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O 1 - CHARACTERIZATION OF HYPERACTIVE ANTIFREEZE PROTEINS

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Ice-binding proteins, IBPs, include those that have the ability to stop ice crystal growth and inhibit ice recrystallization. IBPs can be moderately active or hyperactive according to their thermal hysteresis activity, which is defined as the amount of non-colligative freezing point depression. Inhibition of recrystallization and ice growth by IBPs suggest potential applications of these proteins in preventing frost damage to plants and in cryo-preservation of food and organs. We have used fluorescence microscopy in order to detect the binding planes of different IBPs to ice surfaces and we have compared the interactions of hyperactive and moderately active IBPs with ice crystals. We observed that the hyperactive IBPs have affinity for the basal plane, unlike the moderately active IBPs. This unique trait of the hyperactive IBPs results in several distinctive characteristics. We have also developed microfluidic devices in which the solution around small ice crystals can be exchanged in a temperature-controlled environment. The experiments we performed with the novel microfluidic devices clearly showed that ice crystals were highly stabilized by bound hyperactive IBPs even if the IBP concentration was reduced. Additionally, we used fluorescently tagged IBPs to demonstrate that IBPs stay on ice crystals even after the protein concentration in the solution is reduced. Thus, we found that thermal hysteresis is determined by the surface density of pre-bound IBPs and that these do not equilibrate with IBPs in solution. Furthermore, we observed that surface-adsorbed IBPs also prevent ice from melting, resulting in superheated ice crystals. These results strongly suggest that protein adsorption to the ice surface is irreversible. The techniques we have developed to investigate IBPs such as fluorescently tagged IBPs combined with microfluidics can improve the understanding of how IBPs influence ice growth. We suggest that hyperactive IBPs hold great promise for cryobiology and an understanding of their function is essential for their effective usage.

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O 3 - CRYOCONSERVATION IN STEM CELL RESEARCH: AN UNDERESTIMATED AREA IN TRANSLATIONAL MEDICINE

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Stem cell research is a rapidly progressive field of research connected with high expectations in the area of regenerative medicine to treat diseases by cell replacements in multiple disorders such as Morbus Parkinson and Diabetes mellitus. Next to the omnipotent embryonic stem cells (ESC) lately several groups reprogrammed somatic cells into induced pluripotent cells (iPS) overcoming the immune system's rejection against allogeneic cell types. However, the medical vision of overcoming bottlenecks of transplantable material by differentiated stem cells – somatic, embryonic or reprogrammed – is highly dependent on excellent methods for cryopreservation of cells and tissues. In this context, very different tissue and cell types are cryopreserved for different purposes: Mouse embryonic feeder cells are irradiated and frozen to provide a unique cytokine cocktail to the ESC for long term cultivation; ESC itself should maintain their pluripotency during cryopreservation and differentiated cell types have to survive in a functionally sound manner. Our group focuses on culture, differentiation and cryobiological characteristics of ESC from a small new world monkey, the common marmoset (*Callithrix jacchus*, cjESC) which is readily used in biomedical research and genetically close to humans. In pilot experiments we found a strong effect of different cryopreservative techniques on MEFs influencing greatly the growth and pluripotency of ESCs. Gamma-irradiated MEFs (30 Gy), frozen in 20 mM proline + 2.5% DMSO ESCs maintained the most effective feeder layer for 18 days. However, cryopreservation of cjESC proved to be most effective by vitrification, a method with disastrous survival rates for MEFs. In summary, there will be no general cryopreservation protocol for pluripotent ESCs and its derivatives for regenerative medicine. Thus, it will be important to establish individual cryopreservation protocols for each individual cell type.

O 4 - STEM BASED CELL THERAPEUTIC IMPLANTS: PRODUCTION AND STORAGE

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Human mesenchymal stem cells (hMSC) are qualified for cell therapy. A cell line (hMSC-TERT) has been obtained by transfection of hMSC with a telomerase reverse transcriptase (TERT). The alginate-encapsulated stem cells are an implantable therapeutic cell system, which possess the potential to counteract endocrine deficiencies in vivo. An automatable production process, aiming GMP conformation, includes three steps: (1) the expansion of hMSC-TERT (2), the encapsulation of the harvested cells and (3) the cultivation and possible differentiation of the implants.

Here, we introduce the utilization of fixed bed reactors for step one and two. The filling of the reactor consists of either non-porous glass carriers, which offer a suitable growth surface or the encapsulated cells systems. The non-porosity supports a gentle cell detachment and separation of the cells from the carrier to allow the cells being kept in high vitality. Cultivation and differentiation of the encapsulated cells were carried out in fixed bed bioreactors based on commercial syringes. These disposable plastic syringes were shown to be qualified as small scale single-use fixed bed reactors for the cultivation and differentiation of the encapsulated cells. No disadvantages for the vitality could be observed.

Further development for the storage of these implants by cryopreservation has just started and will be introduced. The implants are cultivated in the syringe and the whole unit should be cryopreserved to then, after thawing, serve as an implantation tool for the cell systems.

O 5 - CRYOPRESERVATION OF SEEDED BONE TISSUE REPLACEMENT MATERIAL

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Introduction: A cryopreservation protocol for soft engineered tissue constructs (mucosa) was successfully developed. This protocol describes the formulation of the cryoprotectant, the temperature profiles for freezing and re-warming and the technical setup used for managing the thermal transport during the profiles.

Materials and methods: These experiences were applied to cryopreserve a hard engineered tissue. Disks from Cerasorb M® (15 mm * 5 mm) were seeded with osteoblasts and cultured up to 35 days. A special fluorescence dye for cellular membranes was used to test the vitality. The ingrowth of the cells into the material was checked by histological methods.

For cryopreservation these disks were placed in special dishes, developed for the cryopreservation of tissues. The cryoprotant was added and after some minutes these dishes were positioned in a special equipment, "rack". This rack has to manage the heat transfer between the environment (freezer) and the tissue during freezing and thawing. The cryoprotocol for the mucosa was successfully applied to this hard tissue. After thawing the constructs were cultured for additional 14 days in order to revise the viability by observation and staining again.

Results: The hard tissue model is in good constitution also after cryopreservation. Histological specimen also showed the cells inside of the disk in a good condition.

Conclusion: The self-developed cryoprotocol works quite well for different tissues. Thereby, the essential feature is the combination of the adapted cryoprotectant, the temperature profile and the thermal managing tools.

The success of the protocol is based by the following:

- The closed rack containing the dishes can be stored at low temperatures as a whole.
- The tissue within the dishes is thawed by a special hot steam re-warming as a closed cycle procedure.

This work was supported by the German Federal Ministry of Economics and Technology.

O 6 - COMPARATIVE EVALUATION OF PROPERTIES OF HUMAN PLACENTA EXTRACTS STORED AT -20°C AND EXTRACTS OBTAINED FROM STORED AT -20°C TISSUES

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Major barrier for clinical usage of human placenta extracts is a short time period between obtaining this material and using it. Low temperatures applied in order to preserve unique properties of placenta extracts. It is necessary to study composition of extracts from placental tissue stored at low temperatures and simultaneously evaluate biological activity of such extracts. Erythrocytes are often used for evaluation of biological activity of various substances at cellular level.

Analysis have revealed that properties of placenta extracts stored at -20°C significantly differ from the properties of extracts, obtained from native tissue, and also differ from the extracts obtained from stored at -20°C placenta tissue.

Storing of extracts at -20°C leads to increasing of relative content of high-molecular proteins, which may be explained by protein aggregation in extracts. Storing of tissues at -20°C leads to increasing in extracts of relative content of proteins with molecular weight of ~80kDa. Increase of malone dialdehyde content is observed in extracts already after a month of storing at -20°C, but not observed in extracts obtained from stored at -20°C tissues.

Freezing and storing of extracts at low temperatures leads to reduction of hemolytic activity if the native extracts have possessed such activity when exposed with erythrocytes. However, long-term storing of extracts at -20°C causes increase of hemolytic activity, which is probably connected with accumulation of toxic POL products that may cause hemolysis. Freezing and storing of tissues also reduces hemolytic activity of extracts, but such property remains unchanged for at least a year of tissue storing.

Also differences between stored at -20°C extracts and extracts obtained from stored at -20°C are observed in biological activity of extracts, resulting in modifications of osmotic and low-pH resistance of erythrocytes exposed with such extracts and in modifications of cytosol microviscosity of erythrocytes.

O 7 - TYPE-DEPENDENT EFFECT OF SACCHARIDE ON THE STABILITY OF FREEZE-DRIED XANTHINE OXIDASE IN DRIED GLASSY MATRICES

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Enzymes are commonly stabilized by freeze-drying in the presence of protectants. Sucrose and trehalose are particularly effective in this regard. Many researches including our previous results 1) showed that despite the lower glass transition temperature (T_g), sucrose is

considerably more effective on protection of enzymes than trehalose. However, no extensive work has been done into the type dependence of saccharide for stabilizing effects. One of differences between sucrose and trehalose is their component monosaccharides. Sucrose is formed by α -glucose and fructose. On the other hand, trehalose consists of two α -glucose units. This causes the differences in structural and chemical properties which may lead to their different stabilizing effects. Therefore, in this study the stability of xanthine oxidase (XOD) freeze-dried in various formulations: sucrose, trehalose, trehalose-glucose, trehalose-fructose, trehalose-glucose-fructose, and glucose-fructose, were compared. The enzyme remaining activity was determined by a UV-VIS spectrophotometer. The Tg of the samples was also examined using differential scanning calorimetry. Trehalose formulation showed the higher Tg relative to that of sucrose. Addition of monosaccharide(s) lowered the Tg of trehalose due to plasticizing effect of the monosaccharide(s). The Tg of glucose-fructose was the lowest value. Sucrose showed the predominant stabilizing effect which 79 % of the enzyme activity was remained, while using trehalose maintained 56 % of the activity. The combination of trehalose and fructose showed the protective ability (78%) nearly as high as that of the sucrose. Approximately 67% of the activity was maintained in trehalose-glucose-fructose sample. In contrast, no improvement of the enzyme stability was observed in trehalose-glucose and glucose-fructose which the remaining activities were identical to that of the trehalose sample.

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O 8 - EFFECT OF METHANOL ON EXPRESSION OF MITOCHONDRIAL DNA REPLICATION FACTORS AND MITOCHONDRIAL DNA REPLICATION IN ZEBRAFISH EMBRYOS

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Cryopreservation of zebrafish blastomeres can damage the mitochondrial genome, which encodes proteins essential for ATP production. Replication of mitochondrial DNA (mtDNA) requires the translocation of nuclear-encoded transcription and replication factors to the mitochondria, a primer generated through transcription by mitochondrial transcription factor A (TFAM) and the mitochondrial-specific polymerase gamma, consisting of catalytic (PolGA) and accessory (PolGB) subunits.

Our studies on zebrafish embryos suggested that the impact on mtDNA was mainly due to the cryoprotectant treatment rather than the temperature decrease. Cryoprotectants have also decreased mtDNA copy number in zebrafish ovarian follicles. We therefore hypothesised that methanol treatment of zebrafish embryos would decrease mtDNA copy number and that mtDNA replication factors may be upregulated to compensate.

Embryos were exposed to 1M-4M methanol for 30 minutes at room temperature and were analysed at 30 mins, 3 hours and 24 hours after removal of methanol. DNA was extracted from and mtDNA copy number was assessed using real time PCR. RNA was also extracted and reverse transcribed into cDNA for real time PCR analysis of mtDNA replication factors.

In control embryos, mtDNA copy number increased during the 24 hour time period whilst those embryos treated with methanol first exhibited a slight decrease in mtDNA copy

number before increasing their mtDNA copy number between 3 and 24 hours after treatment. MtDNA replication therefore appears to be temporarily disrupted. Indeed, at the first two time points, treated embryos appear to contain fewer mtDNA replication factors than control embryos, whilst this is reversed at 24 hours. Although not conclusive alone, these data indicate that mtDNA replication factors may be upregulated to compensate for insufficient/damaged mtDNA molecules. Work is now ongoing to verify these findings and further investigate the impact of cryoprotectants on mitochondria of zebrafish embryos.

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O 10 - CRYOPRESERVATION OF RARE BLOOD IN THE NETHERLANDS: FROM LOW TO HIGH GLYCEROL

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Cryopreservation is a valuable approach for managing an inventory of rare RBC units to provide compatible blood for patients which are negative for blood group antigens with a very high frequency in the population (so called public antigens). In the Netherlands, a stockpiling of rare RBC units is available in the Sanquin Bank for Frozen Blood. Glycerol is the most commonly used RBC cryoprotectant. Depending on the final concentration of glycerol, the RBC unit can be stored at -80°C (40% (w/v) glycerol, high-glycerol method) or below -150°C (19% (w/v) glycerol, low-glycerol method).

The use of thawed RBC units is hampered by a 24-h outdated period due to potential bacterial contamination when a functionally open system is used for addition and removal of the glycerol. A recently developed automated cell processing system, Haemonetics ACP215, uses a functionally closed system to automatically glycerolize and deglycerolize RBC units with 40% (w/v) glycerol. This allows prolonged post-thaw storage for RBCs. We investigated the in vitro quality of thawed, deglycerolized RBC units during storage at 2-6°C in different additive solutions.

O 11 - EFFECTS OF CRYOCONSERVATION ON Γ -IRRADIATED MOUSE EMBRYONIC FEEDER CELLS

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Long term cultivation of pluripotent, induced pluripotent or embryonic stem cells (ESCs) shared one keyplayer - mouse embryonic feeder cells (MEFs). Despite their inevitable use there is very little known about the interaction of stem cells and MEFs. Although a few groups describe long term feeder-free cultures, the majority of researchers still utilize MEF-based culture systems. Lately, our group demonstrated differences in growth behavior of ESCs between Mitomycin C treated and γ -irradiated MEFs. Next to inactivation, cryopreservation greatly influences the quality of MEFs. We speculate that many inconsistent

results from different ESC researchers are caused by quality differences of feeder cell preparations.

This study evaluates different freezing protocols with various cryoprotective agents (CPAs) and routine freezing velocities (100-0.5K/min). As “quality assurance” the following biological parameters were measured after thawing: metabolic activity assessment (MTT assay), survival and re-attachment rates (flow cytometry), semi-quantitative expression analysis of genes essential for cell-cell- and cell-matrix-attachment (RT-PCR) and long term survival analysis.

Fourier transform infrared spectroscopy analysis suggested that γ -irradiation of MEFs does not cause major alterations of the membrane phase properties suggesting that irradiation damages are solely related to DNA and protein alterations. For practical usage we designed a “bench-top” quality control check to ensure consistent feeder cell grade: A 30 gray irradiated NMRI MEF-preparation which is 1) Mycoplasma free (PCR), 2) frozen with 10% DMSO and 1K/min should have the quality of 3) 90% cell survival (87.5% re-attachment) 4) expression of *FN-1*, *Itgb-1*, *Col4a4* $\geq 50\%$ and of *Col-1* $\geq 25\%$ one day after thawing vs. *RPS29*, 5) a metabolic activity of ≥ 1.6 -fold compared to untreated MEFs and 6) will provide for ≥ 16 days an intact cell layer for ESC culture.

Utilizing this “bench-top” assessment with each feeder cell preparation will help to reduce experimental scatter and contradictory results in ESC culture/differentiation.

O 12 - “WHETHER UNIVERSAL VITRIFICATION SOLUTION EXISTS?”

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Vitrification is a promising approach to cryopreservation. This talk will describe grounds of common vitrification solution (40vol% ethylene glycol and 0.6M sucrose) that found to be effective for cryopreservation of variety of cells and tissue. The talk will summarise the author’s experience in vitrification of complex systems involving hepatocytes applied for metabolic support during liver failure (1,2) and tissue engineered constructs involving mesenchymal stem cells (3,4) as well as human oocytes and neuronal stem cells (5,6). Key to success in design of vitrification protocol will be outlined. Several other recognized vitrification solutions will be reviewed.

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O 14 - EFFECT OF DIFFERENT CRYOPRESERVATION TECHNIQUES ON THE QUALITY OF BOVINE EMBRYOS

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Over the last couple of years large improvements have been made in the in-vitro production of bovine embryos. However, there are still remarkable differences in the morphology, the expression patterns of developmentally important genes and the freezeability of bovine in vivo and in vitro derived embryos (Wrenzycki et al., 2005; Lonergan et al., 2006).

The aim of our study was to determine the effect of two different cryopreservation methods on the quality of in vitro produced bovine embryos at the molecular level using a sensitive RT-qPCR assay.

Bovine blastocysts were produced using abattoir ovaries and a standard protocol for in vitro production (Wrenzycki et al., 2001; SOF plus BSAaa). They were randomly either vitrified (PBS plus ethylene glycol and DMSO; VitriStore, Fa. Gynemed) or cryopreserved using a programmable freezer (1.5 M ethylene glycol; Freeze Control; Fa. Minitüb). After thawing, embryos from both groups were cultured for 48 hours. After 24 hours of culture re-expansion rates and after 48 hours hatching rates were documented. After hatching, blastocysts were stored at -80°C for subsequent RT-qPCR analysis. The following gene transcripts were analyzed, HSP70, GLUT-1, GLUT-3, E-CAD, ZO-1, DNMT3a, IFN τ , DCII.

Re-expansion rates were 74.7 % (68/91) and 75.0% (87/116) for vitrified and conventionally cryopreserved blastocysts, and 57.1% (52/91) and 55.2% (64/116) re-expanded embryos hatched. The relative abundances of HSP70, GLUT-1 and ZO-1 transcripts were significantly affected in both groups compared to the control group (hatched blastocysts without cryopreservation). Conventional cryopreservation had a significant effect on the amount of GLUT-3, DNMT3a and IFN τ mRNA, whereas vitrification significantly affected DCII transcripts. E-CAD mRNA expression was similar in all groups of embryos.

These results suggest that not only the cryopreservation process itself but also the method used had a significant influence on the mRNA expression of developmentally important genes in hatched bovine blastocysts.

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O 15 - TOWARDS THE CRYOPRESERVATION OF DROSOPHILA MELANOGASTER LARVAE

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Drosophila melanogaster is very powerful genetic model organism, with over 85,000 unique strains. High-throughput genetic screens are producing new strains at an ever increasing rate and the Stock centres are in crisis. At 25 degrees cultures take 11 days from egg to adult. Maintaining cultures at 18 degrees allows them to be changed once a month. At lower temperatures the life-cycle slows even more but, fungal and bacterial infections become a problem and weak stocks do not survive many passages.

Protocols for cryopreserving eggs were published nearly 20 years ago, but are not suitable for high-throughput applications. Permeabilising eggs to allow entry of cryoprotectants reduces their viability and requires collection of precisely staged material. In practice none of the fly stock centres use these embryonic cryopreservation protocols.

Fly larvae represent an attractive alternative to embryos, not only are larvae more resistant to cellular damage, but they can be fed cryoprotectants directly. In addition, larvae are easy to handle in liquid suspension in 0.25 ml straws. These properties would be a critical advantage in developing high-throughput, automated protocols.

This talk will present data on the freezing dynamics of larvae using DSC and cryomicroscopy, together with viability studies of larvae following controlled cooling rates using a sterling engine freezer.

O 16 - EFFECTS OF A PROLONGED EQUILIBRATION TIME ON CRYOPRESERVED BOVINE SPERM FROZEN WITH EGG-YOLK BASED OR EGG-YOLK FREE EXTENDER

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Bull semen has been cryopreserved for more than half a century for artificial insemination (AI) industry and nowadays it is widely being used all over the world. Therefore, AI industry is strongly interested in improving the quality of frozen sperm. The aim of the study was to determine the suitability of a long time equilibration (24 h) versus 6 h of equilibration at 4°C in two different commercial extenders Bioxcell® (egg-yolk free) and Triladyl® (egg-yolk based). From each of 22 Simmental bulls four ejaculates were collected by using artificial vagina and divided into two equal volumes, which were diluted to a final concentration of 60x10⁶ sperm/ml, using the two extenders as specified above. Extended semen was equilibrated for 6 h and 24 h at 4 °C and frozen in 0.25 ml straws. Sperm samples were analyzed by flow cytometry immediately after thawing at 37°C for 30 s. Plasmamembrane integrity (PMI) and acrosomal damage (AD) were analyzed by using the FITC-PNA/PI assay. DNA integrity (percentage of sperm showing a high DNA fragmentation: DFI%) was quantified by using the sperm chromatin structure assay (SCSA[®]).

The 24 h equilibration showed an positive effects ($p < 0.001$) on the PMI of sperm cells with intact acrosom compared to that cooled for only 6 h in both extenders. The DNA integrity was not affected by holding the semen at 4°C for 24 h. After 24 h, DFI values of sperm samples frozen with Bioxcell showed a less variation compared to those incubated for 6 h with Bioxcell or Triladyl (6 h and 24 h). We concluded that holding the semen for 24 h at 4 °C before cryopreservation results in a higher sperm survival using both egg-yolk based and egg-yolk free extenders.

O 17 - INTERACTION OF DIMETHYL SULFOXIDE AND ETHYLENE GLYCOL WITH CELLS DURING FREEZING

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Dimethyl sulfoxide (Me2SO) and ethylene glycol (EG) are widely used for the cryopreservation of cellular systems. This study delivers more insights in the effects of these cryoprotective agents (CPA) which were studied here by Fourier transform infrared spectroscopy (FTIR) and cryomicroscopy.

Different concentrations of Me2SO or EG ranging from 0 to 2 M in culture media were prepared with and without human pulmonary endothelial cells (HPMEC). FTIR spectra were obtained from 15 mL samples while they were cooled from 4 to -45°C at 1 K/min [1]. Molecular vibrational bands of lipids (~2850 cm⁻¹) and of water (~2300 cm⁻¹) were further analyzed. Cryomicroscopy measurements were performed with comparative cooling protocols.

It was found that Me2SO decreases the wavenumber position of the lipid symmetric CH₂-stretching band arising from the lipid acyl chains. This effect of Me2SO shows a minimum at around 1 M. The effects of Me2SO on the conformational disorder of cellular membranes are temperature dependent and are greater at subzero temperatures. A correlation between low membrane conformational disorder and high cell viability was found. FTIR spectra of the lipid-band with EG revealed lower wavenumbers compared to the Me2SO results. A possible explanation is that EG has a higher number of polar groups compared to Me2SO. The performed cryomicroscopic investigations confirm these findings.

We thank Andrea Deiwick und Claudia Marx for their outstanding technical support. This project was financially supported by the Cluster of Excellence REBIRTH (DFG).

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O 19 - MODULATION OF MEMBRANE STABILITY BY LEA PROTEINS FROM THE HIGHER PLANT ARABIDOPSIS THALIANA

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LEA (late embryogenesis abundant) proteins have first been described almost 30 years ago as accumulating late in plant seed development. They were later found in vegetative plant tissues following environmental stress and also in desiccation tolerant bacteria and invertebrates. Although they are widely assumed to play crucial roles in cellular dehydration tolerance, their physiological and biochemical functions are largely unknown. To come to a full understanding of the role of LEA proteins in desiccation and freezing tolerance, we have initiated a genome-wide characterization of all LEA proteins and their encoding genes in the fully sequenced model plant species *Arabidopsis thaliana*. We have identified 51 LEA protein encoding genes in the *Arabidopsis* genome that could be classified into nine distinct groups based on amino acid sequence similarity (Hundertmark and Hincha, 2008). Expression studies were performed on all 51 genes at different developmental stages, in different plant organs and under different stress and hormone treatments using quantitative RT-PCR. We found evidence of expression for all genes. Expression levels were generally highest in seeds and in vegetative tissues many genes were induced by cold or drought. The majority of the encoded LEA proteins were predicted to be highly hydrophilic and natively unstructured, but some were predicted to be folded. To gain detailed structural and functional information about the *Arabidopsis* LEA proteins, we cloned several LEA genes from *Arabidopsis* and expressed them in yeast to study their potential function in cellular desiccation tolerance and in *E. coli* for recombinant protein production. The recombinant proteins are now used to study their secondary structure in the fully hydrated state and possible structural transitions during dehydration. In addition, we are performing detailed investigations into the effects of selected LEA proteins on the stability and structure of model lipid membranes under freezing and desiccation stress. Examples will be shown, illustrating the wide structural and functional flexibility of these enigmatic proteins.

Hundertmark and Hincha (2008) *BMC Genomics* 9,118

O 20 - CHARACTERIZATION OF WATER MOBILITY AND ICE CRYSTALLISATION IN PLANT TISSUE DURING FREEZING

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To improve the freezing process applied to vegetable and fruit, a better understanding of degradation mechanisms, strongly linked to the texture of the tissue during freezing is needed. Several studies described global texture modifications after freezing on vegetable and fruit (Marti and Aguilera, 1991; Khan and Vincent, 1996; Delgado and Rubiolo, 2005), but none applied multi-scales qualitative and quantitative approach from macroscopic to microscopic level. In this work, different methodologies have been applied to the tissue as mechanical (rheometer), spectral (NMR), thermal (DSC), histological, cytological and chemical measurements, as well as the visualisation of micro-structural modification. As a complementary tool to quantify mechanisms, a mathematical model for the prediction of the water evolution during freezing of vegetable tissue has also been established.

O 21 - VITAMINS C AND E IMPROVE *IN VITRO* RECOVERY OF CRYOPRESERVED BLACKBERRY SHOOT TIPS.

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Oxidative processes involved in cryopreservation protocols may be responsible for the reduced viability of tissues after liquid nitrogen (LN) exposure. We hypothesized that recovery would be improved by antioxidants that counteract these reactions. We studied four critical steps of the PVS2 vitrification cryopreservation technique; pretreatment, loading, rinsing, and regrowth to determine the amount of oxidative lipid injury and the effects of vitamins on shoot regrowth. Two blackberry cultivars with moderate (40-60%) regrowth after standard PVS2 vitrification were tested. Shoot tips treated with vitamin E (tocopherol) and assayed at each step showed greatly increased α - and γ -tocopherols compared to those without added Vit E or for control *in vitro* or field grown plants. Vit E at 11-15 mM added at each of the 4 steps increased regrowth after cryopreservation by 20-30%. Lipid peroxidation measured by the production of malondialdehyde (MDA) was 2 to 4 times higher at each of the first three steps than in fresh untreated shoot tips. Shoot tips treated with Vit E had low MDA, similar to the controls, at each step and improved shoot regrowth after cryopreservation (80%) compared to the controls (50%). Vitamin C (ascorbic acid, Vit C) (0.14-0.58 mM) when added at each of the steps also significantly improved regrowth of cryopreserved shoot tips (90%) compared to the controls (50%). Vit E (11 mM) and Vit C (0.14 mM) combined produced significantly higher regrowth than the untreated control or Vit E alone, but Vit C alone was not significantly different from the combination. These studies determined that lipid peroxidation is involved in the death of plants during the cryopreservation process. This is the first application of antioxidant vitamins for improving cryopreservation of plant tissues. We recommend adding Vit C (0.28 mM) to the pretreatment medium, the loading solution or the rinse solution.

O 22 - CHANGES IN CRYOTOLERANCE AFTER OVEREXPRESSION OF PR10A IN SOLANUM TUBEROSUM CV. DESIREE

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During cryopreservation intracellular ice formation exposes cells to severe osmotic stress by intracellular removal of water. An avoidance of ice crystal formation can only be achieved by artificial dehydration of cells leading to the vitrification of the protoplast which also results in osmotic and possibly salt stress. Previous efforts to investigate the influence of osmotic

tolerance mechanisms on cryotolerance using cell cultures of different potato cultivars showed that cell size may superimpose the effect of anti stress mechanisms.

Recently A. El Banna could show that the overexpression of the PR10a protein in potato cell cultures leads to increased osmotic and salt tolerance. The PR10a protein (formerly STH-2) is known to be induced by biotic stress in potato. Also under abiotic stress conditions like salt and osmotic challenge El-Banna et al. could show that the pathogenesis related protein PR10a was one of the predominant differentially expressed protein spots. In addition overexpression of PR10a led to significantly different stress responses concerning proline accumulation and relative content of oxidized glutathione.

To investigate whether overexpression of PR10 also results in a higher level of cryotolerance cryopreservation of wild type and transgenic cell cultures of *Solanum tuberosum* cv. Desiree were cryopreserved by controlled rate freezing using a minitest system previously worked out at DSMZ. Cells were subjected to different sorbitol concentrations, then incubated in cryoprotector and finally cooled down with a programmed cooling rate of -0,25°C/min to -40°C before exposure to liquid nitrogen. Recovery after thawing was compared after 4 weeks of regrowth. The transgenic cells showed faster regrowth after cryopreservation compared to the wild type. In addition for the transgenic culture equal amounts of surviving cells were determined over the whole range of sorbitol concentrations whereas the wild type showed an optimum of surviving cells at a specific sorbitol concentration range.

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O23 - THE USE OF CRYOPRESERVATION AS A TOOL TO CONSERVE PROTISTAN BIODIVERSITY

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Protists are debatably the most genotypically diverse group of eukaryotic micro-organisms and are found in most ecological niches world-wide. The long-term ex-situ conservation of these organisms has historically involved routine serial transfer; however, since the mid 1970's a growing number of the major microbial Biological Resource Centres (BRCs) have initiated programmes to cryopreserve some, or all, of their holdings. This approach has been particularly successful in the conservation of the prokaryotic cyanobacteria as well as small, simple, unicellular green algae; however, significant challenges remain with many more phenotypically complex taxa.

Through the pioneering work of John Morris and co-workers in the 1970's and early 80's, as well as more recent campaigns CCAP has, to date, cryopreserved over 30% of its holdings, with success in organisms as diverse as freshwater pico-planktonic cyanobacteria, marine flagellate algae and relatively large ciliates. As a result of this experience the CCAP has been active in cryopreservation technology transfer, helping BRC's in Asia, N. America and mainland Europe initiate/ develop their programmes of cryopreservation. This paper will discuss the challenges of technology transfer, the requirement for cross consortium validation of methodologies and standardisation of techniques. The paper will also focus on the need for closer collaboration between the major collections, particularly in the context of the use of

cryopreservation to conserve type material for taxonomic studies and patent depositions under the Budapest Treaty.

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O 24 - CRYOPRESERVATION OF *PINUS NIGRA* AND HYBRID *ABIES* EMBRYOGENIC TISSUES

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Somatic embryogenesis has a great potential for large-scale vegetative propagation of conifer trees. The maintenance of embryogenic tissues is time consuming process. Moreover, this culture method holds risks of contamination and loss of regeneration capacity. Cryopreservation seems to be a suitable and efficient mean for long-term storage of these tissues. The slow freezing cryopreservation method has successfully been applied to many conifer species. Regrowth of embryogenic tissues after cryogenic storage depends on several factors such as pre-treatments, cryoprotective solutions, physiological state of tissues and genetic background. The research based on molecular markers did not reveal changes in genetic fidelity of cryopreserved tissues.

Embryogenic tissues of *Pinus nigra* and *Abies* hybrids have been cryopreserved through slow-freezing. Embryogenic tissues of *Pinus nigra* (altogether 46 cell lines) were cryopreserved and their ability to grow after thawing has been followed. The re-growth of tissues was cell line dependent. Out of 46 cell lines tested, 33 survived short term cryopreservation and their post-thaw growth was not negatively influenced by length of storage in liquid nitrogen. Long-term cryopreservation (20 cell lines were stored for 1 year in liquid nitrogen) resulted in 70% survival.

Embryogenic tissues of *Abies* hybrids (4 cell lines) have been successfully cryopreserved with 100% re-growth and 37 % to 100 % regeneration capacities for individual cell lines.

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O 25 - UNDERSTANDING THE CRYOSURVIVAL OF COLD-HARDENED, WINTER APPLE BUDS – CRITICAL WATER CONTENT AND THE ROLE OF NON-DIFFERENTIATED SECONDARY PRIMORDIA

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Dormant bud cryopreservation is important for genetic conservation as it has the benefits of simplicity and avoids expensive *in vitro* culture yet, for temperate fruit crops e.g. apples and pears, survival can be highly variable within, and between, accessions, cultivars and seasons.

To investigate events during the first part of the cryopreservation protocol¹ (a prolonged dehydration step) winter-collected explants of three apple cultivars were stored for 1-2 weeks at -4°C, allowing water content to reduce from c.45 to c.30% of fresh weight, widely reported as suitable level for subsequent preservation. Using exotherm analysis as a diagnostic tool it was clear that between cultivars significant differences existed in the freezing pattern of bulk water in the dehydrated bud. These related consistently to post-cryopreservation survival. The acclimation history of the explants may be of significance in this regard as bulk water in apple buds is known to increase during the endodormant period [2]. A comparable examination of buds recovered from liquid nitrogen showed similar differences, suggesting that a high, freezable water content at this stage was linked to low survival

Additionally, published survival data typically presents bud outgrowth in some way but ignores the contribution of secondary buds. This study demonstrated that where the primary bud meristem does not survive cryopreservation then secondary bud development and meristem outgrowth can contribute significantly (up to 50%) to overall survival.

1] Toldam-Andersen TB, Nygaard TB and Krogholm KS (2007) *Adv Hort Sci* **21**, 193-197

2] Faust M, Liu D, Line MJ and Stutte GW (1995) *Acta Horticulturæ* **395**, 113-118

O 26 - CRYOPRESERVATION OF COCOA SOMATIC EMBRYOS BY VITRIFICATION

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The need to conserve biodiversity of cocoa has become paramount due to the action of a range of diseases, pests and environmental hazards. In order to develop a vitrification-based cryopreservation protocol for cocoa, floral-derived secondary somatic embryos (SSE) were utilised. To optimise the procedure the effect of preculturing on sucrose and SSE dehydration with plant vitrification solution 2 (PVS2) was studied. SSEs were precultured on embryo development (ED) medium supplemented with either 0.5 or 0.75 M sucrose for 3 or 5 days. After keeping the embryos in loading solution for 20 min they were treated in PVS2 for 45-105 min before they were stored in liquid nitrogen for at least 60 min. The embryos were rapidly rewarmed in a water bath at 42 °C. The PVS2 was removed and SSEs rinsed for 20 min with deloading solution (1.2 M sucrose in ED). Preculturing the embryos on 0.5 M sucrose for 5d and dehydrating them in PVS2 for 60 min led to significantly higher post-cryo survival than any other treatment (74.5±6.4 %). So as to minimise cryo-injury due to cation-induced free radicals formation, nutrient cation sources were removed (cation-free) from the deloading solution and/or the recovery medium (ED) for 3 d, the former treatment resulting in a significant benefit. The protocol was effective across all six genotypes so far tested.

To accelerate bulking up of clones, embryos regenerated following cryopreservation were used as explant sources for the generation of tertiary somatic embryos and the freezing process was not found to have any inhibitory effect on their embryogenic potential. A scanning electron microscopy study showed that, while primary somatic embryos arose from

callus, their descendent SSEs generally originated from cotyledonary epidermal tissue, thereby limiting any additional risk of somaclonal variation that might have been associated with the use of these propagules.

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P 2 - STRUCTURE-DYNAMICAL PECULIARITIES OF MAMMALIAN ERYTHROCYTES AT ARTIFICIAL HYPOBIOSIS AND HIBERNATION

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Interest of cryobiologists to the phenomenon of hibernation is determined by ability of hibernating mammals to survive through subzero body temperatures and adapt to abrupt and severe thermal and metabolic shifts. However, hibernation is genetically fixed adaptation strategy. Usually, the objects of practical cryomedicine and cryoveterinary are organisms, not possessing such adaptive features. Therefore it is interesting to study a model of artificial hypobiosis by Andjus-Bachmetjev-Giaya model (ABG) that is gradual cooling of the organism in a closed vessel. As a result of hypothermia, hypoxia, and hypercapnia an animal develops hypobiosis state – lowering of body temperature down to 16-17°C.

A comparative studying of erythrocyte reactions of homoeothermic (rat) and heterothermic (hamster) mammals on the state of artificial hypobiosis is of our interest.

Dynamic state of cytosol was evaluated by temperature dependences of microviscosity parameter in the range of 37-0°C, using spin probe TEMPON and broadening agent – potassium ferricyanide.

Lowering of body temperature is accompanied with decrease of cytosol microviscosity by 16±2% for hamsters and by 25±2% for rats, smoothing of temperature dependences, and appearing of echinocytes. State of the animals was similar to control already after 2 hours after the impact. However cellular reactions were different. ‘Winter’ and ‘autumn’ hamsters have possessed further lowering of microviscosity (by 28±2% 24 hours after ABG), smoothed temperature dependences, in the way that after 24 hours after ABG dynamic state of cytosol was close to typical for hibernation state. For rats and ‘summer’ hamsters it was close to control.

We suppose that revealed peculiarities are explained by modification of aqueous-protein interactions in cytosol, probably by means of changes in the adjusting of cytoskeleton, because the changes are observed in modifications at 15 and 8°C.

Artificial hypobiosis leads to domination of echinocytes of various types up to 24 hours after the impact.

P 3 - APPLICATION OF SPECIFIC LIPOSOMES FOR PRESERVATION OF RED BLOOD CELLS ALTERS THE CELL MEMBRANE COMPOSITION

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There is an urgent need for the development of red blood cell (RBC) biopreservation techniques that maintain in vitro RBC viability and function. The earliest and most widely investigated approach to RBC biopreservation is hypothermic storage, which allows storage of blood for up to 42 days at 4°C. Cold storage of RBCs leads to a cascade of damaging events including phase changes and phase separation of lipids, lipid peroxidation, and haemolysis. Several studies have shown that small unilamellar liposomes (100-200 nm) composed of specific natural phospholipids stabilize RBCs during hypothermic storage, freezing, and freeze-drying. FTIR-Spectroscopy was used here to study the interaction between liposomes and RBCs. A variety of liposomes composed of different types of saturated and unsaturated lipids (dioleoyl phosphatidylcholine (DOPC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC)) have been incubated with RBCs (4 hours at 37°C) to investigate the effect of liposomes on RBC membranes and vice versa that of RBCs on liposomal membranes. After incubation, the mixture of liposomes and RBCs was separated using Ficoll density gradient separation. FTIR was used to study membrane phase behavior of both the liposome and RBC fraction. Lipid phase behavior was studied by following the temperature dependence of the lipid symmetric CH₂ stretching band. Liposomes composed of unsaturated lipids exhibited signs of lipid transfer, whereas saturated lipids did not. The T_m of DOPC liposomes shifted to higher temperatures after incubation ($\Delta T \approx 10^\circ\text{C}$). The T_m of DMPC increased by about 5°C after incubation, whereas the T_m of DPPC liposomes was not affected after incubation with RBCs. FTIR analysis on ghosts prepared from liposome treated RBCs revealed that liposomes composed of unsaturated lipids have a fluidizing effect on RBC membranes. Taken together, the data indicate transfer of lipid components between RBCs and liposomes.

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P 4 - MODIFYING EFFECT OF CRYOPRESERVATION ON ANTI-TUMOUR ACTIVITY OF FLCs

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Expediency of applying fetal liver cells (FLCs) at oncopathology is stipulated by biologically active substances' content. Cryopreservation as obligatory component of general technology of biomaterial clinical application may be used as way to modify bioobject structural and functional status.

Research aim is comparative evaluation of therapeutic potential of cryopreserved FLCs of different dose in breast cancer (BC) experimental model in C3H mice.

Six months' aged experimental animals were injected with cryopreserved and native FLCs in dose of 1 and 5×10^6 cells per mouse. CBA mice were control. Study of immunological indices, cancer stem cells (CSCs) content in mammary gland (MG), assessment of tumour development frequency were performed in 16 months. Murine fetuses'

FLCs of 14 gestation days were cryopreserved by two-step program under 10% DMSO. Before and after FLCs cryopreservation we assessed FLCs' morphological composition, determined content of hemopoietic stem cells (HSCs) CD34⁺CD38⁻, mesenchymal cells CD44⁺CD73⁺.

When estimating CSCs content in murine MG, in untreated mice even without tumour there were noted CSCs of CD44^{high}, content of other CSCs populations: CD44⁺CD24⁻ and CD133⁺ was significantly higher vs control.

FLCs application reduced CSCs content in MG in all groups of treated animals, but maximum approximating of this index to control values was achieved with cryopreserved and native FLCs in 5x10⁶ and 1x10⁶ doses, correspondingly. The same doses provided in a greater extent decrease in frequency of BC development, stipulated by suppressing effect of immune system cells on CSCs proliferative activity.

Different character of dose-dependent effect manifestation by native and cryopreserved FLCs may be stipulated by modifying effect of cryopreservation on FLCs biological characteristics: decrease in mesenchymal cells CD44⁺CD73⁺ content under HSCs CD34⁺CD38⁻ number increase.

Thus, research demonstrated possibility to apply FLCs for preventive oncopathology treatment. Cryopreservation modified a dose-dependent effect of FLCs, providing higher therapeutic potential to FLCs in higher dose.

P 5 - RESPONSE PECULIARITIES OF EHRlich ADENOCARCINOMA CANCER STEM CELLS TO CRYOEFFECT

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When studying the response peculiarities of malignantly transformed cells to cryopreservation effect, of importance is a change in repertoire of their surface structures, being informative markers, reflecting efficiency of tumour process cryoirradiation. Only the certain part of cells of many tumours hyper-expresses CD44 and is identified as CD44^{hi} cancer stem cells (CSCs). Stemness sings are inherent to their prosperity of different differentiation rates with less density of CD44 marker on membrane in combination with CD24 molecule. Under experimental conditions the convenient model to study cryoeffect in tumour cell subpopulation, including CSCs, is ascitic form of Ehrlich adenocarcinoma (EAC), representing inoculated cell line of mice breast cancer.

Research aim is to evaluate structural and functional indices of CSCs of different differentiation rates in development dynamics of EAC and character of their changes after cryopreservation.

Research object were EAC cells. Cells of 7th day culture of EAC were intraperitoneally introduced to 7 months' old BALB/C female mice and again were obtained to 7th and 14th days (EAC-7 and EAC-14). EAC-7, EAC-14 were cryopreserved in cryoprotectant-free ascitic fluid according to two-step protocol. CD44⁺CD24⁻ and CD44^{high} content, cell viability, cell duplication rate and ratio of their cell absolute number rise in peritoneal cavity were assessed.

There were estimated structural and functional EAC characteristics of 4 cell types: native and cryopreserved EAC-7 and EAC-14.

Material cultured *in vivo* was evaluated to 7th, 14th and 21st days.

Phenotyping EAC cells demonstrated, that CSCs of various differentiation rates differently expressing CD44, changed differently phenotype in response to cryopreservation. Herewith character of these changes depended on EAC culturing term before cryopreservation. Differences of changes in functional status of precursors were manifested in various production rates of CSCs subpopulations. Findings emphasise the value of cryoapplication terms in treating malignant neoplasms.

INFLUENCE OF FREEZING-THAWING ON ANTIOXIDANT ACTIVITY OF WATER-SALINE HUMAN PLACENTA EXTRACTS

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Low temperatures are used in order to preserve unique properties of aqueous-saline human placenta extracts. Antioxidant activity is one of the properties due to which placenta extracts are effectively applied in clinical practice. Study of the influence of low temperatures on antioxidant properties of extracts and their realization at interaction with erythrocytes in condition of oxidative stress are very up to date.

Obtained data had revealed that antioxidant activity, measured using FRAP method is present in native extracts and various fractions, obtained with gel-chromatography method with maximums in fractions with molecular mass in 110 kDa area and less than 5 kDa area. Kinetics of reduction of cytochrome C by native extracts possesses 2-phase character and is probably conditioned by two active centers: one with rapid rate of reduction and the other with slow rate. The first center is identified in fractions with molecular weight of ~ 200kDa. Ability to decompose hydrogen peroxide is mainly located in fraction with 200kDa area. FRAP activity mostly remains after freezing. Lowering of this activity occurs by means of lowering in high-molecular fractions. Ability to reduce cytochrome C lowers depending on the freezing rate. Hydrogen peroxide scavenging activity insignificantly lowers after low temperature application.

Prior exposition of erythrocytes with extracts allows lowering damaging effect of oxidative stress, caused by hypothermic storage of erythrocytes with presence of galactose. Effectiveness of extracts frozen with 300°/min rate is higher then effectiveness of extracts frozen with 1-2°/min rate, and is approximately the same as of the native extracts.

Thus freezing-thawing allows preserving studied extract properties. Lowering of antioxidant activity depends on the freezing rate and occurs due to activity of centers with high molecular weight. Preliminary exposition with extracts allows lowering activity of oxidative stress caused by galactose. Effectiveness of frozen extracts depends on the freezing rate.

P 8 - NANOLUMINOPHORES OF EUROPIUM AND LANTANUM OXIDES AS LONG-TERM MARKERS OF CRYOPRESERVED CELLS

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The research aim is the creation of new nanosized labeling technique for cryopreserved cell cultures at storage stages and prior to the examination or clinical application. Human fibroblast cells and mesenchymal stromal stem cells (MSCs) were stained with luminescent nanoparticles of europium and lanthanum oxide.

The addition of solutions of nanoparticles to the cells in confluent suspension did not change their characteristics. Nanoparticles of Eu_2O_3 and La_2O_3 localized in cell cytoplasm, did not penetrate into nucleus and did not remain on cell membrane.

The experiments on studying the interaction of Eu_2O_3 and La_2O_3 nanoparticles with the cell culture during long-term culturing have been carried-out. The presence of Eu_2O_3 and La_2O_3 nanoparticles in cells within the term of monolayer achieving did not affect proliferation. It has been established that luminescence of Eu_2O_3 and La_2O_3 nanoparticles preserved in cells for 4-5 duplications (observation time). Herewith their localization does not change and luminescence intensity alters insignificantly towards lessening. However SEIRA data were specified on possible conformation changes in DNA and proteins of cells.

The experiments *in vivo* were performed in Balb/C white mice. MSCs suspension was labeled with nanoparticles Eu_2O_3 . The labeled MSCs were injected into murine tail vein in concentration of $1 \cdot 10^6$ cells. The studies were performed at terms of 5 days. There was revealed the presence of luminescent cells in bone marrow and spleen, the concentration made 2.0 ± 0.5 and 3.0 ± 0.5 respectively. The findings testify to the fact that Eu_2O_3 and La_2O_3 nanoparticles have the application potential as labels when operating with bioobjects.

P 9 - MEMBRANE PHASE BEHAVIOR DURING FREEZING OF STALLION SPERM

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Cryopreserved stallion sperm displays a high degree of individual variation with respect to survival after thawing. The mechanisms underlying these differences in cryostability among individuals have yet to be elucidated. We have used Fourier transform infrared spectroscopy (FTIR) to study membrane phase behavior during freezing of sperm from individual stallions, in order to determine if individual differences in cryostability could be related to differences in membrane phase behavior during freezing. Membrane phase behavior was studied by following the temperature dependence of the symmetric CH_2 stretching mode arising from the lipid acyl chains. Stallion sperm cell pellets were cooled at $1^\circ\text{C}/\text{min}$ while the ice nucleation temperature was varied between -2 and -12°C . When ice nucleation was induced at -2°C , membranes displayed a highly cooperative fluid to gel lipid phase transition, which was not observed with nucleation below -10°C . Nucleation at -2°C results in a relatively low residual conformational disorder (less fluidity) of the membranes in the frozen state compared to samples that nucleated below -10°C . The results implicate that high subzero nucleation (seeding at -2°C), which favors cellular dehydration, creates gel phase lipid packing. Lower subzero nucleation (seeding at -10°C), favoring intracellular ice formation, prevents lipid dehydration and leaves the membrane lipids in a relatively fluid state. The effect of the nucleation temperature on the initial rate of membrane dehydration upon ice

formation displays Arrhenius behavior. Activation energies that can be derived from the Arrhenius plots showed differences amongst different stallions. Studies are ongoing to correlate differences in sperm viability after thawing for different stallions with sperm membrane phase behavior.

Source of funding: the Excellence Cluster “REBIRTH” of the Leibniz University of Hannover.

P 10 - EFFECTS OF DIFFERENT EXTENDERS AND CRYOPRESERVATION/THAWING PROCESS ON SUBCELLULAR CHANGES IN BOVINE SPERM DETERMINED BY FLOW CYTOMETRY

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The present study examined the effects of two extenders and computer controlled cryopreservation on subcellular changes in bovine sperm by using flow cytometric assays. Five ejaculates from each of six Holstein bulls were extended to a final concentration of 80×10^6 sperm/ml using two extenders (AndroMed® or Triladyl®). Immediately after the extension and after freezing/thawing, each ejaculate was examined by using flow cytometric assays. Sperm were stained with propidium iodide (PI) to determine subcellular alterations in plasma membrane intact sperm (PMI); DiOC6(3) assay was used to measure the percentage of viable sperm with a low mitochondrial membrane potential (DiOC) and YoPro-1 assay to determine the percentage of moribund sperm (YoPro). Calcium levels (Ca²⁺) and the synthesis of reactive oxygen species (ROS) were determined semiquantitatively by measuring the mean fluorescence intensity of Fluo-4 (Ca²⁺), DCFH and DHR (ROS) in viable sperm. Statistical analyses were carried out using two-way ANOVA. The process of cryopreservation/thawing ($p < 0.05$), but not ($p > 0.05$) the extender type showed effects on PMI values. The parameters DiOC, YoPro, Ca²⁺ and DCFH depended on both factors, extender as well as cryopreservation/thawing ($p < 0.05$). Neither cryopreservation nor the extender type affected DHR levels. The results show that the extension as well as the cryopreservation/thawing induce various subcellular alterations.

P 11 - CRYOPRESERVATION OF HUMAN PULMONARY ENDOTHELIAL CELLS: DETERMINATION OF OSMOTIC PARAMETERS

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During cryopreservation the mass transport of water across the plasma membrane determines the osmotic response of cells. In this study the osmotic response of human pulmonary endothelial cells (HPMEC) has been investigated using a Coulter counter and a cryomicroscope.

For the Coulter counter measurements solutions of NaCl-water or PBS solutions of 100 to 1350 mOsm/kg were prepared. Cell suspensions were mixed with solutions of different osmolalities and cell volume distributions were measured. Boyle-Van't-Hoff plots were

calculated to determine the osmotically inactive cell volumes (V_b). A cryomicroscope was used to investigate the osmotic response of spherical, adhered HPMEC cells at 3 different temperatures 0, 10 and 30°C. After varying the extracellular osmolality from isotonic to 927 mOsm/kg the cell volumes were determined with time and the membrane hydraulic permeabilities (L_p) and the activation energy (E_{Lp}) were calculated.

The Boyle-Van 't-Hoff plots indicated that the cells behave as linear osmometers between 225 and 1350 mOsm/kg. The osmotically inactive volumes, V_b were determined to be $0.26 \times V_o$ in NaCl and $0.31 \times V_o$ in PBS.

The cryomicroscopy studies revealed L_p values of 0.51, 0.43 and 0.06 mm atm⁻¹ min⁻¹ at 30, 20 and 0°C, respectively and E_{Lp} of 22.7 kcal/mol. These parameters can be used to predict the osmotic response and improve cryopreservation protocols of HPMEC cells [1].

We thank Andrea Deiwick und Claudia Marx for their outstanding technical support. This project was financially supported by the Cluster of Excellence REBIRTH (DFG).

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P 12 - INFLUENCE OF LOW-MOLECULAR (BELOW 5 KD) FRACTION FROM CORD BLOOD AND ACTOVEGIN ON PHAGOCYtic ACTIVITY OF DEPRESERVED NEUTROPHILS IN VITRO

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Dependence of phagocytic activity of freshly isolated neutrophils from donor blood on the contents of cattle cord blood low-molecular fraction and actovegin in the incubation media was studied in the work. The concentration diapasons analyzed for cord blood fraction (CBF) and actovegin were 0.15-3.00 mg/ml. The maximal stimulation effects for CBF and actovegin were observed at the concentrations 0.15 mg/ml and 1.5 mg/ml in the incubation media, respectively.

Further we investigated influence of CBF and actovegin on phagocytic activity donor neutrophils subjected to low-temperature preservation (-196oC) with 7.5% DMSO. It was shown that incubation of depreserved neutrophils in rehabilitating medium containing 0.15 mg/ml of CBF substantially activated phagocytic reaction of neutrophils judging by phagocytic number and index of phagocytosis completeness. Actovegin had a similar stimulation effect at the concentration in the rehabilitating medium, which was 10 times as much (1.5 mg/ml) as the concentration of CBF.

NBT test reflecting oxygen-dependent metabolism demonstrated that CBF and actovegin under the above-mentioned conditions increased the percentage of activated neutrophils containing diformazan.

To ascertain a cause of the inequality in CBF and actovegin biological activities we carried out gel-penetrating chromatography on a column filled with polyvinyl gel Toyopeas 1HW-40 Fine. Comparison of the chromatographic profiles of the preparations investigated allowed to assume that this inequality could be due to substantial qualitative difference in them.

Thus, the results obtained allowed to draw the conclusion about advisability of inclusion of CBF and actovegin at appropriate concentrations into rehabilitating media for depreserved neutrophils.

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P 13 - APPLICATION OF COMPATIBLE SOLUTE WITHIN CROYPRESERVATION OF HUMAN ENDOTHELIAL CELLS

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Purposes: A high survival rate of cryopreserved cells requires an optimal cooling/thawing rate and the presence of sufficiently cryoprotective agent (CPA). The most widely used CPAs, dimethyl sulfoxide (DMSO) and glycerol, however are toxic at high concentrations [1]. Therefore, it is of great interest to develop new CPAs. For this purpose we investigated the compatible solutes proline and hydroxyectoine as potential CPAs, since they are effective stabilizers of native proteins against heating, freezing and drying based on the principle of "preferential exclusion" [2].

Materials and Methods: Human pulmonary microvascular endothelial cells (HPMEC-ST1.6R) are used for experiments. Different concentrations of proline (5mM to 100mM) and hydroxyectoine (10mM to 200mM) are being studied in combination with DMSO (0 to 10% v/v). Cells are frozen either directly with freezing medium (FM) containing proline or hydroxyectoine with a 10 minutes equilibration period or after incubation for 48 hours in a proline or hydroxyectoine containing culture medium (CM). Cells are frozen in standard cryovials with $6,65 \cdot 10^5$ cells/cryovial (1,5 ml).

Results: Without proline cell survival rate reaches up to 90% with 10% DMSO. A cell survival rate of 95% was achieved with 20mM proline and 2,5% DMSO in FM. With 5mM proline in CM the cell survival rates could be improved up to 96% with 2,5% DMSO and 85% even with only 1% DMSO. Combinations of hydroxyectoine (10 to 100mM) and 1% DMSO, the amount of viable cells increased compared to cells frozen with only 1% DMSO.

Conclusions: Our results show that proline can be used as additional CPA if a short equilibration time of 10min before freezing is applied. With an extended preincubation time the concentration of DMSO can be further reduced. The investigation of hydroxyectoine as additional CPA shows similarly promising results and will be continued with combinations of other compatible solutes.

Source of funding: The project was supported by the Cluster of Excellence "REBIRTH" (DFG).

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P 14 - CRYODAMAGE AS THE FIRST STAGE IN CREATION OF BIOLOGICAL VASCULAR PROSTHESES

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In this research the effect of low temperatures on morphological structure and biochemical properties of porcine arterial vessels with the aim of creation of acellular xenogeneous vascular scaffolds was studied.

Common carotid arteries and intrathoracic arteries were derived from mature pigs with meeting all the requirements of bioethics. Prepared vessels were subjected to low temperatures by plunging into liquid nitrogen. Biomechanical properties of frozen-thawed samples were estimated by examining the mechanical strength of vessels and static and longitudinal tensile strength as well as measuring the burst pressure. Morphological structure of vessels was estimated using optic microscopy with silver impregnation of intercellular boundaries of endothelium, staining with hematoxylin-eosin and picrofucsin according to Van Gieson.

After freeze-thawing the porcine vessels of small diameter (≤ 6 mm) preserve their mechanical properties, herewith their partial decellularization is observed. Cooling of blood vessels down to -196°C may be one of the stages for creation of acellular vascular scaffolds.

P 15 - CRYOMICROSCOPY AND FTIR STUDIES ON GAMMA TREATED MOUSE EMBRYONIC FIBROBLAST FEEDER CELLS DURING FREEZING

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Mouse embryonic fibroblast feeder cells (MEFs) are used as support layers to culture embryonic stem cells (ESCs). The quality of the MEFs influences long term growth and proliferation of undifferentiated ESCs. Two procedures affect MEF quality before use: (1) Inhibition from proliferation by γ -irradiation and (2) cryopreservation. In this study, cryomicroscopy and Fourier transform infrared spectroscopy (FTIR) were used to investigate why survival rates after cryopreservation of MEFs are so highly variable. The effect of different cryoprotective agents (CPAs) including Me₂SO, glycerol and proline was also tested. Slow cooling protocols resulted in cellular dehydration. In the presence of CPAs, dehydration occurred more gradual. In the absence of CPAs, rapid freezing resulted in intracellular ice formation (IIF), which occurred almost directly after extracellular ice formation. In the presence of CPAs, IIF was not observed directly after extracellular ice formation but was delayed. FTIR studies were done to study membrane phase behavior of cell pellets of gamma treated (30 Gray at 2 Gray/min) and non-irradiated control cells. Membrane phase behavior of γ -treated and control cells was studied by following the temperature dependence of the lipid symmetric CH₂ stretching band during freezing. Membranes displayed a highly cooperative fluid to gel lipid phase transition upon ice nucleation at -2°C , which was not observed with stochastic nucleation at -12°C . The freezing induced fluid to gel

phase transition with nucleation at -2°C directly correlates with the cellular dehydration that was observed by cryomicroscopy under similar freezing conditions. Gamma treatment was found to have little effects on the membrane phase behavior of the cells, suggesting that gamma irradiation does not cause major alterations in membrane phase properties of the cells. Source of funding: Cluster of Excellence "REBIRTH" (DFG).

P 16 - EFFECTS OF DIFFERENT THAWING METHODS ON SPERM QUALITY OF CRYOPRESERVED BOVINE SPERM

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The aim of the study was to examine the effects of two different thawing methods on quality of cryopreserved bovine sperm. The cryopreserved sperm samples were either thawed in a water bath with a temperature of 37°C for 30 s (low temperature: LT) or by using a water bath with a temperature of 70°C for 5 s (high temperature: HT). From each of 22 Simmental bulls four ejaculates were collected and diluted to a final concentration of 60×10^6 sperm/ml in an egg yolk based extender and cryopreserved in 0.25 ml french straws. Plasmamembrane integrity (PMI), acrosomal damage (AD) and inducibility of acrosome reaction (AR) were determined by using the FITC-PNA/PI assay. DNA integrity (percentage of sperm showing a high DNA fragmentation: DFI%) was quantified by using the sperm chromatin structure assay (SCSA™). Measurements were performed immediately after thawing (0h) and after 3 hours (3h) incubation at 37°C . PMI 0h and PMI 3h in sperm samples that with HT were higher ($P < 0.001$) than LT (PMI 0h: 50.8 ± 9.9 vs. 46.9 ± 10.1 and PMI 3h: 45.3 ± 9.9 vs. 41.2 ± 9.7 , respectively). AD 0h and AD 3h at HT were less ($P < 0.001$) than LT (AD 0h: 17.8 ± 5.7 vs. 20.3 ± 5.8 and AD 3h: 29.4 ± 5.5 vs. 32.9 ± 9.7 , respectively). The levels of AR did not differ ($p > 0.05$) between LT and HT (54.5 ± 5.7 vs 55.1 ± 7.8). DFI% values at HT were lower ($p < 0.0001$) than LT (DFI% 0h = $1.8 \pm 0.3\%$ vs $2.6 \pm 0.3\%$ and DFI% 3h = $3.2 \pm 0.4\%$ vs $4.9 \pm 0.3\%$, respectively). In conclusion thawing sperm cells for a short time in a water bath with high temperature leads to an improved sperm quality compared to thawing them for a longer time in a water bath with low temperature.

P 17 - DIMETHYL SULFOXIDE DIFFUSION IN TISSUE-ENGINEERED COLLAGEN SCAFFOLDS VISUALIZED BY COMPUTER TOMOGRAPHY

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While significant progress has been made in cryopreservation of cells, cryopreservation of tissues is still a major problem. Only a few tissues and even fewer organs can be cryopreserved. The diffusion of cryoprotective agents (CPA) into tissue is one of the major hurdles for succesful cryopreservation. In 3-dimensional constructs like biological tissues and cell seeded tissue-engineered scaffolds CPAs like dimethyl sulfoxide (Me2SO) should be

homogeneously distributed. Furthermore, the CPA concentration needs to be sufficiently high enough to cryopreserve cells in the tissue. A local excess of CPA in the construct will damage the cells due to the general toxic effects of CPAs, whereas insufficient CPA concentrations will lead to cryopreservation damage. This study was conducted to measure and visualize the effective diffusion of Me2SO within tissue-engineered collagen scaffolds using computer tomography. Measurements were done by a CT [1] manufactured by Bio-Imaging Research, Inc. (BIR, Lincolnshire, IL).

Collagen scaffolds were manufactured by the power-down method with an average pore size of 100µm (dimension: 30x30x10 mm³) and a porosity of 98% [2,3]. The scaffolds were stored in phosphate buffered saline (PBS). Scaffolds were transferred directly in 10% (v/v) Me2SO in PBS. Computer tomographic images were acquired immediately every 1.5 minutes over a period of 3 hours. Grey scale values that were determined from the images were converted in Hounsfield units (HU). The conversion from HU to DMSO concentration indicates CPA dispersion within the tissue. The Me2SO loading process of the scaffold could thus be measured and visualized in real time. The calibration was done by solutions with Me2SO concentration in PBS between 0 to 10% (v/v). The study showed that incubation times of more than 3h are required to achieve homogenous CPA distribution in collagen scaffolds of this size.

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P 18 - ALTERNATIVE CRYOPROTECTIVE AGENTS FOR THE CRYOPRESERVATION OF HMSC-TERT

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In cell therapy, stem cells gain more and more important. In the last few years, the number of patients treated with stem cells increased so that a solution for cell storage has to be found. For this reason, cells can be conserved at -80°C or -196°C via cryopreservation without losing vitality. During cryopreservation, cells are exposed to osmotic forces and/or dehydration. The addition of cryoprotective agents should prevent the cell from damage and maintain cell vitality. Dimethylsulfoxide is often used as a cryoprotective agent. Some cryoprotective agents, such as dimethylsulfoxide has cytotoxic effects and should therefore be avoided for cryopreservation in clinical use. The aim of this project is to develop a method for cryopreservation of human mesenchymal stem cells with non-cytotoxic biocompatible cryoprotective agents. Therefore glycerine, ectoine and proline were tested as alternatives to dimethylsulfoxide in combination with serum-free media.

P 19 - THE EFFECTS OF CRYOPRESERVATION ON RED BLOOD CELL RHEOLOGICAL PROPERTIES

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Cryopreservation enables RBC storage for longer periods. The rheological properties (aggregability and deformability) of cryopreserved and deglycerolized RBCs remain to be elucidated. Transfusion of rheological impaired RBCs may obstruct the blood flow leading to reduced tissue perfusion in micro-vessels and ischemia.

This study aimed to compare the rheological properties of conventional liquid stored RBCs with cryopreserved RBCs.

Methods: Ten leukoreduced RBC units were anticoagulated with citrate phosphate dextrose. and cold-stored at 2-6°C, according to standard blood bank procedures. Samples were taken from the RBC units for analysis after 21 and 35 days of storage.

Then other leukoreduced RBC units were prepared and cryopreserved with 40% glycerol and frozen at -80 ± 10 °C in a mechanical freezer for 3 years.

RBC aggregability and deformability were monitored in vitro by a laser-assisted optical rotational cell analyzer. Furthermore osmotic fragility index and ATP were determined.

Results: Aggregation was significantly reduced in cryopreserved RBCs compared to liquid stored RBCs ($p < 0.01$).

Deformability was similar in both groups, but the osmotic fragility index, was significantly enhanced during cryopreservation

The ATP values of liquid stored and cryopreserved RBCs reduced significantly with 54.4% and 11.1% respectively. The ATP values of liquid stored RBCs came below the accepted limit of 2.7 $\mu\text{mol/gHb}$.

Conclusion: Our results show that in high glycerol frozen RBC the aggregability was markedly reduced, but the deformability was unchanged. Cryopreserved RBC were more fragile after thawing, which is of concern regarding shear induced damage. ATP values remained close to fresh RBCs, whereas liquid storage reduced ATP.

P 1 - GLASS TRANSITION AND CRYSTALLIZATION OF WATER-PLASTICIZED AMORPHOUS SPRAY DRIED INULINS

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Introduction Sugar glasses are widely used as either cryo- or lyoprotectants for preserving biological molecules. To act successfully as a protectant, the sugars should have a high glass transition temperature (T_g), a low crystallization rate, a poor hygroscopicity, and contain no reducing groups. Inulins, a natural non-digestible fructan, coincide with these requirements. They have been widely used in biopharmaceutics, in addition, in the previous study inulins were confirmed to greatly succeed in stabilizing freeze-dried alkaline phosphatase, which the

activity remained near 100% [1]. Recently, an enzymatically produced inulin, namely Fuji FF, was developed [2]. The Fuji FF consists of fractions with the low degree of polymerization (DP), being less than Ca. 30, which leads to the higher water solubility comparing to the natural inulins. However, up until now the study on physical properties of inulins, which relates to ability as a stabilizer, has not been conducted. On the purpose to understand the stabilizing effect, this study conducted characterizing such properties i.e., water sorption, glass transition temperature, and crystallinity of the enzymatically produced inulin comparing to the natural inulins at different relative vapor pressure (RVP) .

Materials and methods Chicory inulin (Beneo HP; DP 10-60, Beneo ST; DP 3-60, Beneo-Orafti) and the inulin which is produced by using an enzymatic method (Fuji FF, DP 3-30, Fuji Nihon Seito Co., Ltd.) were used. Water sorption isotherm of all inulins were investigated by a conventional desiccator method and analyzed by the Guggenheim, Anderson, de Boer (GAB) model. Differential scanning calorimetry (DSC) was employed to measure the Tg of the inulins. Crystallization was observed from increasing peak intensities of X-ray diffraction (XRD) pattern and an exothermic peak obtained from the DSC.

Results and discussion The value for the monolayer moisture of Fuji FF powder was smaller than those of Beneo HP and Beneo ST, implying that the water sorption ability of Fuji FF powder is lower. From the DSC measurement, all types of inulins were found to be semi-crystalline materials which also showed the existence of a glass transition. The Tg of the inulins decreased with an increase of RVP due to the plasticizing effect of water. In addition, the rate of crystallization increased with increasing RVP and its value was relative with the Tg. Crystallinity of the inulins was low at the low RVP, while at RVP above 53% peak intensities of XRD pattern increased, suggesting that all inulins recrystallized. These were similar to the results obtained from the DSC.

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P 7 - QUALITY OF HUMAN OOCYTES, PROCURED WITHIN ART PROGRAM: STUDY WITH FLUORESCENT PROBES

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Morphofunctional state of oocyte is one of the factors, determining assisted reproductive technologies (ART) results and affecting its capability to fertilisation, formation of morphologically integral embryo, capable for implantation and perspective for cryopreservation. Therefore of great importance from both practical and ethical sides is to have the kit of statistically significant tests, enabling to determine oocyte state at the earliest stage, to forecast ART results and select embryos, valid for cryopreservation.

There were studied 135 unfertilised oocytes from 35 IVF patients (aged about 30-35).

To induce superovulation we used standard "long protocol". Oocyte insemination and embryo culturing were carried-out according to the standard protocols. Fertilisation was controlled in 18 hrs after insemination. Embryo transfer was carried-out to the 2nd-5th days. The investigation of viable human oocytes and embryos is ethically inadmissible, therefore

the normal murine oocytes served as a conditional control. JC-1, JC-9; CFSE, AO, PI, DAPI luminescent probes were used for analysis. Probe kit allowed to estimate changes in functional state of mitochondria, chromatin structure disorder, to reveal membrane permeability and to determine cell esterase activity state.

Probes were added into the medium in 10^{-5} M final concentration and stained by standard technique. Luminescence was estimated with scanning confocal microscope (Carl Zeiss).

All studied oocytes had disordered morphology, characteristic for apoptosis: from minor changes to manifested extent. Abnormal quality of pronuclei (3-5) was in approximately 30% oocytes. In all investigated oocytes we found out an esterase activity, testifying to viability of studied objects. When studying the state of oocyte energetic system with JC-1, JC-9 probes, the level of mitochondrial activity was shown as more or less lower than the control one. This testified to changes in mitochondria and the presence of initial apoptotic stages. AO red staining in 46.2% cells, studied with this dye, indicated to the presence of single- and two-stranded DNA ruptures. Membrane damage, determined by PI, was revealed in 37% cases. DAPI dye showed late apoptotic stages in 65% oocytes. Morphological signs, as well as luminescent microscopic data indicate to the fact, that an early apoptotic stage was observed approximately in fifth part of studied oocytes, average and manifested ones were in 40% studied objects.

The results obtained correlate to the known data about fact that with increase in female age the number of apoptotic oocytes augments. The state of unfertilised oocytes and fertilisation outcome of sister oocytes, used in IVF, were comparatively analysed. The percentage of successful/failed pregnancies was 55:45%. The certain tendency, manifested in the fact, that oocytes, twin to successfully fertilised, were in less manifested apoptotic stages and more integral morphologically. We believe that the presence of manifested apoptotic changes in oocytes is unfavourable forecasting sign both for following fertilisation and survival during following cryopreservation. Nowadays to specify this preliminary conclusion there are performed the studies of characteristics of normal and apoptotic murine oocytes as applied to pregnancy onset in laboratory animals.

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