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Abstracts

ORAL PRESENTATION ABSTRACTS

S1 FOOD TECHNOLOGY AND LOW TEMPERATURES

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Water in foods is often their principal component and water has a significant impact on various food properties. Dehydration of food materials, however, alters food characteristics and behaviour at low temperatures. Dehydration of food solids also occurs during freezing as water transforms to ice and forms a separate phase within a continuous solids phase. Such freeze-concentrated solids phase has a substantial unfrozen water content and affects food stability. Understanding of technological properties of food materials was significantly improved as a result of introduction of glass transition measurements and state diagrams (supplemented phase diagrams). For example, state diagrams provide data for successful freeze-drying (lyophilisation) process control, and a basis for understanding kinetics of food deterioration at low temperatures. Most food materials exhibit nonequilibrium ice formation which involves (i) ice formation in supersaturated, freeze-concentrated solutions in the absence of solute crystallization; and (ii) saturation of hydrogen bonding of amorphous solute molecules with unfrozen water. The unfrozen water-plasticized solute phase exhibits glass transition with onset during heating at T_g' while ice melting onset occurs at T_m' corresponding to saturation temperature of solute-unfrozen water structure and maximum ice formation. Binary solute-water state diagrams are limited in applications to more complex formulations. More advanced tertiary state diagrams for carbohydrate-protein-water systems provide useful data across biological materials application areas as individual component transition data and component unfrozen water contents at maximum freeze-concentration can be obtained. Interestingly, vitrification of unfrozen water within freeze-concentrated complex materials seems to contribute to frozen carbohydrate-protein systems thermal behaviour. Numerous food technology applications, such as preservation of starter cultures, freeze-drying of foods, cryostabilisation of frozen foods, and formulation of frozen desserts, benefit from food materials science advances.

Source of Funding: Not applicable
Conflict of Interest: None to declare

S2 MEMBRANE MATTERS: MEMBRANE PERMEABILITY BARRIER FUNCTION UNDER EXTREME CONDITIONS

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Cellular membranes are exposed to extreme conditions during the processing steps involved in cryopreservation or freeze-drying of cells. In both cases, the first processing step involves incubating cells with a protective agent. Exposing cells to protective agents causes membrane fluxes of both water and the protectant, resulting in cell volume changes and osmotic stress. In addition, protective molecules may interact with membrane lipids which may lead to permeabilization, particularly with membrane permeating protectants. Freezing and drying cause severe physical and chemical stress on cellular membranes. Membranes undergo thermotropic and lyotropic phase transitions during cooling and/or drying, which drastically alters the membrane permeability barrier function. The activation energy for water transport across membranes increases almost three-fold when extracellular ice is formed. In addition, membranes become permeable for molecules for which they are normally impermeable. On the one hand this may result in undesired uptake of sodium and leakage of potassium. On the other hand, one can take advantage of freezing-induced membrane phase and permeability changes for cell preservation. For example, we have shown that disaccharides like trehalose are taken up by cells during freezing. This allows cells to survive freezing and stabilizes DNA in freeze-dried cells during storage. In this talk, the biophysical mechanisms that are involved in stabilization and destabilization of membranes under extreme temperature and dehydration conditions are being discussed, and how this can be applied to cryopreservation and freeze-drying of cells.

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Conflict of Interest: None to disclose

S3 PROMOTION OF ICE NUCLEATION AND INHIBITION OF ICE GROWTH BY MACROMOLECULES

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The formation of ice crystals is a widespread environmental process with beneficial effects such as initiating atmospheric precipitation as well as adverse consequences such as biological frost damage. Several molecules of natural origin exist that can either promote or inhibit ice crystal formation, for example ice-nucleating proteins in bacteria or ice-binding antifreeze proteins in polar fish. This presentation will focus on different mechanisms that influence the kinetics of ice crystal formation and growth. Such processes include the promotion of ice nucleation by suspended particles and dissolved molecules as well as ice growth inhibition by adsorption of antifreeze molecules to ice crystal surfaces. Different experimental techniques for the study of homogeneous and heterogeneous ice nucleation processes will be presented, for example differential scanning calorimetry of water-in-oil emulsions as well as an optical droplet freezing array (BINARY: Bielefeld Ice Nucleation ARraY). Moreover, a recent method to study the kinetics of ice recrystallization in polycrystalline ice

samples will be discussed (IRRINA: Ice Recrystallization Rate INhibition Analysis). It will be shown how IRRINA can be used to quantify the ice recrystallization inhibition efficacy of different molecular inhibitors. Overall, the mechanistic and molecular aspects of the involved processes will be given special consideration.

Source of Funding: This work was supported by funding from the Deutsche Forschungsgemeinschaft through the FOR1525 INUIT research unit (KO 2944/2-2).
Conflict of Interest: None to disclose

S4 MEASURING THE TEMPERATURE OF THE COLDEST LIQUID WATER

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Water, the most abundant liquid on Earth and essential for life, presents many anomalous physical properties, which are enhanced in the supercooled regime, that is, when it remains liquid at temperatures below its normal melting point. The origin of such anomalies is due to the particular structure of hydrogen-bonds between the H2O molecules, although there is no consensus about a detailed explanation at the molecular scale. An efficient technique to produce supercooled liquid water in the laboratory consists of injecting micro droplets into vacuum, which are rapidly cooled by surface evaporation. However it is challenging to obtain a reliable measurement of the droplets temperature under such extreme experimental conditions. Several approaches, like modelling of evaporative cooling or Raman thermometry, have been used in the past. In this lecture, recent experiments [Goy et al., Phys. Rev. Lett. 120, 015501 (2018)] will be presented, where it has been possible to measure the temperature of liquid water microdroplets with great precision, using shape-dependent resonances in the Raman scattering from a train of perfectly uniform water droplets, showing that a fraction of the water droplets, with an initial diameter of 6380 nm, remain liquid down to 230.6 K. Our experiments could provide valuable information on the hydrogen-bond network in liquid water in the hard-to-access deeply supercooled regime.

Source of Funding: Bundesministerium für Bildung und Forschung (05K13RF5), and Spanish Ministerio de Economía y Competitividad (FIS2013-48275-C2)
Conflict of Interest: None to disclose

S5 STRATEGY FOR INHIBITING HETEROGENEOUS ICE NUCLEATION

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Homogeneous ice nucleation in micrometer-sized water droplets at around -40 °C has been successfully investigated at a laboratory level. In most practical cases, however, heterogeneous ice nucleation occurs on solid surfaces. Therefore, it is important to understand and control heterogeneous ice nucleation. There are two common approaches to inhibit heterogeneous ice nucleation; one is changing the bulk water properties by using concentrated aqueous solutions, and the other is changing the properties of solid surfaces, for example, by coating the surfaces with some molecules. Although these two approaches may seem completely different, they possibly rely on the same fundamental strategy of altering the hydrogen bonding network between water molecules required for ice embryo formation near the solid surface. When using concentrated solutions, the solute molecules affect the hydrogen bonding network of water molecules near the solid surface, as well as in the bulk solution. When coating the solid surface with some molecules, the modified surface properties locally affect the hydrogen bonding network of water molecules near the solid surface. Based on this strategy, we tried to inhibit heterogeneous ice nucleation by using traces of ions that might locally affect the hydrogen bonding network of water molecules near the solid surface. Experimental results showed that heterogeneous ice nucleation on silver iodide particles is effectively inhibited by adding some ions to water. We also discussed how ions can affect heterogeneous ice nucleation on silver iodide particles.

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Conflict of Interest: None to disclose

S6 REWARMING PARDOX DURING KINETIC (HYPERFAST) VITRIFICATION

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There are 5 ways to achieve long term biostabilization, namely" 1) slow (equilibrium) freezing (SF); 2) equilibrium vitrification (E-VF) with high concentration of vitrificants, moderately high rate of cooling and very fast speed of rewarming; 3) kinetic vitrification (K-VF) with very high rate of cooling and low (if none) exogenous vitrificants; 4) lyophilization (FD, freeze-drying), and 5) xeroproservation (XP, drying without freezing). We will show using the phase diagram that all 5 ways need to ensure intracellular vitrification, a necessary component for biostabilization of ANY kind. Thus, understanding the ice formation and growth mechanisms is very important, especially for SF, E-KF and K-VF, and at a certain degree, for FD.

Our team has discovered a "vitrification rewarming paradox": at VERY high rate of cooling (kinetic vitrification) one does need NOT very fast rewarming so a mild warming rate would be sufficient. We have developed a phenomenological model of ice formation and growth that not only explains this finding but states that during kinetic vitrification by hyperfast cooling, **the**

FASTER one cools the LOWER the critical rewarming rate actually is.

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Conflict of Interest: None to disclose

S7 ICE BINDING PROTEINS AND THEIR USE IN CRYOPRESERVATION

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Ice-binding proteins (IBPs) are found in organisms such as fish, insects, diatoms, plants, molds, and bacteria that cope with low temperatures. IBPs prevents freezing of organisms in mild supercooled conditions, inhibits ice recrystallization, and enable adhesion to ice. The mechanisms by which IBPs interact with ice surfaces are still not completely understood, and the potential of IBPs as cryoprotecting agents for cryopreservation of cells, tissue, and organs, have not yet been realized. We are investigating the interactions of IBPs with ice surfaces. In particular, we investigate the dynamic nature of the protein&ice interaction using fluorescence cryomicroscopy on a large temperature range. The results show that binding of IBP to ice is irreversible and that the freezing temperature depression is sensitive to the time allowed for the proteins to accumulate on ice surfaces. Our results relate the dynamics and level of activity of various types of IBPs to their ability to bind to specific ice orientations, in particularly to the basal plane of the ice. While in nature most organisms do not exposed to extreme temperatures such as those inflict on samples in vitrification conditions, we find indications that IBPs can function as ice growth repressors at low temperature, and thus open the possibility to reduce ice growth in devitrification. These results contribute to an understanding of the mechanisms by which IBPs act that will be critical for the successful use of IBP in cryobiological applications.

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Conflict of Interest: None to disclose

S8 IMPROVING CRYOPRESERVATION – A MODERN APPROACH FOR AN "OLD" PROBLEM

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Cell-based therapies are rapidly emerging as a critical aspect of modern health care and have shaped new models of care in stem cell therapy, regenerative medicine and transfusion practices. Cryopreservation is becoming increasing important for these therapies, however current cryopreservation protocols are suboptimal and improved cryoprotective agents (CPAs) and protocols are urgently required. During the past fifty years, the field of modern cryobiology has seen tremendous advances and much information has been gained with respect to the physical and biochemical effects when freezing cells, tissues and organs. Despite this understanding, ice formation is the major cause for decreased post-thaw viability and impaired cellular function. The Ben laboratory has discovered several classes of ice recrystallization inhibitors that control ice growth during cryopreservation and increase the post-thaw viability and functionality of many primary cell types including progenitor cells. This presentation will describe the "evolution" of small molecule ice recrystallization inhibitors (IRIs) from generation 1 to 5. Finally, a case study will be presented in which the first *in vivo* translation of the IRI technology will be highlighted. In this study, engraftment capability of HSPCs from umbilical cord blood is greatly improved. While post-thaw viabilities of HSPCs cryopreserved with second and third generation IRIs and DMSO are comparable to that of the 10% DMSO condition, clonogenic assays indicate improved differentiation and proliferation capacity. Increased platelet, progenitor, and B cell levels were observed in immuno-compromised mice transplanted with HSPCs cryopreserved with IRIs in primary and secondary transplant procedures. These data constitute the first step to using this technology as a platform to improve cryopreservation processes.

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Conflict of Interest: Dr. Ben is a co-founder and Director a PanTHERA CryoSolutions Inc.

S9 ULTRA-RAPID FREEZING OF DOMESTIC CAT SPERMATOZOA FOR POTENTIAL APPLICATION TO ENDANGERED FELIDS

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Ultra-rapid freezing (URF) of spermatozoa has proven challenging in mammalian species with most approaches resulting in low numbers of viable spermatozoa post-thaw compared to standard slow freezing methods. In previous research, we demonstrated the feasibility of producing AI kittens following URF of domestic cat sperm, using a soy-based cryomedium containing 0.2M sucrose for direct pelleting into LN_2. With improvement, this approach could be valuable for preserving sperm from endangered felids collected under field conditions. In this study, our specific objectives were to: a) evaluate inclusion of membrane protectants (0.1% BSA or 0.5% Equex) in our soy-sucrose extender; b) compare supplementation with two non-penetrating cryoprotectants

(0.2M sucrose or trehalose); c) assess freezing as pellets vs. cryoloops; and d) investigate addition of seminal plasma (0, 15, 30 or 50%, v/v) on post-thaw (PT) parameters. Ejaculates were collected via artificial vagina from three or four tomcats for each experiment (2-3 replicates/male). After thawing, sperm were centrifuged, resuspended in culture medium and assessed for

acrosome status and motility over six hours. Inclusion of membrane protectants had a neglible effect ($P>0.05$) on acrosome integrity (30±8%, BSA; 20±15%, Equex; 21±7%, sucrose alone; mean ±SD) whereas Equex had a negative impact ($P<0.05$) on sperm motility (1% and 0%; 0 & 6h PT) compared to BSA (25±7% and 8±7%) or sucrose alone (24±7% and 7±3%). Post-thaw motility and acrosome status did not differ ($P>0.05$) for cryoprotectant type or addition of seminal plasma. Sperm freezing in pellets resulted in greater motility (25±9%) and percentage of intact acrosomes (30±6%) than freezing using cryoloops (8±3% motility; 12±5% intact acrosomes; $P<0.05$). Collectively, none of these procedural modifications produced substantial improvement in post-thaw sperm quality compared to our standard URF protocol. Further research of URF with cat sperm is necessary before this approach can be applied routinely to conservation of wild felids.

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Conflict of Interest: None to disclose

S10 SEMINAL PLASMA ADDED BEFORE CRYOPRESERVATION AFFECTS STALLION SPERM BINDING TO BOVINE OOCYTES

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Survival of stallion spermatozoa during cryopreservation varies considerably between individuals. Adding seminal plasma (SP) from good freezers (GF) could aid the survival of spermatozoa from bad freezers (BF). The addition of SP from GF or BF prior to cryopreservation on sperm binding to bovine oocytes was investigated. Ejaculates from 3 GF and 3 BF stallions were processed by colloid centrifugation to remove SP, then sub-divided into aliquots treated with 5% GF SP and 5% BF SP before freezing. After thawing and preparation by low density colloid centrifugation, the sperm pellet was resuspended in modified Whitten's capacitation medium (MW) with $NaHCO_3$ and BSA. Salt-stored bovine oocytes were washed in warm PBS/PVA, equilibrated for 1h at 37°C and transferred to four-well plates containing MW. The following sperm treatments were added at 5×10^6 /mL: G1, GF spermatozoa with BF SP; G2, BF spermatozoa with BF SP; G3, GF spermatozoa with GF SP; G4, BF spermatozoa with GF SP. The plates were incubated for 14-18 h at 38°C in 5% CO_2, 95% humidity atmosphere. The sperm-oocyte complexes were fixed in 2% (v/v) paraformaldehyde in PBS/PVA at 4°C, washed, stained with Hoechst 33342 and mounted on glass slides. The number of spermatozoa bound to the zona pellucida (ZP) was assessed using confocal microscopy at 200x. Data were analyzed by Mixed Model using the SAS® software (version 9.3); significance was set to $P \leq 0.05$. All values are LSMEAN±S.E. The number of BF spermatozoa bound was significantly higher when treated with BF SP than GF SP (G2, 24.4±6.78 vs. G4, 12.05±6.78 $p \leq 0.0001$). No differences were seen when GF spermatozoa were treated with BF or GF SP (G1, 24.75±6.78 vs. G3, 19.92±6.78, respectively). In conclusion, SP-treated stallion spermatozoa binding to bovine oocytes is affected by the origin of the SP and spermatozoa.

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Conflict of Interest: Although JMM is the inventor and patent holder of the colloid used in these studies, the colloid was not compared with other products; therefore this is not considered to be a conflict of interest

S11 MicroRNA NETWORK ANALYSIS AND TARGET GENES ASSOCIATED WITH HUMAN SPERM CRYOPRESERVATION

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Recent studies have shown that sperm delivers many factors including miRNAs during fertilization. There is a lack of information about new aspects of sperm cryobiology such as miRNAs .This is the first study to evaluation the effects of cryopreservation on miRNA profile and target genes in human sperm using the Next-Generation Sequencing. Semen samples were collected from 36 normozoospermic according to the 2010 World Health Organization guidelines. After processing semen samples, each sample divided into 2 equal parts: fresh and frozen. The post-thaw sperm was analyzed for motility parameters, apoptosis status and DNA fragmentation.Total RNAs from each group were extracted with Trizol reagent and purified by using RNeasy mini kit. Single-end sequencing on an Illumina Hiseq ™2500 system (BGI, chin) was performed. Alignment mapped to the human reference genome(hg19). Unique sequences were mapped to specific-species precursors in miRBase 20.0 by BLAST searching to identify known miRNAs. miRNA profiles in groups were compared by Student's t- test. Statistically significant differences were determined at the level of $P<0.05$. TargetScan was used to predict the genes targeted by miRNAs. The gene ontology (GO) of the most abundant miRNAs and target genes were annotated with the Bioinformatic Database. To validate of miRNAs obtained we performed qRT-PCR assays. In comparison

with the fresh spermatozoa, there was a significant decrease in the motility parameters and increase in apoptotic rate in the cryopreserved spermatozoa. For analysis of the miRNAs ,we detected a total of 2,819 reads, and differentially expressed known miRNAs were identified in cryopreserved sperm compared to fresh group. KEGG Pathway showed that the target genes are involved in cancer signaling. We observed their target genes may be involved in early embryonic development.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S12 PROFILIN IS RELATED TO FREEZABILITY OF FROZEN-THAWED BOAR SPERMATOZOA

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Variation in sperm freezability among boars was often reported in the recent literatures.The objective of this work was to look for useful predictive indicators of boar sperm freezability. The comparative proteomics of boar sperm from good freezability and poor freezability boars (n=3, respectively) was carried out using isobaric tags for relative and absolute quantitation (iTRAQ) technology integrated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Among 147 differentially expressed proteins with a threshold of a 5-fold change, profilin presented the highest significant differences between the two groups of good freezability and poor freezability, and its decreased expression was also confirmed at the protein level by Western blot analysis (P < 0.05). The association of profilin with postthaw sperm viability and motility was confirmed using Pearson's linear correlation. It's important to the damage degree of actin cytoskeleton in sperm for its physiological function after cryopreservation. As a actin-binding proteins, profilin involved in actin cytoskeleton regulation can be used as markers of sperm freezability.

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Conflict of Interest: None to declare

S13 EFFECT OF MINOCYCLINE ON ROS-PRODUCTING DURING CRYOPRESERVATION PROCESS ON RAM EPIDIDYMAL SPERMATOZOA

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Reactive oxygen species (ROS: H_20_2, 0_2, OH, ROOH) and antioxidant defenses shown to play an important role in fertility. Direct and indirect evidence indicated that some steps of cryopreservation of semen involve production of toxic ROS. The object of this study was to evaluate the ability of minocycline to decrease ROS level in ram frozen-thawed epididymal sperm. Testis from ten rams were collected from local slaughterhouse. In the laboratory, sperms from epididymis were recovered by flushing and cryopreserved in tris- egg yolk solution supplemented with various concentrations (5mg, 10mg) of minocycline. After thawing, Sperm motility and progressive motility (CASA), malonaldehide concentration (thiobarbiotoc acid), H_2O_2 (dichlorofluorescin diacetate DCFH-DA) and O_2 (dihydroethidium DHE) by flow cytometry were assessed. Extender added with 10mg minocycline yielded the highest results for total (52.95±0.76 %) and progressive motility (15.13±0.64 %), average path velocity, (53.01±0.31, μm/s) and linearity (58.50±0.42%). We could not detect significant changes in other kinematic parameters. The addition of 10mg minocycline provided a significant effect on level of ROS after freeze-thaw process, compared to the control group. Considering membrane lipid peroxidation, the lower MDA concentration were related to 10mg minocycline and control group (0.0022±0.0003 nmol/ml), (0.0025±0.0003 nmol/ml) compared to 5mg minocycline (0.0037±0.0003 nmol/ml). The basal levels of H_2O_2 in 10mg minocycline (18.3±1.11%) and 5mg minocycline (22.8± 1.04%) was significant lower compared to control group (28.03± 0.85%). Also, the mean reflectance fluorescence DHE that indicated intracellular O_2 in 10mg minocycline group (1.5±3.12%) was the lowest compared to the5mg minocycline (1.9±3.12%) and control group (2.43±3.12%) although these differences were not significant. Minocycline-enriched freezing extender improved the post-thaw epididymis ram sperm cells quality. The protection offered by minocycline occurred at mitochondria, involving suppression of cytochrome c release and subsequent decreased levels of intracellular ROS and preservation of mitochondrial integrity and lipid peroxidation.

Source of Funding: The University of Kurdistan
Conflict of Interest: None to disclose

S14 CRYOPRESERVATION OF SPINY MOUSE EPIDIDYMAL SPERM

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Sperm cryopreservation protocols are species specific and there is currently no defined cryopreservation protocol for the spiny mouse. The aim of this study was to define a robust cryopreservation protocol for spiny mouse mature, epididymal spermatozoa. Epididymal sperm samples from adult spiny mice (n=24) were cryopreserved at two different rates (>2000oC/min and 20-40oC/min) in six different cryoprotectants containing varying concentrations of raffinose and skim milk from 10% raffinose, 10% skim milk to 18% raffinose, 3% skim milk. Samples cryopreserved at a slower rate were

aspirated into 0.25mL straws (10uL) and held at -140oC in liquid nitrogen vapour for 10 minutes and then directly plunged into liquid nitrogen. Samples cryopreserved at faster rates were loaded onto fibreplugsTM (2uL) and touched for 3 seconds on a pre cooled block at -196oC and then inserted into pre cooled straws in liquid nitrogen. Motility and membrane integrity was assessed by phase and fluorescence microscopy respectively. Sperm parameters (P>0.05) were significantly reduced in all treatments compared to fresh controls. However, sperm parameters in solutions containing (i) 18% raffinose, 3% skim milk, (ii) 15% raffinose, 3% skim milk and (iii) 15% raffinose, 5% skim milk were not significantly different from each other, but were significantly greater than solutions of 10% raffinose or skim milk regardless of cooling rate. Lastly, motility and membrane integrity in samples cooled at a >2000oC/min were significantly lower than those cooled at 20-40oC/min. Overall, cryopreservation at slower rates allows for successful cryopreservation and storage of spiny mouse spermatozoa for use in future IVF to provide staged embryos for embryo development and implantation studies.

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Conflict of Interest: None to disclose

S15 EFFECT OF FETUIN AND TREHALOSE ON POST-THAWED RAM SEMEN DNA INTEGRITY

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Damage on the DNA (deoxyribonucleic acid) of the spermatozoon significantly effects fertilization. DNA damage can be caused by many factors (free radicals, hormones, mismatch, etc.). For this reason, many detection methods have been developed to determine the damage on DNA and many precautions have been taken to reduce these damages. The aim of current study was to evaluate effect of fetuin and trehalose on post-thawed ram semen DNA integrity. The percentages of TUNEL-positive cells and the cells with DNA damage in randomly selected 200 sperm from two different slides were determined in each sample using light microscopy. All values were expressed as the mean±standard deviation. Mann-Whitney U test was performed to evaluate the % values of the groups statistically for both TUNEL and COMET assays. For TUNEL and COMET values of the groups, it was found that there were statistically significant differences between some of the groups. In light of these differences, some extenders containing trehalose (60 mM) and different doses of fetuin (2.5, 5, and 15 mg/ml) and glycerol (3 and 5%) were effective to prevent double-strand DNA breaks or DNA fragmentation. However, supplementation of glycerol (3%), trehalose (60 mM), and fetuin (5 mg/ml) together was more effective to prevent both double-strand DNA breaks and DNA fragmentation.It was concluded that supplementation of fetuin in semen extenders may provide a dose-dependent protection against sperm DNA damages in cryopreservation. Nevertheless, further analyses are needed to assess the protective effects of different cryoprotectants on DNA integrity of Merinos ram sperm during cryopreservation.

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Conflict of Interest: None to declare

S16 COMPARISON OF ICE NUCLEATION STAGE DURING CRYOPRESERVATION OF CROSSBRED (HF X SAHIWAL) AND BUFFALO BULL (BUBALUS BUBALIS) SEMEN IN TRIS CITRATE EGG YOLK EXTENDER

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Study was conducted to compare the ice nucleation stage during cryopreservation of crossbred (HF x Sahiwal) and buffalo bull (*Bubalus bubalis*) semen in Tris citrate egg yolk extender. Ten ejaculates, each from 3 crossbred (Sahiwal x Holstein Frisian) and 3 buffalo (Bubalus bubalis) bulls, maintained at bull station, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, India (Lattitude/Longitude, 30.55°N, 75.54°E) were collected. Semen was extended in Tris citrate egg yolk extender and equilibrated at 4°C for 4 hours. Straws (0.25 ml) containing 20 million sperms were frozen using programmable freezer by applying freezing rates at -30°C/min (4°C to -15°C) and at -50°C/min (-15°C to -140°C), followed by plunging of straws in into liquid nitrogen. Ice nucleation stage in crossbred and buffalo bull semen was recorded as indicated by peak of latent heat of fusion. Post thaw semen quality was assessed in terms of a) motility, b) viability, c) membrane integrity (hypo-osmotic swelling test; HOST) and d) sperm abnormalities. Data was arc sine transformed and analyzed through one way ANOVA using SPSS software. The ice nucleation temperature was significantly (p=0.04) lower in crossbred bulls (-15.66±0.48°C) as compared to buffalo bulls (-17.58±0.75°C). Individual motility, viability and membrane integrity was significantly higher in crossbred

bulls as compared to buffalo bulls. It was concluded that ice nucleation starts early in crossbred bulls as compared to buffalo bulls under similar conditions during semen freezing.

Source of Funding: Not applicable
Conflict of Interest: None to declare

S17 MESQUITE FLOUR AS A NOVEL INGREDIENT FOR "PANETTONE-LIKE" BREAD: APPLICATION OF PART-BAKING AND DOUGH FREEZING TECHNOLOGY

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Prosopis alba is a leguminous tree widely distributed in several American countries. The flour obtained by grinding the pods, named mesquite flour, contains valuable nutritional and functional components, mainly fiber and minerals, that contribute to enrichment this food ingredient. The main use of this flour is in breadmaking. In the present work, this flour (MF) (from 15 to 35%: MF0, MF15, MF25, MF35) was blended with wheat flour (WF) to obtain composite sweet breads. Part-baking of provides fresh products in minimally equipped sale points with the advantage that the supply is on demand. Part-baking technology was successfully used in formulations with MF since after eight weeks of frozen storage (−18 °C) no changes were observed in the texture parameters of breads in comparison with non-frozen bread. Dough frozen technology did not significantly change moisture and water activity of dough during storage, and no exudates were observed. A significant effect of the level of MF and storage time (up to 12 week) on the elastic modulus G' was detected for freeze dough, while tan d (G"/G') decreased only with the increment of mesquite flour, suggesting the formation of more elastic dough. The maintenance of rheological parameters, associated to the integrity of dough structure during freezing process, is a positive effect observed for MF15 and MF25. Results suggest that MF can protect dough during freezing and consequently would generate breads of good quality.

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Conflict of Interest: None to disclose

S18 FREEZING UNDER PRESSURE: LOW TEMPERATURE AND HIGH HYDROSTATIC PRESSURE IN FOOD TECHNOLOGY

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Pressure is a thermodynamic property, as essential to determine the state and evolution of physical systems (including "biological systems"), as temperature is. However, while variations in temperature are evidently having an effect on anything surrounding us, and specially on living systems, pressure tends to be forgotten, taken for granted at its widespread "atmospheric pressure" level. However, by controlling this parameter, we can induce dramatic effects on many systems.

A significant part of the influence of temperature on biological systems surges in relation to its aqueous content, and for low temperature, as a consequence of water anomalous phase behavior, when both thermodynamic and kinetic aspects of water turning into ice (or glass…) are so relevant to Cryobiology. Pressure has a direct route to come into this play through the different possibilities offered by the complex water pressure-temperature phase diagram.

Solid water, only available at atmospheric pressure as ice I (and amorphous forms), can occur in an increasing number of phases with different temperature-pressure existence regions and distinct properties. Pressure can be used in combination with temperature to act on aqueous systems to obtain novel effects.

Food frequently can be considered as a high water content system, with properties and structure derived from its natural origin and the different technological manipulations suffered. Several uses of the combination high pressure-low temperature are either under study or in application and they may give rise to new products and technological possibilities. In several cases, the ability of high pressure to avoid ice formation at normally freezing temperatures, is exploited: to reduce the size and homogenize the distribution of ice crystals in tissues, with reduced ice damage; to store at low temperature without ice formation, and as effective and mild thawing processes. In other cases, alternative ice forms to ice I are employed.

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Conflict of Interest: None to disclose

S19 WITHDRAWN

S20 ASSESSING MOLECULAR CHANGES DURING PLANT CRYOPRESERVATION BY NOVEL RAMAN AND AUTO-FLUORESCENCE HYPERSPECTRAL IMAGING

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A big interest exists to identify the metabolomic changes that occur in plant tissues or food products that have experienced some modifications like the exposure to environmental contaminants or genetical modifications. To study these metabolic changes and its impact to human health, standard protocols involve cryopreservation. However, the molecular content of plants may suffer changes during cryopreservation. By

comparing cryopreserved and freshly excised plant tissues we can accurately assess the molecular changes that occur by using Raman and auto-fluorescence hyperspectral imaging. By combining hyperspectral imaging and novel chemometric tools we can provide simultaneously spatial and spectral information about the sample.Rice leaves and stems were cut in small sections, embedded in OCT polymer and flash frozen in liquid N_2. 18 µm thick cryosections were obtained and imaged by Raman and fluorescence spectroscopy. Raman spectroscopy and auto-fluorescence techniques are label free and images can be acquired with good spatial resolution. This enables to obtain additional information about the molecular distribution of samples compared with other analytical techniques that require bulk analysis and homogeneous sample extracts. Multivariate Curve Resolution Alternating Least Squares (MCR-ALS) is the chemometric method used to interpret the large amount of information obtained by hyperspectral images. MCR-ALS provides pure spectra profiles and distribution maps for each biological component of the sample. Multiset analysis has been performed to analyze separately images acquired on each of the different conditions (cryopreserved and fresh) in order to interpret better the changes that occur in tissues after cryopreservation. Besides, in plant tissues, the combination between Raman and auto-fluorescence spectral imaging is interesting because they provide complementary information. Overall, we observed a significant increase in the auto-fluorescence signal and different concentration of some molecular components which evidences changes in the molecular composition of tissues during the cryopreservation process.

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S21 WITHDRAWN

S22 CRYOBIOLOGY IN WILDLIFE CONSERVATION

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Close to 25% of species in the animal kingdom face the risk of extinction. A variety of stresses, largely anthropogenic in nature, on the one hand, and lack of sufficient resources and political forces to help counter the accelerated extinction process, on the other hand, suggest that a large number of species will go extinct in the coming years and will be lost for ever, unless we act now to, at least, preserve samples from each and every threatened species. To preserve the world's biodiversity, establishment of genome resource banks is thus desirable. These institutions can preserve gametes, embryos and tissues that, when needed, can be used to replenish genetically dwindling populations or species that are extinct in the wild or, theoretically, recreate extinct species (de-extinction). A wide range of genetic preservation options have been developed over the short history of tissue and cell cryopreservation and success varies between classes and species. The number of species within which attempts were done at cryopreservation of any cell or tissue type is only a small fraction of the total number of species in the animal kingdom. Progress in the field has been slow for lack of resources, insufficient or completely absent basic knowledge about large proportion of species, difficult access to samples, and extensive variations between classes, species and, often, individuals. New technologies are still emerging; thus, when opportunity arises for samples collection, these should be of a wide variety, to cover current and future technologies. Such samples can include (but should not be limited to) gametes, embryos, testicular and ovarian tissues, somatic cells (or primary cell lines), and whole organs or whole animals. In this talk I will attempt to illustrate the current status in the field and the challenges that need to be overcome.

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S23 FROZEN AND UNFREEZABLE WATER DETECTED BY DSC: THEIR ROLE IN PLANT CRYOPRESERVATION

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The aim was to analyse frozen and unfreezable water and clarify its role in cryopreservation of garlic and apple tree germplasm. The amount of frozen and unfreezable water was measured by the Differential Scanning Calorimeter TA 2920 (DSC). Isolated shoot tips of the 'Greensleves' apple tree from *in vitro* conditions were encapsulated in alginate beads and air-dehydrated in a flow box. Isolated shoot tips of garlic 'Djambul' from bulbils were dehydrated by osmotics of cryoprotective mixture Plant Vitrification Solution 3 (PVS3). A lower concentration of PVS3 (glycerol/sucrose ratio 30/30 w/v) and shorter PVS3 treatment resulted in insufficient plant dehydration, high content of frozen water, and plant damage by liquid nitrogen treatment. Unfreezable water in plants is often associated with so-called bound water. The term bound water is often misunderstood and used misleadingly. Unfreezable water in plant tissues was determined as an extrapolation of frozen water determined by DSC to the zero value, in relation to the water content determined by drying at 105 °C. The unfreezable water was 0.73 and 0.42 H_2O/g DW in garlic and apple tree, respectively. The water content was 0.11 - 0.21 g H_2O/g DW and the glass transition temperature was from - 95 to - 17 °C in garlic and 0.24 - 0.72 g H_2O/g DW and the glass transition temperature from - 87 to - 45 °C in apple tree. When comparing the unfreezable water content in garlic it was found out of the water content range where the glass transition occurred. When comparing unfreezable water content in the apple tree, it

was found within the range of water content where the glass transition occurred. Thermal analysis of different water states in the plant samples reveals important information to improve cryopreservation procedures.

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S24 USE OF DESS AS NOVEL CRYOPROTECTANTS FOR EFFICIENT MAMMALIAN CELL STORAGE AND PRESERVATION

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Deep Eutectic Solvents (DESs) are molecular complexes typically formed between quaternary ammonium salts and hydrogen-bond donors. The charge delocalization that occurs through hydrogen bonding between the halide anion and the hydrogen-donor moiety is responsible for the decrease in the melting point of the mixture relative to the melting points of the individual components. DESs share many characteristics of conventional ILs (e.g., nonreactive with water, nonvolatile, and biodegradable) and offer certain advantages. For instance, the preparation of eutectic mixtures in a pure state can be accomplished more easily than that of ILs with no need for postsynthetic purification because the purity of the resulting DES simply depends on the purity of the individual components. Moreover, the low cost of eutectic mixtures based on readily available components makes them particularly desirable (more so than conventional ILs) for large-scale synthetic applications. Herein, we have explored how DESs modify the H-bond network structure of water and hence, act as novel cryoprotectants for efficient mammalian cell storage and preservation.

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Conflict of Interest: None to disclose

S25 COMPARISON OF SUCROSE AND SYNTHETIC ICE BLOCKERS AS ICE MODULATORS FOR VITRIFICATION

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Successful cryobanking of multicellular tissues and organs calls for the avoidance of ice-driven injury by reliance upon ice-free vitrification techniques. The design of cryoprotectant solutions concentrated enough to permit vitrification of multi-milliliter samples, while maintaining tolerable toxicity levels remains challenging. One conceptual approach to the development of new vitrification solutions suitable for large samples is to start with a base solution that has proven utility for smaller samples, and then form new compositions by adding solutes that enhance the solution's stability against ice crystallization and growth. This has been done for the vitrification solution DP6 (3M dimethylsulfoxide and 3M propylene glycol). The application of synthetic ice modulators (SIMs) enables lowering the CPA concentration, thereby reducing the toxicity potential. In turn, its application decreases the critical cooling and rewarming rates and reduces the risk of structural damage to the tissue, as a consequence of thermo-mechanical stresses. We have previously provided evidence that blood vessel specimens display a very high rate of functional recovery after vitrification in various marginal conditions with the application of SIMs. As an extension of our previous work, aimed at scale-up of a baseline blood vessel model, this study compares the relative toxicity and vitreous stability of >10ml samples of DP6 containing either sucrose as a SIM, or the commercial synthetic ice blockers (X1000and Z1000). Using that established protocol, the addition and removal of DP6 + 0.6M sucrose and DP6 + 1%X1000+1%Z1000 were both well tolerated in a pig femoral artery model, when assessed for metabolic recovery and contractility. Using cryomacroscopy, we demonstrate that DP6+0.6M sucrose provided a stable vitrification medium under marginal cooling and warming conditions that resulted in >50% survival. By contrast and under the same conditions, DP6 + 1%X1000+1%Z1000 was subject to visible ice formation during cooling, resulting in a significantly lower recovery of ~20%.

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Conflict of Interest: M. Taylor; Z.Chen; E. Greene; L. Campbell; K. Brockbank were either part-time or full time employees of Tissue Testing Technologies.

S26 A NOVEL POLYSACCHARIDE-BASED APPROACH FOR CRYOPRESERVATION

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Cryopreservation of biological tissue has had a big spotlight in the field of medicine, as it aims for long-term storage of whole organs for transplantation and constant improvement of biobanking methodologies. However, current procedures still struggle with cryoprotectant cytotoxicity, optimal freeze-thaw rates and efficient carrier formulas. The constant need for tailoring the conditions for each individual sample therefore becomes

a strenuous approach in ensuring the highest functional viability.

In this work we implemented a fully biodegradable, bio-based heteropolysaccharide of bacterial origin in cryoprotective formulas. So far, it has been shown to have potential use in food, pharmaceutical, oil drilling and bioremediation applications. In this work, the biopolymer was shown to have antioxidant activity and an antifreeze activity similar to that of antifreeze proteins. When included in cryoprotective commercial formulas, it retained its rheological properties while also securing outstanding colloidal stability of all electrolyte-rich media. Cryogenic studies on microplate-adhered mammalian cells showed that the biopolymer has great promise for implementation in cryoprotective formulas: it shows no significant cytotoxicity, its inherent viscosity does not hinder nutrient diffusion and is able to preserve both the morphological and functional integrity of cells by protecting them against cryoinjury and osmotic shock at an extracellular level.

The ability to freely tailor the biopolymer's properties either for perfusion or immersion solutions and a multitude of beneficial properties renders it as a valuable asset in novel formulations, reducing their cost. The demonstration, for the first time, of its performance in cryobiology is comparable to currently used commercial synthetic polymers, which adds considerably to its value and further drives industrial implementation stemming from bio-production.

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Conflict of Interest: None to disclose

S27 EVALUATION OF ANTIFREEZE PROTEIN III FOR CRYOPRESERVATION OF HUMAN SPERM

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Lower fertility in humans with usage of frozen-thawed semen is attributed because of sperm damage that is believed to be due to the formation of ice crystals during cryopreservation. We hypothesized that addition of antifreeze protein III (AFPIII) to the extender will improve the viability, sperm progressive motility (SPM), plasma membrane integrity (PMI) and DNA fragment index (DFI) of cryopreserved human sperm.

Ejaculated semen was collected from 20 normospermic male and divided into four AFPIII supplemented groups (0.01, 0.1, 1 and 10µg/mL) and one control group without AFPIII. Semen samples were treated with Glycerol-egg-yolk-citrate medium (1:2, at 37○C, final concentration: 40×106 sperm/mL) contained mentioned concentrations of AFPIII, packed into straws. The straws were exposed to liquid nitrogen (LN2) vapor for 10 min and then plunged into the LN2 after a week, straws were thawed at 37○C for 30s and then viability, SPM, PMI and DFI were assessed.

The percentage of sperm viability, SPM and PMI was higher ($p>0.05$) and DFI was lower in 1µg/mL AFP III group compared to control group. Sperm viability decreased ($p>0.05$) in 0.01, 0.1, 10µg/mL AFPIII groups. Other AFPIII concentrations did not show any positive effects on SPM, PMI and DFI parameters.

In conclusion, supplementation of the extender with 1µg/mL AFPIII can improve the results and efficiency of human sperm cryopreservation.

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Conflict of Interest: None to disclose

S28 INVOLVEMENT OF AQUAPORINS IN MAMMALIAN SPERM CRYOPRESERVATION

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Aquaporins (AQPs) are integral, transmembrane proteins that are involved in the transport of water and solutes across lipid bilayers. Thus far, up to 13 different AQPs have been identified in animal cells. These 13 AQPs are classified into three categories, depending on their sequence similarity and substrate selectivity (orthodox AQPs, aquaglyceroporins and superaquaporins). Aquaporins have been identified in the spermatozoa of mice, rats, humans, pigs, cattle and human, the most abundant being AQP3, AQP7, AQP8 and AQP11. In the male gamete, AQPs are related to the regulation of sperm volume, which is required for the differentiation of spermatids into spermatozoa during spermatogenesis, and to their transit along environments of different osmolality (male and female reproductive tracts). Because AQP3, AQP7 and AQP11 do not only transport water but also cryoprotectants, such as glycerol, there is an interest in elucidating whether they could be used for fertility preservation. With this aim, different studies in separate mammalian species (porcine, bovine and equine) investigated whether the presence of these AQPs in spermatozoa could be related to their cryotolerance. In boars, bulls and stallions, AQP3, AQP7 and AQP11 appear to be related, in a species-specific fashion, to the sperm ability to withstand freeze-thawing procedures. In

bulls, relative levels of AQP11 in fresh semen have also been found to be related to the fertilizing ability of frozen-thawed sperm both *in vivo* and *in vitro*. All these data suggest that AQPs are involved in the sperm response to variations of osmolality and to freeze-thawing procedures and indicate that further research on the use of AQPs to improve sperm cryopreservation protocols is warranted.

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Conflict of Interest: None to disclose

S29 THE MOVEMENT OF WATER AND CRYOPROTECTANTS IN MAMMALIAN OOCYTES AND EMBRYOS: MEMBRANE PERMEABILITY AND AQUAPORINS

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The permeability of the plasma membrane to water/cryoprotectants affects the suitable conditions for cryopreservation of the cell. There are two pathways for the movement of water/cryoprotectants across the plasma membrane. One is simple diffusion across the membrane, in which the membrane-permeability is low but its temperature-dependence (the Arrhenius activation energy) is high. The other is facilitated diffusion by a channel process, in which the membrane-permeability is markedly high but its temperature dependence is low. The permeability to water/cryoprotectants of mouse oocytes and 4-cell embryos was low, and the Arrhenius activation energy was high. This suggests that in mouse oocytes and 4-cell embryos, water/cryoprotectants move across the plasma membrane mainly by simple diffusion. On the other hand, the permeability to water, ethylene glycol, glycerol, acetamide, and Me_2SO of mouse morulae and blastocysts was high, but the Arrhenius activation energy was low. This suggests that water and these cryoprotectants move through mouse morulae and blastocysts mainly by channel processes. This high permeability relies on the expression of aquaporins because the suppression of aquaporin 3 expression markedly decreased the permeability to water, ethylene glycol, and glycerol. Furthermore, suppression of aquaporin 9 expression decreased the permeability to acetamide and Me_2SO. Similar results were obtained with bovine and pig oocytes and embryos. Our assessment of the movement of water/cryoprotectants in oocytes and embryos demonstrates that the pattern of movement is stage-specific rather than species-specific, although some species-specific cases exist. For example, acetamide and Me_2SO moved through bovine morulae and blastocysts by simple diffusion, and a marked increase in the permeability to water/cryoprotectants was observed in expanded pig blastocysts but not in pig morulae. The differences in the expression of aquaporins may also be related with species-specificity. Our results may be useful for developing cryopreservation methods for mammalian oocytes and embryos.

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Conflict of Interest: None to disclose

S30 CONCENTRATION DEPENDENCE OF CRYOPROTECTANT PERMEABILITY AND IMPLICATIONS FOR DESIGN OF CRYOPRESERVATION PROCEDURES

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Vitrification has proven to be a promising cryopreservation method for various cell types, including oocytes and embryos. To achieve vitrification, high cryoprotectant (CPA) concentrations are required, which increases the potential for osmotic damage and CPA toxicity. Thus, one of the most important considerations in the design of vitrification procedures is the method for adding and removing CPA. It is common to use cell membrane transport modeling to identify CPA equilibration procedures that avoid osmotic damage, and we recently introduced a new mathematical optimization approach that addresses both osmotic damage and toxicity. The resulting procedures are highly dependent on the permeability of the cell membrane to CPA. In our previous work, we assumed that the CPA permeability was constant and independent of CPA concentration. This assumption is ubiquitous in the cryobiology literature. However, a synthesis of literature data for human erythrocytes, a widely studied model of cell membrane transport, reveals that the CPA permeability decreases by more than a factor of 5 as CPA concentration increases. Human erythrocytes express aquaporin 3, a member of a family of aquaporins (known as aquaglyceroporins) that is permeable to water and small neutral solutes such as glycerol and ethylene glycol. The observed concentration dependence of CPA transport in erythrocytes can likely be explained in terms of saturation of a binding site on the aquaporin channels. Thus, other cell types that express aquaglyceroporins will likely also exhibit a strong concentration dependence of CPA transport. This has broad implications, as many cell types, including oocytes and embryos, have been shown to express aquaglyceroporins.

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Conflict of Interest: None to disclose

S31 EXPRESSION AND DISTRIBUTION OF AQUAPORIN 3 IN HUMAN OOCYTES AND EMBRYOS

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To determine the role of aquaporin 3 (AQP3) isoform in oocytes and embryo, we investigated the expression and distribution of AQP3 in human oocytes and embryos at different developmental stages. Human oocytes were obtained from female who had immature oocytes in in vitro fertilization cycles. 4-cell embryos, 8-cell embryos, and blastocysts were donated by patients who had a successful pregnancy from an in vitro fertilization program at Dalian Municipal Women and Children's Medical Center. Immunofluorescence staining, laser confocal microscopy were used to detect expression and distribution of AQP3 channels in oocytes and embryos. Total RNA was extracted from zona pellucida free oocytes and embryos. The cDNA was prepared reverse transcription, and whole transcriptome amplification was used by REPLI-g WTA Single Cell Kit. Then, the qRT-PCR was used to test quantitatively the expression of gene AQP3 and GAPDH in the oocytes and embryos. Q-RT-PCR shows that the level of Aqp3 transcripts was decreased dramatically at oocytes. And the expression of AQP3 was significantly increased in 8-cell embryos and early blastocysts. The immune-reactive signals were detected in nuclear membrane of the GV oocyte. It was localized in both nuclear membrane and plasma membrane of the MII ooctyte to blastocyst stage. In the blastocyst, AQP3 proteins were localized in both trophectoderm and inner cell mass.

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Conflict of Interest: None to disclose

S32 STABILIZING EFFECT OF FOS DURING FREEZE-DRYING AND STORAGE OF LACTOBACILLUS DELBRUECKII SUBSP. BULGARICUS RESULTS FROM A BALANCE BETWEEN HIGH AND LOW MOLECULAR WEIGHT OLIGOSACCHARIDES

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The aim of this work was to assess the role of mono- and oligosaccharides present in fructo-oligosaccharides (FOS) mixtures as protective agents during freeze-drying and storage of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333.

Different FOS mixtures were enzymatically obtained from sucrose and further purified by removing the monosaccharides produced as secondary products. Their glass transition temperatures (Tg) were determined at 11, 22 and 33 % relative humidity (RH). Bacterial cultures were freeze-dried in the presence of 20 % w/v solutions of the studied FOS. Their protective effect during freeze-drying was assessed by bacterial plate counting, and by determining the lag time from growth kinetics and the uptake of propidium iodide (PI). Plate counting during bacterial storage at 4 °C, and 11, 22 and 33 % RH for 80 days completed this rational analysis of the protective effect of FOS.

Purification of FOS led to an increase of Tg in all the conditions assayed. Microorganisms freeze-dried in the presence of non-purified FOS were those with the shortest lag times. Bacteria freeze-dried with pure or commercial FOS (92 % of total FOS) showed larger lag times (8.9-12.6 h). The cultivability of microorganisms freeze-dried with non-purified FOS and with sucrose was not significantly different from that of bacteria before freeze-drying (8.74±0.14 log CFU/mL). Pure or commercial FOS were less efficient in protecting bacteria during freeze-drying. All the protectants prevented membrane damage. The cultivability of bacteria freeze-dried with FOS decayed less than 1 logarithmic unit after 80 days of storage at 11 % RH. When storing at 22 and 33 % RH, pure and commercial FOS were those that best protected bacteria, and FOS containing monosaccharides were less efficient.

The effect of FOS on bacterial protection is the result of a balance between monosaccharides, sucrose and larger FOS in the mixtures: the smallest sugars are more efficient in protecting lipid membranes, and the larger ones favor the formation of vitreous states.

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Conflict of Interest: None to declare

S33 LIPID METABOLISM REGULATION AND ITS RELATIONSHIP WITH COLD STRESS RESPONSE IN YEAST

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The adaptation and survival of *S. cerevisiae* to temperatures below the optimal requires the deep remodelling of the lipid repertoire. A downward shift in temperature decreases membrane fluidity and permeability, which compromises its functionality and limits cell growth. Thus, homeostasis mechanisms are expected to operate to sustain membrane functionality. Recently, we have demonstrated that a drop in temperature in yeast cells induces decreased plasma membrane $PI(4,5)P_2$ levels, which in turn, modulate the activity of essential regulatory effectors and signaling pathways involved in lipid metabolism. As a result, cold exposure reduces complex sphingolipids abundance in favour of more phospholipids, promoting the formation of a thinner, less compact and more permeable plasma

membrane than that found at higher temperatures. The finding that PI(4,5)P_2 connects different lipid synthesis pathways uncovers a mechanism of how lipid metabolism is coordinated to tailor cell membrane composition and properties to environment temperature changes. The application of this knowledge open the possibility to improve cold tolerance in yeast strains and to obtain new products or ingredients based in yeast biomass or its fractions. In addition, given the prominent role of PI(4,5)P_2 as a modulator of lipid biosynthesis, therapeutic approaches based on the regulation of its synthesis and degradation may also provide new treatments for human diseases based on lipid-disorders.

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Conflict of Interest: None to disclose

S34 TOWARDS MINIATURIZED CULTURE COLLECTIONS: PRESERVATION OF BACTERIA BY FREEZE-DRYING IN MICROPLATES

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Freeze-drying is widely used as long-term preservation technique for bacterial cultures. In culture collections, the freeze-dried product is generally stored in air-tight glass ampules under vacuum to eliminate the hazardous effects of moisture and oxygen. However, the production of these ampules is a manual, time-consuming and expensive operation. In this study, freeze-drying of bacterial cultures in microplates was investigated as an alternative for glass ampules. Three fast-growing strains (*Bacillus subtilis* LMG 13579, *Escherichia coli* LMG 8063 and *Lactobacillus sakei* LMG 9468^T) and 2 slow-growing recalcitrant strains (*Flavobacterium columnare* LMG 10406^T and *Flavobacterium psychrophylum* LMG 13180) were selected as test panel. To obtain highest cell recovery after freeze-drying, different lyoprotectants were tested: horse serum + 10% trehalose (LPA1) and skim milk + 10% trehalose (LPA2) for the 3 fast-growing strains; LPA1, horse serum + 2,5% monosodium glutamate (LPA3), horse serum + 10% trehalose + 2,5% monosodium glutamate (LPA4) and a blend of LPA1 and Modified Shieh broth in a 3:1 ratio (LPA5) for the 2 slow-growing strains. Viability before and after freeze-drying was determined by plate count method and OD600 absorbance values. Bacterial cells, cultured in liquid growth medium, were distributed in 200µl aliquots in flat-bottom 96-well plates, centrifuged and resuspended in 200µl lyoprotectant. The plates were covered with a gas permeable seal, freeze-dried and closed with an air-tight seal for storage at 4°C. The cultures were resuscitated by adding 300µl of liquid growth medium. Best survival rates were obtained after 3 months storage for *B. subtilis* LMG 13579 (96%), *E. coli* LMG 8063 (94%) and *L. sakei* LMG 9468^T (95%) when freeze-dried in LPA1. *F. columnare* LMG 10406^T showed acceptable survival rates for LPA1 (44%), LPA3 (44%) and LPA5 (42%) whereas *F. psychrophilum* LMG 13180 only showed a high survival rate for LPA5 (100%), both after 6 weeks storage.

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S35 USE OF ARCTIC INSECT HEMOLYMPH AS A COMPLIMENTARY AGENT IN APPLIED CRYOPRESERVATION

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The hemolymph of overwintering insects, inhabiting Yakutia (Far East, Russia) rather than to be concentrated in, polyols, fats, amino acids and proteins. Due to the combination of the properties, concentrations and functions of these biomolecules, the hemolymph of such cold hardy insects is characterized by significant antifreeze efficiency. In our research, we used the hemolymph of two Arctic freeze-tolerant insects: A (*Adelocera fasciata* L) and U (*Chrysolina graminis graminis* L) to create a remedy for protection of human skin from frostbite during cold winter seasons. It was shown that insertion of the A-hemolymph into the antifreeze remedy did not cause any cryoprotective effect, while U-hemolymph significantly improved the cryoprotective efficiency of the remedy. The effect of insect's hemolymph in cryopreservation of both human blood lymphocytes and fibroblasts together with Me_2SO was also explored. It appeared that hemolymph from A had a negative effect on freeze resistance of both type of cells, while hemolymph from U acted as a positive effector in cell cryopreservation. The use of Me2SO with insect hemolymph resulted to a significant increase of the amount of intact fibroblasts: up to 85**%.** The cryoprotectant system also was effective at multiply cycles of freezing thawing of the cells at -85◦C. Thus, the research testifies the hemolymph of some cold-resistant insects can be very valuable complementary agent in cell and tissues cryopreservation. However, another one cannot be used for these purposes due to unknown reasons. In this respect, frontal analyzing of the Arctic insects hemolymph perhaps could result to detection of efficient natural cryoprotectants that could be potentially useful for specific purpose in applied cryopreservation.

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S36 BIOPHYSICAL PHENOMENA INFLUENCED BY THE NUCLEATION TEMPERATURE DURING SLOW-FREEZING PROCEDURES

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101) & CRB-ANIM (ANR11-INBS-0003), Marcy L'Etoile, France [2]Université Sorbonne Paris-Cité, Université Paris Descartes, Paris, France [3]INSERM U1148, Paris, France [4]Université Claude Bernard Lyon1, Université de Lyon, IMP, CNRS-UMR5223, Villeurbanne, France. **Corresponding Author*: hugo.desnos@vetagro-sup.fr**

In cell cryopreservation procedures by slow freezing, the non-control of the ice nucleation temperature (T_n), can involve a cell recovery decrease. Among the different explanations that can be proposed, we chose to evaluate the impact of the T_n on different biophysical phenomena associated to ice crystallization. In this purpose, we studied, for different T_n values, the evolution of biophysical parameters in four solutions during cooling (H_2O and IMV®, with or without 10%($^v/_v$)Me_2SO). This study confirmed, with cryostage videos and differential scanning calorimetry (DSC) thermograms, the existence of two dominant ice crystallization modes. The first happens when the solution is supercooled and occurs with a dendritic growth of ice. The second regime starts near to the thermodynamic equilibrium and is determined by water molecules diffusion. We evaluated the dendritic growth speed and the size between two dendrites according to the supercooling magnitude and the solution composition. From DSC measurements, we also estimated the water proportion crystallized in both modes. As the equilibrium crystallization rate evolves with temperature along the liquidus curve in the phase diagram (associated with the solution composition evolution), this proportion is impacted by T_n. Accordingly, we discussed the T_n influence on the solution composition variation when crystallization occurs. Because the theory of slow freezing protocols assumes that the recovery of cells during cryopreservation is possible if cells are packed inside the intercrystalline over-concentrated solution, we studied by CryoSEM the T_n influence on the ice organization in these frozen solutions. We concluded that the T_n could impact this ice organization, but supercooling a given solution of 3 to 10°C led to negligible differences. This observation could not explain alone the cell recovery decrease. This study centered on T_n, highlights how changes in biophysical phenomena can partly explain a cell recovery decrease and could help to optimize cell cryopreservation procedures.

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S37 FREEZING POINT AT HIGH HYDROSTATIC PRESSURE: ROBINSON-STOKES EQUATION EXTENSION TO HIGH PRESSURES

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Pure water freezing point pressure dependence is well known, while ice I-liquid water curve in the phase diagram has been well characterized. However, pure water is scarce in Nature and data on more frequent and interesting aqueous solutions are scarce. Due to technical determination difficulties, only a few sets of independent data are published. A conceptual factor increases difficulties: when speaking of solutions, due to cryoconcentration taking place as soon as any ice fraction (solute free) is formed, we can only properly speak of "initial freezing point". Additionally, the only parameter referable in absolute terms and calculated using thermodynamic laws is the "(initial) equilibrium freezing point", to be well differentiated from "nucleation point", well below equilibrium temperature and following stochastic behavior, depending on ice nucleii formation driven by water molecules Brownian movements.

Freezing point of aqueous systems is required for model elaboration and energetic calculations related to freezing-thawing under high hydrostatic pressure, and natural situations within Geology, Glaciology, Oceanology, Meteorology and Astrophysics realms.

The classical Robinson and Stokes' equation relating solutions water activity (a_w) to its initial freezing point at atmospheric pressure has been modified and extended to higher pressures:
$-\ln a_w = 1/R \cdot (\Delta H(P) - J(P)\, T_0(P)) \cdot (1/T_f(P) - 1/T_0(P))$
(R: gas constant; ΔH: ice molar heat of fusion; J: molar heat capacity difference between water and ice; T_0/T_f pure water/solution freezing temperature). Terms denoted by (P) are taken at a given pressure, P. Assumptions for this derivation include: -a_w constant with pressure and temperature along the phase transition curve, -Ice and water chemical potentials are equal at equilibrium, at any pressure and $T_f(P)$, -J is equal for pure water and solutions, for all pressures.

The extended equation has been successfully tested against a variety of experimentally determined data, showing an average prediction error of 0.4ºC.

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S38 STABILIZING THE AMORPHOUS STATE OF TREHALOSE IN HIGH HUMIDITY ENVIRONMENTS

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Sugar glasses are commonly used in the food and pharmaceutical industry to immobilize and stabilize sensitive molecules and they have recently been explored for the preservation of cellular biologics both in the frozen and dry state. Retention of the amorphous state is very dependent on the local humidity, as water is a known plasticizer that will increase the molecular mobility and lower the glass transition temperature (T_g) of the composition. Water uptake in high humidity environments can result in the T_g falling below the temperature of storage (T_s), increasing the probability that the sample will crystallize within practical time scales and thus shorten the shelf-life of the biological

product. In order to preserve biologics during storage for extended times, delaying crystallization and retaining the amorphous state under adverse moisture excursions is desirable. Additives, such as polymers, other sugars, and salts have previously been used to modify the physical properties to increase the stability of trehalose glasses. Salts are of special interest as electrolytes are often added to formulations that are designed for biological systems. In this work a dynamic vapor sorption method was used to study the crystallization kinetics and construct sorption isotherms for trehalose in combination with a number of inorganic and organic salts. With this knowledge, a superior class of organic salt additives that stabilized the amorphous state of trehalose at high humidity was identified.

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S39 MODULATION OF LIPID BILYAER MEMBRANES CONTRIBUTES TO CRYOPROTECTION UPON RAPID COOLING CRYOPRESERVATION

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Rapid cooling and warming is a promising approach for cryopreservation of living cells. Under the assumption that intracellular formation of ice needs to be completely suppressed to achieve survival, this is often referred to as vitrification. To test this hypothesis, we have measured ice crystal formation directly by cryo-electron microscopy and diffraction in and around cryopreserved HeLa cells. By correlation to survival of such cells, we found that complete vitrification is neither necessary nor sufficient for successful cryopreservation. We found no correlation of the number of ice crystals or the total amount of ice within the cells with cell death. However, re-crystallization to fewer but bigger ice crystals correlated with an increase in cell death. The tolerable amount of re-crystallization depended on the cryoprotective agents used. These results imply that at least part of the cryoprotective effect is not plain suppression of ice crystal formation. Since these cryoprotective agents are known to modulate properties of lipid bilayer membranes, we tested, if we can modulate the properties of the plasma membrane of the cells to tolerate rapid cooling without the addition of cryoprotectants. Increasing the sterol content of the plasma membrane of HeLa cells indeed increased their plasma membrane integrity after rapid cooling and rewarming without addition of cryoprotectants (52.7 +/- 6.7 % intact cells vs 21.5 +/- 5.7 % in controls). Intracellular lysosomes, which did not get enriched with sterols, got damaged under the same conditions. This indicates that a modulation of membrane properties contributes to the cryoprotective effect upon fast cooling.

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S40 BIO-INSPIRED CELL CRYOPRESERVATION USING SYNTHETIC ANALOGUES

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Donor cells and tissue are vital components of modern medicine. Red blood cells can be kept for a maximum of 42 days, platelets for 8 days, and donor organs for just a matter of hours. In principle, cryopreservation can be used to enable the storage of cells and tissue. However, a simple method to preserve biological material does not exist and the development of this process would revolutionize the medical field. Additionally, a simple method to cryopreserve adherent monolayers of cells is currently not available, but the development of this technique could facilitate numerous applications in the field of biomedical engineering, cell line development, and drug screening. Nature has evolved several unique solutions to enable life to flourish at low temperatures including the over-production of small-molecule cryoprotectants during cold stress, such as the production of antifreeze proteins or antifreeze glycoproteins (AF(G)Ps) which act to reduce the rate of ice crystal growth as well as cause a non-colligative depression of the freezing point. Whilst these AFGPs have been implicated to be useful in cell storage, they are expensive and may not be biocompatible. We have explored the use of synthetic polymer mimics, which can reproduce the desirable functions of AFGP but benefit from the tunability of a synthetic system. We have shown that addition of these biomimetic polymers can enhance monolayer cellular cryopreservation, significantly while in the presence of amino acid pre-treatments. Additionally, we have shown success using an adapted polyampholyte that requires no pre-treatment for significant protection during freezing. In this presentation, we will discuss our progress using new bio-inspired polymer mimics of AF(G)Ps and polyampholytes along with their applications in the cryopreservation of red blood cells and attached monolayer mammalian cells.

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S41 DIFFUSION KINETICS OF DIFFERENT CRYOPRESERVATION SOLUTION COMPONENTS INTO OVARIAN TISSUE

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Cryopreservation of ovarian cortex tissue is typically done using mixtures of cryoprotective agents (CPAs).

CPAs protect the tissue during freezing, however, there are toxic effects with exposure to high concentrations as used for vitrification. To rationally design cryopreservation strategies, it is needed to determine permeation kinetics of CPAs to ensure maximum permeation and homogeneous distribution of all protective components, while minimizing the exposure time and toxicity effects. In this study, we have used an attenuated total reflection - Fourier transform infrared spectroscopy (ATR-FTIR) setup to simultaneously monitor diffusion of different components in CPA mixtures into ovarian tissues in real time. Diffusion studies were done with dimethyl sulfoxide, glycerol, ethylene glycol, and propylene glycol as well as with mixtures of these compounds. Diffusion was assessed by determining the increase in solute specific infrared absorbance bands during exposure to vitrification solutions. Clear differences in permeation rates and diffusion coefficients were observed among different CPAs dependent on molecular size and chemical properties. Furthermore, diffusion kinetics of CPAs in single component solutions differed compared to that of the CPA when present in a mixture. Tissue dehydration was monitored by looking at changes in absorbance bands specific for water. Corroborative DSC studies were done to assess the replacement of water in the tissue and the appearance of a glassy state during CPA permeation. When the ice melting peak was analyzed for tissue pieces incubated in CPA solution for different durations, it was found that area (i.e. amount of ice) and the melting temperature gradually decreased with permeation time. Furthermore, with longer exposure durations a glass transition temperature gradually became apparent. Taken together, a combination of ATR-FTIR and DSC provide useful tools to study CPA perfusion of complex CPA mixtures into tissues and to estimate the permeation time needed to form a glassy state.

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S42 DIETARY FATTY ACIDS, VITAMIN E AND HEN OVARIAN CRYOPRESERVATION; APOPTOTIC PATHWAY VIEWPOINTS

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Cryopreservation procedure induces release of apoptotic proteins from the mitochondria that stimulate different downstream effectors such as caspases 9 and 3. In addition, apoptotic pathways are activated after thawing that in turn activate caspases3, 8, and 9. It seems that using nutrients can reduce apoptosis during this process. Moreover, similar histological types of ovarian cellular proliferation have been reported in the hen as compared to women and thus hen may serve a good animal model to study. Our aim was to evaluate the effect of dietary fatty acids and antioxidant on incidence of apoptosis after vitrification and warming in laying hen ovaries. Sixty-eight aged 29 weeks Bovans White laying hens were randomly divided into four groups and were fed for 6 weeks: basal diet+1.5% sunflower oil (n-6); basal diet+1.5% sunflower oil+1.1 IU alpha-tocopherol/hen/day (n-6+E); basal diet+1.5% fish oil+1.1 IU alpha-tocopherol/hen/day (n-3+E) and basal diet+1.5% fish oil (n-3). The ovarian cortex samples were immersed into vitrification solutions (Dulbecco's modified Eagle's medium+15% EG+15% DMSO+0.5 M sucrose+10% fetal bovine serum). After warming levels of apoptotic genes were evaluated using real-time PCR. The expression level of Bak was significantly decreased in n-3+E compared to n-6 ($p<0.05$). Bcl-2 indicated a considerable upregulation in n-6+E and n-3 compared to n-6 ($p<0.01$). Apoptotic index Bak/Bcl-2 was significantly lower in n-3 than the n-6 (2.5 times; $p<0.05$). The expression level of caspase 3 of n-3 hens has dramatically decreased compared to n-6 ($p<0.05$). Also, the mRNA abundance of caspase 8 in n-3 was 1.8 time less than n-6. The expression level of caspase 9 in vitrified ovaries were not affected by diet. Our results show that cryopreservation activates components of both extrinsic and intrinsic pathways. This study provides new insight into the putative mechanism of fish oil action in the reduction of cryopreservation-induced apoptosis in ovarian cell.

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Conflict of Interest: None to disclose

S43 CRYOPRESERVATION OF CAT OVARIAN TISSUE: POST-TRANSPLANTATION EFFECTIVENESS

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The aim of this study was to test three different protocols for cat ovarian tissue cryopreservation and confirm their efficacy by autotransplantation. First, nine pairs of ovaries were obtained after elective ovariohysterectomy in mixed-breed cats. Fragments of ovarian cortex (1x1x5 mm) were subjected to slow freezing with Me2SO 1.5 M, ethylene glycol (EG) 1.5 M or a mixture of both (0.75 M each). All three cryoprotectants showed similar percentages of morphologically normal (MN) follicles at histology (80.56% with EG, 78.7% with Me2SO and 75.87% with EG + Me2SO). After analysis by electron microscopy, the Me2SO protocol showed the best results: absence of oocyte detachment and no cytoplasmic granules or plasmatic membrane rupture. This protocol was selected and autotransplantation was then performed to confirm follicle viability. For this step, four mixed-breed female cats were used. Ovariohysterectomy was performed and fragments of

ovarian cortex were subjected to the chosen slow-freezing protocol. After thawing, five fragments were transplanted to the subcutaneous tissue of the donor cats' dorsal neck. Fragments were removed after 7, 14, 28, 42 or 63 days post-transplantation and processed for histological analysis. The presence of fibrotic tissue after different periods of transplantation was evaluated by Masson's trichrome staining. The cryopreserved tissue showed 31.3, 3.3 and 26.8% of MN follicles after 7, 14 and 28 days of grafting, respectively, but no MN follicles were observed after 49 or 63 days. Moreover, two antral follicles were detected in one cat after 28 days. Fibrous tissue showed a clear increase mainly after 28, 49 and 63 days of transplantation. In conclusion, despite follicle loss after 49 days probably due to ischemia-reperfusion injury, data from electron microscopy regarding the suitability of Me2SO 1.5 M was confirmed, since MN follicles were present up to 28 days of transplantation.

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Conflict of interest: None to disclose

S44 EFFECTS OF DIFFERENT VITRIFICATION SOLUTIONS ON OVARIAN TISSUE CRYOPRESERVATION IN PREPUBERTAL DOGS

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There was no standard protocol associated by preserving follicle reserve in cryopreservation of ovarian tissue. This is the first study that compares the effect of different vitrification solutions in 40 ovarian tissues of prepubertal dogs. All tissues were cryopreserved by needle immersed vitrification (NIV) technique. The tissues were divided into pieces as control (C) and three different vitrification solutions groups [NIV I (12%), NIV II (15%) and NIV III (20%)]. Ovarian tissues were cut into 1 mm^3 pieces and placed in an acupuncture needle. The tissues in all vitrification groups were equilibrated in a solution consisting of 7.5% Ethylene glycol (EG) and Dimethyl sulfoxide (Me2SO) in Dulbecco phosphate buffered (DPBS) supplement with 20% FBS for 10 min. In groups of NIV I, NIV II and NIV III, tissues were equilibrated in cryoprotectant agents with a final concentration of 12%, 15% and 20% DMSO, 12%, 15% and 20% EG and 0.5 M sucrose for 2 min, respectively. The tissues were transferred directly into liquid nitrogen. After one week, the tissues were thawed. The tissues were immersed into Bouins' and glutaraldehyde solution for histological analyses. In NIV I (12%) and NIV II (15%) group, the primordial follicles were more preserved than in the NIV III (20%) group. The number of healthy follicles in the NIV III group was very low and the oocytes were damaged and contracted, their mitochondria were swollen. The linkage of oocyte and follicle cells were detached. The ice damage and vacuoles were noticed in the tissue structure in this group. In NIV I and II groups, there were more normal primordial follicles than in NIV III group. In these groups, it was observed that oocytes' nucleus was preserved, structures of the organelles were normal. The optimal agent percentage (dilution) was determined under 20% for dog vitrification equilibration.

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Conflict of Interest: None to declare

S45 THE EQUILIBRIUM VITRIFICATION TECHNIQUE FOR HUMAN OVARIAN TISSUE CRYOPRESERVATION

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The aim of this work was to test the equilibrium vitrification method for human ovarian tissue cryopreservation. Ovarian biopsies (n=7) from adult women (27 to 33 years of age) undergoing surgery for benign gynecological conditions were used. The samples were fragmented to 1x1x1 mm pieces, which were immediately fixed as fresh controls or placed in 24h *in vitro* culture as described below, and 5x5x1 mm pieces, which were subjected to the vitrification curve performed in a programmable controlled-rate freezing machine (Asymptote, VIA Freeze Research) adapted with a 'liquidus tracking' device. This ensured sample interchange between vials containing cryopreservation solutions with different ME2SO concentrations (15 minutes at each, 10%, 20% and 40% and 135 minutes at 50%), while the temperature was reduced to -40°C. Samples were placed in liquid nitrogen vapor (-150°C) before being submerged and stored (-196°C) until the warming protocol was initiated. The warmed tissue was cultured *in vitro* for 24h in order to re-establish tissue activity and metabolism and observe the cell response to the cryopreservation protocol. The tissue was finally fixed and histologically processed for cell morphology analysis. Despite high percentages of morphologically normal follicles detected in fresh tissue (98.9%), as well as in 24h *in vitro*-cultured fresh ovarian cortex (94.5%) the warmed cultured tissue showed no morphologically normal follicles. Alterations seen included pyknotic oocyte nuclei, degenerated and disorganized granulosa cells, detachment of follicles from the surrounding stroma and absence of an organized basal membrane. Fibroblasts from cortical stroma also showed signs of degeneration. Although the protocol established for bovine tissue did not provide adequate follicle morphology maintenance in humans, analysis of cell ultrastructure and cryoprotectant concentrations in tissue before and after warming is being undertaken in order to identify damage and improve the current protocol.

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Conflict of interest: Nothing to disclose

S46 DIFFERENTIAL GENE EXPRESSION OF FRESH AND CRYOPRESERVED RAT OVARIAN TISSUE GRAFTS

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Ovarian cryopreservation and subsequent autologous heterotopic or orthotopic transplantation has been introduced as an alternative fertility option for female patients who are at the risk of losing ovarian function due to chemo- or radiotherapy. Gene expression changes in cryopreserved (CAT; n=8) and fresh (FAT; n=8) autotransplanted and intact rat ovaries (IO; n=8) were compared using Illumina RatRef12 microarrays. Upregulated genes (>1.5 fold) were used to determine significantly (P>0.001) regulated biological processes (BP) using DAVID Bioinformatics Resources. Comparison of IO and CAT showed that positive regulation of protein kinase activity, positive regulation of cell proliferation, oxygen transport, glomerular visceral epithelial cell differentiation, estrous cycle, response to hypoxia, oxidation-reduction process, embryo implantation biological processes were included in significantly upregulated BP in IO. Some of the significantly upregulated BP in CAT compared to IO were cellular response to transforming growth factor beta stimulus, wound healing, collagen fibril organization, positive regulation of angiogenesis, cellular response to fibroblast growth factor stimulus, cholesterol biosynthetic process, innate immune response, negative regulation of cell proliferation. Comparison of FAT and IO showed that only oxygen transport BP was upregulated in IO. In contrast, upregulated BP in FAT compared to IO were wound healing, positive regulation of angiogenesis, inflammatory response, collagen fibril organization, positive regulation of apoptotic cell clearance, complement activation, cellular response to fibroblast growth factor stimulus, response to estradiol, cellular response to transforming growth factor beta stimulus, immune response, and cholesterol biosynthetic process. There were no BP that was upregulated in FAT compared to CAT. Significantly upregulated BP in CAT compared to FAT included response to drug, muscle contraction, negative regulation of cell-matrix adhesion, immune response, response to estrogen, complement activation, alternative pathway, positive regulation of angiogenesis. Results showed that both FAT and CAT had comparable gene expression and FAT had more similar gene expression pattern to IO.

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S47 WHAT IS NORMAL? RANGE OF SUBSET FREQUENCY IN "NORMAL" PBMC

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Immune monitoring utilizing PBMCs collected from study subjects is a critical component of immunotherapeutic research. Complicating this process is the normal variance in frequency observed across PBMC subsets in the healthy population. Due to the nature of research, it is not uncommon for the same subset of healthy volunteers to donate multiple times; providing control samples for a variety of unrelated studies; bringing to question, what is "normal" as defined by this small subgroup? In an attempt to quantitate this variance, whole blood was collected from six healthy volunteers and processed for PBMCs. Freshly isolated and cryopreserved/thawed PBMCs were assessed for recovery, viability, and analysed by flow cytometry for frequency of lymphocyte populations of T cells, helper T cells, cytotoxic T cells, NK, B cells, TREGs and monocytes. Total number of PBMCs recovered from whole blood ranged from 0.5 – 2.08 million per mL of blood collected, viability 97 – 100%. Post cryopreservation/thaw recoveries ranged from 60 – 99%, viabilities from 97 – 99%. Frequency of PBMC subsets as determined by flow cytometry; T, helper T, cytotoxic T, and TREGs cells yielded values ranging from 26.2 – 81.4%, 27.3 – 84.2%, 12.7 – 63.7% and 6.1 – 9.94% respectively for freshly isolated PBMCs and 53.1 – 84.8%, 29 – 84.7%, 12.6 – 63.8%, and 5.84 – 9.25% for cryopreserved cells. Frequency of NK, B cells, and monocytes ranged from 19.7 – 34.4%, 1.97 – 27.1%, and 6.26 – 15.2% for fresh cells and 31.2 – 64.9%, 8.66 – 43.2%, and 11.5 – 26.5% for cryopreserved cells. In conclusion, variance across PBMC subsets in "normal" donors can be extensive. This may be especially true for the small donor populations that support ongoing research. With standard deviations ranging from 1.3 to 20.36, depending on the subset examined, it becomes important that the investigator be aware that the control being utilized may be representing an extreme value in the "normal" range.

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S48 ERYTHROCYTE OSMOTIC FRAGILITY UNDER COLD EXPOSURES IN RATS OF DIFFERENT AGES

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Environmental stimuli could affect thermal balance in aging via different mechanisms among which the role of erythrocytes and their membrane condition are less determined. The aim of this study was to investigate the effect of long-term constant and short-term rhythmic cold exposures, which increased organism adaptive ability to cold, on erythrocytes osmotic fragility of rats of different

ages. The low-angle light scattering technique was used to study the erythrocyte transformation dynamics from which the osmotic fragility was calculated. Breedless male white rats (6, 12 and 24 mo) were used in the experiments. Long-term cold exposure was performed by keeping a group of rats under the varying ambient temperatures (from 1 °C to 7 °C) for 5 weeks under free-running light conditions with free access to water and food. Rhythmic cold exposure was performed for 2 days with different intensity of the cold stimulus (-12 °C and 10 °C) according to the following protocol: first 15 min of each hour (daylight hours), the animals were exposed to cold, the following 45 minutes they were kept at 26°C (total of 9 exposures per day). We have not found significant variations in erythrocyte osmotic fragility in rats of different ages in the experiments. The osmotic fragility decrease in 6 and 24 mo rats after rhythmic cold exposure with temperature -12 ˚C as well as in 24 mo rats after long-term cold exposure. No changes in osmotic fragility were found in all the rats under rhythmic cold exposure with temperature 10 ˚C.

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Conflict of Interest: None to disclose

S49 ICE RECRYSTALLIZATION INHIBITORS MITIGATE DAMAGE DUE TO TRANSIENT WARMING OF CRYOPRESERVED RBCS

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Red blood cells (RBCs) are typically cryopreserved using the high glycerol method which requires RBCs to be deglycerolized using several wash solutions extending the time required to deliver units to patients. The conventional low glycerolization method allows for post thaw processing time to be reduced, however they must be stored in liquid nitrogen and protected from transient warming events (TWEs). These TWEs can occur during normal operations but allow for ice recrystallization to occur in the presence of low concentrations of glycerol leading to RBC damage and reduced product quality. With the addition of small molecule ice recrystallization inhibitors (IRIs) this damage can be dramatically reduced allowing for higher storage temperatures eliminating the need for liquid nitrogen storage containers. By incorporating fast freezing rates and storage at -80°C, RBCs can be successfully cryopreserved with low concentrations of glycerol in the presence of an IRI. This was evaluating by freezing 2 mL 15% glycerolized RBCs with and without IRIs in liquid nitrogen vapour followed by -80°C storage for 28 days. Immediately post-thaw RBCs without an IRI were 86±3% intact (n=6) compared to 96±1% intact when an IRI was present (n=6). RBCs with an IRI were comparable to those stored in liquid nitrogen with 15% glycerol (96±1%, n=6).When RBCs were exposed to single TWE from -80°C to -40°C and stored for 7 days at -80°C, RBCs with an IRI demonstrated higher intact cells (86±6%, n=8) in comparison to 15% glycerol alone (47±7%, n=8). The effect is also demonstrated when exposing RBCs to multiple TWEs. By preventing damage associated with transient warming, IRIs permit RBC units to be cryopreserved with low glycerol concentrations and stored in conventional freezers. The use of IRIs in RBC cryopreservation solutions can significant simplify the process of storing these cells for clinical and research purposes.

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Conflict of Interest: None to declare

S50 CRYOPRESERVATION OF RED BLOOD CELLS USING ICE RECRYSTALLIZATION INHIBITORS AS NOVEL CRYOPROTECTANTS

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During cryopreservation, ice recrystallization is a significant contributing factor to cellular injury, resulting in reduced cell viability. Consequently, novel cryoprotectants that have the ability to inhibit ice recrystallization are desirable and have the potential to improve cellular viability. It has been demonstrated that small carbohydrate-based molecules have the ability to function as ice recrystallization inhibitors (IRIs) and these molecules are being investigated as novel cryoprotectants for cell and tissue-based systems. Current red blood cell (RBC) cryopreservation protocols employ high concentrations of glycerol (40% w/v) with slow cooling rates. Although 40% glycerol prevents cellular damage during freezing, a major drawback is the lengthy and time-consuming deglycerolization process. Phenolic glucosides β-PMP-Glc 1 and β-pBrPh-Glc 2 have been shown to be effective IRIs and have proven to be beneficial cryo-additives for the freezing of RBCs, demonstrating the ability to increase the percentage of intact RBCs post-thaw when used in combination with 15% glycerol. However, these IRIs yield low post-processing recoveries despite efforts to optimize the deglycerolization procedure. It is hypothesized that 1 and 2 are interacting with the membrane during freezing and as a result, RBCs are susceptible to osmotic stress during deglycerolization. To reduce interactions with the membrane, the amphiphilicity of these IRIs was modulated by incorporating an azide moiety. Azide derivatives of 1 and 2 were synthesized and used to cryopreserve and deglycerolize RBCs. It was shown that RBC recoveries were greatly improved using azide derivatives. In vitro cytotoxicity screening was performed and LD50 values were determined. Further investigation into the mechanism of cell death concluded that these compounds do not initiate apoptosis.

Additionally, in vivo studies were conducted with the most promising IRI and no toxicity was observed. This novel class of IRIs holds great promise as they have demonstrated the ability to successfully cryopreserve RBCs with significantly improved recoveries.

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S51 INTERDEPENDENCY OF FREEZING AND THAWING RATES ON CRYOPRESERVED HUMAN T CELLS

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Cryopreservation for cell therapies is usually performed according to a slow controlled-rate cooling profile (typically -1°C min^{-1}), followed by storage at cryogenic temperatures and eventually thawing in a 37°C water bath before infusion to patients, thereby achieving warming rates of approximately 45°C min^{-1}. Such high warming rates are considered critical for the recovery of cell viability and function post-thaw. However, there is a lack of scientific evidence to support this empirical practice, especially its relationship with the cooling rate applied during freezing. In this work, the interdependency between four different cooling rates using either a VIA Freeze controlled-rate freezer (0.1 and 1.0°C min^{-1}), a Planer controlled-rate freezer (10°C min^{-1}), or a direct plunge into liquid nitrogen (~159°C min^{-1}) and warming rates (113, 45, 6.2 and 1.6°C min^{-1}) on the post-thaw viability and functionality of T cells suspended in 10% Me_2SO in standard 2mL cryovials was studied. Results confirmed that high warming rates (≥ 45°C min^{-1}) lead to higher post-thaw recovery of T cells following a cryopreservation at a high cooling rate (10°C min^{-1}). However, when slow cooling rates were applied (0.1 or 1.0°C min^{-1}), there was no significant impact of the thawing rate. Complementary studies involving cryomicroscopy imaging and differential scanning calorimetry (DSC) suggested that non-equilibrium freezing occurs at rapid cooling rates and this leads to extensive ice recrystallisation phenomena upon slow warming, that could mechanically disrupt cells or cause an osmotic imbalance. On the contrary, equilibrium freezing profiles achieved with slow cooling rates lead to a stable ice crystal structure upon thawing, regardless of the warming rate applied. This data provides a fascinating insight into the crystal structure dependent behaviour during phase change of frozen cell therapies and its effect on live cell suspensions.

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S52 LESSONS FROM ANHYDROBIOSIS IN SEEDS: HOW NOT TO DIE WHEN DRY

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In the so-called orthodox seeds, desiccation tolerance is one of the most fundamental properties that is part of the plant's life cycle. Acquired half way through seed development, desiccation tolerance is a prerequisite that enables dry seeds to survive during storage and provides a means to tolerate environmental stresses, thereby ensuring the dissemination of the species. However, in the dry state, longevity, or life span, of seeds can vary tremendously from years to centuries. Seed longevity is progressively acquired after desiccation tolerance upon further development. Altogether, this suggests that anhydrobiosis requires additional components to be synthesized beyond the capacity to tolerate the total loss of water. This presentation will show that seed development is an excellent model to obtain knowledge on the protective mechanisms and underlying regulatory pathways that are put in place to ensure that seeds can cope with the physical and biochemical challenges accompanying the desiccation process and long-term survival in the dry state. First, we will demonstrate how a systems biology approach can help identify distinct regulatory and mechanistic programs associated with desiccation tolerance and longevity. Next, the development of orthodox seeds will be compared to that of recalcitrant seeds that remain desiccation-sensitive at shedding in order to discriminate putative mechanisms associated desiccation tolerance from those associated with drought tolerance.

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S53 ESTABLISHING AN ORGAN BANKING RESEARCH NETWORK

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Over the past 4 years, broad consensus has developed that advances in cryopreservation and related technologies can revolutionize logistics in a tremendous breadth of areas in biomedicine, such as organ, eye and tissue transplantation, biomedical research (spanning cancer, neuroscience, heart disease, and many other areas), personalized medicine, drug development, regenerative medicine, emergency medicine, and preservation of fertility in pediatric cancer patients. To promote development of a new generation of

cryopreservation technologies, the Organ Preservation Alliance is seeding and fundraising for a bioengineering "Apollo Program" to bank organs and large tissues in a viable, functional state. This network of laboratories and newly established research centers will work in tight collaboration to develop foundational cryopreservation methods that can be applied across a diverse range of organ and tissue systems. While this program is still in its infancy, input is sought from the cryopreservation community on the research directions to be prioritized.

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S54 DIFFERENT APPROACHES TO INCREASE THE NUMBER OF TRANSPLANTS

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Organ transplants save thousands of people daily from death. However, the shortage of organs available remains one of the most important challenges, especially in the case of thoracic organs with very short allograft ischemic time accepted.

According to the World Health Organization, the availability of organs from deceased humans for transplantation is not meeting the demand and one out of every 5 people on the waiting list dies while waiting for a donor, estimating that the transplants performed worldwide cover only 10% of the actual demand originated. In U.S., the need for heart transplantation is more than ten times the waiting list.

There are several strategies to increase the number of transplants: a) increasing the number of donors b) increasing the allograft ischemic time accepted, c) keeping the recipient alive waiting for the right donor, d) xenotransplantation, and e) creating a bank of organs for transplants. Strategies a, b and c are achieved: (a) through citizen awareness and official transplants agencies, (b) through improvements in myocardial protection strategies with new enriched intra and extracellular (blood and crystalloid) cardioplegic solutions, circulatory supports with continuous "ex vivo" organs perfusion, and hormonal therapy in "marginal" donors, (c) by means of circulatory support techniques such as extracorporeal circulation with membrane oxygenation (ECMO), centrifugal assistance, or mechanical ventricular assist with external artificial heart, in patients with terminal heart failure or primary graft failure.
Xenotransplantation, specifically transplantation of organs from genetically-modified pigs, offers immense possibilities and could resolve this problem, but the immunological and pathobiological problems prevent us from its routine clinical use. To date, chimerism by induced Pluripotended Stem Cells (iPSCs) and posterior Xenotransplantation it's just only a hypothetical and experimental option.

The immediate future lies in the creation of an Organ Bank by cryopreservation with cryoprotectant (ice-binding proteins) and associated nanoparticle rewarming.

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S55 ORGAN TRANSPLANTATION: CURRENT SITUATION IN SPAIN AND IN THE WORLD

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Transplantation has become one of the miracles of modern medicine. The World Health Organization estimates that more than 135,000 patients benefit from organ transplantation annually across the world. Although the activity is impressive, it barely covers 10% of the global transplantation needs of patients. As a result of organ shortage, many patients die or endure a poor quality of life while waiting for an organ, and unethical practices as organ trafficking and transplant tourism have emerged in different parts of the world. Organ shortage is therefore the main limitation to the expansion of transplantation therapies.

Since the creation of the Spanish Organización Nacional de Trasplantes (ONT) back in 1989 and the implementation of the so-called Spanish Model on Organ Donation and Transplantation, Spain has been a worldwide leader in organ donation and transplantation activities. The key for success of the Spanish system is an appropriate management of the process of deceased donation -- from the identification of donation opportunities, to the successful transplantation of organs in patients in need. Donor coordinators with the most appropriate profile (intensive care physicians) are designated in every hospital with a potential for organ donation. National and regional health authorities guarantee adequate guidance and continuous training of the donor coordination network. There is also a constant evaluation of performance in deceased donation through a Quality Assurance Programme. A specific relationship with the mass media has replaced any public campaign in support for organ donation. Hospitals are reimbursed to cover all resources required for donation, procurement and transplantation activities. The Spanish Model on Organ Donation and Transplantation has been partially or completely reproduced by other countries with successful results.

The Spanish system has also adapted to a scenario where mortality rates relevant to organ donation fortunately decrease, neurocritical care improves and important changes occur in the care of patients at the end of life. Novel strategies to increase donation opportunities include intensive care to enable organ donation, the use of organs from expanded and non-standard risk donors, and donation after circulatory death. These strategies may inspire other countries to better satisfy the transplantation needs of their patient population.

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S56 KRIOBLAST-3: A THREE-MODULE SYSTEM FOR KINETIC (HYPER-FAST) VITRIFICATION

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At CRYO2017, we presented *KrioBlast-2,* a pilot version of the *KrioBlast*™ platform for cryopreservation by kinetic (very fast) vitrification (K-VF). One of the major advantages of K-VF over the existing approach for vitrification (E-VF) is that K-VF does not need the high concentrations of potentially toxic and intracellular exogenous vitrificants, also called "cryoprotectants", which is not exactly correct in this case. The pilot experiments on human pluripotent stem cells and spermatozoa have shown an equally excellent survival (see a poster by Katkov et al). Here, we present the future development, an industrial three-module system *KrioBlast-3* that comprises 1) the cooling chamber for hyperfast cooling, 2) the intermediate module for shipment or long term storage in liquid nitrogen, and 3) the rewarming module. The second module has two port sites for the cooling and the rewarming modules so the system resembles a space station. All operations of cooling, storage/shipment, and warming are done without any contact of the sample with the ambient environment. The specific cryo containers for K-VF, namely *VitriPlate*^{TM}, *VitriComb*^{TM}, and *VitriScan*^{TM} for vitrification of cells in suspension, packed in straws, and attached to surface in multiwell systems respectively are also discussed.

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S57 HUMAN FIBROBLAST POST-THAW REGENERATION MONITORED BY AFM AND FLUORESCENCE MICROSCOPY

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The stiffness of cells is important phenomenon which reflects cell physiology. We were the first to use atomic force microscopy (AFM) combined with confocal fluorescence microscopy to study the impact of cryoprotectants and freezing/thawing on the stiffness of individual cryopreserved human fibroblasts during post-thawing growth and regeneration. Firstly, we demonstrated that stiffness of non-frozen cells decreased proportionally to the cryoprotectant concentration in the culture medium. Secondly, AFM allowed us to map cellular reconstitution in real time after a freeze/thaw cycle (cells were frozen slowly, in Me_2SO), and to differentiate the regeneration processes at the different depths of the cell and even at different parts of the cell surface (nucleus, edge).Depending on the use of high or low setpoint (and correspondingly, high or low indentation depth), AFM was able to monitor the post-thaw development of cell mechanical properties reflecting cellular processes located in the cytoplasm or in the submembrane region, respectively. Additionally, fluorescence microscopy showed (using actin staining) that the cytoskeleton in fibroblasts damaged by a freeze/thaw cycle was reconstructed after long-term plating (for 4 - 6 h). Also, it revealed (based on lamin staining) structural changes in nuclear envelopes of the frozen/thawed cells. Furthermore, AFM was used to demonstrate that for cells frozen/thawed in polyethylene glycol (a nonpermeating cryoprotectant), the mechanical properties and their temporal development were different from cells frozen/thawed in Me_2SO.

AFM could be used as a non-invasive method to identify thawed cells that regenerate their mechanical properties in an abnormal way. That could be important e.g. for assisted reproduction and future cell-based regeneration strategies.

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S58 NEW INSIGHT OF ANTIFREEZE PROTEINS VIA SITE-DIRECTED SPIN LABELING TECHNIQUE

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Site-directed spin labeling (SDSL) technique was used to examine the antifreeze mechanisms of type-I antifreeze proteins (AFPs). We observed the effects of spin-label groups attached to different side chains of the AFPs on the growth of seed ice crystals and probed the states of water molecules surrounding the spin-label groups via analyses of the variable-temperature (VT) dependent electron paramagnetic resonance (EPR) spectra. The first set of experiments revealed antifreeze activity at the microscopic level, while the second set of experiments displayed that at the molecular level. The experimental results confirmed the putative ice-binding surface (IBS) of type-I AFPs. The VT EPR spectral analysis indicates that AFPs can inhibit the homogeneous nucleation of seed ice crystals down to ~-20°C in their aqueous solutions. Thus, the present authors believe that AFPs protect organisms from freezing damage in two ways: (1) inhibiting the homogeneous nucleation of seed ice crystals, and (2) hindering the growth of seed ice crystals once they have formed. The first mechanism should play a more significant role in protecting against freezing damage among organisms living in cold environments. The VT EPR spectra also revealed that liquid-like water molecules existed around the non-ice-binding side chains of the AFPs frozen within the ice matrices; during the heating process, it was also observed that ice surrounding the spin-label groups melted at subzero temperatures. This presentation concludes with the proposed antifreeze mechanisms of AFPs based on the experimental results.

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S59 SIMPLIFIED METHOD TO STORE EMBRYOGENIC CELLS: SILVER NANOPARTICLES AND CRYOPROTECTORS ELIMINATION EFFECT

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Cryopreservation of embryogenic tissues is an essential requirement to maintain the competence of embryogenic cell lines. In our laboratory, a new method to store *Pinus radiata* embryogenic cell lines at -80ºC was developed successfully using an ultra-freezer. The methodology carried out was simple and similar to the standard cryopreservation protocols used for embryogenic tissues in several plant species: 1.- Pre-culture in a highly concentrated sucrose solution supplemented with Me2SO; 2.- Immersion of the samples in liquid nitrogen for 5 minutes; 3.- Storage at -80ºC. The technique was tested on 62 lines, through the assessment of regeneration capacity and plant conversion ability of the regenerated tissues after a year of storage at this temperature. Although the success of the methodology followed was high, as recovery percentage and plant conversion ability were both above 75%, we sought to improve our results and simplify the methodology. For these purposes, we analyzed the possibility of eliminating the liquid nitrogen step and the removal of Me2SO from preservation solution. The elimination of the two factors mentioned above was satisfactory and a percentage of regeneration of 100% was obtained. Furthermore, in a second experiment we tested if the use of silver nanoparticles in the culture medium and in the preservation solution had a beneficial effect on the regeneration ability of embryogenic cultures. No effect of the use of nanoparticles was observed with respect to the evaluated controls (above 85% in both cases). However the effect of these treatments in the production of somatic embryos and the performance of the resulting plantlets is being currently evaluated. Future experiments will study the combined effect of elimination of Me2SO and the addition of nanoparticles in the long-term preservation of embryogenic cell lines.

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Conflict of Interest: None to disclose

S60 REVERSIBLE CRYO-ARREST FOR IMAGING MOLECULES IN LIVING CELLS AT HIGH RESOLUTION

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Fluorescence imaging with slow acquisition times (e.g. super-resolution imaging and functional imaging) is impeded in living cells by the fast movement of the observed molecules. Images get blurred, which leads to loss of information. Additionally, artifacts can lead to false interpretation. Therefore, chemical fixation is commonly used, which interrupts the out-of-equilibrium state that normally maintains cellular shape. Thereby molecular patterns within cells can get distorted completely. Moreover, chemical fixation is lethal and does not allow to image individual cells at consecutive points in time. We have therefore developed reversible cryo-arrest of living cells on a microscope, which allows us to halt cellular processes at selected points in time. This permits to image the same living cells with practically unlimited acquisition time at consecutive points in time and thereby follow cellular processes like

signal transduction. In our reversible cryo-arrest ice crystallization is prevented by stepwise increase and removal of cryoprotective Me_2SO - on the microscope by a custom-built, temperature-controlled flow-through chamber - during cooling and re-warming of the sample. By this, the toxic effects that high concentrated cryoprotective agents have at physiological temperature are avoided. At the same time, extracellular ice formation and extraction of cellular water are prevented. Survival, general morphology and signaling of receptor tyrosine kinases were found unimpaired during and after cryo-arrest. This method has been applied so far to investigate patterns of activity for different molecules within living, but arrested cells by confocal FRET-FLIM as well as for analysis of distribution of molecules in the plasma membranes by super-resolution microscopy (PALM/STORM). These measurements showed clear artifacts, when they were performed without reversible cryo-arrest.

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Conflict of Interest: None to disclose

S61 CRITICAL DEFECTS IN CRYOPRESERVED CELL NUCLEI: DNA STRUCTURE CHANGES

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We show that frozen/thawed cells (NHDF human skin fibroblasts, and MCF7 mammary carcinoma cells) have altered chromatin structure. The higher-order alterations are cryoprotectant-dependent and correlate with post-thaw viability. Furthermore, replicating cells are susceptible to chromatin fragmentation. We identified DNA structural changes associated with the freeze/thaw process and correlated them with the viability of cells that had been frozen and thawed. We simultaneously evaluated DNA defects and higher-order chromatin structure of frozen and thawed cells with and without cryoprotectant treatment. We found that in replicating (S phase) cells, DNA was preferentially damaged by replication fork collapse, potentially leading to DNA double strand breaks (DSBs), which represent an important source of both genome instability and defects in epigenome maintenance. This induction of DNA defects by the freeze/thaw process was not prevented by any cryoprotectant studied. Both in replicating and non-replicating cells, freezing and thawing altered the chromatin structure in a cryoprotectant-dependent manner. Interestingly, cells with condensed chromatin (above all, cells incubated in Me_2SO prior to freezing) had the highest rate of survival after thawing. The results of our work will facilitate the future design of compounds and procedures to decrease injury suffered by cryopreserved cells.

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S62 IS FREEZER COOLING RATE EQUAL TO SAMPLE COOLING RATE?

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The cooling rates of commonly used freezing devices do not always match the ones indicated by the manufacture. This could crucially affect repeatability of experiments and thus influence the final cryopreservation outcome. The aim of this study was to compare six freezing devices (A to F) with respect to their functioning principle, adequacy of cooling rates and nucleation temperatures. For the temperature measurement thermocouples (Cu-CuNi) were placed in the middle of seven cryovials with 1 ml of bidestilled water. The temperature was recorded using a RedLab device and respective software. The cooling rates were established over the range of 0°C to -30°C with n=3. The devices A and B are insulated containers allowing cooling rates of around 1 K/min by using a -80°C freezer. The controlled rate freezer C provides cooling rates from 0.2 to 2 K/min; here the cooling is based on the Stirling cycle. Other systems, such as device D, E and F utilize liquid nitrogen. This enables expanded cooling rates of 0.2 K/min to 10 K/min for device D and 0.2 K/min to 60 K/min for the devices E and F, respectively. While the controlled rate freezers allow for a programmed cooling rate of 1 K/min in cryovials, The devices A and B show real cooling rates of 0.5 and 0.7 K/min, respectively. Due to the limits of heat transfer, all freezers show a deviation between programmed and real cooling rate in the sample

for higher cooling rates. The insulated freezing containers are a very simple and cheap solution for cryopreservation at around 1 K/min. Device C is compact and works without liquid nitrogen. The devices E and F can provide cooling rates over a wide range and device D is a cryo-workbench that enables the use of three freezing protocols at the same time.

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Conflict of Interest: The institute for multiphase processes was or is collaborating with Asymptote and Askion in projects that are not part of this study.

S63 NIR-LASER MEDIATED MODULATION OF ICE CRYSTALLIZATION BY 2D-NANOSHEETS FOR CELL CRYOPRESERVATION

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Two dimensional (2D) Graphene Oxide (GO) and Molybdenum Disulphide (MoS2) nanosheets (NS) have been widely used as photothermal agents with the potentials for carrying antitumor drugs. Such spatial thermal effect have commonly been explored from the physiological to the hyperthermia temperatures (37 ºC ~ 46 ºC). While, the modulation of the spatial thermal distributions with these nanosheets have significant applications in microstructure control of biomaterials at cryogenic temperatures (<37 ºC ~ -196 ºC), e.g., bioinspired biofabrication by freezing, food and drug freeze-drying, biomaterial cryopreservation, etc. However, such thermal effect of the nanosheets and their applications at cryogenic temperatures have never been fully explored. In this study, the photothermal effect of both GO and MoS2 nanosheets by near-infrared laser were pioneered to suppress the ice nucleation and the growth of the ice crystals in biosamples through both micro- and macro effect during warming. By doing so, the biological cells subjected to fast cooling to deep frozen state (-196 ºC) were successfully recovered with high-survival and full biological functionality. Thus, we provide a nanosheet based effective approach for control the crystallization behaviors of water during warming at cryogenic temperatures, as may have wide applications in both materials science and bioengineering.

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Conflict of Interest: None to declare

S64 EXPLORING DRY STORAGE AS AN ALTERNATIVE BIOBANKING STRATEGY INSPIRED BY NATURE

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Biobanking is done almost exclusively by cryopreservation, followed by maintenance of the samples under liquid nitrogen. Cryopreservation has satisfactory efficiency but it comes with a host of problems, including high costs, need for guaranteed continuous liquid nitrogen and power supply, dedicated staff and storage facilities, complicated transportation, risk of bacterial/fungal/viral contamination and high carbon footprint. The process is also highly species-specific and damaging to the cells. Because of these reasons, biobanking is largely restricted to the Western/Developed World and even there, mainly to relatively large and advanced centres. Like in many other walks of life, we can search Nature for better alternatives. Water content in most organisms is close to 90% yet long-term preservation strategies are more efficient when grounded in water exclusion via controlled drying rather than in water preservation via freezing. Nature also created an assortment of materials that protect organisms from damages during the drying and rehydration processes (e.g. disaccharides, LEA proteins). Once dry, and in the absence of water, metabolism and chemical reactions within the cells are brought to a halt so that these cells can survive extended periods of time and be resistant to extreme environmental conditions. Desiccation is experiencing increased interest in recent years with some exciting developments. Still, the cells normally "die" along the process. Working on spermatozoa and somatic cells from large mammals (cattle, sheep, pigs, humans), we try to understand what drying really does to the cells and what we can do to protect them. Work on this is in progress. Presented here are interim results related to DNA integrity in such desiccated cells. Once the desiccation process matures, it will facilitate long-term sample preservation at ambient temperatures, making banking drastically less costly and accessible to third-world countries, SMEs and may even be available for home storage.

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S65 STRUCTURAL AND FUNCTIONAL DIVERSITY AMONG ARABIDOPSIS LEA_4 PROTEINS

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LEA (late embryogenesis abundant) proteins or their corresponding transcripts have been detected in the seeds and vegetative tissues of all investigated plant species. The genome of the model plant *Arabidopsis thaliana* contains 51 genes encoding LEA proteins. The largest LEA protein family is the Pfam LEA_4 family comprising 18 members. Twelve LEA_4 genes are expressed in seeds, while most of the others are induced by environmental stresses such as salt, drought, cold and freezing. In addition, LEA_4 proteins are commonly found in desiccation tolerant invertebrate animals. All LEA_4 proteins investigated so far are intrinsically disordered proteins (IDPs) in dilute aqueous solutions that (partially) fold into α-helices during drying. For some of these proteins, such as COR15A (compare abstract by Thalhammer et al.), it has been shown that folding can also be induced by chemical agents such as TFE or by crowders such as glycerol. However, no systematic comparative investigations have been reported of the diversity of the structural transitions in LEA proteins. We have therefore expressed six different LEA_4 proteins (COR15A, LEA7, LEA11, LEA25, LEA29, LEA40) in *E. coli* and determined their secondary structure in the presence of different concentrations of TFE, glycerol, and ethylene glycol by CD and FTIR spectroscopy. In addition we used FTIR spectroscopy to investigate protein secondary structure after vapour-phase rehydration of the dry proteins at different relative humidities. Likewise, the proteins showed a wide range of functional properties. They differed in their ability to protect liposomes from damage during a freeze-thaw cycle and to stabilize dry sucrose glasses. Collectively, these data indicate a wide range of folding responses and functional properties among the six LEA proteins. Interestingly, similarities in folding behavior or the degree of liposome protection were not related to sequence similarity.

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S66 LEA PROTEINS – FROM UNDERSTANDING THE BASICS TO TAILORING FOR IMPROVED FUNCTIONALITY

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Water is a key factor of life. On the cellular level, dehydration negatively, often lethally, affects life across all kingdoms. Many organisms have developed strategies to adapt to cellular dehydration when encountering freezing temperatures, drought or high salt concentrations. One of these adaptations is the accumulation of LEA (late embryogenesis abundant) proteins as stress proteins with exceptional structural properties. The cold-induced LEA protein COR15A stabilizes membranes during freezing *in vivo* and *in vitro*. As most LEA proteins, it belongs to the group of intrinsically disordered proteins (IDPs), thus lacking stable secondary structure in the fully hydrated state. It folds into mainly α-helical structure upon full or partial dehydration, as present during freezing temperatures; membrane association modulates this folding, as shown by CD and FTIR spectroscopy and Molecular Dynamics (MD) simulations. The chloroplast localized COR15A interacts with liposome models of chloroplast membranes containing a high percentage of galactolipids, not in the fully unfolded, but only in an at least partially folded state. This interaction was shown by FTIR spectroscopy and SAXS/ WAXS. Specifically, COR15A associates with the membrane peripherally by inserting into the wide headspaces between galactolipid headgroups. This association is stabilized by hydrogen bonding as shown by FTIR spectroscopy and MD simulations. As a consequence, from COR15A-membrane association, target membranes are stabilized during freezing by the suppression of membrane fusion, which we assessed by Fluorescence Spectroscopy and Dynamic Light Scattering. As we found a similar function also for other, highly homologous LEA proteins, we suggest a specific sequence motif to be responsible for membrane stabilization. We use mutational approaches to understand the role of this sequence motif in membrane stabilization in detail. We further investigate the structural consequences of tailoring LEA proteins and try to relate these to functional aspects.

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Conflict of Interest: None to disclose

S67 LIFE IN NO WATER: THE EXTREME DESICCATION TOLERANCE, ANHYDROBIOSIS IN *POLYPEDILUM VANDERPLANKI*

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Anhydrobiosis represents an extreme example of tolerance adaptation to water loss, where an organism can survive in an ametabolic state until water returns. The African midge, *Polypedilum vanderplanki*, is the only insect known to be capable of anhydrobiosis. Trehalose, which accumulates in the larvae up to 20% of the dry body mass, is thought to replace the water in its tissues. Similarly, highly hydrophilic proteins called the late embryogenesis abundant (LEA) proteins are expressed in huge quantities and act as a molecular shield to protect biological molecules against aggregation and denaturation. Trehalose together with LEA proteins forms a glassy matrix, which protects the biological molecules and the structural integrity of larvae in the anhydrobiotic state. However, transduction of trehalose and LEA proteins in desiccation-sensitive cells did not improve viability after long-term storage of the dried cells, indicating that trehalose and LEA proteins would be necessary but insufficient for successful anhydrobiosis. These findings suggest other factors must be involved in induction of anhydrobiosis. The draft genome analysis of this insect has been accomplished. We determine that the genome of the sleeping chironomid specifically contains clusters of multi-copy genes with products that act as desiccation protectants. We established an cell line derived from the anhydrobiotic midge, Pv11, showing ability of complete desiccation tolerance. Pv11 cells can protect an exogenously expressed desiccation-sensitive enzyme preserving the enzymatic activity even after dry storage for over 1 year at room temperature. Since we have developed gene knock-down and knock-out systems for Pv11 cells, this cell line can be used as a tool to investigate the anhydrobiosis at the molecular level. Recently, we revealed heat shock factor 1(HSF1) has a pivotal role for successful induction of the anhydrobiosis. Here we discuss current knowledge of molecular mechanisms underlying the anhydrobiosis in *P. vanderplanki*.

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S68 NEW INSIGHTS ABOUT ANHYDROBIOSIS IN *Polypedilum vanderplanki* (DIPTERA, CHIRONOMIDAE) FROM METABOLOME ANALYSIS

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Larvae of the midge *Polypedilum vanderplanki* (Diptera, Chironomidae) live in small rock pools that appear during the rainy season on granitic outcrops in the semi-arid regions of Africa. At the beginning of the dry season, rock pools dry-up and *P. vanderplanki* larvae desiccate to reach a dormant ametabolic state, called anhydrobiosis, in which they can survive for several months until next rain. Upon rehydration, dry larvae recover normal activity and development. During the induction of anhydrobiosis, *P. vanderplanki* larvae accumulate in their tissues a large amount of trehalose that will form a glassy matrix protecting cellular components and biological molecules against desiccation stress. Nevertheless, larvae experience important oxidative stress during the rehydration process, leading to membrane lipid and DNA damages that are quickly repaired. In this study, we performed metabolome analysis at different steps throughout the induction of anhydrobiosis and the process of rehydration. As a result, strong accumulation of AMP was observed in the anhydrobiotic state, whereas this AMP allowed rapid synthesis of ATP and balanced purine degradation just after rehydration. In addition, pentose phosphate pathway was activated during the few hours following rehydration. These observations suggested that the degradation of trehalose occurring just after rehydration would activate the synthesis of ATP and at the same time fuel the pentose phosphate pathway, thus producing NADPH that would in turn re-boost the antioxidant potential of the larvae. In order to verify this hypothesis, functional analyses based on knock-down by RNAi of the expression of the trehalose degradation enzyme, trehalase, combined with experiments inhibiting the activity of this enzyme were performed on larvae and in cell culture. The results showed that trehalose was actually degraded after rehydration and that its degradation was essential for subsequent survival. Experiments evaluating the antioxidant potential of rehydrated larvae with disrupted trehalase function are ongoing.

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S69 TARDIGRADES USE INTRINSICALLY DISORDERED PROTEINS TO SURVIVE DESICCATION

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Tardigrades (water bears) are a phylum of microscopic, extremotolerant animals renowned for their ability to survive desiccation, freezing, boiling temperatures, intense irradiation, and even prolonged exposure to the vacuum of outer space. The mediators of protection used by tardigrades to survive these extremes and their mechanisms are beginning to be elucidated. Tardigrades possess three novel families of tardigrade-specific intrinsically-disordered proteins (IDPs). We found that members of one of these IDP gene families are upregulated during, and are required for tardigrades to survive, desiccation. Additionally, these proteins increase the desiccation tolerance of heterologous systems (yeast and bacteria), stabilize protein structure, and preserve enzyme function during desiccation. At high concentrations, these IDPs form gels. Simulations, bioinformatics, and biochemical experiments indicate these proteins are comprised of three discrete regions, two terminal regions bridged by an extended linker that together resemble a dumbbell. Dynamic light scattering and bioinformatics suggest the terminal regions are 'sticky.' Based on experimental evidence and simulations, we propose that at low concentrations this dumbbell-like confirmation inhibits intraprotein interactions but at higher concentrations facilitates gelation *via* interprotein interactions. Upon desiccation, these gels vitrify to glass-like solids, and vitrification appears to be mechanistically essential because disruption of the vitreous state correlates with a loss of protective capabilities. Theory suggests that desiccation and freeze tolerance in tardigrades are mechanistically linked. However, transcriptomics indicate tardigrade IDPs are not upregulated during freezing, and transcriptomic responses to these two stresses are highly divergent. Furthermore, tardigrades still survive freezing after RNAi targeting IDP genes. Our results identify the first functional mediators of tardigrade desiccation tolerance, and reveal a potential mechanism of action. More broadly, our findings suggest that desiccation and freeze tolerance are more mechanistically distinct than suspected. These studies provide a platform for pursuing novel methods for stabilizing sensitive biomedical material and engineering stress tolerant crops.

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Conflict of Interest: None to disclose

S70 THE CHALLENGES OF OVARIAN TISSUE BANKING FOR FERTILITY PRESERVATION AND RESTORATION

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Low temperature banking of immature oocytes *in situ* within the ovarian cortex has been successfully used to preserve the fertility of young cancer patients. This approach is possible because of the unique architecture of the mammalian ovary. The ovaries of girls and young women are packed with hundreds to thousands of very early staged primary oocytes enclosed within primordial follicles that are localised in the outermost area of the ovarian cortex. Following the laparoscopic recovery of ovarian biopsies or the whole organ, primordial follicles can be successfully cryopreserved *in situ* within slices of ovarian cortex using either slow freezing or vitrification protocols. Once the patient is in remission and wishes to start her family, her fertility can be restored by auto-transplantation of cryopreserved cortex fragments at either orthotopic or heterotopic sites within the body. The most recent evidence from large animals now suggests that it is also possible to cryopreserve intact whole ovaries and their supporting vasculature for later auto-transplantation and restoration of natural fertility. Considerable research effort has been made to test the efficacy of slow freezing, vitrification and unidirectional freezing of ovarian tissue from a range of species and proof of the clinical utility of ovarian tissue cryopreservation for fertility preservation in cancer patients have been evaluated. Many outstanding research challenges remain. These include the need for: evaluation of the potential for the reintroduction of malignant cells via ovarian autografts; optimisation of graft longevity; quantification of the risk of ovarian damage after chemotherapy drug exposure; maintenance of the architecture and integrity of complex tissues including somatic cells, presumptive gametes and blood vessels during whole organ preservation; and minimising ischaemia following transplantation. Where there is any risk of reintroducing cancer cells via autotransplantation of cryopreserved ovarian tissues then restoration of fertility can only be safely achieved by the complete *in vitro* growth and maturation of oocytes from the preserved tissue. With the development of new multi-phase follicle culture systems it is now possible to activate and grow human oocytes from primordial follicles to maturity *in vitro.* However, the health and genetically and epigenetically normality of metaphase II oocytes derived *in vitro* from cryopreserved human ovarian tissue remains to be proven.

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Conflict of Interest: None to disclose

S71 HUMAN TESTIS CRYOPRESERVATION FROM BODY TO THE DISH

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Testis is responsible for adequate secretion of male hormones and normal spermatogenesis. The general

anatomy of the testis consists of the seminiferous tubules and, among them, the interstitial space. The seminiferous tubules, containing germ cells in different maturation levels depend on age. The pool of germ cells is supported by spermatogonia stem cells (SSC) which are covered by a layer of Sertoli cells embedded in lamina propria. The lamina propria consists of the basal membrane covered by peritubular cells. Differentiated germ cells including spermatocytes and spermatids are located across the tubule towards its centre. The main component of the interstitial space is the Leydig cell population which produces testosterone, but the interstitial space also contains macrophages, lymphocytes, loose connective tissue and neurovascular bundles. Several methods of cryopreservation including slow freezing and vitrification have been applied to preserve this integrated system in patients at risk of long-term persistence on infertility such as cancer patients. Wake Forest institute for Regenerative Medicine (WFIRM) has established and maintains one of the largest active experimental testicular tissue banking to preserve fertility of any types of cancer, urological and genetic diseases compromising fertility. In past two decades, utilizing human cells to create organ-like structures at the laboratory dish that mimic the function of native organs and ultimately develop a "body-on-a-chip" is eagerly desired. At WFIRM, we recently developed a novel *in vitro* 3-Dimensional (3D) human testis organoid (HTO) system from *in vitro* 2D propagated human testicular cells. This HTO system has the potential for *in vitro* differentiation of SSC and androgen production, as well as using as a high throughput screening tool for drug toxicity and environmental studies. Now our laboratory can create HTOs, cryopreserve and ship them to any other laboratory to be used as an *in vitro* human testis model. Cryopreservation of small testicular tissue and HTOs have been established successfully. Future directions should focus on improving the current protocols and developing whole organ cryopreservation methods.

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S72 DEVELOPMENT OF OVARIAN TISSUE VITRIFICATION METHOD BY USING CLOSED DEVICE

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Introduction: Over one hundred live births have been achieved by ovarian tissue cryopreservation and transplantation already in the world since 2006. The standard technique of ovarian tissue cryopreservation is slow freezing. We started developing the vitrification system of ovarian tissue which is the open device system (Ova Cryo Kit Type M (Kitazato, Japan)) since 2006. Now we have also developed the closed device system (Cryosheet (Kitazato, Japan)). Finally, we have three live births using this ovarian tissue vitrification system in Japan. This vitrification system is very easy to use, and we need not long time to perform like as slow freezing technique.

Objective: To demonstrate ovarian tissue vitrification technique using closed (CryoSheet, Kitazato, Japan) device, and thawing procedure for fertility preservation.

Methodology: A step by step video demonstration of ovarian tissue vitrification and thawing procedure. Following oophorectomy, ovary was placed in saline and brought to the laboratory on an ice pack for cryopreservation. Cryopreservation procedure was performed under sterile conditions. Initially, the ovary was transferred to tissue culture media (modified HTF), examined for visible antral follicles and then the observed follicles were aspirated using a 18-21-gauge syringe. The collected follicular fluid was transferred to a petri dish and examined under an optical microscope for healthy oocytes. The identified mature oocytes were directly cryopreserved, while immature oocytes were primarily subjected to in vitro maturation process and matured oocytes were frozen. Subsequently, the ovary was cut into two halves, and the medulla was removed from the ovarian cortex by gentle dissection using a curved scissor until the cortical thickness reaches to approximately 1-mm. Ovarian cortex was cut into 10x10 mm small pieces, and then cortical pieces were transferred sequentially to cryoprotectant solutions which were composed of different concentrations of ethylene glycol and sucrose (Ova Cryo Kit Type M, Kitazato, Japan). At the final step of the cryopreservation, the cortical samples were loaded on to the open and closed devices, inserted directly into the liquid nitrogen, and were stored for future use. For thawing procedure, both open and closed devices loaded with cortical samples were initially placed in step 1 thawing media warmed at 37^{o}C, and then cortical pieces were sequentially subjected to thawing solutions (Ova Thawing Kit Type M, Kitazato, Japan). Conclusions: According to our research, ovarian tissue cryopreservation could be successfully performed via vitrification method using either open or closed devices. We have shown that histological evaluation of thawed samples from vitrified ovarian tissues provided similar follicular viability features to slow-frozen ovarian tissues.

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Conflict of Interest: None to disclose

S73 POLLEN CRYOPRESERVATION IN TROPICAL FRUITS: CHALLENGES AND PROSPECTS

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Conservation through pollen storage in fruit species does not accomplish whole genome conservation, but for a fruit breeder they can have access to a Pollen Cryobank facility for nuclear genetic diversity (NGD) inputs in his breeding program. Besides the already existing role of pollen banks in breeding, there are many promising applications, which have come to focus with the recent advances in allied bio-scientific areas. A pollen cryobank can ensure a constant supply of viable and fertile pollen and can also allow supplementary pollinations for improving seed set. Through exchange of pollen, desired crosses can be made directly on the seed parent, allowing introgression of characters at a much faster rate. The following species pollen were cryopreserved Mango: *M. odorata, M.camptosperma*, Polyembryonic mangos (4 Accessions) pomegranate: nine different accessions Aonla: 5 Varieties Jackfruit: Guava species: *Psidium . guinense*, *P. littorale* Papaya: 5 IC collections, *V.cauliflora, V.parviflora, V.cundinamarcensis* Passion fruit: *P. edulis var. flavicarpa, P. incarnata, P. alata, P. foetida Jamun*: 5 accessions, *Annona squamosa: Balanagar* Pollen collection is usually synchronized with anthesis, between 9 and 11 A.M..After initial viability assessment in vitro pollen samples are packed in gelatin capsules/butter paper packets depending on the method of storage and lowered into a canister of a cryobiological system. The canister is capped with a perforated lid and plunged slowly in liquid nitrogen contained in the cryobiological system. Frequent refilling of the cryoflask with liquid nitrogen at least once every 10 to 15 days, ensures a constant cryogenic temperature. The pollen cryopreservation protocols developed for different tropical fruits mentioned above will be discussed with implications in conservation and fruit breeding.

Source of Funding: ICAR
Conflict of Interest: None to declare

S74 DESICCATION TOLERANCE AND THE HYDRATION WINDOW FOR THE CRYOPRESERVATION OF WOODY SPECIES' POLLEN

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Pollen cryopreservation and use for pollination is widespread among breeders in the horticultural and ornamental sectors to increase crop yield, produce new varieties, and improve species tolerance to pests and diseases. However, it is less commonly applied in wild plant species conservation. Nevertheless, pollen cryopreservation can be an alternative to preserve genes of species that cannot be maintained under conventional seed bank conditions (stored dry at -18°C), including those with seeds that are desiccation sensitive (recalcitrant) or with short longevity when dry (i.e., intermediate seeds). In addition, pollen cryopreservation can be used to support the production of seeds of dioecious species with scattered populations or with asynchronous phenology. The objectives of this study were to study tolerance to desiccation, liquid nitrogen (LN) exposure, and longevity of pollen from diverse temperate shrub and trees species: *Ilex aquifolium, Hedera helix*, *Corylus avellana, Corylus colurna* and *Taxus baccata*. Pollen was equilibrated at different relative humidity (RH) and stored at room temperature (RT) for different times or exposed to LN. Subsequently, germination and vigour were evaluated. Pollen of all the species was desiccation and LN tolerant. *C. avellana* and *C. colurna* pollen had longevities (P50s) at RT and 12% RH of 75 and 37 days respectively; but optimal cryopreservation occurred after equilibration at 37% RH. *I. aquifolium* and *H. helix* pollen had the greatest longevity and optimal cryopreservation at 12% RH (P50 at RT c. 58 days). Pollen samples of each species are now stored in LN at RBG Kew to study their long-term stability. These results encourage the routine cryopreservation of (desiccation tolerant) pollen for biodiversity conservation. This approach can ensure preservation of high genetic diversity in a small space and can be applied beneficially to species with recalcitrant seeds and desiccation tolerant pollen (e.g. *Quercus* sp.).

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Conflict of Interest: None to disclose

S75 CRYO-PRESERVATION OF POLLEN IN HYBRID SEED PRODUCTION

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Hot Pepper or chilli is among main horticultural crops of South East Asia. In peppers, seed production is mainly done using sterile male lines. For seed production, the male lines were grown separately and only male flower buds distributed to growers for pollen extraction and crossing of female buds. An effort was made to preserve pollen from these male buds under refrigeration and cryo-preservation, which can be used to supply pollen instead of buds to longer distances directly. The pollen from these male lines was harvested and stored in average room temperature (25ºC-Control), in deep refrigeration (-20ºC) and liquid Nitrogen (-196ºC). The pollen lost viability after three days and failed to produce seeds when stored under room temperature and after six

days in deep refrigeration. In case of cryo-preservation, the pollen could be stored up to 47 days and produced seeds up to 48 days. The average and economical seed setting are better in cryo-stored pollen the of at least 20 seeds per fruit can be achieved up to 37 days. The other storage methods were failed to achieve economic seed setting beyond six days. The pollen cryo-preservation helps in reducing the cost of seed production and ensures germplasm security.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S76 IMPERILED PLANTS GO COLD: THE HUNTINGTON BOTANICAL GARDENS CASE STUDIES

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Approximately twenty percent of the world's plants are threatened with extinction. Besides their importance to the agricultural, horticultural and pharmaceutical industries, plants are essential for the health of all terrestrial ecosystems. Botanical gardens around the world play a key role in *ex situ* preservation of plant genetic resources and in providing examples of species that have been extirpated from their natural habitat. Although some plant species can be preserved in seed collections, the traditional preservation methods, such as field clonal gene banks, are often costly and risky. An appropriate approach is the use of micropropagation to maintain new collections in addition to the field collections at botanical gardens, such as The Huntington. Furthermore, we can take advantage of cryobiotechnology, which has been used for the preservation of many crop genetic resources. However, additional efforts are still needed for other crop species and many wild plant species in peril. Cryopreservation protocols for shoot tips using droplet-vitrification are being developed at The Huntington botanical gardens, mainly using shoot tips as plant material. In vitro shoots obtained from *ex vitro* buds are used as donor plants, and they are acclimated using osmotic or cold stresses before the shoot tips dissection. Case studies including aloes, agaves, magnolias, avocado and other endangered species, are used to decipher how meristems from different plant species acquire tolerance to cryoprocedure and thus obtain standardized and more efficient methods to assure the long-term preservation of plant genetic resources.

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Conflict of Interest: None to disclose

S77 CRYOPRESERVATION OF JERUSALEM ARTICHOKE CULTIVARS

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Jerusalem artichoke (*Helianthus tuberosus* L.) cultivars are conserved in genebanks for use in breeding and horticultural research programs. As vegetatively propagated collections, Jerusalem artichoke collections are vulnerable to environmental and biological threats. We developed an improved cryopreservation method using four Jersualem artichoke cultivars ('Shudi', 'M6', 'Stampede', and 'Relikt'), that will enable Jerusalem artichoke field collections to be securely conserved in genebanks. Four steps were optimized, and the resulting procedure is as follows: preculture excised shoot tips (2~3 mm) in liquid MS medium supplemented with 0.4 M sucrose for 3 days, osmoprotect shoot tips in loading solution for 30 min, dehydrate with plant vitrification solution 2 for 15 min before rapid cooling in liquid nitrogen, store in liquid nitrogen, rapidly rewarm in MS liquid medium containing 1.2 M sucrose, and recover on MS medium supplemented with 0.1 mg L^{-1} GA_3 for 3 to 5 days in the dark and then on the same medium for 4 to 6 weeks in the light (14h light/ 10h dark). Jerusalem artichoke cultivar 'Shudi' had the highest survival (93%) and regrowth (83%) percentages. After cryopreservation, cultivars 'M6', 'Stampede', and 'Relikt' achieved regrowth percentages ranging from 37% to 53%. No genetic changes, as assessed by microsatellites, were detected in plants regenerated after LN exposure in Jerusalem artichoke cultivar 'Shudi'. Differential scanning calorimetry analyses were used to investigate the thermal activities of the tissues during the cryopreservation process and determined that loading with 2.0 M sucrose and 0.4 M sucrose dehydrated the shoot tips prior to treatment with PVS2. Histological observations revealed that the optimized droplet vitrification protocol caused minimal cellular damage within the meristem cells of the shoot tips.

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Conflict of Interest: None to disclose

S78 CRYOBIOTECHNOLOGICAL APPROACHES FOR THE PRESERVATION OF OAK (*QUERCUS* SP) EMBRYONIC AXES.

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Oaks (*Quercus* sp.) produce desiccation sensitive seeds that cannot be preserved by conventional seed banking (dry storage at -18°C). Cryopreservation of the embryonic axes is the preferable method for their *ex situ* conservation. However, the response to water loss among the embryonic axes of oaks is variable, impacting recovery after liquid nitrogen (LN) exposure [e.g. Ballesteros *et al* (2018) Cryobiology 80: 164]. We have researched embryonic axes *in vitro* survival in 12 *Quercus* species from different environments from the UK, Spain and Lebanon. We measured the response to partial ("flash") drying and exposure to LN in axes excised with a small portion (1 mm) of cotyledon attached that were treated with ascorbic acid after excision. Desiccation had different effects on axes, with the root tip end drying generally slower than the shoot tip. This differential pattern of water loss affected recovery after exposure to LN, resulting in most of the species having more root survival. The two faster cooling rates studied (3-8 and 30-80°C s^{-1}) showed comparable results for embryo cryopreservation. Fast drying and cooling was relatively successful for the cryopreservation of *Q. pyrenaica*, *Q. rubra*, *Q. ilex* and *Q. coccifera* embryos (root + shoot recovery between 25 and 50%), but not for the other species (root + shoot recovery <5%). No clear relation of tolerance to LN and species ecology was found. Glycerol cryoprotection (5% for 1 h + 10% for 1 h) appeared to increase shoot survival in *Q. robur* embryonic axes. Research on the structural changes during drying and cooling are underway to find patterns that explain the tolerance to desiccation and low temperatures within the axis of a species and among *Quercus* species. New innovative cryobiotechnology approaches for embryonic axes of *Quercus* sp. (such as vacuum infiltration vitrification) are also under assessment in our laboratory to improve their cryopreservation.

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Conflict of Interest: None to disclose

S79 CONTRIBUTION OF EMBRYO SIZE AND AGE TO THE SUCCESSFUL CRYOPRESERVATION OF AESCULUS SPECIES.

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Aesculus is a genus of the family Sapindaceae containing 23 species of deciduous trees and shrubs in the northern hemisphere cultivated for centuries as ornamental plants and more recently as pharmaceutical crops. However, some species are threatened in their natural range and long-term *ex situ* conservation options are required to secure the genetic resources of these species. As seeds of *Aesculus* are desiccation sensitive, conventional seed banking is not possible, therefore cryopreservation of embryonic axes (EA) is the preferable method for their *ex situ* conservation. Cryopreservation of EA of recalcitrant seeds can be challenging, and success is often dependent on: (1) the size of the explant to be cryopreserved; (2) differential responses of shoot and root meristems within the axis to stress imposed by partial desiccation and cooling; and (3) the age of the explant. Here we investigated the effects of partial ("flash") drying and exposure to liquid nitrogen (LN) on the recovery response of shoot and root growth *in vitro* for two species of *Aesculus*: *A. hippocastanum* and *A. indica*. In addition, the response to cryopreservation has been evaluated in EAs of *A. hippocastanum* seeds stored hydrated for periods up to 6 months. Our results show that desiccation damage was more pronounced in the larger embryos (*A. indica*) and that the shoot region dried faster than the root region of the axis. This differential pattern seems to affect recovery after exposure to LN, with both *Aesculus* species showing more shoot than root survival, and the smaller EA (*A. hippocastanum*) showing a better recovery after LN. The good response to cryopreservation of *A. hippocastanum* EA's when seeds were freshly harvested was maintained for up to 3 months. These results are discussed in relation to *Quercus* species EA's, which show the opposite differential response of tissues to drying and LN exposure.

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S80 EVALUATION OF SHORT-LIVED SEEDS' CRYOPRESERVATION AS ALTERNATIVE TO CONVENTIONAL SEED BANKING.

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Desiccation tolerant (DT) seeds can be dried to low moisture contents (i.e. equilibrium relative humidity (eRH) 15-25%) and stored in the freezer (e.g. -20°C) for ex situ conservation programs. However, not all seeds survive for long-term (e.g. >20 years) at such conditions, and alternative conservation methods are needed. For seeds with short life-spans (aka short-lived seeds), cryopreservation has been suggested. The optimal hydration window for survival to LN exposure are not fully understood. In addition, it has been demonstrated that diverse germplasm continue ageing at LN temperatures. Short lived propagules (including micro-seeds and fern spores) are routinely duplicated to cryopreservation at the Millennium Seed Bank in Royal Botanic Gardens Kew. We present the preliminary results of a long-term storage experiment on potentially short-lived seeds of 40 species and 28 genera including *Primula*, *Anemone*, and *Corylus*. Seeds previously stored at -20°C were stored in the vapor phase of LN and -20°C after moisture equilibration at 15%, 30%, and 60% RH and tested after one week. In addition, results are also presented for 25+ species retested up to 5 years after duplication to cryo-storage after showing germination decline in -20°C. Results suggest that while the hydration window for initial exposure to LN may be acquired after equilibration above 30% eRH, optimal hydration for maximum longevity at LN temperatures requires further research. Cryopreservation increases seed longevity compared to -20°C for some species, however ageing is still detected. Based in the results from this experiment, the options for the long-term conservation of these species are discussed, including the requirement for modified protocols for some species.

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Conflict of Interest: None to disclose

S81 CURRENT TOPICS IN KIDNEY CRYOPRESERVATION: COOLING INJURY AND BIOCHEMICAL INJURY

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Rabbit kidneys perfused with M22 at -22°C are damaged by cooling to lower temperatures. Cooling to -45°C by immersion in cold M22 resulted in failure to support life after transplantation. This was not due to surface contact between the kidney and M22, but nevertheless, cooling in vapor was successful, although with considerable cooling injury. Cooling in vapor to -80°C failed. Holding kidneys at -22°C for 5 min led to more damage, and holding kidneys at -22°C for 11 min, which is the time required to cool to and rewarm from -45°C, replicated the damage observed after cooling to -45°C. Some kidneys were briefly exposed to a -80°C environment to accelerate the cooling rate and thereby shorten the total exposure time, and then returned to a -45°C environment to complete cooling. Despite shorter overall exposure times, these kidneys did worse than kidneys cooled to a surface temperature no lower than -45°C. To test whether the injury during cooling to -80 and to -45°C was caused in part by surface strain caused by cooling too rapidly, an additional group of kidneys was cooled to an external temperature of -45°C slowly rather than abruptly. This group had the same level of damage sustained by the original -45°C air cooling group, even though mean intra-renal temperatures did not fully descend to ~-45°C. Therefore, cooling injury is indistinguishable from temperature-independent M22 toxicity accumulation during cooling and warming, plus potentially avoidable strain injury caused by faster cooling below -45°C. Because toxicity may be the ultimate problem, we initiated imaging mass spectrometry studies of renal protein populations with and without M22 perfusion to better understand M22 toxicity. Preliminary results indicate that significant molecular changes may be induced by M22 perfusion even at -22°C. Whether these changes are exacerbated by cooling to below -22°C is not yet known.

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Conflict of Interest: None to disclose

S82 EFFECTS OF CRYOPROTECTANT CARRIER SOLUTION TONICITY ON TRANSPORT DURING ORGAN PERFUSION

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There is increasing interest in cryopreservation of organs to extend shelf life and enable more effective utilization of organs for transplantation. To prevent damage from ice formation, cryoprotectants (CPAs) must be delivered throughout the organ, but exposure to CPAs is often toxic. We have approached the problem of minimizing cytotoxicity at the single cell level by mathematically predicting the least toxic CPA equilibration procedure. We found that a key variable affecting the predicted toxicity is the tonicity of the CPA carrier solution. In particular, we found that using CPA in hypotonic carrier solution is advantageous because it induces cell swelling which can accelerate CPA loading and reduce exposure to high CPA concentrations. To examine the potential for extending this approach to organs, we compared perfusion of porcine kidneys with 10% ethylene glycol in either hypotonic or isotonic buffer. The response of organs was analogous to that of isolated cells: the kidney mass initially decreased and then increased, and the hypotonic buffer resulted in a faster and more substantial mass increase. These results suggest faster CPA delivery into the organ using the hypotonic carrier solution, which would be advantageous to reduce CPA toxicity. However, we also observed a dramatic increase in vascular resistance when using the hypotonic carrier

solution, which raises concerns about uniformity of CPA delivery into the kidney. To address this concern, we have adapted a published computed tomography (CT) method for tracking the distribution of dimethyl sulfoxide in real time during kidney perfusion. Preliminary results confirm faster CPA delivery into the kidney when using a hypotonic carrier solution, and suggest that uniformity of CPA delivery is not impaired despite the increase in vascular resistance. These promising results highlight the potential for reducing toxicity during organ perfusion by manipulating the tonicity of the CPA carrier solution.

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Conflict of Interest: None to disclose

S83 FULL FUNCTIONALITY AFTER CRYOPRESERVATION OF BRAIN SLICES BY VITRIFICATION

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The ability to cryopreserve precision-cut brain slices with full functionality is of pragmatic importance and academic interest. The present study demonstrated that rabbit hippocampal slices can be vitrified with subsequent full recovery of electrophysiological function. The cryoprotectant formulas studied were VEG, VM3 and M22. The CPAs were added and removed stepwise at first and later were added and removed continuously. There was no significant difference between naive slices and those vitrified and stored at -130°C for various periods of time. Extracellular, intracellular and optical photo diode array (PDA) recordings all indicated that electrical stimulus-response characteristics including passive membrane properties, field extracellular excitatory postsynaptic potential (fEPSP), network ensemble activity and long term potentiation (LTP) were preserved.

These studies show for the first time that not just viability but also electrophysiological functionality can be cryopreserved by vitrification.

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Conflict of Interest: None to disclose

S84 BREAKTHROUGH OF "ORGAN BANKING": SCOPE OF APPLICATION OF LIMB CRYOPRESERVATION TECHNIQUES

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At the SUMMIT ON ORGAN BANKING held recently in Boston, tissue and organ cryopreservation technology was a hot topic. For whole organs, except for the cryopreservation successes of rat ovary (2002) and hind limb (August 2002), no successful cryopreservations of vascularized animal tissues or organs and their replantation have yet been reported. In December 2002 and December 2003, we successfully replanted amputated fingers after cryopreservation on two patients, demonstrating our first-time success in applying cryopreservation technology to clinical practice worldwide. In our cryopreservation experiment on SD rats, we found that all the replanted limbs that had been amputated above the knee failed, while restoration of blood flow was noted in almost all limbs in Syme's amputation group. The reason for this result is that, in general, small cells with simple structures have a higher survival rate after cryopreservation, while large cells with complex structures have a low survival rate; striped muscle is composed of cells with extremely complex structure. We have conducted histological and electron microscopic examinations on muscle after blood supply restoration to explain this problem so that we can provide our own experience to the knowledge benefit of our colleagues, with the goal of further developing the technology.

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Conflict of Interest: None to disclose

S85 FEASIBILITY STUDY IN THE DEVELOPMENT OF WHOLE OVARY VITRIFICATION AND NANOWARMING

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With over 100,000 childhood and young adult cancer survivors in the US each year and hundreds of thousands more worldwide, reproductive organ banking could preserve fertility and hormone balance for many of these young patients. Early successes suggest that ovarian tissue and even whole ovary banking is possible and could preserve fertility and function. This feasibility study in a porcine model employed a multi-faceted approach targeting human reproductive organ banking including preconditioning, loading of vitrification solution, M22, at sub-zero temperatures, and loading iron oxide nanoparticles (IONPs) for subsequent rapid radio-frequency warming, “nanowarming”. Whole porcine

ovaries were procured and flushed with 10 mL ice-cold heparinized (1000 U/ml) Unisol cold storage solution. Preconditioned ovaries (N=7) were perfused at 4 ± 4 °C with flow rates between 0.5 and 1.2 mL/min, followed by stepwise-loading of M22 at sub-zero temperatures (N=5) using a custom-built multi-thermic machine perfusion (MTMP) system. The MTMP system achieved a cooling rate of -2 °C/minute from 0 °C to -10 °C and 0.2 to 1 °C/min until reaching the coldest temperature, -16.6 °C, during the final step. M22 equilibration was sufficient to achieve vitrification of ovaries (N=4) in a 10 ml volume when plunged in liquid nitrogen. Finally, IONPs were loaded in a dose-dependent manner (N=17) with 5 to 15 g/ml of iron. Particles were observed to be distributed within the vasculature of the theca layer of antral follicles (by histology) and preliminary measurement of warming rates correlated with the infused nanoparticle concentration, achieving a 4X increase (3.58 °C/min) compared to convective warming (0.85 °C/min) from 0° to 22°C. These early results demonstrated feasibility of a comprehensive protocol for whole ovary banking, including hypothermic preconditioning, vitrification, and subsequent nanowarming**.** Additional optimizations could make ovary banking clinically feasible and yield insights applicable to banking testicular tissue, whole testes, and larger organs.

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S86 THE STATE OF CRYOPRESERVATION AND ASSISTED REPRODUCTIVE TECHNOLOGIES FOR THE CONSERVATION OF AMPHIBIANS AND REPTILES

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Amphibians and reptiles are experiencing serious declines around the globe, with the number of threatened species and extinctions growing rapidly as the modern biodiversity crisis unfolds. For amphibians, the panzootic of chytridiomycosis is a major driver. For reptiles, habitat loss and harvesting from the wild are key threats. Cryopreservation and other assisted reproductive technologies (ARTs) could play a major role in slowing the loss of amphibian and reptile biodiversity and managing threatened populations through genome storage and the production of live animals from stored material. These vertebrate classes are at different stages of development in cryopreservation and other ARTs, and each class faces different technical challenges arising from the separate evolutionary end-points of their reproductive biology. For amphibians, the generation of live offspring from cryopreserved spermatozoa has been achieved, but the cryopreservation of oocytes and embryos remains elusive. With reptiles, spermatozoa have been cryopreserved in a few species, but only one protocol has achieved high levels of post-thaw motility, no offspring from cryopreserved spermatozoa have been reported, and the generation of live young from AI has only occurred in a small number of species. Cryopreservation and ARTs are more developed and advanced for amphibians than reptiles. We will discuss the current play for amphibian and reptile cryopreservation and ARTs, outline some programs around the world that are putting these into practice and discuss the future work needed on both groups in order to achieve proof of concept that genome storage and ARTs is a critical tool for the conservation and threatened species recovery of the world's herpetofauna.

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S87 CRYOPRESERVATION OF OOCYTES AND FOLLICULAR CELLS OF THE CANE TOAD, *RHINELLA MARINA*.

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Cryobanking of amphibian germ cells and reproductive tissues could be used to manage threatened species and provide insurance against extinction. However, successful cryopreservation of fully developed amphibian oocytes and whole embryos has not been achieved due to technical problems freezing such large cellular structures. As an alternative approach, we investigated the feasibility of developing protocols for the slow-cool freezing, storage and retrieval of developmentally competent amphibian ovarian follicles containing Stage I and II oocytes that are much smaller in size than later developmental stages. Ovarian follicles from euthanised cane toads were incubated at room temperature in cryodiluents containing either 5, 10, 15 or 20% v/v glycerol or Me2SO for up to 24 hours to assess cryoprotectant toxicity or subjected to slow cooling freezing protocols (1ºC/min to -30 or -80ºC before free fall to LN_2 temperature) in the same diluents. The live cell stain SYBR 14 and its counter stain propidium iodide was used to score the proportion of viable follicle cells before and after cryopreservation. Cryoprotectant type, concentration and exposure time all had significant effects ($P < 0.05$) on the viability of follicle cells, with significant interactions between these variables. Overall,

glycerol was less toxic to follicle cells than Me2SO. At higher concentrations, glycerol exerted high osmotic stress on oocytes, and there was evidence that Me2SO triggered apoptosis in oocytes. The most effective cryopreservation protocol for stage I and II oocyte follicles resulted in a mean post-thaw recovery of 70% viable follicular cells. This protocol involved cryopreservation in 15% v/v glycerol, inclusion of seeding and temperature holding periods during cryopreservation, coupled with rapid thawing in a 30°C water bath. The successful cryopreservation of intact follicles in this study indicates the potential to recover functional ovarian tissues post cryopreservation for continuation of amphibian oogenesis in vitro or in vivo.

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S88 EXPOSURE TO HYPEROSMOTIC SOLUTIONS MODIFIES EXPRESSION OF AQP3 AND AQP7 ON BOVINE OOCYTES

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Successful cryopreservation of mammalian cells requires rapid transport of water and cryoprotectants across the plasma membrane. AQP3, AQP7 and AQP9 are known as channel proteins that can transport water and small neutral solutes. The aim was to evaluate the effect of CPA exposure (hyperosmotic solutions containing EG, Me2SO or sucrose) on the levels of expression of AQP3, AQP7 and AQP9 of *in vitro* matured bovine oocytes.

Cumulus-oocyte complexes were aspirated from slaughterhouse cow ovaries and cultured for 24 h in TCM199 + 10 ng/ml EGF + 10% FCS + 50 mg/ml gentamicin at 38.5°C in a 5% CO_2 humidified air atmosphere. In vitro matured oocytes were treated with TCM199-Hepes supplemented with 20% FCS containing 8% ethylene glycol (EG), 9.5% dimethyl sulfoxide (Me2SO) or 0.5M sucrose for 20 min at 25ºC. After treatment, oocytes were completely denuded, fixed and levels of expression of AQP3, AQP7 and AQP9 were assessed by immunocytochemistry under a fluorescent confocal microscope. Differences on fluorescence intensity were analysed using a lineal model followed by a Tukey test ($p<.05$).

Results showed that AQP7 protein expression was higher than AQP3 protein expression, regardless of the exposure to different hyperosmotic solutions, while no expression of AQP9 protein was observed. Exposure to Me2SO significantly increased AQP3 protein expression. No changes were observed on AQP3 expresion when oocytes were exposed to EG or sucrose when compared to control. Treatment with EG significantly increased AQP7 expression when compared to control, or Me2SO or sucrose treatment. Our study provides the insight that AQP7 and AQP3 in bovine oocytes may mediate tolerance to hyperosmotic stress during cryopreservation. Further studies are needed to understand the importance of AQPs in bovine oocyte cryotolerance.

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S89 A TWO MINUTE PROTOCOL TO PREPARE HUMAN OOCYTES AND EMBRYOS FOR VITRIFICATION

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Vitrification of human oocytes and embryos in differents stages of development is a key element of daily clinical practice of in vitro fertilization treatmets. Despite the cooling and warming of the cells is ultra-fast, the procedure as a whole is time consuming, as the standard preparation protocol to ready the cells for vitrification takes from 8 to 15 minutes. A reduction in the duration protocol is desirable to improve the workflow in the IVF setting and reduce exposure time to potentially cytotoxic cryoprotectants. It is also feasible. In silico and in vivo observations of the osmometric behavior of the human oocyte show that the dehydration upon exposure to commonly employed hypertonic vitrification solutions occurs very fast; the point of minimum volume of the shrink-swell curve is reached within 60 seconds. At that point, the water content is minimum, the permeation of low molecular weight cryoprotectants is almost complete, and as a result the total intracellular solute concentration is high. For that reason, prolonging exposure towards equilibrium up to 10 minutes does not improve the intracellular glass forming tendency significantly. To test this finding, abnormally fertilized human zygotes donated for research were vitrified using the SafeSpeed carrier after 60 seconds of exposure to two standard cryoprotectant solutions (S1: 7.5% v/v EG, 7.5% Me_sSO; S2: 15% v/v EG, 15% v/v Me_2SO, 0.5 M sacarose). All 20 zygotes survived vitrification and 18/20 had resumed cleavage after 24 hours of culture. It shows that the critical intracellular solute concentration necessary for succesful vitrification of human zygotes at the cooling and warming rates attained by the SafeSpeed carrier (120.000 & 200.000 °C/min, respectively) can be achieved in just two minutes. Short steps of exposure to solutions of sequentially higher tonicity seems to be a more time-efficient method for preparation of human oocytes and embryos for vitrification. Modifications of this approach could also be applied to other models.

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S90 OOCYTES VITRIBANKING IN THE DOMESTIC CAT MODEL: DEVELOPMENTAL COMPETENCE IN 3D CULTURE

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In the domestic cat model, vitrified oocytes (VOs) produced promising results, however their potential to develop into late stage embryos is often impaired. Instead of culturing VOs in two-dimensional (2D) microdrops of medium (conventional system), the use of three-dimensional (3D) scaffolds could better maintain their functional integrity, contributing to oocyte *in vitro* outcomes. Therefore, the aim of this study was to investigate survival and embryo development (oocyte competence) of feline VOs cultured in 3D barium alginate microcapsules. Morphologically selected immature cumulus-oocyte complexes (COCs, n=250) were vitrified by Cryotop method, and viable oocytes after warming were *in vitro* matured for 24 hours in 2D (2D VOs) or 3D system, with fresh companion COCs in 1:1 ratio [3D VOs(+)] or without [3D VOs(-)]. After *in vitro* fertilization, presumptive zygotes were cultured for 7 days in 3D or 2D system, according to the maturation conditions. Viability of VOs after warming and number of blastomeres of embryos were assessed by fluorescent stainings (fluorescein diacetate/propidium iodide and bis-benzimide, respectively). Arcsin transformed data were analysed by one-way ANOVA followed by Tukey test, and the level of significance was set at $p<0.05$. After warming, 91.2% VOs were viable. Embryos were successfully obtained at similar proportions in each group [mean ± SD: 3D VOs(+) 0.18 ± 0.17; 3D VOs(-) 0.17 ± 0.12; 2D VOs 0.15 ± 0.09; $p>0.05$], but the use of the 3D system together with companion COCs was determinant for their development, since 3D VOs(+) were the only group to reach the blastocyst stage (mean ± SD: 0.01 ± 0.03, day 7). Degeneration rates did not differ among groups. These positive results prompt the use of 3D enriched culture systems for VOs to exploit this valuable germplasm and widen the potential of vitribanking.

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S91 *ONE-STEP* VITRIFICATION OF MURINE EMBRYOS CHALLENGES CURRENT PARADIGMS OF CRYOBIOLOGY

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Cryopreservation of embryos is amongst the most powerful tools for preserving the genetics of laboratory animals. Vitrification is widely recognized as more efficient than slow freezing e.g. in human assisted reproduction technologies and for preserving murine embryos. Unfortunately, current vitrification procedures require multiple pre-cooling and post-warming exposure steps to dedicated solutions to reach maximum effectiveness, which appears difficult to deal with when many embryos are cryopreserved in one single session. To help solving this issue, we have developed a *one-step* embryo vitrification procedure. Briefly, embryos are exposed to a unique –chemically defined- vitrification solution before plunging in the liquid nitrogen. Subsequent warming is performed by immersing the vitrified embryos directly into the culture medium.

Murine embryos at the zygote, two cells and morula stages have undergone our *one-step* procedure either under aseptic or non-aseptic conditions. After warming, direct *in vitro* survival, development to the blastocyst stage and *in vivo* development to birth were recorded as endpoints. Short exposure times to the vitrification solution (less than 60 seconds) before cooling and direct warming into culture medium led to results equivalent or better than after classical vitrification. Longer exposure times to the vitrification solution (between 90 and 150 seconds) decreased efficiency. These results demonstrate that intracellular vitrification after our *one-step* procedure occurs by combined effects of fast cooling (or warming) and cell dehydration, with minimal, if any, ingress of cryoprotectants. The absence of deleterious effect of warming directly into the culture medium and the relatively low sensitivity to thermal inertia (aseptic vs non-aseptic) of the carrier is a confirmation thereof.

Consequently, beyond bringing a methodological simplification without any loss of efficiency, our patented *one-step* vitrification procedure dramatically lowers if not suppresses intracellular concentration of cryoprotectants and associated toxicity, thereby challenging some commonly accepted concepts of cryobiology.

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Conflict of interest: None to disclose.

S92 SOLID-STATE BIOLOGY AND ITS IMPLICATIONS TO CRYOBIOLOGY

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Preserving viability requires converting cytoplasm from a fluid to solid matrix. The effort is described by terms such as 'vitrification' and 'glass transition' and seemingly competing concepts such as 'bound water' or 'water replacement.' These concepts imply stabilization

of cytoplasmic constituents by immobilizing molecules into a fixed matrix. Much cryobiology research is dedicated to accomplishing the difficult conversion from fluid to solid using chemical additives. We are interested in natural systems that undergo this transition during development and find a wealth of examples in plant reproductive cells, such as seeds, pollen and spores. Solidification of cytoplasm inevitably occurs when cytoplasm dehydrates, and survival depends on cells' ability to tolerate the mechanical stress of shrinkage and molecular compression. Once vitrified, structural and chemical stability of the solid is critical – that is, time-dependent responses manifested by slow movement of molecules trapped within the matrix. Here, we infer molecular mobility from temperature and moisture dependencies of aging rate, and speculate that global mobility of molecules within the solid cytoplasmic matrix becomes severely limited at a temperature well below Tg but well above liquid nitrogen temperatures. At temperatures below this cross-over, local mobility – not global mobility- regulates aging kinetics and temperature effects become minor. Making an analogy with composite materials, we link anomalies in temperature dependencies to differing physical properties of lipids that are surrounded by cytoplasmic solid matrices. These temperature anomalies challenge the effectiveness of conventional storage platforms but are circumvented by appropriate cooling protocols and cryogenic storage. Future work will investigate the role of composition and organization of solidified cytoplasm in regulation of global and local molecular mobility and whether these explain the wide variation of longevity observed in diverse plant germplasm forms.

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S93 CHARACTERIZING DIFFUSION KINETICS TO IMPROVE PANCREATIC ISLET CRYOPRESERVATION

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Determining diffusion kinetics of cryoprotectants into the core of pancreatic islets is essential for design of enhanced cryopreservation protocols. Current protocols which use dimethyl sulfoxide (Me_2SO) achieve suboptimal results and may require additives. We examined the kinetics of diffusion of molecules in aqueous solutions (Me_2SO-free) into islets as a prelude to improving islet cryopreservation by combining Me_2SO with non-toxic cryoprotectant trehalose. Mouse pancreatic islets were isolated and incubated with Hoechst nuclear dye for up to 24h, cryosectioned and the penetration depth was analysed by confocal microscopy. Cryopreservation was performed using 2M Me_2SO, 200mM trehalose or their combination using a programmable freezer. Post-thaw viability was assessed by fluorescein diacetate/propidium iodide staining. Post-thaw islet function was examined using ATP/ADP ratios and glucose-stimulated insulin secretion. Diffusion experiments demonstrated that dye intensity gradient towards the core was present even after 24h of incubation. Several approaches were explored to accelerate the diffusion, including increased hydrostatic pressure, Me_2SO addition and increasing temperature. Optimal diffusion into the islet core was achieved at 37°C, reducing the incubation time to 6h or less. Cryopreservation with trehalose alone did not result in satisfactory cryoprotection for any of the incubation times. Pre-incubation of pancreatic islets in trehalose for 6 hours combined with Me_2SO incubation for 45 minutes resulted in significantly higher viability than Me_2SO alone immediately post-thaw (76.37±7.03% v 58.07±7.25% respectively, n=3, p=0.034, paired t-test). After one day in culture, only 40.63±6.32% of islets number frozen in Me_2SO alone survived compared to 72.65±4.47% using the new protocol. Similar trends were observed in ATP/ADP and glucose stimulation assays. Our results suggest that solute diffusion into dense islet tissue can take up to 6h at 37°C. We demonstrated that pre-incubating pancreatic islets with non-toxic cryoprotectant trehalose before adding Me_2SO results in enhanced cryoprotection. We aim to validate these findings using human pancreatic islets.

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Conflict of Interest: None to disclose

S94 OPTIMIZATION OF PROTOCOLS FOR MICROINJECTION-BASED DELIVERY OF CRYOPROTECTIVE AGENTS INTO FISH EMBRYOS

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Cryopreservation of fish embryos is a promising tool for more efficient aquaculture and conservation of endangered species. However, the presence of structural barriers in the embryos such as the chorion and other membranes constrains the swift penetration and homogeneous distribution of cryoprotective agents (CPAs) inside the embryos. In this study, we optimized key factors for microinjection-based delivery of extenders and CPAs directly into the perivitelline space (PS) and the yolk mass (YM) in embryos of the Japanese whiting, *Sillago japonica*, an emerging model species for fish embryo cryopreservation research. Embryos at all stages from 2~32 cells to tail elongation tolerated dry puncturing through both compartments well but viability

of embryos injected with saline diluents (vehicles for CPA administration) decreased sharply for all stages up to gastrula. Yamamoto solution, fish Ringer, and phosphate buffered solution were found to be suitable extenders for microinjection at volumes up to 2.1 nl in the PS and 15.6 nl in the YM. The extender distributed swiftly within minutes from injection in both the PS and YM as visualized with the aid of trypan blue dye. A comparison of the tolerance of embryos in different developmental stages to microinjection of three common CPAs, dimethyl sulfoxide (Me2SO), 1, 2-propylene glycol (PG), and ethylene glycol (EG) at concentrations of 30-90% into the PS and YM showed that EG was the least toxic followed by Me2SO, that the maximum tolerable concentration was about 50%, and that tail elongation embryos were more tolerant to the CPAs than the other developmental stages. Differential scanning calorimetry (DSC) analysis revealed a significant decrease in the ice nucleation temperature in tail elongation embryos microinjected with EG or Me2SO in the PS and YM compared to untreated embryos. Ongoing research should verify the usefulness of the developed protocol and lead to further refinements.

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S95 'IN AIR' CRYOPRESERVATION OF MESENCHYMAL STROMAL CELLS ON 3D COLLAGEN-HYDROXYAPATITE SCAFFOLDS

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Cryopreservation is a powerful tool for the rapid translation and commercialization of regenerative medicine research through efficient supply of stem cells on demand. Using cryopreservation, stem cells could be stored not only in suspension but also in tissue-engineered scaffolds which greatly reduces storage processing time and improves logistics management. In our previous studies, we demonstrated that conventional slow-freezing using Me2SO and thawing in a water bath developed for suspended cells do not provide sufficient protection of mesenchymal stromal cells frozen in 3D collagen-hydroxyapatite scaffolds. Here, we report on successful cryopreservation of the same tissue-engineered product by adopting 'in air' cryopreservation approach developed by David Pegg, translating sucrose-pretreatment protocol to adherent cells, modifying thawing protocol and combining penetrating and non-penetrating cryoprotectants. Porous 3D scaffolds were fabricated using freeze-drying of mineralized collagen. Amnion-derived MSCs from common marmoset monkey *Callithrix jacchus* were seeded onto scaffolds in static conditions. Cell-seeded scaffolds were subjected to 24h pre-shrinking in 0.1M sucrose and slow freezing in 10% Me2SO/20%FBS alone or supplemented with 0.3M sucrose. Diverse analytical methods were used for the interpretation of cryopreservation outcome such as Raman, cLSM and SEM microscopy, FTIR-spectroscopy, compression testing, cryomicroscopy and DSC. No differences in overall chemical structure of 'in air' cryopreserved scaffolds were revealed. Compressive stress of frozen scaffolds was significantly lower than in the control group presumably due to recrystallization demonstrated by cryomicroscopy. In both groups, cells exhibited typical shape and well-preserved cell-cell and cell-matrix contacts after thawing. Moreover, viability test 24h post-thaw demonstrated that application of sucrose in cryoprotective solution preserves significantly bigger portion of sucrose-pretreated cells (more than 80%) in comparison to Me2SO alone (60%). Scaffolds *per se* showed very low heat capacity compared to that of pre-saturated with tested cryoprotectants. In conclusion, cryopreservation of 'ready-to-use' tissue-engineered products is a promising strategy facilitating their future clinical application.

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Conflict of Interest: None to disclose.

S96 TESTIS CRYOPRESERVATION AND SPERMATOGONIA TRANSPLANTATION AS A TOOL FOR ZEBRAFISH LINE RECONSTITUTION

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Thousands of transgenic zebrafish (*Danio rerio*) lines have been recently created by molecular biology tools so far. The aim of this study was to develop cryopreservation protocols for zebrafish germ cells in order to overcome existing storage shortcomings. Slow-rate freezing (~1 °C/min) of spermatogonia was optimized by testing the effects of various cryoprotectants (dimethyl sulfoxide – Me_2SO, ethylene glycol – EG, propylene glycol – PG and glycerol – Gly), their concentrations (1.0, 1.3 and 1.6 M) and sugar (0.1

or 0.3 M of glucose, sucrose, fructose and trehalose) and protein (1.5% BSA, 1.5% FBS, 1.5% skim milk and 10% egg yolk) supplementation on spermatogonia viability in four consecutive experiments. Vitrification was optimized by testing three different equilibration solutions (ES1–ES3) and three different vitrification solutions (VS1–VS3) containing various concentrations of Me_2SO, PG and methanol (MeOH). Viability of testicular germ cells frozen with the addition of 1.3 M Me_2SO in the cryomedium was significantly higher than the viability of those frozen with other tested cryoprotectants and Me_2SO concentrations. Different sugar and protein supplementation did not significantly affect the viability of spermatogonia. During vitrification, the highest viability was obtained when combining VS3 containing equal concentrations of PG and Me_2SO (3 M of both) and either ES1 or ES3. Both freezing and vitrification protocols demonstrated favourable repeatability since they yielded viability rates above 50% in six different zebrafish lines. To test the functionality of the cryopreserved cells, we sterilized wild-type embryos with *dnd*-moprohlino microinjection and transplanted fresh, frozen or vitrified spermatogonia of β-actin:YFP [Tg(*actb::YFP*)] transgenic zebrafish into the sterilized recipients. Observation of dissected recipients six months after transplantation revealed that YFP-labelled germ cells were present in the recipient gonads (~50% incorporation rates in all groups). Furthermore, viable offspring displaying fluorescent signal was produced indicating the possibility of surrogate-production by transplantation of cryopreserved spermatogonia.

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Conflict of Interest: None to disclose

S97 SHORTENING A CRYOPROTECTANT LOADING PROTOCOL FOR ARTICULAR CARTILAGE VITRIFICATION WITH ENGINEERING MODELLING

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Osteochondral allograft transplantation can be used to treat defects in articular cartilage, but the lack of a long-term storage technique prevents a large quantity of allografts from being available for future transplantation. One promising long-term storage option is vitrification, and a successful 9.5 h loading protocol was previously reported for loading cryoprotective agents into articular cartilage for its vitrification. However, shortening of this protocol is still needed to facilitate clinical uptake. In this work, we first investigate the 9.5 h protocol. We use engineering models to predict the spatial and temporal distribution of cryoprotective agent concentrations and calculate the expected freezing point depression and vitrifiability in 2-mm thick articular cartilage samples attached to bone. We use Fick's law of diffusion, the Gibbs–Duhem equation combined with the osmotic virial equation for freezing point depression, and binary and ordinal predictions of vitrifiability. From the edge of the cartilage down to the bone–cartilage junction, the calculated vitrifiability at the end of the 9.5 h loading protocol exceeds the minimum threshold required for successful vitrification, and thus we can understand why the 9.5 h protocol is successful. Given this knowledge, we propose a new 7 h protocol that is calculated to reach levels of vitrifiability comparable to the successful 9.5 h protocol. To achieve this shorter time, we remove glycerol as a cryoprotectant and we adjust cryoprotectant concentration, exposure time, and temperature during the loading steps. The new 7 h protocol loads dimethyl sulfoxide, propylene glycol, and ethylene glycol into the articular cartilage over the course of three loading steps at progressively colder temperatures, and experimental work on porcine cartilage indicates that this protocol results in cell viability comparable to that for the 9.5 h protocol.

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Conflict of Interest: Some of the authors hold a patent in this area.

S98 OPTIMIZATION OF HYPOTHERMIC CARTILAGE STORAGE FOR CHONDROCYTE VIABILITY AND BIOMATERIAL PRESERVATION.

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Feasibility assessment of two design optimization strategies for hypothermic cartilage storage media was performed. First, testing of doxycycline supplementation of culture medium used for cartilage storage was assessed. Doxycycline has been shown to have beneficial in vivo effects on cartilage in animal models and humans. Second, we compared hypothermic storage in culture medium versus storage in an organ preservation solution (UW). Porcine articular cartilage plugs were incubated for up to 9 weeks at 4°C. At regular intervals the plugs were assessed for viability (alamarBlue assay). Biomaterial properties and gene expression were also

investigated. Retention of phenotype, or not, was assessed based upon RNA expression of a panel of markers. Preliminary results identified 3μM doxycycline combined with 10mM HEPES in DMEM culture medium as significantly better than DMEM alone and no media exchange was better than weekly refreshment. Chondrocyte viability was >80% upon rewarming and recovered to fresh control values during a few days of incubation under physiological conditions. Significantly better retention of fixed charge density was observed with doxycycline ($p<0.05$). No change in hydraulic permeability or water content was observed, aggregate modulus and swelling pressure was reduced. Concluding experiments comparing supplemented DMEM and UW formulations clearly demonstrated superior viability after 9 weeks ($p<0.05$ by ANOVA and $p<0.0001$ by t-test, $n=12$) both before and after 5 days of tissue culture, no significant differences compared with fresh cartilage. UW supported chondrocyte viability for < 1 month. Supplementation of DMEM with doxycycline and HEPES buffer minimized changes in cartilage marker and MMP9 expression reflecting minimal if any potential change in cell phenotype or matrix degradation. In conclusion, supplementation of DMEM with doxycycline and HEPES without media refreshment resulted in retention of cell viability for 9 weeks. In marked contrast, storage in UW solution ± supplements failed to support chondrocyte viability at 4 weeks.

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Conflict of Interest: Kelvin Brockbank is owner and founder of T3LLC

S99 TRANSPLANTED HUMAN THYMUS SLICES INDUCE AND SUPPORT T-CELL DEVELOPMENT IN MICE POST-CRYOPRESERVATION.

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The thymus organ, located in the upper abdominal cavity, is the site for the maturation of T-cells in the body. Children born without a thymus require urgent treatment to reconstitute T-cell immunity. Thymus tissue is removed from infants during cardiac surgery, to allow access to the heart. The thymus is most active during infancy and by adulthood is largely replaced with fibrous tissue, precluding adult donation. This discarded thymus tissue can be transplanted into athymic infants to reconstitute T-cell immunity., After a two to three week culture period to deplete thymocytes, slices of thymus tissue are transplanted into the thigh.
This procedure is life-saving, but recipients often have subnormal T-cell counts, and may develop autoimmunity. It is not practically possible to achieve any degree of MHC-matching transplants between-donor and recipient because of the urgency of performing the procedure. As delays in thymus transplantation could be life-threatening, the procedure would be improved if it were possible to freeze thymus slices for transplantation. Cryopreservation would also open the possibility of partial MHC-matching.
In this study we cryopreserved 1mm thick slices of thymus tissue (as would be used for transplantation) using a VIAFreeze controlled rate freezer and CryoStor cryoprotectant, and found that cooling at 1°C/min was optimal for this tissue, giving less tissue autolysis than either 0.3 or 2°C/min. We show that slices of human thymus tissue that have been frozen and thawed can induce and support T-cell development when transplanted into nude mice. We are currently carrying out a clinical trial comparing transplantation of cryopreserved with fresh tissue in athymic infants. Favourable results of this trial will support the establishment of a cryopreserved bank of paediatric thymus tissue.

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Conflict of Interest: None to disclose

S100 USE OF SUCROSE TO DIMINISH PORE FORMATION IN FREEZE-DRIED HEART VALVES

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Decellularized heart valves hold great promise as alternative for mechanical or bioprosthetic valves to replace a malfunctioning heart valve. In the surgical practice, sufficient availability of decellularized heart valves of different sizes is needed, which requires appropriate preservation methods. If it were possible to dry heart valves by means of freeze-drying without damaging the tissue, this would increase their shelf-life and ensure off-the-shelf availability. Freeze-dried decellularized heart valves can simply be stored at room temperature, are easy to transport, and are ready to use after a simple rehydration step. In this study, heart valves were incubated in solutions of various sucrose

concentrations used as lyoprotectant and subsequently freeze-dried. Histological inspection showed that in the absence of sucrose, freeze-dried valves have pores after rehydration in the cusp, artery and muscle sections. Pretreatment of the valves with sucrose reduced pore formation in a dose-dependent manner, and loading valves in a 40% (w/v) sucrose solution prior to freeze-drying was found to be sufficient to completely diminish pore formation. Multiphoton imaging showed that collagen and elastin in rehydrated freeze-dried cusp and artery sections are well preserved irrespective of the sucrose concentration with multiple layers of collagen bundles visible comprising of apparently symmetrical orientations. By contrast, collagen bundles in glutaraldehyde-fixed valves (resembling the structure in bioprosthethic valves) displayed a wavy structure and a different space arrangement. Fourier transform infrared spectroscopy, and differential scanning calorimetry studies also indicated that matrix proteins were affected by glutaraldehyde treatment and not by freeze-drying. The presence of pores in valves freeze-dried in the absence of protectants was found to coincide with altered biomechanical characteristics in cusp and artery sections, whereas biomechanical parameters of valves freeze-dried with sufficient amounts of sucrose were not significantly different from those of valves not exposed to freeze-drying.

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Conflict of Interest: None to disclose

S101 PRESERVATION OF DECELLULARIZED CARDIOVASCULAR GRAFTS FOR APPLICATIONS IN TISSUE GUIDED REGENERATION

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The rapid evolution of cardiovascular tissue engineering field is progressively moving towards in situ tissue guided regeneration, pushing decellularized scaffolds into preclinical and clinical use. Development of long-term preservation methods for cardiovascular grafts is of major importance to prolong their shelf-life. The objective of this study was to evaluate the suitability of three different preservation methods: slow-rate freezing, vitrification, and freeze-drying for preservation of decellularized bovine pericardial (DBP) scaffolds. Freezing was conducted at ~1°C/min using 10% Me2SO. Vitrification was performed with vitrification solution VS83 and rapid cooling above the vapour phase of liquid nitrogen. For freeze-drying, scaffolds were loaded with sucrose as lyoprotectant and subsequently freeze-dried utilizing a programmable freeze-dryer with temperature-controlled shelves. The impact of the preservation methods on the structural integrity of the scaffolds was assessed using microscopic imaging, biomechanical tests, biochemical characterization, spectroscopic and thermal analysis. Histological staining and microscopic imaging revealed that the extracellular matrix integrity was well preserved after all preservation treatments. In addition, matrix proteins were not affected by any of the preservation methodologies. Biomechanical testing, however, revealed differences among the treatment groups. Frozen scaffolds were found to have a significantly increased collagen phase modulus compared to control scaffolds indicating a decrease in scaffold compliance. By contrast, vitrified and freeze-dried scaffolds displayed similar biomechanical properties compared to the control group. Cytocompatibility studies showed that neither control nor any of the preserved scaffolds display cytotoxicity. It is suggested that freeze-drying, which can be done using non-toxic protective agents and allows easy storage and transport at room temperature, can replace currently used slow-rate freezing and vitrification approaches for preservation of decellularized scaffolds.

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Conflict of Interest: None to declare

S102 IMPACT OF VS83 AND ITS COMPONENTS UPON TISSUE IMMUNOGENICITY

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Banking of large tissues using current tissue banking practices employing conventional cryopreservation by freezing is not feasible due to the well documented damage caused by intra and extra-cellular ice formation. We have used ice-free vitrification for a variety of natural and engineered tissues ranging from epithelial constructs to veins, arteries, heart valves and cartilage. Major constraints for scale-up of cryopreservation by ice-free vitrification included ice nucleation during warming and mechanical forces generated by glasses at low temperatures. In this presentation I will review our first successful strategy for avoidance of ice nucleation using an 83% cryoprotectant formulation, VS83, containing 4.65 mol/L formamide, 4.65mol/L ME_2SO, and 3.31 mol/L 1,2 propanediol. This formulation can be used to retain viable chondrocytes in large osteochondral grafts

or for non-viable cardiovascular grafts with retention of extracellular matrix integrity, depending upon the way in which the formulation is added and removed before and after vitrification. Non-viable cardiovascular grafts with intact matrix have been a major research focus for the last 10 years and both in vitro and in vivo results have demonstrated significantly reduced tissue immunogenicity as well as better in vivo functions. We characterized factors released by human aortic tissue after control rate freezing with 10% ME_2SO and ice-free cryopreservation. Co-cultures with subsets of human peripheral blood mononuclear cells were analyzed to examine functional immune effects triggered by the tissue or released cues. Ice-free tissue exhibited significantly lower release of pro-inflammatory cytokines (IL-6, MCP-1 and IL-8) than frozen tissue, but surprisingly, more active TGFβ. High concentrations of ME_2SO or formamide, but not propanediol, activated latent TGFβ. Less monocyte and T cell migration was detected in a chemotaxis system. These observations combine to demonstrate that VS83 ice-free cryopreservation selectively modulates tissue characteristics and thereby attenuates immune cell attraction and activation.

Source of Funding: Not applicable
Conflict of Interest: Kelvin Brockbank is owner and founder of T3LLC

S103 EVALUATION OF CRYOPRESERVED ACELLULAR NERVE GRAFT

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In order for acellular nerve allografts (ANAs) to be put into broad commercial use, long-term preservation of these biomaterials is crucial during tissue collection as well as during production, sale, distribution and usage. The purpose of this study is therefore to understand the effects of deep low temperature on the histological, mechanical and biological properties of ANAs. It showed no obvious change in morphological appearance or ultrastructure after cryopreservation of human ANA (hANA) for one year. And no significant difference when testing their breaking force (18±2.6N vs 17±3.5N) and cytotoxicity (grade I for both groups). When we applied fresh or cryopreserved rat ANA (rANA) to repair nerve injury in rats, there were no significant changes in interest of integrated optical density (IOD) of NF-200 expression (116201.90±5443.15 vs. 121802.60±4805.15, P>0.05), extent of muscle atrophy (59.89±3.78% vs. 62.78±8.10%, P>0.05) and myelin sheath thickness (4.48±1.70 μm vs. 4.50±0.89 μm, P>0.05) after 28 days post-op. Our study suggests that cryopreservation can greatly extend the storage duration of acellular nerve tissue allografts without concomitant alteration of the physiochemical and biological properties of the engineered tissue to be used for transplantation.

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Conflict of Interest: None to disclose

S104 TGF-α and VEGF EXPRESSIONS IN SKIN GRAFTS CRYOPRESERVED BY ANTARCTIC YEAST ORIENTED ANTI- FREEZE PEPTIDE (Afp1m)

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Skin graft Cryopreservation procedures pose challenges to the clinicians though, tissue cryopreservation is now a century old technique and advancements were made in the field of cryobiology in the current era of live tissue cryopreservation. The use of antifreeze proteins (AFPs) and their derivative peptides (Afp) for tissue preservation were seen as potential discovery during past few decades. Present study was design to describe and assess the cryo-damages caused by subzero temperatures of -10 and -20°C in cryopreserved skin grafts tissue using different Afp1m concentrations of 0.5, 1, 2, 5 and 10 mg/mL for 72 h. Immuno-histochemical scores in three regions of cryopreserved skin grafts, *i.e.* epidermis, dermis and hypodermis were assessed by applying two biomarkers, *i.e.* transforming growth factors (TGF-α) and vascular endothelial growth factor (VEGF) to characterize their expressions in viable skin grafts. This experiment was the part of pre-grafting trails of Afp1m cryopreserved skin grafts evaluation before transplantation. These biomarkers demonstrated that different concentrations of Afp1m at -20 °C were unsuccessful freezing regime and showed significant differences ($p < 0.05$) among the different concentrations at -10 and -20°C. The overall trends observed in all concentrations at -20 °C and at lower concentrations 0.5,1 and 2mg/mL at -10 °C showed low level of TGF α /VEGF viability expressions as compared to the higher concentrations (5 and 10 mg/mL) at -10 °C. In conclusion, the integrity of skin graft layers, showed various changes assed immunohistochemically especially in epithelial layers while deep dermis of cryopreserved skin grafts showed

mild viability expressions. The present study attested that Afp1m is a good agent for the cryopreservation of skin graft which can be optimized to investigate the other cryopreserved organs at subzero temperatures besides the skin which was the main focus of the present work.

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Conflict of Interest: None to disclose

S105 STRATEGY TO IMPROVE POST THAW QUALITY OF BUCK SPERM BY USING ADENOSINE 5' TRIPHOSPHATE

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Cryopreservation affects the plasma membrane, cytoskeleton, motility, mitochondrial membrane, acrosome and the nucleus of spermatozoa. Since mitochondria are the source of the ATP that powers sperm motility. Therefore, the integrity of mitochondrial membrane must be maintained to keep the sperm motile after freezing and thawing. In this background, the present study was aimed to determine the effect of different concentrations of adenosine 5′ triphosphate (ATP) on buck sperm quality after freezing and thawing. Semen was collected from six adult bucks through artificial vagina. After initial evaluation semen samples were pooled and diluted with Tris Citric acid Fructose extender to a concentration of 200×10^6 sperm/mL. The extended samples were split into four equal aliquots and treated with one of the ATP concentrations (0mM 0.5mM, 1mM or 2mM). Then all the aliquots were incubated at 35 ^oC for 15 min. The samples were filled in 0.5 mL French straws, cooled to 4 ^oC within 2hrs and cryopreserved at -196 ^oC following a standard freezing protocol. After freezing two straws from each replicate were thawed and assessed for motility, viability, acrosome, plasma membrane, DNA and mitochondrial integrity. The results showed that mitochondrial and DNA integrity were higher ($P < 0.05$) in ATP treated groups. The sperm viability remained higher in 0.5 mM group compared to other ATP groups. However, the sperm motility, acrosome and plasma membrane integrity remained similar among the groups. In conclusion, addition of ATP in extender enhances the quality of sperm in terms of mitochondrial integrity and viability after freezing and thawing. It can be anticipated that the addition of ATP in extender may enhance sperm cryosurvival by improving metabolic capacity of sperm before freezing.

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Conflict of Interest: None to disclose

S106 SUCCESSFUL SPERM CRYOPRESERVATION AND GENERATED OFFSPRING OF THE ENDANGERED FROG, *LITORIA AUREA*

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Cryopreservation of testicular macerates derived from captive-bred males (n=7) of the endangered Green and Golden Bell frog (*Litoria aurea*) was investigated using 3 concentrations (10, 15 and 20% v/v) of dimethylsulfoxide (Me_2SO) and glycerol in combination with 10% w/v sucrose. In addition, 10% w/v sucrose was tested in the absence of Me_2SO and glycerol. The responses of motility (total and forward progressive motility), vitality and fertility were assessed. Overall, there was no difference in vitality, measured by dye exclusion staining with Eosin-Y, across treatments ($p>0.05$). Intact membranes ranged from 29.5 % ±13.1 (10% sucrose) to 53.4% ±8.2 (15% Me_2SO/10% sucrose). Motility was significantly lower in glycerol treatments than Me_2SO ($p<0.0001$) ranging 15.0% ± 3.0 to 37.0% ±7.3 for total motility in glycerol treatments and 53.7% ± 4.3 to 61.4% ± 7.4 in Me_2SO treatments. Similarly, mean forward progressive motility was highest in 15% Me_2SO, though there was no significant difference between Me_2SO treatments (10.3% ±2.9 to 24.1% ±6.0). In order to test fertility of cryopreserved sperm, 15% Me_2SO and glycerol cryopreserved sperm were tested in an *in vitro* fertilisation system (n=4 females) comparing sperm from fresh macerates and frozen/thawed sperm. It was found that there was no significant difference between fertilisation rates of fresh untreated sperm (85.0% ±4.4) to fresh unfrozen sperm treated with Me_2SO (67.2% ±8.2), but that these treatments were significantly different to cryopreserved sperm treatments ($p<0.05$). Of the frozen/thawed sperm, 15% Me_2SO performed best with 13.9% ±4.2 of ova fertilised and poorest being glycerol with 10.3% ±2.2 fertilised. Taken together, results indicate recovery of motility is higher in sperm cryopreserved in Me_2SO than glycerol, but this does not necessarily translate to difference in post-thaw fertilisation rates. Nevertheless, the data demonstrates viability of sperm cryopreservation as a conservation tool for this species.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S107 INFLUENCE OF TESTICULAR TISSUE AND TESTICULAR CELL SUSPENSION CRYOPRESERVATION ON SPERMATOGONIAL STEM CELL FUNCTION

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Testicular tissue (TT) cryopreservation is increasingly becoming available to prepubertal boys whose fertility is at stake (e.g prepubertal cancer patients). In the case of systemic cancers, the frozen-thawed TT needs to be depleted from malignant cells before use as it represents a high risk of reintroducing cancer cells in the patient. Therefore, the generation of testicular cell suspensions (TCSs) that ought to be cryopreserved the lag time of safety testing is compulsory. For this reason, the cryopreservation of spermatogonial stem cells (SSCs) plays a crucial role for any downstream application (e.g autologous spermatogonial stem cell transplantation (SSCT)). To this date, many studies have solely focused on the cryopreservation of testicular tissues and on the recovery of viable cells after cryopreservation. In this study, we compare the influence of the cryopreservation method (cryopreservation as TT or TCS) on cell viability and SSC function after SSCT. Upon freezing and thawing, the cryopreservation of TTs permitted to recover a significantly higher number of viable cells compared to the cryopreservation and thawing of TCSs ($3.9x10^5 \pm 2.6x10^4$ versus $2.4x10^5 \pm 1.1x10^4$, P=0.002). As the SSCT assay has become the "gold standard" for assessing the stem cell activity of SSCs, frozen/thawed cells from different replicates of both types of samples were transplanted to recipient mice. Twelve weeks after transplantation, frozen/thawed cells following the cryopreservation of testicular cells exhibited a greater stem cell activity, by a higher number of donor-derived SSC colonies (49.25 ± 7.83 versus 20.08 ± 3.36, P<0.0001) and length of colonies (5.76 ± 0.21mm versus 3.08 ± 2.47mm, P=0.0032) compared to its counterpart. Our results indicate an increased recovery of viable cells and SSC activity upon TT cryopreservation. This is probably due to the better preservation of cell-to-cell cooperation within germ and somatic cells during the freeze-thawing events.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S108 EFFECT OF POLYVINYL ALCOHOL ON ANGORA BUCK SPERMATOZOA SURVIVAL AND FUNCTION FOLLOWING CRYOPRESERVATION

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Small quantities of the synthetic polymer polyvinyl alcohol (PVA) were found to inhibit the formation of ice in cryoprotectant solutions. Therefore, we aim to cryopreserve the freezability of semen with 3 different polyvinyl alcohol (PVA). In total, 75 ejaculates from seven Angora bucks were collected by artificial vagina. Samples were diluted with three different PVA co-polymers 9, 18 and 100 kDa; 0.001, 0.01, 0.1, 1 and 2 % added to Tris-egg yolk diluent respectively. Sperm diluent seeding temperature, latent heat plateau and thermal gap were recorded. Semen was diluted and cooled down to + 4 C° in three hours, frozen in a programmable freezing machine. Extender groups showed a large difference upon the ice nucleation point at approximately around -3 up to -35 Cº and 2% glycerol PVA had 8.2 ± 1 C° latent heat plateu difference comparing to control. Highest motility was found in PVA 18 group with 67.72 ± 3.06 % regardless of the dosage (P<0,001). PVA copolymers independently in all other dosage groups 0.001, 0.01, 0.1 (PVA 100, except 0.1%) gained higher motility than control (p <0.001). A statistical difference was not observed among the different PVA derivatives and dose groups in terms of live spermatozoa rate (P>0.694, 0.953). None of the three PVA copolymers used induced spontaneous AR (P>0.001). Mitochondrial activity was higher in all experimental groups comparing to control and was highest at PVA 100 subgroup 0.001% with 73.09 ± 21.8 (P<0.001). Synthetic PVA-derived ice blocking agents can be produced much less expensively than antifreeze proteins, offering new opportunities for improving sperm cryopreservation. PVA copolymer concentrations of 0.001, 0.01, 0.1, and 1 % w/v (PBS: PVA) decreased the concentration of glycerol required to freezing seeding temperature in a 100 ml volume by 0, 1, 1, and 2 % v/v, respectively.

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Conflict of Interest: None to disclose

S109 THE EFFECT OF BLOOD ON PRESERVING ALPACA SEMEN

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Sexual intercourse in alpacas last for more than 20 minutes during which the male positions his penis in both uterine horns ejaculating in a continuous manner even at the vicinity of the cervical external opening, and causes hemorrhage due to having cartilaginous process. Thus, in this study the presence blood in freezing extender during alpaca semen cryopreservation was evaluated. Semen was collected using two methods, first by an artificial vagina, and constituted semen without blood; and second by aspirating it from the vaginal fundus following natural breeding, and constituted semen with blood from the female. Five adult Huacaya males were used during the studies. Semen characteristics evaluated were total motility, vitality, concentration and morphology. The process of freezing semen was an initial evaluation, extension with Tris buffer, refrigerating at 4 ºC for 2 hours, and freezing it by exposing to liquid nitrogen vapor phase after loading semen into 0.5 mL plastic semen straws in a slow manner. The freezing extender contained 7% glycerol, and spermatozoa were in contact with glycerol from the beginning of extension. Data were analyzed using a t-test and a $P \leq 0.05$ was set as being significantly different. There were no differences

(P≤0.05) in semen total motility were 80, 79; 65, 65; 63, 64%, at initial process, extension, and equilibration for semen collected by artificial vaginal and vaginal aspiration, respectively. However, motility in frozen-thawed samples was 35% for semen collected from artificial vagina, and 60% for semen obtained from vaginal aspiration (P<0.05). All other semen characteristics were not different (P>0.05) between semen collected from artificial vagina and vaginal aspiration. The results indicate that the presence of blood into the semen samples may have beneficial effect during the process of alpaca semen freezing.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S110 TRANSCRIPTOME ALTERATION OF SPERM ASSOCIATED ANTIGENS (SPAGs) IN NORMAL HUMAN FREEZED SPERM

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Human sperm membrane protein or sperm associated antigens (SPAG) included 18 types of proteins with a range of 24-71 k Da molecular weight. Some SPAGs (1, 2, 6, 8, 9, 12,13,15) play powerful role in fertility which in case of damage lead to infertility. The rapid freezing of sperm, which is thought to be a tool for ART, may disrupt the expression of these SPAGs. The aim of this study was to evaluate the effects of normal human sperm freezing on the gene expression of SPAGs.4 semen samples were collected from the normospermic cases. After density gradient centrifuge (DGC), progressive motile sperms were washed with HTF (Human tubal fluid) and divided into two groups: fresh and freeze. The freeze group exposed to cryoprotectant solution containing HTF + 70% sperm freeze solution during 10min of equilibration time at room temperature and then transferred on nitrogen vapors for 20 minutes and after that stored in liquid nitrogen. 3 days later, the specimens were warmed in tap water for 5min and then washed with HTF. Then, fresh and freezed sperms pellets, was carried for RNA extraction with TRIzol. cDNA synthesis was performed for evaluation of SPAGs genes expression with real-time PCR technique. Our findings showed a significant decrease in expression of SPAG 6, 8, 12 and 13 genes after warming (P<0.05) and there were no analytically differences in the expression level of SPAGs 1, 2, 9, 15 between fresh and freezed samples. These results indicate that human sperm rapid freezing could alter the gene expression level of sperm associated antigens (SPAGs) which involved in fertility ability of sperm.

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Conflict of Interest: None to declare

S111 CRYOPRESERVATION OF INDIAN RED JUNGLE FOWL SEMEN WITH DIMETHYLFORMAMIDE

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The study was designed to evaluate the effect of dimehtlyformamide (DMF) usage as a permeable cryoprotectant for Indian red jungle fowl spermatozoa on post thaw semen quality and fertility. A total of 40 ejaculates were collected on alternate days from eight Indian red jungle fowl cocks (5 replicates), pooled, diluted in red fowl extender and divided into five aliquots containing different concentrations of DMF (0% (control), 4%, 6%, 8% and 10%). Diluted semen cooled from 37°C to 4 °C @ -0.275 min^{-1}, 20% glycerol added to control and equilibrated for 10 minutes. After equilibration, semen was filled in 0.5 mL French straws, kept over liquid nitrogen vapor (5cm above the level of LN_2) for 10 minutes and plunged into liquid nitrogen. Semen samples were thawed at 37ºC for 30sec. The data on the effect of different stages of cryopreservation and the cryoprotectant concentration were analyzed by ANOVA using two factor factorial design and post-hoc comparisons were made, when F-ratio was found significant (P>0.05). After thawing, sperm motility, plasma membrane integrity, viability and acrosomal integrity were determined. Cryo-survival of Indian red jungle fowl was affected by cryopreservation stages and different concentrations of cryoprotectant used. Highest sperm motility, plasma membrane integrity, viability and acrosomal integrity were recorded in a diluent containing 8%DMF at post-dilution, cooling, equilibration and freeze-thawing. Highest fertility results were obtained after artificial insemination with 8% DMF compared to 20% glycerol. It is concluded that 8% DMF as a permeable cryoprotectant improves the post thaw semen quality and fertility in Indian red jungle fowl and can be used effectively to avoid the contraceptive effects of glycerol.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S112 MODEL PROTOCOL FOR CRYOPRESERVATION OF LIZARD SPERM

USING THE PHOSPHODIESTERASE INHIBITOR CAFFEINE

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We investigated sperm cryopreservation in the yellow-spotted monitor (*Varanus panoptes*), a lizard from northern Australia severely impacted by an invasive toad. The objective of the study was to create an optimised cryopreservation protocol for the spermatozoa of *V. panoptes* testing concentrations of two cryoprotectants (Me_2SO and glycerol) at concentrations of 0.68, 1.35 and 2.7M in PBS. A phosphodiesterase inhibitor (caffeine) was added post-thaw to stimulate motility. We also tested for cold-shock by rapidly cooling unfrozen sperm. Cryoprotectant toxicity was tested by incubating sperm with cryoprotectants at 25°C for five hours. For cryopreservation, sperm were cooled in straws suspended in liquid nitrogen vapour at approximately -32.1°C / minute, before plunging into liquid nitrogen, and later thawing in a water bath at 35°C. Samples were incubated post-thaw for 10 minutes in the presence or absence of 10 mM caffeine. Both cryoprotectant type ($P < 0.001$) and concentration ($P < 0.001$) significantly affected percent sperm motility pre-freezing, with Me_2SO being less cytotoxic than glycerol and motility decreasing at higher concentrations. Cold shock did not significantly affect spermatozoa motility. Both cryoprotectant type ($P = 0.009$) and concentration ($P < 0.001$) significantly affected the motility of post-thawed spermatozoa, with mid-range concentrations (1.35 M) yielding the greatest post-thaw motility for both cryoprotectants, and Me_2SO yielding greater post-thaw motility than glycerol. The best protocols, involving 1.35 M and 2.7 M DMSO with the addition of caffeine post-thaw, resulted in a motility of 48% and 42.3% respectively, comparable to percent motility at similar cryoprotectant concentrations pre-freeze. Addition of caffeine to samples post-thaw significantly increased motility of sperm for both cryoprotectants ($P < 0.001$), with higher stimulation associated with higher cryoprotectant concentrations ($P < 0.003$). Our study established a successful sperm cryopreservation protocol for *V. panoptes*, with increased post-thaw motility by caffeine, indicating that metabolic cryo-injury can be reversed.

Source of Funding: The study was funded by the Jane Fenwick Foundation.
Conflict of Interest: None to disclose.

S113 BIOCHEMICAL CHANGES DURING POTATO SHOOT TIP CRYOPRESERVATION

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Conservation of *Solanum tuberosum* L. genetic resources is essential for breeding. Cryopreservation is used as an effective method for long-term storage of meristematic plant tissue at IPK in Gatersleben, Germany. However, different protocols to cryopreserve potato shoot tips are published. Therefore, the aim of this study was to compare the regeneration of 30 potato genotypes after cryopreservation by using two approaches: a droplet freezing protocol using Me_2SO and a droplet vitrification method using PVS3. Furthermore, an adapted version including a shortened preculture step (PVS3A) was applied to four genotypes and biochemical changes alongside the protocols analysed. Significant different regeneration results were found between Me_2SO Droplet Freezing (49.5 ±7.9 %), PVS3 (57.3 ± 6.8 %) and PVS3A Droplet Vitrification (9.2 ± 2.6 %). Over all genotypes and protocols, soluble sugar content increased during preculture and remained at high levels until rewarming. Differences in mono- and disaccharide uptake from preculture solutions and cryoprotectant agents were found after regrowth between the three protocols. Energetic status, measured as adenosine triphosphate (ATP) content, was reduced after preculture and increased after rewarming during regeneration indicating a complete disruption of energy metabolism during cryopreservation. Differential scanning calorimetry revealed that PVS3 exposure led to typical glass transition, while during Me_2SO treatment ice crystallization appears. The thermodynamic behaviour of remaining water in the shoot tips did not correlate to regeneration ability. To compare regeneration after PVS3 Droplet Vitrification and Me_2SO Droplet Freezing, routine genebank cryopreservation was conducted using 26 potato accessions. Significant higher plant regeneration results and less visible contaminations were observed using PVS3 vitrification. Concluding, physiological changes during explant cryopreservation are strongly depend on the cryogenic procedure and the stress management of relevant accession. Overall, PVS3 influenced positively regeneration but there was no direct correlation between explant regeneration, water, sugar and ATP content.

Source of Funding: Not applicable.
Conflict of Interest: None to disclose.

S114 TRANSCRIPTOMIC, BIOCHEMICAL, AND ULTRASTRUCTURAL ANALYSIS FOR UNDERSTANDING CRYO-STRESS IN GARLIC

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Garlic (*Allium sativum* L.) is a medicinal crop, consumed as a popular condiment and green vegetable. Garlic can only be propagated vegetatively and genetic resources are maintained in vivo or via cryopreservation. The major challenge for cryopreservation is exposing the explant to cryoprotectant-mediated dehydration and ultra-low temperature (−196 °C) thereby inducing a

series of abiotic stress conditions which restrict regrowth. The aim of this study is to understand the influence of abiotic stresses during the cryogenic procedure by using biochemical, physiological and molecular genetic approaches. During explants are dehydrated or immersed in cryoprotective solutions, tissue oxygen drastically decreased from 100 to 1 % of air saturation indicating hypoxic conditions during cryopreservation. LC-MS was used to reveal hypoxia-mediated metabolites in subsequent steps (preculture, dehydration and liquid nitrogen storage) of cryopreservation. In dehydration, 3-phosphoglyceric acid, phosphoenolpyruvate and pyruvate concentrations increased which facilitate changes in the downstream of glycolysis. Reduction in ATP and the calculated adenylate energy charge was significant during dehydration. Oxygen level in different layers of garlic shoot tips and hypoxia was determined in the meristematic dome. This led to the assumption that the ATP level is locally decreased. Ultrastructural analysis using transmission electron microscopy (TEM) revealed major changes after dehydration such as loss of dictyosomes, enlargement of vacuoles and plastoglobules supporting the occurrence of both, oxidative and hypoxic stress conditions. Furthermore, we attempt to reveal this embedded stress using transcriptomics for the de novo assembly with programs such as Trinity, CLC and Bridger. The results obtained in this study will contribute to the garlic cryopreservation program, support garlic cryopreservation and help to understand transcriptomic profiles of non-model plant species using next-generation sequencing technology.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S115 BIOCHEMICAL AND THERMOPHYSICAL STUDIES DURING CRYOPRESERVATION OF VANILLA (*V. INSIGNIS*) APICES

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The accumulation of three osmolytes (sucrose, trehalose and glycerol) supplied through different cryoprotective treatments according to V and D-Cryoplates protocols was evaluated in vanilla (*V. insignis*) apices using gas chromatography-mass spectrometry. Following the same protocols and using Differential Scanning Calorimetry (DSC), the removal of freezable water was calculated after osmodehydration with plant vitrification solutions PVS2 and PVS3 for 30 min at room temperature (V-cryoplate), and after desiccation with silica gel for 2 and 3 h (D-cryoplate). Apices isolated from *in vitro*-grown plants were preconditioned on semi solid MS medium for 7d and on MS medium supplemented with 0.3M sucrose or trehalose during other 7d. After preconditioning, they were encapsulated over cryoplates and loaded with solution 0.4M sucrose and 2M glycerol for 30 min. The highest accumulation of sucrose (112.6 mg g^{-1} FW) and of glycerol (62.7 mg g^{-1} FW) occurred when apices were preconditioned on medium supplemented with sucrose and dehydrated with PVS2. No trehalose was detected in vanilla apices until it was exogenously provided during preconditioning. When apices were preconditioned on medium supplemented with trehalose, the highest contents of sucrose (121.4 mg g^{-1} FW), trehalose (19.8 mg g^{-1} FW) and glycerol (71.7 mg g^{-1} FW) were detected after 2h of desiccation in silica gel. As regards water removal, dehydration with PVS2 eliminated more freezable water than PVS3 when apices were preconditioned with sucrose, and PVS3, when apices were preconditioned on trehalose. The highest water removal was achieved after desiccation for 3h whatever the sugar used in the preconditioning. However, only freezing events were recorded after all the dehydration treatments studied, demonstrating neither the accumulation of osmolytes nor water removal attained were sufficient to avoid ice crystal formation and protect apices during cryopreservation. Post-cryopreservation recovery of *Vanilla insignis* apices has not been possible under any of these conditions.

Source of Funding: Not applicable
Conflict of Interest: None to declare

S116 NON-DESTRUCTIVE MEASUREMENT OF METABOLIC ACTIVITY IN SHOOT TIPS AFTER CRYOPRESERVATION

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The integrity of mitochondrial function within cells is essential for the successful recovery of cryopreserved material but there has been very limited investigation in plants. Mitochondria are the energy production facility in cells, producing adenosine triphosphate (ATP) through the electron transport chain, as well as carbon skeletons required in primary and secondary metabolites. The energy stored in ATP is vital in almost all aspects of cell metabolism, and it is of particular importance for cryopreservation due to its role in providing energy for repairing damaged DNA, the production of new proteins and lipids, and the energy to resume normal cell division and growth after storage. New ASTEC Global Technology Q_2 Oxygen Sensing Technology was used to assess metabolic function after cryopreservation, allowing metabolic activity of individual shoot tips to be assessed and correlated to post-cryogenic regeneration, survival or death. Initial findings indicate an initial lag phase after cryopreservation when metabolic rate is slowed, before shoot tips that survive and regenerate differentiated from the dead shoot tips by its significantly higher metabolic activity. Additionally, it was of interest was that many of the shoot tips considered dead still showed some metabolic activity over the first few days post-cryopreservation, a few showing similar metabolic rates to shoot tips that survived cryopreservation before their metabolic rate reduced.

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Conflict of Interest: None to disclose

S117 WHEAT POLLEN VIABILITY AND FEASABILITY OF POLLEN STORAGE

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Cryopreservation is used to extend the survival of mature pollen during *ex situ* storage and have been successfully applied for fruit crops and ornamental crops. Long-term conservation of wheat pollen could be particularly advantageous to control pollination in hybrid wheat breeding and production. However, wheat pollen have a very short lifetime and protocols for successful storage of viable wheat pollen have not been established until now. The present study aims to investigate physiological, biochemical and genetic factors influencing wheat pollen viability of four varieties in order to extend their longevity during storage. A viability test and evaluation scheme have been developed to examine *in vitro* germination based on pollen tube development. Highest viability of fresh pollen was 73 % on solid medium but varied strongly between the four different varieties. Storage of wheat pollen under four environmental conditions showed that wheat pollen lose germination ability rapidly when stored at room temperature in both, dry air as well as humid air. The percentage of germinated pollen declines from around 30 % of fresh pollen to 1 % within 45 minutes. Cold temperatures (6-8 °C) could prolong pollen longevity. However, an increase of the germination percentage was noted when stored under cold temperatures for 10 to 20 minutes indicating a kind of dormancy release. Therefore, biochemistry and transcriptome in combination with cryopreservation of recalcitrant wheat pollen are in detailed studied to elucidate possibilities of wheat pollen storage.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

S118 SUGAR DETOX: IS HIGH SUGAR CONCENTRATION REALLY REQUIRED FOR REWARMING?

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In many protocols for plant cryopreservation, thawing is done in a Rewarming Solution (RS) with a high concentration of sucrose (0.8-1.2M). A set of 50 publications were reviewed and classified by RS sucrose concentration, species, and regrowth rate prior to starting the study. Of the reviewed publications, 86% reported using relatively high sucrose concentrations of 0.8-1.4M in the RS solution. However, it was questioned if these high sucrose levels were required and/or beneficial. Thus, sucrose concentrations in the RS after liquid nitrogen exposure were assessed with 0.0M, 0.3M, 0.6M, 0.9M, and 1.2M sucrose with a set of 16 potato landraces, cryopreserved with the PVS2-droplet vitrification method. The accessions belong to the five largest taxa of the International Potato Center's in vitro collection: *S. tuberosum* subsp. *andigenum*, *S. stenotomum* subsp. *stenotomum*, *S. phureja*, *S. chaucha* and *S. stenotomum* subsp. *goniocalyx*. The results showed no significant differences for the average regrowth rate (81-87%) between sucrose concentrations of 0.3M to 1.2M. Complete removal of sucrose (0.0M) from RS resulted in a significantly lower average regrowth rate of 67%. Yet, the accessions rewarmed in RS with 0.0M sucrose still showed good regrowth of complete plantlets, ranging from 47-87%. Fourteen of 16 accessions showed their highest regrowth rate with sucrose concentrations between 0.3-0.9M. This study is currently being expanded to a group of ~100 diverse accessions (seven taxa, including a mini core collection of 44 accessions) to determine if lower RS sucrose concentrations can increase the post-thawing recovery rate on a larger-scale.

Source of Funding: Not applicable
Conflict of Interest: None to delcare

S119 OXIDATIVE DAMAGE AND ANTI-OXIDATIVE INDICATORS IN RICE 48H-GERMINATED EMBRYOS DURING CRYOPRESERVATION

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In the current study, reactive oxygen species accumulation (ROS) was investigated in 48h-germinated rice embryos during cryopreservation using vitrification method. We found that cryopreservation procedures using vitrification method significantly affected the ROS levels of germinated embryos, especially superoxide anions. Malonadehyde content was significantly positively correlated with the rate of superoxide anion generation and obtained the highest levels after plant vitrification solution 2 treatment. Our investigation of cell viability in 48h-germinated embryos also suggested significant negative correlations with the rate of superoxide anion generation, malonadehyde content, and electrolyte leakage. Spatial and temporal-specific patterns of ROS accumulation in 48h-germinated embryos were also revealed. Among the treatments of cryopreservation procedures using vitrification method, the preculture treatment was found to stimulate superoxide anion production and to activate the response system. Loading treatments motivated catalase and ascorbate peroxidase activity in 48h-germinated rice embryos. During the plant vitrification solution 2 dehydration treatment, the oxidative stress was the highest, and the antioxidative system positively responded. Specifically, a set of antioxidant enzymes promoted detoxification of reactive oxygen species.

Based on a comprehensive correlation analysis involving ROS accumulation, cell viability, the activities of antioxidant enzymes and their gene expression, the gene expression of *Cu/Zn SOD*, *CAT1*, *APX7*, *GR2*, *GR3*, *MDHAR1*, and *DHAR1* were suggested as the potential and critical indicators which were affected by vitrification treatments. The investigation of these responses in 48h-germinated rice embryos can provide useful information regarding plant cryopreservation using vitrification method.

Source of Funding: Not applicable
Conflict of Interest: None to declare

S120 AUTUMN PHENOLOGY INTEGRATED TO FROST HARDNESS MODELLING OF FRUITS TREES.

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Frost is one of the first causes in the fruits crops yield loss, mostly in autumn and spring. To protect themselves against low temperatures, trees adopt different strategies. Studying trees' responses to frost events in autumn and spring is important to understand those strategies.
To detect trees responses to frost events, we put sensors on four varieties of walnut fruit trees, one species of wood walnut tree and one variety of apple tree. Data acquired with PépiPIAF sensors show us micro-variations of trees' trunks or stems and highlight reactions to freezing events and the decreasing of foliar transpiration until the end of leaf fall. Those data were linked to field observations of leaf senescence and records of temperature variations.
Leaf fall is an autumnal phenological event marking the beginning of dormancy and often occurs during firsts freezing events. In spring, budburst occurs when high temperatures comeback and makes trees vulnerable to late frosts. Therefore, mechanistic models were developed to predict the end of dormancy with budburst in spring but were calibrated mostly on one variety of walnut *Juglans regia* cv Franquette. Data obtained with PépiPIAF and field observations of leaf senescence were linked to dormancy assessment. Thus, the goal was to identify trees reactions to autumnal frosts as phenological markers of dormancy entry and to take it into account to improve models predicting budburst.
Firsts results show differences in trees reactions, leaf fall and dormancy induction between the studied species. Differences and similarities between experimental data and models' predictions may indicate mechanism involved in frost hardness of trees.

Source of Funding: Not applicable
Conflict of Interest: None to declare

S121 ISBER BEST PRACTICES: RECOMMENDATIONS FOR REPOSITORIES, 4TH EDITION

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The International Society for Biological and Environmental Repositories (ISBER) is a global society dedicated to the operation and science behind repositories of biological and environmental specimens. One of the society's key objectives is to develop harmonized principles in the science and management of repositories and it has produced Best Practices guidelines since 2005. The latest edition, the 4th edition, was published in January 2018 and the expanded content reflects the growing diversity of biorepositories represented within ISBER and the advances in biospecimen science, new technologies and requirements for business planning.

This presentation will give an overview of the updates and the areas with expansion and will place the Best Practices in context.

Source of Funding: ISBER
Conflict of Interest: None to disclose

S122 THE UKCRC TISSUE DIRECTORY: ENGAGEMENT FOR A PLATFORM OF UK HUMAN SAMPLE DISCOVERY

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Introduction: The UKCRC Tissue Directory and Coordination Centre coordinates Biobanking in the UK with the aim of improving the visibility and accessibility of human samples. One key way of doing this is via the UKCRC Tissue Directory (UKCRC TD), through which researchers can locate existing sample resources (Biobanks, sample holdings, clinical trials). To continually develop this initiative, we sought to capture the requirements of researchers who use human samples.
Methods: The UKCRC TD is being continually developed under an agile project management process. An ethically-approved survey was circulated to UK based active biomedical researchers from academia or industry of post PhD level. The aim of the survey was to capture research motivators and barriers to human sample access.
Results: The UKCRC TD was launched in 2016, and currently has over 170 Sample resources registered (March 2018). Metrics show around 20 UKCRC TD searches per day, indicating the growing importance of the platform for potential end-users.
Survey results showed that sample users utilised a range of different sample resources, with self-collection being the most common method. Locality and available clinical

data were the most cited reasons for current source choice with quality of sample listed as the sixth reason. 64% of respondents said they would consider using a different sample source in the future, with sample range being the most popular motivator cited, interestingly sample quality was listed as the second most popular reason to change sample resource.
Conclusions: These results have important implications for the development of the UKCRC Tissue directory, by showing that researchers are willing to use new resources that are not local to them, indicating the potential need for a discovery platform. The motivations for sample resource choice will be further dissected in a series of follow up focus groups.

Source of Funding: UKCRC
Conflict of Interest: None to disclose

S123 BIOBANKING FOR CANCER RESEARCH – CHALLENGES AND REFLECTIONS

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The Wales Cancer Bank (WCB) has been collecting tissue and blood samples from cancer patients in Wales for thirteen years. The samples are collected, processed and stored to common Standard Operating Procedures and made available to researchers worldwide for cancer related research. Tissue samples are stored both frozen (-80) and in paraffin wax with all blood and blood products stored at -80 in mechanical freezers.
Frozen tissue samples remain the 'gold standard' for many downstream applications but over the last ten years, only 13% of all samples issued from WCB have been frozen tissue or derived from frozen tissue (DNA or RNA). The move towards stratified or personalised medicine has required the technologies and methodologies to function well with the paraffin block, the staple of diagnostic laboratories around the world and many comparisons have been drawn to elucidate the utility of each preservation method for the varying analytes, markers and molecular signatures in biosamples.
The rising promise of the liquid biopsy has ignited a renewed interest in projects requiring (double-spun) plasma to investigate the reliability and concordance of identifying mutations in circulating 'stuff' in the blood compared with mutations in the tissue sample. Sampling methods have already been developed in Wales and translated into a clinical test to detect the presence of mutations in lung cancer.
This presentation will reflect on the changing landscape of biobanking for cancer research and the requirements of biobanking to be lean and flexible and readily adaptable to remain useful and current for the research community.

Source of Funding: Not applicable.
Conflict of Interest: None to disclose.

S124 COMPARISON OF THREE MULTI-CRYOPROTECTANT LOADING PROTOCOLS FOR VITRIFICATION OF ARTICULAR CARTILAGE

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Vitrification of articular cartilage is an ideal method to preserve cartilage tissue for long term storage. Cryoprotectant (CPA) loading is an essential step for achieving vitrification. In 2012, our group published a successful vitrification protocol for intact human articular cartilage and reported promising cell recovery after using a four-step multi-CPA loading method that required 570 min. In this study, in addition to our published protocol, we used two shorter multi-CPA loading protocols to investigate their impacts on chondrocyte cell recovery after vitrification. Three multi-step CPA loading protocols with different loading times at each step were used (our previous four-step protocol A: 570 min, and two shorter three-step protocols, protocol B: 420 min calculated by mathematical modelling and protocol C: 310 min) including four different CPAs (glycerol, dimethyl sulfoxide, ethylene glycol and propylene glycol). Two CPA loading volumes (15 ml and 50 ml) were used for CPA loading. Healthy femoral condyles from sexually mature pigs were cored into two sizes of 6 mm and 10 mm diameter cartilage dowels with approximately 10 mm bone base, then randomly divided into the twelve experimental groups based on CPA protocol ($n = 3$), dowel size ($n = 2$) and solution volume ($n = 2$) ($N = 4$ samples per group). After vitrification and tissue warming, chondrocyte cell viability was quantified with live-dead fluorescent microscopy and cell mitochondrial function was assessed using AlamarBlue. Chondrocyte cell viability and mitochondrial activity were correlated with CPA loading times, cartilage dowel sizes and CPA loading volume sizes. Protocol B showed similar chondrocyte cell recovery to our established protocol A and it was 150 min shorter. Both protocols A and B showed significantly higher cell recovery than protocol C ($p < 0.05$). This evidence shows the potential of mathematical modelling in shortening the time needed to successfully vitrify articular cartilage.

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Conflict of Interest: Some of the authors hold a patent in this area.

S125 AN AGENT BASED MODEL OF CELL LEVEL TOXICITY ACCUMULATION AND INTERCELLULAR MECHANICS DURING CPA EQUILIBRATION IN OVARIAN FOLLICLES

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The cryopreservation of reproductive tissue allows for storage and transplantation to restore or prolong fertility. Our approach to optimize the process of cryopreservation to increase transplant tissue viability and reduce toxicity is through mathematical modeling. Most current models neglect cell-level damage while modeling mass and heat transport and none incorporate the effects of intercellular mechanics. We hypothesize that these mechanics are critical to understand cryopreservation induced follicular damage as the literature suggests that a significant source of injury is found in damage to the transzonal projections connecting granulosa cells to the developing oocyte. To address existing model deficiencies, here we apply, inform, and test agent-based cell models of cryoprotectant equilibration in secondary preantral follicles. Our focus is on the equilibration of cryoprotective agents (CPAs), a critical part of all successful cryopreservation protocols. In this study, we first derive a model and develop computer code describing CPA equilibration in a 2D tissue model of a secondary stage ovarian follicle. This model accounts for intercellular connections using a modified spring force potential equation, mass transport using a combination of a prebuilt diffusion solver and two-parameter volume equation, concentration based CPA toxicity using a cost function and additional cell mechanics such as motility and cell-membrane rigidity. To do this, we use a highly customizable cell mechanics and interstitial diffusion simulator based on an agent-based model of cells in nonhuman primate ovarian follicles. We inform this model with literature values and explore the defining features of this model numerically. Our simulations show a marked difference in volume change between the granulosa layer and oocyte that may suggest mechanistic explanations for cryopreservation induced damage.

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Conflict of Interest: None to declare

126 TOWARDS NEW PROTOCOLS OF OVARIAN TISSUE CRYOPRESERVATION ASSISSTED BY X-RAY COMPUTED TOMOGRAPHY

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Ovarian tissue cryopreservation is in most cases the only option for female cancer patients to preserve their fertility, especially in the case of prepubertal girls. So far, the cryopreservation of ovarian tissue has been performed mostly by the conventional *slow freezing* method. Even though there are more than 100 live births from transplanted frozen-thawed ovarian tissue, there is evidence of follicle population damage associated with this cryopreservation procedure. The different characteristics between cells suspensions (traditionally cryopreserved by *slow freezing*) and tissues might be the main issue of this follicle loss: tissues are composed of different cells which are densely packed and besides, they have a bigger volume. The purpose of this work was to develop an alternative protocol of ovarian tissue cryopreservation by a *stepped vitrification*, consisting of an increasing in cryoprotectant concentration while decreasing the temperature. This approach aims to reduce cryoprotectant toxicity and avoid permeability problems. CT measurements were performed after the cooling process (below -140 ºC) in order to evaluate the cryoprotectant permeation in the tissues, and after tissues rewarming, to evaluate the cryoprotectant washing process. For these experiments, we used bovine ovarian tissue from one-year old animals. Histological analysis were also performed to evaluate the morphological state of the tissues. Bovine ovarian tissues cryopreserved by *slow freezing* were also compared with ovarian tissues vitrified with the presented method. CT images showed that the equilibration of tissues with the cryoprotectant solution was not complete in most samples of the new vitrification protocol developed, although an equilibration up to 80% of the desired concentration was achievable. Nevertheless, histology results revealed no significant differences between tissues from both protocols. Anyways, X-ray CT technology is proved to provide a very relevant information to adjust the parameters necessary for achieving a successful vitrification method.

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Conflict of Interest: None to disclose.

S127 BIOMECHANICAL MODEL OF CRYOPROTECTANT TRANSPORT IN TISSUES WITH HIGH CELL DENSITY

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Cytotoxicity from exposure to high cryoprotectant (CPA) concentrations is one of the major barriers to the development of effective tissue cryopreservation

procedures. In previous work, our group developed a mathematical approach to minimize CPA toxicity, and applied it to isolated cells and cultured cell monolayers. Our recent efforts have focused on extending this toxicity minimization approach to larger biological specimens. In particular, we have focused on developing a model of CPA transport in tissues that accounts key biophysical phenomena, including mechanical strain and fixed charges in the extracellular matrix. While most previous modeling efforts are based on Fick's law and do not account for tissue size changes or fixed charges, recently a model of transport in articular cartilage was published that accounts for both of these phenomena. However, the published model neglects the effects of cells on interstitial transport. While this is a reasonable assumption for cartilage, most other tissues have much higher cell density. Thus, we have extended this model to account for the inherent coupling between interstitial transport and cell membrane transport. Model predictions reveal two key effects that are expected to affect design of tissue cryopreservation procedures. First, the presence of cells slows down delivery of CPA into the tissue interior. This is because cells act as a sink for CPA. Second, accounting for the presence of cells within the tissue reduces the magnitude of predicted cell volume changes. Physically this can be explained in terms of the interplay between cell size and fixed charge concentration. For instance, cell swelling concentrates fixed charges, which opposes further swelling. The model we present here can be used for tissues with a broad range of cell densities and will serve as the basis for future efforts to mathematically minimize CPA toxicity.

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S128 SIMULATIONS OF OSMOTIC EVENTS IN VITRIFICATION OF EQUINE OOCYTES AND PORCINE EMBRYOS

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High concentrations of cryoprotective agents (CPAs) used for vitrification of equine oocytes and porcine embryos are believed to negatively affect developmental competence. Here, osmotic events were simulated using 2P formalism (Kleinhans, Cryobiology 37, 271–289) to predict effects of reduced duration of CPA exposure and effects of use of non-permeating CPAs. Varying concentrations of permeating CPAs (ethane-1,2-diol and Me_2SO) were used in the first step (equilibration solution, ES) and second step (vitrification solution, VS). In VS, varying concentrations of additional non-permeating solute (sucrose) were used. The duration of the first step (ES) was varied between 0.5-10 min, while exposure to VS was 0.5-1 min. Values for Lp and Ps were based on published values for oocytes and embryos respectively. Typical 'volume excursions' are seen, in which oocytes and embryos rapidly (<0.5 min) shrink in ES due to efflux of water, then start re-swelling due to combined influx of CPA and water. After transfer to VS, the oocytes/embryos shrink a second time, concentrating intracellular solutes including CPA taken up in step 1. In line with that, the simulations show that the allegedly advantageous reduction of the intracellular concentration of permeating CPAs by non-permeating sucrose is only very small. Further, although with a short (0.5 min) step 1 (ES), much less permeating CPA is taken up, yet osmolality equal to that of VS (≈ 'vitrifiability') is reached in the intracellular- and blastocoel space within seconds after transfer to VS, regardless of step 1 duration. But with a short step 1, the cells will be vitrified while being severely shrunken. These simulations (and indeed experimental evidence we obtained in equine oocytes) suggest there may be an optimum balance of the risk of damage due to long exposure to VS and the (assumed) risk of vitrification of embryos in severely shrunken, condensed condition.

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Conflict of Interest: None to disclose.

S129 CRYOTHERAPY TEMPERATURE EFFECTS ON FUNCTIONAL AND ONCOLOGICAL OUTCOMES IN PROSTATE CANCER

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Cryotherapy, using a target temperature of -40°C, is an effective definitive treatment for organ-confined prostate cancer. We sought to determine whether a moderate minimum tumor temperature (below -40°C but above -76°C) is associated with improved quality of life, and/or an increased risk of disease recurrence relative to a very cold temperature (below -76°C).
An IRB-approved database was reviewed for patients who underwent primary cryotherapy for organ-confined prostate cancer from 2004 to 2017. Cohort characteristics were compared using descriptive statistical analysis. EPIC and IIEF quality of life questionnaire responses in the 4 years following treatment; and biochemical recurrence, post-treatment positive biopsy, progression to salvage treatment, metastasis, and overall survival truncated at 6 or 8 years post-treatment (median follow-up 30 [IQR: 33] months) were analyzed and compared using ANOVA, t-tests, Kaplan Meier and log-rank analyses. Patient cohorts were stratified based on whether their minimum tumor temperatures were colder (″very cold″) or less cold (″moderate-cold″) than -76°C, the median minimum tumor temperature for our cryotherapy patients as determined via chart review.

144 patients had moderate-cold minimum tumor temperatures, and 134 had very cold minimum tumor temperatures during cryotherapy procedures. EPIC questionnaire data were available for 52 patients in the very cold group and 64 patients in the moderate-cold group in the 4 years following treatment. The groups with available questionnaires did not differ in age (p=0.66), preoperative PSA (p=0.08), or preoperative Gleason scores (p=0.13). The groups did not differ in patient-reported urinary function (p=0.77) or bowel habits (p=0.15). Moderate-cold minimum tumor temperature was associated with superior (post-operative year 2, p=0.03) and more rapid improvement in sexual function scores relative to the very cold cohort. Moderate-cold versus very cold minimum tumor temperature showed no difference in biochemical recurrence (p=0.60) post-treatment positive biopsy (p=0.95), progression to salvage treatment (p=0.40), metastasis (p=0.47), or overall survival (p=0.06).

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S130 CRYOABLATION IN THE TREATMENT OF LUNG CANCER

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Lung cancer is the most commonly diagnosed cancer in the United States and Europe and it is a major cause of cancer death. Surgical resection, when possible, offers the best chance of healing of NSCLC in selected patients and in early stage. In patients not candidates for surgery, chemotherapy and radiotherapy are mainly palliative. Cryoablation is a minimally invasive technique, highly innovative, which has only recently been used in the treatment of primary and secondary lung tumors. Cell death is obtained as a result of rapid freezing followed by slow thawing that causes necrosis of the target tissue. Cryoablation can be proposed with radical intent (curative) in cases of disease limited to the lung; individual tumors no larger than 5 cm or up to 5 multiple tumors confined to no larger than 3 cm each one. The advantages of cryoablation are due to very precise control of the treated area (display of the iceball) sparing the surrounding healthy tissues. The major risks and complications of pulmonary cryoablation are those deriving from interventional treatment such as: local hematomas, pneumothorax, pulmonary bleeding caused by wrong placement of the cryoprobes and infections.

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S131 AN EXPERIMENTAL AND NUMERICAL APPROACH FOR NODULAR SKIN TUMOUR ABLATION USING CRYOTHERAPY

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Liquid nitrogen spray cooling has been performed to quantify the necrotic zone in the nodular gel phantom. The tissue-mimicking gel is assigned with two different configurations of nodular gel phantom, i.e., first: 4.5 mm depth and 5.5 mm radius; second: 4 mm depth and radius. The spray cooling is carried out using 0.8 mm nozzle diameter, 27 mm spraying distance and 120 s freezing for the experimental study. The multi-block non-orthogonal grid is used for the mathematical model and enthalpy equation is solved with finite volume approach. The variation of temperature and ice front in the nodular gel phantom are evaluated experimentally for both the nodular configurations and corroborated with the numerical study. The lethal temperature (-50°C), measured with the thermocouple is obtained up to 4.5 mm and 4 mm depth in the first and second configuration of nodular gel respectively. The final ice front measured using Image J software in the axial and radial direction for the first configuration is 9.1 mm and 10. 1 mm respectively while for second configuration it is 9.7 mm and 10.4 mm respectively. The ablation volume characterised by -50°C and -25°C is quantified numerically for the application of a malignant and benign tumour respectively. The final ablation volume enclosed by -50°C is 67% and 76% lesser than final ice volume obtained by first and second configuration respectively while for -25°C it is 51% and 61% respectively. The cryogen spray cooling with 0.8 mm nozzle diameter with spraying distance of 27 mm can be suitable for benign skin tumour with both the configurations unlike the second configuration for malignant skin tumour.

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S132 OPEN VS CLOSED ENCAPSULATION-VITRIFICATION OF LEYDIG CELLS

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Vitrification is an economical and rapid method of cryopreserving living cells and tissues for long-term storage and low-cost international transport. However, studies on vitrification of somatic cells are scarce. This study reports open verses close encapsulation-vitrification of somatic cells using sodium alginate micro-beads as pseudo tissue model. Results showed that, 84.0±0.3% viable cells could be recovered after encapsulation into microbeads. The post-warming viability of vitrified cells was found to be 60.0±2.4%, which significantly increased to 82.0±0.9% when PVA30

in the vitrification solution (VS) was replaced with Ficoll70. Size of beads played a vital role in cell viability. Small sized beads (dia: 1.7±0.1mm) resulted in higher post-warming viability (82.0±0.9%) than medium (dia: 2.4±0.1mm; viability: 78.0±0.5%) and large (dia: 4.2±0.1mm; viability: 74±0.4%) sized beads. Metabolic activity of cells after vitrification by open or closed vitrification was observed at day 0, 2, 4 and 7 days interval. It was also observed that vitrification increased the ROS generation in vitrified-warmed cells, which could be annihilated by supplementation of vitrification and warming solutions with Glutathione (GSH) or 2-Mercaptoethanol (2-ME) and resulted in increased post-warming viability in a dose-dependent manner. The best results were obtained at concentrations of 50 mM for 2-ME (85± 2.7%) and 100 mM for GSH (89± 1.6%) in comparison to controls (82± 1.2%) vitrified by open system vitrification ($p< 0.05$). The positive effect of antioxidants on post-warming viability was also observed with closed system vitrification.

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Conflict of Interest: None to disclose.

S133 AN ALLOGENEIC BIOSCAFFOLD FOR THE STORAGE OF HUMAN MESENCHYMAL STEM CELLS.

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MSCs derived from synovial fluid can provide high chondrogenic and cartilage regeneration, emerging as a new alternative stragegy to treat knee ostoearthitis. We have developed an allogeneic and biomimetic bioscaffold composed of Platelet Rich Plasma and synovial fluid that preserve and mimics the natural environment of MSCs isolated from knee. However, cryopreservation of knee-isolated MSCs embedded within the aforementioned biomimetic scaffold to create a reserve of young autologous embedded knee MSCs for future clinical applications remained unsolved. Thus, we tested several cryoprotectant solutions combining dimethyl sulfoxide (Me_2SO) and sucrose, quantifying MSCs viability and functionality after thawing. MSCs embedded in bioscaffolds cryopreserved with Me_2SO 10% or the combination of Me_2SO 10% and Sucrose 0,2 M displayed the best cell viabilities and functionality after thawing, allowing their future clinical use in patients with cartilage defects.

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Conflict of Interest: None to disclose

S134 TOWARDS LARGE-SCALE CRYOPRESERVATION: STERILE VITRIFICATION OF ADHERENT HUMAN INDUCED PLURIPOTENT STEM CELLS AND THEIR NEURAL DERIVATES

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Cryopreservation is still the only possibility to store viable cells for long periods. In general, conventional slow freezing methods are efficient enough when it comes to banking of single cells in suspension for subsequent expansion (e.g. human induced pluripotent stem cells, hiPSCs). However, considering adherent cells or multicellular systems that are increasingly relevant for biomedical research and application, slow freezing shows major limitations. (1) Usually the adherent cells have to be enzymatically or mechanically dissociated to single cells or small aggregates prior to freezing, (2) crystallization-induced damaging mechanisms additionally disrupt cadherin- and integrin-mediated cellular contacts, and especially for hiPSC, (3) the recovered viable cell numbers is dramatically reduced compared to the control. Vitrification provides the possibility to overcome these limitations, but requires skilled handling especially regarding sterile procedures, imply small sample sizes and therefore is considered as unsuitable for bulk storage. To launch vitrification for large cell numbers and thus enabling ready-to-use cryopreserved adherent cell systems, we introduce a sophisticated multi-usage cell culture disposable covering comprehensive cell-based workflows from cultivation/differentiation to sterile vitrification. We validated this disposable by a comparative multi-centre study, examining adherent vitrification in the disposable and of suspension-based conventional slow freezing of six hiPSC lines and the accordant hiPSC-derived neural progenitor cells (NPCs). Viability, cell number, immuno staining and FACS as well as gene expression and raster electron microscopic analysis were performed as post thaw quality controls after one day and four days, respectively. Our data shows superior performance of vitrification over slow freezing of both cell systems. Higher numbers of viable cells and metabolic activity could be detected, while the functionality maintained. Together with the option to parallelize the disposable in multi-well formats, vitrification is applicable for large-scale cryopreservation of adherent multi-cellular systems and enables ready-to-use formats for a variety of biomedical purposes.

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S135 ADVANCES IN CRYOPRESERVATION OF ALGINATE-ENCAPSULATED STEM CELLS AND ANALYSIS OF CRYOPRESERVATION OUTCOME

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Cryopreservation of clinically relevant cells and biologics encapsulated in alginate hydrogels is an efficient methodology to preserve their functionality for further application in regenerative medicine and transplantation. The main advantages of alginate encapsulation for cryopreservation include possibility for process scaling up, encapsulation of required cell numbers within viscous environment, which in turn serves as a reservoir for cryoprotective agents, protects cells from mechanical and osmotic stress as well as provides controlled membrane permeability. In this work, we report on our success in cryopreservation of multipotent stromal cells within different alginate hydrogel formulations (solid and coaxial beads of different sizes), analysis of cell viability using the designed µVision software allowing for spatial reconstruction of cryomicroscopic images as well as ice recrystallization using µCrystal software. The developed approach for cell encapsulation in coaxial alginate beads with varied membrane thickness provides effective formation of tissue-like 3D structures within the core. This approach could serve as a model for cryopreservation of self-assembled structures with cell-cell contacts. For solid alginate beads, optimization of cryopreservation parameters yielded intact alginate hydrogels after thawing according to cryomicroscopic data. High cell viability and recultivation efficiency 24h after thawing (viability 83% by Calcein-AM/EthD-1 staining and 70% by recultivation efficiency) have been achieved. In addition, optimal cryopreservation protocol has been successfully validated for freezing of cell structures within coaxial alginate beads, whereas µCrystal software proved to be efficient for analysis of cryomicroscopic images of ice recrystallization. Taken together, our work provides a comprehensive overview of the main results achieved by the group on cryopreservation of stem cells within alginate solid (microbeads) and coaxial hydrogels.

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Conflict of Interest: None to disclose.

S136 ME2SO- AND SERUM-FREE CRYOPRESERVATION OF MESENCHYMAL STROMAL CELLS USING ELECTROPORATION OF SUGARS

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Cryopreservation is the universal technology enabling continuous availability of cell aliquots to meet regenerative medicine demands. However, safety concerns over Me2SO-induced side effects and immunogenicity of animal serum (main components of standard freezing media), support their replacement with non-toxic substances. Due to multiple cryoprotective properties, selected disaccharides, such as sucrose and trehalose, are widely used as additives to various freezing solutions. Conceptually, combined introduction of sugars into cryopreservation media and their pre-freeze loading into cells serves as a novel alternative to conventional cryopreservation workflow. Among diverse techniques for sugar loading (e.g. fluid-phase endocytosis, genetically engineered proteins or nanoparticle-mediated delivery) electroporation is a preferred method in cryopreservation owing to its high-performance speed, safety and accuracy. In this study, we investigated the effect of electroporation-assisted delivery of sucrose, trehalose and raffinose into human umbilical cord-derived MSCs on their post-thaw survival. The optimal strength of electrical field was determined from permeabilization (propidium iodide uptake) *vs.* cell viability data (AlamarBlue). After sugar loading (0-400mM), cells were allowed to reseal for 10 min and frozen using 1K/min in CoolCell® freezing container followed by storage for 24 h in liquid nitrogen. Cell survival was evaluated using trypan blue assay and FACS. Using sugars as sole cryoprotectants, concentration-dependent increase in cell survival was observed. Irrespective of sugar type, the highest cell survival (up to 80%) was achieved at 400 mM concentration. Cell freezing without electroporation yielded significantly lower survival rates. In the optimal scenario, cells were able to attach 24h after thawing demonstrating characteristic shape and sugar-loaded vacuoles. Application of 10% Me2SO/90%FBS provided cell survival exceeding 90%. None of the sugars demonstrated ice recrystallization inhibition analyzed by cryomicroscopy. However, some differences in osmolality, electroconductivity and glass transition temperatures were detected. In summary, electroporation of sugars is an effective strategy towards Me2SO- and serum-free cryopreservation of stem cells.

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S137 SURFACE ACOUSTIC WAVES BASED MULTISTEP ON-CHIP DEVICE FOR LOADING AND UNLOADING OF CRYOPROTECTIVE AGENTS ON HUCM-MSCS CELLS IN CRYOPRESERVATION APPLICATION FOR SMALL BIO SAMPLES

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Cryopreservation is a widely used technique for preservation of biomaterials in the frozen state with cryoprotective agents (CPAs), and has found many applications in the field of regenerative medicine, tissue engineering, organ transportation, assisted reproduction. CPA loading and unloading is one of the most crucial step during the whole process of cryopreservation. Conventional Multistep loading and unloading is one of the most common and popular used approach. However conventional multistep process is time consuming, often include osmotic shock , mechanical and centrifugal injuries therefore reducing cell viability. So there is a need to provide alternative Multistep loading and unloading method which optimize the CPA loading and unloading process. To this end this is the first report in which we have developed an alternative SAW (Surface Acoustic Waves) Multistep loading and unloading which is fast enough and offer less osmotic shock and help in reducing the mechanical and centrifugal injuries therefore increasing cell viability. The results depicts that because of converting the surface from hydrophilic to hydrophobic surface the power was uniformly acting on the droplet which also help to preserve cell survival. Further analysis indicate that the power and streaming timing in which the device has been operated is not harmful to cells at all. Cell viability indicate that our device help to keep more cells viable as compared to traditional loading/unloading method. Furthermore proliferation results provide the evidence that there is no morphological changes between the SAW based treated cells and fresh cells. We believe that our device is capable of replacing the conventional multistep CPA loading and unloading method for small biosamples, which is fast, easy to use ,offer less osmotic shock due to increased number of steps and also reducing mechanical and centrifugal injuries therefore paving a way for successful cell preservation for use in diverse fields.

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Conflict of Interest: None to declare

S138 NOVEL STRATEGIES FOR STORAGE AND RECOVERY OF CADAVERIC BONE MARROW STEM CELLS

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It was recognized 60 years ago that deceased donor bone marrow (BM) could be a useful source of viable CD34+ hematopoietic stem cells (HSCs) for transplantation. Procedures have since been published for recovery and safe use in immune tolerance clinical trials.

While processing deceased donors to cell suspensions under GMP standards is effective, each process represents substantial work. A strategy that could allow efficient generation of a large bank as well as reduce waste resulting from processing donors that do not meet safety or "immediate utility" criteria would be to cryopreserve the bones with the cancellous marrow matrix intact, and subsequently finish processing post thaw upon need or clearance of test results.

For this study, male and female donors ranging in age from 26-56 years (mean 41.25yrs; n=12) were evaluated. VBs were recovered form organ and tissue donors appropriately consented for research tissue recovery. Upon receipt in the lab, whole VBs were incubated for 24 hr at 4 ^{o}C in a 0.9% saline-based cryoprotectant (CPA) medium with 20% Me_2SO/10% Stemulate human platelet lysate (Cook Regentec). VBs were removed from the CPA and transferred wet into sterile Whirl-Pak bags and placed at -80 ^{o}C inside a polystyrene foam cooler for slow cooling. After 16 hours, the VBs were transferred to LN_2 and stored for 2 weeks and then thawed rapidly at 37 ^{o}C before processing and analysis. BM was obtained from fresh VBs from the same donors for comparison.

Post thawed, processed cells yielded CD45+ viability of 81.79% ± 8.18% of unfrozen controls while CD34+ cells had 90.03% ± 6.27% of controls. The colony forming assay resulted in 71.02% of unfrozen controls based on total colonies.

This is the first report we know of where marrow has been cryopreserved *in situ* within intact bones with subsequent recovery of viable HSCs.

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Conflict of Interest: All authors are employees of Ossium Health, Inc.

S139 SUPPLEMENTING SMALL MOLECULES PROTECTS ISLETS AGAINST CRYO-INJURY AND ENHANCES RECOVERY UPON CRYOPRESERVATION

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The auto-immune destruction of pancreatic β cells leads to occurrence of Type 1 diabetes. Two main therapeutic approaches for the treatment of T1D are islet transplantation and exogenous insulin injection. However both the approaches have not attained significant success. One of the main reasons for the decline in the success of islet transplantation is the shortage of donor pancreas and their poor survival upon transplantation. Hence in the present study we aim to generate ample number of islets

from wharton's jelly mesenchymal stem cells and store them at ultra-low temperature (-196˚C) for islet banking. Cells from Wharton's jelly were characterized as per the ISCT guidelines for their MSC characteristics. Further they were differentiated into insulin like cell aggregates (ICAs) and characterized for pancreatic markers. The ICAs were then cryopreserved in liquid nitrogen in the presence of MSCs, a cocktail of small molecules (EPA+DHA+Metformin) in addition to 5% ME2SO. The ICAs were revived after 30days, cultured for 24h and assessed for their viability, oxidative status and functionality. Viability was determined by MTT and FDA/PI assay and was found that the combinations of small molecules maintained the viability by 4fold when compared to control and ICAs cryopreserved with MSCs. Further the small molecules significantly reduced total ROS ($p<0.05$) (55% reduction), superoxide ions ($p<0.05$) (65% reduction) and nitric oxide ($p<0.01$) (70%) free radicals with enhanced HIF1α expression. The expression of stress markers like CHOP, NOS2a and IL1β, apoptotic markers like p53 and caspase 3 were significantly down-regulated. Western blot analysis confirmed down-regulation of caspase 3 and phospho p38 with up-regulation of anti-apoptotic marker, BCl2. ICAs responded to glucose and showed significant enhancement in insulin secretion in presence of high glucose. Hence our data demonstrates that small molecules and not MSCs protected ICAs when cryopreserved and maintained their functional status for a month.

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S140 ATTEMPTS TO MODULATE APOPTOSIS IN CRYOPRESERVED MOLLUSCAN CELLS

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This work continues our previous studies of cell death pathways in response to a cold stress in marine invertebrate cells and focuses on the functional alterations that occur in larval molluscan cells both in standard culture conditions and in response to cold injury. We used cultivated molluscan and mammalian cells. A relatively high proportion of cryopreserved larval molluscan cells was previously found to be in the early apoptotic stage (up to 24%) after a freeze-thaw cycle. The goal of this study is two-pronged. First, to find apoptotic inducers, commonly used for the chemical induction of apoptosis in mammalian cells, that operate in non-mammalian systems. Second, to reduce apoptosis of molluscan cells after a freeze-thaw cycle. Flow cytometry was used to establish the effects of tested substances on the functional activity, general caspase activation, and loss of plasma membrane integrity. Three apoptotic inducers, staurosporine, camptothecin, and mitomycin C, were tested on molluscan cells. We found that only staurosporine resulted in evident apoptosis. Camptothecin did not increase apoptosis in molluscan cells but did slightly improve their viability after a freeze-thaw cycle. Mitomycin C showed similar results. We suggest that some apoptotic inducers have hereto unknown effects on molluscan cells. Additionally, we tested three apoptotic inhibitors (Y-27632, cyclic pifithrin-α, and CHIR99021), known to mammalian cells. We hypothesized that the use of apoptotic inhibitors could reduce apoptosis after cryopreservation in molluscan cells, however, our attempts were failed. Development of this direction is important for determining cold susceptibility of marine organisms.

Source of Funding: This study was supported in part by the Russian Science Foundation (grant no. 14-50-00034).
Conflict of Interest: None to disclose

S141 CRYOPRESERVATON OF *MYTILUS GALLOPROVINCIALIS* (LAMARK, 1819): TESTING TOXICITY OF CRYOPROTECTING AGENTS ALONG EARLY DEVELOPMENT

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Mussel aquaculture production has increased worldwide during last years. Barriers associated to marine invertebrate breeding diminish economic benefits, so experimental lines try to develop a method to establish a sustainable broodstock reducing economic costs. Cryopreservation techniques are being studied due to potential application in aquaculture industry, providing long-term storage of biological material. When developing a cryopreservation protocol, it is crucial to locate the suitable cryoprotectant agent (CPA) and its optimal concentration to protect cells from freezing damage meanwhile minimizing toxic effects and mortality due to exposure.
These experiments were carried out to test the toxicity of ethylene glycol (EG), propylene glycol (PG), dimethyl-sulfoxide (Me2SO) and glycerol to: fertilized egg, trochophore larvae (18-20h) and D-larvae (48 and 72h) of blue mussel.
Percentage of abnormal D-larvae was calculated as an indicator of toxicity of individual CPAs and to calculate NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) levels.
Results evidenced that ethylene glycol (EG) and propylene glycol (PG) were the least toxic at fertilized egg stage and trochophore larval stage. Toxicity tests of D-larvae did not show expected increase in the percentage of abnormality with increasing concentration of any of the CPAs tested and results were inconclusive. Toxicity tolerance was higher in later developmental stages, which is in agreement with other tests in molluscs. Attending to present results, we recommend PG or EG with concentrations lower than 1M for fertilized egg and equal or less than 3M of EG or PG for

trochophore larvae cryopreservation. Further research must be carried out with D-larvae to understand its response. Preliminary cryopreservation was carried out with trochophores and D-larvae using Ethylene glycol in order to gather information about cryoprotection.

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Conflict of Interest: None to declare

S142 CRYOPRESERVATION OF COMMON CARP (*CYPRINUS CARPIO* L.) SPERMATOGONIAL STEM CELLS

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The aim of the present study was to develop optimal protocol for cryopreservation of common carp spermatogonia and test the possibility of applying interspecific germ cell transplantation for surrogate offspring production. Protocol for slow-rate freezing of carp spermatogonia was optimized by testing the effects of various cryoprotectants and their concentrations, cooling rates, tissue sizes and sugar supplementation in four sequential experiments. Firstly, among the six tested cryoprotectants, dimethyl sulfoxide (Me_2SO) yielded the highest spermatogonia survival rates. Among the five different Me_2SO concentrations (1 - 3 M) and six different cooling rates (1 – 10 °C/min), 2 and 2.5 M of Me_2SO combined with -1 °C/min resulted in the highest cell viability. Sugar supplementation did not have a significant effect on the cryosurvival, while freezing 100 mg of testicular tissue equilibrated for 30 min resulted in the highest viability. The highest cell viability after the first trial was approximately 10%, while subsequent optimization improved the viability up to 40.7%. To test the physiological activity of the frozen/thawed cells, we conducted inter-specific germ cell transplantation of carp spermatogonia into goldfish recipients. Goldfish recipients were sterilized with anti-*dnd* moprohlino microinjection and frozen/thawed and fresh spermatogonia were transplanted into recipient larvae. Two months after transplantation, recipients were dissected and checked for cell proliferation and gonadal development. Approximately 50% of the recipients displayed visibly large gonads which contained germ cells at various stages of development, while none of the sterilized controls displayed such gonads which indicated the donor cells incorporated into the recipient gonads and started to proliferate. We confirmed this observation by detecting PCR amplification of common carp-specific amplicon within the recipient goldfish gonads. Results of this study can serve as an alternative way for long-term preservation of germ plasm in carp which can be recovered through surrogate production technology.

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Conflict of Interest: None to disclose.

S143 EUROPEAN EEL SPERM CRYOPRESERVATION: AN OVERVIEW

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The European eel (*Anguilla anguilla*) is an iconic species with a great commercial value which due to different impacts, has been included in the IUCN red list as critically endangered. Therefore, the development of conservation techniques such as sperm cryopreservation, are necessary to reverse this situation. The development of European eel sperm cryopreservation protocols started in 2003 mirroring the protocol used for cryopreservation of Japanese eel that was developed a few years earlier. Later on, different cryoprotectants have been tested (mainly Me2SO and methanol), as well as different extenders that aim to maintain sperm immotile until activation and to improve post-thawing sperm quality. Furthermore, several additives have been tested including L-α-phosphatidylcholine, glucose, bovine serum albumin or fetal bovine serum with little or no improvement.
The aim of our studies focuses on improving the sperm motility and survival, genetic integrity and fertilization potential of thawed sperm. Our group and collaborators have successfully used cryopreserved sperm for fecundation of European eel and for hybridization with Japanese eel, but using two very different protocols that evidence the need for standardization. Our most recent studies compared those two protocols that include different cryoprotectants (Me2SO and methanol), extenders, dilution rate, cooling rate and volumes, and showed that the use of the protocol containing Me2SO as cryoprotectant led to a hypomethylation of the sperm DNA as well as to sperm activation, whereas the protocol with methanol was innocuous. Furthermore, the viability and motility of thawed sperm using methanol as cryoprotectant were enhanced by using egg yolk as additive obtaining sperm motilities higher than 50%, which is much higher than previously reported. Finally, we have improved the European eel sperm cryopreservation protocol by using different containers (straws, cryovials) of larger volumes, for better management in future fertilization trials.

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S144 PRESERVATION OF DANIO RERIO AND CARP EMBRYOS AND PROLARVA IN CRYOBANKS. PROBLEMS AND PERSPECTIVES

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At the 2004 International conference "Conservation of Genetic Resources" in Saint Petersburg we reported a potential possibility to preserve late embryos and prolarva of Danio-rerio (34 samples in different experiments) in a viable state after deep freezing. However, the results of cryoconservation weretotally unpredictable.
It induced more careful study of different factors contributing to or preventing the emergence of viable embryos or prolarva after thawing.
30 Danio-rerio and carp embryos that survived at the new experiments were at the "eyed egg" stage (fish-breeders permit transportation at this stage), before hatching, and also at the prolarva settling stage, but results were still unpredictable. Their common feature was the absence of proliferative processes, active morphogenetic cell shifts, good water/cryoprotectant permeability of membranes. So, embryos culture should be strictly standardized. Common cryobiological approaches for the choice of effective cryopreservation regimes are not productive. Cytological criterions are introduced in order to define a possible impact level of cryoconservation procedure (first of all overdehydration). It is suggested to judge upon the viability preservation by the reversible protein denaturation of skeletal muscles, observing them in the dark microscope field. Skeletal muscles become milky white under unfavourable conditions when protein is proceeding to IRREVERSIBLE denaturation. Then cryoconservation is impossible.

In 5-10 minutes after thawing some of transparent (not white!) prolarva can also have signs of death (recrystallyzation during the thawing process?).

Perhaps, it can be prevented by increasing freezing/thawing speeds (I. Katkov. Cryoblast 3, a conference report).

Creating teleostei embryos cryotechnologies are important to organize industrial and insurance funds of planting material for cases of man-made or ecological disasters, Low temperature Genobank (LTGB) nets for aquaculture etc.

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Conflict of Interest: None to disclose

S145 CRYOPRESERVATION EFFECT ON STERLET SPERM VIABILITY AND PROTEIN CONTENT AFTER PERCOLL SEPARATION

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In many fish species, sperm cryopreservation procedure leads to deleterious effects and significant decrease of fish sperm viability. After freeze-thawing process, sperm suspension contains viable, non-viable and cryodamaged spermatozoa. Usually, such a suspension is the object of routine cryobiological studies, aiming to evaluate the total cryodamaging effect on spermatozoa. However, the presence of a varying percentage of cryodamaged spermatozoa in the sperm suspension probably obscures the real cryopreservation effects on the fraction of spermatozoa that survived the freeze-thawing process. Therefore, the objective of our study was to compare the sperm motility, viability and proteomes of native and cryopreserved sterlet sperm before and after spermatozoa subpopulation selection using a Percoll density gradient centrifugation. The native sperm of sterlet males as well as cryopreserved one were divided into 2 groups, either with or without application of the Percoll separation. At each step of the experiment, sperm quality was evaluated by video microscopy with integrated computer assisted sperm analysis software and flow cytometry with live-dead sperm viability analysis. Sperm motility and percentage of live cells in the cryopreserved bulk fell to 40% and 63% respectively. However, the percentages of motile and live cells following separation were higher than 90 % in both cases. Using two-dimensional difference gel electrophoresis followed by MALDI-TOF/TOF mass spectrometry, significant changes in 20 proteins were identified when comparing native and cryopreserved sperm, while content of only one protein was significantly changed when comparing native and cryopreserved sperm resulting from the Percoll separation. Thus, the results of this study show that the spermatozoa surviving the freezing-thawing process are much less affected by cryodamages than those in the unselected suspension. However, for a clearer understanding of cryopreservation damaging nature, further researches should be more deeply performed.

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Conflict of Interest: None to disclose.

S146 CRYOBANKING CLONAL CROPS: WHEN KNOWLEDGE AND PRACTICE ARE NOT THE SAME

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Cryopreservation of vegetative parts or embryos offers a reliable practical solution for the long-term conservation of genetic collections of crops that are vegetatively propagated or have recalcitrant seeds. Yet, very few world genebanks utilize this technology in a routine basis to conserve crop collections under their responsibility. Out of 12 vegetatively propagated and recalcitrant seed crops listed in Annex 1 of in the International Treaty of Plant Genetic Resources for Food and Agriculture, only few have well-developed protocols that have been successfully implemented across a wide range of diverse accessions. The major constraints are clearly the lack of funds but also the lack of practical knowledge in how to replicate the laboratory-scale technique at a larger scale and how to make all steps of the process economically affordable without compromising high standards of operation and consistency among replications. Four CGIAR genebanks, Bioversity International, CIAT, CIP and IITA, developed profound experience in cryobanking of clonal crops of their international collections: potato, banana and plantains, cassava and yam. Total amount of material in the cryobanks of these institutes exceeds 5000 accessions and efforts are been made to mainstream the procedures to cryopreserve over 100 accessions per genebank per year. This goal was already overpassed at CIP where 450 potato accessions are cryopreserved annually. As a group of CGIAR cryobank professionals, we present the critical overview of our experience in protocol development and large-scale cryopreservation of the selected crops, and demonstrate how the procedures evolved over time following emergence of new technologies. Among critical steps of the procedure we discuss contamination of the material, protocol development and optimization for multiple accessions, data management and work logistics. We also highlight the importance and difficulties of establishing the quality management programmes that are applicable across cryobanks operating in different cultural, geographical and economic conditions.

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Conflict of Interest: None to declare

S147 STATUS OF CRYOPRESERVATION TECHNOLOGIES IN HARDWOOD FORES TREES

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The main advances made over the last years in cryopreservation of hardwood forest genetic resources are presented here, with special attention to recalcitrant species of the Fagaceae family. The availability of simple, reliable and cost-effective methods for conservation of hardwood forest species will be highlighted in this dissertation. Specifically, emphasis will be given to the following topics: i) cryopreservation techniques; ii) selection of explants for cryopreservation: *in vivo* collection of embryonic axes and dormant buds, and *in vitro* collection of shoot tips and embryogenic cultures. For conservation of recalcitrant seeds, the simplest and recommended method consists of the isolation of embryonic axes, a 20 -25% reduction of water content from their initial level, and direct immersion in LN. A more laborious procedure is required for cryostorage of shoot tips and embryogenic cultures. In the first case, the steps to be taken prior to direct immersion of the explants in LN are, cold hardening of donor shoots (2–4 weeks), pre-culture of isolated shoot tips in sucrose solution for 2–3 days, and pre-cryogenic treatment with vitrification solution for 60–90 min. A similar method must be followed for the cryopreservation of somatic embryos and embryogenic cultures. The most important factor to be taken into consideration in this case is the type of explants selected for cryostorage. Clumps of 2–3 somatic embryos at globular/early or heart stage are highly recommended instead of other materials.

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S148 CHALLENGES IN THE CRYOPRESERVATION OF ENDANGERED WILD SPECIES

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With growing threats to habitat worldwide, *ex situ* conservation is increasingly important as a back-up to threatened plant diversity. For those species that cannot be conserved in seed banks—known as *exceptional* species—alternative conservation technologies are available, and these rely heavily on the methods of *in vitro* culture and cryopreservation. As wild species with a wide range of adaptations, endangered exceptional plants (EEPs) present a variety of challenges to the application of these methods. Of the technologies available, shoot tip cryopreservation is particularly needed for species that have few or no seeds available for banking, but it requires the greatest input of resources and poses the most challenges. Examples will be drawn from several rare species to illustrate the effects of endophytic bacteria (*Crotalaria avonensis*), hyperhydricity *(Cycladenia humilis*), and cryopreservation method (*Hedeoma todsenii*) on survival of shoot tips through LN exposure. Since the goal of *ex situ* conservation is to provide a back-up in the event a species is lost in the wild, the necessity and challenge of capturing sufficient genetic diversity using clonal *in vitro* methods to provide for future restorations will be illustrated by CREW's experience with several hundred

genotypes of *C. avonensis*. Despite the challenges, shoot tip cryopreservation is an effective tool for conservation, as demonstrated in a recent study, in which shoots were successfully recovered from 13 wild species after 4 to 19 years of storage in liquid nitrogen in CREW's CryoBioBank.

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Conflict of Interest: None to disclose.

S149 ASSESSING THE LIMITS OF LIQUID NITROGEN STORAGE: FERN SPORES AS UNICELLULAR MODEL TO UNDERSTANT AND IMPROVE LONGEVITY AT CRYOGENIC CONDITIONS.

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The low temperatures reached during liquid nitrogen (LN) storage are assumed to preserve viability of biological materials "indefinitely". Plant materials (such as seeds, shoot tips, embryos, gametophytes or cells) have been exposed to LN and cryopreserved routinely during the last 30 years, and they have benefited from LN storage. 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 already aged before LN storage, or those of initial low quality, can age and die during LN storage. These results open the debate about the limits of LN storage and challenge the length of "indefinitely". In this paper we present our findings using fern spores. Fern spores are desiccation tolerant (i.e. can be dried until ice-forming intracellular water is removed), so artefacts in the interpretation of the results related to devitrification of hydrated systems during LN storage are avoided. In addition, the single cell nature of fern spores eliminates 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. The use of fern spores reveals that some plant materials can age and die faster than expected, during dry storage at freezing and cryogenic temperatures (from -18 to -196°C). Aging appears to be related to reactions allowed by rotational movements of the molecules trapped within the glassy state. The slow crystallization of storage lipids trapped within the glassy matrix of the cytoplasm also seems to destabilize the glassy structures and may contribute to ageing at such temperatures. A better understanding of the characteristics of intracellular glasses of systems that can maintain viability for longer times in LN, and the role of cryoprotectants in structural stabilization, could help to improve the storability of materials that present limitations during LN storage.

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S150 DESIGN OF EXPERIMENTS (DOE)— HISTORY, CONCEPTS, AND RELEVANCE TO BIOLOGICAL SYSTEMS.

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In vitro technologies for horticultural, plant breeding, or genetic applications often require improving a growth or physiological response of a particularly cell, tissue, or organ culture system – organogenesis, somatic embryogenesis, metabolite biosynthesis, crop improvement responses such as ploidy manipulation, embryo rescue, somaclonal variation, and mutant creation and isolation. *In vitro* biological systems are extremely complex and include the effects of the mineral elements, plant growth regulators, carbon sources, gelling agents, vitamins, environment factors (light type/quality/photoperiod, temperature, gases), and all of their interactions. The result is that determining the effects of these medium components and cultural conditions sufficiently to grow the tissues as they need to be grown can be, and often is, a long and expensive process. Design of experiments (DOE) is a large and well-developed field for understanding, improving, and characterizing complex systems. It is well-suited for *in vitro* culture because the many factors are easily manipulated and the cultures are stored and/or grown under controlled conditions. Because geometry is the underlying framework of DOE, it is useful for understanding the complexity of *in vitro* systems. The geometric perspective of experimentation is highly intuitive and is especially helpful to the researcher in the actual planning, running, and analysis of experiments. The presentation will discuss the underlying concepts of DOE, the geometric basis of DOE, why DOE is a knowledge-based approach (vs data-based), and how DOE concepts and tools are useful in *in vitro* plant culture research.

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Conflict of Interest: None to disclose

S151 THE RISE OF PLANT CRYOBIOTECHNOLOGY AND DEMISE OF PLANT CRYOPRESERVATION?

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Plant cryobiology has evolved over the last 150 years, from innovations in the 19th century using newly-available liquefied gases, to the need to preserve cell lines that efficiently accumulate bioactive, secondary plant metabolites, and the drive to support plant conservation (late 20th century). The 21st century will be an era of rapid advancement in the life sciences,

particularly through developments in systems biology. Knowledge from the 'omics' disciplines will be combined to reveal the regulatory hubs and networks of not only stress tolerance but also recovery growth of tissues *in vitro*. In addition, an understanding of the structural stability of plant cell systems, from single cells to complex tissues, will improve as more powerful biophysical techniques are accessed. Whilst the focus of much of plant cryobiology seems to be on cryopreservation success, such an implementation-centric perspective may inadvertently fail to attract the next generation of scientists and thus limit progress in research and innovation. To embrace this thinking and current developments in science, it is suggested that plant cryopreservation is limited to implementation activities under the wider discipline of plant cryobiotechnology. Since its initial use in the mainstream plant science literature in 2016, other publications have embraced the term cryobiotechnology. Nonetheless, the term remains to be defined clearly. The following definition is proposed: 'cryobiotechnology is the use of modern technologies to understand the response of biological systems to low temperature environments, whether natural or imposed, leading to the production of knowledge, goods and services, including the cryopreservation of organisms, cells and tissues for use by industry, agriculture, medicine and conservation.' The term is validly applied to similar studies on other non-plant taxa.

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S152 MARINE INVERTEBRATE CRYOPRESERVATION: PAST, PRESENT AND FUTURE.

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The number of confirmed marine species has reached an amazing 243,462 by January 2018 according to the World Register of Marine species (WoRMS) but researchers expect oceans to be home to 700,000 to 1 million of species.
A model organism can be defined as a non-human species that has been extensively studied to understand different biological phenomena with the expectation that knowledge made in the model will provide insight into other organisms or processes. Marine organisms had been exceptional model organisms in several research fields.
Tim Hunt won the novel prize 2001 in Physiology and Medicine by research carried out with sea urchins. The discovery of the pandoravirus happened in a sample of phytoplankton in 2013 reaching the cover of Science. Research on the newfound type of cancer in cockles which is transmissible and constitutes a distinct class of infectious agent, was published on the cover of Nature, 2016.
Cryopreservation of marine organisms has very important applications in basic research but also in aquaculture, conservation, ecotoxicology and fisheries management. The current rate of discovery of new oceanic species is approximately 2000/year. The increasing discovery of species, the increasing research on the biology of marine organisms, and the applications resulting from that research should be accompanied by an increase in the number of species being cryopreserved. Research has been published on over fifty different species of marine invertebrates, this has produced over 200 papers. Factors like the lack of knowledge about the reproduction and physiology of many of these organisms, unknown genomes and transcriptomes, the sensitivity to CPAs, the short-time availability of gametes due to reproductive seasonality are factors that had constrained the development of cryopreservation protocols. I would address here some experimental results, the remaining technical constraints, and the immediate future of marine invertebrate cryopreservation.

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Conflict of Interest: None to disclose.

S153 PHYSICAL LIMITS OF LASER GOLD NANOWARMING

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In this work we explore the physical limits of a new technique to rewarm vitrified droplets and zebrafish embryos impregnated with 1064 nm resonant gold nanorods that are irradiated by a Nd:YAG ms pulsed laser. Importantly, the droplets and the embryos loaded with 2 M PG are first cooled by a modified cryotop at rates estimated to be 90,000 °C/min to a visually transparent state in liquid nitrogen. Numerical modeling demonstrates possible differences in warming depending on full mixing (droplets) vs. micro-injection into the yolk of the zebrafish. Experimental measurments based on optical transparency vs. cloudy behavior are then used to judge the physical success of the procedure. From this we present a map of the successful laser power, pulse length, CPA concentrations and gold concentrations that can yield physical success for laser gold nanowarming.

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Conflict of Interest: None to disclose.

S154 SUCCESSFUL CRYOPRESERVATION OF CORAL LARVAE USING VITRIFICATION AND LASER WARMING

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Coral reefs are imperiled globally by warming and acidification of the world's oceans, resulting in the loss of ecosystem function and biodiversity on reefs around the world. As reef degradation accelerates, the need for innovative restoration tools has become acute. Despite past successes with biobanking cryopreserved coral sperm to conserve genetic diversity, cryopreservation of larvae has remained elusive due to their large volume and membrane complexity, which promote the formation of lethal intracellular ice. We hypothesized that this challenge could be circumvented by rapid freezing in viscous cryoprotectants (vitrification) followed by ultra-rapid warming at millions of degrees/min with an infrared pulsed laser. Here we show for the first time that coral larvae can survive cryopreservation and resume swimming after thawing. Vitrification in a 3.5 M cryoprotectant solution followed by warming at a rate of approximately 4,500,000 °C/min with an infrared laser resulted in up to 43% survival of *Fungia scutaria* larvae on day 2 post-fertilization. These survivors continued to develop and swim for at least 12 h post-thaw, many for several days. This important first step, to freeze, store, and revive coral larvae, sets the stage for crucial next experiments to examine symbiosis and settlement of thawed larvae. If managed in a high-throughput manner, where millions of larvae in a species were frozen at one time, this important technology will enable biobanking of coral larvae to secure biodiversity and support crucial research and restoration efforts to diversify wild reef habitats.

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S155 CRYOPRESERVATION OF AQUATIC SPECIES: ACHIEVEMENTS, CHALLENGES AND FUTURE DIRECTIONS

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Cryopreservation of gametes and embryos of aquatic species has been widely covered in the literature in English, however the information published in other languages is less well known. In this presentation, we will bring together the information published both in English and other languages and to provide an overview of the current state of the art on aquatic species gametes cryopreservation. For work published in other languages, we will focus on the Eastern Europe former USSR countries, mainly in the Russian Federation and Ukraine, and Iran as these counties have a large body of publications, functioning cryobanks and sizeable research centres that are working in this field.
Current challenges in aquatic species gamete cryopreservation and future studies will also be discussed. For example, cryopreservation of fish oocytes and embryos represents a significant challenge due to their specific physiology and morphology. The recent reports on successful cryopreservation of zebrafish embryos with the use of golden or other electrically conductive nanorods inserted surgically in each embryo separately following by very fast rewarming may be a step forward although the new method would still need to be developed to address its practicality for conservation of aquaculture species . There is also not clear how very low concentration (fraction of the volume %) of thermoconductive nanoparticles can sufficiently increase cooling or warming rates of the whole sample.
We would like to propose a completely new approach for cryopreservation of fish oocytes and embryos using kinetic (very fast) vitrification (K-VF). The advantage of this method would be that it does not require introduction of permeable vitrification agents or nanoparticles. The engineering solution for K-VF for aquatic species, namely the *KrioBlast-3A* system of hyperfast cooling and moderately fast rewarming will be presented.

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S156 CRYOPRESERVATION OF FISH GAMETES AND ITS APPLICATIONS IN BRAZIL

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Although successful cryopreservation of fish spermatozoa has been achieved with many fish species, cryopreservation of oocytes and embryos has been

challenging with limited success. Cryopreservation and cryobanking of fish reproductive cells offers many benefits to aquaculture and biodiversity conservation. Home to 60% of the Amazon Rainforest, which accounts for approximately one-tenth of all species in the world, Brazil is considered to have the greatest biodiversity of any country on the planet. It has the most known species of freshwater fish and many of them have importance commercial value in aquaculture. However, due to increased human activities e.g. overfishing, pollution and other environmental factors e.g. climate change etc., there has been a significant increase of endangered species in recent years. A 2015 report indicated that 8% of all 4507 assessed fish species in Brazil are endangered. Cryopreservation of fish gametes plays an important role in biodiversity conservation as well increasing the flexibility in brood stock management in aquaculture. An overview of the current status of cryopreservation applications in aquaculture and conservation in Brazil is presented here. The use of cryopreserved semen was crucial for the implementation of the first genetic improvement program of Brazilian native fish created in 2008. Large fish farms have been using frozen semen especially for hybrids production in the Midwest region of the country. Some cryobanks of semen were established and have been used as depository of genetic materials for research at university labs and others as genetic reserve for restocking. Despite not having a long-term successful protocol for fish embryo cryopreservation, we have achieved hatching rates over 70% when using short-term chilling of embryos, which has a practical application in fish stock management.

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Conflict of Interests: None to disclose

S157 THE MODIFICATION OF PHYSICOCHEMICAL CHARACTERISTICS OF FREEZING MEDIA DURING THE HUMAN SPERM CRYOPRESERVATION

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This study was done to apply extremely low repetition rate electromagnetic fields (ELEFs) to change the molecular network of water molecules present in freezing media used for human sperm cryopreservation. For this purpose, different time periods and pulsed electromagnetic field were used to evaluate the physiochemical properties of water. The highest quality of water exposed to ELEF was selected to be evaluated for production of human sperm freezing media. For cryopreservation of sperm, semen samples were obtained from 25 normozoospermia, extended in ELEFs-water made freezing media and cryopreserved according to standard procedure. The lowest rate of hydrodynamic diameter, zeta potential, surface tension, viscosity and density were observed for water samples exposed to ELEF for 60 min. Furthermore, the highest percentages of total motility, progressive motility and viability of thawed sperms were observed in the group treated with ELEF media compared to untreated group, respectively. It seems that modification of physiochemical properties of human sperm cryopreservation media by ELEF is a suitable strategy to improve the outcome of cryopreservation.

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S158 MICROTUBULE DYNAMICITY OF HUMAN SPERM IN CRYOPRESERVATION PROCESS AND 1000 Hz REPETITION RATE ELEF-WATER MADE FREEZING MEDIA

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This study was designed to identify proteomic changes and microtubule dynamicity by applying of 1000 Hz repetition rate extremely low electromagnetic field in human sperm during cryopreservation. ROS concentration was measured using chemiluminescence assay and sperm motion parameters analyzed by CASA. Microtubule dynamicity, secondary and tertiary structure modifications of tubulins and microscopic characteristics were evaluated during cryopreservation process. Total and progressive sperm motility in fresh and treated freezing groups are significantly ($P<0.05$) higher compared to untreated. ROS production in untreated freezing group is significantly higher than fresh and treated. In treated freezing tubulins a decrease in period of nucleation and elongation phase were observed compared to untreated. The emission spectra of untreated freezing group were lower than treated and fresh. Circular dichroism spectroscopy was demonstrated that secondary and tertiary structure of tubulins was affected by cryopreservation while ELEF-water made freezing media moderated these changes. Transmission electron microscopy of microtubules was shown that untreated freezing microtubules length was 418±19 and 460.25±12nm shorter than treated and normal respectively.

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Conflict of Interest: None to disclose

S159 COMPARISON OF POST-THAWED BULL SEMEN MORPHOLOGICAL CHARACTERISTICS STORED IN DIFFERENT TEMPERATURES

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Generally frozen bull spermatozoa are stored in liquid nitrogen at -196ºC.
Although simplicity and mechanical reliability that efficacy of liquid nitrogen in sperm storage has been proven, but the systems based on liquid nitrogen have some problems such as temperature changes, the need of regularly support of nitrogen, moreover its high cost. Consequently, it is needed to find alternatives that avoid the occurrence of complications of traditional technical. Ultra-low temperature freezers is used as a feasible alternative for frozen semen storage at -152ºC. A total of 80 straws collected from 2 bulls on different days. Straws were divided in 2 groups; they had a sample of the same bull semen collected on the same day; also they were thawed and examined in terms of post-thaw morphology by CASA.
As a result, normal spermatozoa ratio was 67.75% for the sperm stored at -196 ºC and 55.42% for the ones stored at -152ºC ($p < 0.001$). While there was no statistical importance between acrosomal anomalies observed in different storage temperatures ($p > 0.05$); cytoplasmic droplet, head, middle and tail anomalies were lower and statistically significant ($p < 0.001$) in semen stored at -196ºC.
In conclusion, it was determined that the post-thawed of frozen bull semen kept at -152ºC had lower morphological parameters values compared to the sperm stored in (-196ºC). In this context, it has been concluded that the storage of frozen bull semen at high temperatures has a negative impact on morphological quality of post-thawed bull semen. Taking into consideration, repetition of similar studies and supplementation with fertility data will increase the accuracy of the results obtained.

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S160 EFFECT OF LECITHIN NANOMICELLES IN EXTENDER ON BULL SPERM CRYOSURVIVAL

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This study was conducted to determine the effect of different levels (1, 2, 3 and 4 % w/v) of nano-micelles or micro-micelles of lecithin in semen extender on post-thaw sperm quality of Holstein bull sperm. Semen samples were collected from six bulls by artificial vagina twice a week during 6 weeks. Soy lecithin nano-micells were prepared with 45 minutes sonication (S-4000 Misonix). The size of particles was determined by dynamic light scattering (z.average diameter=94.6 d.nm). For preparation of micro-micelles, different levels of lecithin were dissolved in buffer by shaking for 30 minutes. Some parameters including sperm motility (CASA), plasma membrane integrity (eosin-nigrosin) and activity (HOST), apoptosis status (Annexin staining), lipid peroxidation (MDA), mitochondrial activity (Rhodamine-123 staining) and acrosome integrity (PSA staining) were measured. All data were checked for normal distribution by Proc Univariate and Shapiro–Wilk test. Then, the data were analyzed by the GLM procedure using SAS 9.1. Total motility after cryopreservation in level of 1, 2, 3, and 4% of micro-micelles or nano-micelles was 54.25, 54.06, 56.07 and 62.05 % Vs. 60.25, 64.64, 66.53, 61.51% (SEM=2.61) respectively. Sperm membrane activity (Host data) in mentioned treatments was 39.44, 36.55, 41.53, 41.87% Vs. 31.16, 43.27, 40.20 and 24.54% (SEM=6.00) respectively. Mitochondrial activity in concentration of 1, 2, 3, and 4% of micro-micelles and nano-micelles was 48.10, 54.60, 55.35, 54.41% Vs. 50.11, 60.49, 65.48 and 57.09 % (SEM=1.40) respectively. The lowest percentage of acrosome integrity (61.86±2.40%) was found in the group containing 1% micro-micelles but there were no significant differences among other treatments. The percentage of viable non-apoptotic sperm significantly increased in treatments containing 3% nano-micelles (78.43±2.28%) and 4% micro-micelles (81.03±2.28%) compared to other groups. In conclusion, for the best cryopreservation of Bovine sperm, the semen extender should be containing 3% nano-micelles of lecithin.

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S161 FREEZING POULTRY SEMEN; EFFECTS OF CPA CONCENTRATION X COOLING RATE; OTHER FACTORS.

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Cryopreservation of chicken semen is used in gene banks for ex-situ conservation of genetic diversity, or in the breeding industry to conserve selection lines. However, post-thaw fertility may be low, especially in endangered breeds. Glycerol is a good cryoprotective agent (CPA), but must be removed before insemination as it acts as contraceptive in the hen. Adequate fertility was obtained earlier with dimethylacetamide (DMA) as CPA. In the present study, a number of CPAs were compared, and DMA concentration, cooling rate, and other variables were studied.
The effect of osmolality of the base extender (no CPA) on sperm cells was first tested during pre-freeze 5°C storage using extenders with equal composition in terms of solute ratios, but having osmolalities ranging from 290-410 mOsm/kg. Higher osmolalities had a strong negative effect on sperm motility, which was only partly reversible, indicating permanent injury of the cells. Six

related CPAs (methylformamide, methylacetamide, dimethylformamide (DMF), DMA, propane-1,2-diol, and diethylformamide) were first pre-screened at 0.6M for freezing semen from individual cocks (n=10) in 0.25-ml straws at a cooling rate of 250°C/min. Post-thaw % motile and % live sperm were highest with DMA and DMF.
Finally, in more detailed factorial experiments, semen from individual cocks or pooled semen was frozen in 0.25-ml straws, using cooling rates (CRs) of 4, 50, 250, and 440 °C/min and [DMA] of 0.4, 0.6, 1.0, and 1.5M. There were clear effects of both CR and [DMA], but no evidence for interaction of CR x [DMA]. Percentage motile and % live sperm were highest for CRs 50-250°C/min. Higher DMA concentrations gave better post-thaw sperm survival, with relative % motile sperm 77±6% (mean±SE; four replicates) at 1.0M DMA and CR 250. However, longevity of the sperm at 1.0 and 1.5M DMA was compromised. Therefore, [DMA] may best be 0.6-1.0M at a CR of 50-250°C/min.

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S162 LIPID PEROXIDATION, ANTIOXIDANT POTENTIAL, DNA INTEGRITY AND MITOCHONDRIAL ACTIVITY OF INDIAN RED JUNGLE FOWL (*Gallus gallus murghi*) SEMEN

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The study was designed to elucidate the impact of cryopreservation on lipid peroxidation, antioxidant activity, mitochondrial activity, viability and DNA integrity of Indian red jungle fowl semen. Semen from eight mature cocks was collected, pooled and diluted at 37°C in red fowl extender using glycerol as cryoprotectant (5 replicates). The diluted semen was cooled to 4 °C in two hours (0.275 min^{-1}), equilibrated for 10 minutes (after the addition of 20% glycerol) and filled in 0.5 mL French straws. The semen filled straws were kept over liquid nitrogen vapors (5cm above the level of LN_2) for 10 minutes and plunged into liquid nitrogen. Motility (%), viability (%; eosin/nigrosin), DNA integrity (%; aniline blue), antioxidant activity (2,2-diphenyl-l-picrylhydrazyl assay; DPPH), lipid peroxidation (malondialdehyde concentration in sperm and seminal plasma) were studied before dilution (fresh semen), after equilibration (processed semen) and post-thawing (frozen semen). Sperm motility, viability, DNA integrity and mitochondrial activity decreased ($P<0.05$) in processed and frozen semen compared to fresh semen. Nevertheless, lipid peroxidation in sperm and seminal plasma were recorded higher ($P<0.05$) in frozen-thawed and processed semen compared to fresh semen. Multivariate regression analysis showed negative impact of lipid peroxidantion in sperm ($R^2=0.90$, Wilk's $\lambda=0.003$, $P<0.01$) and seminal plasma ($R^2=0.84$, Wilk's $\lambda=0.02$, $P<0.01$) on motility, viability, DNA integrity and mitochondrial activity. Nonetheless, total antioxidant capacity had positive impact on sperm parameters ($R^2=0.82$, Wilk's $\lambda=0.96$, $P<0.01$). It is concluded that decrease in motility, viability, DNA integrity and mitochondrial activity of Indian red jungle fowl is associated with higher levels of lipid peroxidation in sperm and seminal plasma during cryopreservation. Furthermore, total antioxidant capacity reduced during freeze-thaw process and found insufficient to protect the integrity of spermatozoa.

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S163 CATALASE ADDED AT THAWING IMPROVES DROMEDARY CAMEL SPERM MOTILITY

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The beneficial effects of antioxidants on sperm cryosurvival suggest that reactive oxygen substances likely contribute to reduced sperm viability following freezing. However, no information is available on the effects of adding antioxidants at thawing to dromedary camel spermatozoa.
Following a pilot study in which 7 antioxidants were considered, the two best (500 IU Catalase or 1 mM Epigallocatechin) were further evaluated for their role in improving dromedary camel sperm cryosurvival when added at thawing. Semen from 6 males (3 ejaculates per male) was liquefied and viable spermatozoa selected by Single Layer Centrifugation (SLC) and frozen in Green Buffer. Antioxidants were added or not (Controls) immediately at thawing. Sperm motilities were assessed by computer aided sperm analysis (CASA: at 1.5 and 3h) and membrane integrity and acrosome status were evaluated with vital stains (1.5 h).
At 1.5 and 3 h post thaw sperm total motility and progressive motility were improved with Catalase (TM $p=0.007$, $p=0.002$; PM $p<0.001$ for both). At 1.5 h, Catalase sperm had higher values of ALH ($p = 0.008$), VAP ($p < 0.001$), VCL ($p < 0.001$) and VSL ($p < 0.001$) than Control and Epigallocatechin sperm. Both Catalase and Epigallocatechin sperm had better STR values than Controls. At 3 h ALH was highest in samples with Catalase ($p < 0.001$). Samples with Epigallocatechin showed higher BCF than Controls. There were no differences between treatments for vitality and acrosome integrity. These results suggest that adding Catalase to thawed dromedary camel sperm benefits motility characteristics.

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S164 VIABILITY OF CATTLE SEMEN AFTER 26 YEARS OF CRYOPRESERVATION

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In 1992 a rancher started a cryopreservation project to introduce specific genetic phenotypes that would optimize his cattle herd. Embryos and semen of a specific Hillcrest Aaron Piedmontese bull were purchased for a mutation in the myostatin (MSTN) gene that results in a desirable change in the phenotype. The MSTN mutation consists of a two base pair deletion in the third exon; which causes a premature stop codon at amino acid 313. Cattle with only one copy of the MSTN mutation have about 14% more muscle mass than other cattle, because they are double muscled. By increasing muscle mass, more beef can be harvested from the herd. Piedmontese cattle originated in Italy and are hard to come by; therefore to introduce Piedmontese genetics into the herd, it was beneficial to use cryopreserved semen and embryos of the bull that won the national championship of genetic traits in Missouri in the early 1990s; enabling the prized bull to father the next generation of the herd. The cryopreserved semen proved to yield successful pregnancies 60-70% of the time. Samples of the frozen semen were analyzed and we found that only 65% of the sperm cells were still viable. The semen has been kept in a 34 Liter LN2 dewar containing liquid nitrogen since September 1992. Throughout the 26 years the semen has been self-maintained by manually checking the level and pouring liquid nitrogen into the dewar with a hose; even though the dewar has reached very low levels of liquid nitrogen. As we do a semen analysis on the cells we find that they are still 60% viable, which is only 5% lower than the original viability of our cattle sperm cells; and can still expect to maintain pregnancy 70% of the time upon post thaw use of these cells.

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Conflict of Interest: None to disclose

S165 NANOPARTICLE AND METAL FORM HEATING FOR IMPROVED CRYOPRESERVATION

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Cryobiologists have demonstrated practical vitrification of 8 – 9 M loaded rabbit kidneys (cm sized system), and 2 M CPA loaded zebrafishembryos. However, these systems routinely fail by devitrification or cracking upon rewarming due to either a lack of speed or uniformity or both. This talk will focus on several collaborative studies lead by our lab to demonstrate that metallic (iron oxide or gold) nanoparticles and metal forms (i.e. stents or foils) can generate the necessary heat to address this problem. Importantly, metallic nanoparticles and stents all strongly absorb electromagnetic energy and are FDA approved for various indications. By careful deployment, tuning and control of warming we now show evidence that we can successfully warm cm scale systems (i.e. tissues and organs) and smaller mm sized systems (i.e. zebrafishembryos).

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Conflict of Interest: None to declare

S166 COLD RESPONSIVE NANOPARTICLES FOR CRYOBIOLOGICAL APPLICATIONS

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Although nanoparticle-mediated delivery of therapeutic agents has been widely studied for thermal therapy utilizing high temperature to destroy tumor for many years, it has not been explored in the field of cryobiology until recently. We have developed novel cold-responsive nanoparticles that release their payload in response to cooling to below room temperature. These nanoparticles can be used to achieve effective intracellular delivery of trehalose, an excellent cryoprotective agent (CPA) that does not enter mammalian cells due to its highly hydrophilic nature. Our data show that the cold-responsive nanoparticle-mediated delivery of trehalose does not compromise the cell viability, and the trehalose delivered into adipose-derived stem cells can effectively protects the cells from cryoinjury without the need of any toxic organic solvent or cell membrane permeating CPAs (e.g., DMSO). Furthermore, these nanoparticles encapsulated with anticancer drugs in combination with ice cooling can be used to overcome cancer drug resistance. This is attributed to the ice cooling induced burst drug release from the nanoparticles and minimization of cell metabolism. The former surpasses the rate of drug efflux via the transmembrane efflux pumps overexpressed on drug resistant cancer cells, and the latter compromises the function of the efflux pump by minimizing the synthesis of ATP that drives the efflux pumps. Moreover, these nanoparticles are shown to preferentially accumulate in (i.e., target) tumor after intravenous injection. When combined with ice cooling, these nanoparticles efficiently inhibit the growth of multidrug resistant tumors in vivo without evident systemic toxicity. Collectively, our novel cold-responsive nanoparticles may find tremendous applications in the field of cryobiology for addressing the key challenges to both cell preservation and cryotherapy.

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Conflict of Interest: None to disclose

S167 FOCUSED ULTRASOUND GUIDED-BY-MRI-THERMOGRAPHY FOR FAST AND CONTROLLED RE-WARMING OF CRYOPRESERVED ORGANS

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Organs transplantation have a great importance in saving and improving millions of lives, but the costs are very high and the unique source of organs is donation. Organ Banking (storage of cryopreserved organs) will reduce notably these costs and alleviate logistic and matching issues. Although during organ vitrification, small ice nuclei can be formed, they are not usually harmful until their growing during the rewarming process. For this reason, it is necessary a warming rate higher than the characteristic time for ice growing. So far, methods used for alleviating the problem has explored using high concentration of low toxic cpa cocktails, liquidus tracking, flow lock approach, microwave rewarming, nano-warming and magnetic ice binding particles, among others. However, although close, none of them have achieved a final solution for organ cryopreservation so far.
Our work is based on the application of High Intensity Focused Ultrasound (HIFU) coupled to thermography by MRI for organ rewarming. Both technologies have been successfully used to treat tumours and neurologic disorders by hyperthermia, because their high capability of very precise control. The characterization of the optimal HIFU system for our purpose has been developed by finite elements with COMSOL Multiphysics. These simulations have been essential to establish the parameters of the transducer: geometry that best suits the target organ, frequency and power. Specifically, the simulations have been made for an organ phantom of 5 cm of diameter, so the focal point of the transducers array must have a similar dimension; in addition, several tests have been carried out at different frequencies and power ranges, from 100 kHz to 2MHz and from 50W to 300W, respectively. Rewarming rates of 100K/min are easily achieved, with gradients of less than 5K. These results make organ warming by HIFU guided by MRI-thermography a promising technology for organ cryopreservation.

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S168 MOLECULAR SIMULATION FOR CRYOPRESERVATION: ICE RECRYSTALLIZATION INHIBITION UNDER COMPUTATIONAL MICROSCOPE

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Molecular interactions between biomolecules, cryoprotectants and water fundamentally determine the outcomes of preservation. While experimental techniques are limited in structural and temporal resolution, molecular dynamics simulations can provide a glimpse into the atomic-level structure and interaction of individual molecules that dictate the macroscopic behavior. Computational research on the systems containing biomolecules, cryoprotectants and water has provided invaluable insights into the development of new cryoprotectants and the optimization of preservation methods. Inspired by nature, antifreeze proteins (AFPs) and their synthetic mimics have been used to inhibit ice recrystallization in cryopreservation, especially for ice-free vitrification. But the fundamental principles of designing AFP mimics are still missing. In this study, we explored the molecular dynamics of ice recrystallization inhibition (IRI) by poly(vinyl alcohol) (PVA), a well-recognized ice recrystallization inhibitor, to shed light on the otherwise hidden ice-binding mechanisms of chain polymers. Our molecular dynamics simulations revealed a stereoscopic, geometrical match between the hydroxyl groups of PVA and the water molecules of ice, and provided microscopic evidence of the adsorption of PVA to both the basal and prism faces of ice and the incorporation of short-chain PVA into the ice lattice. The length of PVA backbone seems to be a key factor dictating the performance of IRI since the PVA molecule must be large enough to prevent the joining together of adjacent curvatures in the ice front. The findings in this study will help pave the path for addressing a pressing challenge in designing synthetic ice recrystallization inhibitors rationally, by enriching our mechanistic understanding of IRI process by macromolecules.

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Conflict of Interest: None to disclose

S169 TOOLS AND TECHNOLOGY FOR FUNDAMENTAL AND APPLIED CRYOBIOLOGY: AN EXPERIENCE IN SEATTLE

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Development of novel tools and technology is essential for both fundamental and applied cryobiology. In this presentation, we will talk about our efforts on the exploration of novel tools and technology for cryopreservation in the last decade, including (1) the development of microfluidic devices with precise control of fluid flow and temperature for the determination of cellular cryobiological characteristics, such as the cell membrane permeability to water and cryoprotective agents (CPAs), (2) cooling rate controlled freezer to

achieve the desired cooling rates for the cryopreservation of different types of cells or tissues, (3) the dilution-filtration system based on hollow fibber cartridge for the automated addition/removal of CPAs, (4) the real-time instant monitoring of CPA concentration during the process of CPA addition and removal with the measurements of electrical conductivity, (5) application of electromagnetic field for the ultra-fast and uniform rewarming for the samples with large volume, and others.

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S170 RAMAN STUDY OF LIPID PHASE STATE IN FREEZING MAMMALIAN EMBRYOS AND OOCYTES

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Cryopreservation of oocytes and early embryos is nowadays used in reproductive medicine, and for Genome Resource Banking. However, some mammalian species with lipid-rich oocytes and embryos are problematic for cryobanking. In particular, the lipid phase transitions and lipid-related cryoinjuries are the challenging aspects of lipid-rich embryos/oocytes cryopreservation due to changes of the phase and conformational state of lipids in cytoplasmic lipid droplets and cellular membranes. The study of the lipid phases and conformational states in freezing cells is essential for our understanding of lipid metabolism downregulation at low temperatures. However, there is a deficiency of knowledge about how lipid structures freeze and a lack of methods that could be used for *in situ* characterization of lipid phase transitions in freezing oocytes and early embryos. Here, we demonstrate that a highly informative non-destructive lipid state monitoring can be realized using Raman spectroscopy approach. It is shown that the onset of the main lipid transition in cat and mouse embryos occurs at temperatures near 0 °C. Evaluated parameters of the lipid phase transition in domestic cat cumulus-oocyte complexes differ from the transition parameters of *in vitro* derived oocytes and early embryos. The capabilities of Raman spectroscopy to investigate the distribution of lipids inside the oocytes/embryos during their freezing are discussed.

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S171 DAY 2 HUMAN EMBRYOS CRYOPRESERVATION: WHICH OF AVAILABLE METHODS IS MOST BENEFICIAL?

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It is rather widely accepted that for day 3 human embryos vitrification method of cryopreservation is most advantageous. However, for day 2 embryos a modified, more efficient method of slow freezing was proposed 9 years ago. To detect the most efficient approach of day 2 human embryo cryopreservation, we compared retrospectively laboratory and clinical effects of standard and modified slow freezing methods with microvolumetric (MSS) vitrification. Standard freezing based on classic cryoprotective agents (CPs) solution consisting of 1.5 M propanediol + 0.1 M sucrose (Medicult, Denmark). Modified CPs solution differs in an increased concentration of sucrose (0.2 M, Vitrolife, Sweden). Freezing curve was standard, including seeding at -6.5°C; 10 min. hold time, 0.3°C/min to -30°C and 50°C/min to -150°C. Air/water standard thawing was followed with 0.5 M sucrose supported CP dilution. Vitrification (closed system) was performed using HSV (CryoBio, France) holders after two step exposition to dimethyl sulphoxide/ethylene glycol (7.5% both) solution, for 10 -12 min. and dimethyl sulphoxide/ethylene glycol /sucrose (15%, 15%, 0.5 M respectively) solution for 55 – 70 sec. (Irvine Scientific, USA). Warming was performed in warm (37°C) 1.0 M sucrose solution followed with additional 3 steps in sucrose solutions of decreasing concentrations. After overnight culture 1 or 2 embryos were transferred to recipient patients. Survival of embryos and pregnancy ratios were calculated and compared using Chi-square or Fisher statistical tests (GraphPad, InStat). Out of 3819, 2932 and 308 thawed/warmed embryos after standard freezing, modified freezing or vitrification 70.1, 85.3 and 97.1% ($P \leq 0.0001$) survived. After embryo transfer, respectively 27.85, 27.84 and 24.31% (NS) of pregnancies was achieved. In spite of insignificantly lower pregnancy rate obtained after vitrified embryo transfer, the highest survival rate of vitrified embryos indicates vitrification as the most beneficial method of cryopreservation of day 2 human embryos.

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Conflict of Interest: None to disclose

S172 CRYO-SURVIVAL, FERTILITY AND SUBCELLULAR STRUCTURAL INTEGRITY OF MOUSE CUMULUS OOCYTE COMPLEXES AFTER CRYOLOOP VITRIFICATION

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Oocyte cryopreservation is one of the fertility preservation options for animals and humans. Vitrification is a relatively novel method of cryopreservation which uses ultra-rapid cooling and eliminates intracellular ice which is often detrimental to oocyte post-thaw survival. However, vitrification might also cause meiotic spindle damage and microfilament

alteration due to exposure of relatively high concentration of cryoprotective agents. The objective of this study is to investigate post-thaw oolemma viability and integrity of subcellular structures of CD-1 mouse cumulus oocyte complexes (COCs) by Cryoloop or French straw (FS) procedure. The whole clutches of COCs from superovulated CD-1 mice were vitrified with either a Cryoloop or FS in the presence of 30% ethylene glycol. Cryosurvival rates of COCs vitrified with FS (19%) were significantly lower than Cryoloop (75%) ($P<0.05$). In-vitro fertilization (IVF) rates of COCs vitrified by Cryoloop (71%) were significantly lower than fresh COCs (78%) ($P<0.05$). Embryo development rates by Cryoloop (56%) were significantly lower than fresh COCs (90%) ($P<0.05$). For the integrity of subcellular structures, 90% of the survived vitrified oocytes had normal spindle for both methods however the actin filament of the oocytes were altered but they were not influencing IVF and in-vitro embryo development rates to blastocyst stage. Our results showed cryoloop vitrification of mouse COCs had high post-thaw survival with high integrity of subcellular structures, IVF and embryo development rates. Cryoloop vitrification method might be an effective way to cryopreserve COCs to preserve mouse strains for genome banking and can be applied to fertility preservation for women.

Source of Funding: The University of Missouri Mutant Mouse Resource and Research Center
Conflict of Interest:

S173 EMBRYO VITRIFICACTION MODIFY THE PROTEOMIC PROFILE OF ADULT RABBIT LIVER, AND IT PERSISTS ACROSS GENERATIONS

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The "Developmental Origins of Health and Disease" hypothesis holds that embryonic and fetal adaptation to suboptimal environments can predispose a series of metabolic disease in adulthood. Assisted reproductive technologies are an example, which include a set of gamete and embryo mechanical manipulation. Thus, vitrification can cause embryo death and is not neutral to survivor cells, involving changes in embryo, foetus and adult individuals, such as modifications in the postnatal liver weight.
To assess the molecular cues of this issue, a proteomic study was performed comparing the liver proteome of adult rabbits derived from natural matting (control group) and from cryopreserved embryos (cryopreserved group: embryos recovered, cryopreserved and transferred into recipient females). These animals constituted the base population (Generation 0, G0). Animals within each experimental group were crossed to produce the next generation (Generation 1, G1). Equally, Generation 2 (G2) were produced from G1. Four liver samples in each group were analyzed through a LC-MS/MS analysis. Statistical differential protein intensities were analysed using InfernoRDN software, while functional classification and kegg analyses were assessed using *DAVID* bioinformatics tools.
When the direct vitrification effect was analysed, 45 differentially expressed proteins were observed. In the subsequent offspring, 41 and 54 differentially expressed proteins were observed in G1 and G2, respectively (transgenerational effects). In the three generations, the PCA and HeatMap analysis grouped the samples according to its lineage (control/vitrified). Studying the differential proteins after vitrification, the modified kegg routes were: "Metabolic pathways" (ocu01100), "Biosynthesis of amino acids" (ocu01230)" and "Carbon metabolism" (ocu01200). These modifications were maintained in their progeny (G1 and G2). Additionally, in G1 "Steroid hormone biosynthesis" kegg pathway appears modified, persisting also in G2. So, these results hold that embryo vitrification technique is not neutral and can induced alterations on offspring, and even up it can be observed in the next generations.

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S174 MOUSE MATURE OOCYTE VITRIFICATION UNDER STATIC MAGNETIC FIELD

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Recently, it was reported that the static magnetic field (SMF) might be a good alternative for cryoprotectant agent (CPA) in cryopreservation solution especially when the CPA concentration is reduced. The cells in SMF obtain better survival rates compared to unexposed controls. Although many studies performed in this area, the detailed mechanism of this action has not yet been elucidated. The aim of this study was to assess the cryoprotective effects on mouse mature complex cumulus oocytes (COCs). This study was conducted on mature COCs of 6-8 wks NMRI mice. COCs were vitrified by two-step vitrification protocol. First, oocytes were exposed for 5 minutes in equilibration medium contains 7.5% EG plus 7.5% DMSO (v/v), then they were put in vitrification solution (15% DMSO, 15% EG, 0.5M sucrose) for 1 minute. Vitrification in the presence of SMF is accomplished like the preceding procedure and samples were placed inside a SMF with 60±3mT magnitude at each stage of freezing or warming. In order to obtain blastocyst (BL) embryos, in vitro fertilization of COCs were performed after recovery post warming. Viability and survival rates and gene expressions (Cdx2, Nanog, Oct4, Sox2) were respectively investigated by trypan blue, Annexin V/PI staining and real time PCR. After warming, the rate of BL formation increased in

vitrification (SMF^+) group (69.99±7.70%) with respect to vitrification group (56.32±12.54%) but no remarkable difference was figured out. The late apoptosis in vitrification (SMF^+) group (8.00±4%) decreased significantly comparison with the other group (10.37±5.78%). The results of real time PCR exhibited meaningful decrease in Cdx2 in SMF^+ group with regards to vitrification group. The mean differences are significant at the 0.05 level. Using of SMF in vitrification had no undesirable effects on fertilization and viability rate of oocytes however the blastocyst rate was improved.

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S175 SUGARS ARE MORE EFFECTIVE IN SUPPRESSING INTRACELLULAR ICE FORMATION THAN PENETRATING CRYOPROTECTANTS

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Clinical applications of cell and tissue preservation require less toxic but efficient cryopreservation methods. Compared to conventional penetrating cryoprotective agents (CPAs), non-reducing sugars such as trehalose are known to be nontoxic. The protective mechanism of trehalose-like sugars has been explained by their excellent glass forming properties and water replacement hypothesis. However, it was unclear whether suppression of intracellular ice formation (IIF) is part of the cryoprotective mechanism of sugars. The objective of this study was to address this question. To this end, the effect of two sugars (i.e., trehalose and raffinose) and three penetrating CPAs (i.e., 1,2-propanediol, ethylene glycol, and dimethylsulfoxide) on IIF was studied using cryomicroscopy and mouse oocytes as a model. Upon superovulation, metaphase II (MII) oocytes were randomly distributed into experimental groups and either equilibrated with 0.1 M concentration of a penetrating CPA or microinjected with a concentrated solution of one of the sugars to obtain 0.1 M intracellular sugar concentration. Subsequently, MII oocytes were cooled to -40°C at 2°C/min on a cryomicroscopy stage in respective 0.1 M CPA or sugar solutions. MII oocytes that were not exposed to any penetrating CPA or sugar served as controls. These experiments were repeated more than three times, and IIF temperatures were determined from recorded cryomicroscopy videos. Statistical analysis of the data revealed that all penetrating CPAs and sugars significantly suppress IIF with respect to controls. Furthermore, sugars were more effective in suppressing IIF than penetrating CPAs. Taken together, these findings further elucidate the cryoprotective mechanism of non-reducing sugars and support their use in development of more efficient cryopreservation methods.

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S176 CRYOPRESERVATION-RELATED STRESSES IN *LACTOBACILLUS DELBRUECKII SUBSP. BULGARICUS*: GLOBAL AND MULTI-SCALE STUDY

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Cryopreservation leads to variable degradation of the biological activity and functionality among lactic acid bacteria (LAB), particularly *Lactobacillus delbrueckii* subsp. *bulgaricus*, a dairy starter of industrial relevance. The aim of this work was to identify cellular markers of cryoresistance or cryosensitivity for better understanding the mechanisms of cell cryoinjury and increasing LAB industrial performances. Cryopreservation was here considered as a combination of cold and osmotic stresses. A particular focus was given to the analysis of the cell membrane, recognised as a primary site of cryoinjury, but also of the cell wall and proteins. Moreover, cells were analysed from the population level down to the single-cell level to quantify the heterogeneity of cell properties within populations. In the first part of this work, bacterial cultivation conditions were compared to identify two *L. bulgaricus* strains with markedly different cell cryoresistance. Moreover, a comparative genomic analysis of the strains was performed to provide some clues for the explanation of their different behaviours. In the second part of this work, the membrane properties were evaluated in response to the cold and osmotic stresses: fatty acid composition, organisation of fatty acyl and phospholipid headgroups, and fluidity. Subcellular membrane fluidity was also characterised by fluorescence microscopy using synchrotron radiation, enabling the quantification of inter- and intra-cellular heterogeneities. Finally, original methodological and technical developments were undertaken to achieve the analysis of individual bacterial cells in an aqueous environment by Fourier transform infrared (FTIR) spectroscopy, for the analysis of the biochemical signature of cells under native conditions. These complementary multidisciplinary approaches revealed different properties and organisation of the membrane of both *L. bulgaricus* strains. It was proposed that different types of interaction between cryoprotectants of the extracellular matrix and the membrane of both strains could be at the origin of cryoinjury for the sensitive strain. Moreover, a high population heterogeneity characterised the cryosensitive strain, ascribed to differences in terms of biochemical composition and organisation of the membrane and cell wall. Altogether, this work suggests some cellular markers to evaluate

LAB cryoresistance and provides methods to characterize population biochemical heterogeneity. These could be applied to any other stressful step of their production process, and should be useful for future production of homogeneous populations of resistant LAB.

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Conflict of Interest: None to disclose

POSTER PRESENTATION ABSTRACTS

P1 SUCCESFUL KINETIC (HYPER-FAST) VITRIFICATION OF SUSPENDIAL CELLS USING KRIOBLAST-2

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On the last Cryo-2017 meeting, we presented KrioBlast-2, a semiautomatic pilot version of the *KrioBlast*™ platform for cryopreservation by kinetic (very fast) vitrification (K-VF). One of the major advantages of K-VF over the existing approach for vitrification is that K-VF does not need high concentrations of potentially toxic and intracellular exogenous vitrificants, also called "cryoprotectants", which is not exactly correct in this case. Here, we present the pilot experiments on of K-VF of human pluripotent stem cells and spermatozoa.
The cells were vitrified in special containers *VitriPlate-1*. Four groups: untreated control (CTRL), all manipulation except for the vitrification (SG), very rapid cooling in KrioBlast **(T-T**), slow rewarming in air (**T-O**), slow freezing in air (**O-O**). "T" and "O" represent transparent and opaque appereance during cooling and rewarming. Five times, three repetitions each (n=15) for both types of cells. Neither sucrose nor speargun introduction produced any sufficient damage to the cells compared to the intact untreated control. K-VF produced equally excellent survival in the range of 90% survival of the non-vitrified control (**SG**) when relatively fast fast rewarming in water bath was performed (group **T-T**). If the samples were not warmed fast enough right after vitrification but exposed to an ambient room temperature for several seconds, it led to recrystallization/devitrification of the sample (group **T-O**); survival of both hESCs and hSPM dropped more than two times in comparison with **T-T** group. If the sample had been already crystallized before super-fast cooling (group **O-O**), practically no cells survived, >90% and cells were TB positive, no moved spermatozoa were observed.
We believe that K-VF can be used for ANY kind of cells loaded into specific cryo containers for K-VF, *VitriPlate*™, *VitriComb*™, and *VitriScan*™ for vitrification of cells in suspension, packed in straws, and attached to surface in multiwell systems respectively will be used.

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Conflict of Interest: None to disclose.

P2 KINETIC (VERY FAST) VITRIFICATION OF LARGE VOLUMES OF CELLS: THE VITRIPLATE-3 SOLUTION

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In the concurrent oral presentations, we discussed kinetic (very fast) vitrification (K-VF) as an attractive alternative to both slow freezing and equilibrium vitrification as K-VF is a simple, robust and quite universal method of cryopreservation that does not require potentially toxic permeable vitrificants such as dimethyl sulfoxide or propylene glycol. We introduced *KrioBlast-2,* a pilot version of the *KrioBlast*™ platform for K-VF and reported very promising results of pilot testing of K-VF on human pluripotent stem cells and sperm. The results were obtaining using a flat cryo container of the *VitriPlate*™ family. We also introduced *KrioBlast-3*, a fully automated three module system as the next step in the direction of practical application of K-VF.
One of the major challenges of K-VF is very fast and uniform cooling of relative large volumes of biomaterial at once. For example, cryopreserving a cord blood specimen, which volume can be as high as 28,000 microliters, is an insurmountable task for the modern methods of vitrification. Here, we introduce a cryocontainer *VitriPlate-3*.The major feature of the *VitriPlate 3* is a meander (spiral-like) inlet that spreads the cooling front much uniformly in cryocontainers, which height (thickness) is substantially large to prevent very high cooling speed in the areas that are far from the major flat surfaces. We then consider partial thermodynamical equations and spatial and temporal distributions of the temperature and the temperature flow with and without *VitriPlate-3*. We show that the meander-spreader sufficiently increases the speed and uniformity of cooling throughout the whole cryocontainer. The applications of the system *KrioBlast-3 + VitriPlate-3* for K-VF of large volumes, e.g., for cord blood banking are also discussed.

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Conflict of Interest: None to disclose.

P3 FREEZE-DRYING AS A NOVEL CEREBROSPINAL FLUID PROCESSING METHOD FOR BIOMARKER MEASUREMENTS

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International consensus guidelines for pre-analytical cerebrospinal fluid (CSF) procedures and processing methods exist but do not include CSF freeze-drying methods, due to the lack of knowledge on the effect of freeze-drying and subsequent storage conditions on biomarkers of interest.
In biological fluids such as CSF, there is a risk of structural and functional damage of proteins, occurring upon freeze-drying. The purpose of this study was to develop a CSF freeze-drying protocol and investigate (1) the impact of freeze-drying and (2) the impact of different storage conditions of the freeze-dried material on stability of CSF-β-amyloid (Aβ42), phospho- and total-tau proteins, using standard ELISAs (INNOTEST, Fujirebio), and CSF metabolites, using the Absolute*IDQ*® p180 kit (Biocrates). The study included 144 CSF samples that had been stored for four years at six different conditions: liquid frozen at -80°C in polypropylene vials, liquid frozen at -80°C in glass vials, lyophilised and stored at ambient temperature, +4°C, -20°C and -80°C in glass vials.
The results showed that our freeze-drying protocol did not alter the CSF proteins and metabolites, but that degradation took place after the freeze-drying process and during long-term storage at non-cryogenic temperatures. Ambient temperature or +4°C cannot be used for long–term storage in the scope of metabolomics. All amino acids were impacted by the storage at 4°C or ambient temperature, with the most significantly affected amino acids being lysine, methionine, ornithine, phenylalanine, tyrosine and biogenic amines, such as putrescine, spermidine and taurine. The Aβ42, phospho- and total-tau proteins also showed significant decrease at ambient and +4°C storage temperatures. Additionally, we found an impact of storage container on the quantity of tau proteins, resulting in lower levels of tau proteins in glass vials as compared to the polypropylene vials.
Our freeze-drying protocol, in combination with cryogenic storage temperatures preserves CSF protein and metabolite biomarkers.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P4 BIOBANKS OF GAMETES AND EMBRYOS FROM AQUATIC SPECIES: APPLICATIONS IN ECOTOXICOLOGY

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Ecotoxicological bioassays using gametes and embryos of aquatic species are commonly used in integrated environmental monitoring programs due to their high sensitivity to the synergistic and/or antagonistic effects of all components interacting with the biota. Cryopreservation provides on demand viable gametes and embryos of homogeneous quality, thus overcoming the main limitations in their use in bioassays. Considering the number of aquatic species for which cryopreservation protocols have been standardized, the creation of a biobank of gametes from both vertebrates and invertebrates from a range of different environments meets the need for innovative and more flexible monitoring tools as well as for ecologically relevant biological indicators; in addition, cryopreserved biological systems could drastically reduce the use of animal models or whole organisms in performing ecotoxicological evaluations.
In the last decades our research group has developed specie-specific and easy-to-perform protocols for the cryopreservation of gametes and embryos from several euryhaline and marine invertebrate (bivalves, sea urchins) and fish species (sea bass, sea bream, common pandora). Successively, the most suitable cryopreserved biological systems have been selected for the creation of a biobank of gametes and embryos to be used in ecotoxicological tests. Spermatozoa from the sea bream *Sparus aurata* are here used as a model to describe the various steps through which the biobanking for ecotoxicological purposes has been carried out. These phases can be summarized as follows: 1) selection of the best freezing/thawing protocol. 2) evaluation of the performances of thawed samples: motility on thawing; motility in the times after thawing; longevity of long-term cryopreserved samples. 3) evaluation of the sensitivity to reference toxicants (identification of effect concentrations, confidence limits, coefficient of variations after exposure to cadmium) and environmental matrices (marine sediments and dumpsite leachate).

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P5 STANDARDIZED DNA AND RNA SAMPLE QUALITY CONTROL

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Many factors have an influence on nucleic acid quality, such as sample source, handling, extraction method and storage condition. The responsibility of the Biobanks to collect, store and ship samples makes it essential to determine sample quality at the point of receipt and release. Sample quality includes concentration and integrity which are both important parameters to ensure that nucleic acid samples are fit for purpose. Nucleic acid quality can be assessed using conventional gel or automated electrophoresis systems.
The DNA Integrity Number (DIN) has been established for genomic DNA (gDNA) qualification with automated electrophoresis systems. It provides an assessment of gDNA sample quality by assigning a numerical score from 1 to 10. A high DIN indicates intact gDNA, and a low DIN degraded gDNA. The DIN enables comparison of samples and allows defining a DIN quality threshold for specific types of samples or preparation. This poster shows examples of DNA sample patterns and correlating DIN across a wide quality range for DNA originating

from blood, fresh frozen tissue and formalin-fixed paraffin-embedded (FFPE) material.
The RNA integrity number equivalent (RIN^e) delivers an objective assessment of total RNA degradation for samples from eukaryotic or prokaryotic origin. The RIN is independent of sample concentration and analyst and allows unbiased confirmation of RNA sample quality. RNA samples extracted from FFPE tissue are typically highly degraded. Many tailored FFPE RNA library protocols use an additional quality metric DV_{200} to define the optimal RNA input amount for successful NGS library preparation. The DV_{200} represents the percentage of RNA fragments above 200 nucleotides. This poster exhibits sample patterns and corresponding quality scores of intact and degraded RNA including FFPE RNA samples.

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P6 CRYOPRESERVATION CAN REGULATE IMMUNOMODULATORY PROPERTIES OF FETAL NERVE CELLS

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The influence of various freezing conditions on the subpopulation composition and the expression of the *ido* gene in fetal nerve cells (FNCs) of rats of 11 days of gestation was studied.
Freezing modes: M1 - rate of 1 deg/min down to -80°C; M2 - 1 deg/min down to -9°C, 10 min stop, cooling of 1 deg/min down to -25°C, 10 deg/min down to -60°C; M3 - 1-2 deg/min down to -60°C with initiation of crystallization. Each mode was completed by an immersion of the samples into liquid nitrogen. Before and after cryopreservation, the content in FNCs of CD 133^+, $nestin^+$, $GFAP^+$ and ß-tubulin III^+ cells and the expression of the *ido* gene were analyzed. The M3 has been shown to preserve all the subpopulations at the control level, M2 has predominantly preserved $CD133^+$ and $nestin^+$ cells, M1 caused a decrease in β-tubulin III^+ content, retained $nestin^+$ and substantially enriched the suspension with glial $GFAP^+$ cells expressing the *ido* gene via the enzyme IDO. This gene participates in catabolism of tryptophan and activation of the suppressor link of immunity. It has been established after thawing the FNCs and 2 days' culturing in the presence of IFN-γ, the level of expression of the *ido* gene using the M1 reached the native control index, slightly exceeded that at the M2 and was significantly higher at M3. In the animals with graft versus host disease, the administration of FNCs cryopreserved according to the M1 caused the highest stimulation of Treg formation and improved clinical status of the recipients. Thus M1 causing the selective enrichment of FNCs by glial cells, provided a higher expression level of the *ido* gene, which might indicate the ability of cryopreservation to act as a factor of directed regulation of a bioobject state with the determined therapeutic characteristics.

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Conflict of Interest: None to disclose

P7 FREEZING CONDITIONS DETERMINE FUNCTIONAL POTENTIAL OF TUMOR CELLS

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The research was aimed to study the effect of freezing conditions on tumorigenic activity of Erlich carcinoma cells. The cells were cryopreserved in ascitic fluid with freezing rate of 1 deg/min down to -80°C and following immersion into liquid nitrogen (Slow Mode 1) and by means of plunging into liquid nitrogen (Rapid Mode 2). After thawing there was determined the quantity of cells in the sample and their viability by means of propidium iodide staining, content of $CD44^{high}$, $CD44^+/CD24^-$, $CD44^+/CD24^+$, $CD44^-/CD24^+$, $Sca1^+$ cells with flow cytometer. Cryopreserved and native Erlich carcinoma cells were cultured *in vivo* during 7 days in peritoneal cavity. The number of grown cells and their subpopulation composition were examined. The Mode 1 was found to preserve higher amount of nucleated cells than the Mode 2. Herewith Mode 2 versus Mode 1 caused higher death in $CD44^{high}$ cell suspension. Cryopreserved *in vivo* according to the Mode 1 the Erlich carcinoma cells formed in 2.8 times less cells for 7 days than native, moreover the content of all the subpopulations did not significantly change. The Mode 2 much stronger inhibited the growth of tumor cells (9 times) and redistribution of the cell subpopulations. The most significant of those was a 4-fold reduction of the amount of $CD44^{high}$ cells. Varying the cooling rates provides the possibility either preserve structural and functional characteristics of tumor cells, or inactivate their tumorigenic potential.

Source of Funding: This work was supported by the National Academy of Sciences of Ukraine (0117U000846).
Conflict of Interest: None to disclose

P8 INDIVIDUAL VARIABILITY ON POST-THAWED SPERM QUALITY IN SAMPLES PERFORMED INDIVIDUALLY OR POOLED

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Cryopreservation allows long-term preservation of sperm cells. However, variations between ejaculates from

different individual animals, in terms of composition of seminal plasma and spermatozoa cryo-sensitivity, could compromise post-thawed sperm survival in samples pooled from several males. The aim of this work was to determine the effect of individual variability on post-thaw quality in samples processed individually or pooled, with same volume and sperm concentration for each male. Sperm samples from three rams were collected by artificial vagina. Samples of each male were diluted in Biladyl® (20% egg yolk) to the same final concentration. Subsequently, one aliquot per male with the same volume was used to form the pool. Sperm parameters of individual and pooled samples were evaluated for both fresh and thawed semen. Mobility parameters were evaluated by computer-assisted sperm analysis and flow cytometry was used to assess sperm viability, acrosome status and mitochondrial activity. Results showed no significant differences ($P>0.05$) between males for most of the mobility parameters tested on fresh sperm. However, linearity showed differences ($P<0.05$) between individual and pooled samples after cryopreservation (50.04; 71.54; 64.65; 60.27). Straight-line velocity was significantly different ($P<0.05$) only among males (57.93; 105.55; 85.48; 79.33). There were no differences ($P>0.05$) in parameters related to cell viability, integrity of acrosome and mitochondrial activity for fresh and thawed samples in both individual and pooled samples. Only apoptotic cells were different ($P<0.05$) among males (13.96; 15.83; 19.34) in fresh semen while no differences ($P>0.05$) were found for thawed samples (11.43; 7.28; 13.79; 11.05). This study suggests that, while there was no individual variability on pre-freezing sperm for most of the parameters assessed, some parameters showed differences between animals on post-thaw quality even after pooled. Therefore, it is necessary to take care of samples performed by the pool of different individuals since may directly affect post-thaw sperm quality.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P9 INVESTIGATION OF ANTIFREEZE PROTEINS APPLICATIONS FOR CRYOPRESERVATION OF BIOLOGICAL SYSTEMS

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Antifreeze proteins (AFPs) are produced by various organisms as an adaptation to subzero temperatures. AFPs have potential applications in cryopreservation as they have the ability to inhibit ice growth. However, a major bottleneck which hinders the extensive use of AFPs in research is the lack of sufficient availability and high cost. Our research focuses on large scale production of recombinant AFPs, investigation of their effect, and underlying mechanism of action.

The fed-batch fermentation technique is employed to obtain high cell density cultures of E. coli producing various recombinant AFPs (Type III AFP, GFP-AFP III, MBP-TmAFP, GFP-RiAFP). A unique protocol was developed to maximize expression yields that reached 1-2 g/L. Production of such large amounts of AFPs enabled us to evaluate their potential application in cryopreservation of cells. We have examined the toxicity effect of Type III AFP and MBP-TmAFP on human epithelial colorectal adenocarcinoma cells (Caco-2) and immortal cell line derived from cervical cancer cells (HeLa). Without freezing, Type III AFP decreased cells viability at high concentrations (>0.62 mM), and had no toxic effect at concentration of 0.12 mM and lower. MBP-TmAFP at the concentration of 0.001-0.13 mM did not show cytotoxic effects. In cryopreservation, low concentrations of Type III AFP (1μM) and MBP-TmAFP (2.5 μM) with 2% of Me2SO showed a slight improvement on cell viability post thaw, compared to control. High concentrations of MBP-TmAFP (>30 μM) and Type III AFP (>10 μM) decreased cell viability post thaw. We surmise that addition of AFP at very low concentrations may further improve cell survival. In vitrification of 36% Me2SO and 1 μM AFP III solution the ice growth from ice nuclei during warming was retarded. Thus we assume that AFPs can diminish devitrification, alleviate ice recrystallization during warming, and increase viability post thaw.

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Conflict of Interest: None to disclose

P10 VITRIFICATION ON EPIDIDYMAL SPERM WITH SUCROSE OR TREHALOSE EXTENDERS IN DOMESTIC CATS

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Reproductive research in the domestic cat could be used as a model for wild felids. Traditional cryopreservation is not the only method used to preserve spermatozoa. Vitrification could be an alternative for cryopreserving sperm. The objective of the present study was to investigate the effect of two different extenders used in the vitrification method for domestic cat sperm. Thirty one (n=31) sexually mature cats were castrated. The sperm recovered was diluted 1:1 with Dulbecco´s PBS-BSA1% extender supplemented with different disaccharides, sucrose or trehalose. The vitrification was carried out in 0.25 ml straws with a total volume of 30 µl and a concentration of 50 x 10^6sperm/ml and directly immersed into liquid nitrogen. Fresh and post-vitrification epididymal sperm was assessed for motility (M), vigour (VI, intensity of movement), viability (V), spermatic morphology and acrosomal integrity (AI). The experiment had been previously approved by the IACUC

of FCV UNLP (26-APR-12). Significant differences were observed in all parameters evaluated between fresh and post-vitrification samples (IM 60±4,1 vs. 5,8±2,06; VI 4,1±0,46 vs. 2,27±0,49; V 62,5±3,2 vs. 41,1±4,6; AI 66,1±2,9 vs. 41,2±0,005; P<0.001). In the extender supplemented with sucrose the sperm M and VI showed higher values than with trehalose (10,5±1,43 vs. 1, 2±1,4; 3,4±0,4 vs. 1,20±0,4, P<0,001, respectively). No significant differences were detected among groups for sperm V and AI. Results support that cat sperm could preserve a percentage of the membranes intact after ultra-rapid freezing using sucrose or trehalose as cryoprotectants, and the best results were obtained when sucrose was used. Although sperm values after ultra-rapid freezing in cat are too low for a practical use in AI, the semen could be used for ICSI or IVF. To our knowledge this is the first study on sperm ultra-rapid freezing using trehalose carried on cat sperm. Thus, more studies should be done to improve M and VI post vitrification.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P11 SPERM ULTRASTRUCTURE AND ABNORMAL MORPHOLOGY IN DOMESTIC CAT (FELIS SILVESTRIS CATUS)

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The domestic cat is classified as teratospermic in relation to the high percentage of abnormal sperm in semen of healthy and fertile cats. The aim of this study was to describe sperm ultrastructure and abnormal morphology in epididymal and seminal sperm in domestic cats. Eighty short-hair mixed breed male cats, aged between 2 and 3 years, and weighing between 3 and 5kg were used. After bilateral orchiectomy, sperm samples were processed and cellular morphology evaluated. The sperm was processed and assessed using light microscopy (LM) and transmission electron microscopy (TEM). The experiment had the approval of the IACUC of FCV UNLP (26-APR-12). Domestic cat sperm consisted of the head and the tail or flagellum. The mean percentage of normal epididymal spermatozoa by LM was 54, 77±2,45% and for semen sperm was 56,63±2,83%. The study using LM allowed to observed abnormalities in the acrosomal as cysts, head swealling, macro and microcephaly, globose, piriformed, elongated and triple head; doubling tail, coiled tail, and Dag defect. Using the TEM the 58,8±4,98% of the heads and 83,5±6,35 % of the tails of the epididymal sperm showed a normal morphology. The 41, 61±6,44% of the heads and 73, 83±7,78% of the tails of seminal sperm showed a normal morphology. The study by TEM allowed to observed acrosomal alterations, Dag defect, nuclear vacuoles, double tails, double heads and incomplete axonemas. Our results show that a variety of sperm abnormalities were similar to those in other large domestic animals suggesting similar underlying pathogenesis of specific sperm defects and similar effects on the fertility. However, fertility tests are necessary to confirm this. To our knowledge this is the first study that describes sperm abnormalities of epididymal and seminal sperm in the domestic cats by LM and TEM.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P12 STUDIES ON VITRIFICATION OF ZEBRAFISH OVARIAN FRAGMENTS CONTAINING EARLY STAGE FOLLICLES

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This study was designed to explore the use of ovarian fragments as alternative to oocytes and embryos in the cryopreservation of fish female germplasm. Zebrafish ovarian fragments containing stages I and II follicles were studied by vitrification approach. The vitrification solution (VS) used was composed of 1.5 M methanol, 6.0 M ethylene glycol (EG) and 1.0 M trehalose in KCl buffer. An equilibration solution (ES) containing 1.5 M methanol in KCl buffer was also used in the study. The ovarian fragments were equilibrated in the ES for 30 minutes and thereafter treated with the VS and incubated for 5 and 10 minutes respectively at room temperature to evaluate the toxicity effect of the VS and determine optimum exposure time. The viability of stages I and II follicles attached to ovarian fragments were then assessed separately by trypan blue (TB) staining. Ovarian fragments for control were incubated in 90% L – 15 medium and assessed for viability after 10 minutes of collection. There was no significant difference between the ovarian fragments exposed to the VS for 5 minutes and the control group. Subsequently, the follicles attached to the ovarian fragments were treated with the VS and incubated for 5 minutes after 30 minutes equilibration in the ES and investigated for vitrification using unsealed 0.25 ml plastic straw and CVA65 cryoloop as loading devices. The viability of the follicles assessed separately by TB staining obtained for both loading devices after vitrification were significantly different from the control group. Stage II follicles had higher membrane integrity compared to stage I follicles. The viability was also higher with the CVA65 cryoloop compared to the 0.25 ml plastic straw. The cryoprotective effect of trehalose in combination with other cryoprotectants commonly used in zebrafish cryopreservation study is promising.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P13 REFRACTOMETRY: A TOOL TO MEASURE ME_2SO REMOVAL FROM CRYOPRESERVED TISSUE ENGINEERED PRODUCTS

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Me_2SO is the most commonly used cryoprotective agent (CPA) and although its benefit at cryogenic temperatures is well accepted, at physiological temperatures the presence of Me_2SO can be potentially toxic. When warming a cryopreserved tissue engineered product for delivery to a patient, it is important to ensure complete removal of Me_2SO.

The Bioartifical Liver Machine (BAL) comprises alginate encapsulated liver spheroids to support patient liver function in patients with acute failure. Cell doses (1.5L biomass) were cryopreserved by controlled slow cooling with 12% Me_2SO in UW solution. After rapid rewarming, it's essential to washout Me_2SO to prevent potential toxicity to the patient. We have developed washing protocols to remove all the Me_2SO and confirmed its removal by measuring the refractive index of the washes.

Refractive index (RFI) was used to measure Me_2SO in cell-free scaffolds of alginate beads. Standard curves of Me_2SO in each washing solution were made. To test CPS removal, 2mL of alginate beads were loaded with CPS at room temperature for 5 minutes. The beads were drained through a 100µm cell strainer and the supernatants collected for RFI. The beads were subsequently washed with reducing concentrations of glucose supplemented DMEM (1M to 0.5M to 50mM to 50mM) in three different bead:wash ratios (1:1, 1:2, 1:3). The supernatants from each wash were assessed by RFI to determine Me_2SO removal.

The 1:3 bead:wash ratio was most effective at removing Me_2SO from the alginate beads, removing 97% of the added DMSO. After 4 washes there were no traceable amounts of Me_2SO, at wash ratios of 1:2 & 1:3. At 1:1 ratio the wash contained 1% Me_2SO by volume. The 1:1, 1:2, 1:3 bead: wash ratios removed 87%, 90% & 97% of the total added Me_2SO respectively.

Measurement of RFI represents an easy and effective technique for ensuring removal of Me_2SO.

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Conflict of Interest: None to disclose

P14 EFFECT OF RECOMBINANT HUMAN SERUM ALBUMIN ON LYOPHILIZAED RED BLOOD CELLS

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The aim of this study was to investigate the effect of recombinant human serum albumin (rHSA) on the recovery rate and function of freeze-dried red blood cells(RBCs). RBCs were subjected to different concentrations of rHSA (1.25%, 2.5%, 5%, 7.5%)(w/v) and then underwent freeze-drying by freeze-dryer. Cell morphology were examined by microscope and number of cells were measured by automatic blood cell counter; free hemoglobin was measured by spectrophotometer. The level of ATP and SOD activity in freeze-dried red cells after rehydration were determined with the method of illumination. Under light microscope, the cell morphology was normal after rehydration. When the concentration of rHSA was 5, 7.5%, the recovery rate of RBCs reached 36.8±0.84%, 39.2±1.21% and the hemolysis rate of erythrocyte was 57.5%±6.32%, 51.2±1.54%, the corresponding ATP level and SOD activity per 10^6 cells were 392±100nM and 410±150nM, 91.7±4.3U/mgHb,93.1±3.6U/mgHb,respectively. To sum, rHSA affects freeze-dried erythrocyte preservation. A 5%, 7.5% concentration of recombinant human albumin has a better protective effect on freeze-dried red blood cells.

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Conflict of Interest: None to declare

P15 IN-VITRO CULTURE OF FREEZE-THAWED HUMAN OVARIAN CORTICAL TISSUE ON AGAR SCAFFOLD

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Nowadays ovarian tissue banks have been set up in many countries to improve chances of cancer patient's child bearing after cure. As transplantation of cryopreserved ovary intensify the possibility of malignant cells reintroduction, researchers are focusing more on ovarian tissue in-vitro culture methods. By tissue engineering and biomaterial improvement, embryologists attempt to use various scaffolds to mimic the ovarian extracellular matrix and preserve the tissue structure in the culture period. Our aim was to achieve an optimum culture system for human ovarian tissue to activate higher numbers of primordial follicles. Therefore, two different

cultivation scaffolds were compared. Donated ovarian tissues from 5 women were dissected and cryopreserved. One tissue strip of each patient was fixed immediately after thawing as the control sample. The cryopreserved-thawed human ovarian cortex strips were cultured on either matrigel coated insert or agar-soaked scaffold in a serum-free culture medium. After 7 days, the cultured strips were fixed and histologically analyzed. The growth evaluation consisted of follicular counts and classification. Although the number of developing follicles was higher in the cultured strips compared to the control group, no significant difference was observed between the two scaffold-cultured groups. Simultaneously, the number of primordial follicles decreased in scaffold-cultured groups compared to non-cultured control groups. Furthermore, the number of atretic follicles was higher after culture in both groups. Since there was no significant difference between these two scaffolds and also because agar is less expensive and more accessible, it is recommended as a suitable scaffold for human ovarian tissue culture.

Source of Funding: Not applicable
Conflict of Interest: None to declare

P16 CHROMOSOMAL ANALYSIS OF VITRIFIED OVINE OOCYTES AFTER INTRACELLULAR CALCIUM CHELATION BY EGTA

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Nowadays ovarian tissue banks have been set up in many countries to improve chances of cancer patient's child bearing after cure. As transplantation of cryopreserved ovary intensify the possibility of malignant cells reintroduction, researchers are focusing more on ovarian tissue in-vitro culture methods. By tissue engineering and biomaterial improvement, embryologists attempt to use various scaffolds to mimic the ovarian extracellular matrix and preserve the tissue structure in the culture period. Our aim was to achieve an optimum culture system for human ovarian tissue to activate higher numbers of primordial follicles. Therefore, two different cultivation scaffolds were compared. Donated ovarian tissues from 5 women were dissected and cryopreserved. One tissue strip of each patient was fixed immediately after thawing as the control sample. The cryopreserved-thawed human ovarian cortex strips were cultured on either matrigel coated insert or agar-soaked scaffold in a serum-free culture medium. After 7 days, the cultured strips were fixed and histologically analyzed. The growth evaluation consisted of follicular counts and classification. Although the number of developing follicles was higher in the cultured strips compared to the control group, no significant difference was observed between the two scaffold-cultured groups. Simultaneously, the number of primordial follicles decreased in scaffold-cultured groups compared to non-cultured control groups. Furthermore, the number of atretic follicles was higher after culture in both groups. Since there was no significant difference between these two scaffolds and also because agar is less expensive and more accessible, it is recommended as a suitable scaffold for human ovarian tissue culture.

Source of Funding: Not applicable
Conflict of Interest: None to declare

P17 CHALLENGES TO PRESERVE BOVINE FREEZE-DRIED FIBROBLAST CELLS AT ROOM TEMPERATURE FOR NUCLEAR TRANSFER

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Freeze-drying has been used for long-term storage of food, drugs, and yeast at ambient temperatures. Recently blastocysts were successfully developed by nuclear transfer (NT) using freeze-dried (FD) somatic cells in sheep, mice and pigs. These results showed that cells are dead biologically after freeze-drying, however, dead cells do not mean "dead DNA". FD somatic cells have been proposed as a new tool for the production of cloned animals, since it can overcome the disadvantages of the current cryopreservation. However, FD cells are difficult to preserve at room temperature. In this study, we investigated the effect of antifreeze proteins (AFPs) into buffer solution to preserve bovine FD fibroblast cells at room temperature and to produce blastocysts by nuclear transfer using FD cells. Bovine fibroblast cells were obtained from ear skin tissue. The cells were suspended into FD solution with or without 1% AFP 1 (NICHIREI), and lyophilized for 11.5 h. Then, FD cells were preserved at 20 degrees for 2, 5 and 7 days. After rehydration, the percentage of DNA damaged cells was evaluated by the alkaline comet assay. Furthermore, FD cells were injected into bovine enucleated oocytes. The rates of DNA damaged cells preserved for 2, 5 and 7 days with AFP 1 were 7.0, 11.3, and 14.7%, respectively. On the other hand, the rates of DNA damaged cells preserved for 2, 5 and 7 days without AFP 1 were 6.3, 21.8, and 46.5%. The blastocyst formation rates using FD cells preserved for 2, 5 and 7 days with AFP 1 were 7.4, 2.4, and 0%. No blastocyst was obtained from FD cells preserved for 2 day without AFP 1. In conclusion, DNA damage on bovine FD fibroblast cells could be decreased by AFPs, and blastocysts were produced by FD cells preserved in 1% AFP 1 solution after NT.

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Conflict of Interest: None to disclose

P18 WOULD ULTRA-DEEP FREEZER BE AN ALTERNATIVE FOR SEMEN STORAGE?

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This study was designed with the idea that using ultra-low temperature freezers could be a feasible alternative to for frozen semen storage at -152ºC. A total of 80 straws collected from 2 stud bulls on different days were used. Straws were divided in 2 so that each group had a sample of the same bull semen collected on the same day. The experimental group of the study consisted of semen stored at -152ºC for 1 week and control group consisted of sperm stored at -196ºC by conventional method. At the end of the experiment period, frozen semen in both groups were thawed and examined in terms of motility, proggressive motility and spermatozoa kinetic characteristics by CASA. Statistically, same group of unit for two different items were used. Such dependant samples result in statistically dependant results. Thus paired sample t test was used. As a result, the motility and progressive motility values (69.66%, 37.61%) of frozen bull semen stored at -196ºC were statistically higher than the sperm stored at -152ºC (54.54%, 28.72%) ($p<0.001$). Between the groups, the percentage of spermatozoa with hyperactivity was 30.79% and 25.61%, respectively ($p<0.05$). There was no statistical significance between the kinetics of spermatozoa and numerical values were close to each other ($p>0.05$). In conclusion, it was determined that the post-thaw bull semen kept at -152ºC had lower motility and kinetic values than the sperm stored in -196ºC. On the contrary, data which was obtained from the study showed that the semen stored at -152ºC stayed above the universal fertility limits. Repetition of similar studies and supplementation with fertility data will increase the accuracy of the results obtained.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P19 SUCCESSFUL STORAGE AND CRYOPROTECTION OF PROTEINS

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Ice formation and growth is a major problem for many different fields including food science, mechanical engineering, agriculture and cryobiology, though little is understood about the underlying mechanisms. Proteins, focused on here, are widely used in molecular biotechnology and as therapeutics, but generally are stored in high concentrations of solvents or osmolytes such as glycerol, which also negatively affect protein function due to cytotoxicity. Insulin is a biologic therapeutic for the treatment of diabetes and is known to irreversibly aggregate upon ‘shaking’ or freezing. Green fluorescent protein (GFP) is a commonly used laboratory reagent for monitoring dynamic processes but also as a fusion tag. When inactivated/denatured there is a reduction in fluorescence, which can be easily monitored. Here we show that a unique antifreeze protein-inspired, ice-recrystallization inhibiting (IRI) polymer can stabilize proteins against stress. Here, ice recrystallization inhibiting polymers are used to reduce ice crystal growth during freezing, thereby preventing protein aggregation and deactivation, as well as preventing aggregation during other stresses. Dynamic light scattering (DLS) was used to measure the aggregation of insulin and fluorescence microscopy was used to measure inactivation of GFP over consecutive freeze/thaw cycles as well as continuous shaking and whether any protein-inspired polymers prevented damage. Different polymer formulations were compared for their protective effect against stressors and also preventing insulin aggregation as well as limited loss in fluorescence compared to proteins in buffer alone. The approach we used is simple and can be applied to other proteins without requiring complicated synthesis or the creation of new therapeutic entities.

Source of Funding: European Research Council
Conflict of Interest: A patent has been filed on this work

P20 MITOCHONDRIAL ACTIVITY IN NATIVE AND DEVITRIFIED BOVINE OOCYTE DURING IN VITRO MATURATION

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The objective of study was to evaluate the effect of pretreatment of cumulus-oocyte complexes (COCs) by fluid from the follicles (FF) d ≤3mm before vitrification on the mitochondrial activity (MA) in devitrified oocytes. MA was evaluated in: native oocytes; devitrified oocytes; devitrified oocytes pretreatment by FF (120 min, 37ºC).Vitrification was performed by equilibration of oocytes in: CPA1:0.7 M dimethylsulphoxide (Me2SO)+ 0.9 M ethylene glycol (EG), 30 sec; CPA2:1.4 M Me2SO + 1.8 M EG,30 sec; CPA3:2.8 M Me2SO + 3.6 M EG + 0.65 M trehalose,20 sec and loading into straws. After thawing COCs washed in 0.25 M,0.19 M and 0.125 M trehalose in TCM-199 and finally in TCM-199. COCs were cultured 24 h in TCM 199 with 10% FCS, 50 ng/ml prolactin, 10^6 granulosa cells /ml. MA was measured by fluorescence probe MitoTracker Orange CMTM Ros, intensity of fluorescence expressed in μA/oocyte, chromatin status have evaluated by Hoechst 33342 (H.Torner et al., Reprod Dom Anim 42, 176–183, 2007). Oocytes were examined using confocal laser scanning system Leica TCS SP5. Chemicals were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by Student's t-test. Chromatin status and MA of 295 oocytes were evaluated (in 4 replicates, 21-24 oocytes/group).

Before cultivation level of MA in native oocytes was significantly higher than in devitrified oocytes (337±17.4μA vs.87±9.2μA and 171±14.5μA, P<0.01). Pretreatment oocyte with FF increased level of MA in oocytes on metaphase-I and II stages (131±10.9μAvs. 201±8.7μAand 109±9.9μAvs. 151±12.8μA, P<0.01). There *were no* differences in the level of MA at metaphase I and metaphase II stages in native oocytes and in oocytes that have pre-treatment by FF (198±12.3μA vs. 201±8.7μA and 148±12.1 vs. 151±12.8μA). In conclusion: treatment of oocyte by FF prior to vitrification has a positive effect on the mitochondrial function in devitrified oocyte.

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Conflict of Interest: None to disclose

P21 AREA OF RAM SPERM HEAD MODIFIED DURING THE ADDITION OF CRYOPROTECTANT AGENTS

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Sperm freezing procedures have considerable variations regarding the steps being employed for addition of cryoprotectants, cooling and freezing-thawing. The area of the sperm head (ASH) is closely related to cell volume, so it was determined for the study of volume changes during the dilution and semen cooling. The effects of the addition of glycerol (4%) and/or trehalose (100 mOsm), either at 30 or 5°C on ASH, were evaluated. The addition of cryoprotectants was observed at 30°C initial time (T_0), at 5°C-90 min (T_{90}) and/or at pre-freezing , 5°C-94 min (T_{pf}). The ASH was determined using Rose Bengal dye and the NIS-element AR® program. At T_0, not differences were observed between cryoprotectants, with an average value of 34.96 ± 0.39 μm^2. At T_{90}, all treatments significantly increased the ASH with respect to the control. At T_{pf}, all treatments showed an increase of ASH, similar to that registered at 90 min, except for glycerol 30°C + trehalose 5°C treatment, which reduced this parameter to values similar to the control. The analysis of the addition of cryoprotectants at T_{pf} with respect to those at T_0 allows to affirm that the diluents containing trehalose reduce the flow of water into the sperm cells, while the diluent containing only glycerol, added at T_{pf}, showed a significant increase in ASH, and consequently an increase in cell volume (35.52 ± 0.33 μm^2 at T_{90} vs. 36.89 ± 0.32 μm^2 at T_{pf}, $p < 0.05$). When the cells are immersed in a solution with glycerol, in a few seconds the cell loses water. The presence of a non-permeable solute such as trehalose in the diluent at T_{pf} exerts an osmotic counterforce such that the volume changes produced by glycerol are reduced. This condition would be more favourable to support the freezing-thawing process.

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Conflict of Interest: None to disclose

P22 DEVELOPING A CRYOPRESERVATION PROTOCOL FOR AVOCADO (PERSEA AMERICANA MILL.) SHOOT TIPS.

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Avocado (*Persea spp.*) diversity is traditionally maintained in field collections because seeds are highly heterozygous and recalcitrant to long-term storage. However, field banks are constantly exposed to abiotic and biotic stresses. Moreover, the size of the gene pool, number of replications and quality of maintenance are restrained by the local environmental conditions, space and funding. Cryopreservation offers a necessary, complementary method that is safe, cost-effective and allows for the long-term storage of *Persea spp*. *in vitro*. Although cryopreservation of somatic embryos has been reported for *Persea* germplasm, they are heterogeneous to their mother-stock. Cryopreservation of shoot-tips of *Persea* spp is an alternative for the long-term conservation of clonal collections, thus maintaining the genetic identity of mother-stock trees. One critical problem in developing a cryopreservation protocol for avocado shoot tips is overcoming oxidation caused by the abiotic stresses applied during the cryo-procedure. This work focused on the regeneration of shoot tips dissected from in vitro shoots of avocado cv 'Reed'. We studied the effect of various antioxidants (ascorbic acid, PVP, citric acid and melatonin) on reducing browning of fresh and treated shoot tips (vitrification solutions). Shoot-tips were scored for browning at 0, 2, 3 and 4 weeks and results were also recorded after 8 weeks for shoot-tip survival and regrowth, biomass and water content. Ascorbic acid at 100 and 250 mg/L reduced browning of tips to the greatest extent. Shoot-tips exposed to 1.5 g/L PVP showed normal growth and reduced browning. However, tips treated with citric acid showed vitrification and abnormal growth and while melatonin at 0.2μM was effective in controlling browning, the shoots were stunted and lacked vigour. Moving forward ascorbic acid at 100 and 250 mg/L was selected to be used in regrowth media for further optimization experiments.

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Conflict of Interest: None to disclose

P23 OVARIAN TISSUE CRYOPRESERVATION BY STEPPED VITRIFICATION AND VIABILITY ANALYSIS

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Nowadays cancer is the second leading cause of death globally, and was responsible for 8.8 million deaths in 2015 (WHO). However advances in diagnosis and treatment have increased the life expectancy of premenopausal women. Ovaries are very sensitive to chemotherapy and different fertility preservation possibilities can be offered, but ovarian tissue cryopreservation is the preferred option.*Slow-freezing* technique is, currently, the most popular procedure used. This process has been carried out with an unchanging concentration of cryoprotectant agent (specifically 10% Me_2SO) and cooled down at 0.3 ºC/min. With this approach, after the transplant most of the follicles become damaged and unusable and a low efficiency (5-10%) is obtained, even though the technique is standardized and is carried out under the best conditions.The purpose of this research was to provide a new method which could overcome these obstacles. This technique was called *stepped-vitrification* and the viability of the whole procedure has been compared with the results obtained from the *slow-freezing* method and with a control group which did not undergo any cryopreservation process. *Stepped-vitrification* consists in slow decreasing the temperature while increasing the Me_2SO levels. Moreover, a 50% Me_2SO concentration could be reached, which would prevent ice nuclei formation, without severely affecting the tissue, as other protocols do. For this reason, a new device has been designed to introduce simultaneously several fragments of tissue and in each solution. After the cryopreservation process, samples were incubated for 2h at 37 ºC; this allowed the recovery of the samples to their basal metabolic rate. Finally, they were fixed with paraformaldehyde 4%. A wide variety of histological and immunochemistry analysis were conducted, mainly comparing the number of viable and degenerated follicles in the cryopreserved and control groups. Preliminary results are very promising showing a good efficiency in this technique.

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Conflict of Interest: None to declare

P24 INDUCTION HEATING OF CRYOPRESERVED OVARIAN TISSUE

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Due to the great advances in chemotherapy and radiotherapy, the survival rate of cancer patients has increased during the last decades. However, ovarian functional recovery after cancer treatment is still an issue, due to the loss of follicles which results in an impaired hormone balance and infertility. Thus, ovarian dysfunction does not only affect the reproductive capacity of the patient but may also induce psychological side-effects as well as cause life-threatening conditions. Ovarian tissue cryopreservation before cancer treatment is considered a promising means of preserving fertility and may be seen as the solution to providing patients with a normal and improved life. In this work, we undertook the challenge of improving the current methods for ovarian tissue cryopreservation. Cow ovarian tissue samples (5x5x1 mm^3) were cryopreserved through the technique Slow Freezing and, subsequently, rewarmed by using inductive heating of magnetic nanoparticles. The rewarming device consisted of a 6 cm diameter, 7-turn coil and 300W RF inductive heating coil system operating at a frequency of 100 KHz. Moreover, histological and immunohistochemistry analysis were carried out in order to test the viability of the samples after the whole cryopreservation process. We report 10-100 times faster and more uniform warming rates than that with other proven approaches. In addition, viability tests showed a reduction of the thermal mechanical stress and cracks in the ovarian tissue. These results are evidence of the suitability of the inductive heating by using magnetic nanoparticles as a favorable and promising method for ovarian tissue cryopreservation. This technique would certainly be a step forward towards treatment for cancer and will make Organ and Tissue Banking a reality.

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Conflict of Interest: None to disclose

P25 WATER CONTENT OF STRYCHNOS GERRARDII DETERMINES VITRIFICATION IN CRYOPRESERVATION: BIOPHYSICAL APPROACHES

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Vitrification is an effective freeze-avoidance mechanism and living tissue cryopreservation is, in most cases, relying on it. As a glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to metabolic inactivity and stability over time. However, the various procedures (prior to, and immediately after) ultra-low temperature storage generate reactive oxygen species (ROS) which often preclude success, in most cases associated with lethal effects on the shoot apical meristem. Testing the success of new cryopreservation protocols, or their adaptation to different species, varieties or tissues, by checking the actual recovery after cryostorage, is long, costly and tedious. Not only a considerable time is involved, but for obtaining statistically significant data, a large number of specimens must be employed. To check for successful vitrification, simplified biophysical approaches can reduce length and cost of the study.In order to check the effect of cathodic water (a treatment proposed to reduce oxidative damages) on the correct vitrification of *Strychnos gerrardii*, zygotic axes submitted to a frequently employed cryopreservation protocol were monitored through its different stages by low-temperature scanning electron microscopy (cryo-SEM) and differential scanning calorimetry (DSC). Axes were immersed for 30 min in cathodic water following excision and after the dehydration stage in the protocol. DSC allowed to quantify the frozen water content reduction and to detect the eventual glass transition. In parallel, the vitrification of the specimens cellular content was ascertained by cryo-SEM. Cathodic water treatments were shown not to affect negatively the *Strychnos gerrardii* zygotic axes vitrification. Results show how tissues at intermediate treatment steps (with decreasing water content) develop ice crystals during liquid nitrogen cooling, while tissue vitrification is successfully achieved after the final protocol stage.

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P26 EFFECTIVE CRYOPRESERVATION APPROACH FOR ANDEAN POTATO SHOOT TIP IN VITRO CULTURE

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A droplet vitrification protocol was employed to set up the long term conservation of the potato collection at the Potato Genebank (EEA Balcarce-INTA, Argentina). The study objective was to evaluate the effects of different pre and post-treatment conditions for shoot tips regeneration during cryopreservation. Plantlets of the Andean potato variety 'Chaqueña' were cultivated on MS medium with 25 $g.L^{-1}$ sucrose, 4.5 $g.L^{-1}$ phytagel and vitamins. Nodal segments were precultured in two conditions, **A**: 22°C, 16 h/8 h (control) and **B**: 22°C, 16 h and 10°C, 8h, both photoperiods with light intensity 45 $\mu mol.m^{-2}.s^{-1}$, for four weeks. Isolated apical shoot tips were cultivated overnight on MS medium containing 0.3 M sucrose, then transferred to loading solution for 20 min, and dehydrated with PVS2 for 20, 25 and 30 min. Shoot tips were placed in PVS2 droplets onto aluminium foil strips, then plunged into cryovials with liquid nitrogen. The recovery was tested in five conditions (in dark; **I**: 5, **II**: 7, **III**: 14 and **IV**: 21 days, or **V**: 7 days dark + 7 days diffuse light) and tips with and without cryogenic exposure was assessed after 8 weeks. Shoot tips and cryoprotecting solutions were analysed by Differential Scanning Calorimetry to investigate their thermal behaviour and the eventual ice formation during the protocol. Also, the frozen and unfrozen water fraction was determined for all stages at each protocol.
The **B** pre-treatment increased the samples quality after post-cryo recovery. The best regrowth results were for **III** and **V** conditions, with 40 and 58 %, respectively. Dehydration during 20 min was enough to vitrify the samples during cooling to liquid nitrogen temperatures, as detected by DSC. However, survival and re-growth results were lower than those obtained with 25 min in PVS2, attributed to traces of frozen water present in samples dehydrated at 20 min.

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P27 USE SOME LYOPROTECTANTS FOR SHELF LIFE IMPROVEMENT OF A LYOPHILIZED INTRAVESICAL IMMUNE BCG

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Intravesical immune BCG (Bacillus Calmette-Guerin, strain: 1173p2) vaccine as an immune response modifier agent against superficial bladder cancer is being produced in Iran with high potency in form of ready to use (suspension) at once but also requires storage and

distribution in frozen state with extra care and cost. Since successfulness of lyophilization is a strain and technical dependent process, efficacy of some admissible lyoprotectants (LPs) including sucrose, lactose, trehalose, glucose, dextran-40, and monosodium L-glutamate (MSG) upon biological and physical characteristics of the lyophilized BCG bacilli was investigated with aim to provide a more stable lyophilized product through and after lyophilization and, during long storage period. Bacterial bulks was formulated with different amounts of Lps and submitted to freeze drier with three different primary drying stages ranged below the theoretically glass transition temperature (Tg) of the bulk for the best results. Required characteristics have been significantly improved by a sample contained a combination of lactose, MSG, dextran-40 and tween-80 so that the satisfactory results for viability, moisture content, appearance, reconstitution time, and shelf life period were achieved.

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Conflict of Interest: None to disclose

P28 DEVELOPMENT OF 3D PRINTED MICROFLUIDIC PERFUSION DEVICES TO MEASURE CRYOPROTECTANT PERMEABILITY

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We need new cryopreservation protocols with novel cryoprotectants since many endangered Australian plant species are resistant to existing cryopreservation protocols. Although over 40 molecules have been identified as having some cryoprotective ability, most current cryopreservation media are based on the cryoprotectants such as Me2SO and Glycerol, and the toxicity of these molecules remains a serious issue. So, it is important to study the biophysical response of the cells to different potential cryoprotectants in order to develop "cell-type specific" novel cryopreservation protocols. One of the key parameters in a molecule's cryobiological potential is cell permeability. Various devices have been developed using conventional photolithography techniques to measure the permeability. However, this technique has a number of limitations for applications related to biological systems. The development of soft lithography based on Poly Di Methyl Siloxane(PDMS) by the Whitesides group has complemented conventional photolithography and enabled more microdevices to be fabricated for biological applications. These devices have been employed in the study of cell osmotic behavior for many years. One disadvantage of soft lithography is that it requires photomasks to create the initial pattern, which are comparatively expensive for pattern features <25µm. In the past decade, the technological advances in 3D printing have enabled the easy fabrication of inexpensive microfluidic devices. Using 3D printing, a master mould can be made which can be repeatedly used for creating PDMS replicas with soft lithography. Here we report the development of 3D printed microfluidic perfusion devices for the investigation of biophysical response of individual cells to various cryoprotectants. Using these devices, we could successfully trap individual cells and analyse the cell shrink-swell kinetics with improved accuracy.

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Conflict of Interest: None to disclose

P29 PRESERVING BACTERIA WITH OLIGOSACCHARIDES AND ECO-FRIENDLY PROCESSES (PREMIUM)

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Many microorganisms' potential remains unexploited due to the current inability to preserve them at an industrial scale. Lactic acid bacteria (LAB), are a group of microorganisms widely used for producing a wide diversity of fermented foods. The market of concentrated cultures (starters) is continuously growing due to the development of health benefits products, the use of plant origin proteins as fermentation substrate (instead of milk proteins) and also to lactic acid bacteria's ability to convert by-products of green chemistry. The manufacture of starters requires the application of successive operations that generate stresses, including storage and delivery, as well as reactivation (thawing, rehydration). These operations usually lead to cellular damage and loss of functionalities, in particular following the stabilisation processes: freezing, freeze-drying, spray drying. In addition, these processes generate environmental impacts, due to energy consumptions and use of the cold chain. Taking this into account, the process of LAB preservation needs to be completely revisited integrating all the steps and the three dimensions involved: product quality, process efficiency and environmental impact, in order to develop original and innovative alternatives to companies and society. In this context, a four-year multidisciplinary project funded by the European Commission, PREMIUM project, involving academic and industrial partners from France, Argentina, Portugal, Spain and the United Kingdom, proposes to develop new strategies to preserve lactic acid bacteria from laboratory to industrial scale. Using multicriteria analysis will

identify the most promising strategies for industrial eco-friendly preservation of micro-organisms. Moreover, after validation from lab to industrial scale on a small number of strains, other micro-organisms and mammalian cells will be tested. The initial emphasis on mammalian cells will be on stem cells and mesenchymal cells for clinical application. The project will thus pave the way for future commercial exploitation of new protective formulations (composed of oligosaccharides), by extending to other cells and by drawing up a feasibility study for spin-off activities regarding the sourcing of new protective molecules to the standards required for clinical application.
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Conflict of Interest: None.

P30 EFFECTS OF CRYOPROTECTANTS ON THE HATCHING RATE OF *COLOSSOMA MACROPOMUM* EMBRYO AFTER COOLING

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Successful cryopreservation protocols for fish embryos remain absent; however, the embryo cooling technique has shown acceptable results with efficiency and practical application. Temperature reduction causes lethal damage to cells and the cryoprotectants are essential for cell survival during freezing and rewarming. This study aimed to assess the effects of eight cryoprotectant solutions on the cooling of tambaqui (*Colossoma macropomum*) embryos. After artificial fertilization, the embryos were placed into a 200 L incubator where the embryo development was observed. After six-hour fertilization at 27 ± 0.5 °C, 75 to 90% epiboly stage was visible and viable embryos were selected. Viable embryos, in the post-gastrula phase, were exposed for two minutes to several different cryoprotectant solutions. Cryoprotectant solutions included permeating cryoprotectants (ethylene glycol, glycerol, methanol and dimethyl sulfoxide in concentration of 10%) combined with two levels of sucrose (8.5 or 17%). Embryos were placed into 0.5 mL plastic straws and immediately began the gradual reduction of temperature (1°C/min) until reaching -8ºC. After six-hour storage, embryos were cultured. Hatching rate was significantly higher ($P<0.05$; 77%) in the solution composed by 17% sucrose and 10% methanol when compared to other treatments, suggesting that on *C. macropomum* embryos the cryoprotectant solution containing sucrose (17%) and methanol (10%) reduces the cell damage caused by cooling. After hatching, all embryos showed larvae with regular and vertical swimming. All treatments containing cryoprotectants showed translucent embryos, with no whitening after warming. White coloration characterizes ice crystal formation; therefore, the cryoprotectant agents protected the embryos from crystallization. The results of the present study confirm previous studies that showed a significantly higher embryo hatching rate when sucrose (0.5 M) was added to metanol (2 M). Therefore, the addition of sucrose to methanol was essential for tambaqui embryo survival at low temperature storage.

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Conflict of Interest: None to disclose

P31 CYSTEINE SUPPLEMENTATION ON THE QUALITY OF CRYOPRESERVED SPERM OF SOUTH AMERICAN CATFISH

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The aim of this study was to assess the effects of different concentrations of cysteine on post-thaw sperm motility, morphology and membrane integrity in the South American silver catfish (*Rhamdia quelen).* Sperm collected from nine fish, was cryopreserved in extenders (50 g/L, powdered milk 50 g/L and methanol 100mL/L diluted in distilled water) containing different cysteine concentrations (0, 2.5, 5, 10 and 20 mM). Sperm pool, with motility greater than 80%, was diluted at the ratio of 1:3, filled in 0.25 mL straws, frozen in nitrogen vapor, and plunged into liquid nitrogen. After thawing (25°C for 10 s), motility (total motile, 0-100%), morphology (Bengal Rose Staining) and membrane integrity (Eosin-Nigrosine) were measured. The concentrations tested affected the parameters of motility and sperm morphology, but not membrane integrity ($P=0.8164$). The higher concentration of cysteine (20 mM) had very low motility (8.75%). The best results for sperm motility were obtained in the control (0 mM; 35%) and in the solution containing 10 mM (40%). The lowest percentage of normal cells was observed in the cryoprotectant solutions containing 2.5 (17.15%) and 5.0 mM (17.61%). However, there was no difference among control (0 mM), 10 and 20 mM treatments in evaluation of normal morphology. Samples with 5.0 mM showed the highest percentage of strongly coiled tails (15.86%). Samples with 2.5 mM showed the highest percentage of fractured tails (10.39%) and macrocephaly (31.54%) and the lowest percentage of bent tails (8.89%). However, samples containing 20 mM showed the highest percentage of distally coiled tails (19.58%). The most frequent types of morphological alteration were macrocephaly and distally coiled tails. The findings of this study showed that addition of cysteine (2.5, 5, 10 or 20 mM) has no beneficial effects to silver catfish sperm motility, morphology or membrane integrity during frozen-thawed process.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P32 CRYOPRESERVATION OF MUSSEL TROCHOPHORE LARVAE AND LONG-TERM EFFECTS: FROM LARVAL REARING TO SETTLEMENT.

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Due to the economic importance of the mussel *Mytilus galloprovincialis* in the Spanish aquaculture of molluscs, there is a high interest in improving the methods of production of breeding seed, to ensure the supply of biological material in good conditions and to avoid the environmental problems that suppose an economic risk for this sector. The aim of this study was the development of a cryopreservation protocol for the larvae of *Mytilus galloprovincialis*, Spain's main molluscs species and long-term study of cryopreservation effects on their culture. Post-freezing survival and resistance studies to the toxicity of cryoprotective agents are in higher advanced stages of development, therefore we chose throcophore larvae development stage (24h p.f) and we evaluated the effect of Ethylene Glycol (EG) for cryopreservation following the preliminary protocol that E. Paredes developed for *M. galloprovicialis* mussel trochophores in 2013 and extended the study to larval rearing and settlement to juveniles. The cryopreservation protocol consisted on: holding at 4ºC for 2 min, then cooling at 1ºC / min to -10ºC, holding for 5 min to ensure seeding, then cooling at -35ºC with a rate of 1ºC / min, then plunging into liquid nitrogen. Thawing was performed on a 28ºC water bath. With this protocol E. Paredes reached a 48.9 ± 7.6% D-larvae (48 hours incubation) with 10% EG + 0.2 M Trehalose (TRE). The initial test of 2013 only studied the trochophore larvae until they reached the D-stage (48h old), here we furthered the study and also carried out a larval rearing with cryopreserved trochophore larvae in comparison to fresh larvae. This experiment allowed a comparative post-freezing study. The data revealed survival until settlement of the cryopreserved larvae of 27.74%, Over time, growing-of control larvae was faster than growth of cryopreserved larvae (3.91% smaller), Attending to settlement, we obtained a 64% of cryopreserved larvae respect to the control.

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P33 EFFECTS OF ECHINACEA EXTRACT ON THE FREEZABILITY OF RAM EPIDIDYMAL SPERM CELLS

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During last years, many experiments reported from the antioxidant activity of Echinacea purpurea extract. In general, antioxidant activity of E. purpurea extract is high then of other medicinal and aromatic herbs which could be ascribed to the polyphenolic components, such as flavonoids phenolic acids or phenolic diterpenes. The objective of the present study was to evaluate the ability of the E. purpurea extract to protect ram sperm cells from freeze- thaw damages. The sperm cells separated and recovered in the laboratory by flushing from ten ram testes, and cryopreserved in tris- citrate solution supplemented with various concentration of E. extract (5-10 and 20 mg/L). The motility and velocity parameters, plasma membrane functionality, viability, response to hypo osmotic swelling test (HOST) and malonaldehide (MDA) concentration were as sperm cell characteristics assessed. The results indicate that the addition of 10 mg E. purpurea extract to the extender presents significantly an enhancement of total motility (81.34±1.2%), progressive motility (50.25±0.14 %), average path velocity (VAP, 57±0.36, µm/s), as compared to the other treatments. Regarding the plasma membrane functionality, the extender supplemented with 20 mg E. purpurea extract increased significantly (90.04±2.4%) as compared to extenders containing 5 (81.06±1.9%) and 10 mg (82.04±1.6%) E. purpurea and control (81.79±1.3%). No significant differences were observed for the percentage of acrosome integrity. between the treatments. Additionally, the obtain results showed that MDA concentration was significantly lower in extenders added 10 mg (0.0055±1.55 nmol/ml) and 20 mg E. purpurea (0.0057±1.55 nmol/ml) treatments as compared to 5 mg E. purpurea (0.0078±1.55 nmol/ml) and control (0.0080±1.55 nmol/ml), respectively. Our data imply that supplementation of E. purpurea extract in an appropriate level has a beneficial effect on post- thawed ram sperm cell parameters without any deleterious effect on sperm motility and velocity. However, further studies are necessary to confirm present findings.

Source of Funding: University of Kurdistan
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P34 EFFECT OF DIFFERENT CONCENTRATIONS OF PENTOXIFYLLINE ON GOAT SPERM AFTER FREEZING-THAWING

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The goal of this study was to investigate the effect of different concentrations of pentoxifylline (PTX) on frozen-thawed goat spermatozoa. Semen was collected from four mature goats (3-4 years, 55-65 kg) by electroejaculation. Only semen samples with motility more than 70% and sperm concentration more than 3×10^7sperm/ml were pooled and used for cryopreservation. The pooled samples were then divided into four equal parts, diluted with Tris-based extender containing different concentrations of PTX (0, 3, 6, 9 mM), filled in 0.25-ml French straws, and then frozen 4 cm above nitrogen vapor after cooling to 5˚C in 2.5 h.

The frozen spermatozoa were thawed at 37°C for 30 sec. The thawed semen samples have been evaluated for total motility and progressive motility by CASA, and viability using eosin-nigrosine staining method. The results showed that the treatments supplemented with PTX indicate significantly (P<0.05) an increase in progressive motility compared to control. Moreover, the observed data enhanced significantly (*P*<0.05) total motility and viability when extenders contained 3 and 6 mM PTX, compered to control and diluent added 9 mM PTX. However, regarding total motility and viability, the results of control and diluent contained 9 mM PTX present significantly (P>0.05) no differences. Subsequently, comparing the diluents supplemented with 3 concentrations of PTX demonstrated that 3 mM was the best extender, and presented the most beneficial effect on frozen-thawed goat spermatozoa. Therefore, we suggest using this concentration for freezing of goat spermatozoa, however, further studies seem to be necessary to confirm present findings.

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P35 SPERM SELECTION BY BOVIPURE™ AFTER THAWING IMPROVES QUALITY PARAMETERS IN WILD SHEEP

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This study examines the effectiveness of Bovipure™ density-gradient centrifugation (DGC) in both ejaculated and epididymal frozen-thawed sperm samples from European mouflons (*Ovis musimon*) using two different freezing methodologies. Samples were cryopreserved in a TEST based extender (TES, Tris, glucose, 6%-egg-yolk) by two methods: conventional freezing in straws (CF, glycerol as cryoprotectant) and ultrarapid freezing in pellets (UF, sucrose as cryoprotectant). CF and UF frozen-thawed samples were subjected to the DGC (centrifuged at 300g for 20min) and the pellet containing the purified sperm was recovered. The following quality parameters were assessed before and after the purification: sperm motility, viability, acrosome integrity and morphological abnormalities. Concerning ejaculated sperm, the viability and acrosome integrity increased after purification in CF (40±3.2% *vs* 51.8±3.5% and 82±2.2% *vs* 91.7±1.4%) and UF (20±2.1% *vs* 27.8±3.1% and 72±2.9% *vs* 84.3±1.9%) samples (p<0.05). Curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) decreased while linearity (LIN) and wobble (WOB) increased after purification of FT ejaculated sperm. Regarding epididymal sperm, total motility (CF: 30.5±4.4% *vs* 48.8±7.0%; UF: 21.7±2.4% *vs* 43.2±7.4%), progressive motility (CF: 12.0±2.6% *vs* 30.6±4.9%; UF: 8.7±1.6% *vs* 25.6±5.0%), straightness, LIN and WOB increased after purification (p<0.05). Moreover, viability increased in purified UF samples (18.1±1.8% *vs* 37.9±6.3%; p<0.05). The decapitated sperm percentage increased after purification in UF ejaculated (1.7±0.7% *vs* 5.3±1.4%) and in epididymal sperm (CF: 3.7±1.6% *vs* 10.8±2.7%; UF: 5.1±1.7% *vs* 16.2±3.5%; p<0.05). To summarize, the acrosome integrity increased after the DGC in ejaculated sperm but not in epididymal sperm while total and progressive motility increased in epididymal sperm but not in ejaculated sperm. CASA kinetic parameters were modified after the DGC in epididymal sperm and CF ejaculated sperm but not in UF ejaculated sperm. In conclusion, the use of Bovipure™ is recommended to increase the post-thaw quality of wild sheep sperm.

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P36 DEVELOPMENT OF MOLECULAR MARKERS FOR TELEOST BREEDER SELECTION PREVIOUS TO CRYOPRESERVATION

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The availability of molecular markers in sperm samples able to predict reproductive success and successful embryo development it is highly valuable in reproductive management. Cryopreservation could increase the presence of reactive oxygen species and could decrease overall sperm quality after the process. Interestingly molecular alterations have been also described in the spermatozoa after freezing-thawing even when optimized cryopreservation protocols are used. For these reasons, the use of optimal sperm samples for gene banking purposes it is relevant and highly recommended. In this work we study the validity of different RNAm and miRNA for their use as molecular markers for teleost sperm samples. We used *Danio rerio* as a model species and *Solea senegalensis* as an example of species with high commercial value. The set of mRNA and miRNA derived from this study could be used as complementary analysis to determined the molecular status of a specific sperm sample, together with traditional analysis such as motility and viability.

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P37 EFFECT OF SPERM QUALITY AND GERM CELL CRYOPRESERVATION ON DNA METHYLATION PATTERN IN TELEOST

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Cryopreservation is a technique commonly used for gene banking purposes in aquaculture. Molecular modifications potentially produced by cryopreservation on key transcripts could be relevant for fertilization success after cryopreservation. The reduction of some transcripts or even the elimination of some of them as a consequence of cryopreservation has also been reported by our group in different species. DNA methylation is a major epigenetic modification that plays an important role in regulating gene expression. Bisulphite sequencing analysis of CpG methylation in the promoter of *cxcr4b*, *pou 5f1*, *sox3* and *vasa* genes in fresh and cryopreserved zebrafish genital ridges demonstrated that cryopreservation could produce an increase in methylation that could be correlated with gene downregulation. When global methylation of teleost sperm samples (from *Danio rerio* and *Solea senegalensis*) with different qualities was analyzed using a commercial kit "epiJET DNA Methylation analysis kit" that uses the MspI and HpaII restriction enzymes, no significant differences were found, probably due to the high level of methylation in sperm cells. To provide more precise information, we analyze embryo methylation pattern after fertilization with different quality sperm samples, in order to discard the possibility that molecular alterations that could not be detected using this technique in the hypermethylated sperm cells, could be altering embryo development after fertilization.

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P38 KIDNEYS DEVELOPED FROM VITRIFIED METANEPHROI EXHIBIT ENDOCRINE FUNCTIONALITY AFTER ITS TRANSPLANTATION

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Renal transplantation represents the optimal treatment to restore lost renal function in patients with end-stage kidney disease (ESKD). However, kidney transplantation is currently compromised by donor availability, risks of graft rejection and by immunosuppressive therapy toxicity. Additionally, the lack of successful organ preservation protocols aggravates these situation, because long-term conservation is essential to organise staff, facilitate the organ transport, and to perform the necessary laboratory tests. Metanephroi from 15-days-old embryos were recovered and vitrified following the minimum essential volume method using Cryotop® as a device and VM3® as vitrification solution. After 3 months of storage in liquid nitrogen, 18 metanephroi were warmed and transplanted using minimally invasive laparoscopic surgery into retroperitoneal fat of 5-month-old immune-competent New Zealand rabbits. In the same way, 16 fresh metanephroi were transplanted. Twenty-one days after transplantation, developed kidneys were recovered and evaluated morphologically, histologically and endocrinologically (mRNA). The primers used were: Renin forward primer: 5′-GGGACTCCTGCTGGTACTCT-3′, Renin reverse primer: 5′-CTGAGGGCATTTTCTTGAGG-3′; Erythropoietin forward primer: 5′-ACGTGGACAAGGCTGTCAGT-3′, Erythropoietin reverse primer 5′-TGGAGTAGATGCGGAAAAGC-3′; GAPDH forward primer: 5′-GCCGCTTCTTCTCGTGCAG-3′, GAPDH reverse primer 5′-ATGGATCATTGATGGCGACAACAT-3′. Host kidney were used as controls. A total of 9 kidneys developed from vitrified metanephroi (50%) and 7 from fresh metanephroi (44%) were recovered. Significant growth and fully differentiated mature glomeruli were observed in all kidney graft explants recovered. In addition, the relative levels of erythropoietin mRNA expression in vitrified (0.73±0.258 a.u.) and fresh (1.12±0.346 a.u.) metanephroi were consistent with the expression in host kidneys (0.77±0.446 a.u.). Also, renin mRNA expression in vitrified (1.48±1.295 a.u.) metanephroi was comparable with the control kidneys (0.23±2.242 a.u.). Metanephroi survive vitrification, become vascularized and develop morphologically normal glomeruli after their allotransplantation, exhibiting normal endocrine function. Based on these results, creating a long-term bio-bank of kidney precursors as an unlimited source of organs for transplantation would be possible, opening new therapeutic possibilities with ESKD.

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P39 MINIMUM VOLUME VITRIFICATION DEVICES USED FOR CRYOPRESERVATION OF RABBIT EMBRYOS

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Today, vitrification is being widely utilized in livestock and human embryos, with very satisfactory outcomes by means of methodologies that use a minimal volume, which allow to increase the cooling/thawing rate improving the outcomes. Many techniques have been developed to reduce sample volume with an explosion of methods appearing in the literature over the last decade. With this aim, more than 20 vitrification devices are commercially available, but these devices are rather expensive since it was intended for human use. In addition, devices designed to reduce the volume are

difficult to produce in-house. This study was therefore designed to compare the efficacy of the Cryotop® and nylon mesh devices for embryo vitrification by analysing the subsequent *in vitro* development rate in rabbit.
To assess this issue, rabbit embryos (72 hours post-insemination) were vitrified using cryotop® (n=110) and nylon mesh (n=103). Fresh embryos (n=110) were used as a control. Embryos were vitrified in a two-step addition procedure; equilibrium (10% EG + 10% Me2SO + 10% Dextran) for 2 minutes and vitrification (20% EG + 20% Me2SO + 10% Dextran) for 1 minutes. After thawing, embryos were cultured for 48 hours, evaluating the in vitro developmental rate until hatching/hatched blastocyst. In this context, rates of embryo development were similar between vitrified groups (Cryotop® and nylon mesh devices), which are lower than in the fresh group (Cryotop®: 73±4.2%; nylon mesh: 66±4.7%; control: 97±1.6%; $P<0.05$). We conclude that nylon mesh could be applicable in rabbit embryos vitrification and our results suggest that it could also be widely applicable in others species, at negligible cost.

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P40 DOES HIGH PRESSURE VITRIFICATION IMPROVE SURVIVAL RATES OF FELINE OOCYTES?

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Preservation of genetic diversity in endangered species is extremely important. In particular, combination of cryopreservation and assisted reproduction may contribute to conservation efforts. In many species, the method of choice to preserve female gametes is vitrification, but in felids the oocyte survival after warming is still very low. One interesting approach might be a self-pressure freezing method, which is used in electron microscopy to maintain ultrastructure by preventing ice crystal formation. We assumed that the use of self-pressurization combined with vitrification might minimize damaging effects on feline oocytes. For vitrification we used micro aluminium tubes which were tightly closed after the loading of cells. Immature cumulus–oocyte-complexes (n=121) from domestic cat ovaries were vitrified with our 3-step protocol, consisting of ethylene glycol, Me2SO, fetal calf serum, trehalose and Ficoll PM-70 and inserted into aluminium tubes (outer/inner diameter 0.6 mm/0.3mm). Tightly closed tubes were plunged into liquid nitrogen. Unexpectedly, when decreasing cryoprotectants´ (CPA) concentration until 20% none of the oocytes (n=60) were able to resume meiosis. When the usual 40% CPA was employed (n=61), 22.9% of the oocytes matured *in vitro* and 14.3% cleaved after fertilization by ICSI. Suspecting detrimental effects of the method due to lower survival compared with standard vitrification, gametes were placed into media with and without CPA and subjected to the tubes without vitrification. Developmental capacity of the oocytes thereafter decreased significantly in the presence of CPA (59.1% vs. 13%, n=45). In addition, cat sperm cells showed a significant decrease in motility after 4 minutes incubation in CPA medium which was preincubated in the aluminium tubes, compared to fresh CPA medium (25.8% vs. 13.2%, n=1914). These results suggest that CPA extract toxic elements from the aluminium tubes. In future we will test self-pressurization with other tube materials that might not be harmful for gamete survival.

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P41 OPPORTUNITIES OF THE LARGEST FISH SPERM CRYOBANK IN RUSSIA

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The largest Russian fish sperm cryobank was created in All-Russian Research Institute of Freshwater Fishiries in 1989. Cryobank collection consists of 32 species and 38 breeds among which there are species included in the Red Data Book. Cryopreservated sturgeon sperm is of great value in the collection, because sturgeon's natural population condition is critical. In the cryobank there are some samples stored there 28 years ago which were taken from the males from natural bodies of water. The total volume of the stored sperm is 28849,18 ml, which includes sperm of the Acipenseridae − 15751,65 ml from 697 males, Salmonidae − 2192,32 ml from 540 males, Cyprinidae − 10459,99 ml from 1029 males, Coregonidae − 401,97 ml from 322 males, Mugilidae – 43,25 ml from 26 males, etc.. A 28-year safety monitoring of the cryopreservated sperm shows that storage time doesn't influence on the sperm quality. Usage of the defrosted sperm allows to reach fertilization percentage compared to the native sperm. Cryopreservated sperm is used successfully to fertilize large volume of oocytes. The main tasks of our research are: improvement of the basic methods of the fish sperm cryopreservation and development of new ones, collecting sperm of the endangered species, development of the gene expression realization methods from cryopreservated sperm using molecular biology techniques, conduction of experiments to solve problems of the cryopreservation of embryos. Cryobank opportunities are saving endangered species, supporting genetic variety of aquaculture fish introducing wild fish genes, strengthening the most valued characteristics, reproducing extinct species by dispermic androgenesis or by intermediate stage of interspecific hybridization, increase in reproducing male quantity by the previously frozen sperm, determination of the fish genetic specification at the DNA level, prevention of diseases, reception of noninfected larvae, reception of offsprings with no mature males.

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P42 STUDY OF NATURAL ICE-NUCLEATING AGENTS FOR CRYOPRESERVATION OF 3D TISSUE-ENGINEERED SCAFFOLDS

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Ice formation at high subzero temperatures triggered by ice-nucleating agents is a natural strategy to avoid supercooling that can potentially be used for cryopreservation of cells in tissue-engineered scaffolds This could provide high cell recovery and scaffold stability for transplantation needs in a 'ready-to-use' format. In this work, we evaluated the impact of several natural ice-nucleating substances (Snowmax™ and crude leaf extract from *Hippophae rhamnoides*) as well as AFPIII, with supercooling and ice recrystallization inhibition activity, on freezing/thawing profile of 3D porous collagen-hydroxyapatite scaffolds. To address this issue, we used infrared video thermography, cryomicroscopy and differential scanning calorimetry. Scaffolds saturated in solutions of either ice-nucleating substances or AFPIII were subjected to slow cooling. The probability of heterogeneous nucleation was higher in cryoptotective solutions with scaffolds in comparison to that of without scaffolds. Heat capacity of hydroxyapatite-containing scaffolds was lower than that of scaffolds made of pure collagen. Both leaf homogenate from *Hippophae rhamnoides* and Snowmax™ promoted slow ice crystallization at high subzero temperatures alone and in combination with AFPIII increasing its supercooling point. At the same time, ice recrystallization inhibition activity of AFPIII in a mixture with both ice-nucleating substances was preserved as analyzed by cryomicroscopy. Application of natural cryoprotective substances with different mechanisms of action is a promising approach for future optimization of cryopreservation protocols for long-term storage of tissue-engineered scaffolds.

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P43 EFFECTS OF HIGHLY DISPERSED SILICA NANOPARTICLESON THE CRYORESISTANCE OF THE BOVINE CUMULUS-OOCYTE COMPLEXES

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Products of nanotechnology are expected to revolutionize cell technology and biomedicine. The aim of our study is to identify the effect of highly dispersed silica nanoparticles (HDSn) on the developmental competence of devitrified oocytes. Four groups of COCs were evaluated: native oocytes (C-1);native oocytes were cultured with HDSn (E-1);devitrified oocytes (C-2);devitrified oocytes were cultured with HDSn (E-2).Before vitrification oocytes of experimental group (E-2)were incubated for 120 min (38ºC, 5% CO_2) in TCM - 199 with 0.001% of HDSn(Chuiko Institute of Surface Chemistry, Ukraine). Vitrification was performed by equilibration of oocytes in: CPA1:0.7M dimethylsulphoxide (Me2SO)+ 0.9M ethylene glycol (EG), 30 sec; CPA2:1.4M Me2SO + 1.8 M EG,30 sec; CPA3:2.8 M Me2SO + 3.6M EG + 0.65 M trehalose,20 sec and loading into straws. After thawing COCs washed in 0.25 M,0.19M and 0.125M trehalose in TCM-199 and finally in TCM-199. COCs were cultured 24 h in TCM 199 with 10% FCS, 50 ng/ml prolactin, 10^6/ml granulosa cells (C-1,2), medium of experimental groups (E-1,2) were added by 0.001% of HDSn. Chemicals were purchased from Sigma-Aldrich (Moscow, Russia). Data were analyzed by χ2-test. Chromatin status (staining by Hoechst 33258), morphology of 429 oocytes and developmental competence of 412 fertilized oocytes were evaluated (in 4 replicates, 21-24 oocytes/group). Indicators of the cryoresistance markers of native oocytes were significantly higher than in devitrified group. The percantage of devitrified oocytes with high expanded cumulus was significantly lower than in devitrified oocytes treated by HDSn [31%(33/107) vs. 59% (64/109), P<0,01]. Portion of the matured oocytes and cleavage rate were significantly higher in E-2 than in C-2 [61%(66/109) vs. 41%(44/107) and 56%(57/101) vs. 29% (29/99), P<0,01, respectively]. In conclusion, treatment of oocyte by 0.001% of nHDS have a positive effect on the oocyte cryoresistance. The mechanism of the influence of HDSn on other biomarkers of cryoresistance remains to be explored.

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Conflict of Interest: None to disclose

P44 MONOZYGOTIC TWINS RESULTED FROM FROZEN THAWED BLASTOCYST GENERATED FROM FROZEN THAWED EGG

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OBJECTIVE: Vitrification techniques have been used widely in cryopreservation of human oocytes (l,2,3,4,5)and blastocysts(6,7,8). Healthy babies from re-vitrified embryos in ovum donation cycles conducted with vitrified occytes have been reported (9). We are reporting a live birth of healthy monozygotic twins that resulted from a vitrified oocyte that was vitrified again after fertilization and culture to a blastocyst. DESIGN: Case report. SETTING: Private IVF practice. PATIENTS: An infertility couple, 42-year-old woman and 36-year-old man. MATERIALS and METHODS: Oocytes from a 30-year-old donor were vitrified on vitristraws (REF: VS-1212, SciTech Invention) in Vit Kit-Freeze medium (lrvineScitific) by using the simplified oocyte vitrification protocol (lrvineScintific). Seven of eight oocytes survived after thawing in Vit Kit-Thaw medium (lrvineScintific) on November 16, 2013 by following the simplified warming protocol (lrvineScintific). On day 1, there was 100% fertilization (7 of 7 oocytes) after ICSI. Four out of seven fertilized eggs developed to blastocysts by Continuous Single Culture Medium (SCSM, lrvineScintific) for 5 days. Two blastocysts were transferred, resulting not in pregnancy. The other two blastocysts were re-vitrified on a vitristraw by using the embryo vitrification protocol (lrvineScintific). Those two re-vitrified blastocysts were thawed and assisted hatching was performed. The blastocysts were then transferred to the patient on May 8, 2014. RESULTS: The patient achieved a normal pregnant on her second embryo transfer. On June 14, 2014, on ultrasound scan, there were two heartbeats in one gestational sac. Two healthy monozygotic boys (body weight 2466 g and 2353 g) were born on January 13, 2015. CONCLUSION: The extra embryos can be re-vitrified in frozen egg donation cycles, and as with any re-frozen blastocyst may result monozygotic twining.

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Conflict of Interest: None to declare

P45 QUALITY ASSESSMENT OF STURGEON JUVENILES OBTAINED BY CRYOTECHNOLOGIES

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Nowadays interest to the fish sperm cryopreservation methods for the usage in fish farming is increased. However, at the moment these methods have not been widely applied in practice, perhaps because of the fish grower's fear that the obtained juveniles will differ from those traditionally obtained.The aim of the work was to receive life-resistant Russian sturgeon offspring using cryopreservated sperm and to assess the offspring quality.In case of the fertilization by defrosted sperm of Russian sturgeon in industrial conditions, in the experimental batch the percentage of fertilization was 60%, in the control batch – 80%. In the analysis of morphometric indicators fish obtained by using cryosperm had an advantage in comparison with the traditionally obtained individuals. Behavioral responses of the offspring were evaluated in the «open field» test, which was conducted by placing one individual (pre-larvae, larvae or juveniles) in a special installation, with coordinate grid, where the number of the coordinate lines intersections by the object was registered. The fish were affected by the following stimulus: light, illumination 20 Lx, low-frequency signal (frequency 20 Hz), bright light (100 Lx), high-frequency signal (300 Hz), vibroacoustic stimulus. The test was subjected to 30 individuals in the experimental and control groups at the 1st, 8th and 15th day after hatching.No differences were revealed in the evaluation of the reactions of pre-larvae, larvae and juveniles, obtained by traditional technology and with the use of cryopreservated sperm.The usage of cryopreservated sperm makes it possible to obtain resistant juveniles and can be recommended for using in artificial reproduction hatchery. This will allow to obtain genetically diverse offspring, will reduce the area and the cost of males maintaining, thereby allowing to increase the production of females stock.

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Conflict of Interest: None to declare

P46 EFFECTS OF ACTIVATING SOLUTIONS ON SPERM QUALITY OF THE THREATENED AMAZONIAN FISH *HYPANCISTRUS ZEBRA*

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Our research team has been working over the last years to establish a germplasm bank and contribute for conservation of this endemic Amazonian fish species. The aim of this study was to assess the effects of activating solutions on the quality of *Hypancistrus zebra* sperm. Semen (1 µl) collected from seven males was placed on a glass slide and activated (10 µl) by three different test solutions: 0.29% NaCl, distilled water and 1% $NaHCO_3$. This procedure was repeated three times to each male to assess motility rate (%), swimming vigor (1-5 score) and motility duration (min).
When *Hypancistrus zebra* sperm was activated by 0.29% NaCl or distilled water, there was no significant difference ($p > 0.05$) in motility rate (85.71 ± 7.87% and 88.57 ± 13.45%) and swimming vigor (3.14 ± 0.38 and 3.29 ± 0.49), respectively. On the other hand, the above-mentioned parameters were negatively affected when sperm got in contact with 1% $NaOH_3$. The longest

motility duration ($p < 0.05$) was found when using 0.29% NaCl (14.27 ± 9.24 min) as activator. Distilled water (5.17 ± 3.45 min) and 1% $NaOH_3$ (1.78 ± 1.40 min) dropped down the motility duration significantly. This is the first study to assess the quality of *Hypancistrus zebra* sperm using different activating solutions. The solution containing 0.29% NaCl was the most efficient in activating the spermatozoa and ensuring the longest motility duration. Based on these results, we have moved forward and we are testing the cryopreservation protocols in order to create a germplasm bank for this Amazonian endangered fish.

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Conflict of Interest: None to disclose

P47 INFLUENCE OF FREEZE-DRYING ON PROBIOTIC PROPERTIES OF MICROORGANISMS ISOLATED FROM KEFIR

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Considering that orange juice could be a healthy alternative for probiotic delivery, the aim of the present work was to obtain freeze dried strains to be included in a novel functional food. The resistance of Lactobacillus plantarum CIDCA 8327 and Kluyveromyces marxianus CIDCA 8154 to the freeze-drying process in milk, sucrose 10 %w/v or PBS, and their survival in dried-powder at 4ºC were evaluated. The freeze-dried strains were individually added to reconstituted orange-juice–powder to a final concentration 107-108 CFU/ml. The viability of the lactobacilli and yeasts in juice was determined on MRS-agar and YGC-agar plates respectively; the turbidity, color, odor and overall acceptability of the products were evaluated by a non-trained panel of 30 individuals using a nine-point Hedonic scale. The resistance to low pH (2.5 at 37 °C for 3 h) and bile salts (0.5 % w/v), and the adhesion to Caco-2/TC7 cells of the strains after the freeze-drying process and after their inclusion into orange juice were also studied. L. plantarum was more resistant than K. marxianus to the freeze-drying process. The use of sucrose 10 %w/v as cryoprotector improved the survival of both strains and allowed the obtaining of a powder with constant microorganism concentration during 75 days of storage at 4 ºC. Once included in the juice, the viable number of both strains remained constant during 8 h of storage at room temperature. No significant differences were observed in the color, turbidity, and overall acceptability between the control and the juice added with L. plantarum. The freeze-drying of the L. plantarum CIDCA 8327 and its inclusion to orange juice did not change its ability to resist bile salts and to adhere to Caco-2/TC-7 cells indicating that orange juice could be a promising non-dairy vehicle for their delivery.

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Conflict of Interest: None to disclose

P48 SEASONAL EFFECT ON SPERM CRYOPRESERVATION IN *EPIDALEA CALAMITA* (ANURA: BUFONIDAE)

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An understanding of how seasonality impacts amphibian sperm quality could be crucial for the establishment of genetic resource banks from threatened species. We examined the effect of cryopreservation on sperm DNA integrity and fertilizing capability both within and outside the breeding season of the common Natterjack toad as a model. Spermic urine was collected (n=23 males/season) following intraperitoneal injection of 10IU of human Chorionic Gonadotropin per gram of body weight. Fresh sperm samples were assessed for motility, concentration and DNA integrity. Sperm was mixed 1:1 with chilled cryodiluent (10% sucrose + 10% dimethylformamide) and cryopreserved in LN2. Then, sperm was thawed, diluted 1:1 in distilled water for activation, evaluated for DNA integrity and used for artificial fertilization. Eggs were mixed with 100µl of activated sperm collected either within (n=14) or outside (n=11) of breeding season and evaluated for embryonic cell division. More males presented sperm after hormone administration during the breeding season (12 vs. 18; 52.2% vs. 78.3%), although the difference was not significant ($p>0.05$). Fresh sperm motility and integrity of DNA were not different between in and out of season ($p>0.05$). However, sperm concentration was higher ($p=0.043$) during the breeding season (5.2 vs. 2.9x106 spermatozoa/ml). The fragmentation of sperm DNA increased after freeze-thaw in both seasons ($p<0.01$) and was higher ($p=0.009$) in samples obtained during the breeding season (58.1% vs. 35.5%). Fertilization experiment found that morulas were observed in 9/14 (breeding season) and 8/11 (outside breeding) samples. Five days post-fertilization, live embryos were observed in 2 (3±0.2% embryos, outside season) and 9 (38.4±6.7% embryos, breeding season) samples. DNA integrity was not a predictor of fertilizing capability as shown by a 3% correlation. Overall, sperm obtained during the breeding season maintained a better fertilization capacity after freeze-thaw. Therefore, protocols must be adjusted for necessary collections outside of breeding season.

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Conflict of Interest: None to disclose

P49 SUCCESFUL POSTMORTEM REFRIGERATION OF EPIDIDYMAL SPERM CELLS FROM A KOMODO DRAGON

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The Komodo dragon (*Varanus komodoensis*) is the largest lizard in the world and currently listed as threatened with extinction. However, there is almost no knowledge of its gametes. In order to reinforce the conservation of endangered populations through germplasm genetic resource banking, this work aimed to evaluate whether Komodo dragon sperm could be successfully preserved by refrigeration. Thus, semen was collected one day after death from epididyma and deferent ductus from an adult Komodo dragon (13 years old) by slicing the tissue. The sperm cells were collected and diluted using a commercial extender for semen without permeable cryoprotectants (Gent A; Minitub Iberica, Spain), and maintained at 4˚C for 0, 24, 48, 72, 96 and 120 hours. Sperm viability was assessed by counting viable sperm cells after eosin-nigrosin staining. Initial viability was 96% (0h) and remained above 80% for 96h of refrigeration, whereupon 60% viability was recorded at 120h. Motility features were assessed using a computer-assisted sperm analysis system (CASA system; Proiser SL, Valencia, Spain). Average motility after collection was 87.1% with 75% of progressiveness. Motility decreased over cooling time ($R2=0.93$; $p<0.01$) and remained above 55% for 96h, whereupon 32.9% was recorded at 120h of chilling. Progressiveness decreased over cooling time ($R2=0.98$; $p<0.01$) and remained above 55% for 48h, whereupon a first drop to 34.7% (72h) was recorded and later critically dropped to 7.1% (120h). Our data suggest that epididymal spermatozoa from Komodo dragon can be stored at low temperatures without cryoprotectants maintaining sufficient levels of sperm motility for several days. This should allow transport of semen between institutions and theoretically be sufficient to achieve artificial insemination in Komodo dragons.

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Conflict of interest: None to disclose.

P50 AMPHIBIAN ART OVER THE GENERATIONS: FROZEN SPERM OFFSPRING PRODUCE VIABLE F2 GENERATION

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Sperm cryopreservation is a key element in the Assisted Reproductive Technologies (ART) toolbox for stabilizing genetic diversity of captive and wild amphibian populations threatened with extinction. When expressed as spermic-urine, mature amphibian spermatozoa have characteristically long needle-shaped heads with a high proportion of surface membrane to cytosolic volume, making the cells extremely fragile and susceptible to osmotic stress. Despite these challenges to sperm cryopreservation, we have produced F1 generation offspring from cryopreserved spermic-urine in both caudates (Tiger salamander, Ambystoma tigrinum) and anurans (Mississippi gopher frog, Lithobates sevosa; Boreal toad, Anaxyrus boreas). A key question now presents itself- what is the reproductive capacity of this frozen sperm produced F1 generation? In 2015, male and female A. tigrinum were treated with human chorionic gonadotropin and gonadotropin releasing hormone for gamete development and release. Frozen sperm (5% Me2SO+0.05% BSA) thawed for IVF, yielded F1 offspring (n=21). In 2018, a mature F1 female and two males were analyzed using ultrasound for oocyte and testicular development and hormonally treated to obtain gametes. The F1 female produced 550 viable eggs; a subset(n=45) underwent IVF. Fertilization, cleavage and early embryonic development of the F2 generation to blastula stage was achieved(n=8/45;18%). The same sperm sample yielded blastula stage embryos(n=74/174; 42%) from an F0 female, control. Similarly, in 2013 spermic urine from hormonally induced L. sevosa that was frozen(10% dimethylformamide+10% trehelose) and thawed for IVF resulted in F1 offspring (26.3% frozen; 45.6% control). In 2018, spermic urine from adult F1 males and a control F0 male were used to fertilize eggs from an F0 female. The development and survivability of the F2 generation to advanced stage tadpoles/metamorphs was similar ($p> 0.05$) between F1 frozen males (9%, 7%) and F0 control male (8%) for the same egg mass. Thus, a frozen sperm F1 generation can produce viable gametes and offspring.

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Conflict of Interest: None to disclose

P51 OPTIMAL CRYOPRESERVATION YIELDS INTACT ALGINATE MICROSPHERES AND HIGH CELL VIABILITY AFTER THAWING

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The possibility of long-term storage of clinically relevant cells combined with daily availability of genetically stable material gives cryopreservation a high potential in modern cell therapeutics. Although cryopreservation of alginate-encapsulated cells is very promising for further clinical application, there is still no optimal cryopreservation protocol for freezing and thawing of

cells within intact and mechanically stable alginate micro-spheres. Alginate-based semipermeable capsules allow for permanent protection of cells against physical damage caused by the cryopreservation process. In this work, a range of parameters such as alginate cross-linking time, concentration of dimethyl sulfoxide, its loading time and cooling rate were analysed for freezing of 300, 800, 1000 and 1500 µm alginate micro-spheres. The micro-spheres were generated using electro-spraying (300 µm) and air flow (800, 1000 and 1500 µm) encapsulation methods. The structural integrity of cell-free alginate micro-spheres was evaluated upon freeze-thaw cycles using an Axio Imager M1m microscope with Linkam cryostage to identify an optimal combination. Verification of optimal cryopreservation protocol yielding intact capsules has been performed using multipotent stromal cells derived from the common marmoset (Callithrix jacchus). Freezing of alginate-encapsulated cells has been conducted using a controlled-rate freezer Planer Kryo 560-16 and Askion C-Line workbench. Analysis of cell viability after thawing has been performed using Calcein-AM / EthD-1 cell viability assay following image analysis using µVision software. Among the optimal conditions, the following ones yielded the highest cell viability (83%) and recultivation efficiency (70%) 24 h after thawing: cross-linking time 15 min, 10% (v/v) Me2SO, loading time at 4°C of 45 min and a cooling rate of 2.5 K/min. The results of this study demonstrate a high potential for clinical application of mechanically stable alginate capsules for efficient treatment of rare diseases.

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P52 EFFECT OF VITRIFIED-WARMED BOVINE OOCYTES ON THE BLASTOCYST HATCHABILITY

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This study evaluates the effect of vitrification of blastocyst which derived from IVF following vitrified-warmed matured bovine oocytes. During experiment, IVM oocytes or blastocysts were exposed to medium containing 5% dimethylsulfoxide (DMSO) and 5% ethylene glycol (EG) for 30 sec, followed by exposed to medium containing 12% DMSO, 12% EG and 0.25M sucrose for 30 sec, finally exposed to vitrification solution containing 20% DMSO, 20%EG and 0.5M sucrose for 30 sec. Oocytes or blastocysts were loaded on a paper container before plunged into liquid nitrogen. Oocytes were conducted into four groups; A) fresh MII oocytes and blastocysts, B) vitrified IVF-derived blastocysts, C) vitrified MII oocytes only and D) vitrified both MII oocytes and blastocysts. The results revealed that blastocyst production rates of group A, B, C and D were 15.7, 16.5, 9.4 and 9.4%, respectively, which was not significant difference. In case of blastocysts hatchability rate, the blastocysts in group A, B, C and D were 73.3, 56.5, 66.6 and 16.7%, respectively. The blastocysts hatchability rates of blastocyst of group C and D were not difference when compared with group A. However, blastocysts hatched rate in group C (6.3%) was significantly ($p<0.05$) lower than that of Group A (12.1%), but significantly ($p<0.05$) higher than that of group D (1.6%). We estimated that the oocytes survival rate in group C was significantly correlated with cleaved rate whereas the blastocysts hatchability rate in group A showed significant correlation ($P<0.01$) with its survival rate. In conclusion, the blastocyst hatched rate was seriously affected by double vitrification on oocytes and blastocysts.

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P53 GLYCEROL AND POSTHYPERTONIC SHOCK OF ERYTHROCYTES WHEN VARING MEDIUM TEMPERATURE AND OSMOLALITY

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In this research the effect of glycerol on sensitivity of human erythrocytes to the action of posthypertonic shock (PHS) was studied. PHS was initiated by transferring the erythrocytes from dehydration medium (1.5 mol/L NaCl) into rehydration one (0.15 mol/L NaCl) The experiment was carried out in two versions: 1) the erythrocytes pre-treated with glycerol (37°C, 20 min) were subjected to PHS (control); 2) glycerol pre-treated cells were also exposed to PHS, but glycerol was present in the dehydration medium. In the presence of glycerol (15%) the incubation time of erythrocytes at the dehydration and rehydration stages of PHS were 20 and 5 mins' respectively. At 37°C, when the glycerol concentrations (5, 10 and 15%) were varied, an increase of hemolysis level of erythrocytes was shown with using the cryoprotective agent (CPA) in a high concentration (compared to cells without CPA). The presence of glycerol in the dehydration medium led to an increase posthypertonic hemolysis of erythrocytes approximately in 1.5 times at 10 and 15% CPA. At 0°C the addition of glycerol (5, 10 and 15%) resulted in the development of a high level of posthypertonic hemolysis of the control cells and erythrocytes, which were transferred to the dehydration medium containing CPA. The obtained temperature dependencies of posthypertonic hemolysis of cells pre-treated with CPA (15%) show that under low

temperatures (0 - 5°C) the hemolysis values are approximately 3.5 times higher than those for untreated with CPA erythrocytes, and within the temperature range from 5 to 30°C there is a uniform decrease in the hemolysis values from 90 down to 25%.

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P54 CATHEPSIN B-LIKE CYSTEINE PROTEASE APCATHB ADVERSE IMPACTS CELL VIABILITY IN CRYOPRESERVATION

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Cryopreservation-induced cell death is regarded as an important drawback faced by cryobiologists. We isolated a gene, encoding cathepsin B-like cysteine protease from Agapanthus
praecox ssp. orientalis named ApCathB, whose expression pattern had a positive correlation to the occurrence of programmed cell death (PCD) during cryopreservation. The mature ApCathB protein possess in vitro cathepsin B activity and its activity could be inhibited by E-64 and Leupeptin. Overexpression of ApCathB in A. praecox embryogenic callus (OXEC) reduced the survival rate from 45.68% to 18.33% after cryopreservation. Along with the reduced cell viability, the H2O2 and O2·- significantly accumulated in OXEC. The DNA fragment detection showed intensified signal by TUNEL and higher ratio of DNA ladder during OXEC cryopreservation. The cis-elements of TCA-element, ERE, MBS and ARE were responsible for driving the ApCathB expression during cryopreservation treatment. This indicated that the expression of ApCathB was induced by the environment stress of drought and hypoxia and the hormone signals of salicylic acid and ethylene. We analyzed the differentially expression genes (DEGs) of transgenic (OXEC) and non-transgenic (NTEC) embryogenic callus treated with dehydration, dilution and 24 h-recovery to investigate the downstream events effected by ApCathB during cryopreservation. The oxidation reduction process and MAPK cascade were altered in OXEC cryopreservation. Overexpression of ApCathB weakened the antioxidant ability of the cells. In addition, as another partial of stress defense system, the second metabolism and signal transduction pathways were also down-regulated in OXEC. In cryopreservation-induced PCD pathway, ApCathB aggravated the protein proteolytic and inhibiting the anti-apoptosis genes expression. This work verified that ApCathB negatively impact the cell viability through mediate the PCD in plant cryopreservation and provided a novel insight into the mechanism of PCD in cell viability during cryopreservation.

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P55 CRYOPROTECTIVE AGENTS AND LOW TEMPERATURE AFFECT THE ERYTHROCYTE SURFACE MARKER CD44

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Changes in surface markers induced by cryopreservation stresses can be an important sign of cell injuries. This study aims to examine changes in the marker CD44 in erythrocytes under the effect of cryoprotective agents (CPAs) and the impact of cross-linked modification of membrane-cytoskeleton proteins on CD44 over the cell exposure to CPAs, as well as the assessment of consequences of erythrocyte cryopreservation for CD44 alterations. Prolonged exposure of erythrocytes (20 h) with glycerol, Me2SO, sucrose and PEG-1500 led to a reduced CD44 expression and decrease in the CD44+-cell amount in contrast to the short-term exposure, when no changes were found. Extracellular CPAs exhibited a more pronounced effect on erythrocyte CD44 in comparison with the examined endocellular substances. Modification of membrane-cytoskeleton proteins with protein-linking reagent diamide intensified the revealed tendencies. Meanwhile, short-time incubation (1h, 37oC) of frozen -thawed erythrocytes caused a significant decrease in the CD44 expression and in the CD44+-cell amount. These changes were more pronounced in erythrocytes cryopreserved with PEG-1500 than with glycerol. Evidently, time discrepancies in the manifestation of the CD44 changes in erythrocytes exposed to CPAs and cryopreserved ones specify the different processes affecting the cell stability. The changes revealed during cell exposure to CPAs may attest subtle adjustments in protein-protein interactions in erythrocyte membrane-cytoskeleton complex, which are able to increase the cell resistance. Whereas the CD44 alterations in cryopreserved erythrocytes can attest membrane injuries. Since cryopreserved erythrocytes that survived after CPA removal were found to have similar CD44 characteristics as fresh erythrocytes indicating that changes in erythrocytes covered only part of the cells, which were instable and lysed during the washing process. Thus, changes in surface markers of erythrocyte membranes caused by CPAs and freeze-thawing processes can be important for the assessment of structural and functional cell integrity.

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Conflict of Interest: None to disclose

P56 CONVOLUTIONAL NEURAL NETWORK-BASED CELL SEGMENTATION DURING FREEZING IN CRYOPRESERVATION

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For most of the cells water dehydration and cell membrane plasma transportation during cooling plays a significant role in the practical optimization of successful cryopreservation. It is essential to effectively measure the permeability of the cell during subzero temperature. So far, there is no perfect segmentation techniques to be used for this kind of image processing task accurately. The ice formation and variable background of the cells during freezing posed a challenging task for a numbers of conventional segmentation processes. Thus, we need to present a novel approach that can consistently extract cells from the extracellular ice, which are attached to the cell boundaries and its surrounding background. Hence, we propose a Convolutional Neural Networks (CNNs) similar to U-Net. This U-Net like structure comprise a contracting and expanding paths to achieve a high resolution output image. The CNN model is employed to predict the results of cell boundaries and overcomes some of the difficulties encounters by manual cell segmentation. Due to the functional learning ability of our CNNs model, complex performance of cell border contour from the background in it freezing state was more coherent and effective as compared with the traditional segmentation approaches.

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Conflict of Interest: None to disclose

P57 CRYOPROTECTIVE AGENTS DO NOT ALTER CRYOPRESERVATION BUT CHANGE FROZEN-THAWED BOAR SPERMATOZOA CRYOSURVIVAL

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Cryopreservation of boar semen decreases mitochondrial membrane potential (MMP). It impairs the sperm viability, especially in protocols of one-step-method. Since the cryoprotectant agent (CPA) have higher toxicity at high temperatures. Boar semen are currently cryopreserved with two steps protocols. To cryopreserve boar semen in one-step-method, it is necessary a study of different CPAs determines which one fits the best for the new protocol. Thus, the aim of the present study was to find the better CPA to cryopreserve boar semen in one-step-method. The CPAs used was: 2% glycerol and 2% methylformamide (CPA1); 5 % acetamide (CPA2); 5% dimethylformamide (CPA3); 1% glycerol and 4% dimethylformamide (CPA4). Three sperm-rich fractions from three boars (n=9) were collected. The semen was extended in BTS 1:2 (semen: extender) and held t 17ºC (holding time– HT) during 24 hours. After the HT, the samples were centrifuged (500g/10 min at 17ºC) and extended in CPA according to the treatments to obtain 600x106 spermatozoa/mL. The extended semen was packaged in 0.5mL straws. Semen was frozen in an automatic system using a rate of -0.5°C/min until 5°C, -20°C/min until -120°C and immersed in liquid nitrogen. Two straws were thawed in water-bath (37°C/30seg). The semen MMP were accessed by flow cytometer (JC-1/Propidium iodide). The data were analyzed by SAS program, subjected to analysis of mixed models, treatments were evaluated using Tukey Test. Statically, significant differences were considered when $p<0.05$. The results (mean ± SEM) for sperm cells alive with high potential were (16.68±4.59a; 6.38±2.32c; 5.82±1.22c; 15.14±4.10b) for CPA1, CPA2, CPA3 and CP4 respectively. The lowest levels of MMP were obtained in the semen cryopreserved using the CPA 2 and CPA3. In conclusion, the CP1 can be used in future for cryopreservation in one-step-method. Since it increases live cells with high potential mitochondrial.

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P58 CRYOPRESERVATION OF BOAR SPERMATOZOA: HOLDING TIME AND CAPACITATION-LIKE CHANGES ON SPERM MEMBRANE

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Spermatozoa cryopreservation lead to functional and structural capacitation-like changes. Boar spermatozoa is very sensitive to cold shock because its membrane composition. Thus, this study aims to elucidate the effect of holding time on membrane permeability and peroxidation. Three sperm-rich fraction were collected from five boars (n=15). Samples were divided in seven aliquots and extended 1:2, semen:BTS. Samples were maintained on holding time at 17ºC for 0, 4, 8, 12, 24, 28 or 32 hours. After each time, samples were centrifuged (2100xg/3min) and extended in freezing extender (2% glycerol, 2% methylformamide) to obtain 600x106spermatozoa/mL. The extended semen was stored in 0.5mL straws and frozen in an automatic system using a rate of -0.5°C/min until 5°C, -20°C/min until -120°C and immersed in liquid nitrogen (-196°C). Two straws were thawed in water-bath (37°C/30seg). An aliquot was extended (TALP) to 5 x 106spermatozoa/mL, dyed to following assays: Yo-Pro1/Propidium iodide (PI – membrane permeability) and C11Bodipy/PI (membrane peroxidation). Samples were analyzed by flow cytometer. The data were analyzed by SAS program, subjected to analysis of mixed models, treatments were evaluated using Tukey Test. Four hours of holding time decrease membrane permeability changes (1.50 ± 0.42c,d; 0.87 ± 0.12d; 1.38 ± 0.16b,c,d; 1.42 ± 0.31c,d; 2.74 ± 0.37a; 1.76 ± 0.26b,c; 1.89 ± 0.25a,b to 0, 4, 8 12, 24, 28 and 32 hours of holding respectively). The lowest levels of membrane peroxidation were obtained

on holding time of 0, 4 and 8 hours, and at 32 hours the highest levels (1404.15 ± 139.07b; 1411.19 ± 139.05b; 1435 ± 163.31b; 1549.83 ± 161.30a,b; 1545.63 ± 152.68a,b; 1636.94 ± 106.38a,b; 1808.38 ± 173.54a to 0, 4, 8 12, 24, 28 and 32 hours of holding respectively). In conclusion, to avoid early changes on sperm membrane is necessary use four hours of holding prior to cryopreservation of boar spermatozoa.

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Conflict of Interest: None to disclose

P59 SPERMATOZOA CRYOPRESERVATION IN SMALL FELINE SPECIES

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The aim of this study was to compare different methods of feline semen freezing using domestic cat as a model species and to apply these methods for the purposes of Far-Eastern wildcat (Prionailurus bengalensis euptilurus) biodiversity conservation. Two different commercially available freezing media, i.e. CaniPlus Freeze (CPF) (Minitube, Germany) and Sperm Freeze (SF) (Ferti Pro, Belgium) were tested for epididymal domestic cat spermatozoa freezing. The use of a commercial product has the advantage to be standardized and easy to perform in field conditions. The viability of domestic cat epididymal spermatozoa evaluated by the VitalScreen test was 68.7 ± 3.0% for nonfrozen group; 51.2 ± 6.3% for CPF group and 54.4 ± 3.1% for SF group. In vitro fertilization (IVF) of domestic cat oocytes with nonfrozen and frozen-thawed spermatozoa produced developing embryos. The same freezing method based on CPF freezing medium was applied to Far-Eastern wildcat ejaculatory spermatozoa at the Experimental Station of A.N. Severtsov Institute of Ecology and Evolution (near Moscow) where this species is bred in captivity. The frozen spermatozoa were transported to Novosibirsk and analysed. The viability of Far-Eastern cat frozen-thawed spermatozoa was 36.7 ± 6.5%. These spermatozoa fertilized in vitro matured oocytes of the domestic cat and 35.5 ± 15.0% heterologous embryos developed successfully. The cell number per embryo was 26.9 ± 5.1 on the 5th day of the in vitro culturing. The results of this study demonstrated the applicability of CPF-based semen freezing method for conservation of rare felid species.

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Conflict of Interest: None to disclose

P60 EFFECTS OF SPERMINE AND SPERMIDINE ON SPERMATOLOGICAL PARAMETERS OF FROZEN STALLION SEMEN

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In this study, we aimed to determine the effects of polyamines on post-thaw total motility, progressive motility and morphology of stallion spermatozoa. Spermine and Spermidine were added to standard stallion freezing extender (SFE; INRA 96, egg yolk and glycerol) either 100 µg, 200 µg, 400 µg or 1 mg, 2 mg, 4 mg levels. Semen were collected from 4 adult stallions and collection was repeated 5 times. Each sample was centrifuged with 600 g force for 10 minutes to remove seminal plasma and then sperm pellets were diluted (200x106 spermatozoon/ml) and split into 13 aliquots. Sperm samples were frozen as a control group that extended with SFE; and experimental groups that were extended with SFE supplemented with various concentration of Spermine and Spermidine. Post-thaw total motility, progressive motility and abnormal spermatozoon rates were evaluated by CASA system. One-way analysis of variance with a subsequent Tukey's test was used to compare the mean values of various treatments at a significance level of $p<0.05$. It was determined that 100, 200, 400 µg and 2 mg Spermine and Spermidine supplemented extenders had higher total (ranging from 54.39% to 59.07%) and progressive (ranging from 20.02% to 22.83%) motility than the others ($p<0.001$). SFE having 4 mg spermidine gave the lowest total (29.54%) and progressive motility (9.12%) in all groups ($p<0.001$). While the lowest abnormal spermatozoa rate was obtained when sperm samples were frozen in SFE containing 200 µg spermidine (23.75%), the highest rates of abnormal spermatozoa was obtained when sperm samples were frozen in SFE containing 4 mg Spermine (29.90%) and Spermidine (29.70%) ($p<0.05$). Our results suggest that while SFE having 200 µg Spermidine significantly improves post-thaw total motility, progressive motility and abnormal spermatozoa rates, 4 mg Spermidine have detrimental effects on stallion semen cryopreservation.

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Conflict of Interest: None to disclose

P61 REDUCING WATER LEAKAGE BY FRUCTO-OLIGOSACCHARIDES ENHANCING TREATMENT IN STRAWBERRIES

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Strawberries, highly appreciated for their excellent flavor, have relevant economic and nutritional benefits. However, their quality deteriorates rapidly due mainly to water loss. Water is a very important component in fruit, mainly in strawberries that exhibit a large quantity of water. More than moisture content it is of great interest to quantifiy the amount of freezable and unfreezable water fraction in strawberries. Some varieties are classified as a high CO2 tolerant fruit and atmospheres with high CO2 concentrations have been used successfully to control fungal decay and to increase flesh firmness. Interestingly, the metabolic adjustment caused by high CO2 treatment involved the enhancement of hydrophilic compounds, facilitating water retention, thereby improving the quality of chilled and frozen strawberries. The aims of this work were first, to determine how protective short treatment such as 20 kPa CO2 for 3 days at low temperature, modifies fruit water status. For that, the freezable and unfreezable water content in strawberries from two different harvesting time were studied using differential scanning calorimetry. Second, to analyze how changes in unfreezable water fraction was correlated with endogenous fructo-oligosaccharides levels and cell structure in strawberry fruit stored under high CO2 atmosphere. Finally, in the present work we also tried to determine whether the changes in the levels of unfreezable water content in CO2-treated strawberries might affect the thawing behaviour of fruit tissues. The water-holding capacity of strawberries after thawing was determined by analysing the drip loss of CO2-treated and untreated samples. Our results suggest that by increasing the concentrations of fructo-oligosaccharides, protective high CO2 concentrations reduce the amount of water available to be frozen, thus reducing water leakage associated with the freezing-thawing process.

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Conflict of Interest: None to disclose

P62 INDIVIDUALIZATION OF THE CRYOPRESERVATION PROCEDURE OF BIOPSIED EMBRYOS ALLOWS TO INCREASE THEIR SURVIVAL

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Pre-implantation diagnostics (PGD) is required for the couples with a risk of genetic diseases in order to avoid transfer of embryos with monogenic diseases or chromosomal abnormalities in infertility treatment cycles. Cryopreservation of embryos after biopsy for PGD is a necessary procedure, due to the impossibility of carrying embryo transfer in a fresh cycle. However, the protocols used to vitrify embryos with and without biopsy are common, that may affect the cryopreservation outcome. The main reason of this fact is the speed difference of cryoprotectant flow into the embryo. The purpose of this work was to evaluate the osmotic behaviour of embryos with biopsy for PGD during exposure with cryoprotective media and their survival rate after cryopreservation. Blastomeres biopsy were carried to day 5 embryos, obtained by the intracytoplasmic sperm injection in to the oocyte. Afterwards the embryos were vitrified (Kuwayama et al.) on Cryotec carriers (CryoTech, Japan). It was found that dehydration of biopsied embryos occurred in shorter time than in embryos without biopsy observing of their osmotic behavior during exposure with cryoprotective media. After warming the survival rate of embryos without biopsy was higher (96.4%) then the one in the biopsied embryos (68.2%, $P < 0.05$). The pregnancy rate after transfer to vitrified embryos without biopsy was 54.2%, and with biopsy was $55.6 \pm 4.5\%$. Individualization of exposure time of biopsied embryos in cryoprotective media depending on the onset of their shrinkage and re-expansion allowed to increase the survival rate up to 88.7%. Based on the work done, it can be concluded that blastocytes after biopsy have a different osmotic response compared to intact ones. These individual characteristics must be taken into account to increase their survival rate by selection of individual time intervals for exposure of biopsied embryos with cryoprotective media.

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Conflict of Interest: None to disclose

P63 EFFECT OF MELATONIN ON SPERMATOZOA MORPHOLOGY IN Leporinus obtusidens CRYOPRESERVED SEMEN

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This work aimed to observe the morphological changes of the Piapara (Leporinus obtusidens)´s spermatozoids cryopreserved in solutions containing melatonin. All procedures were approved by the Ethics Committee for the Use of Animals of UFLA (n° 039/13). Semen of fifteen animals, hormonally induced, was used. Sperm motility was measured subjectively, using only samples with motility greater than 80%. Semen samples consisted of four "pools" from 3 animals each. The semen was diluted 1:9 (semen: solution) in the following treatments: (T1): 10% methylglycol (MG) + 5% Beltisville Thawing Solution (BTS); (T2): 10% MG + 5% BTS + 1mM melatonin and; (T3): 10% MG + 5% BTS + 2mM melatonin. After dilution, the samples were bottled in reeds of 0.5mL, sealed and frozen using the nitrogen gas cylinder (dry shipper: -35.6°C/min), where it remained for 24 hours and then it was transferred to a liquid nitrogen cylinder until 60 days. Samples were thawed in a water bath at 60°C for 5 seconds and the semen was fixed in 4% formaldehyde-citrate solution for morphology. The mean percentage of normal

spermatozoa and altered spermatozoa (primary and secondary alterations) was compared between "in natura" semen and cryopreserved T1) and also among the treatments (T1,T2 and T3). Data were submitted to analysis of variance and Tukey's test ($P<0.05$). About the effects of cryopreservation, the percentage of normal spermatozoa was reduced from 49,0% (in natura) to 34,7% (T1). About the morphological alterations, in freezing process, T1 presented a higher rate of altered spermatozoa than "in natura" semen (23,6%; 9,5%, respectively), while between the solutions, there were more sperm primary alterations in T1 (7,7%) than T3 (3,6%) and in the sperm secondary alterations the treatments with melatonin presented the highest values (T2:32,4%; T3: 33,1%) when compared with T1 (24,6%). In conclusion, 2mM of melatonin influenced the sperm morphology.

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Conflict of Interest: None to disclose

P64 A SYSTEM TO KEEP LONG-TERM PERFUSION WITH CONSTANT SPEED IN MICROFLUIDIC DEVICE

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In cryobiology and cryopreservation, long-term perfusion with constant (or controlled varied) speed is needed, but still remains challenging in the currently-used syringe pump setup. In this work, we designed a system to keep microfluidic perfusion at constant speed for a long time through cooperating two syringe pumps and implementing it on an integrated microfluidic platform with the C# programming language. Briefly, the system is run as follows: Firstly, we prepared the two syringe pumps into an initial state before the cooperating, and computed the corresponding parameters needed; Secondly, the first syringe pushed at a fixed speed from the state of full, meanwhile the second syringe pulled at full speed, until it refilled this syringe. Thirdly, the two syringes began the first cooperation when the remaining distance of pushing of the first syringe was equal to the cooperating distance, the first syringe began to push at uniform deceleration until it reached zero, then its state was changed into pulling, until the syringe was fully refiled. At the same time, the pushing speed of the second syringe began to accelerate from zero at constant acceleration, and the speed maintained a fixed value when it reached the working speed. Finally, the second cooperation began when the remaining distance of pushing of the second syringe was equal to the cooperating distance, and so on, cyclically alternating for a long time. The results of experiments showed the effectiveness of this system.

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Conflict of Interest: None to disclose

P65 SUGAR-MODIFIED ALGINATE EFFECTS ON IMMOBILIZED PROBIOTICS VIABILITY AFTER LOW-TEMPERATURE STORAGE

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The aim of our study was to increase the survival of probiotic cells immobilized in a modified sodium alginate gel after low-temperature storage. The subjects were probiotic strains of bifidobacteria, lactobacilli, lactococci and yeast S. cerevisiae (var S. boulardii). Probiotics were immobilized in 1% sodium alginate gel granules by ionotropic gelling with calcium ions. The diameter of the granules was 2.2 mm. The gel was modified by addition of lactose, maltose, trehalose. The samples were frozen by rapid or slow cooling and stored at –80 and –196 °C. Slow cooling with the rates of 1 – 10 degrees per minute provided higher preservation of microorganisms during freezing. One year storage (observation period) at –80 and –196 °C did not cause additional cell death. The presence of sugars in the gel increased its cryoprotective properties. The maximum protective effect was provided by alginate gel modified with trehalose.

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Conflict of Interest: None to disclose

P66 -1.2 °C/MINUTE BRAIN COOLING VIA FLUOROCARBON LUNG LAVAGE IN A DOG SURVIVAL MODEL

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Rapid brain-cooling in large mammals requires circulatory forced convection. A method of blood-cooling by lung lavage with cold fluorocarbon (FC) liquid is efficient, as ventilation is carried on simultaneously with oxygen gas foam.
33 dogs (25.0 ± 0.88 kg, age 6.8 ±0.53 yrs, N=33) were divided into 4 Groups G1-G4 according to FC liquid type used. Groups were G1 (FC-84/FC-40 2:1; N=6), G2 (FC-40/HFC 7200DL 1:1; N=12), G3 (FC-40/HFC 7200DL 2:1; N=8), and G4 (FC-3283; N=8). The FC liquids were chosen for low vapor pressure and viscosity. Anesthetized dogs received, per #10 endotracheal tube, ~

50 lavages (liquid breaths) of 6.5 mL/kg FC at < 1 ºC , over 7 minutes (total 340 mL/kg). A custom machine delivered cold FC and suctioned warm foam. Lavage cycle was 3.5 seconds infusion and 4.5 seconds suction. 500 mL O_2 gas delivered by bag-valve accompanied each infusion. After lavage, 1-hour temperature equilibration (T_{equil}) was followed by recovery. 20 dogs had arterial blood gases (ABGs) drawn from femoral arterial cannulae.
Tympanic temperatures (T_{tymp}) cooled by -6.5 ±0.28 (mean ± S.E.M),-6.8 ±0.41, -6.3 ±0.38, and -6.5 ±0.54 ºC for groups G1-G4. T_{tymp} minimum (nadir) occurred at -7.7 ±0.2 minutes with mean cooling rate to nadir -0.83 ±0.27 ºC/minute. Maximum cooling rate was -1.2 ±0.051 ºC/minute, which occurred at 2.7 ±0.19 minutes. T_{equil} was -3.8 ±0.10 ºC. ABGs before lavage end, were pO_2 279 ±21 and pCO_2 31 ±1.9 torr. One G2 dog fibrillated at 7.5 minutes. The remainder survived to symptom-free ambulation. G4 had significantly faster recovery from post-lavage airway obstruction syndrome.
Lung lavage has some advantages over total liquid ventilation. With O_2 gas insufflation at 3 times lavage size, thermal transfer efficiency is high (60%), thermal dead space is suppressed by turbulence, and no oxygenator or CO_2 absorber is needed. Cooling may be as fast as cardiac-bypass. In addition, we find that FC-3283 (perfluorotripropylamine) is a particularly benign lung lavage liquid.

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Conflict of Interest: None to disclose

P67 A HYBRID REWARMING SYSTEM CONSISTING OF ELECTROMAGNETIC HEATING AND CONVENTIONAL THERMAL CONDUCTION FOR LARGE VOLUME BIO-SPECIMEN

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Rapid and uniform rewarming are needed for successful cryopreservation of biological cells and tissues, especially for large-volume samples. In order to achieve ideal rapid rewarming rate and homogeneous temperature distribution for large size biomaterials, a hybrid rewarming system that consists of electromagnetic heating and conventional thermal conduction was developed. The hybrid rewarming system was realized by a designed single-mode electromagnetic resonant cavity and a special sample solution holder with a central post to provide auxiliary heat to the cryopreserved materials. We tested the rewarming of 20 mL Jurkat cell suspension from -80 °C to 0 °C using this hybrid rewarming system. An average warming rate at 120 °C/min was achieved and the temperature distribution was confined within 0.1°C/mm. In contrast, conventional water bath rewarming method can only achieve an average warming rate at 40 °C/min. The maximum temperature gradient is 0.25 °C/mm. The average recovery rate for the cryopreserved Jurkat cells was significantly increased from 68% (water bath) to 82% (hybrid EM-conduction system).
The system demonstrated here can create sufficient heating to penetrate large samples and generate uniformly volumetric rewarming. It is expected that; this hybrid system may be applied to rewarm large vitrified tissues and organs.

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P68 EFFECT OF DIFFERENT STRUCTURES OF LECITHIN NANOPARTICLES ON BULL SPERM CRYOPRESERVATION

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The aim of this study was to investigate the effect of different structures of lecithin nanoparticles on bull sperm quality after freezing and thawing. We have tested nanomicelle (NM) and nanoliposome (NL) structures of lecithin as an extender could improve the results of bull sperm after freezing and thawing. Different ratios of nanomicelles to nanoliposomes (3:0, 2.5:0.5, 2:1 and 0:3 for NM:NL of lecithin) were used to freeze bull sperm. Ejaculates were obtained from six Holstein bulls. For producing nanomicelle, extenders were made by adding soy lecithin to a Tris-citric-fructose buffer, put in slow shaking in order to get a lecithin emulsion and then sonicated to a particle size in range of nanometres. For producing nanoliposome, we used the thin film preparation method. Post-thaw sperm quality was evaluated as motility (CASA), plasmalemma functionality (HOS test), abnormal forms, and acrosome and plasmalemma integrity (flow cytometry). Data were analyzed using GLM (SAS) to determine the effect of nanostructure of lecithin. The result of dynamic light scattering (DLS) shows that the average particle size used in the present study was 73.7 nm. Results are showed as mean±SEM or percentages (%). Total and progressive motility, plasmalemma functionality, plasmalemma integrity and acrosome integrity were higher in samples frozen with extender based on tris containing of 3% nanoliposome of lecithin (0:3 nanomicelles: nanoliposomes) (82.16%, 48.16%, 70.3%, 82.04%, 70.01% respectivaly). This data shows that with increasing nano-liposome ratio in the extender, sperm damage was reduced and the motility and plasmalemma integrity was improved. It seems that the use of liposomal structures alongside the micelles and the particle size below 100 nm can be a good way to improve the efficiency of lecithin as a cryoprotective for

cryopreservation of domestic animals' sperm.

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Conflict of Interest: None to disclose

P69 ENRICHMENT OF A NANOLIPOSOME WITH REDUCED GLUTATHIONE FOR BOVINE SEMEN CRYOPRESERVATION

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The aim of this study was to investigate the effect of enrichment of liposome as a cryoprotectant with reduced glutathione (GSH) during bovine semen cryopreservation. We have tested the effect of different levels of GSH as an antioxidant at 0 (control), 1 mM, 2.5 mM and 5 mMon concentration on quality of bull sperm after a freezing and thawing process. 36 ejaculates were obtained from six Holstein bulls. The thin film preparation method was used to prepared liposomes containing GSH which were then sonicated to reduce the particle size to the range of nanometres. Post-thaw sperm quality was evaluated including motility (CASA), plasmalemma functionality (HOS test), membrane integrity (Eosinophilic test) and abnormal forms. Data were analyzed using GLM (SAS) to determine the effect of GSH concentration. Total and progressive motility, plasmalemma functionality, and membrane integrity were the highest in the samples which were frozen at a concentration of 2.5 mM GSH (45%, 27%, 57%, and 60.24% respectively). In conclusion, enrichment of the nanoliposome with GSH could improve sperm cryosurvival in Holstein bull.

Source of Funding: This work was supported by funding from the University of Tehran.
Conflict of Interest: None to disclose

P70 GLASS VISUALIZATION IN CELLS AND TISSUES BY LOW-TEMPERATURE SCANNING ELECTRON MICROSCOPY (CRYO-SEM)

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Many cryopreservation protocols rely on vitrification, after water being removed by evaporation, cryoprotectant addition or external ice formation followed by quench-cooling. Vitrification guarantees long-term survival because ice nucleii formation is impaired in these extremely viscous solutions. Specimen vitrification monitoring allows optimizing cryopreservation protocols for different species and tissues. However, physical changes associated to glass transition, Tg, are small and difficult to detect. Differential scanning calorimetry (DCS) is frequently used: Tg appears as a small jump in the thermogram base line. DSC yields global information for the whole pan content, and the absence of ice in the particular cells required for survival cannot be acknowledged.
When observing biological samples by low-temperature scanning electron microscopy (cryo-SEM), the "etching" protocol increases contrast: after initial sample cooling at liquid nitrogen conditions, temperature is raised under high-vacuum to induce ice sublimation. After metallization, image appears as a distribution of black "indented" regions (ice crystals), separated by clearer areas, not sublimating.
An application of etching-cryo-SEM can detect vitrified areas within a specimen. If solutions in its tissues are viscous enough to become vitrified upon introduction in the microscope cryo-chamber, etching is not effective, as vitrified water sublimation rate is very low.
As illustrated with both models (vitrification solutions at different dilutions) and real samples (mint axes at different stages of cryopreservation protocols), the "border" between ice forming and vitrifying conditions shows extremely clear. Increasingly dehydrated and viscous tissues produce increasingly clear and detailed images (due to the smaller size of their ice crystals). When dehydration is enough to allow vitrification upon cooling, this detailed image is briskly lost.
There is a perfect agreement between the dehydration conditions producing ice thermal events or Tg base line-displacement by DSC with those yielding detailed images or not-etched ones. For real mint samples, there is also agreement for specimen survival.

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Conflict of Interest: None to disclose

P71 THE EFFECT OF MELATONIN AND COOLING TO -5°C ON BULLDOG SPERM CRYOSURVIVAL

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Artificial insemination (AI) in dogs has been increasing in recent years especially for some breeds like the Bulldog. AI is usually carried out employing fresh semen collected immediately before use; thus, males should always be available and close to the females. The use of frozen spermatozoa may facilitate AI when required; however, cryopreservation reduces sperm fertility. Use of antioxidants such as melatonin (MLT), and cooling to subzero temperatures have improved, in some cases, cryosurvival of sperm from different species. To assess

the effect of MLT, and cooling to -5°C before freezing on Bulldog sperm cryosurvival, semen was collected from 4 adult males. Semen was assessed, centrifuged and resuspended in a freezing medium (EYT, 3% glycerol, 400x10^{6}cells/mL). Sperm was cooled to 5°C in 2 hours, and EYT (7% glycerol) was added to reach a final concentration of 200x10^{6}cells/mL and 5% glycerol; then MLT (0, 1, or 2 mM) was added, and sperm from the different treatments were packaged in 0.5 mL plastic straws. Half of the straws at 5°C was exposed 15 min to nitrogen vapor (4 cm above liquid nitrogen), and then were immersed in liquid nitrogen. The other half (at 5°C) was further cooled to -5°C (0.15°C/min) employing saline ice at −12°C, and frozen as mentioned. Straws were thawed in a water bath at 37°C for 30 sec; then, progressive motility, plasma membrane integrity, capacitation status, and acrosome integrity were assessed. Data were arcsine transformed to normalize them before ANOVA between MLT levels; "t" test was used to compare cooling temperatures. There were significant differences ($P<0.05$) between cooling temperatures regarding non-capacitated acrosome intact, capacitated acrosome intact, and acrosome intact spermatozoa. There were no significant differences between MLT levels, or the interaction cooling*MLT. In conclusion, pre-freeze cooling to -5°C improved Bulldog sperm cryosurvival but MLT did not.

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Conflict of Interest: None to disclose

P72 EFFECT OF HYPOTAURINE ON VITRIFICATION OF HUMAN SPERM DURING CRYOPRESERVATION

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The use of vitrification process with antioxidants such as hypotaurine is the stuff of the cryobiology for treatment of male infertility. In spite of cryopreservation advantages, this process cause functional and structural damage to the sperm. Each sample was divided in two groups consist of vitrification (control) and hypotaurine group. After semen preparation with density gradient centrifugation (DGC), total and progressive motility (analyzed by Computer-Assisted Sperm Analysis), morphology (stained by papanicolaou), acrosome reaction (FITC-PSA labeling) and malondialdehyde levels were studied in two groups. Total and progressive motility in hypotaurine group compared to the control group was not significant. The normal morphology in the hypotaurine group was significantly higher than the control group (7.02±2.66 and 10.05±3.31%). The acrosome reaction in the control group was significantly decreased compared to hypotaurine (56.60±10.49 and 41.75±10.38%). Changes in the lipid peroxidation level in the control group in the respect of hypotaurine group are not significant during vitrification. Data in this experiment demonstrates that usage of hypotaurine is an important cryoprotectant during vitrification and therefore improved sperm recovery.

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Conflict of Interest: None to declare

P73 EFFECT OF EDTA AS CHELATING AGENT IN EXTENDER ON POST THAW QUALITY OF BUFFALO SPERMATOZOA

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Spermatozoa are prone to mechanical and biochemical damages during cryopreservation. Influx of Ca^{++} causes early capacitation and production of reactive oxygen species. Hence, calcium chelators should be added to halt these reactions. The present study was designed to evaluate the effect of EDTA (Ethylenediaminetetraacetic acid) as a calcium chelator in a semen extender at four different groups respectively (0, 0.1, 0.2 and 0.3%). A total of n = 5 (replicate = 3) mature bulls were used for this experiment. *Tris*-citric egg yolk extender was used for semen extension. Semen from each bull was subjected to initial evaluation (> 500 million spermatozoa/ml and > 60% motility) and were pooled and then divided into four aliquots for extension in four different concentrations of EDTA. Semen was extended at the 50×10^6 motile spermatozoa per ml. Aliquots were then cooled to 4 ºC in 2 hours. At 4 ºC semen was kept for 4 hrs to be equilibrated. Cooled semen samples were frozen by programmable freezer. Post thaw motility, velocity distribution and kinematics were assessed through computer assisted semen analyzer while plasma membrane integrity, sperm viability and acrosome integrity, chromatin integrity were assessed by supra vital, Dual Trypan blue-Giemsa and toluidine blue stains respectively. All the data were analysed by analysis of variance. Group III (0.2%) improved visual motility % (64 ± 0.26), progressive motility % (34.6 ± 2.43), plasma membrane integrity % (42 ± 1.88). Results of BCF, VCL, VSL and VAP were higher in group III, which disagreed with the other three groups significantly ($P < 0.05$). Therefore, it can be concluded that 0.2% EDTA improves majority of the semen parameters observed, hence it should be included in a semen extender to have the better post thaw quality of Nili-Ravi buffalo bull semen.

Source of Funding: National Agricultural Research Centre, Islamabad, Pakistan
Conflict of Interest: None to disclose

P74 SEMINAL PLASMA OXIDATIVE STRESS BIOMARKERS DO NOT PREDICT BOAR SPERM FREEZABILITY

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Porcine sperm are very sensitive to oxidative stress (OS) and seminal plasma (SP) plays a key role in regulating the OS intensity. This study aimed to evaluate whether OS biomarkers of SP could predict sperm freezability of boar ejaculates. Ejaculate samples (n = 38) from 5 boars were split into 2 aliquots; one was immediately centrifuged twice (1500 xg, 10 min) to obtain SP and the other remained stored overnight at 17 ºC embedded in its own SP (diluted in BTS, 1:1; v:v) before being frozen with a standard 0.5-mL straw protocol. Biomarkers of OS in the SP-samples were evaluated in terms of advanced oxidation protein products (AOPP), ferrous oxidation-xylenol orange (FOX) and total oxidant status (TOS) using an automated analyzer (Olympus AU600). The values ranged from 1.15 to 77.25, 0 to 22.06 and 1.15 to 15.30 µmol/L for AOPP, FOX and TOS, respectively. Sperm quality was assessed before freezing (samples stored 24 h at 17 ºC) and at 30 and 150 min after thawing (in a water bath at 37 ºC) in terms of total and progressive motility (CASA system) and sperm viability (flow cytometer [H-42, PI and FITC-PNA]). The data of post-thaw sperm quality were normalized to the values before freezing and expressed as recovery rates. Multiple linear regression analysis showed that SP-OS biomarkers had a weak predictive value, since they only explained a 25.5%, 10.7% and 15.2% (adjusted R^2) of the variance of recovered rates of total and progressive motility and sperm viability, respectively. In conclusion, the assessment of SP-OS biomarkers did not seem an effective procedure to predict the sperm freezability of boar ejaculates.

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Conflict of Interest: None to disclose

P75 CRYOPRESERVATION MODIFIES THE PROTEIN PROFILE OF BOAR SPERMATOZOA

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The fertility of pig sperm decreases substantially after cryopreservation. Modifications in the protein profile experienced during the freezing and thawing (FT) process could be one of the main causes. The present study tries to identify putative changes between the proteome of fresh and FT spermatozoa. Manually collected ejaculated sperm-rich fractions from 5 fertile boars were centrifuged (1,500 g 10 min) twice and the resulting sperm pellets pooled and then split into 2 aliquots: one was preserved fresh and the other was frozen and thawed. Freezing followed a standard protocol for 0.5 mL straws, and the FT sperm was passed through Percoll gradient to remove egg-yolk remnants. Both sperm samples (fresh and FT) were further split into 3 technical replicates, all subjected to a combination of one-dimensional gel electrophoresis, liquid chromatography tandem-mass spectrometry and sequential window acquisition of all theoretical spectra (SWATH) for protein identification and quantification. After bioinformatics analysis, 1,102 proteins were quantified and the penalized linear regression LASSO (Least Absolute Shrinkage and Selection Operator) revealed 5 proteins overexpressed in fresh respect to FT-sperm: Fibronectin, Thrombospondin 1, Heat shock protein 90 alpha family class A member 1, Nexin-1 and Protein disulfide-isomerase. These five proteins are related to capacitation, acrosome reaction, oxidative stress and motility and their underexpression in FT-sperm may help explain why FT porcine sperm are less fertile than fresh sperm.

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Conflict of Interest: None to disclose

P76 IMPROVEMENT OF HUMAN SPERM QUALITY AFTER SUPPLEMENTATION ENRICHED SERUM IN FREEZING MEDIA

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Background: The importance of freezing sperm has been shown in previous studies. It seems that the use of ingredients such as antioxidants and fatty acids are effective in improving sperm quality after freezing this study was conducted to determine the combined effects of adding enriched serum in frozen media in human sperm. **Objective:** This study was performed to evaluate the effects of enriched serum (5%) contains these nutrients in commercially freezing media for cryopreservation of human sperm. **Materials and Methods**:To produce enriched serum, 16 rams (n=4) were fed diets as follows: Control (CTR), vitamin E (VITE; 200 IU/ram/day), fish oil (FO; 40 g/ram/day) and fish oil + vitamin E (FO+VITE). In the second phase, semen samples were collected from 25 normospermic men and then were divided into six equal experimental groups for freezing media containing enriched 5% serum as mentioned above. To evaluate the effects of enriched serum on the quality of frozen-thawed sperm, several parameters such as motion characteristics (CASA), viability, DNA fragmentation and total ROS were recorded. Data were analyzed using SPSS. **Result**: The highest significant (p<0.05) percentage of sperm motility and viability were observed in groups containing VITE (49.78±1.96 and 44±8.03) and FO+VITE (51.28±2.71 and 48±7.74) compared to control group

(38.66±1.89 and 37±8.47), respectively. Moreover adding serum contains FO+VITE to freezing media improved (VCL) than other group. ROS concentrations were not significantly (p>0.05) affected by the serum supplementation. Flow cytometry parameters in this study confirmed our results related to motility and viability. **Conclusion**: This improvement is mostly related to characteristic of antioxidant activity of vitamin E and omega-3 fatty acids. This combination has several characteristic that may improve the quality of sperm such as membrane flexibility and improve signal transduction during cryopreservation.

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Conflict of Interest: None to declare

P77 EFFECTS OF ANTIFREEZE PROTEINS ON CRYOPRESERVED STERLET, *ACIPENSER RUTHENUS* SPERM QUALITY

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The effects of antifreeze proteins on sterlet *Acipenser ruthenus* sperm quality and fertility ability were investigated during cryopreservation in present study. Two antifreeze proteins (AFPI or AFPIII) were used at concentrations of 0.1, 1, 10, and 100 µg/mL. The motility, velocity, plasma membrane integrity and fertilization rate of fresh, cryopreserved sperm with/without antifreeze proteins were compared. Fresh sperm showed 85 ± 4 % motility and 160 ± 2 µm/s curvilinear velocity, respectively. A significant decrease of motility in frozen-thawed sperm without addition of antifreeze proteins was observed (44 ± 9 %) following cryopreservation, compared to the fresh sperm. The highest motility of frozen-thawed sperm was obtained in cryopreserved sperm with addition of 1 µg/mL of AFPIII (58 ± 14 %). No significant differences were observed in curvilinear velocity between fresh sperm and cryopreserved sperm with/without addition of AFPI or AFPIII. The flow cytometry analysis revealed that 94.5 ± 6 % of fresh sperm had intact plasma membrane, while only 26.6 ± 14 % of cryopreserved sperm without addition of antifreeze proteins had intact plasma membrane. Supplementation of 10 µg/mL of AFPI in cryopreservation medium was the most effective treatment for improvement of sperm plasma membrane integrity (65.4 ± 12 %). Additional fertilization experiment with 200 000 spermatozoa per egg showed that no significant differences was detected in fertilization rate between fresh and cryopreserved spermatozoa with/without addition of antifreeze proteins, except the sperm cryopreserved with 100 µg/mL of AFPIII. Thus, addition of antifreeze proteins to cryopreservation medium could be considered to improve the post-thawed sperm quality of sterlet.

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Conflict of Interest: None to disclose

P78 SPERM CRYOPRESERVATION IN *Prochilodus lineatus* THROUGHOUT THE SPAWNING SEASON

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Cryopreservation of fish sperm is a technology used for the conservation of genetic resources, especially for endangered species.However, this process causes cryoinjuries to the cell and can reduce sperm fertilization capacity, which can be predicted by post-thaw sperm quality. Thus, the aim of this study was to evaluate the best period for the collection and cryopreservation of *Prochilodus lineatus* sperm throughout the spawning season. Sperm was collected during the spawning season (2017/2018) on November (n=7), December (n=8), January (n=9), February (n=11) and March (n=8). After collection, the sperm was diluted in BTS® and methyl glycol [$CH_3O(CH_2)_2OH$] solution, at a ratio 1:8:1, drawn in straws (n=3 per male), frozen in a nitrogen vapor vessel (freezing rate of -36.5 °C/min) and transferred to a cryogenic tank within 24 h for storage. After thawed at 60 °C for 8 s, the sperm was activated in 150 mOsm/kg glucose solution, and the CASA system (Microptics) was used to evaluate the rate of motility (%) and curvilinear velocity (VCL; µm s^{-1}) 10 s post-activation. Data were compared by ANOVA, followed by the Tukey test (P<0.05). Significantly higher (P<0.05) motility rate was observed when samples were cryopreserved from January to March (71-72%) compared to those from November (44%), samples cryopreserved on December yielded intermediate motility value (63%). There was no difference in VCL among samples cryopreserved from November to March (P>0.05), which ranged from 46 to 59 µm s^{-1}. *P. lineatus* sperm presents higher motility when cryopreserved between the months of January and March, which is the recommended period for this species semen cryopreservation.

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Conflict of Interest: None to declare

P79 THE EFFECT OF MELATONIN AS ANTIOXIDANT FOR THE CRYOPRESERVATION OF BOAR SPERMATOZOA

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During cryopreservation spermatozoa suffer a number of physical and biochemical changes, such as oxidative stress and lipids phase transitions, which compromise their morphology and physiology. Uses of melatonin (MLT) as antioxidant, and pre-freeze cooling to sub-zero temperatures have improved sperm cryosurvival. To assess the effect of MLT, and pre-freeze cooling (+5° or -5°C) on boar sperm cryosurvival, semen was collected from 4 boars (18 ejaculates). Semen was diluted in a commercial extender to be transported (34°C, 90 min); sperm were assessed for: progressive motility, viability and morphology, concentration, plasma membrane integrity and fluidity, acrosome integrity, and capacitation status. Semen was centrifuged, and pellet was resuspended in BF5 freezing medium without glycerol. In the first stage, MLT (0, 1, or 2 mM) was added to sperm samples at: i) 23°C, before cooling; ii) 5°C, before addition of glycerol or iii) 5°C, after addition of glycerol. In the second stage, MLT (0, 1, or 2 mM) was added to sperm samples (200 x10^6 cells/mL, 1% glycerol) that had been cooled to 5°C. Diluted sperm were packaged in 0.25 mL plastic straws, half of which was frozen over nitrogen vapors and immersed in liquid nitrogen; the other half was further cooled to -5°C, and frozen. Straws were thawed in water bath at 39°C for 30 sec; data was analyzed by ANOVA and "t" test. Addition of MLT at 5°C was better after than before addition of glycerol, and better than at 23°C. There were differences ($P<0.05$) between cooling treatments in plasma membrane integrity, non-capacitated acrosome intact, and capacitated acrosome intact spermatozoa (+5 better than -5°C). There were no differences in sperm quality between MLT treatments; also, there were no differences in any of the combinations of MLT and cooling. In conclusion, pre-freeze cooling was more important than MLT in boar sperm cryopreservation.

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Conflict of Interest: None to disclose

P80 SOLVENT-FREE CRYOSTORAGE OF MICROORGANIMS USING ICE GROWTH INHIBITNG POLYMERS

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In the fields of micro- and molecular biology it is vital to successfully store, bank and transport micro-organisms with maintenance of membrane integrity and viability. Glycerol is currently the cryoprotectant of choice, however it is intrinsically toxic to many micro-organisms. Here we introduce the use of ice recrystallization inhibiting (IRI) polymers, inspired by extremophile species which survive freeze-thaw cycles, to enable solvent-free cryostorage of bacteria.
Our group is developing synthetic polymer mimics of antifreeze (glyco)proteins (AFPs) with the aim of reproducing their ice recrystallization inhibition (IRI) activity. A key benefit of polymeric formulations is that they are low cost and highly tunable in terms of structure and hence function. We have previously shown that addition of these polymers to DMSO mediated cell cryopreservation can dramatically improve the post-thaw viability. In this work, we have developed an IRI active polymer formulation to investigate the cryopreservation of bacteria.
The unique polymer formulation was observed to provide a synergistic effect for all strains, giving equal or better levels of recovery than glycerol. The formulation results in a 4-fold increase in *Escherichia coli* yield post-thaw at 1.1 weight percent compared to the customarily used 25 weight percent of glycerol. These results expose the versatile nature of this polymer formulation, as the method is appropriate for Gram negative, Gram positive and Mycobacteria strains.

Source of Funding: European Research Council
Conflict of Interest: Patent application has been filed on this work

P81 CRYOPRESERVATION OF BOVINE GRANULOSA IN 96-WELL PLATES ENHANCED BY ICE NUCLEATION CONTROL

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Control of ice nucleation during cryopreservation of cells is a crucial factor as excessive supercooling of cryopreservation media during controlled rate freezing can lead to intracellular ice formation and cell death. Small volumes of liquid (100's of microliters) have a far greater propensity to supercool to a high degree than the relatively large volumes (several millilitres) typically used for cryopreservation. Cryopreservation in the highly convenient 96-well plate format, with sample volumes per well of only 100s of microliters, has therefore been challenging to date. We present results of cryopreservation experiments using primary mammalian cells in 96-well culture plates using a mineral nucleator

(Asymptote IceStart™ arrays) and conduction based cooling system (Asymptote VIAFreeze). This applies a discovery from the atmospheric sciences where it was found that certain mineral dusts nucleate ice efficiently. Primary cultures of bovine granulosa cells were cultured to confluence in McCoys 5A medium supplemented with antibiotics and 10% foetal calf serum in 96-well plates. After removal of culture medium and addition of cryoprotectant and IceStart™ arrays the plates were cooled to -80 °C in a controlled rate freezer at 1°C/min and then stored for at least 3 days. The cryoprotectant medium used contained 10% Me_2SO and 0.1M trehalose. The impact of cryoprotectant treatment in the absence of freezing, and post-cryopreservation and thawing on viable cell number per well were quantified using the vital dye neutral red. Preliminary results showed that plates cryopreserved using a nucleator demonstrated enhanced survival rates compared to those without; typically 30% compared to 15%. In plates where ice nucleation was triggered using a liquid nitrogen cooled freezing tip ~60% survival was observed. These preliminary results suggest that control of ice nucleation is critical for successful cryopreservation of primary bovine granulosa cells in 96-well plates and that control can be achieved using mineral nucleator-based arrays.

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P82 REWARMING OF CRYOPRESERVED *C. ELEGANS* BY INDUCTION HEATING WITH ALTERNATING MAGNETIC FIELDS.

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Standard technique of cryopreservation of *C. elegans* nematodes is possible with larvae specimens achieving survival rates of less than 40 % when using slow freezing protocols. However, the preservation of adults is difficult with this approach, where the common survival rates are around 3%.The main issues in the cryopreservation processes used so far is to achieve high warming rates and homogeneous thermal fields. To date, these goals have only been achieved for small volume samples such as cells or small tissues (where thermal differences and thermal mass are not significant) but not for bulky samples. The standard rewarming method consists in using convective air flows or warm water baths on certain occasions. These methods could cause excessive temperature gradients for bigger samples. In this work, we have tested the inductive rewarming as an alternative to standard rewarming. When particles are exposed to alternating magnetic fields, they turn into small heat sources. As long as the particles are homogeneously distributed, it is possible to achieve uniform thermal fields in the rewarmed volumes. In addition, we can manage the warming rates by controlling the power supplied to the induction device. This method was tested by using 1ml vials with *C. elegans* suspended in a freezing solution consisted of 15% glycerol in M9 buffer. The vials were stored at -70 ºC during 2 weeks. Then, they were rewarmed by using both standard and magnetic induction methods. After the rewarming process, samples were added to 100 mm diameter Petri plates, which had been previously marked and by zones (to facilitate the subsequent nematodes counting). 24 hours after rewarming, we counted the specimens that were crawling. In adults, we achieved an average survival rate of 43.9% with maximum of 84.5% and warming rates around 100 ºC/min with thermal gradients lower than 10 ºC.

Source of Funding: Cryobiomed Research Institute
Conflict of Interest: None to disclose

P83 CHANGES OF CRYOTOLERANCE DURING SEED DEVELOPMENT IN POMELO AND GRAPEFRUIT

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Pomelo and grapefruit are commercially important tropical and subtropical fruit crops and both produce intermediate seeds. Their seeds can be desiccated to below 10% moisture content (fresh weight basis) safely, but significant seed viability losses (≥15%) were detected after only 12-months' low-temperature storage at both 4ºC and -18ºC, thus cannot be stored in conventional seed bank for long-term germplasm conservation, and cryopreservation is the only method to ensure this purpose, necessitating development of cryopreservation protocol. To develop cryopreservation protocol, there is a critical question to be answered firstly: *When to harvest the seeds?* Our studies revealed changes in cryotolerance of pomelo and grapefruit seeds during development. Although no seeds survived cryo-exposure at early stages of their development, 20% postthaw seeds emerged around 150 days after flowering; cryotolerance increased with seeds development, and maximized around 250 days after flowering, with only a small fall in cryotolerance at the last stage of seed development. This cryotolerance development pattern differs from those of orthodox and recalcitrant seeds previously reported. Mature pomelo and grapefruit seeds had cytological traits characterized by orthodox seeds, i.e., organelles concerning metabolism were rare except sporadic mitochondria, embryonic cells arrested at an inactively metabolic status, but poor in accumulation of soluble and heat-stable proteins, indicating cytological and biochemical basis of cryotolerance. This knowledge can be a help for seed collection when developing cryopreservation protocol for pomelo and grapefruit seeds.

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Conflict of Interest: The author has declared that no competing interests exist.

P84 BIOTECHNOLOGY FOR VIRUS-FREE PLANTING STOCK OF APPLE AND TREE NUTS USING CRYOTHERAPY

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Improved fruit and nut production is dependent on healthy planting stocks. Many traditionally propagated cultivars have accumulated a heavy virus load, making them less productive. The goal of this project was to use cryotherapy to eliminate viruses from important cultivars. *In vitro* apple cultures infected with one or more viruses, *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* and *Apple mosaic virus*, were subjected to cryotherapy. For cryotherapy shoot apices (0.8 mm) were aseptically isolated from 3-week cold acclimated shoots (8 hours at 22°C, light intensity 10 $\mu E \bullet m^{-2} \bullet s^{-1}$/16 hours at -1°C in the dark). Apices were cultured on Murashige-Skoog medium with 0.3 M sucrose for 2 days under cold acclimation and cryopreserved using a PVS2 vitrification protocol. After immersing in liquid nitrogen, shoot tips were rewarmed and placed on multiplication medium for regrowth. On average, 40.1% of the tested materials were virus-free after one cryotherapy procedure. Virus elimination varied from 12% to 100% among the genotypes. Cryotherapy efficiency depended on apple cultivar studied and virus (or viruses) infected. 'Rennet Landsbergskyi' mix-infected by ACLSV and ASPV retained both viruses in all shoots after cryotherapy whereas 'Arm 18' infected by ACLSV was totally virus-free following cryotherapy. Since apple is a clonally propagated crop, even a relatively low percentage of virus elimination can provide virus-free cultures for producing stock plants. Recovered shoot tips were micropropagated, rooted, and transferred to containers with sterile soil and covered with plastic caps. Virus-free apple stocks were obtained, and a similar procedure was used for virus elimination in hazelnut and walnut shoots. The resulting *in vitro* collection will serve as the basis for the creation of a germplasm cryobank, and for obtainining super elite plant stocks for distribution to nurseries.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P85 DEVELOPMENT OF THE D CRYO-PLATE METHOD FOR CRYOPRESERVATION OF GARLIC (*ALLIUM SATIVUM*)

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The D cryo-plate method was successfully adapted for the cryopreservation of garlic plants (*Allium sativum*). The optimal D cryo-plate method for garlic is as follows. Shoot tips were collected from harvested bulbs and precultured for 1-3 days at 25°C on solidified MS medium containing 0.3 M sucrose. Precultured shoot tips were placed in wells on an aluminum cryo-plate and embedded in calcium alginate gel. Osmoprotection was performed by immersing the cryo-plates for 30 min at 25°C in LS (2.0 M glycerol and 1.0 M sucrose). The optimal dehydration time of shoot tips by air dehydration under the air current of a laminar flow cabinet for 30 to 180 min at 25°C. Cooling was performed by transferring the samples in uncapped 2 ml cryotubes held on a cryo-cane which was directly plunged into liquid nitrogen. Regrowth rate of cryopreserved shoot tips was 100%. Furthermore, shoot tips treated as described above but then air-dried for 120-180 min can be stored at -80°C for at least 1 week without a significant decrease in survival rate, which is convenient for most laboratories that have a -80°C freezer but not a liquid nitrogen container for long-term storage. These preservation techniques for *Allium* spp. should increase their availability in the breeding community.

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Conflict of Interest: None to disclose

P86 *IN VITRO* STORAGE AND CRYOPRESERVATION OF CLONALLY PROPAGATED PLANT GERMPLASM IN KAZAKHSTAN

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In vitro storage and cryopreservation are now considered reliable ways to provide medium and long-term *ex situ* conservation of clonally propagated plant germplasm. The Institute of Plant Biology and Biotechnology is developing techniques for cryopreservation of shoot tips for several fruit, berry, tuber and nut-bearing crops. Aseptic *in vitro* cultures were established by testing for endophytic infection on the 523 detection medium. The PVS2-vitrification protocol optimized for *Malus* germplasm was successful with few modifications for diverse germplasm cryopreservation. The modifications included variations of *in vitro* shoot cold acclimation (temperature regime, constant or variable temperatures, duration of cold acclimation), pretreatment media composition for isolated shoot tips, and PVS2 exposure time and temperature. For apple and barberry genotypes tested three or four weeks cold acclimation of shoot cultures at alternating temperature (22°C with 8 h light (10 µmol / m^2 / s)/ -1°C 16 h darkness), preculture of isolated shoot tips on Murashige and Skoog (MS) medium with 0.3 M sucrose for two days at cold acclimation conditions, and PVS2 treatment of shoot tips

on ice for 80 min were required for successful cryopreservation. Cold acclimation of potato shoot cultures was not needed. Preculture of shoot tips on MS medium with 0.3 M sucrose at 24°C and PVS2 treatment of shoot tips for 30 min at 24°C were effective for potato. Recovery of shoot tips of 15 apple cultivars ranged from 55.6 to 84.6%, 10 rootstoks from 68.2 to 95.4% and 14 wild forms of *Malus* from 70.8 to 92.1%, 10 wild forms of *Berberis* from 50.6 to 83.2% and 20 potato cultivars and hybrids form 41.2 to 68.5% following liquid nitrogen exposure. The first cryogenic bank at -196°C for preservation of valuable clonally propagated plant germplasm has been established in Kazakhstan.

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P87 CRYOPRESERVATION OF HUMULUS GERMPLASM

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Cryopreservation is a process of plant conservation under ultra-low temperatures of 196 °C under zero in a liquid nitrogen. It is used for conservation of plant germplasms, which are multiplied in a vegetative way. 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 depreciation caused by biotic and non-biotic stresses if multiplied in ex vitro conditions.The hop plant, *Humulus lupulus*, L., is a dioecious perennial species, and only female clones are used for beer brewing. Hops are used to impart bitterness, flavour and preservation properties to modern beers.*In vitro* cultures were derived from extracted meristems tips. Nodal cuttings were acclimated by low temperature and sucrose treatment. Isolated shoot tips were loaded with 0.7M sucrose for overnight and subsequently 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. The methods used and results are presented on Czech variety Kazbek and new breeding material of hop number 13966 and 14516, 13971.

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Conflict of Interest: None to declare

P88 EFFECT OF BLANCHING, FREEZING AND THAWING ON OLIVE FRUITS QUALITY

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Freezing of olives includes an initial blanching step, followed by freezing itself and thawing. Fresh olive fruits (*Olea europaea* L. vars. *Manzanilla* and *Hojiblanca*), collected at the appropriated ripening stage, were immersed in a water bath at 95 °C for 2.5 min, subjected to calcium treatment, frozen by forced convection with air at -30 °C, packed under slight vacuum, stored at -24 °C (48 h) and thawed in sodium hydroxide (NaOH) solution at 1%. Olive firmness was determined through a texture analyzer equipped with a 2-mm diameter cylinder probe. Blanching operation had the most negative effect on alterations in drupe consistency. Compared to fresh samples, significant decreases in the cuticle hardness and pulp consistency were observed varying from 8.98 to 5.55 N and 9.66 to 6.19 Nmm, respectively in *Manzanilla* fruit and from 12.6 to 7.53 N and 11.3 to 6.48 Nmm, respectively in *Hojiblanca* one. However, subsequent freezing and thawing only cause an additional loss of cuticle hardness and pulp consistency, with respect to mechanical properties of fresh fruits, of 6.9 and 9.2% in *Manzanilla* and 8.0 and 12.8%, in *Hojiblanca* olives, respectively. Total chlorophylls content (TCC) and total carotenoids (TC) were determined by a spectrophotometric assay. TCC was similar in both fresh fruits (49.0±5.57 and 55.6±5.29 µg/g fw in *Manzanilla* and *Hojiblanca*, respectively). Blanching did not significantly change the TCC in *Hojiblanca* olive, but significantly increased (23%) in *Manzanilla*. TC was significantly higher in *Hojiblanca* than in *Manzanilla* (90.5±6.68 and 68.4±4.34 µg/g fw, respectively), and blanching increased ($p < 0.05$) TC by 68% and 14% in both cultivars, respectively. Complete processing maintained the TCC of both fresh drupes, but increased by 70% their initial TC. Results were dependent on cultivar and can help in designing blanching and freezing operations which can be applied in olive industry.

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Conflict of Interest: None to disclose

P89 LONGEVITY OF SEEDS OF SIX BRITISH ORCHID SPECIES IN CRYOPRESERVATION AND CONVENTIONAL STORAGE AT THE MILLENNIUM SEED BANK (ROYAL BOTANIC GARDENS, KEW)

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Seeds ≤ 0.2 mm are known as micro-seeds. Long-term survival of micro-seed collections stored in ex-situ seed banks is not clearly understood and data is lacking to

inform an optimal strategy for seed conservation. Orchids (family Orchidaceae) are economically and culturally valuable plants which produce micro-seeds. Although orchid's seeds are desiccation tolerant (and so able to be stored using conventional storage methods at ~15% eRH and -20°C), it is thought that they may have short life spans at such conditions and cryopreservation (storage at ~-197°C) may be needed to extend seed survival rates in long-term storage. As such, since 2012, the protocol for the curation of micro-seeded species at the Royal Botanic Gardens, Kew's Millennium Seed Bank (MSB) is to duplicate the collection in both conventional storage and cryopreservation. We have analysed routine viability test results of six collections of British orchid species (*Dactylorhiza fuchsii, D. viridis, Gymnadenia borealis, Liparis loeselii, Ophrys apifera* and *Orchis mascula*) to compare longevity between storage methods following two to three years storage. At the point of storage, each collection was duplicated to both conventional and cryo-storage at ~15% eRH. Where germination data is unavailable, a Fluorescein Diacetate (FDA) test is used to indicate seed viability. All germination tests were performed using the standard MSB protocol for temperate, terrestrial orchids on BM1 medium. Preliminary results from on-going tests indicate that seeds stored in conventional storage are retaining comparable viability when compared to those stored in cryo-preservation over this two to three year period. More time may be needed to assess whether storage conditions have impacted on seed longevity for these collections in the long-term and hence the suitability of the current protocol for the ex-situ conservation of orchid seed in the MSB.

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P90 MULTI-CRYOPROTECTANT EFFLUX FROM PORCINE CARTILAGE AFTER A SHORTER LOADING PROTOCOL WITH ADDITIVES

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Our group has published a successful 570 min vitrification protocol for intact human articular cartilage, and cryoprotectant (CPA) efflux modeling has been used to validate the amount of CPA permeation into human articular cartilage for this protocol. In this study, our goal was to investigate multi-CPA efflux from porcine articular cartilage after CPA loading with and without additives. Firstly, we measured multi-CPA efflux after a shorter CPA loading protocol that requires 310 mins and checked the validity of our previously established 2-dimensional mathematical model for this protocol. Secondly, we evaluated multi-CPA efflux after CPA loading with three additives to confirm that the additives have no effect on CPA loading/efflux processes. Multi-CPA solutions prepared with dimethyl sulfoxide, ethylene glycol and propylene glycol were used for the 310 min multi-step CPA loading (total solution concentrations from 6 M to 9 M). Three additives: ascorbic acid (AA), chondroitin sulfate (CS), and glucosamine (GlcN) were added separately to the CPA solutions. A control solution consisted of CPAs without additives. Healthy femoral condyles from sexually mature pigs were randomly divided into four groups (N = 6). After CPA loading, 10 mm diameter full thickness cartilage disks were removed from the condyles and immersed for two sequential washes in 10 ml PBS at room temperature for 30 mins each. Removal solution osmolality was recorded as a function of time. The multi-CPA efflux predicted using our mathematical model agreed with experimental measurements on porcine articular cartilage within 7%, which was better than predictions of previous experimental measurements on human articular cartilage. There were also no significant differences in the measured total osmolality in the control, AA, CS and GlcN groups after the two 30 min effluxes ($p > 0.05$). The inclusion of additives to optimize vitrification protocols for articular cartilage may be beneficial without altering the protocols.

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Conflict of Interest: Some of the authors hold a patent in this area.

P91 HISTOLOGICAL EVALUATION OF ANTIFREEZE PEPTIDE CRYOPRESERVED SKIN GRAFTS BEFORE AND AFTER TRANSPLANTATION

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Cryopreservation of tissues and cells has witnessed a magnificent attention in the scientific society since 1949. With the progress of the use of autogenic and allogeneic tissues, the need for developing new compounds and techniques is increasing, but the freezing damage represent one of the biggest challenging issues. All approaches for cryopreservation aim at overcoming the biological, chemical, mechanical and thermal stresses of ice crystal formation and re-crystallization. The objectives of our study is to establish a cryopreservation technique to preserve living tissues using antifreeze

peptide type 1m (Afp1m) and to evaluate its effects on the histological properties of skin grafts before and after transplantation in a rat animal model. The histological examination of skin samples before transplantation showed that there was mild and moderate tissue damages in the tissue at epidermal, dermal, and hypodermal regions of the skin cryopreserved with 3 and 5 mg/ml Afp1m up to 72 hours at -4 and -8°C as compared to glycerol however, the skin graft cryopreserved in 5mg/ml at 24, 48 and 72 hrs have the best preserved structural integrity and tissue architecture morphologically. Statistical analysis showed significant difference ($P< 0.05$) among different treatment groups. The in vivo study was performed on 24 rats. The rats (n=24) were divided into four groups. One-two circular full-thickness 2.5 cm diameter wounds were created on the backs of rats. Non-cryopreserved and cryopreserved auto skin grafting were placed into the wound area and were stitched. Grafts were bandages. Wounds were monitored macroscopically and evaluated clinically at days 5, 8, 11, 14, 17, 20 and 22 post-operation. All skin grafts were subjected to histological examinations at the end of experiment. The histological results revealed a significant difference among 4 groups in epidermal integrity, dermal-epidermal junction. In conclusion, Afp1m was found beneficial which could serve as an efficient agent for skin grafts cryopreservation.

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Conflict of Interest: None to disclose

P92 LONG TERM STORAGE OF VITRIFIED STRATIFIED ADIPOSE-DERIVED STEM CELL SHEETS

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Adipose-Derived Stem Cells (ADSC) are mesenchymal stem cells used in the field of regenerative medicine and can differentiate in any type of cells. In this study, ADSC were seeded in culture dishes. When they reached confluence (2 days) or formed a stratified ADSC cell sheet (10 days), cells were frozen using ethylene glycol solution and preserved in liquid nitrogen (LN2) for 116 days for the multilayer cell sheets and for 122 days for the monolayer cell sheets. Cells were then thawed using complete culture media, supplemented with sucrose, and re-cultured again. Multilayer and monolayer ADSC cell sheets were harvested 2 and 14 days respectively after thawing. The vitrification of the cell sheets did not impair their properties to be harvested. However, it is important to notice that monolayer ADSC cell sheet needed 14 extra days to be ready for harvesting, when only 8 extra days were necessary when monolayer ADSC cell sheets were not thaw. Using immunofluorescent staining, the cell sheets were tested for negative (CD19, CD45R and HLA-DR) and positive markers (CD29, CD73, CD105 and HLA-A), who are markers used to identify ADSC. Compared to the control a non-vitrified ADSC cell sheet, the expression of negative markers, such as CD19, CD45R and HLA-DR, were not altered. The expression of CD29, CD73, CD105 and HLA-A were preserved. This demonstrated that a complex multilayer ADSC cell sheet structure could be preserved without damage beyond 100 days in LN2. Our cryopreservation methodology preserved the expression of stem cell markers and most likely, their capabilities to differentiate into different types of cells, such as adipocytes, chondrocytes and osteoblast.

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Conflict of Interest: Joan Oliva and Yutaka Niihara are employees of Emmaus Medical, Inc.

P93 THE CRYOPRESERVATION OF DOG OVARIAN TISSUE WITH NEEDLE IMMERSED VITRIFICATION TECHNIQUE

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Ovarian tissue (OT) cryopreservation is a promising approach for fertility preservation. The objective of this study was to investigate the effect of "needle immersed vitrification" technique for cryopreservation of dog OT. The OT (n= 40 dogs) were collected by elective ovariohysterectomy procedure and each OT was divided into 2 pieces as fresh control and vitrification group. Ovarian tissues slices (1 mm^3 pieces) were vitrified in L15 medium supplemented with 10% FBS, 50 IU/ml of penicillin, 50 mg/ml of streptomycin and placed in an acupuncture needle. The OT were exposed to cryoprotectant solution consists of 7.5% ethylene glycol (EG) and dimethyl sulfoxide (Me_2SO_4) in Dulbecco phosphate buffered (DPBS) supplement with 20% FBS for 10 min. The OT were then transferred into a vitrification solution consists of 15% EG, 15% Me_2SO_4 and 0.5 M sucrose for 2 min and then plunged directly into liquid nitrogen and stored at least 1 week. For thawing, needles were removed from the cryovial and quickly plunged into 1, 0.5 and 0.25 M sucrose solutions at 37°C for 5 min sequentially. The thawed OT fragments were incubated in DPBS supplemented with 20% FBS for 20 min at 37°C. Both fresh and vitrified-warmed OT were fixed in Bouins' solution and 2% glutaraldehyde solution for further histological analyses. Histological sections were examined under both light and electron microscope. Large number of primordial follicles (average ten primordial follicles in the 0.16 µm2) were counted in OT obtained from prepubertal dogs, whereas very few follicles were observed in OT of pubertal dogs. It was challenging to assess the presence of less follicular structures in vitrified-warmed pubertal

dogs. It has been suggested that OT vitrification studies in dogs are more suitable during the prepubertal period. This is the first study showing that dog OT is suitable for needle immersed vitrification technique.

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Conflict of Interest: None to disclose

P94 INVESTIGATION OF EFFECTS OF CRYOPROTECTANT AGENTS' TEMPERATURE ON DOG OVARIAN TISSUE VITRIFICATION

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The vitrification of ovarian tissue has the potential to preserve the fertility. The purpose of the present study was to reveal the effects of cryoprotectant agent temperature on the morphological and ultrastructural features of follicle in dog ovarian tissue. The ovarian tissues were classified as control and two vitrification groups. The forty tissues were exposed to cryoprotectants at 4°C (Group I) or room temperature (RT) (Group II). To vitrification procedure, 1 mm^3 pieces of ovarian tissue were placed in an acupuncture needle. The tissues were exposed to 7.5% ethylene glycol and 7.5% dimethyl sulfoxide (Me_2SO_4) for 10 min and then 15% ethylene glycol and 15% Me_2SO_4 for 2 min, either at room temperature or at 4°C and stored in liquid nitrogen at least 1 week. After then the tissues were thawed and immersed into Bouins' and glutaraldehyde solution for analysis. The tissues which were vitrified at 4°C were more preserved than at the room temperature. Lots of healthy primordial follicles were observed in the Group I. In Group II, there were very few normal follicles. In the ultrastructural examination in the Group I, it was observed that the double membrane structure of the oocytes' nucleus was preserved, the distributions and structures of the organelles were normal. In Group II ultrastructural observation, the oocyte cytoplasm was damaged and the linkages between oocytes and the follicle cells were detached. The ice damage was noticed in the tissue structure in this group. Oocytes vacuoles and interstitial vacuoles were seen. The optimal agent temperature was determined as 4°C for dog vitrification equilibration.

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Conflict of Interest: None to disclose

P95 MOTILITY AND MEMBRANE FUNCTIONALITY OF POST-THAW BULL SPERM UNDER EFFECTS OF LOW CONCENTRATIONS OF HYDROGEN PEROXIDE

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Sperm freezing is an important method that is widely used for long-term storage of sperm. But during the freezing process, the sperm is subjected to biochemical, osmotic, physical, mechanical and oxidative stress. Free radicals such as *reactive oxygen species* (ROS), are one of the causes of cryo-damages that can adversely affects the sperm performance. However, in recent years, very low concentration of ROS could improve the quality of post-thawed sperm in certain mammalians. The aim of this study was to consider the effects of very low concentration of hydrogen peroxide on parameters of motility and membrane integrity after freezing process. Motility and membrane integrity were assessed using computer aided sperm analysis (CASA) and Hypoosmotic swelling test, respectively. Semen sample in fresh group, control freezing group and a freezing group containing very low concentration of hydrogen peroxide were assessed (18 samples from 3 bulls). The results of the study showed that applying very low concentration of hydrogen peroxide improves the motility of bull sperm after thawing.There was no significant difference in membrane integrity between the fresh, control freezing and treatment freezing groups. It is well-known that ROS has a dual role in the biology of sperm and can improve the cryo-tolerance of bull sperm if the very low concentration was used.

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Conflict of Interest: None to disclose

P96 MOTION CHARACTERISTICS AND MITOCHONDRIAL MEMBRANE POTENTIAL OF BULL SPERM CRYOPRESERVED IN HOME-MADE SOYBEAN BASED EXTENDER

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Cryopreservation has been applied as a routine technique for processing bull sperm for artificial insemination. However, around 30-40 % of the thawed sperm are not viable. Therefore, the use of an optimal extender for cryopreservation of sperm appears to be necessary. This study assessed the effect a home-made extender containing soybean lecithin for cryopreservation of bull sperm (18 samples from 3 bulls). Motion characteristics and mitochondrial membrane potential of bull sperm were assessed after thawing. The indicators of motion characteristics were assessed using computer aided sperm analysis (CASA) and mitochondrial membrane potential using the JC1 probe. Data were analyzed using general

linear model procedure using SAS 9.1 and Tukey's test was used to determine statistical differences among groups ($P \leq 0.05$). Result showed that there was no significant difference between sperm motion characteristics (VAP, VSL, VCL, ALH and LIN) between soybeans based extender and control extender. But mitochondrial membrane potential was higher when soybean lecithin-based extender was used. Beneficial effects of soybean lecithin in the extenders for cryopreservation of bull semen need to be further investigated.

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Conflict of Interest: None to disclose

P97 PROTECTION AGAINST CELL DEATH DURING CRYOPRESERVATION WITH WHEAT PROTEINS: WHICH MODE OF CELL DEATH?

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Hepatocytes are a good physiological model for pharmaceutical and clinical domains. They are important for drug toxicity testing and are also useful for human cell transplantation as an alternative to liver organ transplantation. Freshly isolated hepatocytes are used immediately or stored by cryopreservation for later use. This approach requires cryoprotective chemicals to avoid damage caused by the freeze / thaw process and prevent loss of viability and metabolic functions. One of the most commonly used cryoprotective agents is Me_2SO, which is cytotoxic and decreases viability, metabolic functions and attachment efficiency. Our goal is to develop new cryoprotective agents that are less toxic, based on nature's freezing tolerance-associated proteins that are present in wheat. These proteins contribute to the wheat plants' capacity to withstand cold and freezing conditions. Two recombinant wheat-derived proteins, enolase and 2-cys peroxiredoxin, were used as cryoprotectants and improved hepatocyte post-cryopreservation viability and metabolic functionality, when compared to cells cryopreserved with Me_2SO. This study investigates mechanisms of cell death induced during cryopreservation with plant proteins compared to Me_2SO. While levels of reactive oxygen species (ROS) increased during cryopreservation with Me_2SO, this did not occur when hepatocytes were cryopreserved with the recombinant plant proteins. These proteins therefore appear to play an antioxidant role. Given that ROS are activators of apoptosis, we investigated whether different apoptotic pathways were activated in hepatocytes during cryopreservation. The principal type of cell death that occurred was apoptosis, although some necrosis occurred. Me_2SO caused considerably more apoptosis, whereas the plant proteins provided protection. These results improve our understanding of how cells die during cryopreservation and should help to optimize cryopreservation protocols to limit loss of cell viability and metabolic functions, and diminish cellular stress.

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Conflict of Interest: None to declare

P98 CRYOTHERAPY COMBINED WITH CORD BLOOD INJECTION IMPROVES MYOCARDIUM STRUCTURE IN OBESE RATS

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The numbers of obese people is still growing in developed countries. Obesity is often associated with an increased risk of coronary heart disease and hypertension. The goal of our study was to assess and compare influences of sole and combined use of rhythmic extreme whole-body cryotherapy (RE WBC) and cord blood nucleated cells (CBNCs) injection on myocardium structure in obese young rats. The experiments were approved by the Committee on Bioethics at the Institute for Problems of Cryobiology and Cryomedicine of NAS of Ukraine. The whole cooling cycle included 9 procedures (3 procedures/day) of cooling (2 min each) for 5 days with a day interval. Immediately after cooling the skin temperature declined within the range of 3–5 °C, the rectal temperature did not change significantly. Thawed CBNCs preparation was injected intraperitoneally in dose of $(1–3)\times10^5$ $CD34^+$-cells/kg (after the ninth procedure in RE WBC+CBNCs group). The preparation was made up to 1 ml volume with plasma autologous to the cells. The rats were decapitated 30 days after the last cooling procedures and/or the injection, and the pieces of myocardium tissue were taken for a histological study. Myocardium structure was assessed in histological specimens stained with hematoxylin and eosin, van Gieson's picrofuchsin. The signs of circulatory disorders, myocardium hypertrophy, interstitial and perivascular edema, and focal cardiosclerosis were indicated in obese rats. After combined application of RE WBC and CBNCs these signs were poorly expressed. The morphological signs of neoangiogenesis were noted. This means improving blood flow and trophism of the heart, hindering the development of ischemia and necrosis, which allows maintaining an increased number of cardiomyocytes, as well as providing their needs for hypertrophy of the myocardium, directly associated with the diet-induced obesity. Thus, the combined application of RE WBC and CBNCs can improve myocardium structure in obese young rats.

Source of Funding: Not applicable
Conflict of Interest: None to disclose

P99 USING CRYOPROTECTIVE AGENT (CPA) FOR EFFECTIVE CRYOGENIC THERMAL STORAGE UNIT USING WATER

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There are numerous cryogenic engineering applications that require low temperature for certain duration of time without utilizing cryogenic refrigerators. Depending on the temperature, heat duty, and time, various thermal storage units (TSU) have been conceived to explore large latent heat or sensible heat of materials. Some are also practically developed in many cryogenic fields such as low temperature detectors in space, superconducting coils, and liquid air energy storage (LAES) systems. Although solid nitrogen is a very good candidate for cryogenic thermal storage unit due to its high specific heat and phase transformation heat, the gas handling at room-temperature does not make it attractive for engineering applications. Liquid form such as water is much more convenient and preferred due to its high specific heat, but its solid form which is ice, may cause unnecessary mechanical stress and thermal strain when the TSU with water is implemented. Cryoprotective agent (CPA), therefore, is a good medium to alleviate or eliminate thermal stress during cool-down process by inducing vitrification of water. This research focuses on finding appropriate CPA concentration in water and exploring mixture effect of CPA to obtain zero thermal contraction of TSU material. Experimental investigation with aluminium container and strain gauge attached on the wall identifies the proper medium for cryogenic engineering applications. Effect of recrystallization and stability of glassy state after vitrification is also discussed from the engineering view point.

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P100 STUDIES ON EFFECT OF ASPHALTUM ON SEMEN QUALITY AND FREEZABILITY OF LOHI RAMS

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The study was conducted to examine the effect of asphaltum on semen characteristics and post thaw sperm motility and velocity of adult Lohi rams. Fifteen healthy and clinically normal rams were distributed into three groups 1, 2 and 3, in each group there were five rams. Control group was referred to as Group 1. Asphaltum was dispensed per oral to the Lohi Rams of groups 2 and 3 at a dose rate of 750 mg/animal and 1500 mg/animal, respectively on daily basis for eight weeks. The experiment lasted for ten weeks. At the start of the experiment and then from 6th week (twice a week) onwards semen was collected, up to the 10th week and was assessed for physical properties and semen was then filled in straws and was cryopreserved in liquid nitrogen vapour and then immersed into the liquid nitrogen container and seven days after collection, the straws of semen were thawed in water bath at 37°C, for 30 seconds. The sperm characteristics (the sperm motility and the velocity) were assessed with the help of microscope. Data was statistically analyzed and was subjected to ANOVA, two factor CRD and LSD at 5% level of significance. The present study concluded that asphaltum lead to significant (P<0.05) increase in semen volume, number of sperms, sperm motility percentage, mass activity and decrease in semen pH and dead percentage of sperms and post thaw motility and velocity of sperms.

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Conflict of Interest: None to declare

P101 CHARACTERIZATION OF ANTIMICROBIAL AGENT-LOADED CHOLESTEROL IN SITU FORMING MATRIX AS PERIODONTAL POCKET TARGETED DELIVERY SYSTEM FOR PERIODONTITIS TREATMENT

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Cholesterol has been widely employed as biomaterial agent in the drug delivery devices including pharmaceutical implant owing to its good biocompatibility. It is an essential structural component of animal cell membranes which it has been used as a lipid phase in liposome. A local antibiotic agent-loaded intra-pocket delivery system could promote a high drug concentration in the human gingival crevicular fluid to improve the drug efficacy for periodontitis treatment. Solvent exchange induced-cholesterol *in situ* forming gels (isg) comprising doxycyclate hyclate (DH) as antimicrobial agent for periodontitis treatment by injection into periodontal pocket using *N*-methyl pyrrolidone (NMP) as a solvent were prepared and investigated for their properties including gel formation, drug release and antimicrobial activities. The topography characteristic of them was also investigated with cryo-SEM which was imaged at temperatures below -140^{o}C. The burst drug release from a DH-loaded isg was reduced when the amount of benzyl benzoate was increased which the transformation from solution into matrix-like structures was attained effectively. The crystalline structure in prepared isg increased apparently when the amount of benzyl benzoate was increased. The amount of NMP was reduced when the amount of benzyl benzoate was increased, and there was a higher concentration of DH. Therefore, the cool environment initiated the higher DH crystal formation in cryo-SEM micrographs. These DH-loaded cholesterol isg systems inhibited *Staphylococcus aureus* ATCC 6853P, *Streptococcus mutans* ATCC 27175 and *Porphyrommonas gingivalis* ATCC 33277 effectively. DH-loaded cholesterol isg comprising 10% benzyl benzoate was the most suitable owing to its sustainable release manner for 10 days; thus, it was the interesting formulation for periodontitis treatment.

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P102 AN ULTRA-RAPID FREEZING TECHNIQUE FOR VITRIFICATION OF CELL SUSPENSION

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Vitrification is one of effective methods for cell cryopreservation. An ultra-rapid freezing rate is beneficial to improve the vitrification level of the extra and intracellular solution and decrease the concentration of cryoprotectant. This study develops an ultra-rapid freezing technique which utilizes thin film evaporation of liquid nitrogen on a microstructured surface instead of pooling boiling that cell sample is directly immersed in liquid nitrogen in conventional cell vitrification methods. The dimenthylsulphoxide cryoprotective solution is cooling using this new method and the pool boiling. Experimental results show that the average freezing rate of the new method reaches approximately 140000°C/min in a temperature range from 0°C to -60°C. And, that of the pool boiling is only approximately 6,500°C/min. The freezing rate with thin film evaporation is 23.8 times higher than that with pool boiling. The ultra-fast cooling rate can remarkably improve the vitrification tendencies of the cryoprotective solution. This methodology opens the possibility for more successful cell vitrification cryopreservation.

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P103 NATURAL BIOCOMPATIBLE OSMPROTECTANTS AS EFFICIENT CELL CRYOPROTECTANTS

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Cryoprotectants (CPAs) are essential for successful cell cryopreservation because they can decrease mechanical damages and osmotic shocks to cells. The current state-of-the-art CPAs are mainly based on organic solvents Me_2SO. However, the limitations of Me_2SO, especially the toxicity, remain serious concerns for clinical application. Herein, inspired by nature, we explored a series of natural osmoprotectants including proline, glycine and taurine and betaine as novel CPAs. Results showed that among them, proline and betaine exhibited more stronger abilities to protect cells from osmotic injury as well as depress water freezing point, thus mitigating ice injury to cells. As a result, we found that these two molecules exhibited high efficiency in cryopreservation of different types of cells (GLC-82, Hela, NIH 3T3 etc.) with an ultra-rapid freezing protocol. Moreover, these natural small molecules showed excellent biocompatibility, superior to the toxic CPA Me2SO. A potential mechanism was also proposed: they could enter or exit the cells rapidly via transport proteins, which could significantly simplify the cryopreservation procedures. This work sheds light on new opportunities for exploring novel CPAs and offers a new solution for cell storage, transportation and distribution.

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P104 USE OF AQUEOUS SOLUTIONS OF FULLERENE C60 IN CRYOBIOLOGICAL STUDIES

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Microalgae (cyanobacteria) *Spirulina platensis* are a valuable food, constitute a significant part of world aquaculture.
This research aim was to study the possibility of preservation and hypothermic storage of the culture of *Spirulina platensis* cells in C60 fullerene solutions. *S.platensis* cells were washed of the growth medium and placed into aqueous solutions of chemically unmodified hydrated fullerene C60 FWS. Solutions of fullerene C60 FWS of the following concentrations were used: 10^{-5}M; 10^{-7}M; 10^{-9}M; 10^{-17}M. Control was *S. platensis* cell suspension in distilled water. The test samples were stored at + 8°C. To determine the viability of *S. platensis* cells, we applied the methods of vital differential staining and analyzed the spectra of own fluorescence of *S. platensis* cells.
It has been shown that storage of *S. platensis* cells at + 8°C for 7-10 days resulted in complete destruction of all the cells in the control. In the solutions with a concentration of fullerenes 10^{-5}M; 10^{-7}M; 10^{-9}M; 10^{-17}M the number of living cells was 80-90%. After 4 weeks of hypothermic storage, the maximum number of viable cells, (80% of the initial), was observed only in a C60 FWS solution with 10^{-5}M concentration. This is confirmed by analyzing the *S. platensis* cell suspension fluorescence spectra.
Fullerene C60 FWS solutions have protective properties and can be used for hypothermic storage of *S. platensis* cell culture, which makes it possible to develop the protocols for low-temperature storage of microorganism cultures.

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P105 OPTIMIZATION AND PREPARATION OF NANOCAPSULES FOR FOOD APPLICATIONS USING TWO METHODOLOGIES

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This study investigated preparation of Nanocapsules (NCs) containing food-grade ingredients using two experimental designs: a one-factor-at-a-time method (OFATM) and an optimization method (OM). Response surface methodology (RSM) was used to optimize the process. The variables explored were concentration and type of polymer wall, using polycaprolactone (PCL) and polyethylene glycol-polybutylene adipate-polyethylene glycol (PEG-PBA-PEG) (1.0–4.0 mg) polyester triblock copolymer; food oil, using olive and avocado oil (0.5–2.0 mg); solvent, using acetone and ethyl acetate (6–12 ml); and surfactant concentration, using Tween 80 and Tween 60 (1–5 mg). The optimum conditions to obtain NCs were found to be 2.0 mg of PCL and 1.65 mg of PEG-PBA-PEG, olive oil (0.5 and 0.88 mg), acetone (6 and 10.25 ml), and Tween 60 (3.0 and 4.25 mg), with 90.9 and 71.9 nm for OFATM and OM, respectively. This research was to investigate the use of NCs in the manufacture of fruits.

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P106 THE CREATION AND MANAGEMENT OF TUMOR BIOLOGICAL SAMPLES BANK IN NANTONG, CHINA

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Background: The genetic resource of cancers have not been collected and protected well; therefore, to protect and utilize the tumour samples resources fully and provide a suitable number of tumour samples for studying tumour, an information-based management of tumor database was established in Tumor Hospital Affiliated to Nantong University. Methods: The tumor tissues, tumor-adjacent tissues, distal normal tissues removed from surgery, and blood were collected. Specimens of the tissue, serum, plasma, white cell and red cells, etc. were respectively conserved in liquid nitrogen or -80 ºC refrigerator. Meanwhile, a database of information management of tumor samples bank was established, including positions of sample placement, specimen use, comprehensive query, follow-up and management of specimen disposal, etc. Results: Sets of tissues and blood samples have been collected over 30,000 cases. The bedside health survey and long-term follow-ups have been done over30,000 times. The tumor samples data base was established and managed, which functioned with good results. Conclusion: The establishment of tumor samples database enables the quantitycontrol of the specimens and information-based management, as well as conservation of infrequent specimens and resource sharing. .A standard and fully informative tumor tissue bank and a set of related methods and managements have been established and formed for supplying a service platform of cancer research in the future,in China. Key words: tumour, biological samples bank, blood.

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Conflict of Interest: None to declare

P107 MEASUREMENT OF OOCYTES AND EMBRYOS PERMEABILITY TO ME2SO THROUGH X-RAYS CT

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In recent years, oocytes and embryos vitrification is a technique that has acquired great importance in Reproductive Medicine. In this technique oocytes and embryos are usually placed in two different solutions with different concentrations of cryoprotectants. They are generally composed of Me_2SO, EG and sucrose. The exposure to the first cryoprotectant solution last around 10 minutes; then the oocytes and embryos are transferred to the second cryoprotectant solution for 1 minute. Finally, they are usually plunged in liquid nitrogen in order to get vitrified. Due to the high toxicity of the cryoprotectants it is important to develop novel protocols in order to reduce the exposition to these agents. The diffusion rate of Me_2SO into oocytes and embryos is difficult to determine. An estimation of this parameter can be done by observing the variations of oocytes and embryos volumes: first, they reduce their volume by losing water and then they are re-expanded through the diffusion of Me_2SO. In this work, X-Rays CT (resolution of 50 μm) of oocytes and embryos has been performed in order to know the diffusion rate of Me_2SO with greatest precision and help to improve the current oocyte and embryo vitrification protocols. X-Rays CT allows measuring Me_2SO due to the attenuation produced by the sulphur atom into the Me_2SO molecule. Oocytes and embryos images have been taken by X-Rays CT in a drop and inside the vitrification system (SafeSpeed). In addition, the diffusion rate of Me_2SO has been studied at different temperatures since it increases while the temperature does. The temperatures chosen have been: 37 ºC, 25 ºC and 4 ºC. Through these experiments it has been possible to obtain the diffusion rate of Me_2SO inside oocytes and embryos at different temperatures with greater precision than it has been possible until this moment.

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