SLTB 2016 in Dresden on Wednesday September 7th

Back-to-back with

International Institute of Refrigeration (IIR) workshop on the 8th and 9th September

The Society will be holding its annual meeting in Dresden, capital of the eastern German state of Saxony, on the 7th of September, as a pre-workshop satellite to "Cold Applications in Life Sciences", organized by the Institut für Luft- und Kältetechnik gGmbH (ILK Dresden) and supported by the International Institute of Refrigeration (IIR). SLTB delegates can register for the IIR meeting at the rate for IIR members.

Our meeting will be held at the ILK (Institut für Luft- und Kältetechnik Gemeinnützige Gesellschaft mbH), Dresden, and will include a session based around current challenges in the application of cryopreservation to practices and process in regenerative medicine. Effective cryotechnologies are essential in this area to meet many of the major challenges in 21st century clinical practice.

Additionally, there will be a traditional, open session embracing all aspects of low temperature biology. Further details of the meeting are enclosed with this letter together with links to helpful internet sites. More complete details of the programme will be circulated shortly.

SLTB delegates are invited, and encouraged to join the IIR workshop that will cover '*Cell/ Tissue Cryopreservation and Storage Challenges'* and *'Freezing and Storage of Active Agents for in-vitro and in-vivo Applications'*. A more detailed programme can be found at

http://www.ilkdresden.de/en/cryobio-workshop/objectives/.

Getting together in Dresden will be a great opportunity to listen, to share information and make new/renew old connections. It will also be an opportunity for you to put forward your view of the future for the Society and help direct the activities for 2017.

So, on behalf of the committee, we look forward to meeting up with you later in the year.

Brian Grout, Chairman SLTB

SLTB Programme

8.15 **Registration and coffee**

9.15 **Official Greeting from ILK**
R Herzog

9.30 **Welcome to SLTB & Tribute to Dr Peter Mazur**
B Grout

Free communications

9.50 Quality of fresh and cryopreserved silver carp sperm after stimulation by commonly used hormonal preparations compositions
K Butskyi

10.10 Cryomicroscopic ultrasound ice induction for cryopreservation approaches
L Lauterboeck, W Wieprecht,R Jung, B Glasmacher

10.30 Mimicking long-term biobanking of placental multipotent stromal cells by temperature fluctuations during cryopreservation
D Pogozhykh, O Pogozhykh, V Prokopyuk, A Goltsev, C Figueiredo, T Müller

10.50 Metabolic changes and hypoxic conditions during garlic cryopreservation
K Ulagappan, N Heinzel, H Rolletschek, ERJ Keller, M Nagel

11.10 Ovarian tissue cryopreservation by means of x-ray computed tomography
A Corral, M Clavero, M Balcerzyk, M Gallardo, CA Amorim, A Parrado-Gallego, MM Dolmans, R Risco

Coffee

Poster Teaser Talks

11.50 Viscosity measurement in solutions of interest in cryobiology at variable temperatures by Brownian motion analysis
H Desnos, A Baudot, M Teixeira, L Gavin-Plagne, G Louis, L Commin, S Buff, P Bruyère

11.55 Project outline to study wheat pollen viability and the opportunities for long-term conservation
D Impe, A Senula, M Nagel

12.00 Project outline to study oxidative stress in potato shoot tips during cryopreservation
C Köpnick, M Grübe, M Nagel

12.05 Effects of repeated cycles of rhythmic extreme whole body cooling on indices of neurohumoral regulation in rat's aging

Yu V Martynova, VG Babiychuk

12.10 Cryopreservation and redox state of cord blood nucleated cells
OO Mykhailova, O Ye Makashova, PM Zubox, OL Zubova, LA Babikchuk

12.15 Advances in cryopreservation of feline and canine erythrocytes
D Pogozhykh, Yu Pakhomova, B Glasmacher, G Zhegunov

12.20 Beneficial effect of platelet lysate during manufacturing and banking of mesenchymal stem cells
O Rogulska, O Tykhvynska, Y Petrenko, A Petrenko

12.25 Cryopreservation of mint – some key factors during the droplet-vitrification process
A Senula, D Büchner, J Keller, M Nagel

12.30 Equipment for cryopreservation. Ukrainian experience practice
O Snurnikov

12.35 Survival rate of human vitrified blastocysts depending on chosen collapsing method
MP Petrushko, VI Pinyaev, TA Yurchuk

12.40 ATP levels during freezing and thawing across the diversity of life
F Bajerski, J Stock, B Hanf, T Darienko, E Heine-Dobbernack, S Eberth, M Lorenz, L Naujox, ERJ Keller, R MacLeod, M Schumacher, H-P Mock, O Kniemeyer, T Friedl, J Overmann

12.45 Effect of cryopreservation on functional potential of fetal neural cells
Ie Porozhan, M Ostankov, T Dubrava, A Goltsev

12.50 Regenerative potential of cryopreserved mesenchymal stromal cells of various origin
NA Volkova, AN Goltsev

12.55 Cryopreservation without ice crystal formation - the fourth arrangement of water in biological glass
J Zámečník

13.00 Cryobanking of hop germplasm in the Czech Republic
M Faltus, P Svoboda

13.05 Sucrose preculture improves regeneration of aloe after droplet vitrification
R Folgado

Lunch break & poster session

Cryopreservation in Regenerative Medicine Part I

14.30 Influence of cryopreservation on survival of placental, umbilical cord, and fetal membrane explants, as well as placental cells within spheroids and alginate microspheres
V Yu Prokopyuk, D Pogozhykh, O Pogozhykh, IB Musatova, LG Kuleshova, OS Prokopyuk

14.50 Utilization of a new freezing protocol containing higher concentrations of DMSO to cryopreserve human ovarian tissue: preliminary results
CA Amorim, F Paulini, M Balcerzyk, ALP Gallego, CM Lucci, MM Dolmans, R Risco, A Corral

15.10 Hypothermic Treatment to Improve Culture Expansion
KT Mahbubani, P Kilbride, NKH Slater, K Saeb-Parsy, J Morris

15.30 A cryogenic cold chain for clinical delivery of regenerative medicines
J Morris

Coffee break and tour of ILK

Cryopreservation in Regenerative Medicine Part II

16.20 Cryosensitivity of tissue-engineered collagen constructs with multipotent stromal cells
VV Mutsenko, O Gryshkov, S Knaack, M Gelinsky, A Yu Petrenko, B Glasmacher

16.40 High performance vitrification technology potentially suitable for stem cells
R Risco, MG Vilches, M Gallardo, D Barranco, A Corral, A Lucas, M Hebles, P Sanchez

17.00 Application of plant-derived anti-freeze proteins in MSC cryopreservation
Y Naaldijk, A Friedrich-Stöckig, A Stolzing

17.20 Towards an understanding of heterogeneous ice nucleation in cryobiology
TF Whale, BJ Murray, TW Wilson, MA Holden, J Morris

17.40 Developing a commercially feasible cryopreservation process for a bioartificial liver support device
A Picken, Q Liu, A Iftimia-Mander, M Ginai, S Lamb, A Stolzing, J Morris, N Medcalf

18.00 AGM & Business Meeting

19.00 Dinner

QUALITY OF FRESH AND CRYOPRESERVED SILVER CARP SPERM AFTER STIMULATION BY COMMONLY USED HORMONAL PREPARATIONS COMPOSITIONS

K Butskyi*

Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkiv, Ukraine.

An important task of cryobiology is to improve the of available fish sperm cryopreservation methods. Fresh sperm quality is very important for cryopreservation. It can be difficult to create favorable conditions for the fish breeding, so now the selection of the optimal hormonal stimulation type of the final gametogenesis stage is important (1). The aim of this work is to compare the effect of different hormonal stimulation on the ATP concentration in semen and its quality and cryoresistance.

Materials and Methods: Experiments were performed on males of white silver carp (Hypophthalmichthys molitrix). A drugs that are used to stimulate the maturation of sperm in fish farms were used for injection: 2.5 mg/kg of carp pituitary suspension; 1 μg/kg surfagon (MosAgroGen, Russia) - a synthetic analogue of gonadotropin-releasing hormone for mammals; 1 μg/kg "surfagon" + 5 μg/kg "metoclopramide" - dopamine receptor blocker; 0.5 granules/kg commercial drug "ovopel" (Hungary). Determination of ATP is carried out by enzymatic method with conversion of NADH to NADPH system of coupled enzymatic reactions. Cryopreservation was performed by the standard method for carp sperm (2).

Results and discussion: The experiments showed the following levels of mobility before and after cryopreservation: 80 ± 17% / 33 ± 10% when stimulated by the pituitary gland; 53 ± 27% / 32 ± 15% for stimulation with a mixture of "surfagon" + "metoclopramide"; after "ovopel" injection 85 ± 13% / 30 ± 15%, male, injacted by the "surfagon" have no sperm generated. Motility time had no significant differences and was ~45s. It was shown that sperm with the highest level of motile cells obtained after stimulation hormonal drug "ovopel". Although after stimulation of males with a mixture "surfagon" + "metoclopramide" it had received a lower level of sperm motility, white silver carp cryopreserved sperm quality had no significant differences. This indicates a greater cryoresistance sperm after stimulation with a mixture of "surfagon" + "metoclopramide".

Because the energy characteristics associated with cryoresistance (3), to explain differences the ATP concentration in the fresh silver carp sperm was investigated: after stimulation with a mixture of "surfagon" + "metoclopramide" there is almost twice bigger ATP level.

Conclusions: After silver carp hormonal stimulation by a mixture of "surfagon" + "metoclopramide" it was the highest concentration of ATP in sperm that was one reason of higher cryoresistance.

(1) Kayim M, Bozkurt Y, Ogretmen F. (2010). *J Anim Vet Adv*, **9**, 2589–92. **(2)** Kopeika EF (1986) Instruction of carp sperm low temperature conservation, Moscow: VNIPRH **(3)** Kopeika EF, Cherepanov VV, Dzyuba BB (1997) *Problems of Cryobiology*, 4, 41–5.

CRYOMICROSCOPIC ULTRASOUND ICE INDUCTION FOR CRYOPRESERVATION APPROACHES

L Lauterboeck[1,*], W Wieprecht[2], R Jung[3], B Glasmacher[1]

[1]Institute for Multiphase Processes, (IMP), Leibniz Universität Hannover, Hannover, Germany,
[2]Atmospheric Chemistry and Air Quality, Brandenburg University of Technology Cottbus, Cottbus, Germany
[3]Bandelin electronic GmbH & Co. KG, Berlin, Germany

In recent years induced nucleation is used to improve poorly preserveable cells such as iPSCs. Different methods were developed to active control the nucleation temperature such as seeding, electro-freezing and ultrasound. For the first time the coupling of ultrasound with a cryomicroscope was designed at IMP in Hannover. The Bandelin electronic company (Berlin, Germany) developed a special sonotrode which can be used for cryomicroscopic ultrasound induction. This device was coupled with a Linkam cryo-chamber BSC 196. To avoid cavitations, which are damaging to cells, a frequency of 28.6 kHz was used and transferred to a poly(methyl methacrylate) pan via a stainless steel tongue. To observe the induction and its influence on the crystallisation process 5µl distilled water or 1M sucrose in combination with 5µm glass beads and a cooling rate of 5°C/min until -30°C was used. Different intervals and intensity were investigated to induce nucleation at least 5°C above the spontaneous nucleation. As a control, no induction was performed during cooling.
Beads with 5µm diameter were used to visualize ultrasound intensity in the sample. Bead velocity showed a positive relationship to pulse length and intensity. Nucleation was induced in water up to 10°C higher than spontaneous nucleation and 8°C in sucrose. Intensity and duration of the impulses did not have an effect on reducing ice crystal size. In summary, ultrasound ice nucleation in a cryomicroscopic setup has been developed which can effectively induce ice nucleation in small samples.

This project was partially funded by ZIM (Zentrales Innovationsprogramm Mittelstand, KF2285503) and REBIRTH (EXC/62).

MIMICKING LONG-TERM BIOBANKING OF PLACENTAL MULTIPOTENT STROMAL CELLS BY TEMPERATURE FLUCTUATIONS DURING CRYOPRESERVATION

D Pogozhykh[1,2,*], O Pogozhykh[1,2], V Prokopyuk[2], A Goltsev[2], C Figueiredo[1], T Müller[1]

[1]Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany
[2]Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkov, Ukraine

Current progress in stem cell research and regenerative medicine highly increased the demand for readily available cellular suspensions. In particular, cell therapies with application of multipotent stromal cells (MSCs) have proven clinical efficiency in wound healing, regulation of the endocrine system as well as in the treatment of a range of other severe pathologies. Placenta and its components are among the most promising sources of MSCs due to major advantages such as the possibility of obtaining autologous material without invasive surgery, relative simplicity of retrieving large amount of cells, low immugenicity, high plasticity and proliferation capacity. Cryopreservation is the only effective approach for long-term preservation of viability and unique properties of such cells. Nevertheless, various maintenance, transportation, technical and stocking events, associated with practical biobanking, are often accompanied with repeated temperature fluctuations or interruption of a cold chain. Though biochemical processes are anticipated to be on hold during cryopreservation, such temperature fluctuations may lead to accumulation of stress in the samples as well as result in periodic release of water fractions (1). Accumulation of frequency of such events may lead to alterations in vital parameters after long-term storage. Comprehensive analysis of alterations in survival, vital parameters, and plasticity of placental MSCs after temperature fluctuations at subzero temperatures, mimicking long-term preservation in conditions of practical biobanking, transportation, and temporal storage has been performed. The temperature in the samples was increased from 196°C to -80°C, -100°C, and -150°C with 5, 10, 20, 30, 40, and 50 temperature cycles for each temperature range. Viability and metabolic parameters of placental MSCs were not significantly altered after less than 20 cycles of temperature fluctuations in the range between 196ºC and 100ºC in comparison to constant temperature storage. Increasing the number of cycles as well as increasing of the temperature range to the 80ºC higher temperature end point during cycles, resulted in significant lowering of these parameters after thawing. Apoptotic changes are observed with increasing of the number of cycles of temperature fluctuations. Moreover, capability of cells to adhesion after thawing is significantly compromised in the samples with temperature fluctuations during cryopreservation. Both cryopreservation with constant end temperatures and with temperature fluctuations did not compromise plasticity of placental MSCs after thawing. Interestingly, regulation of various genes after cryopreservation was observed. Therefore, potential temperature fluctuations in the samples should be considered in practical biobanking during long-term preservation of placental MSCs. While plasticity and transgene expression are not compromised after mimicked long-term storage conditions, alterations in vital parameters and gene regulation are observed. The comprehensive evaluation of the MSCs viability and functionality after cryopreservation is crucial to ensure their optimal therapeutic effect in modern regenerative cell therapies.

(1) Vysekantsev IP, Gurina TM, Martsenyuk VF *et al*. (2005) *Cryo Letters*, **26**, 401-8.

METABOLIC CHANGES AND HYPOXIC CONDITIONS DURING GARLIC CRYOPRESERVATION

K Ulagappan*, N Heinzel, H Rolletschek, ERJ Keller and M Nagel

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, D-06466 Gatersleben, Germany

Garlic (*Allium sativum* L.) is a medicinal crop, consumed as a popular condiment and green vegetable. Garlic cultivars do not produce seeds and, therefore, can be propagated only by vegetative mode. Maintenance of garlic germplasm via cryopreservation is increasingly applied worldwide (1). Dehydration and ultra-low temperature (−196 °C) cause a series of abiotic stress conditions including hypoxia (2). Under dehydration or immersion in cryopreservation solutions, tissue oxygen was drastically decreased from 100 to 1 % of air saturation. To reveal hypoxia mediated metabolic fluxes, LC-MS was employed in subsequent steps (preculture, dehydration and liquid nitrogen storage). During dehydration, 3-phosphoglyceric acid, phosphoenolpyruvate and pyruvate concentrations increased which facilitate changes in the downstream of glycolysis (3). In addition, increased levels of AMP, decreased levels of ADP and ATP and a depletion of the calculated adenylate energy charge were observed during dehydration step. Oxygen level was measured in different layers of garlic shoot tips and hypoxia was characterized. This led to the assumption that the ATP level is locally decreased. In attempts to control the redox homoeostasis in the plant tissue, we found that the oxygen environment, especially deficient when using the cryoprotectant PVS3, could be oxygen-enriched by external aeration. This will be used to elucidate the effect of oxygen during cryopreservation.

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OVARIAN TISSUE CRYOPRESERVATION BY MEANS OF X-RAY COMPUTED TOMOGRAPHY

A Corral[1,*], M Clavero[2], M Balcerzyk[1], M Gallardo[4], CA Amorim[3], A Parrado-Gallego[1], MM Dolmans[3], R Risco[1,2]

[1] National Center for Accelerators (CNA), Seville, Spain
[2] Engineering School of Seville (ETSI), Seville, Spain
[3] Université Catholique de Louvain, Louvain, Belgium
[4] Ginemed, Seville, Spain

Cryopreservation of ovarian tissue is, in most cases, the only fertility preservation option for cancer female patients (0). The main advantages over other alternatives are that it can be performed at any time in the menstrual cycle, in pre-pubescent patients, or in cases of hormone-sensitive tumors, requiring minimally invasive surgery. Even though the number of successful cases is very limited, thawing and transplantation of the cryopreserved tissue can restore the ovarian function and the ability of achieving a pregnancy (2, 3). Among the current cryopreservation procedures for ovarian tissue, vitrification involves a faster and affordable method, however it is limited to small fragments of tissues and requires higher cryoprotectant (cpa) concentration. Further research in vitrification protocols is still necessary. So far most successful cases of ovarian tissue cryopreservation have been achieved by the traditional slow freezing procedure (0).

X-ray Computed Tomography has been proved to be an excellent tool to assess tissue cryopreservation (0). In a previous work (0) we analyzed ovarian tissue cryopreserved by slow freezing with this technology. Even though results showed a good global tissue equilibration, images showed that the cpa loading was non-homogenous and some ice crystals have formed in regions with lower cpa concentration.

As an alternative, we have studied a vitrification method by slow cooling that allows vitrifying larger samples avoiding the formation of extracellular ice and high levels of toxicity. The procedure consists in increasing the cpa concentration while lowering the temperature in steps. Bovine ovarian tissue was cut in slices of 5x5 mm^2 and 1 mm thickness. The tissue was loaded with increasing Me_2SO concentrations (up to 50% v/v) at decreasing temperatures in a cooling device consisted of a methanol bath with programmed ramps until -40^oC. After that, samples were cooled to -150 ^oC in vapors of liquid nitrogen and stored at -196^oC. Different protocols were studied by varying the loading time for each concentration. Tissues were then analyzed in a NanoCT device at low voltage (75 kV). For tissues well equilibrated in Me_2SO we performed an immunostaining study in order to assess tissues viability. Caspase 3 and Ki-67 protocols were used to observe apoptosis and cellular proliferation. Cryopreserved tissues of one of the protocols achieved similar results to the fresh ones, meaning that the cooling process did not cause extra damages. In this way CT technology allows developing new alternative protocols of successful ovarian tissue cryopreservation.

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VISCOSITY MEASUREMENT IN SOLUTIONS OF INTEREST IN CRYOBIOLOGY AT VARIABLE TEMPERATURES BY BROWNIAN MOTION ANALYSIS

H Desnos[1,2,*], A Baudot[2,3], M Teixeira[1], L Gavin-Plagne[1], G Louis[2,3], L Commin[1], S Buff[1], P Bruyère[1]

[1]Université de Lyon, VetAgro Sup, UPSP ICE (2011-03-101) & CRB-ANIM (ANR11-INBS-0003), Marcy L'Etoile, France
[2]Press Sorbonne Paris-Cité, Université Paris Descartes, Paris, France
[3]INSERM U1148, Paris, France

Viscosity is a major parameter for scientists who want to study freezing processes in cryopreservation. Its value directly influences molecules' diffusion process as predicted by the Stokes-Einstein law. Diffusion is thus involved in the growth of ice dendrites or in the displacement of materials (for example intra/extracellular exchanges).
A well-known technique to determine the dynamical viscosity of a complex solution is based on Brownian motion analysis. Indeed, following the motion of perfectly spherical balls with controlled size, at a selected temperature, allows to calculate the complex solution's dynamical viscosity.
We propose here to use this technique on a cryostage device (Linkam BCS196) coupled with an Olympus BX51 microscope equipped with a Fujitsu CELSIUS W520 workstation. This device allows the observation of the 2D motion of latex balls (Polybead® Carboxylate Microspheres - 2.022µm in diameter) inside 5µL of solution at a selected temperature.
First, we describe our tracking method of balls and the viscosity calculation which ensues from it. Next, to validate this method, we compare our results with published data for widely studied solutions. Then, we use this technique inside our solutions of interest and we follow, before ice appearance, the evolution of the viscosity with temperature ranging between 20°C and -15°C. We present viscosity data for the following solutions: IMV®+10%(v/v)DMSO, IMV®+5%(v/v)DMSO and IMV® (IMV holding medium).
This simple method hardly consumes any solution and is quick to implement. It allows to make a comparative study of the viscosity. This information could help to adapt and improve slow freezing protocols of cryoprotective solutions, as it allows the understanding of diffusion processes during freezing.

Acknowledgment: Michael Dang

PROJECT OUTLINE TO STUDY WHEAT POLLEN VIABILITY AND THE OPPORTUNITIES FOR LONG-TERM CONSERVATION

D Impe*, A Senula, M Nagel

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstraße 3, 06466 Gatersleben, Germany

Longevity of pollen varies among species and depends on environmental factors such as temperature, wind and humidity (1). Storage in liquid nitrogen (cryopreservation) is used to extend the survival of mature pollen to unpredictable period and have been successfully proven for fruit crops and ornamental crops (2). However, similar to seeds, pollen can be distinguished in orthodox and recalcitrant types, which, respectively, survive desiccation over long periods or die immediately after release. Mature wheat pollen lose viability after anther extrusion (3, 4), which challenges crossing management in breeding programs. Mostly staining methods are applied to indicate wheat pollen viability but correlations to pollen germination and tube growth are lacking. Therefore, the present project aims to study physiological, biochemical and genetic factors affecting pollen viability and storability in wheat. Stresses induced by desiccation after anther extrusion and the influence on subsequent performance of pollen are examined. Comparisons within *Poaceae* family will elucidate dependencies on pollination system and support the investigation on specific metabolic pathways and genes for recalcitrant behaviour of wheat pollen. The gained know-how of pollen physiology and biochemistry is assumed to improve germplasm preservation, breeding programs and crossing management.

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PROJECT OUTLINE TO STUDY OXIDATIVE STRESS IN POTATO SHOOT TIPS DURING CRYOPRESERVATION

C Köpnick*, M Grübe, M Nagel

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstraße 3, 06466 Seeland, OT Gatersleben, Germany

Potatoes are an important staple food and have high potentials for the non-food industry. The maintenance of biodiversity is a prerequisite for potato breeding. Cryopreservation is a method for long-term preservation of the genetic resources of *Solanum tuberosum L.* varieties and wild species (1). The IPK houses one of the world largest cryocollections with more than 1,400 potato accessions. Conventionally, the IPK applies the droplet-freezing using dimethyl sulfoxide (DMSO) as cryoprotectant. The aim of the following study is to compare the DMSO with the droplet vitrification method using PVS3. First results indicate that both approaches differ in regeneration performance and the ability to form callus. Accountable for these variations are assumed to be different osmotic, mechanic or cold stress conditions, which occur during the cryoprocedure and lead to an increasing production of reactive oxygen species (ROS) (2). These oxidative reactants play significant roles in stress response mechanisms and plant defense strategies. For continuously improvement of the cryopreservation procedure, it is indispensable to find critical points during the procedure when oxidative stress conditions emerge and lead to the oxidative destruction of the cell, which will implicate a diminished recovery after cryo-storage (2, 3). Thus, analysis of soluble sugar, antioxidant enzymes and ROS in potato shoot tips by UV/VIS spectrometry can give a first instance of screening, in which parts of the methods more reactive oxygen species will be formed and how genotypes can be differentiate.

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EFFECTS OF REPEATED CYCLES OF RHYTHMIC EXTREME WHOLE BODY COOLING ON INDICES OF NEUROHUMORAL REGULATION IN RAT'S AGING

Yu V Martynova*, VG Babiychuk

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine

Whole body cryotherapy is widely used to treat various diseases and disorders (5). An extreme whole-body cooling (WBC) improves a functional state of the body and does not harm a healthy body (1, 6). An application of 10 sessions of the WBC, traditionally used, is not enough to achieve favorable adaptive changes (2). We have previously reported that rhythmic extreme (–120°) whole body cooling (RE WBC), used from a young age, promotes an improvement of microscopic (4) and ultramicroscopic (3) structure of myocardium tissue at the later age.

The aim of this study was to examine the influence of repeated cycles of RE WBC (–120°C) on the indices of neurohumoral regulation in dynamics of rats' aging.

The investigation was carried out in 55 white male rats during their natural aging, starting from young age (6 months) up to 18-month age with the control point at 12 months. When animals were 6-month-aged, they were divided into test and control (intact) group, studied simultaneously during their aging. The whole cooling cycle included 9 procedures of a 2 min cooling (-120° C) for 5 days. Every 6 months the cooling cycle was repeated. The functional state of neurohumoral regulation and the activity of its various units were assessed by spectral analysis of heart rate variability (HRV), ELISA for thyroid (thyroxine, triiodothyronine) and steroid hormones (testosterone, estradiol, dehydroepiandrosterone sulfate) and spectrophotometry for the stable end products of nitric oxide (by the Griess reaction) in the rat's blood serum. All measurements were performed 30 days after the last cooling procedure.

The present investigation showed that the use of RE WBC significantly increased the HRV indices in rats of different ages, testosterone as well as testosterone-estradiol ratio level in 18-month-aged rats, but decreased testosterone and testosterone-estradiol ratio level in 6- and 12-month-aged rats, thyroxin level in 18-month-aged rats. The levels of dehydroepiandrosterone sulfate, triiodothyronine and NO metabolites did not change significantly.

We can assume that RE WBC, used from a young age, has a stimulating effect on the functional state at the later age, but not on the whole animal's neurohumoral regulation system. Additionally, RE WBC causes a decrease some endocrine indices at young ages.

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CRYOPRESERVATION AND REDOX STATE OF CORD BLOOD NUCLEATED CELLS

OO Mykhailova*, O Ye Makashova, PM Zubov, OL Zubova, LA Babijchuk

The Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine, Kharkiv, Ukraine.

Successful transplantation of cryopreserved human cord blood (CB) nucleated cells (NCs) largely depends on total cell amount and their quality after long-term storage (1). DMSO, which commonly used as cryoprotectant for CBNCs, can be toxic under certain conditions, in particular, it can break the oxidant-prooxidant balance, enhance the formation of reactive oxygen species (ROS), contribute to the development of oxidative stress and cell death (2). An excessive accumulation of ROS in cells could be prevented by supplementation of antioxidants to cryoprotective media (3). One of those is N-acetyl-L-cysteine (NAC).

The aim of this study was to determine the redox state of CBNCs, as well as their viability at each stages of cryopreservation in cryoprotective solutions containing different concentrations of DMSO and NAC.

Dextran-isolated CBNCs were treated with 5; 7.5; 10% DMSO and cooled according to standard program at 1-3 deg/min down to -80 deg, and immersed into liquid nitrogen. Prevention of ROS overproduction was achieved by adding 5, 10, 15 or 30 mM NAC into the cryoprotectant medium. Assessment of the intracellular content of reactive oxygen species (ROS) ($DCFH_2$-DA) and cells viability (7AAD) were accomplished via flow cytometry.

After cryopreservation of CBNCs with 5 mM NAC in cryoprotective medium significant change in the number of cells with an increased content of ROS was not revealed. Similar to the low concentration, 30 mM NAC did not demonstrate any antioxidant activity and even contributed to the prooxidant effects, while 10-15 mM NAC reduced the number of cells with an increased content of ROS. The least number of DCF-labeled cells was observed in combination 10 mM NAC with 7.5% (8.7±1.5% (without NAC 20.6±3.2%)) or 10% DMSO (8.3±0.9% (without NAC 21.2±1.9%)). The inverse relationship between the number of preserved cells and cells with increased content of ROS was revealed. Thus, cell suspension supplementing with NAC contributed to the improving of the cells preservation after freeze-thawing and was the most pronounced in presence of 10-15 mM NAC. Percentage of viable cells did not show significant differences between the experimental groups. However, recalculation on the total amount of preserved cells showed that the cryopreservation with NAC raised the total number of vital NCs: the highest results were obtained in the presence of 10-15 mM, i.e. up to18-22% augmentation.

Thus, supplementation of cell suspension with 10-15mM NAC reduces the number of cells with increased content of ROS and improves total number of viable cell after cryopreservation.

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ADVANCES IN CRYOPRESERVATION OF FELINE AND CANINE ERYTHROCYTES

D Pogozhykh[1,2,3,]*, Yu Pakhomova[3], B Glasmacher[2], G Zhegunov[4]

[1] 1Institute for Transfusion Medicine, Hannover Medical School, Hannover, Germany
[2]Institute for Multiphase Processes, Leibniz Universitaet Hannover, Hannover, Germany
[3]Institute for Problems of Cryobiology and Cryomedicine, National Academy of Sciences of Ukraine, Kharkov, Ukraine
[4]Kharkiv State Zooveterinary Academy, Mala Danylivka, Kharkov Region, Ukraine

Increase of the number of hematological diseases in small companion animals, in particular cats and dogs, raises demand in veterinary transfusion approaches. Erythrocyte concentrate transfusion allows successful correction of various hematological pathologies, severe bleeding, and etc. However, low number of donor individuals, complexity of selection of blood according to variety of blood groups and other phenotypic characteristics, as well as limited storage period at 4°C require readily available supplies of the donor material. Therefore, reliable biobanking with application of cryopreservation technologies for long-term storage of the blood components is highly essential. While several research groups demonstrated approaches to cryopreserve red blood cells (RBCs) of dogs, there is a lack of data on cryopreservation of cat erythrocytes. In this study we perform comprehensive comparative analysis and optimize parameters for low temperature storage of RBCs of both species. Efficiency of single-component and multicomponent cryoprotective media with penetrating and non-penetrating cryoprotectants (CPAs) as well as influence of pre-incubation time prior to rapid freezing is analyzed. It was found that glycerol was not sufficient for cryopreservation of RBCs of cats and dogs under studied conditions. Application of 10% DMSO allowed significant reduction of hemolysis of cat and dog erythrocytes after thawing. Hydroxyethyl starch (HES) in 17.5% concentration possessed the highest cryoprotective activity for both species. It was found that incubation for 30 min at 22°C in cryoprotective media prior to freezing is required for efficient survival of dog RBCs under studied conditions, while for erythrocytes of cats 20 min was already sufficient. Combination of CPAs was less effective in comparison to single-component solutions. Presented data may be utilized in transfusion veterinary practice and blood banking optimization.

BENEFICIAL EFFECT OF PLATELET LYSATE DURING MANUFACTURING AND BANKING OF MESENCHYMAL STEM CELLS

O Rogulska[1,*], O Tykhvynska[1], Y Petrenko[1,2], A Petrenko[1]

[1]Institute for Problems of Cryobiology and Cryomedicine of National Academy of Science of Ukraine, Pereyaslavskaya 23, Kharkov, 61016, Ukraine
[2]Institute of Experimental Medicine AS CR, v.v.i, Vídeňská 1083, Prague 4-Krč, 14220, Czech Republic

Therapeutic application of mesenchymal stem cells (MSCs) in clinically relevant doses requires safe and efficient procedures for cell expansion, cryopreservation and banking. The aim of this study was to develop serum- and DMSO-free protocol for manufacturing and banking of human MSCs using platelet lysate (PL) and sucrose.

Human MSCs expansion was carried out in media supplemented either with fetal bovine serum (FS) or human PL. MSCs were cryopreserved without DMSO and FS, using sucrose pretreatment protocol (1). 24 hours prior to cryopreservation procedure cells were pretreated with 100 mM sucrose in complete culture medium. After 24 hrs of pretreatment MSCs were detached and resuspended in 1 ml cryopreservation solution. The basic composition of cryopreservation solution was α-MEM supplemented with 200 mM of sucrose. Cell suspensions were cryopreserved with the cooling rate of 1 degree/min down to −80°C with following plunging into liquid nitrogen. Cryopreserved samples (1×10^6 cells/cryovial) were stored at −196°C and thawed on water bath at 37°C before further studies. On each stage of manufacturing process MSCs were tested for survival (Trypan Blue test), viability (MTT test), proliferation and ability to multilineage differentiation.

On expansion stage replacement of FS with PL accelerated proliferation in 2.6 times and shortened population doubling time from 89.2 ± 4.3 to 39.1 ± 4.7 hrs. Inclusion of PL into the DMSO-free cryopreservation media enhanced post-thaw cell survival by 14 ± 4%. Viability rate assessed 2 hrs after thawing was also significantly enhanced when FS was substituted by PL. Post-thaw recovery of MSCs during culture in PL-containing media allowed replacing damaged cells faster than using traditional approach based on FS application. MSCs cryopreserved in DMSO-free media possessed normal fibroblast-like morphology and had capacity to differentiate into adipogenic and osteogenic directions.

This study demonstrates the benefits of using PL for safe and high quality manufacturing and banking of mesenchymal stem cells.

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CRYOPRESERVATION OF MINT – SOME KEY FACTORS DURING THE DROPLET-VITRIFICATION PROCESS

A Senula*, D Büchner, J Keller, M Nagel

Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstraße 3, 06466 Stadt Seeland/OT Gatersleben, Germany

In genebanks, the maintenance of vegetatively propagated mint is mainly realised by field cultivation or *in vitro* storage. Cryopreservation is an efficient and long-term alternative and has been elaborated at IPK for ten years. It coincides with the development of a simple droplet-vitrification protocol using *In vitro* plants as source material, the plant vitrification solution PVS 2 as cryoprotectant and aluminum strips as carrier material (1). Recently, the number of accessions exceeded 140; hence, 17 mint species are safely cryopreserved and show on an average 60% regeneration after rewarming. In course of the routine cryopreservation, selected factors were investigated. The variability of regrowth results is mainly caused by the initial plant quality, the duration of multiplication phase of *in vitro* plants prior to the cold hardening, precise shoot tip preparation and the avoidance of endophyte outbreak after rewarming. The best starting material for cryopreservation were *in vitro* plants from slow growth storage at 2 °C or 10 °C. Highest plant regrowth, up to 100%, was achieved when *In vitro* plants coming from 10 °C cold storage, were prepared for nodal culture and cultivated under changing temperatures at 25 °C/-1°C. The direct use of cold stored plants for explant preparation, without nodule culture, was not efficient. Under these conditions, multiplication and hardening was realized in a short-term period of two weeks before cryopreservation and endophytes outbreak could almost completely be avoided after cryopreservation. No relationship was found between regeneration results and the *in vitro* storage duration prior to sampling for cryopreservation. However, results significantly decline in dependence on the length of the last cold subculture (2). Other factors like genotype, recovery medium and addition of antioxidants to the preculture and dehydration steps had only minor impacts on overall regeneration. Furthermore the incubation time of explants in loading solution (20 min-120 min) and in PVS2 for 20-40 min at ambient temperature did not have significant effect on the regrowth. Comparison of the PVS2 incubation phases between room temperature and on ice, and between cooling of aluminum strips directly in LN or protected inside tubes, revealed no significant differences in regrowth. The final developed method is simple, suitable for routine use and applicable to all mint accessions.

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EQUIPMENT FOR CRYOPRESERVATION. UKRAINIAN EXPERIENCE PRACTICE

O Snurnikov*

"Interradiofizika" JSC, Kharkiv, Ukraine

The cryogenic instrument making industry is known to be a new branch of cryogenic technology to preserve and store various biological objects within many years. Among the preserved and successfully recovered biological objects used in practice there are the cell suspensions including nucleated red blood cells and bone marrow, as well as embryonic cell cultures, sperm, viruses (1). Of great importance is use of this equipment for the agriculture related studies (2). All these processes occur in cryobanks which are considered as hardware systems to ensure the cryopreservation, long-term storage and sale of biological objects, cryobiological researches. The main role in cryobanks is played by a programmable freezer, providing programmable, controlled, recorded, full-scaled reproduced cooling of the object down to a predetermined temperature. The history of low temperature equipment in Ukraine starts in the 6os of last century. The Kharkov Transport Equipment Plant has the experience of producing and developing the cryovessels since 1965 (3). Here there was comparatively analyzed the available publications starting from 1978, when the first all-purpose freezer allowing to automatically implement any cooling protocol within a wide temperature range was produced in Kharkiv (Ukraine). During the years the experience of applying this type of freezers had shown a contradiction between the tasks set and the device itself. The main problem was a human factor error. That time a cryoprotectant crystallization initiation was manually operated. (4) This paper covers the quick review of existing now cryogenic devices producing in Ukraine. These are the ZPM series, mainly applied nowadays in medical, biotechnological, veterinary, R&D centers. So, this goal of this paper was the attempt of summarizing the experience of using these devices in Ukraine. The attention is emphasized to describe the certain experience of Ukrainian scientists and technicians in this field.

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SURVIVAL RATE OF HUMAN VITRIFIED BLASTOCYSTS DEPENDING ON CHOSEN COLLAPSING METHOD

MP Petrushko, VI Pinyaev, TA Yurchuk*

Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv, Ukraine

Vitrification is the most promising method to cryopreserve the preimplantation human embryos. An embryo at the expanded blastocyst stage is a multicellular structure with the presence of blastocoel filled with plenty of liquid, making a successful cryopreservation difficult. In this connection, it is an expedient use the procedures helping to reduce blastocoel cavity prior to vitrification.

The purpose of this study was to evaluate the survival rate of human embryos at the stage of expanded blastocyst using various methods of collapsing prior to vitrification.

Blastocysts were vitrified on carriers («CryoTech», Japan) using the medium containing ethylene glycol, sucrose and dimethyl sulfoxide. Collapse of the blastocysts was performed by chemical, mechanical or laser methods. The survival rate after freeze-thawing of blastocysts was assessed by morphological criteria [Gardner D. et al., 1999], recovery of their volume and *in vitro* development. Cryopreservation efficiency was examined by the frequency of pregnancy onset.

Blastocysts before vitrification were divided into four groups: 1 - without collapse (control), 2 - 4 - with a collapse of the blastocyst cavity by chemical, mechanical or laser methods.

Clinical scores of patients in the studied groups (age, experience of infertility, number of aspirated oocytes) fertilization rate, the morphological characteristics of the embryos and the rate of their morphogenesis had no statistically significant differences in all groups studied.

The survival rate of blastocysts after freeze-thawing in the control Group 1 was (82.8 ± 4.4)% and Group 2 - 4 made (79.5 ± 6.8); (93.2 ± 5.3); (92.4 ± 4.7)%. Moreover, the index increase in the Groups 3 and 4 was significantly higher than in the first two groups.

The pregnancy rate in groups with chemical (71%) and laser (63%) type of a collapse was higher than in the control group (58%) and the group with mechanical collapse (54%).

Based on these findings we can conclude that the use of chemical and laser collapse of human expanded blastocysts before vitrification increases the survival rate of embryos and the pregnancy rate.

ATP LEVELS DURING FREEZING AND THAWING ACROSS THE DIVERSITY OF LIFE

F Bajerski[1,*], J Stock[2], B Hanf[3], T Darienko[4], E Heine-Dobbernack[1], S Eberth[1], M Lorenz[4], L Naujox[1], ERJ Keller[2], R MacLeod[1], M Schumacher[1], H-P Mock[2], O Kniemeyer[3], T Friedl[4], J Overmann[1]

[1]Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany
[2]Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany
[3]Leibniz Institute for Natural Product Research and Infection Biology e.V., Hans-Knoell-Institute (HKI), Jena, Germany
[4]Experimental Phycology and Culture Collection of Algae at the University of Goettingen (EPSAG), Goettingen, Germany

Organisms are exposed to low temperature and temperature shifts in their natural habitats, like in the deep sea, certain other waters, the polar regions, high altitude alpine sites or during diurnal/nocturnal shifts. The effects of cold stress and cryostress vary a lot within the tree of life and particularly the specific cellular processes during cryostress remain poorly understood. When temperatures drop below the freezing point, cells undergo several freeze-thaw cycles which induce thermal, mechanical and osmotic stresses. These effects occur also during cryopreservation. The present study, performed within the framework of the Leibniz Association-funded KAlT-project (acronym deduced from the German title: "**K**ryostress – **A**npassung der Ze**l**le an **T**iefsttemperaturen), aimed to improve the understanding of cellular processes during ultra-deep freezing using cryopreservation as a practical example to address the effects of freezing and thawing. Using ATP measurements as a universal physiological parameter, representatives of bacteria, fungi, algae, plant tissue, plant cell line and human cell line react similar to cryostress during freezing and thawing, despite completely different preconditions. Cryoprotectant treatment and freezing generally led to a decreased level of intracellular ATP, whereas low osmotic stress increased ATP in plant cell lines. Directly after thawing, ATP decreased further or slowly started to recover (fungi and bacteria). After a phase of regrowth, all cells reached the basic ATP level under optimum growth conditions. ATP is also increased during plant tissue development or in the exponential growth phase of bacteria. For the analyzed human cell line the changes were not significant. We deduce a four-step stress response that is reflected in the intracellular ATP level values: (1) At optimum growth, ATP is constant. This state is kept as long as possible. (2) At initial or low stress, ATP increases in preparation for the ensuing stress response. (3) At continuing stress, ATP decreases due to ATP consumption during the stress response reaction and ATP synthesis may decrease due to lower temperature. (4) If the stress maximum is reached, cell metabolism and division is shut down until cell death occurs. For two organism groups, viz. bacteria or algae, a different stress response in mesophilic and cold-adapted species was observed. The success of plant- survival in cryopreservation strongly depends on (osmotic) pre-acclimation. Monitoring the cryopreservation process with ATP as a universal physiological marker may help to predict regrowth success after cryopreservation.

EFFECT OF CRYOPRESERVATION ON FUNCTIONAL POTENTIAL OF FETAL NEURAL CELLS

Ie Porozhan*, M Ostankov, T Dubrava, A Goltsev

Institute for Problems of Cryobiology and Cryomedecine of the National Academy of Sciences of Ukraine

Cryopreservation is one of the technological steps to apply the fetal brain cells in clinical practice. This procedure enables storing a certified biomaterial within different time periods under low temperature bank conditions and using it as a demand. However, the cryopreservation causes a multi-vector effect on biological object's genomic profile, structure and function. The peculiarity of fetal neural cells (FNCs) when administered into a recipient is their capability to correct an immune status of recipient's body at autoimmune diseases, for example, multiple sclerosis (MS), via inhibition of T-cell reactivity. Of note is the fact that the FNCs are heterogeneous by composition of cells, responding differently to cryopreservation, that ultimately determines their functional potential. Therefore, our study was aimed to investigate a subpopulation composition, *ido* gene expression in FNCs cryopreserved by different regimens and their therapeutic effect in the model of experimental allergic encephalomyelitis, analogue of MS in human.

Experiments were implemented in FNCs of rats of 11 gestation days. The FNCs were cryopreserved with 10% DMSO by 2 regimens: R1 comprised the cooling with the rate of 1°C /min down to -80°C, and immersion into liquid nitrogen, R2 consisted of cooling with the rate of 1°C/min down to -9°C, stop for 10 min and then cooling with 1°C/min rate down to -25°C, 10°C/min down to -60°C and immersion into liquid nitrogen. The expression of FNCs phenotypic markers was assessed with flow cytometer (FACS Calibur, USA) using monoclonal antibodies to nestin, GFAP, β-tubulin III, CD44, CD106 and CD133 antigens.The *ido* gene expression in FNCs was assessed using RT-PCR. Experimental allergic encephalomyelitis (EAE) was induced by introducing homologous spinal tissue homogenate (50 mg) with complete Freund's adjuvant containing 2-4 mg/ml of Mycobacterium tuberculosis into the footpad. The disease pattern severity was evaluated by a five-point scale. The FNCs were administered intraperitoneally in a dose of $5x10^6$ cells per 100g of animal weight to day 14 after EAE induction. The intact animals and those with introduced adult neural cells at the same concentration were the control. In EAE animals prior to and after FNCs administration we determined a number of cells with $CD4^+Foxp3^+$ T-regulatory cells (Tregs) phenotype and their total fluorescence intensity (TFI). The pathology progressing and the therapy implemented were judged by a number of sensitized immunocompetent cells (ICCs) in the test system of neural cell culture.

The cryopreservation regimen R1 was shown to cause no effect on a number of cells with markers of $CD133^+$ and $nestin^+$-stem cells, however, it resulted in 1.7-fold reduction of the content of β-tubulin III^+ cells, referred to FNCs, differentiated into neuronal direction. Despite an increased in 11.4 times concentration of $GFAP^+$ glial cells, a number of $CD106^+$ and $CD44^+$ cells remained unchanged. When using the cryopreservation regimen R2 a number of β-tubulin III^+ cells reduced twice and the one of $GFAP^+$ cells increased in 4.7 times. In addition, a number of $CD133^+$ and $nestin^+$ cells increased in 3 and 2.4 times, respectively, and $CD106^+$ and $CD44^+$ cells did in 6.3 times. The very adhesive fraction of FNCs with $CD106^+$ and $CD44^+$ markers has the capability to produce the *ido* enzyme and activate Tregs. Our findings showed a 3-fold increase of *ido* gene expression in FNCs cryopreserved with R2. In the FNCs cryopreserved with R1 no significant changes in this gene expression were observed. The administration of FNCs, cryopreserved with both regimens provided a

desensitizing effect. However, the most pronounced immune-correcting effect was inherent in the FNCs, cryopreserved with R2. After their introduction a number of sensitized lymphocytes decreased 3 times as compared with the index in untreated animals. In addition, namely in this group there was a significant increase in a number and TFI of Tregs $CD4^{+}Foxp3^{+}$ to day 21 of EAE development. As a result, to day 35 we observed the maximum reduction of EAE clinical manifestations in the animals with administered FNCs, cryopreserved with R2.

Our findings testify to the fact that the varying of cryopreservation regimen enables a selective change not only of a cell composition of FNCs by phenotypic characteristics, but their functional activity as well. In particular, the redistribution of cell subpopulations towards increasing a number of stem and adhesive cells occurred in the R2 cryopreserved FNCs. There was an increase in the *ido* gene expression in FNCs, that stipulated the augmentation of functionally active Tregs in recipient's body. Namely this fact may explain a high degree of ICCs desensitization and the maximum reduction of EAE clinical manifestations. We consider a possible application of cryopreservation as a methodical approach in mastering the MS therapy.

REGENERATIVE POTENTIAL OF CRYOPRESERVED MESENCHYMAL STROMAL CELLS OF VARIOUS ORIGIN

NA Volkova*, AN Goltsev

Laboratory of Biotechnology and Applied Nanotechnology, Department of Cryopathophysiology and Immunology, Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkov, Ukraine

The aim of research was to comparatively evaluate the regenerative potential of cryopreserved mesenchymal stromal cells (MSCs) from bone marrow, fat and tendon.

MSC cultures obtained from rat bone marrow, fat and tendon were cryopreserved under protection of 10% DMSO and 20% FBS with cooling rate of 1 deg/min down to -80°C followed by plunging into liquid nitrogen. The thawing was done on water bath at 40°C up to the appearance of liquid phase. Proliferative characteristics were examined by MTT-test at 1, 3 and 10 hours. An assessment of type I and III collagen content was performed with monolayer MSCs using monoclonal antibodies to type I collagen with CF^{TM}488A and monoclonal antibodies to type III collagen with Goat anti-rat IgG Alexa fluore 647 conjugate according to the manufacturer's instructions. The fluorescent microscopy was performed by a laser scanning microscope LSM 510 META. The ability of MSCs to migrate was determined by filling the defect monolayer (0.2 cm^2) for 3 days. The results were processed with Student's t-test using Excel software.

Cryopreserved MSC from bone marrow had a slow proliferation (67.2 ± 4.5% monolayer), low migration ability and a high level of collagen type I and III synthesis (89.6 ± 2.7% and 73.6 ± 1.4% positive stained cells respectively). Cryopreserved cultures MSCs of tendon and adipose tissue had high proliferative activity (90.5 ± 3.1% and 96.2 ± 2.7% monolayer culture on day 10, respectively) and the ability to fill a defect in comparison with bone marrow MSCs. Synthesis of collagen type I in MSCs tendon and adipose tissues was lower than in bone marrow MSCs (52.6 ± 2.1% and 63.4 ± 2.7% of positively stained cells, respectively). Cryopreserved MSCs from bone marrow, fat and tendon kept ability in various degrees to adhesion, proliferation, migration and synthesis collagen I and III type.

Summarizing the obtained data we can conclude that while this study is not of a direct clinical significance, it should be considered as one of the attempts to explain the action of MSCs derived from bone marrow, fat and tendon, which are focused on the potential application of these cell sources for regenerative medicine.

CRYOPRESERVATION WITHOUT ICE CRYSTAL FORMATION – THE FOURTH ARRANGEMENT OF WATER IN BIOLOGICAL GLASS

J Zámečník*

Crop Research Institute, Prague, The Czech Republic

New cryopreservation methods are based on biological glass formation. Low water content is the prerequisite for involvement the glassy state in plant cytoplasm. The level of dehydration is close to the vital amount of water. The resistance to dehydration is crucial for higher regeneration of thawed plants from ultra-low temperatures. Knowledge of the glass transition temperature is useful not only for improving cryo-methods based on glassy state but also for conditions for long-term storage of shoot tips. The temperature induced glass is called the glass-transition temperature (Tg); molecular motion nearly ceases and the liquid becomes a glassy solid, important status for plant long-term storage. Glass transition (vitrification) is an example of the second order transition. Definition of the phase transition of the second order is when the first derivative of these variables (heat capacity and expansion coefficient) changes by jump. Three methods for glass transition temperature were used: standard Differential Scanning Calorimetry (DSC), Temperature Modulated DSC and Quasi-isothermal Temperature Modulated DSC. Both the TMDSC and the QITMDSC methods based on temperature modulation gave less ambiguous results, the QITMDSC gave better results for distinguishing between heat capacity for endothermic ice melting and glass transition. It is believed that plants can survive ultra low temperature by formation of biological glass, a biological matter excluding formation of ice crystals. So, successful cryopreservation methods are those which involve biological glass formation by dehydration or adding cryoprotectant (1,2). High plant vitality, after storing them at ultra low temperature, is important for safe plant biodiversity keeping.

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CRYOBANKING OF HOP GERMPLASM IN THE CZECH REPUBLIC

M Faltus[1,*], P Svoboda[2]

[1]Research Institute of Crop Production, Prague, Czech Republic
[2]Hop Research Institute, Co., Ltd., Žatec, Czech Republic

Hop (*Humulus lupulus* L.) belongs to the most important crops in the Czech Republic. This crop is propagated and maintained vegetativelly. Conservation of hop germplasm in the field collections increases the risk of accidental lost of valuable genotypes. This risk can be reduced using the method of cryopreservation, which allows safe storage of the plant samples at ultra-low temperatures. This method contributes to the conservation of genetic stability and prevent from ageing. It is used for conservation of genetic resources of cultural and wild plants in such virus free material, which is endangered by biotic and abiotic stresses if multiplied in field conditions. Simple cryopreservation method was used for cryopreservation of selected genotypes of the Czech hop germplasm collection. Nodal cutting were acclimated by low temperature and sucrose treatment. Isolated shoot tips were loaded with 0.7M sucrose for overnight and simultaneously dehydrated above silicagel for approximately 100 minutes on aluminium plates. Shoot tips were plunged directly into liquid nitrogen. Control explants were thawed at 40 °C water bath and regenerated on medium for 8 weeks. Altogether 42 hop genotypes have been cryopreserved with average recovery rate of 40%. 79% of accession showed higher plant recovery than 30%. The minimal number of plants to recover for each cultivar was calculated as a sum of minimal numbers of viable plants in particular cryopreservation procedures according to a probability tool developed by Dussert et al. (1). Altogether of 7720 hop apical shoot tips were stored in cryobank. The methods used and results are presented.

Acknowledgement: This study was supported by the research project QJ1630301 of the Ministry of Agriculture of the Czech Republic.

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SUCROSE PRECULTURE IMPROVES REGENERATION OF ALOE AFTER DROPLET VITRIFICATION

R Folgado*

The Huntington Library, Art Collections and Botanical Gardens, 1151 Oxford Road, San Marino, CA 91108, USA

All *Aloe* species (except for *A. vera* L.) are potentially threatened due to restricted distribution ranges, habitat destruction and fragmentation, collecting for horticultural purposes, invasive alien encroachment, and harvesting for medicinal uses. *Aloe veseyi* Reynolds is confined to the southern end of Lake Tanganyika. It is a cliff-dwelling aloe that grows hanging from rocks and vertical cliff-faces at altitudes of 840–1500 m. A protocol for cryopreservation of shoot tips of *Aloe veseyi*, using a droplet-vitrification procedure was developed. Apical shoot tips of 1 mm size from 5-week-old *in vitro* aloe plantlets were exposed to loading solution for 20 min at room temperature, dehydrated with PVS2 for different times (from 0 to 60 min) at 0 °C, transferred to aluminium foil strips and directly plunged into liquid nitrogen. For re-warming, aluminium strips were rinsed in unloading solution for 20 min at room temperature. Explants were transferred to recovery medium and kept in the dark for 1 week. The best results were obtained after 45 min of exposure to PVS2. In a second assay, *in vitro* donor plants were cultured in control medium or sucrose-supplemented medium. After two weeks of treatment, water content and biomass were recorded, and the results showed the donor plants respond to the osmotic stress. Also, shoot tips were excised and cryopreserved following the method described above using 45 min of exposure to PVS2. Shoot tips regrowth was observed within 2 weeks (control, 73 %; Sucrose, 97 %) and new shoots were regenerated within 5 weeks (Control 37 %; Sucrose 63 %). Plant recovery was recorded 3 months after the rewarming: Control, 10 % and Sucrose, 33 %. The plant recovery was affected by oxidation during the recovery time. The preculture of donor plants in sucrose-supplemented medium improved the regeneration of the explants. However, the shoot tips oxidation was not entirely overcome, and some of them died during the recovery due to blackening. The addition of antioxidants to the recovery medium could minimise the damage during the regeneration of the explants after rewarming.

INFLUENCE OF CRYOPRESERVATION ON SURVIVAL OF PLACENTAL, UMBILICAL CORD, AND FETAL MEMBRANE EXPLANTS, AS WELL AS PLACENTAL CELLS WITHIN SPHEROIDS AND ALGINATE MICROSPHERES

V Yu Prokopyuk, D Pogozhykh*, O Pogozhykh, IB Musatova, LG Kuleshova, OS Prokopyuk

Institute for Problems of Cryobiology and Cryomedicine NAS of Ukraine
Institute for Transfusion Medicine, Hannover, Germany

Current development of regenerative medicine and cellular biology raises importance of cryopreservation of cells and tissues of placenta, umbilical cord, and fetal membranes. Such biomaterial is widely used in the stem cell generation, pharmacological studies, auto-banking, as well as in the wide range of clinical and scientific research. Fetal membranes are successfully used as scaffolds for tissue engineering constructs, and allografts are used in reconstructive surgery and ophthalmology. The aim of the study is to compare the survival of placental, umbilical cord and membrane explants, as well as spheroids and alginate microspheres containing placental cells after cryopreservation.

The explants of human placenta, umbilical cord and fetal membranes were obtained after normal delivery from 12 placentas of women aged 18-35 years with their informed consent. Spheroids were obtained using hanging drop method. Alginate microspheres were obtained by polymerization of 1% sodium alginate containing 10^6cells/ml in 10% $CaCl_2$. Morphological evaluation of survival was performed with application of vital staining techniques with trypan blue, propidium iodide, neutral red, fluorescein diacetate. Functional state was analyzed, using the MTT and resazurin reduction tests, evaluation of the level of glucose in incubation medium, activity of lactate dehydrogenase and alkaline phosphatase before and after cryopreservation. Cryopreservation protocol with 10% DMSO in the culture medium and 1K/min cooling rate was applied.

Cryopreservation by the developed technique allows preserving the structure and metabolic activity of placental tissue, umbilical cord and fetal membrane explants. The integrity of spheroids was compromised after cryopreservation, but the individual cell viability has been retained. The integrity of the alginate microspheres has been retained, however, the number of viable cells was significantly reduced. Thus, intercellular substance has an effect on the safety of cells and tissue structure.

MTT-test, resazurin reduction test and absorption of glucose from the incubation medium were the most informative methods for assessment of studied explants, as the objects for low-temperature biobanking. Histological studies were sufficient for assesment of survival of placental villi. Vital staining may be used as an express test for tissues, e.g. placental villi and amniotic membranes, where individual cells are visualized in the native preparations.

UTILIZATION OF A NEW FREEZING PROTOCOL CONTAINING HIGHER CONCENTRATIONS OF DMSO TO CRYOPRESERVE HUMAN OVARIAN TISSUE: PRELIMINARY RESULTS

CA Amorim[1,*], F Paulini[1,2], M Balcerzyk[3], ALP Gallego[3], CM Lucci[2], MM Dolmans[1], R Risco[3,4], A Corral[3]

[1]Pôle de Recherche en Gynécologie, Université Catholique de Louvain, Brussels, Belgium
[2]Physiological Sciences Department, Institute of Biological Sciences, University of Brasília, Brasília, Brazil
[3]National Center for Accelerators, Seville, Spain
[4]Engineering School of Seville, Seville, Spain

Conventional freezing protocols containing low concentrations of DMSO have been applied to preserve ovarian tissue from cancer patients needing to undergo chemo/radiotherapy. However, several studies have reported that such techniques can damage granulosa and stromal cells and have a negative impact on the formation of theca cells. The goal of this study was therefore to evaluate if modifying our freezing protocol (1) could improve follicle survival after cryopreservation and xenotransplantation. For this, we used ovarian tissue from 8 adult patients, frozen either using our original protocol [1] (protocol 1) or a modified version thereof (protocol 2), containing a higher concentration of DMSO (20% instead of 10%), larger volume of cryopreservation solution (1.8ml instead of 0.8ml), and lower seeding temperature (-11°C instead of -8°C). After thawing, the ovarian fragments were xenotransplanted to four SCID mice for 3 weeks. After grafting, follicles at all stages of development were found. A total of 4600 follicles were counted and classified in both protocols. In protocol 1, 55% (63/114) of follicles from fresh biopsies were primordial, 39% (44/114) primary, and 6% (7/114) secondary. In frozen-thawed and grafted ovarian tissue, 45% (442/973) of follicles were primordial, 53% (511/973) primary, and 2% (20/973) secondary. In protocol 2, 57% (493/863) of follicles from fresh biopsies were primordial, 40% (345/863) primary, and 3% (25/863) secondary. In frozen-thawed and grafted ovarian tissue, 34% (749/2194) of follicles were primordial, 57% (1241/2194) primary, and 9% (204/2194) secondary. A significant decrease in the primordial follicle population and significant increase in the population of growing (primary and secondary) follicles were observed with both protocols after xenografting compared to fresh controls. Although a statistically lower proportion of primordial follicles was found after freeze-thawing and xenografting with protocol 2 compared to protocol 1, the percentage of follicle activation (Ki67-positive primordial follicles) was significantly higher with the first protocol. Evaluation of follicle apoptosis by caspase-3 immunostaining showed the number of atretic primordial and primary follicles to be higher after xenografting when protocol 1 was applied for ovarian tissue cryopreservation. Our preliminary results show that it is possible to freeze human ovarian tissue using higher concentrations of DMSO. However, further analyses (follicle healthy status, stromal tissue quality and vascularization) are ongoing to determine whether such a protocol may prove more appropriate for the preantral follicle population.

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HYPOTHERMIC TREATMENT TO IMPROVE CULTURE EXPANSION

KT Mahbubani[1,2,*], P Kilbride[3], NKH Slater[2], K Saeb-Parsy[1], J Morris[3]

[1]Department of Surgery, University of Cambridge, Cambridge, United Kingdom
[2]Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, United Kingdom
[3]Asymptote Ltd., St John's Innovation Centre, Cambridge, United Kingdom

Culture expansion of mammalian cells will likely play a significant part in the development of personalised and regenerative medicine. To ensure it meets the potential, it is critical that culture processes are scalable and able to produce clinically relevant biomass rapidly with minimal time and cost whilst still maintaining critical biological functionality.

While hypothermia is known to result in many negative conditions – such as decreased viability, metabolic damage, apoptosis and necrosis – all of which are commonly seen after cryopreservation or after extended periods of hypothermia. It seems that short-term exposure to hypothermic conditions results in a deviation from this expectation.

Freshly passaged flasks of Chinese hamster ovary cells were cultured as a serum-free suspension either experienced hypothermic conditions (placed in an ice bath at 4°C) or were kept as a control (held at room temperature at 20°C) for up to 15 minutes prior to returning to the incubator to allow for cell expansion. Cells were enumerated daily using a haemocytometer and trypan blue exclusion stain to determine viability. Three days post-treatment, CHO cells exposed to the hypothermic conditions showed an increased growth rate of up to 12% while maintaining cell viability above 85%. The control condition also maintained this high cell viability with a lower growth rate.

These findings could rapidly decrease time for culture expansion and ultimately impact the associated costs. Additionally it further raises the question about the associated effects on downstream processes, not just in industry but also in clinics such as how cells and tissues are handled when being transferred. Optimisation of the exposure time to hypothermic conditions is being considered alongside other cell types to determine a clearer understanding and answer these questions.

A CRYOGENIC COLD CHAIN FOR CLINICAL DELIVERY OF REGENERATIVE MEDICINES

GJ Morris*

Asymptote Ltd., St John's Innovation Centre, Cambridge, United Kingdom

Asymptote Ltd is developing the equipment necessary for a Good Manufacturing Practice (GMP) compliant cryogenic cold chain with particular application in regenerative medicine (RM). This cold chain will exploit Stirling cryocooler technology and will thus overcome the contamination issues associated with use of liquid nitrogen. Compared with conventional liquid nitrogen systems this equipment is more accurate, allows active control of the freezing process and can be integrated with robotic systems. GMP compliant devices are required at many stages in the cold chain for regenerative medicine:

Autologous sourced cells: cryopreservation in the operating theatre of the source material for autologous treatments will optimise cell quality. Cells are either subsequently isolated and expanded (T cell therapies) or transplanted (ovarian tissue, parathyroid). This requires an operating theatre compliant controlled rate freezer with theatre compliant data collection and validation via a tablet.

Allogeneic cells: master cell banks (MCB) and working cell banks (WCB), consisting of 50 to 500 cryovials need to be processed using a controlled rate freezer with minimum sample to sample variation and validation of the process parameters.

Manufacturing: for many allogeneic and autologous treatments it will be necessary to cryopreserve the cell manufactured product, this may require cryopreservation in cryovials (up to 500); bags (5 to 500ml fill volume) or cassettes (up to 2000 ml).

High quality outcomes (functionality and viability) from the controlled rate freezers require controlled ice nucleation: Asymptote has identified suitable materials to achieve this which can either be added to the cryocontainers in the form of beads or may be coated on the freezing containers (cryovials, bags, etc).

In all applications validated equipment for controlled cell thawing, especially of large cell volumes, is required by the end user. Current practice often involves water baths, which generally are not validated and also cannot be used in clean rooms.

Data will be presented with ovarian tissue, human embryonic stem cell lines, T cells, engineered T cells, and alginate encapsulated hepatocytes processed in this new equipment.

CRYOSENSITIVITY OF TISSUE-ENGINEERED COLLAGEN CONSTRUCTS WITH MULTIPOTENT STROMAL CELLS

VV Mutsenko[1,3,*], O Gryshkov[1], S Knaack[2], M Gelinsky[2], A Yu Petrenko[3] and B Glasmacher[1]

[1]Institute for Multiphase Processes, Leibniz Universität Hannover, Hannover, Germany
[2]Centre for Translational Bone, Joint and Soft Tissue Research, Technische Universität Dresden, Dresden, Germany
[3]Institute for Problems of Cryobiology and Cryomedicine of National Academy of Sciences of Ukraine, Kharkiv

The current trends in bone tissue engineering demonstrate that to produce functional bone tissue substitutes the most perspective is to use biomaterials with close structural resemblance to human bone tissue and multipotent stromal cells with high osteogenic potential. From a translational perspective, there is a growing need in logistically efficient low temperature storage strategies for such products to meet experimental and clinical needs. Following these ideas, we have produced a novel 3D collagen-hydroxyapatite porous scaffolds and have shown their biocompatibility with the non-human primate *Callithrix jacchus* amnion-derived multipotent stromal cells (MSCs). Accordingly, the research aim of the present work was to investigate the possibility of cryopreservation of such tissue engineered constructs as a model for providing "ready to use" bone substitutes.

Scaffolds has been prepared by the method comprising simultaneous collagen fibril reassembly and mineralization. Amnion-derived MSCs were seeded onto scaffolds with a static seeding method (2×10^5 cells/scaffold). The cells were allowed to colonize the scaffolds within 24 h and subsequently equilibrated with culture medium-based cryoprotective solution containing 10% Me_2SO/20%FBS on ice for 10 min. The samples were frozen in a standard cryovials using Mr. Frosty™ Freezing Container and stored for 5 days at -152°C followed by thawing at a water bath. Cell viability within constructs was evaluated by live/dead staining followed by quantitative analysis of confocal microscopy images using ImageJ software.

After cryopreservation the viability of attached MSCs reduced by 20% when compared to non-frozen samples. Moreover, partial disintegration of scaffolds *per se*, affected cell cytoskeleton and cell detachment were observed by SEM and confocal microscopy. In addition, a 2D model with a cell monolayer on glass coverslips has been utilized to determine the response of adherent MSCs to freezing injury using cryomicroscopy. Intensive intracellular ice formation and subsequent significant reduction of cell viability estimated by trypan blue were observed.

The findings suggest that MSCs frozen in attached state within 3D scaffolds are much more cryosensitive when compared to cells frozen in suspension. The future work will be directed towards optimization of cryopreservation protocol to provide off-the-shelf availability of 3D stem cell-based tissue-engineered bioequivalents.

HIGH PERFORMANCE VITRIFICATION TECHNOLOGY POTENTIALLY SUITABLE FOR STEM CELLS

R Risco[1,*], MG Vilches[2], M Gallardo[3], D Barranco[1], A Corral[1], A Lucas[1], M Hebles[3], P Sanchez[3]

[1]National Acceleratos Center (CSIC-US), C/ Thomas Alba Edison, 41092 Seville, Spain
[2]Complejo Hospitalario Torrecardenas, C/ Hdad. de Donantes de Sangre, 04009 Almeria, Spain
[3]Ginemed Clinicas, C/ Farmaceutico Murillo Herrera 3, 41010 Sevilla, Spain

Human oocytes and adult C. elegans nematods had in the past extremely low recovery rates when slow freezing was used. These two very different biological systems have now recovery rates 100% circa when SafeSpeed technology is used. This technology has been developed for being independent of the biological system. This wide range of application of this technology compel us think that its use in other fields, when practically possible, shows the same good results. Non-equilibrium vitrification systems developed until now relies upon the fact of achieving high cooling rates. Therefore, open systems were designed in the past under this framework. However, its opened character makes them quite unpractical for same situations, not to say about the potential risk of contamination. The technology presented here relies on a very high performance in the *re-warming* rate, not in the cooling rate, and has the important property of being closed, making them suitable for a wide range of practical applications, from adult C. elegans cryopreservation to human embryos and oocytes, not only for its aseptic storage conditions, but also for its practical way of loading the sample, contrary to opened systems. We will display here how it was used in human oocytes, embryos and worms, and the results obtained. Then we will see possible scenarios in regenerative medicine where it can be useful. After incubation of the sample in the chosen cryoprotectant, it is loaded inside SafeSpeed by aspiration. Then this device is sealed at both ends and quenched into liquid nitrogen. Two important parts of this container are a devoted area for writing the sample identification (patient, …), and a slide-able protector to guarantee its robustness inside the storage tank. For re-warming, the protector is removed, the container is moved from liquid nitrogen to a 37 ºC water bath, two both ends opened and the sample expelled into the washing solutions. The results are, for oocytes (with which afterward an ICSI was performed) and embryos, the same as the fresh control group, giving rise to the standard rate of live born babies in case of non-cryopreserved material; for larvae and adult C. elegans, above 90% recovery rate in both cases. Even the memory of the worms was shown intact after cryopreservation by this technique.

APPLICATION OF PLANT-DERIVED ANTI-FREEZE PROTEINS IN MSC CRYOPRESERVATION

Y Naaldijk[1], A Friedrich-Stöckig[2], A Stolzing[1,3,*]

[1]Leipzig University, Germany
[2]Fraunhofer Institute for Cell Therapy and Immunology, Leipzig, Germany
[3]Centre for Biological Engineering, Wolfson School, Loughborough University, UK

Regenerative medicine requires the cryopreservation of a range of cell types, many of these are sensitive to freezing. Mesenchymal stem cells (MSC) are investigated for many different therapies and tissue engineering applications. Storing and banking of MSC is an important step to minimize costs in allogeneic applications. Conventional cryopreservation solutions include use of cryoprotectants such as dimethylsulfoxide (Me2SO), but have not prevented ice-induced damage to cells during freezing. Antifreeze proteins (AFPs) may provide a solution for icing problems as they can protect cells by inhibiting ice formation. We have investigated AFP derived from various plants induced to produce AFPs. AFP production was introduced either by chemicals or environmental clues in root cultures. Cryoprotectant solution cocktails containing combinations of AFPs and M2SO were used to freeze MSC using slow or fast freezing protocols. M2SO concentration could be reduced to 1% without any loss of viability post-thaw or differentiation ability of MSC. These findings support the potential of incorporating AFPs in solutions used to cryopreserve MSC.

TOWARDS AN UNDERSTANDING OF HETEROGENEOUS ICE NUCLEATION IN CRYOBIOLOGY

TF Whale[1,*], BJ Murray[1], TW Wilson[1], MA Holden[1], J Morris[2]

[1]School of Earth and Environment, University of Leeds, UK
[2]Asymptote Ltd. Cambridge, UK

Ice formation is a process of vital importance in the majority of cryobiological procedures and systems (1). Ice nucleation, which necessarily precedes crystallisation of ice will be caused by heterogeneous ice nucleation in the vast majority of cryobiologically relevant systems as the presence of container walls and biological entities will usually induce nucleation before sufficiently low temperatures for homogenous nucleation are reached. While the ice nucleating efficiencies of many substances are known it is generally not known why some substances nucleate ice better than others (2). As a result, it is very difficult to predict the temperature at which ice will nucleate in any given system. Given that ice formation, and therefore ice nucleation, are such key processes throughout cryobiology improved understanding of ice nucleation is potentially of much interest.

Here, we present the results of several new studies aimed at improving the understanding of heterogeneous ice nucleation by certain substances. While this work has been principally directed at species relevant to ice nucleation in clouds much of what has been learnt is potentially of relevance to cryobiological systems as well. It has been shown that features related to certain types of defects are largely responsible for ice nucleation observed on some minerals. Such knowledge may lead to better understanding of what causes ice nucleation in cryobiological systems and may therefore facilitate control of ice nucleation in such systems. Additionally, it has been shown that solutes have the potential to profoundly affect ice nucleation by a range of different ice nucleants. Solutes can both enhance and weaken ice nucleation efficiency. The relevance of these new findings to cryobiological procedures is discussed, in particular the potential importance of cryoprotectant choice for control of ice nucleation temperatures.

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DEVELOPING A COMMERCIALLY FEASIBLE CRYOPRESERVATION PROCESS FOR A BIOARTIFICIAL LIVER SUPPORT DEVICE

A Picken[1,*], Q Liu[1], A Iftimia-Mander[1], M Ginai[1], S Lamb[2], A Stolzing[1], J Morris[2], N Medcalf[1]

[1]Centre for Biological Engineering, Wolfson School of Mechanical, Electrical & Manufacturing Engineering, Loughborough University, Leicestershire, United Kingdom
[2]Asymptote Ltd, Cambridge, United Kingdom

Liver failure is a deadly consequence of several liver diseases including alcoholic liver disease and cancer. If acute liver failure cannot be reversed through supportive treatment, then liver transplantation is the only viable treatment option. Donor livers are in finite supply, with an uncertain duration between onset of disease and the identification of a suitable donor organ. Bioartificial liver (BAL) support aims to provide the biosynthetic and metabolic functions of the liver to liver failure patients as a bridge to transplant, or to support native liver regeneration in the absence of transplant.

BALs contain living cells and consequently require an effective preservation method to avoid degradation of the product during storage & shipping after their manufacture. Examples of chilled (+4°C to +8°C; ELAD, Vital Therapeutics, Inc.) or warmed (37°C; AMC-BAL; Hep-Art Medical Devices B.V.) preservation have been described. However, these preservation strategies do not facilitate the long-term (>12 months) stability necessary for a truly "off-the-shelf" BAL.

Here we describe our efforts to develop a commercially feasible cryopreservation process for alginate-encapsulated HepG2 cell spheroids in cryobags. We consider challenges beyond lab-scale feasibility, including scale-up; process operability; patient risks (e.g. from raw material selection); and commercial risks, which include manufacturing cost and the costs associated with regulatory compliance. In particular, we highlight a pragmatic development approach incorporating (1) improved devices for LN2-free cryobag freezing; (2) solid-state thawing; (3) Ice-nucleation control using IceStart™; (4) Design of Experiments; (5) analytical method suitability and (6) use of Cost-of-Goods modelling to guide process development.