA repair from damage caused by different kinds of xenobiotics and environmental stresses (Table). Although we have at hand an extensive scale of results of almost all aspects of the method studied, a practical outcome has not yet been proposed.. Thus in this report we would like to discuss possibility for recovery and enhancement of growth of industrially stored seeds. There is also a significant possibility for the use of this method in seed-banks.

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DOES A BYSTANDER EFFECT PLAY A ROLE IN THE APPLICATION OF THERAPEUTIC LASER?

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The biological effect of laser on tissue has been proved in a large number of experiments and there is no doubt about its analgetic, anti-inflammatory and stimulating effect. A mechanism of effect of therapeutical laser in deeper level of tissue (successfully used in locomotive apparatus treatment) has not been still explained. A direct influence is not possible according to on the laws of physics (absorption).

An explanation of this mechanism may lie in the bystander effect. Molecules synthesized after irradiation are supposed to be transferred from the irradiated cells to non-irradiated ones by means of a gap junction of approximately 2kDa. Then adjacent cells can be influenced by substances which are released from irradiated cells into cellular environment (e.g. peroxides of lipids, cytokines).

An energy transfer from the laser source into keratinocytes can trigger a local non-specific stress reaction. It is known that cellular stress comprises many changes, for example in nuclear factor kB, stress activated protein kinase, heat shock protein 70 activation. Knowledge of the relationship between laser effects and intracellular homeostasis should start an improvement in non-invasive laser therapy.

PALEOPATHOLOGICAL FIND OF A SACRAL NEURILEMMOMA FROM ANCIENT EGYPT

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A large cavity moulded by pressure of a relatively hard, globular and lobulated tissue mass was found inside the sacrum of the middle-aged woman Imakhetkherresnet, sister of the lector-priest Iufaa, whose unmolasted tomb dating late 26th-early 27th Dynasties was discovered by the Czech Institute of Egyptology in 1994-98 at Abusír - Egypt.By help of macroscopic and radiographic examination as well as histological analysis, the diagnosis of the originally present benign tumour, originating in nerve myelin sheath and called neurilemmoma, coult be firmly established.

The material was fixed, dehydrated and embedded in paraffin wax.The histological sections were stained by hematoxylin and eosin. For transmission electron microscopy matrial was fixed by glutaraldehyde, the blocks were dehydrated and embedded in Epon.Epoxy semi-thin sections were stained with toluidin blue. Immunohistochemically,nerilemmomas should be positive for epithelial membrane antigen EMA and for glial fibrillary acidic protein GFAP.

Because the macroscopic and radiological shape of the cavity shaped by presure of the intrasacral tumour suggested as the first option diagnosis of a neurilemmoma, we looked for its characteristic features. It has two components, known as Antoni A and Antoni B tissue.Antoni A tissue is cellular and consists of spindle-shaped Schwann cells, set in a variably collagenous stroma showing nuclear palisading, whose parallel arrays are known as Verocay bodies. Antoni B areas consists of Schwann cells arranged in net-works suspended in myxoid or microcystic matrix, accompanied by blood vessels with hyaline walls and lipidized areas.All these features were attested in our palaeopathological samples which were moreover, compared with features of a recent case of neurilemmoma.

The diagnosis of the originally present benign tumour, originating in nerve myelin sheath and called neurilemmoma, could thus be established. This is the first description of this kind of tumour in sacral location in the palaeopathological literature.

THE CHROMOSOME END REPLICATION: LESSONS FROM MITOCHONDRIAL GENETICS

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The widespread occurrence of linear mitochondrial genomes evokes intriguing questions concerning the evolutionary origin and mechanisms leading to the emergence and stabilization of linear DNA genophores. The study of their replication strategies opens a unique possibility to discover alternative solutions to the end-replication problem and to elucidate how these mechanisms have appeared in evolution. The analysis of linear mitochondrial genomes in organisms belonging to different phylogenetic lines indicates that their evolutionary emergence was accompanied by the generation of various types of terminal structures, the adaptation of existing replication machinery and by the application of different strategies of the telomere replication. This scenario is illustrated by the molecular anatomy and replication of the linear mitochondrial genome in the opportunist yeast pathogen *Candida parapsilosis*. Recent studies have revealed the existence of extragenomic minicircular molecules derived from the telomere repeats that seem to participate in the novel pathway of telomere maintenance. Importantly, several lines of evidence indicate that a similar mechanism may also be involved in the alternative, telomerase-independent, maintenance of nuclear telomeres in higher eukaryotes, including human telomerase-negative tumor cells.

ER-TO-CELL SURFACE SIGNALLING: CALRETICULIN AND CELL ADHESION

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Cell shape, adhesion, and motility are affected by Ca-regulated pathways, which depend on Ca-binding proteins. One such protein is calreticulin, a ubiquitous and major Ca-binding protein, resident in the ER of eukaryotic cells. In the lumen of the ER, calreticulin is a lectin-like chaperone, sharing this function with an ER-membrane protein, calnexin. Calreticulin also functions as an ER-lumenal Ca store and plays a central role in intracellular Ca homeostasis, including the regulation of store-operated Ca influx via plasma membrane and ER Ca channels. Calreticulin also affects processes outside of the ER; most notably, it modulates expression of several genes, some of them adhesion related, such as vinculin and fibronectin. Curiously, changes in the expression level of calreticulin strongly affect tyrosine phosphorylation of cellular proteins, which is known to affect many adhesion-related functions. Consequently, calreticulin affects cell adhesion via the regulation of expression of proteins important in adhesion, as well as via its effects on intracellular signalling pathways. One of the proteins differentially phosphorylated in a calreticulin-dependent manner is b-catenin, a structural component of cadherin-mediated adhesion complexes and a part of the Wnt signalling pathway. We suggest that the observed changes in cell adhesiveness may be due to calreticulin's influence on a signalling pathway from the ER, which includes the b-catenin/vinculin protein system. Differential expression of calreticulin may affect the phosphorylation status of b-catenin by either inhibition of specific phosphotyrosine kinase(s) or activation of phosphotyrosine phosphatase(s). This is likely to affect the balance between complexed and free b-catenin and impinge further down on the Wnt signalling. At present, the mechanism by which calreticulin affects gene expression can only be speculated upon, but our data indicate that calreticulin, *via* its effects on Ca release from the ER, may indirectly control the expression of several genes by interfering with calcineurin activity and the ability of the transcription factor, NFAT-3, to translocate to the nucleus. The activation of calcineurin depends on the sustained release of Ca from ER stores, which is dependent on calreticulin.

In summary, we propose that calreticulin may be a centrally located connector molecule in a signalling network in the lumen of the ER. Calreticulin is uniquely endowed for such regulation because it is a multifunctional protein that interacts with several other ER proteins in a Ca-dependent manner, suggesting that it may function as a signalling "toggle switch". We therefore hypothesize that calreticulin regulates gene expression by participating in an "ER-to-nucleus" signalling pathway, which parallels an "ER-to-cell surface" pathway based upon post-translational events.

NUCLEAR IMPRESSIONISM: HOW THE ACTIVE GENOME CREATES THE VERY CANVAS ON WHICH GENE EXPRESSION IS PAINTED

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This paper concerns the functional architecture of the cell nucleus. Though it is DNA that carries our literal blueprint, our ancestry includes the nucleus itself, passed down through the 2.5 billion year evolutionary history of the Eukarya. Nuclear structure is presented here as two contrasting possibilities. In one case, the nucleus is envisioned as being built upon a backbone of protein filaments, analogous to the cytoskeleton. In this conceptual framework, the chromosomes are considered to passively adopt locations that are dictated by their attachments to the imagined skeleton, and their activity is postulated to be the result of such attachments. In the other case, nothing in the architectural design of the nucleus is more deterministic than the chromosomes themselves, and their activity**.** Here, gene activity is thought to be based on the binding of DNA sequence-specific activator or silencing proteins that arrive at their target sites by diffusion. Moreover, additional elements of nuclear structure are viewed as arising from the very action of the genes themselves, such as nascent mRNAs packaged into ribonucleoprotein particles as well as large, heterotypic molecular machines involved in RNA processing. In this case, termed the "genome-centric model", the observed structure of the nucleus is not based on some underlying, pre-fabricated skeleton, but is in fact the actual ongoing cytological manifestation of genes in action. Upon careful analysis of all the evidence, the genome-centric model enjoys favor at the present time. However, we are still in kindergarten days in our understanding of the cell nucleus and, as always, it is wise to keep an open mind. New advances in biophysical, nanotechnology and systems biology approaches to nuclear architecture encourage us to believe that we may soon graduate into the gymnasium – if not university, level of our nuclear education. Viewed metaphorically as art (as in the playful title of this paper), we understand the paint at every atom of pigment on the palette – i.e., the covalent genome, the DNA. It is the final, creative work as applied to the gene expression canvas itself that we must now strive to know

THE PROLIFERATIVE COMPARTMENT OF FREE-LIVING FLATWORMS (TURBELLARIANS): STEM CELLS, NEOBLASTS, THEIR CHARACTERIZATION AND LOCALIZATION

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A survey of the current knowledge on stem cells and cell proliferation in turbellarians is presented, with special focus on recent results obtained by the authors when studying cell kinetics and cultivating neoblasts from various species. Differentiated somatic cells do not divide in flatworms. In these animals neoblasts constitute a proliferative compartment. These cells are rather uniform when viewed through a light microscope, but constitute a heterogeneous population comprising actual stem cells, progenitors and early differentiation stages. Cell kinetics were monitored by the incorporation of 5-bromo-2´-deoxyuridine and by immunocytochemical staining for mitoses in the marine microturbellarians *Macrostomum*, *Microstomum* and *Convolutriloba*. Distribution patterns of proliferating cells within the body were documented, and a tentative interpretation of the differences observed is given. Correlation to the nervous system and the mode of reproduction seems to exist To analyse neoblasts quantitatively and establish primary cultures, neoblasts from the freshwater planarians (triclads) *Dugesia* and *Schmidtea* were isolated, purified and subjected to various culture conditions, with the ultimate but not yet achieved goal of establishing a permanent stem cell line.

THIOLIC ANTIOXIDANTS INCREASE THE PHAGOCYTOSIS AND DECREASE THE ADHERENCE IN VITRO OF MOUSE PERITONEAL LEUCOCYTES

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Leucocytes can suffer damage because of the oxygen free radicals that they use to support their functions. These deleterious effects of the free radicals can be neutralized by antioxidants and in fact, the levels of endogenous antioxidants decrease with leucocyte activity (Hernanz et al. 1990). An increase of oxygen radicals in addition to a decrease of antioxidant defences causes an oxidative stress, which is responsible for a functional

deterioration of leucocytes. For this reason, we have studied in the present work the effect in vitro of several thiol antioxidants, such as glutathione (GSH), n-acetylcysteine (NAC) and thioproline (TP) on two functions of leucocytes: adherence to tissues and phagocytosis, which increase or decrease with oxidative stress, respectively.

GSH, NAC and TP at 0.5, 1 and 5 mM were used. The peritoneal suspensions from Swiss mice were obtained by a procedure previously described (De la Fuente, 1985). Adherence to fibronectin and collagen was evaluated incubating aliquots of 100 ml (adjusted to 10^6 cells/ml) with the different concentrations of antioxidants and the fluorescent probe BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) (1µM) in a fibronectin (20µg/ml) or type I colagen (10 µg/ml) (pre-coated plate for 15 min at 37 °C). After that time, plates were washed to eliminate non-adhered cells, and incubated again 10 min with Tris (50 mM) + SDS (0.1%). The fluorescence of wells was read in a fluorescence plate reader at 485 nm excitation and 535 nm emision. Results were expresed as adherence index (A.I.) obtained according to the following equation: A.I. = ((S-A)/T) x100, with *S* being the fluorescence units in the sample, *A* - the fluorescence units corresponding to the autofluorescence of the cells incubated without BCECF, and *T* being the fluorescence units in non-washed wells, representing maximum adhesion. The phagocytosis assay of inert particles (latex beads) was carried out according to a modification of a technique prevously described (De la Fuente, 1985). Aliquots of 200 µl of the peritoneal suspensions were incubated in culture plates for 30 min. After this time 20 µl of latex beads (1.09 mm of diameter) were added as well as 20 µl of the different concentrations of antioxidants or phosphate-buffered saline (PBS) solution (control samples). After 30 min of incubation the plates were washed, fixed and stained, and the number of particles ingested by 100 macrophages (Phagocytosis index, P.I.) as well as the percentage of ingesting macrophages (Phagocytosis Eficiency, P.E.) were determined by counting in an optical microscope.

The results show a stimulation of phagocytic capacity, both the P.I. and the P.E., with the lower concentrations used (0.5 and 1 mM) of NAC, TP and GSH. No effect was observed with the highest concentration of 5 mM. With respect to the adherence capacity, the antioxidants decrease adherence to fibronectin and collagen at 0.5 and 1 mM concetrations. The 5 mM concentration did not have any effect, with the exception of TP on adherence to collagen, which was also decreased.

The concentrations of 0.5 and 1 mM of GSH, NAC and TP improve the function of peritoneal leucocytes, since they increase phagocytic activity and decrease adherence to tissues. A very high adherence to tissues prevents the arrival of leucocytes to their place of action.These effects show that at the concentrations used the thiolic antioxidants studied neutralized the oxidative stress in mouse peritoneal leucocytes. Since we have indicated that the functions of these leucocytes may be a good marker of health and longevity, the use of thiol antioxidants could find some usefulness in the maintenance of subject health.

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EFFECTS IN VITRO OF THIOLIC-ANTIOXIDANTS ON APOPTOSIS AND SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES OF MOUSE PERITONEAL LEUCOCYTES.

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An excess of oxygen free radical production by leucocytes to support their functions, can be neutralized by endogenous antioxidants. However, the increased levels of oxygen radicals with respect to the antioxidant defences, leads to an oxidative stress, with harmful effects on the cells. For this reason, the addition of antioxidants, in vitro or as a supplementation to the diet, has been used to improve leucocyte functions (Del Rio et al. 1998, De la Fuente 2002). These effects of antioxidants could be due to their capacity to neutralize oxidants, to increase the endogenous antioxidant defenses, and consequently to preserve the viability of leucocytes. The effects in vitro of thiol antioxidants, concretely glutathione (GSH), n-acetylcysteine (NAC) and thioproline (TP) on apoptosis and antioxidants defenses such as superoxide dismutase (SOD) and catalase (CAT), are not known. In the present work we have studied the effect in vitro of several concentrations of those antioxidants on the viability and apoptosis as well as on the SOD and CAT activities.

GSH, NAC and TP at 0.5, 1 and 5 mM were used. The peritoneal suspensions from Swiss mice were obtained by a procedure previously described (De la Fuente 1985). Apoptosis was induced incubating leucocytes with H_2O_2 (50µM) during 6 hrs at 37^0C. Detection of apoptosis was carried out with a fluorescent

probe, JC-1, measuring the mitochondrial membrane despolarization (λ excitation of 485 nm; λ emission of 530 nm). The results were expressed as percentage with respect to the fluorescence units of the control (100%). Cellular viability was measured at 1, 6, 24, 48 and 72 hrs by the trypan blue exclusion test. The SOD activity was determined spectrophotometrically (420 nm) measuring the percentage of inhibition of the autoxidation of pirogallol by superoxide anion. CAT was determined spectrophotometrically (240 nm), following the transformation of H_2O_2 into H_2O.The results of enzyme activities were expressed as activity units per mg of protein.

GSH, NAC and TP decrease the apoptosis of peritoneal leucocytes, both basal and induced by H_2O_2. The viability of cells was increased with respect to the controls at 72 h of incubation with TP (1 mM). GSH stimulates SOD activity at all concentration used, and CAT was increased in the presence of the NAC (0.5 mM), TP and GSH (0.5 and 1 mM).

The thiolic antioxidants studied improve the activity of antioxidant enzymes such as SOD and CAT in peritoneal leucocytes, preserving and decreasing their viability and apoptosis, respectively.

This work was supported by a MCYT (BFI 2001-1218) grant.

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BIOLOGICAL ACTIVITY OF BINARY MIXTURES OF 2,4-D WITH SOME AMINOPHOSPHONATES

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A series of cyclic and acyclic aminophosphonates was synthesized for agrochemical application. The compounds differed in the substituents at the carbon, nitrogen and phosphorus atoms. Their potential biological activity was checked by studying their hemolytic potency, since hemolysis of erythrocytes by various compounds was found earlier to be a good indicator of their pesticidal efficiency. A series of hemolytic experiments permitted us to check the pesticidal efficiency of aminophosphonates and to determine what structural features of aminophosphonates are responsible for it.

Parallelly, we studied the hemolytic efficiency of binary mixtures of the aminophosphonates with the well-known herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). The aim was to check if potential biological efficiencies could be enhanced in comparison with those found for individual components due to interactions between aminophosphonates and 2,4-D. An analysis of the effects binary mixtures was carried out by constructing graphs for both components of binary mixtures that give the same hemolysis (the isobole method).

SPERMATOGENESIS IN FISH: A MODEL SYSTEM FOR THE ANALYSIS OF SEX DIFFERENTIATION AND FERTILITY

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The medaka (*Oryzias latipes*) is an attractive model system for analysing the mechanism of sex determination and sex differentiation and the effects of xenobiotics that may interfere with these processes. For example, when genetic males (XY) are exposed to estrogens during early juvenile development fertile females develop. We have studied the expression of sex-specifically expressed genes in normal and sex reversed XY females produced by exposure to 17ß-ethynylestradiol. While female specific genes (e.g. Fig *a)* become induced in such animals, a crucial gene involved in male sex determination, Dmrt1b(Y), remains expressed in sex reversed females. The long-term goal of these studies is to unravel the molecular mechanisms of sex determination using this model vertebrate.

In order to analyse the cellular and molecular processes of spermatogenesis ***in vitro*** we have established primary cultures of medaka (*Oryzias latipes*) and tilapia (*Oreochromis niloticus*) testis cells. Within 48h of culture medaka or tilapia spermatocytes differentiate into mature spermatozoa. By means of flow cytometry we have developed a method to quantify both the proliferation of spermatogonia and meiotic and post-meiotic

differentiation processes. The effects of hormonal disrupters or natural compounds (for example flavonoids) on spermatogenesis has been subject of our studies. Furthermore, the function and physiology of spermatozoa has been assayed with respect to the endogenous production of reactive oxygen species (ROS), the mitochondrial membrane potential of mature spermatozoa, changes in free Ca^{++} and the effects of these physiological parameters on sperm motility and capacity to fertilize ovulated medaka eggs.

THE ESTABLISHMENT OF POLARITY IN *C. elegans* EARLY EMBRYO

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The anterior-posterior polarity is essential for the asymmetric cell division of the *C. elegans* early embryos. The sperm entry point defines the posterior pole and the polarizing cue is thought to be the sperm centrosome. In response to this cue, PAR-1 and PAR-2 become enriched on the cortex nearest to the sperm asters, while the PAR-3/PAR-6/PKC-3 complex becomes enriched on the opposite pole. Concomitant with the establishment of the polarity, the embryo undergoes cortical rearrangements, resulting in a smooth posterior cortex and a contractile anterior cortex separated by an ingression. For PAR localization and for the polarity of the cortex an intact actomyosin cytoskeleton is required. Chemical disruption by Cytochalasin D causes mislocalization of the PARs and abolishes the cortical contractions. The underlying mechanism of PAR polarity and its relationship to the cortical dynamics is not well understood. However, we observe that the PAR-2 domain is confined to the smooth part. We are interested in knowing whether modulation of the actomyosin cytoskeleton affects the PAR-2 localization.

RNAi of Rho family members was performed by injection into GFP::PAR-2

Members of the Rho GTPase family are known to alter actin cytoskeleton. We found that due to RNAi of *rho* and *cdc-42*, PAR-2 is found on the entire cortex. However, their effect on the cortex is different. *Rho(RNAi)* abolishes the cortical asymmetry, whereas *cdc-42(RNAi)* does not. RNAi of a putative GAP for Rho GTPases causes expansion of the PAR-2 domain into the anterior and induces hypercontractility of the anterior cortex.

These results show that altering the activity of Rho family members affects the correct size of the PAR-2 domain formation.

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PRIMARY CULTURES OF NEOBLASTS (STEM CELLS) FROM THE PLANARIANS *Dugesia tahitiensis* AND *Schmidtea polychroa* (PLATYHELMINTHES: TURBELLARIA – TRICLADIDA)

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In flatworms differentiated somatic cells do not divide, and a separate proliferative compartment is competent for all cell renewal during growth, physiological and reparative regeneration (Baguñà 1998). It is made up of cells termed neoblasts that are scattered throughout the parenchyma and form a heterogeneous population (see Peter 2001, 2002, Peter et al. 2003). The actual stem cells are probably a minority among them. One approach to learn more about their totipotency and their pathways of commitment and differentiation would be an established cell line. On the way to this goal, we stimulated primary cultures of purified neoblasts prepared from *Dugesia tahitiensis* (Schürmann et al. 1998). This was done by the addition of 2.5% of Embryonic Stem Cell Qualified Foetal Bovine Serum (Life Technologies) and differentiated feeder cells from planarians to an isotonic medium (Schürmann and Peter 2001). In such cultures, mitoses persisted for up to seven days. When the serum was omitted, the metaphase index was lowered by roughly one fifth. Without the presence of differentiated cells, no mitoses were observed and neoblasts maintained their undifferentiated state. In earlier experiments with the related sexual species *Schmidtea polychroa*, cultures of purified neoblasts could be kept alive for up to 31 days; there were no signs of differentiation. The composition of the substratum influenced shape and behaviour of the cells. On polystyrene or glass surfaces, cells failed to adhere and stayed spherical. Layers of rat collagen I or IV and coatings prepared from the supernatant after the disintegration of regenerating planarians induced adherence

and flattening of the cells. Some of them developed processes of varying length. These were transitory and could be observed for one day at maximum in a given cell. There were uni-, bi- and multipolar cells. Similar changes in cell shape could be induced by a moderately hypotonic medium (88 mOsmol/kg, compared to 125-128 mOsmol/kg for planarian tissues). Being an exclusively asexual species, *Dugesia tahitiensis* offers as a preferential source particularly rich in neoblasts. There are no germ cells that might divide and be intermingled with stem cells (Peter et al. 2001).

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MICROBIAL INTERACTION AND GROWTH DYNAMIC OF THE THERMOPHILIC BACTERIAL STRAINS *BACILLUS* AND *THERMUS*

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The degradation potential of a mixed population of thermophilic bacteria can be used in waste degradation. To choose a good species composition in microbial population, knowledge about their behavior and interaction is necessary. The growth characteristic and interaction were measured on agar plates with strains of *Bacillus stearothermophilus* CCMI 237, *Bacillus acidocaldarius* CCM 3497, *Thermus species* CCM 4199 and *Thermus aquaticus* CCM 3488, using Luria-Bertani medium.

The growth dynamics and the interaction between all of them were obtained in the temperature range from 40°C to 60°C and for the 60°C, respectively. The colonies of microorganisms were photographed in a 12-hour period over 7 days and photographs were then analyzed with LUCIA Imagine system.

New criteria for characterization of colonies and their changes were introduced, such as the criteria of division and the criteria of interaction. The criteria, the area, and the theoretical and real perimeter of colonies were then compared and demonstrated as graphs.

The maximum growth rate was observed for *Thermus sp*. (area 4.11 cm^2 and real perimeter 9.12 cm) at 60°C and minimum for *Thermus aq.* (area 2.76 cm^2 and real perimeter 9.89 cm). The first eye-visible colonies were observed after 12h for all above mentioned strains, only for *Bacillus stearothermophilus* after 20h. At temperature 40°C no visible colonies were apparent after 156h. The interactions were predominantly based on substrate competition. No other significant relations were observed.

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LOCALISATION OF IMPORTED PROTEINS IN THE CHLOROPLAST COMPARMENTS OF *Euglena gracilis*

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About 90% of plastid proteins of flagellate *Euglena gracilis* are nucleus encoded and synthesised as a preproteins in the cytosol of the cell. Preproteins are transported from the Endoplasmic reticulum *via* Golgi to the chloroplasts integrated in the vesicule membranes (Sulli *et al.* 1999).

For more detailed determination of where the imported proteins are located, fractionation of chloroplasts into stroma, envelope membranes and thylakoids was achieved. The major components of stromal fraction correspond to the small and large subunits of the enzyme ribulose diphosphat carboxylase/oxygenase (Rubisco). The polypeptide profiles obtained for the envelope membranes showed most proteins in the regions of the gel corresponding to the size of 30 to 65 kDa (unidentified proteins). The major components of thylakoid fraction are analogous to the groups I and II polypeptides associated with photosystems I and II, respectively. Further studies of the structure and function of separated inner and outer envelope membranes required their isolation free of other cellular components. The polypeptide profile of each fraction was distinctive, the inner envelope membrane contains fewer proteins than the outer one.

Localisation of LHCPII (light-harvesting chlorophyll *a/b* binding protein of photosystem II) and SSU (small subunit of Rubisco) proteins was determined by Western blot analysis with monoclonal antibodies. Signal for LHCPII protein was obtained from the thylakoid fraction at about 2 5 kDa, signal for SSU protein was detected in the stromal fraction at about 15 kDa.

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GLYCOPHENOTYPE OF SQUAMOUS EPITHELIA: FROM LABORATORY TO CLINICAL PRACTICE

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The epidermal stem cell plays a pivotal role in the function of squamous epithelia in physiological as well as in pathological conditions such as cancer. This review summarizes data about the glycobiology of normal squamous epithelia and related tumors with respect to epithelial cell differentiation and search for a glycophenotype specific for epidermal stem cells using labeled plant and endogenous lectins. Although the glycophenotype typical for epithelial cells at the stage of low differentiation level were found, no typical cell surface saccharidic markers of stem cells were detected. The nuclear binding of galectin-1 seems to be specific for the keratinocyte population prepared from hair follicles enriched for multipotent stem cells. The close topographical relationship of nuclear galectin-1 binding sites with SC35 splicing factor suggests some role for these glycoepitopes in pre-mRNA splicing. The data shown in this paper can be employed for diagnostic purposes and for cell therapy of skin defects and indicate the importance of the use of endogenous lectins as probes in biology and medicine.

FURTHER NOTES ON NUCLEI AND NUCLEOLI IN THE PROGRAMMED CELL DEATH AND PRECEDING TERMINAL MATURATION

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As generally known, terminal maturation and programmed cell death - apoptosis - are accompanied by characteristic nuclear and nucleolar changes which are related to the inactivation of biosynthetic activities. On the other hand, it seems to be established that programmed cell death may and may not be preceded by terminal maturation.

The present study was undertaken to provide more information on both natural and induced apoptotic process with and without preceding terminal maturation using erythroid and granulocytic precursors as the cell models since their developmental or maturation stages are well known and morphologically defined.

The physiological cell terminal maturation of erythroblasts preceding programmed cell death is accompanied by chromatin condensation, the transformation of nucleoli to micronucleoli and the formation of nuclear HERDS (heterogeneous ectopic ribonucleoprotein derived structures). The apoptotic process is terminated by the loss of the nucleus frequently containing a micronucleolus. Then the free nucleus is engulfed by a macrophage and the cytoplasm is circulating as an immature erythrocyte (reticulocyte). The pathological

clone of erythroblasts, i.e. ringed sideroblasts of refractory anemias in patients suffering from myelodysplastic syndrome entering to premature apoptosis, exhibited marked structural abnormality of nucleoli as well as their loss. In addition, HERDS were noted even in erythroblasts within bone marrow macrophages.

In cultured leukemic granulocytic precursors originated from the early stages of the granulocytic development of acute myeloid leukemia represented by HL-60 cells, the apoptotic process was induced by photodynamic treatment without previous terminal maturation. In these cells, characteristic chromatin condensation was not accompanied by the nucleolar transformation to micronucleoli but only by the marked reduction of AgNORs. In contrast, the photodynamic treatment, carried out under the same conditions, did not induce the apoptotic process in leukemic granulocytic precursors represented by K 562 cells. These cells mostly did not exhibit any signs of terminal maturation or apoptotic process. It should be mentioned that they also originated from the early stages of the granulocytic development similarly as HL-60 cells but from chronic myeloid leukemia.

The presented observations clearly indicate that in physiological or pathological clones of erythroblasts the apoptotic process is preceded by terminal maturation in which the transformation of nucleoli to micronucleoli or loss of nucleoli together with characteristic gradual chromatin condensation and HERDS formation apparently represent the preapoptotic state. Such a preapoptotic state might be absent after the induction of the apoptotic process as demonstrated on HL-60 leukemic granulocytic precursors. The different behavior of leukemic granulocytic precursors originating from acute (HL-60 cells) and chronic myeloid leukemia (K 562 cells) after the photodynamic treatment demonstrates that the apoptotic process primarily depends on the cell type and not on its inducer.

EXPRESSION OF PKCd DURING PORCINE OOCYTE GROWTH

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Protein kinase C (PKC) is a very important multifunctional molecule that mediates a number of diverse physiological functions (Bement, 1992; Liu and Heckman, 1998). This monomeric protein includes twelve various serine/threonine protein kinases divided into three groups according to the presence of C1 domain for DAG and C2 domain for Ca^{2+} binding in regulatory domain. PKCd isotype is a member of the novel PKC that are calcium independent, but they can be activated by DAG and phorbol ester (Kanashiro and Khalil 1998, Bement 1992, Liu and Heckman 1998). PKC δ activated by distinct mechanisms plays different roles and contributes to both general and cell type-specific functions (Kikkawa, Matsuzaki, Yamamoto, 2002).

PKCδ was found in rat and mouse oocytes and it participates in the regulation of meiosis I and meiosis II transition in mouse oocytes (Viveiros *et al.* 2001, Gangeswaran and Jones 1997, Raz *et al.* 1998). Only limited data are available about the role of PKCδ in porcine oocytes.

The aim of our study was to verify if PKC δ is expressed in the porcine oocytes during their growth.

The oocytes were collected by aspiration of follicles. Growing oocytes were categorized to three groups according to their size (<90 μm, 100-110μm, fully grown). Proteins were separated by SDS-PAGE on 10% separating gel. The proteins were detected using ECL Western blot method.

PKCδ was found in both of the categories of growing oocytes (90 μm and 100-110 μm) as well as in fully grown oocytes. On the basis of this observation we assume that PKC participates in the regulation of porcine oocyte growth.

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CROSS-TALK BETWEEN THE CELL WALL AND CYTOPLASM

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The cell wall is no longer considered an inert and silent structure on the outside of the cell but instead, seems to be involved in an extensive cross-talk with the cytoplasm. Turgor, osmosensing, mechanical stress and other strains – all are mediated through the wall. The plant cell senses these signals through integral plasma membrane proteins whose extracytoplasmic domains are extended to the wall and intracytoplasmic domains are coupled with the components of cytoplasmic signal pathways. With its dynamic interactions the cell wall is comparable with the extracellular matrix of animal cells. Only a small part of the sensory machinery has been revealed in both plant and fungal cells.

PROTECTIVE EFFECT OF PHENOLICS AGAINST H_2O_2-INDUCED DAMAGE OF KERATINOCYTES

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UV exposure to skin induces the extensive generation of reactive oxygen species (ROS), which can react with DNA, proteins, fatty acids, and sacharides and can cause their oxidative damage. These injuries result in many harmful effects, including disturbing cell metabolisms, morphology and ultrastructural changes, attacks of regulation pathways, differentiation, proliferation and apoptosis of skin cells. The results of these processes can be photoageing and skin cancer development.[1,2] Hydrogen peroxide is one of the most frequent form of ROS produced in dermal cells after UV exposition, mainly as a result of UVA action (320-400 nm). Therefore H_2O_2 is often used as agent simulating cell UV damage.[3]

One approach to protect human skin against harmful effects of UV irradiation is using an antioxidant as a photoprotective. In recent years naturally occuring herbal compounds have gained considerable attention as protective agents.[4,5] In our study we tested selected flavonoids, and the known antioxidants: silybin, dehydrosilybin, quercetin and taxifolin.

The human normal keratinocyte cell line (HaCaT) was cultivated in concentration 1×10^5 cells/cm^2 for 48 h in 7% fetal calf serum (FCS). HaCaT were 30 min pretreated with tested compounds (1-50 ㎜) in medium without FCS and then H_2O_2 (0,5 mM; 4 h) was applied. The cytoprotective effect of the tested compounds against H_2O_2 was evaluated by the following methods: LDH assay, neutral red retention assay, MTT assay, intracelullar level of ATP (cell energy capability).

All tested compound exhibited an ability to reduce H_2O_2-induced keratinocyte damage in the following order: dehydrosilybin > kvercetin > silybin = taxifolin. Double bond in ring B (C_2 - C_3) of dehydrosilybin and quercetin is responsible for better cytoprotectivity. At the tested concentration range (1-50 ㎜) silybin, quercetin and taxifolin had no-toxic effect. Although dehydrosilybin showed low cytotoxicity (20 %) at 50 ㎜ concentration we found that at lower concentrations (1-25 ㎜) it proved much more active than other tested compounds.

These results show that the phenolics studied may be used in photoprotective dermatological preparations and skin care cosmetics.

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BIOLOGICAL ACITIVITY OF FLAVONOIDS CORRELATE WITH THE DEGREE OF HYDROXYLATION

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The biological activity of eleven structurally closely related flavonoids (flavones and flavonols) with different number of hydroxyl groups was analysed and an attempt was made to correlate the observed effects with structural features of the tested compounds. Using human myeloid leukemia (HL-60) cells we investigated the toxicity of the flavonoids, their effect on cell proliferation (by metabolic conversion of Resazurin and by flow cytometry) and their protection against reactive oxygen species (ROS) using the fluorescent indicator 2',7'-Dichlorofluorescein-diacetate (quantified by flow cytometry).

Among the substances tested 3,3',4',5,5',7-Hexahydroxyflavone (Myricetin), 3,3',4',5,7-Pentahydroxyflavone (Quercetin), 3,3',4',7-Tetrahydroxyflavone (Fisetin) and 3,4',5,7-Tetrahydroxyflavone (Kaempferol) are characterized by the highest degree of hydroxylation and these compounds induced apoptosis after 48h of culture in a dose dependent manner while Flavone, 3-Hydroxyflavone (Flavonol), 5-Hydroxyflavone (Primuletin) and 7-Hydroxyflavone were ineffective. A similar correlation was apparent when cell proliferation was quantified in the presence of the test compounds (20µM). For example, Quercetin increased the percentage of non-cycling cells to 60±3% while the compounds Flavone and Primuletin did not inhibit cell cycle progression at all. The analysis of the ROS scavenging activity showed a correlation with the degree of hydroxylation. Myricetin, Quercetin, Fisetin, or Kaempferol lowered the ROS level by a factor of 3-5 while Flavone was ineffective. The observations are difficult to interpret at present but altered hydrophobicity by hydroxylation and the possible consequences for the intracellular distribution must be taken into account as well as the different binding properties of strongly hydroxylated flavonoids with cellular proteins.

DENSITY OF TRABECULAR BONE ASSESSED BY IMAGE ANALYSIS

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Computational modelling of the biomechanical problems requires qualified assessment of input data. Due to insufficient experimental data, the material characteristics of trabecular bone are often elicited from that of the compact bone. The aim of our work was to assess the density of bone trabecules and lamellae in selected sections through spongy bone tissue.

We cut eight samples of human humerus, femur, and tibia in frontal, sagittal and horizontal plane. The meshwork of trabecules and lamellae constituting the very surface of the cutting plane were painted with water-colour. Each sample was scanned at 600 dpi and three squares (1×1 cm) representing the spongy bone underwent image analysis. We assessed percentage ratio of the highlighted bone in the sectional area, using the software AREA (Sofo Brno, Czech Republic).

The results revealed the following average values of the spongy bone density: 19% in humerus, 38% and 25 % and 27 % in three different samples of head of femur, 28% and 37% in two samples of distal femur, 28% in femur condyle, and 39% in distal tibia.

The static data describe neither dynamic stress-adaptation, nor directionality or anisotropic architecture of the bone trabecules. The results are ready to be used for the data input estimation in biomechanical modelling of the spongy bone.

This study was supported by the project CEZ J13/98:111400001.

MICROSCOPIC IMAGE ANALYSIS OF ELASTIN NETWORK IN HUMAN CHILDREN'S AND ADULT ABDOMINAL AORTA COMPARED TO ASCENDENT AND DESCENDENT THORACIC PORCINE AORTA

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The elastin system in the tunica media of the aorta appears as a network-like complex of bundles with many sections through the branched bridges interconnecting the neighbouring membranes. The aim of our work was to assess the morphology of micrographs of this network.

We analyzed tissue samples of the anterior wall of normal non-atherosclerotic human adult abdominal aorta (n=19), normal human children's abdominal aorta (n=6, age 1-2 years), porcine ascendent aorta (n=41) and descendent thoracic aorta (n=44). Serial sections were stained with green trichrome with Verhoeff's iron hematoxyline. Thresholded and segmented micrographs of elastin with constant magnification underwent 2D fast Fourier transform (FFT). The shape of the power spectrum was assessed by the Feret's ratio of its polar coordinates histogram.

The results revealed following average values of the Feret's ratio (with standard deviation at confidence level of 90%): 94(1.1) in children's aorta, 80.6(0.4) in human adult aorta, 90.5(0.2) in porcine ascendent aorta, and 91.1(0.2) in porcine descendent aorta.

With increasing age, the abundance ratio of elastin and its complexity decreases, even in normal human aorta. We found no significant differences between samples of ascendent and descendent thoracic porcine aorta. Assessment of elastin by means of the 2D FFT of its thresholded micrographs proved itself to be a useful method of elastin network description, reflecting its an/isotropy.

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YEAST TELOMERES: HOW TO IGNORE ESSENTIAL DOUBLE-STRAND DNA BREAKS?

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DNA looping is one of the mechanisms involved in telomere maintenance. It probably provides a solution not only to 'the end-replication problem', but also for the protection of chromosomal ends against degradation enzymes and, as typical double-strand breaks, from DNA repair machinery. Telomeric loops (t-loops) formed by an invasion of protruding 3' overhangs into the double-stranded telomeric regions were observed in a variety of organisms ranging from ciliates to mammals. Genetic data indicate that looping also occurs at the telomeres of *Saccharomyces cerevisiae*, suggesting its importance for telomere function in yeast. However, several observations argue against the presence of 'true' t-loops in the budding yeast telomeres (e.g. the lack of TRF-like protein, heterogeneous telomeric sequences). Instead, telomeres in *S. cerevisiae* appear to form fold-back structures mediated by protein-protein interactions. To directly visualize the telomeric structure in budding yeast, we developed a system based on a mini-chromosome carrying an array of *lac* operator sequences allowing its purification by the *lac* repressor affinity column. In contrast to budding yeast, the fission yeast *Schizosaccharomyces pombe* contains a homologue of the human telomeric protein TRF2, designated Taz1p. As the TRF2 protein has been implicated in remodelling telomeres into t-loops, the ability of Taz1p to promote t-loop formation is examined by electron microscopy using purified protein and synthetic templates containing a double-stranded fission yeast telomeric tract. Our studies should shed some light not only on telomeric architecture in yeast, but should also be instrumental in deciphering detailed telomeric structure in higher eukaryotes.

THE ROLE OF Slb/Wnt11 IN CONTROLLING CELLULAR POLARITY AND MOVEMENTS DURING ZEBRAFISH GASTRULATION

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During vertebrate gastrulation, highly coordinated cellular rearrangements lead to the formation of the three germ layers ectoderm, mesoderm and endoderm. Recent studies have found that *slb*/*wnt11*, which belongs to the Wnt family of secreted glycoproteins, plays an important role in regulating vertebrate gastrulation movements.

In *Xenopus* and zebrafish, *slb*/*wnt11* signals through a pathway that shares significant homologies with the pathway specifying planar cell polarisation of epithelial tissues in *Drosophila*, suggesting a role for cell polarisation in the regulation of gastrulation movements.

FORMATION OF NUCLEAR SPLICING FACTOR COMPATMENTS IS INDEPENDENT OF LAMINS A/C

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Nuclear lamins are the major components of the nuclear lamina and they have been implicated in the functional organization of the nuclear interior possibly by providing structural support for nuclear compartments. Here, we have specifically tested whether lamins A/C contribute to the intranuclear organization of pre-mRNA splicing factor compartments. Using embryonic fibroblasts from lamin A/C knock-out mice we show that loss of lamins A/C has no significant effect on the cellular distribution of several pre-mRNA splicing factors and does not affect the compartment morphology as examined by light and electron microscopy. The association of splicing factors with the nuclear matrix fraction persists in the absence of A-type lamins. Live cell microscopy demonstrates that the relative intranuclear positional stability of splicing factor compartments is maintained in the absence of lamins A/C and that the exchange dynamics of SF2/ASF between the compartments and the nucleoplasm is not affected by loss of lamin A/C. Our results demonstrate that formation and maintenance of intranuclear splicing factor compartments is independent of lamins A/C.

ULTRASTRUCTURE OF A MICROSPORIDIUM *BRACHIOLA GAMBIAE* N. SP. PARASITISING A MOSQUITO *ANOPHELES GAMBIAE*, A MALARIA VECTOR

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In 1959 Fox and Weiser published the case of a *Nosema* identified at that time as *Nosema stegomyiae*, isolated from rearings of *Anopheles gambiae* in Liberia, preventing development of malaria parasite in infected mosquitoes. The microsporidian caused destructive epizooties in mosquito rearings in the insectary. This parasite reduced susceptivity of the mosquito to development of malaria parasites and their transmission to man. It infects most tissues of adult male and female (no larvae) mosquitoes, destroying the midgut, Malpighian tubules, the fat body, muscles, hypoderm and connective tissues. We used this old material in Canada balsam for study in electron microscope.

Slides were opened, the balsam was removed and sections were transferred into alcohol and water. The sections were closed in 2% agar, refixed in 2% glutaraldehyde in cacodylate buffer, postfixed in 1% osmium tetroxide, washed in buffer and dehydrated in alcohol. The material was embedded in Epon and ultrathin sections were contrasted in uranyl acetate and lead citrate and inspected in a Philips CM 100 electron microscope (Kodak electron microscope films 4489).

In the re-fixed and stained ultrathin sections we found stages of the end of schizogony, early and late sporonts and mature spores. The diplokaryotic schizont is closed in a fine continuous smooth membrane of electron dense plasmalemma. The outer surface of the plasmalemma is covered by a thin layer of fine electron dense granulation, without irregular secretory processes. Thick walled sporonts with smooth walls have no electron lucent layer between the wall and the plasmalemma. The cytoplasm is a dense granulated mass without distinct nucleus, visible only as a confluent vacuolated system in the centre. Internal structure were destroyed during the Bouin's fixation in 1958. Spores (2.2-2.5 x 1.4-1.6 µm) are oval to pyriform, with deformations and compressions from fixation. On the surface of the spore are indistinct plasmalemma, thick electron lucent endospore and thin electron dense exospore. In the centre are two electron dense nuclei. The ribosomal system and the Golgi system are indistinct. The polar sac encloses the polaroplast with irregular remains of its structure. The coiled part of the filament is anisofilar, has 6 broader and 3 narrow cross-sections. In the group of normal spores on its perifery are some larger spores, usually 1 in 30, with all structures equal to normal spores, but with up to 13 turns of polar filament.

The striking similarity of the appearance of the 1958 material in ultrastructures with members of the genus *Brachiola* (Cali *et al.* 1998, Lowman *et al.* 2000) invites a correction of the old identification and a proposal for a new name for the pathogen of *Anopheles gambiae* and *A. melas* in Liberia interactive with transmission of malaria parasites in mosquitoes., *Brachiola gambiae* n. sp.

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EFFECTS OF NATURAL SUBSTANCES ON ENDOTHELIAL CELLS

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Endothelial cells (EC) are constantly exposed to the possibility of oxidative damage from reactive oxygen species and such damage to the endothelium is considered to be one of the principle mechanisms in the pathogenesis of atherosclerosis. To study atherogenic damage *in vitro*, EC isolated from the human umbilical vein (HUVEC) are used in experiments.[1]

Table. **The cytotoxicity of tested benzo[c]phenanthridine alkaloids**

Compound / Methods	Incubation (h)	IC_{50} (μmol/l)	
		MTT	NR
Sanguinarine	24	2.3 ± 0.6	1.8 ± 0.1
	48	3.2 ± 0.4	3.5 ± 0.7
	72	2.6 ± 0.2	5.5 ± 0.1
Chelerythrine	24	5.5 ± 0.6	4.6 ± 0.3
	48	5.1 ± 0.3	4.8 ± 0.3
	72	5.8 ± 0.9	8.7 ± 0.9

The aim of our work is to standardize the cultivation conditions of EC and toprepare this model for the study of:

i) cytoprotective effects of extracts from *Prunella vulgaris* and *Smallanthus sonchifolius* (rich in phenolics and recommended for the prevention of oxidative stress)[2,3] against oxidative damage of the EC. The effects of the plant extracts were compared with the phenolics caffeic and rosmarinic acids.

ii) cytotoxicity of quarternary benzo[c]phenanthridine alkaloids sanguinarine (SA), chelerythrine (CHE) and their action on the caspase cascade. These compounds are used in dental care applications and as additives in feed for farming animals. The medical applications of these alkaloids in relation to their biological activities are discussed.[4]

Cell line (HUVEC) was obtained from Promocell and cultured in a Endothelium Cell Basal Medium supplemented with growth factors (Promocell). Cells were used at passages 2–7 and cultivated in flasks coated by 0.2% gelatine. At confluence, cells were trypsinized, transferred into 96-well or 6-well plates coated by 0.2 % gelatine at a density of $0.25x10^7$ cells/plate.

The cytotoxicity of benzo[c]phenanthridine alkaloids (0.1–2 μmol) and cytoprotectivity of extracts from *P. vulgaris* and *S. sonchifolius* (1–1000 μg/ml) were determined using MTT and neutral red assay (NR). SDS electrophoresis and Western blot were used to study the effect of SA, CHE, and extracts from *P. vulgaris* and *S. sonchifolius* on caspase-3 activation.

The model of HUVEC was successfully optimized for cytotoxicity and cytoprotectivity studies. The cytotoxicity of tested benzo[c]phenanthridine alkaloids is shown in the table and is no different from data using other proliferative cells. The data are expressed as means ± SD, n = 3.

Neither *P. vulgaris* and *S. sonchifolius* extracts nor the phenolic acids exhibited significant toxicity in the concentrations used. Moreover, most of these substances were able to prevent hydrogen peroxide induced

caspase-3 activation. This effect was most pronounced for ***P. vulgaris*** extract at 500 μg/ml and this finding supports the use of these plants for medical application.

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COMPARISON OF DIFFERENT DETECTION SYSTEMS IN THE POSTANATAL FUNCTIONAL DEVELOPMENT OF POLYMORPHONUCLEAR LEUKOCYTES

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The aim of this work was to establish the development of the respiratory burst, which is characterised by the creation of the reactive oxygen species – ROS, during the postnatal development of a pig. The results will be compared with our recent findings concerning the development of the superoxide anion production. Furthermore, the expression of some other surface CD markers was determined.

The peripheral blood from piglets of 1, 7, 18, 31, 66 and 100 days after birth was used in the experiment for the assessment of the respiratory burst. The production of the ROS was established by the luminometric technique from the whole peripheral blood. The blood was diluted in 1:100 ratio in order to decrease the quenching influence of red blood cells. This procedure was more advantageous than the application of certain separation methods due to the elimination of unwelcome activation of white blood cells. Luminol was used as substrate. The ROS production was enhanced by the opsonized zymosane or PMA. The expression of CD18 and CD45 was determined using the monoclonal antibodies against porcine CD18 (APC conjugated) and CD45 (FITC conjugated).

The ROS production per one millilitre of blood does not show any dependence on the age after the activation by either zymosane or by PMA. On the other hand, the values recounted for 10^6 of phagocytes have a decreasing trend from 1st to 66th day with a sudden rise from 66th to 100th day after activation by zymosane. The activation by PMA displays a similar trend without the eventual increase between 66th and 100th day. The ability of zymosane to activate the respiratory burst was almost five times higher than analogous ability of PMA.

Our recent investigations prove an increase in the production of the superoxide anion after the activation by zymosane or myristate even without the activation between 17^{th} and 26^{th} day of life. Moreover, a sudden increase in the production of the superoxide anion between 70^{th} and 105^{th} day was detected. Furthermore, the changes in the spontaneuos production of the superoxide anion between 26^{th} and 30^{th} day indicate that it is altered by the influence of weaning. The ability of zymosane to activate the superoxide anion production was two times lower than analogous ability of PMA.

The results suggest that changes in the representation of individual ROS occur during the postnatal development. Whereas the production of the superoxide anion (which is an initial substrate for the creation of other ROS) grows, the overall production of the ROS displays a steady decrease.

The development of the expression of CD18 and CD45 will be discussed.

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INSULIN RESITANCE: A PHOSPORYLATION-BASED UNCOUPLING OF INSULIN SIGNALING

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Insulin resistance refers to a decreased capacity of circulating insulin to regulate nutrient metabolism. It is associated with the development of type 2 diabetes, a 21^{st} century epidemic. Recent studies reveal that agents that induce insulin resistance exploit phosphorylation-based negative feedback control mechanisms otherwise utilized by insulin itself, to uncouple the insulin receptor from its downstream effectors and thereby terminate

insulin signal transduction. This talk will describe recent findings that present novel viewpoints of the molecular basis of insulin resistance, focusing on the cardinal role of Ser/Thr protein kinases as emerging key players in this arena.

One of the key modes of action attributed to inducers of insulin resistance is their ability to enhance Ser/Thr phosphorylation of either the insulin receptor itself or of its downstream effectors. This reduces the Tyr kinase activity of the insulin receptor (IRK) and its ability to Tyr phosphorylate substrate proteins {reviewed by Zick 2001). Our recent studies reveal that prolonged insulin treatment enhances Ser/Thr phosphorylation of Insulin Receptor Substrate (IRS) proteins. This impedes their interaction with the juxtamembrane region of the receptor and in such a way turns them into poorer substrates for IRK. Impaired Tyr phosphorylation eliminates the ability of IRS proteins to recruit downstream effectors such as PI3K and inhibits insulin signal transduction. Hence, Ser/Thr kinases, stimulated by insulin, act as negative-feedback regulators to turn off insulin signals under physiological condition. Ser phosphorylation and reduced binding of IRS proteins to IR occurs at a slower rate, when compared to their rate of insulin-induced Tyr phosphorylation. This suggests that initiation of negative-feedback control mechanisms in the form of Ser/Thr phosphorylation and the dissociation of receptor-substrates complexes, is designed to commence with a delayed onset, thus allowing enough time for the insulin signal to propagate, before it is being turned off.

Importantly, inducers of insulin resistance such as phorbol esters and TNFa take advantage of this regulatory process. By activating Ser/Thr kinases, these agents enhance Ser/Thr phosphorylation of IRS proteins, at the same 'inhibitory' Ser sites, which impairs the interactions of IRS proteins with the insulin receptor. Such impaired interactions abolish the ability of IRS proteins to undergo insulin-induced Tyr phosphorylation and further propagate the insulin signal, thus providing us with a molecular basis for the induction of an insulin resistance state. Given the large number of stimuli, pathways, kinases, and potential 'inhibitory' Ser sites involved, it appears that Ser/Thr phosphorylation of IRS proteins represents a combinatorial consequence of several kinases, activated by different pathways, acting in concert to phosphorylate multiple sites. Devising of effective means to prevent the phosphorylation of this 'inhibitory' sites could turn beneficial in attempts to promote insulin action, and protect against the adverse effects of inducers of insulin resistance.

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AZIMUTH RELIEF CONTRAST MICROSCOPY: A REFINEMENT OF THE RELIEF CONTRAST AFTER HOSTOUNSKÝ MICROSCOPY

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Attempts to obtain a 3D image of objects under observation date back to the very first version of a binocular microscope blueprint (Chérubin d'Orléans 1677 in Needham 1958). A number of solutions have been suggested and implemented. These include a stereoscopic microscope for small magnification; the use of Ramsden's discs, including the dynamic 3D microscopy (Zeiss) for high magnification; and laser confocal microscopes equipped with computer processing of optical sections, where, however, it is not possible to perform the observations in real-time. Real-time observation has so far been possible only with special microscopic techniques providing a 3D imaging effect, such as Differential Interference Contrast after Nomarski (D.I.C.), Hoffman's modulation contrast, or Relief Contrast after Hostounský (R.C.H.). Yet another option is the novel, presently tested Azimuth Relief Contrast (A.R.C.) which itself represents a refinement of the R.C.H. in terms of maximizing the 3D effect. So far, we have published several accounts of the R.C.H. microscopy and its application in studying the morphology of microorganisms (Žižka *et al.* 1999, 2001) and reptile integument (Žižka 2002). The present paper focuses on the use of A.R.C. microscopy in studying fungi and algae.

The fungi we have been using were obtained from the Culture Collection of Basidiomycetes, Institute of Microbiology, Academy of Sciences of the Czech Republic and cultivated in a standard way, as described elsewhere (Gabriel *et al.* 1994). The algae/other organisms were collected in the wild (ponds/residential areas in the vicinity of Hostivice).

The equipment used for examining the microorganisms consisted of a laboratory microscope, DN 45 – BH 51 model (Lambda Ltd. Praha, Czech Republic, formerly Meopta Ltd. Praha, Czechoslovakia) fitted with achromatic objectives and a special condenser (ARC 1) whose relief diaphragm can be rotated clockwise or counterclockwise (optional) by a stepper motor ($0 – 360^0$, step size 3.6^0; angle recorded and displayed digitally). Images were recorded with Lambda FS – 1 microphotography equipment fitted with Minolta X – 300 S camera

(35 mm film, Agfa Vista 200 or Fuji Color Superia 100) or CCD colour camera (Sony SSC – DC 398 S, 1/3 inch, 480 TV line scans) with an output on a TV screen or a PC monitor.

In fungi, cell morphology and mycelial pellets were examined; in prolong cells, azimuth setting of the relief diaphragm made it possible to optimize (augment) the 3D effect, prolong structures and to examine reproductive stages (vacuoles, nuclei, basidia, spores etc.). The intracellular architecture in algae (cell walls, vacuoles, chloroplasts, granules, isthmuses, semicells etc.) was also well discernible when examined with this particular equipment capable of augmenting the 3D effect.

To conclude, we would like to point out that the novel A.R.C. condenser features higher resolving power and contrast, and a stronger 3D effect than the standard R.C.H. condenser. Moreover, it enables precise setting of the relief diaphragm angle (within the entire range of $0\text{-}360^0$). These features are of particular interest when studying prolong structures that are present in random orientations in the specimen, e.g. fungal hyphae and algal filaments and/or their inner architecture.

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