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THERMAL ANALYSIS FOR CRYOPRESERVATION METHOD IMPROVEMENT

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Changes in thermal characteristics of plant tissues are important for explants successful cryopreservation. These changes can be influenced by genotype of plant, preculture conditions of explants and osmotically active solution used. The effect of genotype was studied in three grapevine varieties with different sensitivity to dehydration. The effect of preculture conditions was evaluated in poplar explants by means of three different ways of preculture: 1) no treatment, 2) low temperature treatment, 3) simultaneous low-temperature and osmotic treatment. Grapevine shoot tips were acclimated osmotically on media with gradually increased content of sucrose (0.3, 0.5, 0,75M) during three days. Shoot tips of both species were subsequently transferred into series of cryoprotective solution: LS - Loading Solution, 50%PVS3 and 80%PVS3. In case of grapevine experiments, one more treatment was included between LS and 50%PVS3 treatments: VLS - *Vitis* Loading Solution (1). Thermal characteristics - melting point temperature and proportion of freezable water (percent of the fresh weight) were determined using a differential scanning calorimeter Q2000 (TA Instruments, USA). Different sensitivity of grapevine varieties to dehydration influenced significantly their tolerance to cryopreservation. A significant effect of explants acclimation on their tolerance to dehydration before cryopreservation was detected in poplar explants. Thermal analysis allowed control of the whole dehydration process, including optimal hydration level of explants before cryopreservation. This work was supported by the research project of Ministry of Agriculture of the Czech Republic QJ1630301.

(1) Wang QC *et al.* 2003 *CryoLetters* **24**, 293-302.

CRYOPRESERVATION WITHIN THE MARINE ENVIRONMENT: THE SEA URCHIN

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Asahina and Takahashi (1) on sea urchin sperm are the pioneers in sea urchin cryopreservation. It was from the 90's onwards when the number of references began to increase research in this topic. Nowadays, 11 species of sea urchins have been studied for cryopreservation: the most common are *Hemicentrotus pulcherrimus, Strongylocentrotus nudus and Stongilocentrotus intermedius* , *Tetrapigus niger*, *Evechinus Chloroticus* or *Paracentrotus lividus*.

Paracentrotus lividus has been the main focus of our research since 2008; we have developed a cryopreservation protocol for early blastula embryos. This species is used for research, ecotoxicology studies and it actively recollected in the Spanish northwest coasts generating a direct annual profit of 1 million euros just in the Spanish region of Galicia.

Paredes and Bellas (2,3,4,5) established a protocol for blastula cryopreservation using 1.5M Me_2SO with 40 mM trehalose obtaining larvae that developed more slowly than unfrozen controls within the first 48 hours, then the larvae grow achieving in average 50% the size of the unfrozen controls after 96 hours. The percentage of abnormalities during larval development is minimal and once incubated to juveniles, after 20 days post-thaw 25% larvae settled into juveniles. These embryos were used for embryo-larval ecotoxicological bioassays with several different pollutants and Paredes and Bellas (3) proved that

they can obtain a sensitive response to pollutants and obtain a Dose-Response curve to calculate parameters like EC50 EC10. Cryopreserved embryos produced a more sensible response to pollutants than fresh embryos.

Sea urchins are a very useful model organism: easy to obtain from nature, easy to work with, very fertile and their embryos and larvae are easy to culture. On the negative side due to their marked reproductive seasonality it is impossible to collect mature sea urchins all year-round and maintaining a stock of mature sea urchins all year round is very difficult and expensive when possible. The establishment of biobanks in those facilities where sea urchins are used as model organisms would help overcome the constraint of biological material supply all year round.

An intense research effort is needed towards widening the knowledge of sea urchin membrane parameters, modelling the addition and dilution of cryoprotecting agents, studies of membrane composition, long term viability of cryopreserved cells in order to increase the number of species/cell types that are available to biobank and use when necessary.

(1) Asahina E, Takahashi T (1977) *Cryobiology* **14**, 703. **(2)** Paredes E, Bellas J (2014) *CryoLetters* **35-6**, 482-494. **(3)** Paredes E, Bellas J (2015) *Chemosphere* **128**, 278-283. **(4)** Paredes E, Bellas J, Costas D (2015) *Aquaculture* **437**, 366-369. **(5)** Paredes E (2015) *J.Mar.Sci.Eng.* **3**, 1-x manuscripts; doi:10.3390/jmse30x000x.

UTILIZATION OF A NEW FREEZING PROTOCOL CONTAINING A HIGHER DMSO CONCENTRATION TO CRYOPRESERVE HUMAN OVARIAN TISSUE

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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 11 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 six SCID mice for 3 weeks. After grafting, follicles at all stages of development were found. 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. Results regarding follicle density, development, ultrastructure and function were similar between treatments. Moreover, results of tissue fibrosis and vascularization did not show any difference between protocols. In conclusion, our study showed that while the higher DMSO concentration did not improve the survival of preantral follicles, it also did not seem to induce any major toxicity to the follicle population.

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TISSUE AND ORGAN CRYOPRESERVATION BY MEANS OF X-RAY COMPUTED TOMOGRAPHY

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One of the main goals in Cryobiology is the cryopreservation of tissues and bulky organs. The economic and social benefits of having a biobank are numberless. Nevertheless, the complexity and dimensions of these samples make their cryopreservation procedures very challenging, being crucial to have an exhaustive control of the heat and mass transfer processes (1). We have used X-ray Computed Tomography as a technique to monitor some of the parameters involving the cryopreservation procedures. The main characteristic of our device is the use of a low voltage compared with conventional CTs (75 kV), although a medical CT scanner (120 kV) has also been tested for this purpose. This imaging technology allows us to assess the concentration of vitrifying solutions containing dimethyl sulfoxide (Me_2SO), one of the cryoprotectants most used in the last decades. The sulfur atom of this molecule has a higher atomic number (Z) than those of other cryoprotectants, usually alcohols (C, O, H). Therefore, the X-ray attenuation obtained for Me_2SO solutions is proportional to its concentration, either at room and cryogenic temperature (below -140 ^{o}C) (2). Based on the same principle, the difference of the attenuation between the cryoprotectant and water, it is possible to detect the ice formation within tissues during the cooling and warming processes. On the other hand, the CT technique is also able to give important information in the case of eventual fractures due to mechanical stresses, which becomes more relevant in samples of big volumes such as tissues and organs.

We have studied different applications of this technology to Cryobiology. We monitored the perfusion of a rabbit kidney with a Me_2SO solution through the vascular system and visualized the formation of ice crystals (up to 2 µL) inside vitrified kidneys. We have also used X-ray Computed Tomography for ovarian tissue cryopreservation: we optimized the current slow freezing procedures used to date and developed a different procedure based on a slow vitrification, consisting of a gradual increase of the vitrification solution concentration while decreasing the temperature. Finally, we have characterized the cooling process for a Me_2SO solution in order to minimize the formation of fractures by studying the influence of some parameters: cooling rates, type of vials and insulating containers, degasification and orientation of the container.

To summarize, X-ray CT technique has been proved to be an excellent tool to monitor and optimize any procedure of tissue and organ cryopreservation.

FEATURES OF HUMAN RED BLOOD CELLS MEMBRANES CHARACTERISTICS IN THE TEMPERATUTE RANGE FROM 12 TO 8°C

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Due to high cholesterol content calorimetric and X-ray tests do not reveal thermotropic transitions in human erythrocyte membranes in the range of positive temperatures. However a break in Arrhenius plots for different characteristics with a change in the slope indicates the existence of such structural

transitions. We studied temperature dependence of water molecules exchange time by erythrocytes. Our results show that in the range of temperatures from 12 to 8°C Arrhenius graphs of this characteristic undergo a break with a significant increase in the activation energy of the process at temperatures below 8°C (1). We have also demonstrated Arrhenius plots fractures at temperatures from 12 to 8°C for the human erythrocytes permeability to cryoprotectants with an increase in the activation energy in penetration of these substances in the sub-zero temperature range (2). Data on the erythrocytes spherical index distribution also confirmed the existence of temperature dependent changes in the erythrocyte membranes that affect their shape. The most significant changes were observed in the temperature range from 12 to 8°C. The number of cells with a small spherical index significantly increased. In this regard we carried out microscopic analysis of erythrocytes. The temperature of the sample was gradually decreased from 37 to 0°C. We observed sudden rouleaux disruption at 12°C (3). We assumed that at temperatures from 12 to 8°C the ratio between the area of the outer and inner membrane monolayers of the erythrocyte membrane changes. In accordance with the hypothesis of a bilayer pair, this leads to the rapid formation of stomatocytes by mirror bulging of one of the erythrocyte central concavity. The repulsion forces that arise during the instantaneous transition from a discocyte to stomatocyte are sufficient for the rouleaux disintegration. In agreement with our light microscopy observation, electron microscopy data confirmed a sharp increase in the stomatocytes number to 30% at 12°C and up to 50% at 8°C compared to 1-3% at 37°C. Using the known value of human red blood cells surface charge density, we have calculated erythrocyte surface potential in a wide range of temperatures. Our calculations show the existence of the peculiarities in the temperature dependence of the surface potential in the range from 12 to 8°C (4). We suggest that disturbance of the balance of electrostatic interactions on the membrane outer monolayer surface may be a trigger mechanism for rearrangements and changes in the interactions between the protein and lipid components of the membrane.

SMART FREEZING SYSTEMS FOR CRYOBIOLOGY

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Besides application of cryoprotective agents, the process of cryopreservation of cells and living tissues includes application of cooling rate for freezing of sample as well as heating rate for thawing upon request. These parameters significantly must be controlled precisely. The most commonly used devices for freezing include freezing containers such as CoolCell® LX and Mr. Frosty™, which allow freezing at slow cooling rates up to 1 K/min in a -80°C freezer without an active control over the cooling rate. If precise control of cooling rate is required, the following liquid nitrogen based devices can be used, namely CM-2000®, Planer® Kryo 560-16 and Askion C-line® WB 230. Other systems such as Asymptote VIA Freeze™ are based on Stirling process, which does not require any liquid nitrogen supply. In this work, a comparison of different commercial freezing systems regarding cooling rates, precision, special functions and running costs has been performed to show main similarities, differences and possible areas of application. For example, CoolCell® LX and Mr. Frosty™ are suitable for cooling rates around 1 K/min. The Asymptote VIA Freeze™ can be used in the temperature range between 0.2 to 2 K/min. The controlled rate freezers CM-2000® and Planer® Cryo 560-16 show their strengths in the region of higher cooling rates. The Askion C-line® WB 230 is particularly suitable for nonstop usage. The measurements have shown that the ice nucleation temperature depends on the applied freezing device. At 1 K/min the temperature of ice formation of water is between 0°C and -8°C for Planer® Kryo 560-16, CoolCell® LX and Mr. Frosty™, whereas the ice formation takes place between -5°C and -15°C for Askion C-line® WB 230 and Asymptote VIA Freeze™. It has to be considered that the configured cooling rates of all systems are often apart from the real cooling rates in the middle of the samples (cryovial). This effect is more pronounced at higher cooling rates. It is important to note that the manufacturers often use different temperature ranges to define the cooling rates of their devices. In summary, user assisted measurement of

cooling and thawing rates have to be performed while using the above-mentioned devices to gain comparable results.

CHARACTERISATION OF CELLS DERIVED FROM CRYOPRESERVED PLACENTAL TISSUES

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Placenta, foetal membranes, umbilical cord, and multipotent stromal cells (MSCs) of placental origin are increasingly attracting the attention of researchers and clinicians worldwide. Possibility of obtaining large amounts of tissues without invasive and traumatic procedures, high proliferation potential and low immunogenicity of the cells of placental origin, capacity of isolation of MSCs in amounts sufficient for therapeutic purposes are among numerous advantages of placental material.
Currently there is a demand for development of efficient technologies for cryopreservation and storage of placental tissue, umbilical cord, and foetal membranes with possibility of further extraction of the living cells, their cultivation, expansion, and characterisation.
At this work we analysed the possibility to cryopreserve various tissues of placental origin. Life-dead staining showed significant amounts of viable cells in the studied samples after cryopreservation. Therefore, additionally, we showed the possibility of cell isolation from cryopreserved placenta, amnion, and umbilical cord tissues. Obtained cell populations were characterized for MSC origin. It was shown, that cells extracted from cryopreserved tissues possessed similar phenotypic characteristics to the cells extracted from the native (non-cryopreserved) tissues.

VITRIFICATION FOR STEM CELLS – NEW PROCEDURES AND TECHNOLOGIES

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Cryopreservation is still the only method for long-term storage of cells and provision of a readily available, reliable and safe supply of material. In recent years many efforts have been made to improve the cryopresevation protocols in terms of efficiency, long-term stability and GMP-compliance for therapeutic cells. As result, freezing procedures and equipment are now available that enable reproducible cryopreservation of high cell quantities and safe storage of samples mainly by freezing cells in suspension with slow-rate protocols using -1 to -10 °C/min. Nevertheless, in the field of regenerative medicine and tissue engineering, freezing protocols for cell-scaffold constructs or multicellular model systems are needed to efficiently store the constructs after a long-lasting preparation, differentiation or cultivation procedure. But the three-dimensional structure of the constructs results in suboptimal heat dissipation and permeation of cryoprotectants using standard slow-rate protocols. This increases the risk of large ice crystal formation within the multicellular structures, disruption of cell-cell and cell-matrix contacts and serious cell damage.

One method to reduce ice crystal formation during cryopreservation are ultra-fast freezing protocols (vitrification), transferring the cells into a glass-like state. Ice nucleation in the cell sample is inhibited during cooling by increasing the viscosity until a glass is formed. To achieve that, vitrification protocols are based on extremely high concentrations of cryoprotectants and fast cooling rates using very small sample volumes (< 50ul) and direct contact with liquid nitrogen. Based on standard vitrification

protocols in open pulled straws, we have developed an adapted protocol for adherent cells without the need of direct and therefore possibly contaminating liquid nitrogen. This procedure already showed the efficient cryopreservation of adherent human embryonic and induced pluripotent stem cells and could also be used to freeze cell-matrix constructs, for example retinal pigment epithelial cells on amniotic membranes.

For future applications of this vitrification procedure, not only efficient protocols are necessary, but also freezing substrates that enable high cooling rates due to their material characteristics and procedures that can be integrated in automated workflows. Therefore we are currently developing a multi-well-plate for vitrification and storage of adherent cell networks for ready-to-use assays and vitrification protocols that can be performed in automated liquid handling platforms.

With these developments, automated preparation and storage of ready-to-use assays and scaffolds will improve future applications in the fields of tissue engineering, regenerative medicine, and drug development.

CRYOCHAIN EQUIPMENT AND CONSUMABLES FOR CLINICAL DELIVERY OF REGENERATIVE MEDICINES

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For many cell-based therapies (CBT) the long term business model assumes timely delivery of a consistently reliable and effective therapy to the point of clinical use. Successful cryopreservation together with an effective cryochain of storage and supply are essential elements for making such deliveries.

In addition to the research and development necessary to ensure a clinically effective product there are regulatory issues that must be accommodated, as cells for parenteral application (administered by injection) are treated as medicines. These issues include:

- Minimised potential for contamination of the sample.
- Hermetically sealed samples.
- Reproducibility – all samples to have the same viability and efficacy on thawing.
- Traceability throughout the entire cold chain, right up to the patient.

It is essential to minimize any potential ‘bottleneck’ effect that cryopreservation may have on the clinical delivery of such regenerative medicines. In this talk the critical factors to be considered in the development of successful cryopreservation protocols will be discussed with the examples of autologous treatments such as CAR T immunotherapies and allogeneic treatments such as MSCs.
It is helpful when developing a cryopreservation protocol to consider the process from the point of clinical delivery to the patient backwards to cell manufacture as the mode of delivery to the patient will, for example, affect the type and size of the container cells are frozen in.

COMPLEX TREATMENT OF BABESIOSIS IN DOGS WITH APPLICATION OF CRYOPRESERVED RED BLOOD CELLS

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Babesiosis is a quickly progressing parasitic disease of dogs associated with the rapid destruction of red blood cells (RBC), requiring specific and immediate treatment. In severe cases of development of this pathology, blood transfusion is the only possibility to save the animal.

We have developed and applied a novel approach in the complex treatment of babesiosis in dogs by transfusion of cryopreserved packed red cells. Previously, we have shown the possibility to cryopreserve RBCs of cats and dogs (1). In this work, it was found that the blood transfusion of cryopreserved erythrocytes gives a positive result, both in acute and chronic course of the disease in a complex scheme of treatment of babesiosis. It was shown that by the tenth day of treatment all studied clinical and biochemical parameters of blood of convalescent dogs have returned to physiological norm and all the symptoms of babesiosis were eliminated.

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CRYOPRESERVATION OF AMNION-DERIVED MULTIPOTENT STEM CELLS WITHIN ALGINATE- AND COLLAGEN-BASED POLYMERIC MATRICES

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Historically, cryopreservation of cells in suspension has been widely studied and optimized for many types of stem cells and reproductive cells. However, there is no unified cryopreservation protocol enabling commercial availability of clinically relevant tissue-engineered products (TEPs) and their further use on demand. Either cryopreservation of isolated cells entrapped in polymer matrix or cells attached to polymer matrix meet a number of challenges dictated by complexity of the 3D environment, which, in turn, determines cryobiological nuances of ice propagation. Therefore, this work is devoted to the validation of freezing and thawing parameters enabling efficient cryopreservation of various 3D TEPs seeded and/or enclosed by multipotent stromal cells (MSCs) derived from amniotic membrane of the common marmoset *Callithrix jacchus*.

As model objects we used two different 3D cell configurations and two different polymers: cells attached to the matrix made of collagen-hydroxyapatite porous scaffold and MSCs embedded into a matrix of alginate microbeads. As quality assurance parameters, we determined recultivation efficiency after 24 h post-thaw and mechanical properties.

For alginate encapsulated stem cells, we focus on preservation of initial uniformity and homogeneity of alginate microbeads sized from 300 up to 1500 um. The microbeads were generated either using high voltage or air flow approach. Structural integrity was first analysed for cell-free microbeads using cryomicroscopy (Carl Zeiss Axio Imager M1m microscope with FDCS 196 Linkam cryostage). The optimal conditions (cooling rate, concentration of dymethyl sulfoxide (Me_2SO) and its loading time) were then utilized for cryopreservation of alginate encapsulated cells using controlled rate freezers (Planer Kryo 560-16 and Askion Workbench C-Line WB230). In parallel, we successfully validated our protocol for cryopreservation of cell-seeded collagen-hydroxyapatite scaffolds applying Me_2SO with and without sucrose.

Collagen and alginate 3D structures were frozen without an active control over the temperature of ice formation using slow cooling. In both cases, viability of cells exceeded 60%. In turn, mechanical properties of collagen-based scaffolds were compromised after thawing.

In summary, we assume that for successful cryopreservation of TEPs much attention must be paid to provide ambivalent preservation of high cell viability and viscose-elastic properties of polymeric matrices.

A TALE OF TWO EXTREMES: EXTREME COLD AND WARM YEARS LIMIT THE REPRODUCTIVE PERFORMANCE OF ANTARCTIC ECHINODERMS

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The ongoing impacts of climate change are well established and predicted to continue at an unprecedented rate. Superimposed on this backdrop of environmental change is an increase in the frequency and severity of extreme weather events, a trend that is forecasted to escalate in many areas of the climate system under future global change scenarios (1, 2, 3, 4). Fluctuations in extreme events have been implicated as major mechanistic drivers of ecological responses to climatic trends, often being more relevant than variances in mean climate (5, 6). They are, however, generally underestimated owed to their infrequency (7, 8), and research being focused on the average rate of warming and warming impacts (9, 10, 11). In addition, ecologists have historically focused on quantifying the effects of locally measured components of weather as opposed to regional modes of climate variability (e.g. El Niño Southern Oscillation (ENSO)) that integrate over multiple local variables and climate extremes, and facilitate a more holistic approach when researching climate change effects. Here we investigate the impact of large-scale climate cycles and local environmental conditions on a key indicator of ecological performance, reproductive potential, in two Antarctic echinoderm species over 17 years of monthly samples. We report long-term trends recorded in reproductive performance, measured as gonad index and egg size, and observe extreme El Niño and La Niña signals in the reproduction of both polar taxa. We find strong empirical support that both species are limited in extreme El Niño years (cold) years. However, we observe variable responses in reproduction in extreme La Niña (warm) years, where reproductive potential is limited in the more stenothermal brittle star, but not in the more heat tolerant sea star. We identify the complex interactions between food supply effects, seawater temperature, and the energy allocation and bet-hedging strategies of both echinoderms as key drivers of reproductive responses, where climate extremes underpin long-term trends in biological flexibility. Therefore the infrequency of extreme weather events underscores the need for long-term biological time series to better describe and reliably predict faunal responses to future climate change, especially in terms of variability. Consequently, interannual variability of extreme events and cold conditions may be more important drivers of population dynamics in rapidly changing environments than previously recognized.

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METABOLIC CHANGES UNDERPINNING THE LIFE PHASE TRANSITIONS OF SNOW ALGAE IN ANTARCTICA

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Snow algae communities consist mainly of green algal species of the *Chlamydomonas* and *Chloromonas* genera. They have a bi-phasic life cycle consisting of an active reproductive motile stage seen as green patches in the snow and a dormant encystment phase during which the cells accumulate the red keto-carotenoid astaxanthin, giving rise to red snow patches. We measured the metabolic composition of snow algae in both green and red phases, from samples taken in the field at various locations in Antarctica (compared to laboratory grown samples) during the 2014/15 austral summer season. We also tested for evidence of spatial variation in the metabolic composition of each algal patch.

Our data showed that the metabolic composition of the cells was the same (when expressed on a per cell/dry weight basis) from four geologically distinct but spatially close locations in the vicinity of Ryder Bay. However, our data also show a high degree of patchiness in the snow melt between the locations where snow algae communities occurred, from mean values of 0.5g dry algae per L of snow melt at Lagoon Island to 3.25g dry algae per L of snow melt at Léonie Island. There was a corresponding site variation in the chlorophyll and lipid content of the snow melt between Lagoon Island and Léonie Island. Our lipidomic data showed that the snow algae were rich in 16:4, 16:1, 16:0, 18:1 fatty acids and that Fourier transform-infrared spectrometry (FT-IR) and gas chromatography-mass spectrometry (GC-MS) were able to detect detailed changes in lipid, carbohydrate and protein chemistry in the cells during encystment. This provides the means to study environmental factors important in the green-red transition that characterises the life cycle of these snow algae.

FEATURES OF HEART RATE VARIABILITY IN HUMANS DURING WINTERING IN THE ANTARCTICA

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The study of Environmental Cryobiology in Antarctica implies the presence of the researchers under appropriate conditions and their adaptation to these conditions. We analyzed the changes in heart rate variability (HRV) in 12 men aged 22-62 years, who overwintered for 12 months at the Ukrainian Antarctic station "Akademik Vernadsky". Also, seven winterers took part in the experiment with the 3-min cold pressor test (CPT) (1). The CPT was performed by immersing a right hand into cold water (10.8±1.4°C) for 3 min. Air temperature was 24.4±1.5°C. Blood pressure, axillary temperature and the temperature for both hands and forehead before, just after and 3 minutes later the CPT were monitored. The HRV was calculated before, during and after the CPT.

A posteriori we found that the winterers can be divided into 2 groups. In first group (5 men) the average heart rate (HR) decreased by 7.9% and the average total power (TP) increased by 28%. In the second group (7 men) the HR increased by 7.7% and TP fell by 20%. The age and professional factors did not affect the type of response.

In the CPT the temperature of hand back side immersed into cold water was reduced by 47%, and afterwards after 3 minutes it was restored up to 66% of initial values. As we predicted according (1), the participants were divided into 2 groups according to the HR response after CPT, but these groups did not coincide with the previous ones.

In the first group (4 people), we observed a 5% decrease in HR during the CPT, which did not recover the baseline within 3 minutes after the CPT ended. At the same time, the TP increased by 9%

during the CPT and remained at the same level in 3 minutes. In the second group (3 persons) during the test the HR increased by 6%, and after the cessation of the cold effect, it fell down to 98% of the baseline. TP during the test increased by 70%, and in 3 minutes after the stop of cold effect, it recovered to its baseline.

The existence of at least 2 strategies in response to cold exposure in healthy people was reported by different authors (1-4), but the real mechanisms of these responses have remained unclear as well as the relationship of these responses to the initial vegetative status of a person. Therefore, further research is needed in this area.

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LEA PROTEINS: INTRINSICALLY DISORDERED PLANT STRESS PROTEINS – LINKING STRUCTURAL TRANSITIONS TO THEIR FUNCTION IN FREEZING TOLERANCE

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Late embryogenesis abundant (LEA) proteins accumulate in seeds and vegetative plant tissues, especially after exposure to abiotic stresses and in desiccation tolerant bacteria and invertebrates. Their expression is directly linked to cellular dehydration as arising during freezing or desiccation. Most LEA proteins are intrinsically disordered under fully hydrated conditions and fold during drying (1). We focus on two cold-induced *Arabidopsis thaliana* LEA proteins, COR15A and COR15B. Functionally redundant, COR15A and COR15B stabilize membranes during freezing *in vitro* and *in vivo* while they do not stabilize selected enzymes during freezing *in vivo* (2). Both proteins are disordered in solution, but fold into amphipathic α-helices in the dry state, as shown by CD and FTIR spectroscopy and *in silico* analysis. We used glycerol as a low-molecular weight crowding agent to model reduced water availability during freezing. Experimentally, we found a concentration dependent gain of α-helical structure in solutions containing glycerol, which is enhanced in the presence of lipid membranes (3). The unfolding process of both COR15 proteins in differentially hydrated environments was modeled by MD simulations, which identified H-bonding interactions of the protein backbone with surrounding water molecules as the major driving force for protein unfolding (4). FTIR spectroscopy, X-ray diffraction and MD simulations showed that the proteins interact with artificial membranes exclusively in a partially folded state.

Collectively, our findings suggest that the COR15 proteins protect plants during freezing by associating with labile membranes following a protein folding - membrane binding - enhanced folding mechanism. Thereby an initial dehydration-induced folding step is necessary to render the COR15 proteins competent for membrane interaction while a second folding step takes place during membrane association (5).

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ELUCIDATING THE MECHANISMS OF LEA PROTEIN INDUCED MEMBRANE STABILIZATION DURING FREEZING

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Plants are fascinating organisms that developed many ways to cope with environmental stresses to improve their survival on earth. Global warming has led to harsh winters in the northern hemisphere; and the southern region, already predisposed to arid conditions, has already seen an additional increase in soil salinity. Both abiotic stress factors have a major impact on plant survival and hence productivity in agriculture as they lead to dehydration of the plant cell (1, 2). However, some plants are able to acquire a higher freezing tolerance upon cold acclimation (3) During this process, major cellular changes take place that, for instance include the accumulation of small molecular weight solutes such as different types of sugars or hydrophilic proteins (4). A group of these highly hydrophilic proteins are Late Embryogenesis Abundant (LEA) proteins, many of which are encoded on cold responsive (COR) genes (5). My PhD research project will enhance the structural and functional knowledge about COR15A, a member of the LEA_4 family proteins from Arabidopsis thaliana. COR15A is an intrinsically disordered protein in solution but undergoes structural transitions into predominantly α-helical structure upon dehydration, similar to the closely related LEA proteins LEA11 and LEA25 (6, 7). In such a folded state, COR15A associates with inner chloroplast membranes and thereby stabilizes them in vivo and in vitro (6). I am investigating folding of COR15A, LEA11 and LEA25 on different structural levels in response to water deficiency in the presence and absence of membranes, as well as protein-membrane interaction and the resulting effects on membrane organization using a variety of biophysical and biochemical methods. I identified a consensus motif within the three target proteins, which is putatively responsible for membrane binding and stabilization. I will use the abundance of this motif in further, to date uncharacterized LEA proteins and several COR15A motif deletion mutants for validating its putative functional impact. These findings will pave the way for an application of chimeric COR15-like proteins as cryo-protectants in medical approaches.

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PLANT CRYOPRESERVATION: AXES TO SEEDS

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As a complementary strategy to *in situ* conservation, seed storage provides a mechanism by which plants can be preserved from biodiversity loss. Many national programs have therefore been initiated for banking desiccation-tolerant seeds of crops and wild species for long-term storage. However, these are not without challenges. Based on longer-term studies in three genebanks, around one third of collections of orthodox seeds may have half-lives < 100 years, making cryopreservation more appealing (1). This is particularly the case for oily seeds that have relatively high temperature lipid transformations that known to show reduced viability at -20ºC. The success of whole seed cryopreservation depends on various factors such as seed physiology, developmental stage, size and tolerant level to desiccation. Seed developmental stage is a very important criterion in cryopreservation as complex physiological changes take place as the seeds mature. It was reported that drying of very young seeds, induced rapid deterioration of cell membranes as indicated by high electrolyte leakage (2). Conservation of species with recalcitrant seeds also poses challenges. Generally large in size, such seeds have to be handled as whole embryos or excised

embryonic axes. The capacity of desiccated immature embryos to survive cryopreservation is reported to depend not only on the degree of desiccation reached by artificial drying, but also on development stage at harvest (3). Therefore, harvesting these seeds at the correct maturity is the key to successful cryopreservation. For many seeds, excision of the embryos could cause wounding injuries which are exhibited as phenolic compound production. Excision followed by rapid dehydration prior to cryopreservation can also induce superoxide production and additional oxidative stress (4). Cryopreservation protocol development for sensitive germplasm therefore requires the consideration of both cryogenic and non-cryogenic factors. Thermal analysis using differential scanning calorimetry is often applied to help to optimize vitrification-based cryoprotection strategies (5). The use of cryoprotectants such as Plant Vitrification Solutions has been adapted for extremely recalcitrant axes. However, tissue heterogeneity in morphology, physiology and cellular chemistry can compromise survival post-cryopreservation. To overcome this problem and to reduce stress during cryopreservation procedures, vacuum infiltration vitrification has been proposed as a generic, high throughput cryopreservation technique (6). In summary, research in the last 25 years has provided great insights into optimization of seed and embryo cryopreservation that enables wider plant genetic resources conservation providing future food security and protection of species at risk of extinction and disease threats.

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PLANT CRYOPRESERVATION: CELL SUSPENSIONS TO SHOOT TIPS

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Plant cryopreservation is a complex multi- component process where cryogenic and non-cryogenic factors influence the storage outcome. When developing a protocol for the cryopreservation of a particular plant species and cell/tissue type there is generally a focus on the factors directly associated with a given cryopreservation protocol. For example the assessment of the effectiveness of different cryoprotectant cocktails used in controlled rate freezing approaches for the cryopreservation of plant cell suspension cultures and different plant vitrification solution exposure regimes for shoot tip cryopreservation. However, the success of a cryopreservation protocol is also reliant upon the availability of suitable cell/tissue material and the optimization of an efficient plant regeneration system. This presentation will consider the importance of such factors and their significant influence on the successful cryopreservation of plant cells and tissues. There will be a focus on two aspects of such non-cryogenic factors. Specifically:

a. The physiological status of cells and explants selected for cryopreservation and how this is influenced by the character of the explant and the effect of *in vitro* culture.

The use of specialised *in vitro* culture conditions during the immediate period after rewarming to support the survival of cryoinjured cells and tissues and subsequent cell and tissue regrowth.

LIFE AND DEATH AT CRYOGENIC CONDITIONS: USING FERN SPORES AS UNICELLULAR MODEL TO EVALUATE BENEFITS AND LIMITS OF LIQUID NITROGEN STORAGE

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The low temperatures reached during liquid nitrogen (LN) storage are assumed to stop ageing and preserve viability of biological materials indefinitely. However there are few validating data sets (1, 2). The use of plant propagules to test these assumptions is important because other cryopreserved systems lack quantitative measures of viability to allow comparisons among timed points (1, 2). Plant materials (such as seeds, shoot tips, embryos, gametophytes or cells) have been exposed to LN and cryopreserved routinely during the last 30 years (3), and they have benefited from LN storage. For example, cryopreservation is essential for the long-term storage of plant materials that do not tolerate sufficient drying to remove ice-forming intracellular water (e.g. embryos of desiccation sensitive seeds, shoot tips, cells, gametophytes) (4). In addition, storage at LN temperatures is needed to extend longevity of seeds that tolerate enough desiccation to remove ice-forming intracellular water but present short life spans when stored above zero or in the freezer (ca. -15 ± 5°C) (5). However, the analysis of desiccation tolerant seeds stored dry in LN for more than 15 years has showed that some seeds, particularly those with inherently short-lifespans, those aged before LN storage, or those of initial low quality, can age and die during LN storage (1, 2). These results open the debate about the limits of LN storage, and challenge the length of "indefinitely". In this work we present our preliminary findings using fern spores. Fern spores are desiccation tolerant (i.e. can be dried until ice-forming intracellular water is removed) (6), so artefacts in the interpretation of the results related to devitrification of hydrated systems during LN storage are avoided (1). In addition, the single cell nature of fern spores eliminate confounding effects of differentiated cell structures. Moreover, some fern spores are very short-lived, so viability decrease at LN temperature can be monitored in a tractable time frame (6). The use of fern spores reveals that some plant materials can age and die during storage at cryogenic temperatures (from -80 to -196°C), and that this aging may be related to reactions allowed by rotational movements of the molecules trapped within the glassy state, as has been demonstrated to occur in dry seeds even at LN temperatures (7). A better understanding of the systems that can maintain viability for longer times in LN and the characteristics of their intracellular glasses could help to improve the storability of materials that present limitations during LN storage.

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CRYOPRESERVATION OF SUCCULENT PLANTS (CACTI, ALOE, AGAVE) AT THE HUNTINGTON

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Besides their importance to the horticultural industry, succulent plants are sources for food, fibers, medicines and cosmetics. Human activities, such as over-collection in the wild, are the main threats for the survival of many wild populations of succulent plants (1). The Huntington Desert Garden is one of the largest and oldest field collections of succulent plants in North America. However, traditional propagation methods for the living collections do not assure the long-term preservation of these old, often historic, succulent plant collections. Therefore, besides the seed cryopreservation of cacti, agave and aloe species, protocols are being developed for the clonal preservation of type plants. Seed germination tests have been performed for several species to check their tolerance to liquid nitrogen. Besides, experiments of droplet-vitrification based techniques (2) have been used to cryopreserve clonal accessions of aloes and agaves. 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 aluminum foil strips and directly plunged into liquid nitrogen. For re-warming, aluminum 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. In additional experiments, shoot-tips excised from donor plants pre-cultured onto sucrose supplemented media were also submitted to cryoprocedure. Preculture of donor plants with sucrose-supplemented medium improved the regeneration of the cryopreserved explants. The optimized protocol that has been developed for *Aloe fievettii* (with 70% of plants recovered after cryopreservation), is being tested for other *Aloe* and *Agave* species. Regenerated plants were transferred to *ex vitro* conditions. The tests with seeds are also promising, and therefore, we will be able to establish a seed-bank into liquid nitrogen.

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DEVELOPING CRYOPRESERVATION PROTOCOLS FOR COCONUT AND ESTABLISHING CRYO-GENEBANK AT RDA WITH THE BIOVERSITY INTERNATIONAL

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Coconut (*Cocos nucifera* L.) is one of the most important palm crops in the world, being primarily cultivated on about 12 million hectares of land. Coconut palms produce highly recalcitrant seeds that are sensitive to desiccation and that show no dormancy. As such, they cannot be stored through traditional ways such as in a seed bank. Therefore, they are currently conserved as whole plants in field collections. Maintenance of such a collection in the field is risky since it is prone to exposure to unknown climatic factors and diseases (1, 2). Cryopreservation of plant tissues in liquid nitrogen (-196°C) is currently the only method allowing safe and cost effective long-term conservation of recalcitrant seed species such as coconut (3). Bioversity International has supported the development of a global strategy for conserving coconut germplasm that aims to cost-effectively optimize conservation of as much representative coconut diversity as possible. The Rural Development Administration (RDA) of Korea and Bioversity International, as part of their partnership to enhance the sustainable use and conservation of genetic resources in the Asia Pacific region signed a Memorandum of Understanding (MOU) in 2009 that included an R&D partnership for coconut cryopreservation. In connection with this MOU, end 2015 an RDA funded international cooperative project was developed. This project aims at developing and validating robust cryopreservation protocols, starting with coconut and then exploring the potential for cryopreserving other priority species. Three LOAs were finalized, relating to work for cryopreserving coconut genetic resources with 1) Sunchon National Univ.(SNU) of Korea on developing a cryopreservation protocol for zygotic embryos of coconut; 2) KU Leuven of Belgium on developing a cryopreservation protocol for plumules and meristems tips of coconut using droplet vitrification, and 3) the Philippine coconut authority (PCA) of the Philippines to supply KU Leuven and Sunchon University with good quality zygotic embryos. A kick-off meeting was held in November 2016 hosted by the PCA at Legazpi, the Philippines, gathering 16 experienced coconut tissue culture and cryopreservation scientists from 13 different countries. The meeting i) presented the project to the coconut community ii) defined priority research areas; iii) exchanged experience and ideas on coconut tissue culture; iv) decided on and negotiated with the coconut zygotic embryo provider; v) gave a practical demonstration on coconut embryo and tissue culture and hosted a field trip. Through the former collaborative project between SNU and the PCA, the two protocols that are preculture-desiccation of intact embryos and vitrification of plumular cube, were proposed as a candidate for routine implementation of cryobanking for coconut collections. We present an overview of existing cropreservation work as well some preliminary results.

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POTATO CRYOPRESERVATION IN GERMANY - BIOCHEMICAL CHANGES DURING CRYOGENIC PROCEDURES

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The federal *ex situ* collection of agricultural and horticultural plants at IPK Gatersleben houses more than 3,000 *in vitro* and 1,500 cryopreserved potato accessions. Initially, DMSO droplet freezing was established to preserve potato shoot tips, whereas vitrification protocols were successfully applied to garlic and mint. DMSO and PVS3 protocols were applied to 26 genebank accessions and revealed significant higher plant regeneration and less callus formation after cryopreservation with PVS3. The aim of the study was to compare regeneration results and biochemical changes between the DMSO droplet freezing (DMSO) and two droplet vitrification methods using PVS3 as cryoprotector (PVS3, PVS3A). On the average of four genotypes, significant differences were found in the regeneration of plants, between the adapted protocol PVS3A with 9,2 %, while DMSO generates 49,5 % and PVS3 57,3 % plant regrowth, respectively. The analysis of soluble sugars and ATP concentration performed along the cryopreservation protocols. Over all protocols, sucrose, glucose and fructose content showed a drastic increase during preculture and remained at high levels until rewarming. The ATP content of the explants reduced during preculture but increased after rewarming indicating active stress response mechanisms. Differential scanning calorimetry (DSC) elucidated that the exposure of shoot tips to the PVS3 solutions generate the typical glass transition, whereas DMSO treatment cannot completely prevent ice crystal formation. Nevertheless, this thermodynamic behavior does not correlate to the regrowth of explants. Summarizing, the genotypes differed in regeneration performance and the tendency to form callus, due to the three approaches. These variations are affected by different genotypic compositions and osmotic, mechanic or cold stresses. However, expected relationships between regeneration, glass transition temperature, sugar and ATP content was not detectable.

CRYOPRESERVING CASSAVA MERISTEMS BY DROPLET VITRIFICATION; TRYING TO SOLVE THE REGENERATION PROBLEM

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Cassava is an important root crop cultivated in the tropics and source of starch and animal feed. Pests, diseases and monocultures pose a major threat to its genetic resources. Through cryopreservation, we can protect the wide variety that exists in this species.

Cryopreservation, using 2 different droplet vitrification methods was investigated and the response of 3 different accessions of *Manihot esculenta* (Cassava) was compared. Apical and axillar meristems were excised from 4, 8 and 16 weeks old in vitro grown plants. Higher viability, shoot growth and plant regeneration percentages were observed for apical shoot tips compared to axillar shoot tips. Overall viability and shoot growth rates where high (average of 78.3% and 73.56%). Plant regeneration percentages, however, varied considerably for the 3 accessions (between 1.26% and 30.9%; average of 11.7%).

Subsequently three different regeneration media were investigated by using apical and axillar meristems. Again, the overall viability and shoot growth were high (respectively 91.1% and 77.1%), while the plant regeneration achieved only 10.7%. When the regeneration of the three different accessions are compared, the M. Bra 856 scores significant the best with a percentage of 24.0%, while the other two accessions CM 3306-4 and CM 507-37 reached only a regeneration percentage of respectively 4.4% and 3.9%. Regeneration frequencies on the three different culture media show no significant differences.

A cyto-histological study shows some damaged meristematic zones after the shoot tips underwent cryopreservation protocol. This could be linked to the observation that despite high viability and even high shoot growth frequencies are obtained; the formation of rooted shoots is still difficult.

WHAT EFFECT DOES THAWING RATE HAVE ON POST-THAW RECOVERY OF CRYOPRESERVED ALGINATE ENCAPSULATED LIVER SPHEROIDS?

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The bioartificial liver (BAL) is a form of extra-corporeal organ designed to supplement the function of the liver in patients with acute liver failure. Thereby allowing time to find a suitable donor or for the liver to undergo self-repair. The BAL consists of liver cells (HepG2) encapsulated in alginate, which have formed spheroids (Alginate encapsulated liver spheroids (AELS)). The AELS demonstrate upregulated function compared to monolayer. In order to form the spheroids and achieve suitable cell density the AELS are grown in a fluidised bed bioreactor (FBB) for 12 days until a cell density of ~3e7cells/ml is reached. However, it is important in the case of acute liver failure that a treatment is available "off-the-shelf" and not after 12 days. To that end we have endeavoured to develop a cryopreservation protocol to preserve a large biomass and recover it after thawing within 4 days.

A freezing profile has previously been developed, however it displays reduced cell viability and cell proliferation post thaw. We investigated the effect of freezing in a cryobag vs a 60ml polypropylene bottle in order to assess the effect of fast and slow thawing on post thaw viability and cell proliferation. The small bottle is a model of the large cryobag (1.3L and 3cm thick) mimicking the large scale model, improvements in the polypropylene model could be applied to the larger scale.

AELS were grown in an FBB for 12 days to a viable cell density of 2.6e7cells/ml after which they were frozen in 55ml samples, either in a 500ml cryobag (to a thickness of 3mm) or a 60ml polypropylene bottle (to a thickness of 3cm). They were thawed in a 37°C waterbath and placed into a small FBB in order to recover.

The AELS frozen in the bottles recovered to pre-freeze viable cell number after 3 days post thaw (2.8e7cells/ml). The AELS frozen in the cryobag did not recover to pre-freeze viable cell number, even after 4 days (2.0e7cells/ml). Results show that freezing in bottles produces better post thaw recover than when the same volume biomass is frozen in a cryobag. We hypothesise that this could be due to the effect that the thickness (3mm) of the bag has on the freezing rate and thawing rate of the biomass, potentially altering kinetics of ice crystal growth. However, further study is needed.

OPTIMIZATION OF IN VITRO GERMINATION MEDIUM AND CRYOPRESERVATION PROTOCOL FOR SEEDS OF DENDROBIUM ORCHID SPECIES

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Projections for the impact of ecosystem degradation on flora over the world argue for enhanced efforts toward *ex situ* conservation as an insurance policy against loss of populations *in situ*. *Ex situ* conservation is less costly than *in situ* conservation (1), allowing affordable support to ensure long term conservation. It is suggested that some orchid species have complex behavior; they are tolerant to low moisture content (2, 3) but appear to be short-lived compared to other species (4). Consequently, cryopreservation becomes a viable option for orchid seed conservation. Determining a suitable germination medium for a particular species is an essential prerequisite for any orchid *ex situ* conservation programme (5). *In vitro* germination and seed development of three *Dendrobium* species; *D. strebloceras*, *D. lineale* (Indonesia) and *D. cunninghamii* (New Zealand) were compared on four different media: Murashige and Skoog (6), Vacin and Went (7), Norstog (8), and control (agar + sucrose 20 g/l) medium. Seed germination (%) was compared with seed viability using the Tetrazolium (TZ) chloride staining test (9). The preliminary results showed that Murashige and Skoog (MS) medium produced the best seed development of the three *Dendrobium* species. Subsequently, MS medium was used to germinate seeds following cryopreservation. Recovery following cryopreservation without cryoprotectant and with Plant Vitrification Solution 2 (PVS2) was assessed on two Dendrobium species; *D. lineale* and *D. cunninghamii*. PVS2 vitrification treatment was compared over four durations (0, 20, 50, 70 min) at two temperatures (on ice and room temperature). The optimum regrowth media for each species tested and the recovery comparing different cryopreservation methods will be discussed.

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CRYOPRESERVATION AND RECOVERY OF A CLINICAL SCALE BIOARTIFICIAL LIVER (BAL) BASED ON ALGINATE CELL SPHEROIDS

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Background and Aims: There is a need for a bioartificial liver (BAL) to support patients with liver failure (LF) while their liver recovers or a donor liver becomes available. The marketability of a Bioartificial Liver (BAL) as a clinical product is related to its immediate availability, due to the unpredictability of disease progression of acute liver failure, and the rapid deterioration of patient health. The aim of this study was BAL biomass cryopreservation and recovery, for an off-the-self product.

Methods: HepG2 cells were encapsulated in alginate, and 3D-cultured in a Fluidised bed bioreactor (FBB) for 12-14d. Cryopreservation of >1L of alginate cell beads (~500µm diameter) was achieved in a segmented CryoStore™ conical bag using a controlled rate freezer (CRF, Kryo750 Planer) (n=3). A final concentration of 12% DMSO in Viaspan (Belzer UW, Bridge to Life) was used as cryoprotectant (CPS) with 0.2-0.02% cholesterol as nucleator. Several freezing profiles were evaluated: a linear decrease at -0.3°C/min; and multi-step profiles with linear decrease (-0.5 or -1°C/min) interspersed with holding step at -40°C or -50°C. Cell beads were thawed in a water bath at 37°C, kept on ice until all CPS was removed through DMEM washes. Encapsulated liver spheroids (ELS) were recovered in FBB or a rotary-cell-

culture-system (RCCS). Cell viability was assessed by metabolic live-dead cell staining (FDA/PI), and image analysis.

Results: Cell number in encapsulated beads was 3.4-4.8x10^{10} at a density of 26-39x10^{6}/ml of beads with a cell viability >97%. After cryopreservation and thawing, there was an average 4.5% drop in viability and 33% drop in cell number, which were both restored to pre-freeze levels by day 5. During freezing the recorded cooling rates were on average between minus 0.27 to minus 0.35°C/min. A total biomass of ~3.2-4.8x10^{10} cells was recovered after freezing.

Conclusions and discussion: We describe a protocol for cryopreservation of the encapsulated liver cell spheroids on a large scale, about 16-48% of a total human liver size. Since a biomass of 30% liver cell number is likely required for a BAL, this is of the required order of magnitude. The use of micro-organoids within an alginate matrix is an interesting choice for large-scale cryopreservation evading problems associated with large tissue freezing. A more rapid recovery would be ideal, thus we are exploring further improvements. This process is easily scalable by freezing more bags in the Kryo750 freezer and by using the same fluidised-bed bioreactor for cell recovery.

SLEEP-ACTIVITY ONE YEAR MONITORING DURING RESIDENCE AT THE UKRAINIAN ANTARCTIC STATION "AKADEMIK VERNADSKY": A PILOT STUDY

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Overwinterers of Antarctic expeditions are subjected to the combined effect of marked fluctuations in meteorological conditions, the season inversion, the time zone shift, photoperiodic characteristic changes (from polar day to polar night) and light spectral composition peculiarities during winter/summer, physical inactivity, social isolation, sensory and sexual deprivation (1-3). Even separately each of the factors mentioned may affect sleep and circadian rhythmicity of the organism's function that in due turn is reflected in performance and organism adaptability changes of the crew members.

The aim of this pilot study was to investigate changes in sleep-activity in winters during 21-st Ukrainian Antarctic expedition (during 2016-2017) at the "Academic Vernadsky" station.

Four people (43, 51 and two of 23 years old) participated in the study. All of their expedition routine was connected to daily outdoors activity as well. Personal smartphones with installed Sleep as Android app (Urbandroid Team) were used for nightly sleep-activity cycle registrations. Total sleep time (TST), deep sleep time (as program determined) (DST), its percentage (DS%) and number of the sleep cycles data were calculated by the program and were extracted from it for the further analysis.

All the subjects in the study had individual peculiarities of sleep-activity pattern as well as both in TST and DST changes during the year. There was a slight decrease in TST and DST in one person over the year even in winter (the darkest period), the second one had slight decrease in TST and DST till the end of the winter (August) following with slight increase and "plateau-like" period, the third person had initial increase followed by "wave-like" changes in TST as well DST and the fourth one had an initial decrease with following stable increase both in TST and DST after August. Also, there was a tight correlation between TST and DST monthly changes in all the subjects examined.

As was elegantly noticed by (4) "Antarctica, like all extreme environments, offers only small samples of hardly generalizable data, for each expedition or winter over will have its own peculiarities". As far as the main health complaint in overwinteres in Antarctic expeditions concerns to sleep (1-4) its monitoring and early disturbances evaluation may be of very importance for the whole mission completion. It was the first sleep investigation at the Ukrainian Antarctic expedition and further study will be needed for sleep-activity changes determination as well as utilizing more comprehensive devises for the purpose.

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INVESTIGATING ICE NUCLEATION TEMPERATURE EFFECTS ON CELL RECOVERY FROM CRYOSTORAGE

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Introduction: Haemophilia A (HemA) is caused by a genetic deficiency in the production of clotting factor VIII (FVIII). The HemAcure consortium seeks to use blood outgrowth endothelial cells (BOECs) from HemA patients and correct the FVIII gene within these cells. These corrected BOECs shall be transplanted back into the patient in a previously implanted prevascularized device (Sernova's Cell Pouch™) releasing FVIII into the blood. Storage of FVIII corrected BOECs must result in high functionality and be cost effective as the patient might need several transplantss over a life-time. For commercialisation of the final cell product, Good Manufacturing Practice (GMP) must be observed. Hence, a freezing protocol must be adapted to include controlled rate freezing. By including GMP compliant ice nucleators, the temperature at which ice forms can also be manipulated to further control the thermal gradient. In addition, a GMP-compliant, chemically defined growth-medium (GM)was developed by one of the consortium members and tested, to further reduce batch variation, cell impairment and to increase the safety of the cell product. By understanding the influence of temperature variation due to ice formation in a high throughput setting, this allows for the combination of ice nucleators and non-toxic serum free medium to be investigated for the creation of a stable GMP process.

Methods: 4,500cells were seeded into 96-well plates in chemically defined growth medium (GM). This was then replaced with either GM+10% DMSO or a commercial serum- and xeno-free cryo-medium (PromoCell Cryo-SFM). Ice nucleators were introduced into the freezing medium (IceStart, Asymptote) and then the plate transferred to a controlled rate freezer (ViaFreeze, Asymptote). A cooling gradient of 1^{o}C/minute was used. Plates were thawed quickly (<10 minutes to 37^{o}C) and cultured for 8 days. Cell recovery was measured using AlamarBlue at day 1, 4 and 8.

Results: A significant increase in the mean temperature of nucleation was observed with nucleators, resulting in a more gradual cooling gradient after nucleation. A corresponding increase in BOEC recovery over 8 days post thawing was also observed in conditions with the higher nucleation point, implying a more controlled, higher quality process.

Conclusion: GMP conditions require high quality production by ensuring that the processes of manufacturing are controlled and produce as little functional changes in the end product as possible. The addition of active nucleation sites reduces the damaging variation observed during a standard freezing process without optimisation of cryomedium and in a high throughput setting. This will allow for the storage of FVIII gene corrected BOECs in acontrolled state and which will positively affect recovery for therapeutic use.

CONSERVATION OF THE CZECH HOPS BY CRYOPRESERVATION

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Hop (*Humulus lupulus* L.) belongs to the most important crops in the Czech Republic. 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

In vitro cultures were derived from extracted meristems tips according to procedure described by (2) and maintained by (2,3).

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 45 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 (4). The methods used and results are presented.

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INITIAL ATTEMPTS TO PRESERVE VALUABLE GENETIC MATERIAL OF ENDEMIC CHESTNUT POPULATIONS IN CRETE WITH THE CRYOPRESERVATION OF SOMATIC EMBRYOS

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Attempts are being made to support *ex situ* conservation of selected, endemic populations of *Castanea sativa* by the cryopreservation of somatic embryos. The aim is to preserve unique genotypes of local varieties from the Prefecture of Chania (Crete) and enhance the marketability of harvested chestnuts.

Fruits of four local chestnut populations, namely Rogdiani, Strovliani, Koutsakeri and Katharokastania, were harvested and stored at 3±1°C for 2 months. The initial water content of isolated embryos was 59.6±2% of fresh weight. After storage the shells were removed and the fruits surface sterilized according to the protocol developed by Corredoira *et al.* (1). It should be noted that surface sterilization of excised axes was lethally damaging.

Thereafter, embryonic axes were separated from their cotyledons and dehydrated in a laminar flow cabinet for 0, 2, 4, 5 or 6 hours. The water content of the axes for each treatment was determined prior to direct immersion of naked embryos in liquid nitrogen (LN). The axes were thawed in a water bath (30°C) and then placed on a solidified Murashige & Skoog recovery medium for 24h. Subsequently, they were transferred onto a solidified M&S cultivation medium and placed in a growth chamber at 25±2°C with a 12 h day/night photoperiod. The number of the embryos that survived the process was scored after

2 weeks, with an optimal treatment of 4h dehydration delivering a cryopreserved axis survival of ≥53% (3 replicates of *n*=10). The survival rate achieved is in most cases comparable with the survival rate obtained when the axis where cryopreserved in cryovials. Likewise, the survival of the control was equal or greater of the survival post cryopreservation except in the case of Koutsakeri, where many of the control explants where lost due to contamination. It can be concluded that successful cryopreservation, adopting the 40% survival lower limit for genetic conservation (2) is feasible for the *Castanea* genotypes under investigation.

Occasionally, surviving axes did not show normal root/shoot development and produced callus tissues (aprox. 15%), indicating a level of non-lethal damage during the cryopreservative process. A limited level of difficulty was also found with microbial contamination from surviving axes, suggesting the presence of systemic infections in some of the material. The culture of apical meristems excised from the axes, before or after cryopreservation, is being considered.

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BLASTOMERE BIOPSY OF INCOMPLETE COMPACTED HUMAN MORULA CAN IMPROVE THE EMBRYO CRYOPRESERVATION OUTCOME

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The study of embryo cryoresistance at morula stage is crucial for the assisted reproductive technologies, since at this stage of *in vivo* development the embryo enters an uterine cavity. Post-cryopreservation embryos with an incomplete compaction of blastomeres may have a different survival rate. Probably, removal of aberrant sites will allow increasing the embryo cryopreservation outcome.

The aim of this study was to estimate the survival and blastocyst formation rate of post-cryopreservation embryos at the morula stage with preliminary biopsy of blastomeres and fragments non-included in morula compaction.

The human embryos on day 4 of *in vitro* development were divided into 4 categories according to the level of blastomeres compaction: 1- fully compacted morula; 2, 3, 4 - incomplete compacted morula; percentage of blastomeres and fragments not included in morula compaction occupy 25, 50, 75 % of embryo volume, respectively.

A biopsy of blastomeres and extracellular fragments was carried out by micromanipulator Narishige (Japan) using Micropipet Cook (USA) after double mechanical hatching. Embryos were cryopreserved in vitrification media based on a mixture of ethylene glycol, dimethylsulfoxide and sucrose solutions on Cryotec carriers (CryoTech, Japan). After warming the survival rate, developmental kinetics of embryo *in vitro* were evaluated.

Fresh morulas in categories 1-4 developed into optimal blastocyst in (98.8 ± 7.9), (85.3 ± 8.2), (69.7 ± 11.7), (22.4 ± 4.9) % of the total, respectively. Survival rate of post-cryopreservation morulas in these categories was (97 ± 3.2), (85 ± 4.1) and (50 ± 5.8) and (38.2 ± 4.4) % and and blastocyst formation rate after freeze-thawing was (95.4± 4.6) %, (71 ± 6.2), (44 ± 9.9), (12 ± 5.8) % respectively. Removal of blastomeres and extracellular fragments of incomplete compacted morula prior to embryo cryopreservation, led to an increase their post-thawing survival rate up to (93.1 ± 4.1) and (75 ± 8.8) % and blastocyst formation rate up to (85.2 ± 10.4), (59,4 ± 5.2) in categories 2 and 3, respectively. Morulas with even greater aberrant compaction had the lowest survival rate (35.2± 5.4) % and cleavage potential after freeze-thawing was (19.7±4.8) %. Investigated indexes in post-cryopreservation incomplete compacted morula depends on the aberrant compaction degree is most likely due to the negative effect of necrotic factors of the damaged blastomeres and fragments non-included in embryo compaction. Biopsy of these parts can prevent negative cryopreservation impact and improve survival and blastocyst formation rate of incomplete compacted morulas category 2 and 3.