CryoLetters 42(6), 366 – 379 (2021)

ABSTRACTS

56th Meeting of the Society for Low Temperature Biology
7-9 October 2020 (online), in association with UCLouvain, Belgium

RECOVERY OF ERYTHROCYTE OXYGEN TRANSPORT FUNCTION AFTER HYPOTHERMIC STORAGE UNDER THE ACTION OF HUMAN CORD BLOOD LOW-MOLECULAR FRACTION AND THE ACTOVEGIN DRUG

O Gulevskyy[1], Yu Akhatova[1], Ye Zharkova[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine

The cord blood-based drugs are known to increase the energy of human donor blood cells (1). They can be used in transfusiology to restore the functions of erythrocytes after long-term storage or at pathological conditions associated with impaired oxygen transport function (2-3). In this research the effect of low-molecular fraction (below 10 kDa) derived from human cord blood (CBF) and Actovegin on the content of hemoglobin forms in erythrocytes stored in hypothermia was studied. Evaluation of the ratio of hemoglobin forms in erythrocytes stored in hypothermia for 7-21 days, showed that in the control there is a redistribution between the content of oxy-, deoxy- and methemoglobin towards increasing the proportion of the latter two. After incubation in a rehabilitation medium with CBF there was a significant increase in oxyhemoglobin (15-20%) and a parallel decrease in deoxy- and methemoglobin (18-20% and 25-60%, correspondingly). The resulted ratio of the indices was restored to the initial level, as evidenced by an increase in the haemoglobin oxygenation coefficient in an average by 1.5 times.

After using Actovegin as a rehabilitative medium, the same pattern was found. But it should be noted that its effect on the content of deoxyhemoglobin was more pronounced compared to the first series of experiments. Thus, on day 21 of storage, a probable decrease of this index by 30% was recorded. At the same time the content of oxyhemoglobin increased by 19%. At the final term, the hemoglobin oxygenation coefficient increased more than 70%, which exceeded even the native level.

Thus, by incubating erythrocytes stored in hypothermia in a medium with CBF or Actovegin was able to strongly increase the oxyhaemoglobin content. Both rehabilitation media affected similarly the ratio of haemoglobin forms. In general, the findings indicate the normalization of oxygen transport function of erythrocytes and increased blood oxygen capacity.

(1) Gulevsky AK et al. (2015) *Biotechnologia Acta* **6**, 63-70. **(2)** Henkelman S et al. (2015) *Vox Sang* **108,** 103-112. **(3)** Cavezzi A et al. (2020) *Clin Pract* **10**, 1271.

CRYOPRESERVATION OF TISSUE-ENGINEERED CONSTRUCTS IN CRYOBAGS USING COMMERCIALLY AVAILABLE FREEZING SOLUTIONS

O Gryshkov[1], V Mutsenko[1], B Glasmacher[1]

[1] Leibniz University Hannover, Institute for Multiphase Processes, An der Universität 1, 30823, Garbsen, Germany

Cryopreservation is gaining far more attention in ensuring on-demand availability of tissue-engineered constructs (TECs) for the transplantation and drug testing. In this regard, the recent advancements in the development of less toxic and animal serum-free cryopreservation solutions for cell suspensions (1,2) gave us the new impetus towards establishing similar biobanking strategies for TECs. In this attempt we have screened the selected set of commercially available freezing solutions on cell-seeded electrospun scaffolds as model TECs using custom-made cryobags as freezing containers.

SAOS-2 cells (1×10^5 cells) were seeded on UV-sterilised fibrous electrospun scaffolds (diameter 16 mm, thickness 100 µm) produced from a blend of polycaprolactone (PCL, 100mg/ml) and polylactide (PLA, 50mg/ml). On day 3, the cell-seeded scaffolds were loaded with the respective freezing solutions such as CELLBANKER®1 (S1), CELLBANKER®2 (serum-free) (S2), STEM-CELLBANKER®-GMP (S3) and STEMCELLBANKER®-GMP-DMSO-FREE (S4) (all from AMSBIO) on ice for 10min. Afterwards, the cell-seeded scaffolds were frozen in cryobags in a Planer Kryo 560 controlled-rate freezer at 1 K/min to -100°C. After storage at -150°C for at least 3 days, the cell-seeded scaffolds were thawed in a 37°C water bath and re-cultivated for 24h. The viability and metabolic activity of cell-seeded scaffolds before cryopreservation and post-thaw was analysed using live-dead (day 1 post-thaw) and Resazurin reduction (day 1, 3 and 7 post-thaw) assays, respectively.

The collective effect of freezing in cryobags utilizing indicated solutions revealed the following tendencies in cell viability decreasing in the order S1 (79±16%) > S2 (76±12%) > S3 (74±11%) > S4 (67±12%) compared to the non-frozen controls (97±1%). The similar trend was observed in metabolic activity of cells after cryopreservation according to the results of the Resazurin reduction assay. In summary, combination of commercially available freezing solutions developed for cell suspensions with cryobags represents a significant step forward to efficient biobanking of clinically relevant TECs.

(1) Mutsenko V et al. (2019) *Cryobiology* **91**, 104-114. **(2)** Lauterboeck L et al. (2016) *Biopreserv Biobank* **4,** 530-538.

INTEGRATED CRYOBIOTECHNOLOGY OF THE GENUS *Aesculus*.

D Ballesteros[1], P Butel[1], N Fanega-Sleziak[1], HW Pritchard[1]

[1]Royal Botanic Gardens Kew, Wakehurst Place, West Sussex, RH17 6TN, United Kingdom

Aesculus (horse chestnuts) comprises 23 species of northern hemisphere's deciduous trees and shrubs often cultivated as ornamental plants or pharmaceutical crops. However, some species (including the iconic *A. hippocastanum*) are threatened with extinction, and long-term *ex situ* conservation options are needed to secure its genetic resources (1). Species in this genus produce desiccation sensitive seeds, hindering their preservation in conventional seed banks (i.e., dry storage at -20°C). There has been some success in cryopreserving the embryonic axes (EA) in a few species (2). However, the efficacy of such an approach across the genus remains unknown. To enable the development of gene bank strategies globally, we investigated the *in vitro* growth of EA after partial ("flash") drying and exposure to LN in six *Aesculus* taxa (five species, one hybrid) across the phylogeny. *Aesculus* sp. EA lost water from the root and the shoot tip at a similar rate; however, shoot tips were more sensitive to drying than root tips.

This differential response to desiccation affected recovery after LN exposure, with species generally having more root survival. All species studied tolerated desiccation down to 0.30 $gH_2O \cdot g^{-1}DW$ (23 % FW). Below this limit, desiccation damage to the EA was evident in all species except in the hybrid, *Aesculus x arnoldiana*. Maximum plant growth after LN exposure was achieved after predrying to 0.25-0.35 $gH_2O \cdot g^{-1}DW$ for all taxa studied, with *A. californica* and *A. hippocastanum* showing the highest recovery rates (>70%) and *A. indica* the lowest (<10%). Furthermore, we investigated the cryobiotechnology of pollen from seven *Aesculus* taxa. All tolerated dehydration to <0.08 $gH_2O \cdot g^{-1}DW$ and LN exposure, with good levels of subsequent *in vitro* germination. These results suggest that, as for *Quercus* species (3), an integrated cryobiotechnological solution for the *ex situ* conservation of *Aesculus* genetic resources is available using EA and pollen storage.

Acknowledgements: Part-funding from the Garfield Weston Foundation. RBG Kew receives grant-in-aid from Defra, UK

(1) Rivers MC et al. (2019) *European Red List of Trees. Cambridge, UK and Brussels, Belgium: IUCN. 2019*. **(2)** Pence VC et al. (2020) *Biological Conservation* **250**, 108736. **(3)** Ballesteros et al. (2019) *Acta Hort* **1234**, 37-46.

OLIGOASTHENOTERATOZOOSPERMIA. HOW TO IMPROVE THE CRYOPRESERVATION OUTCOMES?

M Petrushko[1,2], V Piniaiev[1,2], T Yurchuk[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine
[2] Ukraine ART-clinic for human reproduction, Gagarina av. 38b, Kharkiv, Ukraine

Cryopreservation of spermatozoa from men with oligoasthenoteratozoospermia (OAT) leads to an increase level of lipid peroxidation (LPO) and DNA fragmentation rate which negatively affect the male gametes fertilization capacity (1, 2). The aim of the study was to assess the antioxidant system state of spermatozoa in men with OAT after cryopreservation, as well as fragmentation DNA rate and LPO level of cryopreserved OAT spermatozoa in the medium with glycerol and antioxidant superoxide dismutase (SOD) supplement. Motile sperm fraction from men with normozoospremia and OAT was isolated and divided into three portions: fresh, cryopreserved in medium with glycerol final concentration 5% and cryopreserved in this medium supplemented with 200IU/ml of SOD. The LPO intensity was assessed in spermatozoa by the level of malondialdehyde (MDA), as well as DNA fragmentation rate, SOD and antioxidant activity (AOA) levels. It was shown that the MDA, SOD levels and DNA fragmentation rate were significantly higher in fresh OAT spermatozoa compared with fresh normozoospermic cells. Increasing of SOD level was observed in OAT spermatozoa probably due to the compensatory response to the ROS increasing. After cryopreservation the MDA level increased and was the highest in OAT group while SOD and AOA levels were decreased compared with fresh normozoospermic and OAT spermatozoa. The addition of SOD to the cryopreservation medium led to decrease in the spermatozoa MDA level and DNA fragmentation rate by 1.6 and 1.3 times respectively and to increase AOA by 1.5 time. The data obtained make it possible to use an optimized cryopreservation medium for spermatozoa from OAT men in further research on improving the fertilization rate and embryo development in ART programs.

(1) Al Omrani B et al. (2018) *Reprod Biol Endocrinol* **16**, 49. **(2)** Petrushko MP et al. (2017) *Cytol Genet* **51**, 278-281.

EVIDENCE OF METABOLIC ACTIVITY DURING LONG-TERM TRANSPORT OF OVARIAN TISSUE

JMV Vilela [1], MCNM Blackman[2], P Sonveaux[2], Ana Luisa Miranda-Vilela[3], MM Dolmans[1,4], CA Amorim[1]

[1] Université Catholique de Louvain, Pôle de Recherche en Gynécologie, Institut de Recherche Expérimentale et Clinique, Avenue Mounier, 52 bte B1.52.02, 1200 Brussels, Belgium
[2] Université catholique de Louvain, Pôle de Pharmacologie et thérapeutique (FATH), Institut de Recherche Expérimentale et Clinique (IREC), Avenue Hippocrate 57 box B1.57.04, 1200 Brussels, Belgium.
[3] Independent researcher, Brasília, DF, Brazil
[4] Cliniques universitaires Saint Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium

Due to the increasingly strict European regulations, ovarian tissue cryopreservation is a strategy that a numbered hospitals and reproductive centers can offer to the patients. In order to increase patient access to this alternative, ovarian tissue transport can be proposed. Currently, ovarian tissue transport has been validated for up to 24 h, but little is known about the metabolic context of the ovaries during transport. The aim of this study was to understand how media affect cell metabolism during ovarian tissue transportation. Ovaries from 4 cows obtained from a local abattoir. Fragments were incubated in 20 mL of either PBS, IVF-medium or L-15 medium for 1 or 24 h at 4°C. Media was analyzed for glucose, lactate and pyruvate concentrations, and cell death rates were assessed by flow cytometry (FACS) using annexin V and propidium iodide. There was no consumption of glucose or pyruvate in IVF and L-15 after 24 hours of incubation ($p>0.05$), but there was a significant lactate release in all media ($p<0.05$). FACS showed a mean of 4% of necrotic cells in all media and 15-19% of apoptotic cells after 1 hour of incubation, but less than 1% of necrotic cells and 2-6% of apoptotic cells after 24 h in all media. Our results suggest that cells are using internal sources of energy for lactate release. Since they need their internal energy source for the post-grafting period before revascularization, these findings indicate that 1) such long period of transport may have an impact on follicle and stromal cell populations and possibly influence transplantation outcomes; 2) we might improve post-graft viability by taking into consideration tissue needs.

(1) Vilela, JMV et al. (2020) *JARG* **37**, 2477–2486. DOI: 10.1007/s10815-020-01935-y.

COMPARISON OF SUBNORMOTHERMIC AND HYPOTHERMIC STORAGE OF PLACENTAL CELLS, TISSUES, AND TISSUE ENGINEERED CONSTRUCTS

V Prokopiuk[1], A Tkachenko[2], O Prokopiuk[1], O Pogozhykh[1,3], R Safonov[2], V Lazurenko[2], C Figueiredo[3], D Pogozhykh[3].

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine
[2] Kharkiv National Medical University, 4 Nauky Avenue, 61022, Kharkiv, Ukraine
[3] Institute for Transfusion Medicine and Transplant Engineering, Hannover Medical School, Carl-Neuberg-Straße 1, 30625, Hannover, Germany

Tissues, cells, and tissue-engineered constructs are promising candidates for efficient application in regenerative medicine. Placenta is among the most prospective sources, capable of delivering a wide range of clinically relevant material, from tissues and mesenchymal stem cells to extracts, lysates and

conditioned medium (1). The challenges in transportation of placental derivatives between a clinic and a laboratory or biobank could be solved without the necessity of additional equipment and toxic reagents, by using subnormothermic (20°C) or hypothermic (4°C) storage (2).

The aim of the study was to compare the possibility of subnormothermic and hypothermic storage of a range of placental derivatives.

Placental tissue explants and cells were isolated from human term placenta; alginate beads were received using calcium solution; spheroids were received by a hanging drop method. Structure and metabolic activity of placental derivatives where studied in fresh samples and after hypothermic and subnormothermic storage for up to 4 days.

It was found, that hypothermic storage allows maintaining structure, viability and metabolic characteristics for all placental derivatives no longer than 24 hours. Subnormothermic storage is effective for explants, placental cells in suspension and in alginate microspheres for 48 hours, and for 24 hours for spheroids. Cell suspension was the most stable among the studied samples. Spheroids were the most sensitive to the storage; they quickly disintegrated into separated cells and lost metabolic activity.

In conclusion, subnormothermic storage appeared to be more effective for short-term storage of placental explants, cells in suspension, spheroids and cell encapsulated in alginate beads, in comparison to hypothermic.

(1) Pogozhykh O et al. (2018) *Biomaterials* **185**, 39-50. **(2)** Giwa S et al. (2017) *Nat Biotechnol* **35**, 530-42.

TIME DEPENDENT OSMOTIC DAMAGE IN SEA URCHIN OOCYTES

D Olver[1], P Heres[2], E Paredes[2], JD Benson[1]

[1] Department of Biology, University of Saskatchewan, Saskatoon, SK
[2] Department of Ecology and Marine Biology & Marine Biological Resources Functional Preservation Service at ECIMAT marine Station, University of Vigo, Vigo, Spain

Most cryobiological protocols require loading and unloading of cryoprotective agents (CPAs) to mitigate ice damage during the freezing and thawing process. However, CPAs change the osmolality of the solution creating an osmotic gradient across the cell membrane, causing large volumetric changes. Classically, mechanical damage due to swelling or shrinking have been thought to correspond to constant osmotic tolerance limits (e.g., 20% reduction in survival of the population at a given hyper and hyposmolality), which are crucial in determining optimized cryoprotocols. Here we show that osmotic damage is not dependent solely on volume deviance for sea urchin (*Paracentrotus lividus*) oocytes, but instead osmotic damage is time-dependent. We exposed urchin oocytes (n >= 100 per treatment with 3 replicates) to hypertonic treatments of 1500, 2000, and 2500 mOsm/kg made by adding either NaCl or sucrose to seawater, or hypotonic treatments of 800, 700, 600, and 500 mOsm/kg made by diluting seawater with DI water. The exposure duration periods were for 2, 6, 15, 30, 50, 75, and 90 minutes. After exposure, oocytes were returned to isosmotic holding media, *in vitro* fertilization was performed, and development to the 4-arm-Pluteus stage was assessed at 48 h. We fit these data to a mathematical model of population cell death that is proportional to the integration of the deviation of cell volume from isosmotic volume. This model works well to describe osmotic related damage across multiple concentrations and solution types (adjusted R2 values of 0.97, 0.96, and 0.76 for DI water, NaCl, and sucrose respectively). We then derive continuous and stepwise vitrification protocols and compare its predicted efficacy against standard osmotic tolerance limit models when determining optimal CPA equilibration protocols using cytotoxicity cost functions. This may result in more accurate models of cell damage, and more optimized protocols for loading and unloading of CPAs.

No conflict of interests declared.

Acknowledgements: Funding for this project was provided by NSERC RGPIN-2017-06346 (JB), a Mitacs Globalink award (JB & DO), the Gabriel Dumont Graduate Bursary (DO), and Assemble+, grant from the European Union's Horizon 2020 research and innovation programme (No. 730984) (EP).

APPLICATION OF A CELL-DENSE MULTIPHASIC MODEL TO CRYOPROTECTANT EQUILIBRATION

R Shuttleworth[1], R Warner[2], A Eroglu[3], AZ Higgins[2], JD Benson[1]

[1]University of Saskatchewan, Canada
[2]Oregon State University, USA
[3]Augusta University, USA

Cryoprotectant (CPA) equilibration in tissues remains challenging due to the risk of both mechanical strain and cryoprotectant toxicity damage. Many models have been developed to describe the loading of CPAs into either individual cells, or into a thin slab of tissue, however there have been few reports of the two models being combined. Here we propose a model that builds upon a triphasic model for articular cartilage introduced by Abazari et. al. in 2009, using a system of partial differential equations to describe the mass transport of each component, namely, water, CPA, salt, and the solid matrix. Within this system we incorporate the well-known two-parameter model to describe the cell membrane transport of both water and CPA within individual cells. Combining these two systems allows us to investigate the stress placed on the tissue by considering the interactions at both an extracellular and intracellular fluid level.

In addition, this general model allows us to specify properties of a tissue, ranging from its structure and composition, i.e., the spatially dependent percentage of tissue solids and cells or the hydraulic conductivity and CPA permeability of the embedded cells. By defining a bath solution containing a CPA concentration, our model is able to predict the CPA, salt, and water concentrations inside and outside of cells as a function of space and time in the tissues. This allows calculation of the spatial dependence of stress/strain in the tissue during CPA loading as well as the local CPA toxicity. We can adapt the percentage of cells in our tissue to investigate how cell density affects the overall stress/strain placed on a tissue during CPA loading. Ultimately, we will use our results to predict and test optimized loading protocols to reduce the overall strain and CPA toxicity during CPA loading in different tissues.

Acknowledgements: This work was supported by funding from the Canadian National Science and Engineering Research Council (RGPIN-2017-06346) and the US National Institutes of Health (5R01HD083930 and R01 EB027203).

CRYOPRESERVATION OF MOUSE RETINA AT -10°C USING DIFFERENT FREEZING MEDIA

S Bojic[1,2], R Bauer[3]

[1] School of Computing, Newcastle University, Newcastle upon Tyne, UK
[2] Department of Genetics, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[3] Department of Computer Science, University of Surrey, Guildford, UK

Retinal degenerative diseases are one of the main clinical causes of severe, progressive vision impairment. Due to limited availability of retinal tissue the ideal solution would be the formation of retinal cryobanks. Stored retinas could be used for research purposes, epidemiological studies, validation of newly developed drugs etc. Retinal cryopreservation is underresearched, with only two studies published1,2, both studying immature murine retinal tissue. We focused on mature mouse retina and investigated the impact of a slow-cooling to -10°C using different freezing media (FM). Retinas from adult C57BL/6 mice were preconditioned on ice using mouse artificial cerebrospinal fluid (maCSF) with addition of 10% DMSO for 4 hours with a constant supply of carbogen. After preconditioning, samples were transferred to different FM: (1) 90% retinal organ culture media (50% DMEM-GlutaMAX, 25% HBSS, 25% FBS, 5.75 g/l glucose, and 0.1M sucrose) with 10% DMSO (2)

90% FBS and 10% DMSO; (3) Cryostore; (4) Nutrifreeze; and (5) Bambanker. Samples were cooled down at 1°C per minute to -10°C using a VIA Freeze TM Research controlled rate freezer (Asymptote, UK) and kept cool for 24 hours. Afterwards, the samples were warmed to 37°C, washed and transferred for recovery to maCSF with a constant supply of carbogen for 24 hours at room temperature. After recovery, the tissue was fixed and processed for immunofluorescent and hematoxylin & eosin staining. Taking into consideration macroscopic and microscopic damage, we found that cooling with Nutrifreeze best preserved retinal layer organisation and tissue morphology, including all retinal cell types. Our findings indicate that murine retinas can be effectively stored at -10°C for at least 24 hours without significant impact on the tissue organization and cell morphologies using Nutrifreeze, or DMSO in combination with either FBS or retinal organ culture media. Further experiments will confirm functionality of these tissues.

(1) Aramant R and M. Seiler (1991) *Brain Res Dev Brain Res* **61**, 151-9. **(2)** Jensen S et al. (1987) *Cryobiology* **24**, 120-34.

CRYOPRESEVATION OF ALLOGENEIC HAEMATOPOIETIC PROGENITOR CELL (HPC) CONCENTRATES BEFORE TRANSPLANTATION – TEMPORARY OR PERSPECTIVE SOLUTION

P Měřička[1], J Gregor[1], M Jandová[1], M Lánská[2], D Vokurková[3], I Fátorová[2], P Paterová[4], J Radocha[2], L Štěrba[1]

[1] Tissue Bank, University Hospital Hradec Králové, Czech Republic
[2] IV.th Department of Internal Medicine Charles University Faculty of Medicine Hradec Králové, Czech Republic,
[3] Department of Clinical Immunology and Alergology, Charles University Faculty of Medicine Hradec Králové
[4] Department of Clinical Microbiology, Charles University Faculty of Medicine Hradec Králové, Czech Republic

While autologous HPC transplantations are tightly connected with cryopreservation of HPC concentrates use of cryopreservation techniques in allogeneic HPC transplantations has been limited to rare cases when coordination of HPC collection and preparation of the patient for transplantation failed. On the other hand, cryopreserved donor lymphocytes (DL) have been increasingly used in guided GvD after allogeneic HPC transplantation. This situation has changed this year as the EBMT recommended to freeze the collected allogeneic HPC concentrates to prevent COVID-19 transmission. The Tissue Establishment (EU TE CODE CZ000426) has performed cryopreservation of 8 allogeneic HPC concentrates. They were obtained from collection centres inside European Union (EU) in 7 cases (Czech Republic 3, Germany 3, Poland 1), and in one case from the centre outside EU (Turkey). Cryopreservation was started in 2 cases within 24 hours, in 6 cases within 34 hours after collection. A standard operating processing and cryopreservation procedure using clean room facility, 10% DMSO and controlled rate freezing with storage in vapour phase of liquid nitrogen was used. In all cases a part of collection was frozen separately for future DL infusion. On the next day control samples were thawed for blood count including detailed WBC differential, CD34+ and CD3+ counts and viability assessment. As the Transplantation Unit required to obtain the results of performed tests within 4 days after cryopreservation the standard sterility test was supplemented by quantitative bacteriology. All cryopreserved concentrates were found suitable for clinical application (median CD34+ dose/kg of patient's weight 5.93 x 10^6, mean post-thaw nucleated cell viability 89 %, no signs of bacterial contamination). The EBMT recommendation has been suspended recently and remained limited to imports from countries with severe epidemiological situation, nevertheless the gained experience represents an important tool for solving similar situations in future.

DEVELOPMENT OF A RELIABLE CRYOPRESERVATION PROTOCOL FOR THE COMMON COCKLE

P Heres[1],2, J Troncoso[1,2], E Paredes[1,2]

[1] Marine Biological Resources Functional Preservation Service, Estación de Ciencias Mariñas de Toralla, Universidade de Vigo, Illa de Toralla, 36331, Coruxo, Vigo, Spain
[2] Departamento de Ecología y Biología Animal, Facultad de Ciencias del Mar, Universidad de Vigo, Vigo, Spain

The common cockle (*Cerastoderma edule*) is an important commercial mollusk for several European countries. The diseases incurred by parasites and the disseminated neoplastic disorder have sharply affected the natural populations, with significant repercussions on the economy. The aim of this work was to develop a reliable cryopreservation protocol for larval stages to enhance the restock of wild populations and improve the hatchery production. First, toxicity tests were carried out on 48 and 72 h-old D-larvae to determine the suitable Cryoprotecting Agent (CPA) attending to harmful effects. Larvae were exposed to increasing concentrations of Ethylene-Glycol (EG), Propylene-Glycol (PG), Glycerol (GLY) and dimethyl-sulfoxide (Me_2SO). Considering toxicity results, two CPA combinations a) 10% EG + 0.4 M Trehalose (TRE) and b) 10% PG + 0.4 M TRE (final concentrations) were considered to cryopreserve both larval stages. In addition, the effects on survival and shell size of increasing equilibrium times (15, 30 and 60 minutes) were evaluated. After equilibration, larvae were cryopreserved using a controlled freezer, as described: holding at 4°C for 2 min, then cooling at -1°C/min to -12°C (seeding), holding for 2 min, then cooling at -1/°C/min to -35°, then plunging into liquid nitrogen. Thawing was carried out by immersion of straws into a water bath at 35°C for 6 seconds. The obtained data evidenced the high tolerance of this specie to cryopreservation. Although no significant differences were found, the CPA solution with PG was better for the cryopreservation of the earlier development stage, obtaining 100% of survival with 60 minutes of equilibration. The successful cryopreservation experiment using 72 h-old D-larva provided survival rates closer to 100% regardless of the treatment selected. In both cases cryopreservation yielded a delay on larval size. Further research should focus on the long-term effects and the capacity to produce cockle spat from cryopreserved larvae.

CRYOBIOLOGICAL TECHNOLOGIES IN PROVIDING TOLEROGENIC POTENTIAL OF CRYO-CONSERVED AND LYOPHILIZED HUMAN CORD BLOOD CONCENTRATE

A Goltsev[1], O Lutsenko[1], L Ostankova[1], K Yampolska[1], M Ostankov[1], I Grisha[1], L Sokil[1], M Bondarovych[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine

Regulatory T cells (Treg) belong to a subpopulation of CD4 + T cells with suppressor properties and play an important role in maintaining autotolerance. Currently, cord blood (CB) is considered as one of the sources of Treg for immunotherapy. This necessitates the establishing of the CB reserves using cryopreservation and lyophilization methods.

The research purpose was to comparatively assess the structure and functions of Treg as well as the content of IL-10 in cryopreserved and lyophilized leukoconcentrates of human CB (HCBL).

The cryopreserved HCBL (cHCBL) was obtained after freezing of HCBL according to a two-stage program (1); lyophilized HCBL (lHCBL) – as reported by A. Goltsev et al. (2). The amount of Treg, i.e. CD4 + CD25high, the average and total fluorescence intensity of the CD25 marker, and the Foxp3

protein content in cells was determined by flow cytometry (FACS Calibur, BD, USA). The concentration of IL-10 in HCBL was determined by enzyme-linked immunosorbent assay.

Differences in functional parameters of Treg after cryopreservation and lyophilization of HCBL were found. The amount of Treg decreased by 2.2 fold in cHCBL, while it increased in 1.6 fold in lHCBL compared with their quantity in a fresh preparation, which did not affect the content of IL-10. At the same time, after cryopreservation and lyophilization, an increase in the average fluorescence intensity of the CD25 marker in Treg was noted, which may indicate a higher readiness of cells to implement their immune suppressive potential. In addition, the total fluorescence intensity for the CD25 marker and the Foxp3 protein content were maximal in Treg from lHCBL.

The findings indicate the ability of cryopreservation and lyophilization to stimulate the Treg functional potential, increasing the immune regulatory properties of HCBL. Moreover, after lyophilization such an activity is manifested to a greater extent.

(1) Tsutsayeva AO, Grischenko VI, Kudokotseva OV, Shcheglov AV, Tupchienko GS, Prokopuk OS (inventors); Institute for Problems of Cryobiology and Cryomedicine, assignee. [Method of cryopreservation of hemopoietic cells of cord blood] Ukrainian patent 31847 A 1998 November 5. Ukrainian. **(2)** Goltsev AM, Taranik GC, Grisha IG, Sokil LV, Bondarovich MO, Ostankov MV, Lutsenko OD, Goltsev KA, Ostankova LV (inventors); Institute for Problems of Cryobiology and Cryomedicine, assignee. [Method of lyophilization of leukoconcentrate of cord blood]. Ukrainian patent 113006, 2017 Jan 10. Ukrainian.

AMPHIPHILES PROTECT HUMAN ERYTHROCYTES UNDER POST-HYPERTONIC SHOCK

O Chabanenko[1], Natalya Yershova[1], Natalia Orlova[1], Natalia Shpakova[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine

This research is devoted to a comparative study of the effect of amphiphiles, differing by physicochemical properties on the sensitivity of erythrocytes under post-hypertonic shock. Post-hypertonic shock simulates the influence on cells of cryodamage factors, acting at erythrocyte thawing stage, as well as when that of cells, cryopreserved under protection of penetrating cryoprotective agent are transferred into bloodstream (1). Post-hypertonic shock (PHS) of erythrocytes was initiated by transferring cells from dehydration medium (1.65 mol/L NaCl) into rehydration one (0.15 mol/L NaCl) at 0°C. The various classes of amphiphiles (anionic sodium dodecyl sulfate, non-ionic decyl-β,D-glucopyranoside, and cationic trifluoperazine) were used. Amphiphiles were supplemented into rehydration medium prior to transferring the cell. Amphiphiles were shown to protectively affect erythrocytes in various extents. Under these conditions, decyl-β,D-glucopyranoside and trifluoperazine were more effective, for which maximum antihemolytic activity are recorded at a level of ~60%, in contrast to sodium dodecyl sulfate, which maximum antihemolytic activity was 2 times lower (~30%). A comparative study of the efficiency of amphiphiles under post-hypertonic shock of erythrocytes showed the differences in the size of concentration plateau and the values of effective concentrations. A narrowing of the plateau was revealed in a series: decyl-β,D-glucopyranoside > trifluoperazine > sodium dodecyl sulfate. Decyl-β,D-glucopyranoside characterize the maximum effective concentration (1,000 μmol/L) and the minimal one for sodium dodecyl sulfate (50 μmol/L). The obtained results suggest that the effectiveness of amphiphiles under PHS conditions of erythrocytes is associated with their ability to integrate into membrane, possibly, to the defect formation areas. This is accompanied by an increase in membrane surface area and, therefore, in critical hemolytic volume of cells, which allows them to swell to a larger volume in rehydration medium.

(1) Semionova YA et al. (2017) *Problems of Cryobiology and Cryomedicine* **27**, 51-60.

PROLIFERATION AND APOPTOSIS IN WOUND GRANULATION AND CONNECTIVE TISSUES AFTER SKIN CRYOABLATION USING PIGLETS CRYOPRESERVED SKIN FRAGMENTS EXTRACT

O Vlasov[1], G Kovalov[1], M Myroshnychenko[2]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine
[2] Kharkiv National Medical University, 4 Nauky Avenue, 61022, Kharkiv, Ukraine

The piglets cryopreserved skin fragments extract is known to stimulate the wound healing after skin cryoablation, but the mechanisms of this stimulation have been poorly understood. Apoptotic (p53) and proliferative (Ki-67) cell activity was studied on days 7, 14, and 21 after skin cryoablation in hairless rats, with intraperitoneal administration of saline (group 1) or piglets cryopreserved skin fragments extract (group 2). In groups 1 and 2, at all periods of the experiment in granulation and connective tissues, p53 and Ki-67 expressed predominantly fibroblastic cells, vascular endothelial cells and immune cells. In group 1, the number of Ki-67- and p53-positive cells on days 7, 14, and 21 was 18.7 ± 2.8 and 9.5 ± 1.5, 23.4 ± 3.6 and 12.9 ± 2.0, 28.5 ± 4.6 and 15.9 ± 2.5, respectively. In group 2, the number of Ki-67- and p53-positive cells on days 7, 14, and 21 was 27.4 ± 3.9 and 14.8 ± 2.1, 32.2 ± 5.1 and 17.8 ± 2.9, 37.3 ± 5.8 and 22.5 ± 3.7, respectively. In both groups, firstly, the proliferative activity of the cells prevailed over the apoptotic one, and secondly, with a rise in the duration of the experiment, the processes of apoptosis and proliferation increased. In group 2, compared with group 1, at all periods of the experiment, the absolute number of Ki-67 and p53-positive cells was higher. Intraperitoneal administration of piglets cryopreserved skin fragments extract in a balanced manner activates the apoptotic and proliferative activity of fibroblastic cells, vascular endotheliocytes, immune cells in granulation and connective tissues, filling the wound cavity after skin cryoablation.

OSMOTIC RESPONSE OF FRESHWATER FISH SPERMATOZOA AS AN INDICATOR OF THEIR CRYORESISTANCE

A Puhovkin[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine

Fish spermatozoa cryopreservation protocols are characterized by high species specificity. A unique method of their low-temperature storage should be created for each species (1). Taking into account that research is often carried out in the field, there is a need to use simple and universal tools to determine the functional characteristics of spermatozoa.

In order to solve this problem, we designed a method that allows studying osmotic response of spermatozoa using photoelectric colorimeter, based on changes in the optical density of the suspension (2). We described the osmotic response of spermatozoa using such parameters as membrane permeability and activation energy of water transfer through the membrane. In addition, we used cells osmotic resistance as cells lysis time in osmotic shock.

To determine the membranes permeability of fish spermatozoa to water or cryoprotectant molecules, spermatozoa were added to the cuvette, filled either with NaCl aqueous solution or NaCl based solutions of ethyleneglycol, 1,2-propanediol, methanol, glycerol or other cryoprotectants. Permeability coefficients of spermatozoa plasma membranes for either water (L_p) or cryoprotectant (K_p) molecules were determined by fitting the experimental dependences of relative cell volumes versus time and the solutions of theoretical model equations. The activation energy (E_a) of substance transfer through

cell membranes was calculated from the $\ln L_p$ or $\ln K_p$ dependencies versus temperature, the slope of those according to the Arrhenius equation was E_a/R, where R was the universal gas constant.

Thus, we investigated the spermatozoa osmotic response of such freshwater fish as carp, silver carp, crucian carp, tench, grass carp, pike perch, pike, sterlet, Russian sturgeon and some others.

There is a tendency for membrane permeability to correlate with sperm cryoresistance with some restrictions and conditions. This is consistent with previously obtained data (3). Also, determining the value of this parameter allows the selection of suitable cryoprotective media.

(1) Kopeika EF, Kopeika JE (2007) in *Fish Spermatology*, (eds) Alavi SHM, Cosson. Coward JK & Rafiee G, Alpha Science International Ltd. Oxford, pp 347–396. **(2)** Puhovkin AY, Kopeika EF (2015) *Cryobiology* **71**, 567. **(3)** Dzyuba B et al. (2013) *Cryobiology* **66**, 192–194.

EMBRYO DEVELOPMENT RATE AFTER FERTILIZATION WITH FRESH VS. FROZEN SPERM IN GOATS

A Bogdaniuk[1], V Garkavii [2]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine
[2] LLC «Institute of Contemporary Veterinary Technologies», Cherevky, Ukraine

In vitro embryo production and embryo transfers in goats are a developing industry in Ukraine. Introducing effective technique for *in vitro* fertilization would lead to better results in goat reproduction. The objective of this study was to compare embryo development after oocyte fertilization with fresh versus frozen sperm in Saanen goats. 3 mature Saanen goats (84 ± 8 kg) were used as donors. Estrus synchronization was performed using intravaginal sponges with 45mg flugestone acetate for 14 days. On day 7 after sponge insertion, ovarian stimulation was induced by injecting intramuscularly 5 doses of follicular stimulating hormone (4D – 500IU, 1D - 250IU) with intervals of 24 h and administrating a single intramuscular injection of 1000IU of a pregnant mare's serum gonadotropin 36 h before sponge removal and oocyte aspiration. Laparoscopic ovum pick-up was performed using a 23G Aspic needle connected to 20mL syringe. Following *in vitro* maturation, 29 recovered oocytes (9.7 ± 2.1 per goat) were randomly divided into groups (20 and 19 oocytes) and *in vitro* fertilized using washed fresh and frozen-thawed semen derived from Saanen buck. After 7 days of *in vitro* culture 11 blastocysts (55%) and 9 blastocysts (47.4%) were developed in groups with fresh vs. frozen sperm consequently. To conclude fertilization with fresh sperm leads to better embryo development rate than with frozen-thawed sperm in goats.

CRYOPRESERVATION OF NON-MOBILIZED PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) – CURRENT SITUATION AND FUTURE PERSPECTIVES

P Měřička[1], M Jandová[1], J Gregor[1], M Lánská[2], D Vokurková[3], I Fátorová[2], P Paterová[4], J Radocha[2], L Štěrba[1]

[1] Tissue Bank, University Hospital Hradec Králové, Czech Republic
[2] IV.th Department of Internal Medicine Charles University Faculty of Medicine Hradec Králové, Czech Republic ,
[3] Department of Clinical Immunology and Alergology, Charles University Faculty of Medicine Hradec Králové
[4] Department of Clinical Microbiology, Charles University Faculty of Medicine Hradec Králové, Czech Republic

Treatment by means of donor lymphocyte infusions (DLI) after allogeneic haematopoietic progenitor cell (HPC) transplantation represents, now the main indication for cryopreservation of PBMC. Usually a small part (20-30 ml), surplus of the collected allogeneic HPC concentrate is frozen in several units for administration in increasing CD3+ cell doses (standard scheme once or twice: $0.5x10^5$ /kg, 1x 106 /kg, 5x 10^6 /kg,1x 10^7 /kg of the patient s weight) to achieve guided GvD. If the sufficient number of cell doses cannot be achieved, additional collection of non-mobilized PBMC must be performed in an original HPC donor. Results of cryopreservation of concentrates collected in 16 donors (originated from Czech Republic in 9 cases, from Germany in 5 cases, from Poland in 2 cases) and sent to the EU TE Code CZ000426 are presented. The standard operating processing and cryopreservation procedure using clean room facility, 10%DMSO, and controlled rate freezing with storage in liquid nitrogen vapour phase was used. The WBC count including detailed differential, CD3+ count, cell viability and sterility tests were performed from the collected suspension and the thawed control samples. The median values of collected suspensions were: volume 152 ml, NC concentration 75.2 x 10^9 /l, TNC 144.2 x 10^8, MC percentage 96.4. The MC post- thaw viability was 95 %, post-thaw recoveries: viable TNC 88.25%, viable TMNC 87,93%, viable CD3+ cells 88.62%. Annual DLI application rate has shown a slowly increasing trend between 20 to 79 infusions in the last 5 years. The authors plan to use the described experience in a near future in cryopreservation of non-mobilized autologous PBMC as starting material for manufacturing of genetically modified advanced therapy medicinal product Kymriah (tisagenlecleucel) CTL 019, Novartis that should expand the spectrum of therapeutical tools in treatment of malignant lymphomas, multiple myeloma and certain types of leukaemia.

THAWING REGIME OPTIMIZATION FOR SEMINIFEROUS TUBULES OF RAT TESTES AFTER VITRIFICATION USING FIBRIN GEL

N Volkova[1], M Yukhta[1], L Chernyshenko[1], L Stepanyuk[1], L Sokil[1], A Goltsev[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine

The aim of the study was a comparative assessment of the thawing regimes on the morphofunctional characteristics of the immature rat seminiferous tubules after vitrification. The samples of immature rat testicles of 2-3 mm^3 size were exposured in cryomedia (Me2SO+glycerol+sucrose) of increasing concentrations based on a fibrin gel for 5 min at 4°C and then were vitrified by rapid immersion in liquid nitrogen. The following regimes were used for thawing: I - at 22°C; II - at 50°C; III - at 70°C by successive transfer of samples to a sucrose solution of decreasing concentration (1M, 0.5M, 0.25 M, 0M) for 5 min. Intact control was used as a comparison group. Histomorphological data, MTT-test, total antioxidant defense system (TAS) activity were studied. The results were processed with Kruskal-Wallis ANOVA test with multiple comparisons. The indicates of MTT-test and TAS activity in the samples warmed at 50°C were higher by 1.9 and 1.7 times respectively relative to regime I. The use of regime II and III did not significantly differ from each other in the investigated parameters. However, these indicates in all experimental groups remained significantly lower than in intact control. The warming at regime I negatively affected histological structure seminiferous tubules: there were extensive destruction zones, and spermatogenic epithelium lost its organization. The samples warmed at regime II retained their architecture (slight retraction and vacuolization of the nuclei of individual cells, which were located throughout the epithelial layer). After thawing of samples at regime III, the spermatogenic epithelium was characterized by a sharp retraction and desquamation cells, which resulted in defects of the spermatogenic layer. The thawing at 50°C allows effective preserving of the morphofunctional properties of vitrified seminiferous tubules. These findings relate to reproductive medicine and can be used for development of effective thawing regimes for vitrified seminiferous tubules.

METHOD OF THERMAL ANALYSIS FOR THE DETERMINATION OF THE CONTENT OF FREEZABLE WATER IN THE BREEDING MATERIAL OF POTATOES AND HOPS

M Faltus[1], P Svoboda[2], J Domkářová[3], V Horáčková[3], J Zámečník[1], V Nesvadba[2], A Bilavčík[1]

[1] Crop Research Institute, Prague, Czech Republic
[2] Hop Research Institute Co., Ltd., Žatec, Czech Republic
[3] Potato Research Institute, Havlíčkův Brod, Czech Republic

The aim of this work was to verify the possibility of using the cryopreservation method in the breeding process of potatoes and hops in the form of explant cultures and pollen. Two methods of dehydration of potato shoot tips were tested: 1) dry air over silica gel, 2) PVS3 cryoprotective solution and the only the first mentioned method for hop shoot tips. Thermal characteristics were determined using a Q2000 differential scanning calorimeter (TA Instruments) at isolated shoot tips of potatoes or hops before or after dehydration specified above. In the case of potato explants, dehydration of the growth peaks with a cryoprotective solution was found to be more effective compared to dry air dehydration. In the case of hop explants, it was found that the optimal dehydration of the growing peaks occurs in the range of 1.5 to 2 hours, when a decrease in the proportion of frozen water to 6 to 4% was found. When comparing the results of dehydration of potato and hop explants, the presence of two endotherms was found when the proportion of frozen water decreased by 18%, regardless of the method of dehydration of plant material and plant species. While an endotherm that has been detected at a higher temperature appears to be related to the presence of free water, the significance of the second endotherm is unclear and will be the subject of further research. Thermal analysis of the pollen did not detect the presence of frozen water in the potatoes or hops. The presence of a wide glass transition in the range of -55°C to 0°C with a mean value of approximately -25°C was detected in all tested materials. This finding suggests the possibility of widespread use of the cryopreservation method for preserving potato and hop pollen for its use in the breeding process.

Acknowledgement: This study was supported by research projects of the Ministry of Agriculture of the Czech Republic QK1910277 and MZE RO0418.

EFFECTIVENESS OF CERIUM DIOXIDE (CeO_2) NANOPARTICLES IN CULTIVATION AND CRYOPRESERVATION OF FIBROBLASTS

O Polivanova[1], O Sidorenko[1], O Falko[1], V Chizhevsky[1]

[1] Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Pereyaslavska str. 23, 61016, Kharkiv, Ukraine

The current state of nanotechnologies development allows the obtaining of multifunctional nanomaterials, applicable in cryobiology, such as nanocrystalline cerium dioxide (CeO_2NPs), which can be used as an auxiliary component of cryoprotective media to reduce the Me_2SO concentration, commonly applied to freeze cells, but being toxic. However, CeO_2NPs can also exhibit toxic effects, and therefore determination of CeO_2NPs non-toxic concentrations is important.

Two series of experiments were performed: detection of toxic CeO2NPs concentration for L929 mouse fibroblast cells by MTT test and evaluation of apoptosis / necrosis in L929 mouse fibroblast cells cryopreserved in media containing CeO_2NPs using Annexin V and 7AAD dyes by flow cytometry (FACS Calibur, USA). In the first series of experiments, the cells were cultured in the nutrient medium with the addition of CeO_2NPs at final concentrations of 1.0; 0.2 and 0.02 g/l, followed by their evaluation using the MTT test on the fourth cultivation day. In the next series of experiments, the fibroblasts were cryopreserved in a protective media containing 1% and 5% Me_2SO. As an additional component 1.0 g/l

and 0.02 g/l of CeO_2NPs were added. Control were the cells cryopreserved in a medium containing 1% Me_2SO without the addition of CeO_2NPs.

According to the MTT test, it was found that only 1 g/l was toxic of all three studied CeO_2NPs concentrations added to the medium during cell culture. However, according to flow cytometry, at this CeO_2NPs concentration there is a decrease in the number of Annexin V+/7AAD+ cells after cryopreservation in a medium containing 1% and 5% Me_2SO. CeO_2NPs at a concentration of 0.02 g/l and 0.2 g/l have no any toxic effect during the cultivation of fibroblasts but also do not have a cryoprotective effect. Thus, further research may be aimed at finding a nontoxic concentration of CeO_2NPs having a cryoprotective effect.