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Keynote Lecture

TAPb, CREATING A SUSTAINABLE INFRASTRUCTURE TO PATIENT-CENTRIC RESEARCH AND BIOBANKING

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ABSTRACT

Involving patients and their tissues in the research pathway is crucial for future heath innovations. There are many challenges including sustainability, logistical organisation whilst maintaining robust ethical oversight. For over a decade, TAPb has developed to become a research infrastructure to facilitate patient-centric studies across the research pipeline (from bench science, translation to human tissue and the sample component of interventional clinical trials). The TAPb platform groups researchers across academic, commercial and clinical trial sectors, and clinical services. This spreads the costs of the core team, and introduces capacity to provide specialised research services in an incremental way. TAPb is a not-for-profit, full cost recovery model that has been implemented across 7 patient clinical services within the Royal Free London NHS Foundation Trust. It supports an office of 6 fulltime staff, complemented by a number (currently about 7) part-time staff, to reflect the fluctuations in research demand. The researcher journey starts with TAPb linking NHS standard of care and research requirements to aid study design and feasibility. TAPb then provides suitable ethics and project management to develop timescales and delivery methodologies according to evolving research requirements, throughout the delivery phase. Samples and link-anonymised clinical data, and disease interpretation is provided to place samples in the correct research context. This presentation will provide an overview of the important steps which TAPb office have learnt which can make the infrastructure sustainable. Some examples of the significant research impact will be given as an illustration.

Acknowledgements: Parts of TAPb development have been supported by UCL Enterprise Award & Royal Free Charity of Royal Free London NHS Trust

DEVELOPMENT AND CRYOPRESERVATION OF HUMAN OVARIAN AND FALLOPIAN TUBE SPHEROIDS: PRELIMINARY RESULTS

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ABSTRACT

WHO ranks female infertility as the 5th most severe cause of disability worldwide (1), with 10-15 % of reproductive-age women suffering from impaired fecundity. Its incidence is rising due to multiple factors, including environmental pollutants and the off-target effects of drugs. How this infertility is caused is largely unknown. In vitro studies are limited to 2D cultures that do not mimic the in vivo physiological condition. The goal of this study is, therefore, to develop ovarian (OS) and fallopian tube spheroids (FTS) to better replicate the 3D structure of these organs. Ovaries and fallopian tubes from two post-menopausal multi-organ donors (UCLouvain's Institutional Review Board approval on May 25, 2019 (IRB reference 2018/19DEC/475)) were used for cell isolation (2). Cells were counted and seeded onto agarose micro-well molds (3). After 6 days of OS and FTS formation, 20 spheroids were individually transferred to ultra-low attachment plates, in vitro cultured for 4 days and morphologically evaluated. Several condensed-packed spheroids were formed from both ovarian and fallopian tube cells. The analyzed spheroids showed homogeneous composition, well-delimited borders, and different diameters. After 2 days of in vitro culture, OS diameter significantly increased (D0: 189.0±9.2 µm; D2: 296.2±15.2), maintaining its values until 4 days (D4: 264.6±17.7 µm; $p<0.0001$). Similarly, FTS diameter also increased on day 2 (D0: 238.4.0±42.5 µm; D2: 359.8±106.3,) being sustained on day 4 (D4: 346.8±105.5 µm; $p<0.001$). Ovarian and Fallopian tube cells can be successfully assembled into spheroids using scaffold-free agarose micro-well molds. We are now developing a cryopreservation protocol to generate a cryobank of these structures for their future application and transport.

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DIRECT IN STRAW THAWING OF BOVINE BLASTOCYST AFTER VITRIFICATION USING EITHER CRYOTEC OR OPEN POOLED STRAW

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ABSTRACT

Despite the fact that the efficiency of cryopreservation of bovine embryos by vitrification method is much higher than slow freezing, it does not dominate on farms using assisted reproductive technologies in breeding. The main reason for this is the need for gradient washing of embryos from the cryoprotectant solution before embryo transfer, which requires the presence of an appropriate specialist and equipment. The purpose of this work was to compare the effectiveness of direct in straw thawing after vitrification using either Cryotec or open pooled straw (OPS) and after slow freezing. Bovine embryos were obtained from oocytes out of the slaughterhouse ovaries. Vitrification was performed using 15% DMSO, 15% EG, 0.65M sucrose media in OPS or Cryotec. Thawing was carried out in the French straws filled with two solutions of 033.M sucrose and PBS and heated to 38 °C. Slow freezing was performed in straws for direct transfer filled with 2 columns of 0.3M sucrose on both sides and an embryo in 10% EG in the middle. The straws were thawed on a water bath at 38 °C. The re-expansion and the hatching rate were evaluated after 24-48 hours. DNA fragmentation rate was evaluated in hatched embryos using TUNEL-test. Our results showed that the rate of re-expansion and hatching was 78.3 and 69.6%; 96.0 % and 72.0%; 78.0% and 57.0% for Cryotec, OPS and slow freezing groups, respectively. The DNA fragmentation rate was 11.1±2.1, 21.3±7.8 and 10.9±1.8% for the same groups. Thus, the embryos vitrified in Cryotec or OPS and directly thawed in straws had better development rate than after slow freezing. The highest embryo survival and DNA integrity rate were after using OPS. This simplified technic can be successfully used on the farm for bovine cryopreserved embryo transfer or in the field for wild cattle.

CRYOPRESERVATION OF HIPSCS-DERIVED NEURAL ORGANOIDS AS A BASIS FOR THE DEVELOPMENT OF CRYOPRESERVATION ROUTINES FOR 3D CELL SYSTEMS

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ABSTRACT

Organoids derived from human induced pluripotent stem cells (hiPSCs) are three-dimensional (3D) cell systems exhibiting superior functionality compared to 2D cultures and thus are of increasing interest. With their organ-like physiology, they pave the way for innovative opportunities as human model system in biotechnical research and medical applications. Hence, the demand for such 3D cell culture models is high while differentiation and maturation of iPSC-derived organoids is complex, often not standardized and time-consuming. Taking into consideration that the time window for application is additionally short, there is a demand for "ready-to-use" 3D systems. An efficient cryopreservation routine for organoids would face these challenges, offering both, stock-keeping as well as a fast "ready-to-use" supply at the same time. However, cryopreservation and thawing procedures for heterogeneous 3D samples still lack in efficiency and the damaging regimes are insufficiently examined. Main obstacles are the inhomogeneous distribution of cryoprotectants and heat throughout the organoid during freezing and thawing. These gradients cause osmotic and mechanical stress leading to loss of cell contacts, tissue integrity, and function. Our work provides new insights into the underlying mechanisms of cryoinjuries in early neural stem cell organoids as homogenous precursor model of midbrain organoids. In this context, the organoids were comparatively cryopreserved via conventional slow rate freezing and ice-free vitrification approach. The validation of the recovery efficiency is based on various quality controls including morphology, ultrastructure (SEM), viability and proliferation studies as well as gene and protein expression analyses. Additionally, we introduce a workflow for Raman micro-

spectroscopy to derive diffusions rates of Me2SO into organoids with different diameters (300-600 μm) and maturation states. This method enables, the adaptation of Me2SO incubation times prior freezing based on organoid parameters to improve cell survival while reducing cytotoxicity.

UNDERLYING CHALLENGES OF SPHEROID PRESERVATION

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ABSTRACT

Spheroids represent physiologically relevant models that mimic the complexity and behavior of cells in natural environment. Efficient spheroid preservation methods are needed to improve experimental reproducibility, enable biobanking for clinical applications, and quality control. Being relatively large structures composed of multiple cells, spheroids are a challenging object for low-temperature storage.
In the current study, we aimed to assess the effect of short- and long-term preservation on the structural integrity of spheroids, viability, and functional activity of cells in their composition. Spheroids were made of human mesenchymal stem cells (MSCs) by hanging drop technique. Cryopreservation was carried out using a slow-freezing method with a cooling rate of 1°C /min to - 80°C and following plunging into liquid nitrogen. For hypothermic storage, original trehalose- and sucrose-based solutions, and commercially available Custodiol-CE and HypoThermosol were applied. To assess the preservation of spheroid characteristics, their structural integrity, size and fusion ability, cell survival, and metabolic activity were compared to non-stored control. Paracrine activity was assessed using ProcartaPlex immunoassay. Culture of cells in spheroids boosted their paracrine activity and altered metabolism. Cryopreservation decreased the packaging density of cells in spheroids and led to the partial disintegration of 10% of samples. Application of 7.5% or 10% Me2SO gave comparable results and preserved up to 75% of viable MSCs. 3-day-long hypothermic storage in all tested solutions did not significantly affect cell survival and metabolic activity. Prolonged 7-day storage decreased the share of viable cells, the best results were achieved using trehalose-based solution. Despite the relatively high survival of MSCs after cryopreservation and hypothermic storage, they showed a significant delay in post-thaw spheroid fusion indicating disruption of intercellular junctions and decrease in migratory ability. To sum up, although current preservation technologies uphold high cell viability, the functionality of spheroids is compromised and requires the development of recovery strategies.

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PCL/PLA ELECTROSPUN SCAFFOLDS FOR THE HYPOTHERMIC PRESERVATION OF CELLS

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ABSTRACT

Electrospun scaffolds represent a suitable approach for the regeneration of tissue defects. Due to their fibrous structure, these scaffolds mimic the native extracellular matrix and thus can be seeded with various relevant cells. Due to the limited shelf-life of biological materials such as cells, two different preservation strategies can be applied to store the living material for a define duration of time. Cryopreservation is chosen for the long-term storage of samples. Compared to the cryogenic storage, lower technical requirements are necessary to store samples at hypothermic temperatures. Thus, hypothermic preservation is a method to sufficiently store living material for a short term. We fabricated polymeric scaffolds with different concentrations from two different polymers (polycaprolactone (PCL) and polylactic acid (PLA)) via horizontal and vertical electrospinning. The morphological, thermal, and chemical properties of the obtained fibre mats were characterized with scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR), respectively. A random fibre alignment with a mean fibre diameter between 1.7 µm and 4.2 µm was observed. The electrospinning process had an influence on the crystallinity of the scaffolds. For PCL this was in the range of 44% to 71% and for PLA between 40% and 50%, depending upon the blend ratio. FTIR measurements showed the characteristic bands of the polymers and detected no residual solvent. Additionally, we seeded human bone marrow stem cells (hBMSCs) onto the scaffolds and successfully cultivated them up to 14 days. Cell viability and metabolic activity were maintained until day 10. The cell investigations showed the superiority of PCL/PLA blended scaffolds compared to pure PCL. Current investigations will reveal the preservation outcome of hypothermic and cryogenic preserved cell-seeded electrospun constructs in different preservation media.

PRESERVATION STRATEGIES FOR MESENCHYMAL STROMAL CELL-BASED SPHEROIDS

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ABSTRACT

Mesenchymal stromal cells (MSCs) based spheroids demonstrate enhanced stemness and paracrine activity and need effective preservation technologies for their implementation in practice. Cryopreservation is the only approach for banking of readyto use product while non-freezing storage is more safe and attractive for short-term transportation. Herein we aimed to develop protocols for MSCs-

based spheroids vitrification, slow cooling preservation, and storage at ambient temperature. Spheroids were formed by hanging drop method. Vitrification was performed in vitrification solution (15% dimethyl sulfoxide (Me2SO), 20% ethylene glycol, 20% 1.2-propanediol, 1% polyvinyl alcohol, 0.5 M sucrose) in accordance with early developed protocol (Trufanova et al., 2020) with modified multistep addition procedure. Spheroids were also cryopreserved by conventional protocol under protection of Me2SO. Effects of CPA concentration and cooling regimens were analyzed. Besides, spheroids were stored in sealed vials at 22°C in alpha-MEM with 10% of fetal bovine serum. Viability (FDA/EB staining), metabolic activity (Alamar Blue), attachment to the tissue culture plastic, abilities for cell spreading, growth, and differentiation were examined before and after cryopreservation, and storage at ambient temperature. MSCs-based spheroids were successfully preserved by vitrification (80.3±1.4% viability, 78.7±5.6% attachment, 65.7±4.5% metabolic activity compared to control group), slow cooling with rate 0.75 °C/min under protection of 7.5% Me2SO (92.3±1.5% viability, 100% attachment, 76.8±5.4% metabolic activity), and 7-days non-freezing storage (77.4±6.7% viability, 80.3±5.6% ability, 79.8±3.2% metabolic activity). Sensitivity to Me2SO concentration and cooling rates was revealed – 10% Me2SO and higher cooling rate caused cell damage. After deconservation spheroids in all groups required time for injuries repair. Ability for differentiation in osteogenic and adipogenic directions was preserved after vitrification, slow cooling, and non-freezing storage. Different preservation strategies were developed for MSCs-based spheroids which can be used both for long-term and short-term storage in medical practice.

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STUDY OF SLEEP IN THE ANTARCTIC WINTERERS

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ABSTRACT

For a long stay in polar conditions, people are regularly exposed to various stressors. Various sleep disturbances are often one of the main symptoms of the "Antarctic syndrome". It is difficult to single out the influence of individual factors in extreme conditions, and it is undeniable that temperature in such conditions is one of the key factors affecting the human body. Therefore, in our opinion, during a long stay in Antarctica, it is extremely important to keep in mind the possible influence of cold on any disorders of human homeostasis. During 2016-2022, we were studying the effect of cold on some features of autonomic regulation in winterers at the Antarctic station "Akademik Vernadsky". Crew members of the 21st, 23rd-26th Ukrainian Antarctic Expeditions (54 men and 4 women, average age 38) participated in the study. In particular, the quality of sleep in winterers was evaluated quarterly using psychophysiological questionnaires. Some people additionally monitored their sleep patterns with portable gadgets. The analysis showed that the peak values of typical sleep disturbances (trouble falling asleep -56%, restless sleep -65%) occurred in the middle of the Antarctic winter. Waking up too early was minimal in spring and summer (near 10%). Also, in the summer, an increase in the duration of sleep was observed, due to the addition of daytime sleep - up to 36%. It is worth noting that the dynamics of

subjective problems of sleep quality were characterized by individual features and did not significantly depend on the experience of previous participation in the Antarctic expedition and profession. Separate winterers could not adapt to the time zone change and their sleep-wake regimen throughout the year remained as close as possible to the time zone of Ukraine. The study was carried out with the support of the National Antarctic Scientific Center of Ukraine.

CAN ANY HYDROPHILIC POLYMER PROTECT PHAGES DURING CRYOPRESERVATION?

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ABSTRACT

Bacteriophages have a lot of biotechnological and therapeutic applications. Similarly to other biologics, phages must be banked for storage/transportation, typically using cryopreservation. This cryopreservation step is particularly important in the context of phage cocktails, to ensure a consistent post-thaw composition, which matches the pre-frozen. Currently, phages are routinely stored using 10 % glycerol as a cryoprotectant, but poly(ethylene glycol) has recently emerged as an alternative that can also protect phages during freezing (**1**). Macromolecular cryoprotectants are rapidly emerging for a range of biologics, with specific requirements for each biological sample. In contrast to cells and proteins, the design criteria for polymeric phage cryoprotectants is not currently known, nor has the chemical space been explored. Here we screen a panel of polymers, prepared by RAFT polymerization, for their phage cryoprotective activity. Using five distinct phages we show that, unexpectedly, all tested polymers provide benefit, even polymers which are phage-static (phage reversibly inhibiting), once diluted away. A particular benefit of a polymeric cryopreservation formulation, over glycerol is that the polymers do not act as carbon sources for the phage hosts (bacteria) and hence do not interfere with post-thaw measurements. This work shows that unlike other cryopreservation challenges, such as cells/proteins, phage are amenable to protection with hydrophilic polymers and opens up new opportunities for advanced formulations for future phage therapies.

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NATURAL DEEP EUTECTIC SOLVENTS IN CRYOPRESERVATION: PHYSICAL APPROACHES BY DSC AND ATR-FTIR SPECTROSCOPY

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ABSTRACT

Natural deep eutectic solvents (NADES) are solvents composed of natural metabolites with a deep eutectic behaviour, i.e., a significantly lower freezing temperature than those of their components. They

consist in combinations of hydrogen bonds donors and acceptors, and show a counter-intuitive transformation from solid mixtures to clear, homogeneous liquids upon formation. Their wide solvent capacities, non-toxic properties and biodegradable, non-pollutant and inexpensive components are making NADES popular for a wide applications variety. The search for new approaches for improving cryopreservation procedures, confronting current challenges (toxicity and mutagenicity of cryoprotecting agents, low-specimen size, high cooling/warming rates…), has suggested the employment of NADES for these purposes. As part of a preliminary characterization, the low-temperature behaviour of four NADES (composed of β-alanine and either DL-malic acid or citric acid, and choline chloride and ethylene glycol or glycerol) was investigated by differential scanning calorimetry (DSC). Alanine-based NADES, in relation to their 3/5 molar water content, show glass transition events between -40 and -80°C. Meanwhile, cholin-based NADES (without water), present no thermal events in the 0° to -80°C range. Increasing water amounts addition causes the apparition of glass transitions in all NADES (at decreasing temperatures, with increasing water content), as well as devitrifications and freezing events. Calcium alginate beads with high sucrose content were used as a crude model for biological systems for cryopreservation. Beads were incubated in NADES, and their low-temperature properties characterized. Even short incubation times gave rise to radically different behaviours from native beads, evidencing the NADES effect. Attenuated total reflectance with Fourier transformed infrared spectroscopy (ATR-FTIR) yielded a complex picture of the interaction among the different components of the systems studied by DSC (NADES, added water and sucrose calcium alginate beads), which allowed to identify the weakening of NADES components interactions, as water and other components are included.

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NEW INSIGHTS ON ERYTHROCYTE PHYSICOCHEMISTRY AND PERMEABILITY TO CRYOPROTECTANTS FROM EXPERIMENTS AND MODELING

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ABSTRACT

Effective use and development of cryoprotective agents (CPAs) rests on quantitative determinations of the permeability coefficient characterizing their rates of transfer through the membranes of the cells being frozen, hence the high interest in this membrane property for existing and potential new CPAs (1). The osmotic shock method for measuring the permeability coefficient involves immersion of cells in hyperosmotic CPA solutions for varied prescribed times, over which varying amounts of the CPA penetrate following initial crenation (shrinkage) of the cells, followed by immersion in isotonic saline causing osmotic shock. The fraction of cells hemolyzed ultimately indicates the amount of CPA that entered the cell, and thence the permeability coefficient (2, 3). We present a comprehensive revisitation of this method, and the modeling of cell volume needed for data analysis, within the context of erythrocytes (RBCs) of various species. Our experiments and modeling yield three significant conclusions. First, it turns out that there is no disconnect between the experimental facts that the Boyle–van't Hoff plot is well-described by an apparent osmotically inactive volume fraction of ~0.5, and that the dry (non-water) volume fraction is ~0.3 (4). The difference between these two volume fractions has

long fueled belief in a significant amount of osmotically inactive water (5), now shown to be invalid. Second, the initial crenation step makes erythrocytes more fragile to an extent which correlates with their crenated (shrunken) volume (4). Third, the increased fragility can be corrected for in determining the CPA permeability coefficient. We also demonstrate a novel fit of osmotic shock data in which both the increased fragility due to the initial crenation and the membrane permeability coefficient are simultaneously determined. A comprehensive bibliographic analysis positions our new analysis within the context of past literature in the field, and we clearly explain its relevance to characterization and development of CPAs (1).

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PROLINE-CONDITIONING AND CHEMICALLY-PROGRAMMED ICE NUCLEATION PROTECTS SPHEROIDS DURING CRYOPRESERVATION

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ABSTRACT

Cell culture is an indispensable tool for studying biochemical pathways, drug discovery, and biologics production. Continuous cell culture often leads to phenotype drift and requires cryopreservation to facilitate banking and distribution. While cryopreservation has been successful for cells in suspension, storing 2-D monolayers and 3-D models (spheroids and organoids), poses significant challenges. Spheroids, which mimic 3-D tissue niches more accurately than traditional cell cultures, offer great potential in various research fields. However, cryopreserving spheroids remains challenging, as conventional cryoprotectants do not adequately mitigate all damage mechanisms. In this study, we propose a novel approach combining chemically-programmed extracellular ice nucleation to prevent supercooling and proline pre-conditioning to improve post-thaw recovery of spheroids. Our results demonstrate that this synergistic strategy enhances spheroid recovery rates from 40% to 70%. Moreover, the use of specific biochemical inhibitors and extracellular nucleators, beyond standard cryoprotectants, addresses both biochemical and biophysical damage pathways. Our findings indicate that cryopreserving spheroids using the proposed approach leads to reduced reactive oxygen species and increased F-actin polymerization, thereby promoting improved functionality. Notably, cryopreserved HepG2 spheroids exhibited enhanced function in a toxicology assay, validating the effectiveness of our strategy. Additionally, we observed that spheroid density impacts functionality, highlighting the importance of considering this parameter in future studies. By leveraging the discovery and application of chemical compounds and materials, this research represents a significant step forward in advancing the banking and deployment of 3D models for both basic and applied research purposes. In conclusion, our study demonstrates the successful implementation of chemically-programmed extracellular ice

nucleation and proline pre-conditioning to enhance post-thaw recovery and functionality of cryopreserved spheroids. This work underscores the need to explore innovative cryopreservation approaches that address both biochemical and biophysical damage pathways, ultimately facilitating the broader utilization of 3D models in various research fields.

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PASSIVE MECHANICALLY INDUCED HYDROSTATIC PRESSURE AND ION FLUX DRIVES VOLUME REGULATION IN HEPG2 CELLS

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ABSTRACT

Creating models that can accurately predict cellular volumetric response to anisosmotic conditions is critical for the success of cryoprotocols. Cells are generally assumed to behave as ideal osmometers with their water volume linearly proportional to the inverse of osmotic pressure. This relationship between volume and environmental osmotic pressure is known as the Boyle van 't Hoff (BvH) relation. However, the BvH relation is simple and does not include many cellular phenomena. Here we present alternative models that account for mechanical resistance to volumetric expansion, ion-osmolyte leakage, and active ion pumping. The human hepatoma cell line, HepG2, was investigated in this study. Using the BvH plot, the BvH relation failed to describe cellular volumetric response to anisotonic conditions. However, the BvH relation was appropriate to describe cell volume when restricted to hypertonic solutions. With use of the return-to-iso plot, we showed neither the leak nor the turgor model properly accounts for cellular osmotic behaviour. Finally, we observed HepG2 cells undergo regulatory volume decrease at both 20 °C and 4 °C, indicating regulatory volume decrease to be a relatively passive phenomenon. We determined the turgor-leak model, that accounts for mechanical resistance and ion leakage, best fits this observation. These results show that mechanical resistance and ion leakage are critical mechanisms for HepG2 cellular volumetric response to anisotonic conditions and regulatory volume decrease. This turgor-leak model is critical for understanding non-ideal loading and unloading of CPA when cells actively resist volumetric change and ions are able to cross the membrane through mechanosensitive channels.

Keynote Lecture:

A MATTER OF STRUCTURE: METHODS FOR MORPHOLOGICAL AND ULTRASTRUCTURAL ASSESSMENT IN TISSUE CRYOPRESERVATION

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ABSTRACT

Tissue cryopreservation is widely recognized as an effective method for preserving organic functions. To enable the future reestablishment of tissue function, researchers have focused on identifying the most suitable preservation techniques. An essential component of this research involves assessing tissue morphology, which serves as a reliable, inexpensive, and accessible method for confirming the efficacy of both established and novel cryopreservation methods. The application of light microscopy, employing specific staining protocols and immunoreactions, offers a straightforward approach to analyzing structural aspects. Furthermore, electron microscopy provides a powerful method for in-depth analysis of cell membranes and extracellular compounds, which are particularly vulnerable during the cryopreservation process. However, it is crucial to emphasize that tissue processing and preparation for morphological analysis require meticulous attention. This presentation highlights the significance of tissue morphology assessment as a tool for elucidating specific cellular and extracellular tissular aspects, providing a comprehensive methodology and practical tips for preparing tissues intended for both light and transmission electron microscopy. By incorporating innovative techniques, we will explore how tissue preservation has been improved, enabling excellent morphological tissue assessment. Through disseminating these findings, researchers and practitioners in the field of tissue cryopreservation can gain valuable insights into effective preservation methodologies and cutting-edge techniques. This knowledge will further advance the development of improved cryopreservation protocols, ultimately enhancing the potential for successful tissue preservation and subsequent functional restoration.

ULTRASOUND REWARMING OF CRYOPRESERVED ARTERIAL ALLOGRAFT

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ABSTRACT

Late advances in organ cryopreservation and banking are coming as a result of several decades of research and expertise. In particular, our group has pointed its efforts towards using High Intensity Focused Ultrasound (HIFU) for solving the currently existing problems in the rewarming process (1). Recently, we have migrated from the *in silico* assay of HIFU rewarming (2) to real experiments with living beings (3), with great success. This has encouraged us to test our technology in tissues of greater size using the murine model. Vascular tissue, fundamentally arteries, is of great importance as segments are used often for coronary disease treatment and for transplants. The state of the art currently employs water or saline warm baths as the preferred rewarming method, providing around 100 ºC/min warming rates. In this work, we reproduce standard cryopreservation protocols for aortic section preservation, introducing our HIFU equipment for twice as fast (~208 ºC/min) warming rates, with an average cell viability of 74%, peaking at 100% for optimal assays. Viability was assessed through cell count and integrity was evaluated through H&E staining under light microscopy. These results were contrasted with the ones obtained after standard warm bath rewarming. DMSO was chosen as the standard cryoprotecting agent, diluted to a 15% final concentration v./v. in Krebs-Henseleit Buffer. The samples were passively cooled to -80 ºC at a -0.6 ºC/min cooling rate. These were obtained from CD1 strand mice (n = 24), cut into 1 mm long rings and stored in custom made agar wells inside a Petri dish, in which it was cooled. These results embody yet another successfully taken step towards the goal of organ banking at the same time as it opens new paths for vascular and other tissue preservation, which hopefully can benefit medicine and research in cardiovascular diseases and cancer.

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HIGH INTENSITY FOCUSED ULTRASOUND FOR THE RECOVERY OF MOUSE HEARTS AFTER HYPOTHERMIA

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ABSTRACT

Fast rewarming is considered beneficial for organ recovery after preservation, both in hypothermia and cryogenic storage. In hypothermia, it is able to shorten the second warm ischemia prior to anastomosis and, therefore, the organ will be healthier to be transplanted. In the case of cryogenic storage, its advantage is associated with the inhibition of recrystallization. Recently we demonstrated (1) the possibility of reaching high warming rates using High Intensity Focused Ultrasound (HIFU) as rewarming source. Indeed, fully recovery of *Caenorhabditis elegans* was achieved when rewarmed by HIFU after cryopreservation. In the present work our aim is to show the benefits of HIFU also for hypothermia in the case of mammalian organ. Ten mouse hearts were taken to deep hypothermia (- 6 °C) with 5% (v/v) DMSO as cryoprotectant solution, half of them in PBS and half in Krebs-Henseleit. After that, they were rewarmed by means of HIFU (140 °C/min) until reaching the physiological threshold (29 °C). To assess the viability, physiological performance in Langendorff organ bath system, microscopy analysis and ECG monitoring were carried out. The results were that (i) all ten hearts got back their pre-cool beating rate (98±3%), (ii) they presented the same ECG pattern as the fresh controls, and (iii) no evidence of damage appeared in the morphological analysis. The outcomes were compared to the control group (slower rewarming), in which no sign of recovery was observed.
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LIQUIDUS TRACKING APPROACH FOR MOUSE AORTA RINGS CRYOPRESERVATION

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ABSTRACT

Liquidus Tracking represents a promising strategy for effectively cryopreserving large samples. This technique consists of reducing the temperature in a controlled manner while increasing the supply of cryoprotectant concentration. This method successfully decrease the toxicity of cryoprotectants to cells and tissues. While 3D cell cultures (1) and human cartilage (2) have been successfully recovered using standard liquidus tracking, cryopreserving whole organs remains as a challenge. In consequence, murine aorta rings was used by our group as an initial model before attempting cryopreservation of organs. To achieve successful cryopreservation of mouse aorta rings, we developed a stepwise system that has proven highly fruitful preserving the nematode *Caenorhabditis elegans*. We adapted this setup for mouse aorta rings, which required the design and manufacture of a cryo-reactor specifically tailored to our needs. In our protocol, we employed Ethylene Glycol (EG) as the cryoprotective agent to reach deep hypothermia, having managed to reach – 20 °C, where from this point the cryoprotectant is considered practically non-toxic. The protocol includes precise holding times during the cooling process to ensure proper diffusion and equilibration of the cryoprotectant solution into the muscular and endothelial tissue layers of the aorta. Throughout the cooling process, we closely monitored the formation of ice crystals. To rewarm the samples, they were brought back to air room temperature (22 °C). Viability was assessed by comparing cell counts to fresh samples using Trypan Blue staining. As a result, a preliminary 45% viability has been reached with no ice formation inside the cryo-reactor. Work is in progress by modifying the cooling and the CPA concentration profiles as a function of time with the aim of finding a better recovery.

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COMPUTER SIMULATIONS BY FINITE ELEMENTS FOR ULTRASOUND REWARMING OF CRYOPRESERVED AORTIC TISSUE

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ABSTRACT

High Intensity focus ultrasound (HIFU) is a pioneering technology in cryopreservation. It has been successful in preserving larval and adult C-elegans (1). HIFU has potential for tissue and organ cryopreservation when combined with multiple transducers and medical imaging (2). However, preliminary data on transducer performance is necessary in the early stages of research. Understanding the pressure and temperature map produced by a transducer is important in determining effective combinations of warming rates, volumes, and transducer characteristics. Our group has previously demonstrated good practices in this area (3). Finite element simulation is a useful tool for accurately and quickly studying these factors. In this study, we apply these practices to cryopreservation of vascular tissue using HIFU. The simulations involve replicating the geometry of the experiment and solving a set of differential equations under our experimental conditions. These conditions include using a 60W, 1.224MHz transducer to apply ultrasound to a 1 mmx1mm mouse aorta ring stored at -80ºC. The simulated waves pass through different materials involved in the actual experiment, such as Ethylene Glycol, agar, water (ice), and muscle. The results of the computer modelling align well with experiments performed in our lab using real aortas, particularly in terms of warming rates and homogeneity of

heating: the simulated aortas reached 0ºC within 60 seconds of ultrasound application, with minimal thermal gradient within the volume of interest.

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STUDY OF THE PROTEOME ALTERATIONS IN *Mytilus galloprovincialis* OOCYTES INDUCED BY CRYOPRESERVATION

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ABSTRACT

To date, cryopreservation of marine invertebrates' oocytes is a challenge with an extremely low success rate. The reasons for this poor outcome are still unclear, as it remains unknown whether it is caused by the toxic effects of the cryoprotectants or by the cryopreservation technique employed. Examining the molecular changes induced by either of these factors could provide further insight into this question. Such information would be useful firstly to reveal the potential mechanisms underlying the observed results, and subsequently to develop a successful cryopreservation process for mussel oocytes. We present the first quantitative shotgun proteomic analysis of *M. galloprovincialis* oocytes treated with two cryoprotectants (dimethyl sulfoxide, DMSO and ethylene glycol, EG) and a monitored cryopreservation protocol. Results revealed that changes induced by the differential cryoprotectants toxicity derived in a higher proteome-wide impact than those caused by cryopreservation itself. This strongly suggests that the inability to cryopreserve mussel oocytes may be directly affected by the cryoprotectant's toxicity on key protein factors in fertilisation. Candidate proteins and molecular pathways affected will be discussed from their potential implication in toxic effects on mussel oocytes and then in further observed non-viability of cryopreserved oocytes used for *in vitro* fertilisation. These findings may contribute to shedding some light on understanding and solving current limitations to the future success of marine invertebrate oocyte´s cryopreservation.

EFFECTS OF CRYOPROTECTING AGENTS ON THE ULTRASTRUCTURE OF *Paracentrotus lividus* OOCYTES

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ABSTRACT

Cryopreservation has revolutionized the preservation of various species of marine invertebrates, but the majority of existing protocols focus on sperm or larvae. Oocytes of aquatic organisms present unique challenges for successful cryopreservation due to their large volume, low surface-to-area ratio, high

water and lipid content, limited permeability to water and cryoprotectants (CPAs), high sensitivity to CPA toxicity, and susceptibility to chilling (1,2). This study aims to shed light on the damage incurred by *Paracentrotus lividus* oocytes when exposed to different cryoprotecting agents (CPAs), employing electron microscopy techniques. By analyzing the obtained images, we strive to gain a deeper understanding of the existing obstacles for oocyte cryopreservation. Oocytes were exposed to 0.5M and 3M concentrations of Dimethyl Sulfoxide (Me2SO), Ethylene Glycol (EG), or Propylene Glycol (PG). Toxicity tests were conducted either through a single-step incubation of oocytes with CPAs for 15 minutes or by gradually adding CPAs in 15 equimolar steps of 1 minute each. The electron microscopy images revealed that, regardless of the CPA or addition method employed, low concentrations did not inflict significant damage on the oocytes. The eggs maintained their spherical shape, exhibited visible microvilli on the surface, and retained the presence of Ca2+ granules, as observed in control cells. Conversely, exposure to the 3M concentration resulted in irreparable damage, with the oocyte surface destroyed, and the intracellular material becoming disorganized. This comprehensive investigation unravels the complex dynamics between cryoprotectants and cryoprotection in *Paracentrotus lividus* oocytes. By studying damage, we take a step closer towards overcoming the obstacles that currently hinder successful oocyte cryopreservation in marine invertebrates.

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CRYOPRESERVATION AT THE ROSCOFF CULTURE COLLECTION (RCC)

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ABSTRACT

Created in 1998 to conserve and distribute marine microorganisms, the Roscoff Culture Collection (RCC) at the Roscoff Biological Station (France) now maintains more than 7000 strains, mostly by regular transfer of active cultures. In order to offset genetic mutation and reduce the workload involved in culture transfer, since 2010 the RCC has successfully cryopreserved over 2600 strains of unicellular eukaryotes and prokaryotes. Using a standard cryopreservation protocol, high success rates have been obtained for species belonging to several phylogenetically divergent eukaryotic lineages (*Mamiellophyceae*, *Trebouxiophyceae*, *Chlorophyceae*, *Pyramimonadophyceae*, *Noelaerhabdaceae*, *Phaeocystaceae*, and *Pavlovaceae*). For cyanobacteria, the same protocol has successfully been applied to species with different cell types (single cells to trichomes). Factors such as cell size clearly influence cryopreservation success rates, while there is no obvious link to geographic origin or the temperature at which strains are maintained. Several groups of eukaryotic phototrophic microorganisms, such as dinoflagellates and cryptophytes, are still considered to be recalcitrant to cryopreservation techniques. However, no extensive studies have been carried out to find a way to cryopreserve them. In this context, the RCC is working on cryopreserving recalcitrant strains using new methods, such as vitrification or the use of different cryoprotective agents. In addition, research is focussed on developing methods for cryopreserving complex microbial communities in natural samples collected in the marine environment.

CRYOPRESERVATION OF *Platynereis dumerilii* LARVAE

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ABSTRACT

The marine annelid *Platynereis dumerilii* has emerged as a significant model organism for both developmental biology and evolutionary biology research. Consequently, investigations involving *Platynereis dumerilii* have the potential to greatly enhance our understanding of biology. Ensuring continuous access to high-quality biological material is crucial for advancing research with this model organism. Currently, there is only one species of polychaete, *Nereis virens* (1), for which cryopreservation protocols have been developed for their larvae. The objective of this study was to establish a cryopreservation protocol for the larvae of *Platynereis dumerilii* that ensures long-term preservation and facilitates proper growth and development after thawing. We examined the influence of larval age on cryopreservation, identified suitable cryoprotecting agents for this particular larval type, determined optimal cooling and warming rates, and assessed the equilibration time for cryoprotecting agents. As a result, we have successfully devised a cryopreservation protocol that yields a survival rate ranging from 1% to 6% for larvae capable of developing segments over an extended period. Subsequent research endeavours will focus on enhancing survival rates and evaluating the effectiveness of this protocol in various mutant strains.

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MODERN CRYOBANK AS AN IMPORTANT SUPPORT OF CLINICAL CELL AND TISSUE TRANSPLANTATION PROGRAMMES

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ABSTRACT

The importance of the cryobank is documented on experience with the support of haematopoietic progenitor cells (HPC) and allogeneic vascular tissue transplantation (VT) programmes. The cryostability data verifying 5-year shelf life of cryopreserved HPC and VT products are presented as well. Storage of cryopreserved HPC and VT was performed in the vapour phase of liquid nitrogen in the containers equipped with automatic filling, continuous temperature monitoring and inventory software. High temperature alarm was adjusted at -160 °C. The data of the number of grafts delivered from the cryobank and of the number of performed transplantations were taken from the annual reports. The HPC nucleated cell (NC) membrane integrity and the cell potency measured by granulocyte-macrophage colony formation (CFU-GM) at infusion after 5 years were compared with the pre-freezing data in 9 patients. The cryostability of cells of vascular wall was assessed in 6 great saphenous veins using in situ vital staining by fluorescence dyes and confocal microcopy. The total of 964 autologous and 161 allogeneic 100mL HPC units were delivered from the cryobank in the last 5 years and used at 209 autologous and 25 allogeneic HPC transplantations. The 5-year cryostability study showed the median post-thaw NC viability of 85 %. The median of NC recovery was 96 %, the CD34+ cell recovery 117 % and CFU-GM recovery 88 %. At the same time, the total of 71 VT grafts were delivered. The median post-thaw cell viability of 79 % was found at longitudinal sections and the viability of 91.03 % in perpendicular sections of the vessels thawed slowly for 2 hours. The cryobank was able to meet the growing needs of clinical transplantation programmes and to assure safe storage of cryopreserved HPC and VT products for at least 5 years.

REGULATING BIOCHEMICAL PATHWAYS TO IMPROVE T-CELL CRYOPRESERVATION

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ABSTRACT

Cryopreservation allows cellular long-term storage by cooling to low sub-zero temperatures and is essential to allow the safe delivery of emerging cell-based therapies to patients. To prevent fatal ice-related damage, cells are traditionally frozen in suspension with 5-10 % dimethyl sulfoxide (DMSO) as a cryoprotectant. Despite this being a widely used method, cell recovery and functionality remain low in some cell types, especially in post-thaw cultures. One reason for low post-thaw cell health is the activation of apoptosis (programmed cell death). For chimeric antigen receptor T-cell therapies, optimal cryopreservation is especially relevant, as patient outcomes depend on optimal cell viability and efficacy, and freezing is necessary at several manufacturing stages. This study used Jurkat cells, a T-cell line, to explore and mitigate the impact of apoptosis on cell viability and recovery after cryopreservation in 5 or 10 % DMSO. In both conditions, early apoptosis peaked at 4- and 8-hours post-thaw, with around 20% of cells undergoing early apoptosis. Late apoptosis and cell death peaked at 24 hours post-thaw. Incubation with 2.5 µM Fasudil hydrochloride, a rho-associated protein kinase (ROCK) inhibitor, significantly increased cell yield from 51.5 % ± 5.6 to 71.1 % ± 8.5 after cryopreservation with 10 % DMSO. Flow cytometry was used to calculate the number of post-thaw apoptotic cells after treatment with Fasudil, however, no significant population differences between untreated and treated samples were found. Therefore, reactive oxygen species were measured as an additional indicator of cellular stress. This was assessed by loading cells with carboxy-H2DCFDA, which is fluorescent when oxidized, and measured by flow cytometry. After cryopreservation, fluorescence notably increased in comparison to unfrozen control cells, and treatment with Fasudil

decreased fluorescence intensity. Overall, cell yield was increased by reducing post-thaw cellular damage in a T-cell line, without modifying the cryopreservation process itself.

PRESERVATION OF MULTIPOTENT MESENCHYMAL STROMAL CELLS, THEIR SECRETOME AND 3D SCAFFOLD-BASED CONSTRUCTS FOR REGENERATIVE MEDICINE

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ABSTRACT

The use of multipotent mesenchymal stromal cells (MSCs) shows great potential in regenerative medicine and tissue engineering. Besides the enhanced immunomodulatory properties and potential for multi-lineage differentiation, these cells have unique paracrine activity, which supports tissue regeneration and repair. Considering these features, the MSCs are applied as suspension for cell therapies, components of the 3D-engineered grafts, or preparation of cell-free secretome therapeutics. The manufacturing process for MSC-based therapies usually involves preservation strategies, either of the intermediate or the final product. In this work, we aimed to study the effect of different biopreservation strategies for MSC suspensions, cell-free secretome, or generated 3D grafts, considering their potential for clinical application. We have evaluated the characteristics of MSCs derived from different human sources and analysed their paracrine activity, metabolic features, and ways of cell activation. We have tested several conditions for the hypothermic storage and cryopreservation of MSCs using xeno-free supplements. Additionally, we have collected the MSC-derived secretome, and assessed the effect of lyophilisation and long-term storage at different conditions on the content of growth factors and cytokines. Finally, we have established the 3D MSC-based cultures using clinically approved hyaluronic acid-based scaffolds (Hyalofast®) and evaluated the effect of hypothermic storage and cryopreservation on the viability of MSCs within this 3D bioengineered graft. The results of the study provide valuable insights into the strategies for the biopreservation of MSCs, their secretome, and 3D MSC-based constructs for regenerative medicine applications.

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GMP-COMPLIANT SIMPLE CRYOPRESERVATION OF CELLS FOR CLINICAL USE

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ABSTRACT

Timely delivery of cell-based advanced therapy medicinal products (ATMPs) maintaining their high-quality standards to the site of the transplantation is critical for successful clinical outcomes of cell

therapies. Large distance from the site of production to the clinical site or complications during the transportation causing delay of the delivery may lead to serious problems including failure to perform the treatment. Successful cryopreservation at very low temperatures represents a resolution of such undesired events. GMP-compliant cryopreservation is also required for on-shelf cellular medicinal products, including those intended for allogeneic transplantation while being prepared for multiple recipients as well as for autologous cells planned for repeated therapeutic applications. We developed a GMP-compliant cryopreservation solution that allows direct administration of the thawed cell product to the patient without the need for its removal or washing the cells. Since the solution simultaneously acts as the product`s excipient, the cell products are exposed to no further manipulation at the site of transplantation. Therefore, no related equipment is necessary at the clinical site, the risk of contamination of the product is minimalized, and the regulatory requirement of as-simple-as-possible handling of the ATMPs is satisfied. The cryopreservation solution is suitable for both local and systemic applications. Moreover, the frozen cell suspension can be thawed and kept at 0-4°C for up to 24 hrs before the transplantation allowing the delivery and immediate application of the ready-to-use ATMP at the clinical site.

QUALITY OF STORAGE AND SHIPMENT SYSTEMS FOR SMALL BIOBANKS

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ABSTRACT

Biobanks have shown that in storage there might be a progressive build-up of microbial contamination in storage containers from biobank staff, leaking sample containers and the environment. This hazard is almost inevitable due to the widespread use of traditional storage vessels for which cleaning involves considerable effort and risk to samples which may partially thaw or become damaged in the process. Dry-shippers are similarly challenging and like storage vessels, are often not periodically cleaned. Stability of stored biological materials is crucial to their long-term utility for good quality research. This relates to the viability and/or structural integrity of the stored cells/samples. It has been shown that temperature excursions above glass transition temperature, can cause a decline in the viability of samples. Furthermore, samples stored at sub-optimally high or uncontrolled storage temperatures, whilst still appearing to be frozen can degrade due to a range of effects including part thawing and chemical reactions. In medical applications the risks of the accumulating microbial contamination have been addressed through strict physical and procedural controls. Also, stable storage conditions can be managed where there are appropriate resources and expert understanding of preservation science. However, small and less well-resourced biobanks (often agricultural) are typically constrained due to lack of appropriately trained staff and funding. Typically, these also contain diverse and scientifically precious biosamples for research, germplasm and breeding that would represent a significant loss to science if compromised. They also potentially conceal diverse pathogens and lack the necessary cleaning and biosecurity infrastructure. Standards such as the ISO20387 general standard for biobanking, tend to provide generic guidance which does not necessarily reach small biobanks or provide them with the detail required. We consider what measures small biobanks can take to economically address the vital issues for sustaining the quality of their holdings in support of future research.

INFLUENCE OF DIFFERENT CAR-T THERAPY PRODUCT MANUFACTURING STRATEGY ON LOGISTICAL CHAINS USED BY COOPERATING TISSUE ESTABLISHMENT

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ABSTRACT

CAR-T therapy based on the genetic modification of T-lymphocyte receptors is currently a modern method of patient treatment suffering from B-cell malignancies, however, there is a challenge in the translation of CAR-T therapy to other tumor types, including solid tumors. Available advanced therapy medicinal products can be divided into registered and investigational products. The leukapheresis of starting material, its processing, storage, and release for the manufacture takes place in the authorized Tissue Establishment (TE). The starting material is usually fresh mature peripheral blood mononuclear cells, which are sent to the manufacturing site in a chilled state, or they can be cryopreserved and then sent in a frozen state. Individual manufacturers use a different cold chain in the production of their products. Sometimes the starting material is frozen by the manufacturer and the final manufacture is carried out before the actual administration to the patient as a fresh suspension. However, the most common variant is production using the cryopreservation of the final product and sending it to the place of the administration. We present our experience with procurement of starting material for advanced therapy medicinal product manufacture, its cryopreservation and transport to the manufacturing site. In particular we reflect on the potential impact of different manufacturer protocols for the cryo-chain. Receipt of final products takes place in cooperation with representatives of the Hospital Pharmacy, followed by storage in a vapour phase of liquid nitrogen in Tissue Bank. Until now, we have received 14 registered and 4 investigational products to store. Transport and thawing before administration, including special rules for investigational products in the regime of genetically modified organisms, is also presented.

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CRYOPRESERVATION OF EQUINE MONOCYTE DERIVED DENDRITIC CELLS AND THE EFFECT OF VARYING THAWING TEMPERATURES AND PRESERVING CONDITIONS

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ABSTRACT

Cell therapies have gained increasing importance in various treatments over the last decades. Cancer treatment with dendritic cells as an alternative to conventional therapies is being actively researched and shows promising potential. Clinical availability of this treatment may be improved through cryopreservation of the cultivated cells. The objective of this study is the validation of a control protocol for the cryopreservation of equine monocyte derived dendritic cells by using a cryoprotective agent consisting of 10% (v/v) DMSO and 90% (v/v) autologous serum a cooling rate of 1 K/min and storing the samples for up to 12 weeks in liquid Nitrogen (LN2) and then thawing them in a water bath at 310.15 K. After that we validated the implementation of higher thawing rates by employing passive warming techniques, the utilization of passive cooling devices instead of controlled rate freezers and storing the samples at 193.15 K instead of LN2. We were able to demonstrate that thawing samples in a water bath at relatively high temperatures (323.15 K and 338.15 K) with continuous manual stirring enhanced the recovery rates significantly. Additionally, we showed that the experimented freezing and storage methods did not affect the recovery rate. For the future we aim to establish a DMSO-free cryopreservation protocol and to validate it for the above-mentioned conditions. We also aim to verify novel thawing methods like using ultrasound waves to accelerate the thawing process.

CATASTROPHIC CRYOSTORAGE FAILURE

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ABSTRACT

The security of low temperature storage of biological material is a prime objective for all working in the field. The increased demand for bulk capacity, attendant equipment complexity, monitoring, local, remote alarms and back-up strategies demand rigorous quality management. Nonetheless, catastrophic cryostorage failure with the loss of irreplaceable therapeutic and research cell and tissue samples occurs every year due to human error or equipment failure. This presentation will review the experiences of cryostorage in the stem cell transplantation laboratory at UCLH and include: 1) An early report of vapour phase storage failure to preserve bone marrow longer than six months in an autograft canine model (1) led to our adoption of ‘under liquid’ storage but this resulted in a contaminated tank and a

HepatitisB outbreak in transplant patients (2) and adoption of -140˚C mechanical freezers. 2) Our own and other published evidence of stem cell stability in myeloma patients receiving tandem autografts from their initial stem cell harvest, equally divided and cryopreserved for the first and then second transplant up to 9.6 years later without affecting haematological recovery (3, 4). 3) Stability data of stem cells stored at -80˚C and the risks of transient warming events. 4) Lessons learned from the freak failure of a -80˚C mechanical freezer failure to alarm locally or remotely without rescue by the liquid CO_2 back-up supply. 5) Thaw colony forming cell assays to avert discarding irreplaceable stem cells after a suspected 'bad freeze' or temperature excursion (5, 6, 7, 8). Finally, a series of reported cryostorage disasters from the UK and USA. The most dramatic of these was the loss of thousands of eggs and embryos by two independent USA fertility centres on the same day, March 2018, which was followed by a drive to improve cryostorage QC (9) and studies to predict the impending failure of Dewars and vapour phase tanks (10).

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Keynote Lecture

HOW THE INSTITUTE FOR PROBLEMS OF CRYOBIOLOGY AND CRYOMEDICINE OF THE NAS OF UKRAINE SURVIVES IN WARTIME

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ABSTRACT

Institute for Problems of Cryobiology and Cryomedicine of the NAS Ukraine (IPCC) is based in the city of Kharkiv, the second largest city of Ukraine, just 30 km away from the Russian-Ukrainian border. Since the very first days of Russian military invasion the city of Kharkiv and the entire North-Eastern part of Ukraine suffers from constant shelling and bombing by Russian aggressors, all this brings mortality and disability of population, destroys buildings, infrastructure and ecology of the whole region. As a consequence, a major part of the IPCC employees and residents left the city. Many young scientists were hosted by European universities and research centres due to different scientific programs and now they are able to continue their research. We greatly appreciate such a huge support. Those who stayed in Kharkiv and came back after ceasing an acute phase of war make every effort to defend Ukraine, keep the Institute infrastructure and continue research. The government supports IPCC as much as possible. Thus, in 2023 the National Research Foundation of Ukraine provided the grant support, which financial coverage was suspended in 2022. This emphasizes that the priority of our government is aimed at strengthening the defence capability, and the financing of science is quite short-term now. However, the Institute is trying to maintain the research trends and to be involved in new ones that emerged as a challenge to this cruel war. Russia's invasion of Ukraine affects the ecosystem,

from water pollution to loss of biodiversity. So, we are going to be involved more actively in cryobanking of genetic resources and other biological materials for restoring the country and bringing its scarred landscape and ecosystems back to normal.

Acknowledgements: This study was supported by National Research Foundation of Ukraine (project Number 2021.01/0276).

LONG-TERM CONSERVATION OF AMERICAN MAGNOLIAS USING CRYOBIOTECHNOLOGY

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ABSTRACTS

It is concerning to note that a significant number of Magnolia species are currently at risk worldwide, as highlighted in the Red List of Magnoliaceae (1). To ensure the long-term preservation of these species beyond their natural habitats, cryobiotechnology techniques such as plant tissue culture and cryopreservation can be utilized. At The Huntington, we have successfully established and multiplied shoot cultures from adult Magnolia spp, which were later subjected to cryopreservation experiments. Apical shoot tips from microshoots of two American magnolias were submitted to cryopreservation using a modified droplet vitrification method (2), with the donor plants exposed to cold and osmotic stresses before the cryoprocedure. The Plant Vitrification Solution 2 was also modified during dehydration to study its impact on survival and regeneration. Encouragingly, shoot tip regrowth was observed four weeks after exposure to liquid nitrogen, indicating that the preculture conditions could enhance regrowth following exposure to liquid nitrogen. Further experiments will be conducted to test the optimized method on other Neotropical magnolias, specifically focusing on plant recovery after cryopreservation.

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FROM BIOSPHERE TO ATMOSPHERE AND BACK: THE ICE NUCLEATING MOLECULES OF PLANT POLLEN

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ABSTRACT

Primary biological aerosol particles can rapidly alter the composition of mixed phase clouds by nucleating ice from supercooled water droplets, and thereby impact weather and climate. Pollen, the male gametophyte of seed-bearing plants, is known to carry soluble molecules capable of nucleating ice at relatively warm sub-zero temperatures. Recent studies indicate that the atmospheric impact of this activity is augmented by the generation of sub-pollen particles, from pollen bursting under atmospheric conditions, which may act as persisting carriers for the ice nucleating molecules. Our droplet ice nucleation measurements of extracts from >50 species' pollen, over a range of plant types, native climates, pollination times and methods, reveal that the ice nucleating activity of these molecules is more widespread and diverse than previously thought. Amongst these, we have noted particularly active pollen samples; with microlitre droplet mean freezing temperatures as warm as -8 °C (*Pinus mugo* pollen solution) compared with -28 °C for purified water. It is not yet clear whether these ice nucleators are adaptive or incidental. Remarkable similarities in the infrared absorption spectra of soluble components from fern spores and pollen suggest that the molecules responsible are of ancient origin and that these are conserved across the phylogeny. In contrast to the proteinaceous ice nucleators of bacteria and fungi, evidence suggests that polysaccharide units are primarily responsible for the ice nucleating activity of plant pollen but that proteins may play a role in enhancing this activity. We have shown that ice nucleating macromolecules, extracted from plant pollen, can significantly improve outcomes in the cryopreservation of cells by inducing extracellular ice nucleation. Better understanding the nature of these biological ice nucleators is crucial for the development of advanced cryoprotectants to achieve greater control over ice nucleation.

CHANGES IN LOW-TEMPERATURE PHASE TRANSITIONS OF DORMANT BUDS OF FRUIT PLANTS AFTER THEIR DEHYDRATION

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ABSTRACT

Development of crystallization processes and their localization in plant tissues are decisive factors in survival under the influence of low temperatures and they limit the distribution area of plant species (1). This study aimed at studying the low-temperature phase transitions in fruit plant one-year-old twigs and their changes during dehydration. Thermal analysis of *Prunus armeniaca L.*, *Rubus idaeus L.* and *Vitis vinifera L.* tissues was performed using differential scanning calorimeter (2). It was revealed that crystallization events in the tissues of studied plants did not occur simultaneously. During cooling of dormant buds, an intense main peak of water crystallization was first recorded at temperatures from -5 to -18 °C, depending on species and varieties, and a small peak of crystallization of supercooled water was recorded at lower temperatures (-37 – -41 °C). During heating, only one intense peak of ice melting was recorded in all plant samples. In woody stem tissues, the crystallization peak of supercooled water had significantly higher enthalpy than in buds. Crystallization peaks close to temperature of homogeneous ice nucleation in woody tissues (-38 °C) may be due to xylem parenchyma cells not contain nuclei for heterogeneous crystallization and freeze as individual units or small groups of cells (3). A significantly smaller amount of crystallized water and a lower crystallization temperature were found in *Rubus* tissues than in *Prunus* tissues. The onset temperature and enthalpy of crystallization decreased with decreasing water content. During cooling, a superposition of several low-intensity peaks was recorded, indicating the absence of a crystallization front, and crystallization occurred separately in different buds and wood compartments. Thus, the process of crystallization in plant tissues is complicated due to the properties of cell wall, which promotes supercooling and prevents the spread of ice from neighbouring cells.

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Poster Session

EFFECT OF CRYOPRESERVATION ON PHENOTYPICAL AND MORPHOLOGICAL CHARACTERISTICS OF PRIMARY MURAL GRANULOSA CELLS.

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ABSTRACT

Granulosa cells are an important entry point for assessing follicular development. They can convert androgens to estrogens, direct progesterone synthesis (1). Low temperature preservation is necessary for establishing the stock of mural granulosa cells (MGCs) for their further use as a model object for toxicology studies. Aim of work: to estimate the effect of cryopreservation on phenotypical and morphological characteristics of primary MGCs. Human MGCs were isolated by the centrifugation of the follicular fluid (2, 3). Cryopreservation was performed under 10% DMSO protection with the addition of 10% fetal bovine serum. The cryotubes were placed into the Mr. Frosty container and cooled for 24 hours to –80°C and thereafter transferred to liquid nitrogen. Cryovials were thawed up in a water bath at 37°C until a liquid phase appeared. Cell viability (trypan blue), phenotypical (HSD17B1, IGFBP5, COL3A1), morphological characteristics were determined on the 10th day of cultivation. Fluorescence microscopy was performed using an Olympus BX61 microscope. The results were analysed with Student's t-test using Excel software. After cryopreservation, the number of MGCs with intact membrane integrity decreased by 26.6±4.8%. Fresh and cryopreserved MGCs cultures were characterized by the presence of round cells, sail-shaped, stellate and spindle-shaped cellular elements. The growth dynamics of the studied cultures was similar, but an increase in cell proliferation was significantly higher in the fresh MGC compared with cryopreserved cultures throughout the observation period. At the same time, the relative number of cells expressing HSD17B1 (44.2±5.1%), IGFBP5 (53.5±4.8%), COL3A1 (28.3±3.7%.) proteins did not significantly differ between the fresh and cryopreserved samples on the 10th day of cultivation. The obtained data can be used to develop new reproductive biotechnologies for the application of cryopreserved MGCs as an experimental system for *in vitro* functional studies in the field of toxicology or inter-cellular communication.

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CRYOPRESERVATION-INDUCED ALTERATIONS IN FATTY ACID COMPOSITION OF HIGHLY MOTILE SPERM FRACTION IN STERLET (*Acipenser ruthenus*)

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ABSTRACT

Sperm freezing-thawing procedures often result in the emergence of viable, lethal, and sublethal-damaged subpopulations, which are extensively studied to evaluate the impact of cryopreservation on sperm biochemical parameters. However, the presence of differentially damaged subpopulations can obscure the true effects on the viable, highly motile sperm crucial for fertilization. To overcome this challenge, it is essential to investigate the highly motile sperm fraction separated from the post-thaw sample. However, in fish species, this approach presents difficulties due to the immotility of their ejaculated sperm. Consequently, only a limited number of fish species have developed methods for sperm fractionation. In this study, we examined the sperm of sterlet (*Acipenser ruthenus*), a representative species of sturgeon fishes known to experience oxidative stress during cryopreservation. Recently, a method for separating highly motile sperm subpopulations was developed. We hypothesized that freezing and thawing-induced changes in fatty acid composition would be less pronounced in highly motile spermatozoa. Sperm samples from six males were frozen using a conventional method with a 15% methanol-containing medium. Fractionation was achieved through Percoll density gradient centrifugation, and gas chromatography was utilized to analyze fatty acid composition. The results indicated that cryopreservation significantly reduced the total content of polyunsaturated fatty acids while increasing the saturated fatty acid content. However, no differences were observed between sperm subpopulations after cryopreservation, leading us to reject our initial hypothesis. Future studies should focus on elucidating the biological implications of changes in fatty acid composition in highly motile spermatozoa and their impact on fertilizing ability. These findings will provide valuable insights into the effects of cryopreservation on fish sperm and its reproductive potential.

Acknowledgements: We gratefully acknowledge the financial support of the Czech Science Foundation (project N 22-14069S) and the Ministry of Education, Youth and Sports of the Czech Republic, specifically the CENAKVA project (LM2018099).

BIOAVAILABILITY OF QUERCETIN IN LOW-TEMPERATURE STORAGE OF AQUATIC PRODUCTS

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ABSTRACT

Quercetin at a concentration of 2.0 g/l prolongs the low-temperature storage period for aquatic products (Shao et al., 2022, Wei et al., 2021). The event mechanism is implemented via antioxidant and antibacterial effects. The possibility of modifying quercetin to increase its bioavailability and activity remains unclear. To test this hypothesis, we compared the influence of quercetin and its water-soluble

complex with 2-hydroxypropyl β-cyclodextrin (QC), being cyclic oligosaccharides and widely used in the food industry. This complex is well water-soluble, contains 5.4% quercetin and in contrast to poor water-soluble quercetin has a higher bioavailability that grants the use of lower quercetin concentrations in similar effect achieving. Cyprinus carpio fresh muscle tissue fragments of 4x5x2 cm were kept in an aqueous solution of 2.0 g/l quercetin and 2.0; 1.0; 0.4 and 0.2 g/l QC for 30 min at 20°C. The control was the fish muscle tissue without any influence. Then the fragments were removed from the solutions, dried from excess moisture, placed in plastic containers and stored for 7 days at 4-5°C. The samples were evaluated by a 5-point organoleptic scale on days 3, 5, and 7. Research material was obtained keeping the bioethics rules. We showed that on color, smell and consistency, the best preservation was noted for tissue in a 0.4 g/l QC solution before storage. It was established that the control group samples deteriorated already on storage day 3. In the samples treated with 2.0 g/l quercetin and 2.0; 1.0; and 0.2 g/l QC, the first signs of spoilage were noted on storage day 5. In the samples exposed to 4.0 QC, the first signs of deterioration were noted only on day 7. We have shown that the water-soluble QC with 2-hydroxypropyl β-cyclodextrin is twice as effective at hypothermic storage of fish that is perspective for "green" food additives in healthy nutrition. We believe more research and discussions should be done to explore this topic deeply.

COMPARISON OF COOLING AND FREEZING PROTOCOLS FOR CRYOPRESERVING GOAT BUCK SPERM: IS THERE ANY COMBINATION SUPERIOR TO THE OTHERS?

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ABSTRACT

Cryopreserved sperm plays a pivotal role in dairy goats breeding programs. The processes of cooling to 4 °C (C) and freezing (F) in liquid nitrogen (LN) vapor may be achieved by using different methods some of them more economic and simpler (beaker (B) in a cooling chamber at 4 °C and polystyrene (P) box with LN) and some others more expensive and sophisticated (programmable refrigerated water bath (WB) and freezer (PF)). In this study, we compared the quality of goat sperm cryopreserved with a skimmed milk-glucose-glycerol extender and four cryopreservation protocols: B-P, B-PF, WB-P, WB-PF. Five goat males from Murciano-Granadina breed were used, processing two ejaculates per male. Total (TM, %) and progressively motile sperm (PM; %) as well as the average path velocity (VAP; mm/s) were determined with a CASA system and acrosome reacted sperm (AR; %) was determined with the fluorescent stain FITC-PNA and flow cytometry (1). Data were analyzed using ANOVA with C (2 levels) and F methods (2 levels) and their interaction, session (5 levels) and male (5 levels) as fixed effects. Interaction effect was not significant. Similar values for the two C (B and WB) and the two F methods (P and PF) were observed for TM (61 to 62% ± 1.9), PM (44 to 46% ± 2.2) and VAP (68 to 71 mm/s ± 3.2). The two C methods gave similar AR sperm (7.6 for B and 7.3 for WB) but more AR sperm were observed ($p < 0.05$) when freezing in P box (7.8% ± 0.27) than in PF (7.1 ± 0.27). In conclusion, any of the protocols tested is equally effective for cryopreserving goat buck sperm. Acknowledgements: AMURVAL, GVA-IVIA (52201K) co-funded by the EU through OP ERDF of the CV 2021-2027 and CEU Universities (INDI2140).

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IN-PLATE 3D TISSUE CRYOPRESERVATION – A NOVEL CRYOSTORAGE CONCEPT FOR ORGANOTYPIC IN VITRO MODELS

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ABSTRACT

Drug discovery and safety testing depend on predictive *in vitro* models, including multicellular tissues with organotypic functions. However, efficient long-term storage of 3D tissues is difficult, as established cryogenic procedures hamper survival and don't follow scalable industrial labware standards. Here, we demonstrate a new cryopreservation method for multicellular systems that prevents tissue-damage by avoiding harmful ice crystal formation and is applicable to standard industrial processes required for *in vitro* testing. Pre-formed scaffold-free tissues (spheroids, organoids) are equilibrated with cryoprotective agents while a recipient vessel is pre-cooled with liquid Nitrogen. Utilizing Hamilton's MagPip pipetting technology, the tissues are spotted onto the vessel within small droplets (≤1 µl volume) and instantly freeze upon contact with the cold surface, reaching high cooling rates required for vitrification. Liquid Nitrogen is used for long-term storage. To avoid harmful re-crystallization during thawing, rapid warming is achieved by applying pre-warmed medium and additional ventilation of the vessel. Tests with standard 96-well microwell plates and pre-formed liver spheroids (comprising primary human hepatocytes and non-parenchymal liver cells) demonstrated compact and round morphology of frozen tissues, similar to non-frozen controls. H&E staining and immuno-staining for CD68 (Kupffer cells), Albumin (Hepatocytes), and BSEP (canalicular structures) revealed similar patterns in both groups. 2 and 7 days after thawing, ATP levels reached 85% − 75% of controls. Both groups showed very similar Albumin secretion, CYP450 activity, and response to three known liver-toxic compounds. These results demonstrate the successful cryopreservation of highly differentiated spheroids based on primary cells in standard microplates, enabling long-term storage without loss of function. Enabled by unique sub-microliter liquid-handling capabilities (MagPip technology), such a scalable process could be a game-changing method for on-stock production of complex 3D cell culture models, further expanding InSphero's ARCTis™ product portfolio (Always Ready Cryo Tissues) for researchers in academia and industry.

OPTIMIZING A CRYOPRESERVATION PROTOCOL FOR D-LARVAE OF *Mytilus galloprovincialis*

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ABSTRACT

The specie *Mytilus galloprovincialis* Lamarck, 1819 is one of the most cultivated species in the world, as well as one of the most important in aquaculture in Spain, where its culture has a determinant socio, cultural and economic impact, especially in regions such as Galicia responsible for over 94% of Spanish mussels and 50% of the worldwide. However, in recent years, its cultivation has been limited due to decreasing recruitment events, regional restrictions on natural seed recollection which is key for the traditional culturing techniques in mussel rafts. This work aims to improve the current cryopreservation protocol developed by *Heres, 2022*, for 72h old D-larvae of *M. galloprovincialis*. We proposed to modulate factors such as temperature and salinity, by creating four pre-freezing treatments (40 ‰-16ºC; 40 ‰-20ºC; 42.5 ‰-16ºC and 42.5 ‰-20ºC) where our larvae develop during the first 72 hours. These pre-freezing treatments, with high salinity and temperature, have already shown very positive results in other marine invertebrate complex larvae, as in the cryopreservation of 48h pluteus larvae of the species *Paracentrotus lividus* (Lamarck, 1816), as reported by Lago & Paredes, 2023. These pre-freezing treatments have been designed based on the results obtained from different salinity and temperature bioassays, which have made it possible to determine the optimum range of larval growth within these factors. The aim of this work is to produce an optimized cryopreservation protocol, increasing the larval survival rate (Currently 77± 4.31%) calculated as percentage of normality of the larvae 48 h post thaw compared to the current protocol according to *Heres, 2022*.

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STORING MSCS IN ALGINATE CORE-SHELL CAPSULES AT AMBIENT TEMPERATURE: INVESTIGATING VIABILITY AND METABOLIC STATE

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ABSTRACT

Human mesenchymal stromal cells (MSCs) are a promising cell type for tissue engineering and regenerative medicine. To realize the potential of MSCs it is necessary to create effective ways to store

these cells. Storage at ambient temperatures may simplify transportation and overcome disadvantages of cryopreservation. Here we studied efficacy of human bone marrow MSCs storage at 22°C in α-MEM with 10% (v/v) fetal bovine serum in five different forms: monolayer, suspension, encapsulated in alginate microspheres (AMS), and core-shell alginate capsules with the addition of fresh porcine blood plasma or human amniotic membrane (hAM) extract. 2.5% (w/v) low-viscosity alginate was used for the AMS and capsules production. Viability (Trypan Blue, FDA/EthD dual staining) and metabolic activity (Alamar blue) were assessed on day 1, 3, 5 and 7 of storage. Cell cycle was studied with the Premo™ FUCCI Cell Cycle Sensor. Viability after 7 days of storage was 32.8±2.5% in monolayer, 58.3±1.9% in suspension, 84.2±3.6% in AMS, 87.3±2.8% in capsules with hAM extract, and 83.1±1.3% with porcine blood plasma. The metabolic activity of cells in AMS comprised 55.0±1.3%, in capsules with hAM extract - 58.1±1.2%, and in capsules with porcine blood plasma 64.0±0.3% from storage start level at 7th day of storage, respectively. Metabolic activity in monolayer and suspension decreased sharply and was 27.4±1.0% and 16.75±0.3% on the 7th day. Cell cycle analysis showed that MSCs were completely arrested in G1 phase 2 days after encapsulation. Conclusion: encapsulation techniques provide effective storage of MSCs at ambient temperature for transportation and further use.

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SUPPLEMENTATION OF BSA IN SEMEN EXTENDER MAINTAINS MOTILITY OF SEA URCHIN SPERMATOZOA DURING REFRIGERATED STORAGE AT 4°C

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ABSTRACT

Long term refrigerated storage of sperm simplifies the transport of the genetic materials, and extends its use for research. This study examined the effect of bovine serum albumin (BSA) in semen extender during refrigeration of sea urchin sperm at 4°C for 48h. Semen samples were collected from ten sea urchins, *Paracentratus lividus*, and stored in three media: dry (undiluted) or diluted in sea water (SW) with or without BSA (3% final concentration; w/v). Sperm Total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL), straight line velocity (STR), average path velocity (VAP) were analyzed with a CASA system (AI Station, SpermTech, Spain) at collection time (0h), 24h and 48h after cold storage. Data was analyzed using GLM of the SPSS. Model with two factors (time, medium) and double interaction was used. Dry sperm retained the highest TM and PM (TM; 64.2, 53.4, 47.7, PM: 57.0, 60.7, 56.6 at 0, 24 and 48 h respectively) after refrigeration while VCL and VAP were higher for sperm stored in BSA. Motility parameters in BSA at 24h and 48h showed no significance difference with dry and 0h collected semen. On the contrary, sperm total motility decreased significantly after 24h when stored in SW alone (TM: 64.1, 18.9 at 0h and 24h). TM were considerably lower between semen stored in SW and the other groups after 24h ($p<0.05$). These results showed that BSA could have a protection role against cold shock in sea urchin sperm cells diluted in SW.

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EVALUATION OF IN SILICO-DESIGNED VITRIFICATION PROTOCOLS: ASSESSMENT OF THE EFFICACY OF ETHYLENE GLYCOL AND PROPYLENE GLYCOL PERMEABILITY IN PRESERVING MEIOTIC SPINDLE INTEGRITY OF BOVINE IN VITRO MATURED OOCYTES AT VARIOUS TEMPERATURES

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ABSTRACT

Current protocols for bovine oocyte vitrification rely on a combination of ethylene glycol (EG) and dimethyl sulfoxide (Me_2SO). Because Me_2SO has been associated with high cytotoxicity, propylene glycol (PG) has been proposed as an alternative cryoprotectant (CPA). In previous studies using *in silico* and *in vitro* osmotic observations, we observed that the original oocyte volume was recovered within 1min-35sec at 38.5°C and within 4min-15sec at 25°C upon exposure to the equilibration solution (ES) containing 7.5%PG-7.5%EG. The aim was to compare the optimized times of CPA addition by examining effects of CPA toxicity and the vitrification/warming process on oocyte spindle morphology. Bovine oocytes were *in vitro* matured for 22h and exposed to ES for 4min-15sec at 25°C or for 1min-35sec at 38.5°C. Half of oocytes in each group was vitrified/warmed using the Cryotop method (VIT25 and VIT35.8, respectively) while the other half were only exposed to the vitrification and warming solutions to assess CPA toxicity (CPA25 and CPA38.5, respectively). Fresh, non-vitrified *in vitro* matured oocytes served as the Control group. Oocytes were fixed, immunostained and spindle configuration was classified as described in Garcia Martinez et *al.* (2022) (6 replicates). Percentages of bovine oocytes reaching the MII stage did not differ between treatments. While percentages of normal meiotic spindle configuration were significantly lower in the VIT38.5 group (47,83% n=58) when compared to fresh control (88,06% n=90), percentages in CPA38.5 (91,18% n=68), CPA25 (83,33% n=48) and VIT25 (63,96% n=45) groups did not differ from the control fresh group. Significantly higher percentages of disorganized chromosomes were found in those oocytes vitrified/warmed at 38.5ºC (40,86%) when compared to other groups. To conclude, exposure to a combination of EG-PG for the proposed times prior to vitrification/warming preserved the spindle configuration of oocytes exposed to the ES at 25ºC but had a detrimental effect at 38.5ºC.

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HIGH RECOVERY RATES OF PRESERVED *Caenorhabditis elegans* THROUGH OPTIMIZATION OF CRYOPROTECTANT SOLUTION AND IMPROVE REWARMING

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ABSTRACT

Caenorhabditis elegans is an important animal model in molecular and developmental biology. The traditional procedure for cryopreservation based on slow freezing, as described by Brenner (1974) (1). The standard method uses 15% glycerol in *S Buffer*. However, only the larval stages L1-L2 recovered at a rate of 30-35%. In some mutant strains of *C. elegans*, these survival rates are even lower (2). The ultra-rapid vitrification technique offers recovery rates exceeding 90% (3). However, it has the disadvantage of significantly modifying the nematode handling process. This departure from common laboratory practice necessitates a change in the procedural training for research personnel. In this study, we designed a protocol that achieves high recovery rates for *C. elegans* while preserving the conventional slow freezing method. To achieve this, the following improvements were implemented: **i)** modification of the cryoprotectant, **ii)** pre-incubation of worms in the cryoprotectant solution at a temperature of 0 °C for 1.5 minutes, and **iii)** rewarming in a water bath for 1.5 minutes. In our work, we have developed an alternative protocol based on a 15% ethylene glycol (EG) in *S Buffer*. In this case, the thawed cryovials rewarmed by immersion in a water bath, unlike the traditional method that is performed at room temperature. With this protocol, the recovery rates for L1-L2 larval stages are 75% compared to the 35% achieved with the conventional method, without the need to acquire new equipment or modify their handling.

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