ABSTRACTS

IN PERSON AND VIRTUAL MEETING

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Joint Meeting of the **Society for Cryobiology** and the **Society for Low Temperature Biology**

Society for Cryobiology
International Society for Low Temperature Biology and Medicine

SLTB

Fáilte Ireland

MEET IN IRELAND

S1 ATP-BIO, A NEW CENTER USING NANOMEDICINE TO PRESERVE BIOLOGICAL

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Advanced Technologies for the Preservation of Biological Systems (ATP-Bio): An NSF Engineering Research Center, United States

Advanced Technologies for the Preservation of Biological Systems (ATP-Bio) is a 2-year old Engineering Research Center funded by National Science Foundation (NSF) that synergizes research undertaken at five world-class research institutions in the field of bio- and cryo-preservation: University of Minnesota, Massachusetts General Hospital, University of California Berkeley, UC Riverside and Carnegie Mellon. ATP-Bio aims to "stop biological time" and radically extend the ability to bank and transport biological testbed systems across multiple scales: from cells and tissues to whole organs and even whole organisms. Ultimately, "off the shelf" biopreservation technology of living products will be readily available across the globe to advance healthcare, biodiversity, food supply and sustainability. ATP-Bio will also train the next-generation biopreservation workforce in both industry and academia and make it a reflection of the demographics of our world. This will support and strengthen a vibrant and growing innovation ecosystem that will revolutionize cell therapy, regenerative medicine, aquaculture, and organ and tissue markets. An Ethics and Public Policy pillar guides ATP-Bio research and development to manage risk and secure societal benefit. In this presentation, we will review the founding of the center and discuss its structure and its overarching goals, emphasizing the integration of its engineering pillar with workforce development, culture of inclusion, innovation, and ethics and public policy. We will also present some of our most significant accomplishments thus far and share our ambitions for the future especially as it overlaps with the field of cryobiology.

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S2 DAVID PEGG'S INSIGHTS INTO FREEZING INJURY IN CELLS, INCLUDING THE 'UNFROZEN FRACTION' DEBATE WITH PETER MAZUR

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In addition to his impressive body of work on organ preservation, David Pegg also made significant contributions to our understanding of freezing injury in cells. Indeed, many of his experiments with cells helped to shed light on the mechanisms of cryoinjury in organs. This talk will focus on three areas, which to a certain extent are inter-linked: the influence of cooling and warming rates on nucleated cells, the cell-packing effect in erythrocytes, and the role of the unfrozen fraction in the survival of frozen cells. While it had been known for many years that cooling rate had a major impact on cell survival, the role of warming rate was typically overlooked. Work on red blood cells (RBCs) had already shown that there was not necessarily a single optimum cooling rate when warming rate was varied. David investigated this in great detail using mouse L-cells and found two similar peaks of survival with the conventional

combination of 1°C/min and rapid warming and when cells were both cooled and warmed slowly. Further experiments on cell packing, by varying the haematocrit of RBCs before freezing showed that haematocrits above 50% were associated with lower cell survival. The implications for organs were clear given their high cell density and, because of their size, the requirement for low rates of cooling and warming. When David studied the influence of cooling and warming rates on the packing effect, he initially felt the results could be explained by Mazur's recent unfrozen fraction hypothesis. However, Mazur's experiments required cells to be in a range of solutions that are not isotonic before freezing and David demonstrated that cells that differed in initial volume before freezing also differed in their susceptibility to the changes in solute composition occurring during freezing and thawing. There ensued a fascinating, evidence-based debate between these two giants of cryobiology, providing a profound example of how strongly held interpretations of similar data could and should be discussed without rancour or loss of friendship.

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S3 CRYOBIOLOGY OF MULTICELLULAR SYSTEMS: ROLE OF ICE AVOIDANCE, TOLERANCE, AND PROMOTION IN TISSUE CRYOPRESERVATION

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It is widely acknowledged that the modern era of cryobiology began with the discovery of the protective effect of glycerol in 1949 after which decades of research led to successful cryopreservation of a wide range of cell types and an understanding of the fundamental mechanisms of cryopreservation and cryoinjury. The anticipated extrapolation of these protocols and freezing techniques from single cell-based systems to multicellular tissues and organs was not easily realized and became a research focus of David Pegg's group in the 70s and 80s. He defined several additional mechanisms of cryoinjury in multicellular systems, of which the role of extracellular ice was recognized to be of paramount importance. This subsequently led to studies to *control ice formation* by various means and provided a fundamental contribution to the development of successful methods of tissue cryopreservation. This presentation will highlight some of the salient contributions which remained a focus of David's academic research for several decades and continue to this day to be relevant and provide the basis for ongoing research to develop new and improved methods of organ banking.

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S4 DAVID PEGG AND THE COLD CHAIN FOR ORGANS: A DECADE OF OBJECTIVE AND INSIGHTFUL SCIENCE AT THE DAWN OF CLINICAL TRANSPLANT SERVICES

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As solid organ transplantation moved beyond research investigations towards an important clinical reality, in the 1960's, David Pegg was developing his own interests in the value of cryobiology in

medicine. When he collaborated with Roy Calne at the Westminster Medical School in London UK, they were searching for a way to ensure donor kidneys, when removed from the body, would retain good physiological function and avoid severe ischaemic injury which would render them useless for transplantation. David's interests and reputation led him to become Director of the newly-forming Division of Cryobiology in new premises at the Clinical Research Centre in Harrow, where he undertook a number of important studies linking both the engineering and biological factors to maintain organs suitable for transplantation. A block of 4 papers, published in Cryobiology between 1972-1978, examined in detail the different important aspects of physiology and biochemistry which subsequently have been recognised as the cornerstones of modern donor organ preservation. He also acted as a focus point in that era for other experts in this novel field, and over the next decade hosted 3 published symposia [Organ Preservation and Organ Preservation II (both Churchill Livingstone Press), and Organ Preservation – Basic and Applied Aspects (MTP Press)], where not only were published the important individual research papers, but also the live discussion sessions which were recorded and transcribed (in a time without computing power and internet), providing a unique and important historical legacy in the field. This talk will explore this unique contribution which show how organ preservation has developed into the global activity in the 21st century, and what founding principles were developed which can guide the topic further in coming years. It will also introduce how the studies aided the progression towards David's organ cryopreservation studies, which are also highly regarded within the Society for Cryobiology.

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S5 DAVID PEGG, PIONEER OF ORGAN CRYOPRESERVATION AND ELECTROMAGNETIC HEATING

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David Pegg, MD, FRC path, was an early and outstanding advocate for the development of methods for cryopreservation and banking of transplantable organs. He was among the first scientists to study the problem methodically and in-depth. His work at Audrey Smith's laboratory (1967-), and with his own groups at Cambridge (1978-) and the University of York (1993-) established foundational knowledge of organ cryopreservation that we take for granted today. Studies included composition of perfusates, perfusion methods, effects of different cryoprotectants (organ permeation, toxicity, perfusate compatibility), how to load and unload cryoprotectants from organs without lethal osmotic effects, and of course the effects of freezing. Achievements included the loading and unloading of rabbit kidneys with 3 M, and in some cases 4 M, glycerol, followed by return to normal function a month after transplantation, and cooling of unfrozen kidneys to -25 degrees Celsius while loaded with 6M glycerol. In the mid-1980s, David's group began collaborating with Cambridge physicist and Antarctic explorer, Stanley Evans, to methodically study the problem of radiofrequency (RF) electric field warming of cryopreserved organs. Insights included the importance of frequency selection for adequate penetration depth and avoidance of wavelength effects, the electrical properties of organs being strongly influenced by perfusates, and the preferability of

dielectric (dipolar) heating over ohmic (ionic) heating and resulting considerations for the composition of perfusates. David came to believe that successful organ cryopreservation would likely require the avoidance of ice by either metastable or stable (liquidus tracking) vitrification. Building on this pioneering knowledge, Greg Fahy's group has been able to load and unload rabbit kidneys with 9.4 M mixtures of cryoprotectants, and reproducibly recover them from -45 degrees Celsius and even lower. RF dielectric warming guided by the research of David and his collaborators has permitted apparent recovery after vitrification at -130 degrees Celsius in isolated instances. Work continues to make methods robust, reproducible, and ultimately scalable to larger organs as David envisioned.

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S6 DAVID PEGG AND THE DEVELOPMENT OF THE SCIENCE OF TISSUE BANKING AND ITS REGULATION IN THE UK

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David's research interests in multicellular systems which, in the mid-80s, led to insights into the role of extracellular ice in tissues had, by the early 90's, led him to successfully tackle a widespread problem in tissue banking: that of the development of fractures in cryopreserved small arterial and venous grafts.
The contacts developed during this period proved fruitful, when, with the demise of the Cambridge-based Medical Cryobiology Group in 1992, David setup the East Anglian Tissue Bank (EATB) which became the first tissue bank within the UK National Blood Service. Service provision developed rapidly to include both bone and heart valve banking as well as cryopreservation services for haematopoietic cells.
At this time, tissue banking in the UK was mainly a "cottage industry" comprising predominately small, single-tissue "banks" often serving a single hospital. Larger multi-tissue banks of the kind seem in the US were few in number and there was no co-ordination of service provision nor application of agreed standards and practices: indeed, at this time, there was no statutory regulation across the sector at all. David saw this as a fundamental impediment to the safety and future development of tissue transplantation. His response was to drive the establishment of the British Association for Tissue Banking (BATB): developing published standards and guidance documents covering the main tissue banking sectors. This subsequently led to a UK Department of Health Code of Practice; forming the basis for inspection and accreditation in the UK until the adoption of the EU Tissue and Cells Directives in 2004. It is noteworthy that this Code of Practice was used as an exemplar in the development of the EU Directives.
This talk will explore the twin themes of research and regulation and the influence David had on both of these within the tissue banking sector.

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S7 HIGH AND LOW SUBZERO ENGINEERING APPROACHES FOR ORGAN PRESERVATION

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This talk will focus on engineering approaches to improve organ preservation,

a key area of David Pegg's legacy in the field of Cryobiology. Organ preservation remains a top priority for overcoming perpetual shortages as identified by interagency science panels, the American Society of Transplantation, and other major transplant societies. Whole organ banking will significantly reduce the 700,000 annual US deaths attributable to end-stage organ disease and also enable matching and application of advanced immunotolerance protocols to improve transplantation outcomes. Although the first CPA (glycerol) was discovered in 1949 and the development of Me_2SO occurred over 60 years ago, we have only recently learned that *high subzero* (≥ -20 °C) preservation (either in a supercooled or partially frozen state) can significantly increase storage times (3-6 fold or more in organs) over simple hypothermia (< 1 day) while avoiding traditional problems of toxicity and chilling injury. Likewise, *low subzero* preservation (or *vitrification*) (< -140 °C) effectively stops biological time and can be achieved by using either extremely rapid cooling rates or high concentrations of CPA cocktails. *Isochoric* (or constant volume) *systems* have the potential to precisely control ice formation and, in some cases, even eliminate the need for high CPA concentrations. The Figure below captures the range of preservation temperatures being used by several groups world-wide. We will also review new rewarming technologies to bring chilled and vitrified organs back for transplant or biomedical use.

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S8 CONTACTS AND LINKS BETWEEN SLTB, CRYOBIOLOGY AND IPCC. GROWTH AND COLLABORATION TO CHAMPION CRYOBIOLOGY

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This year marks the 50th anniversary of the establishment of the Institute for Problems of Cryobiology and Medicine in Kharkiv, Ukraine (IPCC). At that time (1972)the Society for Low Temperature Biology (SLTB) was exploring and developing links with cryobiology groups in both Europe and the USA (notably the Society for Cryobiology). In 1975 a link between SLTB and IPCC was established, with an invitation to visit Kharkiv made to the late David Pegg (then as always a major player in SLTB). On the back of the growth of this link an SLTB group travelled to Kharkiv in Spring of 1983 for a joint symposium with IPCC. This laid the foundations for further, important interactions including joint participation in a UNESCO symposium in 1988, the development of plans for publishing in cryobiology,the establishment of the UNESCO Chair in Cryobiology and joint annual Young Scientist meetings at IPCC. Events such as these have helped to drive the international recognition of cryobiology as a science of great value. The timeline and significance of these collaborations will be outlined together with thoughts on the potential and planning of future development and growth between cryobiology institutions.

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S9 THE IMPACT OF THE UNESCO CHAIR IN RESEARCH COLLABORATIONS

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The *Centro Binacional (Argentina – Italia) de Investigaciones en Criobiología Clínica y Aplicada (CAIC)* (Binational Center (Argentina – Italy) for Clinical and Applied Cryobiology Research) was established in 2009 as a scientific cooperation between the Rosario National University (Rosario - Argentina) and the Italian Liver Foundation (Trieste - Italy) for research related to liver preservation. As one of its main objectives, spreading of scientific knowledge related to Cryobiology, both within academia and general society, has also provided the basis for broadening international collaborations. In year 2004, professors Rodriguez, JV and Guibert, EE, former directors of CAIC, were invited to participate in the activities of UNESCO Chair in Cryobiology established at the Institute for Problems of Cryobiology & Cryomedicine of the National Academy of Sciences of the Ukraine (Kharkov, Ukraine). Since then, collaboration between CAIC and UNESCO Chair members has been focused in production of scientific reviews related to fundamental aspects of low temperature cells and organ preservation. Recently, this partnership also addressed the, essential although usually overlooked problem of translational cryobiology in economical deprived countries.
Within the activities of training and professional development, CAIC has created an official post-graduate career on biopreservation. Participates in various short-courses and scientific meetings and provides regular positions for grade and PhD students.
Nowadays, under direction of Dr. Maria Celeste Robert, CAIC has expanded its research focus to other areas of cryobiology and low temperature science, such as therapeutic hypothermia, stem cells and laboratory animal embryo cryopreservation but remains actively engaged in science communication and stimulation of scientific vocations among high school students. This presentation aims for sharing the experience of CAIC in the framework of the UNESCO Chair in Cryobiology, looking forward to find new opportunities for scientific and academic collaborations.

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S10 INSTITUTE FOR PROBLEMS OF CRYOBIOLOGY AND CRYOMEDICINE OF THE NAS OF UKRAINE: FUTURE OUTLOOK

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This year we celebrate the 50th Anniversary of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (IPC&C). During this half of the century, the IPC&C serves as a unique research center, which combines all the cryobiology disciplines under one roof and significantly contributes to the overall development of the cryobiology field and training of new professionals.
The war against Ukraine brought significant destruction of civil infrastructure in Kharkiv. Today, the IPC&C faces limited working capacities, which forces significant migration of younger researchers who represent the future of the Institute.

The unavoidable stagnation of the Ukrainian economy expected in the nearest years drives us to search for new alternative strategies for future maintenance and the development of the IPC&C. In the presentation, we will show an overview of possible ways of IPC&C development. The proposed strategies include several main aspects: a) boosting the international cooperation and multisided researchers exchange; b) improvement of the managerial structure through the organization of International Advisory and Supervisory Boards; c) re-assessment of research directions, including fundamental and translational studies; d) searching for the mechanisms, supporting the return of IPC&C young researchers and attraction of new scientists into the IPC&C research; e) searching for infrastructural funding and investments possibilities.
We strongly believe that the joined efforts of the World Cryobiology Community will allow overcoming the current threats and support the further development of the cryobiology field in Ukraine.

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S11 FISH SPERM CRYOBANKING IN THE VIEW OF INTERNATIONAL COOPERATION

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Fish sperm cryopreservation is traditional and well-developed field of modern cryobiology. Protocols for fish sperm cryopreservation were developed worldwide by many scientific teams, and now there is quite a strong background for applying existing technologies and their further improvement. As fishes are the most taxonomically diverse group of vertebrates, the characteristics of their reproductive system, including sperm biology, are also various. Currently, sperm cryopreservation protocols are well designed for salmonids, percids, and carps and can be used on the "large-scale" aquaculture level. In addition, the taxa-specific methods of sperm collection, improvement of sperm fertilizing ability *in vitro* and storage before cryopreservation are developed for sturgeons and silurids. That also strengthens the potential for applying cryopreserved sperm in these species. It is clear that the set of these species-specific approaches allows practical application of sperm cryopreservation in aquaculture and biodiversity preservation. International cooperation may help fish sperm cryobanking to be more effective. Czech-Ukrainian collaboration can be an excellent example of this, as these countries have a long history of activities in fish sperm cryobanking, and the potential for application of fish sperm cryobanking is evident.

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S12 THE CONTRIBUTION OF THE UKRAINIAN SCIENCE TO THE DEVELOPMENT OF HUMAN REPRODUCTIVE CRYOBIOLOGY

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Significant progress in human infertility treatments through research, development and application of cryobiology to gamete and embryo preservation has been made since the Department of Cryobiology of Reproductive System at the Institute for Problems of Cryobiology and Cryomedicine, Kharkiv, Ukraine has been established almost four decades ago.
In our recent studies, we have shown that sperm morphology and DNA integrity decrease significantly in cryopreserved sperm at normozoospermia and especially at such spermatogenesis pathology as oligoasthenoteratozoospermia (OAT). Therefore, in such cases it is necessary to select the morphological normal gametes and use IMSI method for oocyte fertilization to prevent abnormal development of embryos. Also we developed a unique cryopreservation method for OAT spermatozoa without washing step after thawing.
Despite the fact that oocyte vitrification is currently a common practice for female fertility preservation, the research of the cryopreservation effects on oocyte genome is of great interest. We have shown that vitrification of oocytes did not increase the overall level of aneuploidy in embryos, but the aneuploidy level of certain chromosomes changes. Thus, increased aneuploidy of chromosome 13 and decreased aneuploidy of chromosome 18 and sex chromosomes was found.-This is new data in long-term research efforts on the selective effect of cryopreservation on certain oocytes on their ploidy chromosome set number.
In the study of susceptibility to damage by factors of cryopreservation of embryos, its change was found depending on their stage of preimplantation development. A differential approach to cryopreservation of embryos depending on the intervention in the *Zona Pellucida* to increase the survival rate is proposed. Taken together, our studies indicate the prospects for the use cryobiology and cryoconcervation strategies in reproductive medicine and further in-depth study of cryoregulation and epigenetic changes in the genome of gametes and embryos.

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S13 THE IPC&C NAS OF UKRAINE CONTRIBUTION TO THE IMPLEMENTATION OF THE STATE PROGRAM OF RESEARCH IN ANTARCTICA

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Ukrainian fundamental and applied research in Antarctica is conducted in accordance with the State Targeted Scientific and Technical Program. Employees of IPC&C NAS of Ukraine have joined the research in the areas of “Biology” and “Medicine and Physiology” of this program since 2016. During this time, 2 employees took a direct part in the annual winter at the Ukrainian Antarctic Station “Akademik Vernadsky” in 2016-17 and 2021-22.
One of the projects is a comprehensive study of the body's adaptation to cold in the conditions of the Antarctic sojourn and the impact of the combined action of cold and light changes on some indicators of sleep in winterers. Analysis of sleep quality

indicators in winterers can be useful to elucidate the mechanisms of the "Antarctic syndrome" and help normalize the psychophysiological state of the winterers during the winter. In the framework of this project, we identified 2 types of changes in the autonomic regulation of heart rhythm during the winter in winterers, in particular, these types of responses correlate with the response to cold exposure. We also investigated concentrations of osmolites in the blood of Antarctic fish. It should be noted that our results indicate a probable dependence of the concentrations of osmolites in the blood associated with latitudinal fluctuations in water temperature.

A separate area of research is cryopreservation of cells and whole organisms. Also during the winter of 2021-22, a study was launched to determine the cryoresistance of Antarctic fish gametes.

As the involvement of scientific institutions in the implementation of research in Antarctica is carried out on a competitive basis, an important lever is international cooperation and the implementation of joint scientific projects with foreign organizations.

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S14 THEORETICAL APPROACHES IN CRYOBIOLOGY

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Studies of the processes occurring in biological systems at different stages of low-temperature preservation followed several directions. The mass transfer through cell membranes play an important role in processes which take place at cryopreservation of biological systems and have to be taken into consideration. Based on the fundamental principles of linear non-equilibrium thermodynamics, a model of transmembrane passive mass transfer was developed. According to this model we developed algorithms for the experimental determination of the cell membranes permeability coefficients for the molecules of water and cryoprotectants. Differences in the plasma membrane transport characteristics of various cell types lead to the existence of particular optimal cooling rates. We determined the optimal modes of cryopreservation of various cells taking into account the obtained characteristics of cell membranes (cell membrane permeability coefficients for water and cryoprotectants and the activation energy of their penetration) and using a physico-mathematical model that determines the probability of cryo-damage of cells based on two-factor theory of cryo-damage, thermodynamic theory of homogeneous crystallisation and the general theory of activation-type processes.

We also developed a physico-mathematical model of elastic deformation of biological membranes. This model accounts for the hierarchy of free energy values corresponding to different deformation types: bending, shear in the membrane plane and isotropic tension. According to the thermodynamic principle of free energy minimum, if external conditions allow, it is energetically advantageous for the membrane to take one or another shape not due to isotropic stretching, but by shifting in the plane of the membrane or bending. On the other hand, if the deformation results in isotropic stretching of the membrane, the other two types of deformation can be disregarded. Based on this principles, specific physical

phenomena occurring in biological membranes during their deformation were described.

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S15 LOW-TEMPERATURE STABILIZATION OF PROTEINS

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Due to the increasing application of proteins in biomedicine, pharmaceutical sciences and clinical diagnostics, protein stabilization, i.e. the preservation of their structure and functionality, during storage, is of enormous importance. Determining the conditions for the preservation of isolated proteins in solutions, incapsulated in alginate microspheres and immobilized on a substrate, as well as preservation of complex protein mixtures (i.e. placenta extract, blood plasma and serum) are therefore the main research interests of the Cryobiophysics Department of IPC&C. The influence of different freezing and storage protocols on the structure and functions of isolated proteins as well as on the properties of complex protein mixtures has been studied at the department for decades. It was demonstrated that the nature of the low temperature effect on isolated proteins depends on the peculiarities of protein structure and freezing protocols. The antioxidant activity of proteins can serve as a parameter for evaluating structural stability: Unfolding as a result of freezing and thawing leads to an increase in the antioxidant activity of proteins, while a decrease in this activity may be associated with the aggregation of macromolecules. Encapsulation of proteins in alginate microspheres before freezing and storage prevented protein oxidation and allowed preservation of protein functions. Storage of immobilized proteins as part of a biosensor required cryoprotective substances in the freezing medium to preserve functionality. The results obtained for complex protein mixtures show that rapid freezing and storage at -196 °C is necessary to avoid a liquid phase in the sample and to minimize protein damage during storage.

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S16 THERAPEUTIC EFFECT OF WOUND DRESSING CONTAINING CRYOPRESERVED COMPONENTS

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During the treatment of gunshot, burn and combined wounds impaired wound healing and purulent-inflammatory complications are often observed. Current protocols for superficial wound healing do not provide effective prophylaxis of wound infections, caused by antibiotic resistant pathogens, which contribute to chronicity of wounds. To avoid these complications, wound dressings containing bioactive compounds are very promising. The main requirements to these dressings are stimulation of synthesis of connective tissue intercellular matrix and growth factors, in addition to suppression of pathogens. A key challenge for the utility of these dressings is stabilisation of their properties in storage.
We have developed a wound dressing containing fibroblasts, gel-immobilized

drugs and bacteriophages (Patent UA 116072). The bioactive components of the dressing were stored at −196°C.
We aimed to study the therapeutic effect of the wound dressing to which cryopreserved components were added after thawing at 37°C.
The base of the designed dressing was methylcellulose gel which was the carrier for the bioactive agents and prevented wound desiccation. Alginate gel beads containing antibiotic, immunomodulating agent, bacteriophages virulent to *Staphylococcus aureus* and *Pseudomonas aeruginosa* were placed into cryovials and frozen by liquid nitrogen immersion. Rat fibroblasts suspended in bovine serum with 10% Me_2SO were cooled down to −70°C at a rate of 1°C per minute and immersed in liquid nitrogen.
Wounds were modelled on rats by subcutaneous administration of acetic acid. The wounds were infected with antibiotic resistant isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.
Prior to dressing application on the wounds thawed drug-loaded alginate beads as well as fibroblasts were introduced into methylcellulose gel.
It has been found that the cryopreserved components of wound dressing retained their therapeutic efficacy. They stimulated the wound healing process, prevented wound infections and shortened the duration of healing. The therapeutic efficacy exceeded that observed at standard wound treatment protocols.

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S17 THE OVERVIEW OF STEM CELL RESEARCH IN THE INSTITUTE FOR PROBLEMS OF CRYOBIOLOGY AND CRYOMEDICINE

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The stem cells (SC) research direction was initiated in the Institute for Problems of Cryobiology and Cryomedicine (IPC&C) in 1980th with studies related to bone marrow transplantation and characterization of hematopoietic stem cells (HSCs) in culture. In late 1990th ¬¬– early 2000th much attention has been focused towards the evaluation of properties of fetal liver HSCs, their potential for treating various disorders and optimization of cryopreservation strategies. Later, the umbilical cord blood HSC research triggered the establishment of first Ukrainian Cord Blood Bank. In 2002 we isolated mesenchymal stromal stem/progenitor cells (MSCs) from various adult and fetal tissues and evaluated their immunophenotypic and differentiation properties, introducing new and exciting field of MSC-based regenerative medicine and tissue engineering. Many research projects have been focused to study the MSC growth and differentiation in a variety of biomaterial scaffolds of different nature and architecture – from polymeric beads, porous scaffolds to carbon fibres and nanomaterials. The preservation of 3D cell-based constructs for therapy or disease modelling is still a challenge and much effort is put into the development of cryopreservation, vitrification and hypothermic storage of cells and engineered tissues. The unique expertise of IPC&C team in SC research and banking, tissue engineering and regenerative medicine in combination with appropriate funding, optimization of infrastructure and

harmonization of research and development activities to EU standards may drive the new era of IPC&C as Centre of Excellence in Stem cell research, regenerative medicine and cryobanking within the Ukraine and East European region.

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S18 TREE POLLEN DERIVED ICE-NUCLEATING MACROMOLECULES ENABLES CRYOPRESERVATION OF MAMMALIAN CELL MONOLAYERS IN 96-WELL PLATES

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Sub-milliliter volumes of aqueous solutions tend to deeply supercool before ice nucleation occurs, often freezing at temperatures below -20°C. This makes cryopreservation of biological material in these small volumes challenging as deep supercooling generally favours intracellular ice formation, impairing cell health post-thaw. The ice nucleation ability of pollen washing water (PWW), which is produced by exposing water to tree pollen grains then filtering off the pollen grains, has previously been examined due to its potential to cause ice formation in clouds and thereby impact weather and climate. In this study we demonstrated that use of small quantities of PWW raised ice nucleation temperatures of 100 µl liquid volumes in 96-well plates from ≈ -13°C to ≈ -7°C. We further demonstrated that use of PWW during cryopreservation increased post-thaw metabolic activity of adherent monolayers of A549 immortalized lung carcinoma cells cryopreserved in 96-well plates from 1.6% (95% CI [-6.6% - 9.79%]) to 55.0% (95% CI [41.6% - 68.4%]). Similar increases in post-thaw viability were found for a range of other adherent cell types. Post-thaw metabolic activity of various suspended cells was also enhanced by use of PWW, although to a lesser extent. Cryomicroscopy revealed that use of PWW tended to reduce the likelihood of intracellular ice formation during cooling by raising ice nucleation temperatures. We have also demonstrated that PWW is sterile and non-cytotoxic meaning it is straightforward to incorporate PWW into existing cryopreservation protocols. In total, PWW can be viewed as a new kind of soluble cryoprotectant, which acts by increasing ice nucleation temperatures and so reducing the likelihood of intracellular ice formation during cooling.

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S19 NEOTERIC SOLVENTS FOR CRYOPRESERVATION

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Cryopreservation is an important method of preserving biological materials for extensive periods of time. Assisted reproductive technologies, stem cell therapies, and blood banks all rely on the use of cryopreservation and highlight the need for effective cryoprotectants. Cryoprotectants are added to biological samples to promote vitrification to minimise freeze damage. Glycerol and dimethyl sulfoxide (Me_2SO) have been the cryoprotectants of choice since their discovery over a half a century ago. However, their toxicity and inability to work as effective cryoprotectants for many cell types inherently limits the application of cryopreservation. Tissues and organs cannot be cryopreserved because the cryoprotectant is not given sufficient time to penetrate to deeper cell layers on account of its toxicity. Novel, non-toxic cryoprotectants are required to reduce this reliance on Me_2SO and glycerol and expand the use of cryopreservation to other cell types and subsequently, other applications. Neoteric solvents such as ionic liquids and deep eutectic solvents are alternatives to common organic solvents (such as Me_2SO and glycerol) and have been minimally investigated as potential cryoprotectants. We have characterised the permeability, toxicity, and thermal properties of a number of neoteric solvents. The interactions of the neoteric solvents with mammalian cells have been investigated, as well as their potential as cryoprotective agents. This work assesses the viability of neoteric solvents as a new class of cryoprotectants and consolidates a systematic method of testing the potential of a solvent to be an effective cryoprotectant for mammalian cells.

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S20 INFLUENCE OF ICE FORMATION ON THE DYNAMIC AND THERMODYNAMIC PROPERTIES OF AQUEOUS SOLUTIONS

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Liquid water cooled below its standard freezing point remains typically liquid for a few degrees below 273 K and then forms solid hexagonal ice. If the water is very pure and cooled quickly (supercooling at ~ 100 K/min), the liquid water may supercool further, to approximately 235 K. This temperature corresponds to the homogeneous ice nucleation, i.e., when water spontaneously crystallizes. On the other hand, if liquid water is cooled fast enough (at rates about 10^6 K/s) it becomes a non-crystalline solid, i.e., a glass. However, on heating glassy water crystallizes at approximately 150 K. This is why the temperature window between 150 and 235 K is called "No man's land" (it is inaccessible to liquid water). However, for water in solutions (the so-called soft confinement) or water confined to porous

materials (hard confinements), it is possible to access this temperature range without crystallization. In some cases, crystallization can be avoided only during the cooling but not on the heating. That phenomenon is called cold-crystallization. The formation of ice crystals generally destroys the biological tissue. Therefore, the dynamics of water and ice in biological and synthetic solutions has enormous importance in various technological, biological and basic research fields. In this work, we apply broadband dielectric spectroscopy combined with calorimetric measurements to elucidate the dynamics of water in aqueous solutions, where all the water was fully amorphous or partly crystalline. The crystallinity degree was obtained by performing different annealing at temperatures between the glass transition and the cold-crystallization. We elucidated how the dynamical behavior of the water is altered both during and after the annealing. For partially crystalline aqueous solutions, after the annealing, it was found that the amorphous water dynamics and glass transition temperature were affected by the crystallinity, but that these effects can almost completely be explained by freeze concentration of the solution. Thus, the water dynamics of the partially crystalline sample becomes the same as for a fully amorphous sample with the same water concentration as the amorphous part of the partially crystalline solution.

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S21 POLYMER-MEDIATED CRYOPRESERVATION OF BACTERIOPHAGES

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Bacteriophages (bacteria-targeting viruses) are an abundant type of viruses with biotechnological and therapeutic potential for the treatment of internal bacterial infections (phage therapy). Application as pure or heterogeneous mixtures requires a robust mechanism for transport and storage, with different phage types having different stability profiles across the storage conditions. Cryopreservation can be employed to bacteriophages long-term storage with the addition of cryoprotectants to mitigate cold-induced damage. In this study, we report that poly(ethylene glycol) can be used to protect bacteriophages from cold damage, functioning at concentrations as low as 10 $mg.mL^{-1}$ (~1% wt. %) outperforming glycerol, the currently used phage cryoprotectant. Protection is achieved at both -20 and -80°C. Polymer concentration required leads to frozen solutions at -20 °C, whereas 50% glycerol result in liquid solution. At both freezer temperatures, post-thaw recoveries close to 100% plaque-forming units were afforded after 2 weeks of storage with our method and bacterial host eradication assays confirmed the lytic function of the phages. Initial control experiments with other hydrophilic polymers also showed cryoprotection, but the exact mechanism of this protection cannot be concluded at this stage. Nonetheless, it does show that water-soluble polymers offer an alternative tool for phage storage. Ice recrystallization inhibiting polymer (poly(vinyl alcohol)) was found to provide no additional benefit, in contrast to their ability to protect proteins and microorganisms susceptible to recrystallization damage. PEG's solubility, low cost, low toxicity/immunogenicity, and

fitness for human consumption make it ideal to help translate new approaches for phage therapy.

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S22 AMPHIPHILES AS A TOOL OF STUDYING THE STATE OF CELLS UNDER TEMPERATURE-OSMOTIC SHOCK

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The research is aimed to compare the efficiency of amphiphiles belonging to different classes of surface-active substances under hypertonic shock (HS) and posthypertonic shock (PHS) conditions of human erythrocytes. Hypertonic hemolysis simulates the affecting factors of cryodamage, acting at the freezing stage, in PHS model the affecting damage factors were implemented at the thawing stage. Anionic sodium decyl sulfate (C10) and cationic trifluoperazine (TFP) were used. Posthypertonic shock was performed by transferring the erythrocytes from dehydration medium (1.65 mol/L NaCl) into rehydration one (0.15 mol/L NaCl), while hypertonic shock was done by transferring the erythrocytes into 4.00 mol/L NaCl at 37 and 0°C. Under PHS conditions (at 0°C), amphiphiles reduced hemolysis level of erythrocytes. Antihemolytic activity of C10 and TFP is 74% and 60%, respectively. At 37°C no protective effect of amphiphiles was revealed. Under HS conditions the amphiphiles reduced hemolysis level at 37 and 0°C, and antihemolytic activity at 37°C was higher (~90%) than at 0°C (40 – 50%). Comparative analysis showed significant differences in the effective concentrations of amphiphiles under different types of temperature-osmotic shock. Under PHS of erythrocyte the effective concentrations of C10 and TFP were higher (by 40 and 6 times, respectively) compared with HS. Based on the above the different mechanisms of protective action of amphiphiles under PHS and HS conditions of erythrocytes can be assumed. Thus, if mechanism of action of amphiphiles under HS was associated with perturbation of erythrocyte membrane, then under PHS it was due to its stabilization at the moment of transmembrane pores formation under shock.

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S23 THE IMPORTANCE OF OVARIAN TISSUE TRANSPORTATION FOR FERTILITY OUTCOMES

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Chemotherapy and radiotherapy are successful to treat many types of cancer, but not without serious consequences, such as infertility and sterility. To overcome these issues, some fertility preservation

techniques have been developed, such as ovarian tissue cryopreservation (OTC). This technique, however, presents its own limitations and challenges, and thus, improving the transportation of ovarian tissue is a way to increase the access to OTC. In order to create fertility networks for this goal, it is necessary to understand the impact of transportation conditions. Studies in different animals' species and humans show an important impact of temperature, medium and duration of transportation. Increased duration of transportation of the ovary showed progressive decrease in morphologically normal follicles (MNF), as well as lower blastocyst yield, and progressive increase of reactive oxygen species (ROS) and apoptosis. Meanwhile, additives such as superoxide dismutase and melatonin improved viable cumulus-oocyte complexes, cleavage rates and embryo quality. In ovarian tissue, duration of transportation increased DNA fragmentation and more complex media showed a higher percentage of MNF. Additives such as antioxidants and plant extracts also improved the percentage of MNF and showed lower DNA fragmentation. Transportation of the tissue similarly affected implantation and live birth rates of mouse ovaries after transplantation and in vitro fertilization, showing the importance of defining a standard protocol for transportation that meets the needs of the ovary during this period.

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S24 DRIED FOR THE FUTURE: LONG-TERM STORAGE OF ANIMAL GERMPLASMS AT AMBIENT TEMPERATURES

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Long-term preservation of viable spermatozoa, eggs, embryos, and gonadal tissues of good quality is essential in human reproductive medicine and for the population management of livestock, laboratory, and wild species. The integrity structure, composition, and function of preserved samples is usually ensured by storage at freezing temperatures, which requires specialized equipment and expensive maintenance. Therefore, a lot of research is conducted to develop simple and cost-effective options for long-term storage. Encouraging findings indicate that structures and functions of germ cells or gonadal tissues can be preserved in trehalose glass after dehydration and then stored at supra-zero temperatures. This body of research has been inspired by the capacity of some small organisms to accumulate trehalose intracellularly and resist dried conditions. However, vertebrate cells have lost that capacity, so different approaches have been developed in laboratories to incorporate this natural sugar into the cytoplasm. Following trehalose exposure, different dehydration techniques have also been explored (from lyophilization to evaporative drying to microwave-assisted desiccation) to reach a stable glass stage. Despite early successes in sperm drying and storage at ambient temperatures, cellular and molecular sensitivity or resilience to dehydration is not well characterized in germplasms. This major limitation must be addressed to develop safe and reliable desiccation and storage strategies at ambient temperatures for any cell types. Lastly, while biobanking at ambient temperatures is actively studied, the advantages, needs, and implications of these future storage options must be carefully examined. This goes from biosafety to operational aspects.

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S25 CRYOPRESERVATION OF IMMATURE TESTICULAR TISSUE AS A FERTILITY PRESERVATION STRATEGY FOR PREPUBERTAL BOYS: CURRENT STRATEGIES

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Cryopreservation of immature testicular tissue (ITT) containing spermatogonial stem cells (SSCs) is an ethically accepted and increasingly proposed experimental fertility preservation strategy for prepubertal boys who are at risk of losing their reproductive potential due to a gonadotoxic treatment.
Freezing of ITT can be done in the form of small tissue fragments or as cell suspensions using either slow freezing (controlled and uncontrolled) or vitrification. Cryopreservation of small ITT fragments is the favored approach worldwide and controlled slow freezing using Me_2SO as a main cryoprotectant is the most commonly applied protocol.
Several studies have already demonstrated the ability of human SSCs to survive the freeze-thaw procedure, keeping their capacity to proliferate and initiate differentiation in various xenografting and organ culture models. However, restoring spermatogenesis in vitro and in vivo remains challenging.
Although no studies have demonstrated to date whether human frozen ITT has the potential to restore a man's fertility in adulthood, animal studies are very encouraging. Indeed, it was shown that frozen SSCs remain functional and able to restore spermatogenesis even after being frozen for up to 20 years. Furthermore, offspring was obtained in pubertal primates after autografting of their frozen-thawed ITT.
Nevertheless, while the research in the field is very active, it is of note that all freezing protocols used to date are based on small comparative studies and that so far no protocol demonstrated its superiority over another in experiments dedicated to fertility restoration approaches.

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S26 TRANSPORTATION OF HUMAN OVARIAN TISSUE: A SYSTEMATIC REVIEW

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Ovarian tissue cryopreservation (OTC) is becoming a routine female fertility preservation technique. Setting up a cryobank to store the ovarian tissue, however, is not without its challenges in that it is expensive, requires intensive training of personnel and strict regulations. A viable solution is creating fertility networks and improving transportation of

ovarian tissue. The aim of this study was to gather and evaluate current evidence on ovarian tissue transportation. This review was carried out according to the Preferred Reporting Items for Systematic Review and Meta-Analysis Protocols (PRISMA-P) and registered on PROSPERO (CRD42020155900). A total of 46 studies were found, of which four were conducted on humans. The temperature at which the tissue was transported ranged from 4-10°C. Studies show that time, temperature and media affect follicle survival and morphology. Low temperatures tend to better preserve follicle morphology and there was a positive impact of more complex media and additives, such as antioxidants. Moreover, there is evidence of tissue metabolism during 24h transportation and there are no set standards for ovarian tissue transportation. Data showed that transportation protocols have an impact on fertility outcomes, both in ovarian tissue and isolated follicles. With increasing demand for OTC and transplantation around the world, creating standard protocols for efficient transport is imperative in order to reach more patients, in whom other methods of fertility preservation are not feasible.

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S27 ADDRESSING THE NEEDS FOR CONSERVING THREATENED EXCEPTIONAL SPECIES—THE CRITICAL ROLE OF CRYOPRESERVATION

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The importance of ex situ conservation of threatened plants is increasing, and much is being accomplished effectively through seed banks. However, for a subset of species, known as exceptional species, cryo-banking of seeds or tissues will be needed. The conceptual framework of exceptionality is built around the steps of conventional seed banking (collecting, processing, storing, and recovery) and the roadblocks that can occur at each step. Using this definition, the first iteration of the Global Working List of Exceptional Status has identified 775 species as exceptional, less than 3% of the number predicted. A recent survey of plant cryopreservation literature using Web of Science™ revealed that research has largely focused on economically important species. Taxonomic groups such as tropical woody species, which are predicted to include many threatened exceptional species, were highly under-represented. There were exceptional species families not represented in the search at all, many from the tropics or Southern Hemisphere. When Google Scholar was searched for cryopreservation literature for these families, fewer than half had one or more references in literature not covered by WoS, including less-cited journals, workshops, proceedings, and theses. Cryopreservation research has been also heavily skewed toward the cryopreservation of in vitro tissues, while there has been comparatively less research with embryos, pollen, dormant buds, and other tissues, although this does not include desiccation tolerance and longevity studies, which are needed for seeds, embryos, and pollen before cryopreservation can be undertaken effectively. Overall, while there has been significant work on plant cryopreservation, there are critical gaps in knowledge for large groups of taxa that

need to be filled in order to effectively manage the needs of conserving exceptional species.

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S28 CAN THERMAL FINGERPRINTS OF MEDITERRANEAN TERRESTRIAL ORCHID SEEDS BE USED TO OPTIMIZE EX SITU CONSERVATION?

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Orchidaceae is one of the largest plant families in the world but also one of the more threatened mainly due to habitat degradation. This research aims to improve the prospects for the successful conservation of European orchids.
Ex-situ seed storage offers a potential solution to mitigate the extinction risk. Terrestrial orchid seeds are orthodox in storage behaviour, so their ex-situ conservation in seed banks can be a cost-effective tool to provide a long-term backup of their genetic diversity. However, dry seed longevity under conventional seed bank conditions (-20°C) is known to be extremely variable, with orchids tending to be relatively short-lived. But why? Seed lipids have long been thought to be a determinant of seed ageing, with lipid composition impacting differing susceptibility to oxidation and to variation in thermal behaviour. In particular, the thermal characteristics of lipid melting and crystallisation are hypothesized to influence the storage stability of oily seeds. So, seed lipid thermal fingerprinting could prove to be a useful tool for identifying and potentially predicting, longer-term cold storage problems in lipid-rich seeds, helping to inform storage practices.
Orchid seeds are minute (weighing µg) and have lipid as the main, high-energy storage compound. Only very few data are available on the thermal properties of orchid seeds. In this study, we have used differential scanning calorimetry to explore potential links between poor storage performance and lipid thermal fingerprints of seeds of 15 Mediterranean terrestrial orchid species. In particular, we determined the temperature spread and complexity of the lipid melt (co-operativity), peak temperature and enthalpy, as well as the short-term crystallization kinetics at ca. -70°C and -20°C. We interpret our data in relation to the risk of lipid crystallisation during cold storage and attempt to answer the question: what is the optimal storage temperature for the long-term preservation of orchid seeds?

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S29 MODIFICATION OF STRESS FACTORS AND ICE

CRYSTALLIZATION BEHAVIOUR FOR SUCCESSFUL CRYOPRESERVATION OF DUCKWEED SPECIES

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The Lemnaceae are a family of aquatic plants that comprise 5 genera (36 species). They are among the fastest growing flowering plants and have a high potential for starch/protein production and wastewater management. They typically reproduce asexually by developing meristematic pockets from which clonal daughters bud off and detach; hence, the conservation of fronds (vegetative bodies) would be a good choice for the preservation of the Lemnaceae genetic diversity as resource for scientific research and future applications. To maintain viable fronds over long-term, cryopreservation would be the only approach for preservation of the desiccation sensitive organs.
For successful cryopreservation of living organs, vitrification approaches including application of dehydration, cryoprotection, ultra-low temperatures (-196°C) and pre- and post-cryopreservation treatments are often applied. However, these treatments induce mechanical stress provoked by excision, osmotic stress induced by the pre-culture or cryoprotection of the explants and thermo-mechanical stress that occur during cooling/rewarming. All these stress factors affect the ability of explants to survive cryopreservation.
To study the sensitivity of duckweed fronds towards diverse stress factors, we examined the success of cryopreservation along with (1) the type, concentration and exposure time of cryoprotectants, (2) effect of the microbiome and (3) on light treatments after cryopreservation. We used differential scanning calorimetry (DSC) and a range of different cryopreservation protocols and media compositions and analysed the regrowth behaviour after different combinations of non-axenic/ axenic culture, non-excised/excised fronds, non/low/moderate sucrose concentrations, Me_2SO /Plant Vitrification Solution 2 (PVS2)/PVS3 and various light treatments. Although duckweed fronds tolerated only short-term dehydration and showed ice crystallization during Me_2SO droplet freezing, sterile fronds of *Lemna minuta* and *Lemna gibba* survived Me_2SO freezing and PVS 3 droplet vitrification. Based on these promising results, we plan to develop a stable and reliable cryopreservation protocol that is applicable to a range of Lemnaceae species.

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S30 CRYOBIOTECHNOLOGY FOR THE CONSERVATION OF BRYOPHYTES

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Bryophytes (mosses, liverworts, hornworts) comprise around 20,000 predominantly ectohydric plants occupying most biomes (from deserts to polar regions) and habitats (from naked rocks to aquatic systems). Recent research suggests that they are the living representatives of the monophyletic sister lineage to all other land plants. These unique ecological and phylogenetic features make bryophytes very important in fields like ecology, physiology, and evolution. However, bryophytes are threatened in many cases. For example, the moss *Orthodontium gracile* has been pushed to the brink of extinction in Europe and is part of diverse conservation programs in the UK. The ex situ conservation of bryophyte species is possible through in vitro culture and cryopreservation. Thus, cryobiotechnology is needed to understand the traits that make cryopreservation of a wide range of species possible, particularly those that are threatened. Here we explore two aspects of bryophyte cryobiotechnology with a focus on British species: 1) the tolerance of spores to desiccation and storage at sub-zero temperatures, 2) the survival after long-term cryostorage of *Orthodontium gracile* and *Dittrichum cornubicum* gametophytes. The final goal is to secure ex situ conservation of these species by supporting the establishment of bryophyte spore banks and the use of cryopreserved gametophytes in restoration programs. Our results show that bryophyte spores can be stored dry at sub-zero temperatures due to their tolerance to desiccation and exposure to cryogenic temperatures. However, the presence of chloroplasts in their cells and storage lipids with particular thermal properties could potentially reduce their longevity during storage at the conventional temperatures of seed banks (-20°C). *Orthodontium gracile* gametophytic tissues survived 15+ years of cryogenic storage, developing new gametophytes that were successfully transplanted onto rocks used in restoration programs. These results highlight the potential of ex situ collections of spores and gametophytes for protecting the bryophyte genetic diversity in the long-term.

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S31 MANAGEMENT OF GAMETE AND EMBRYO CRYOBANKS DURING MILITARY OPERATIONS

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Cryobank management is known to strongly affect the success of infertility treatment by the assisted reproductive technology using cryopreserved biological material. Effective management involves constant monitoring of the processes ensuring safe and efficient storage of biological samples. Selection of cryopreservation equipment and documentation of biomaterial storage processing together with audit of components, all need to be properly and regularly managed.
However, Russia's military aggression against Ukraine has significantly impacted the routine management of cryobanks in many clinics of our country. The aim of the research was to supplement the existing requirements for quality management

biobanking, namely the procedure for organizing the evacuation of cryopreserved samples during military operations based on our own experience. If there is a threat or need to evacuate the cryobank, a disaster recovery algorithm should be developed, which includes the following steps if possible:
1. access to equipment for liquid nitrogen generation,
2. access to a back-up source of liquid nitrogen for emergency evacuation,
3. a clear decision about sample packing density of the container because of the possibility for liquid nitrogen overconsumption
4. electronic documents on the samples in the cryobank in addition to paper versions
5. appropriate changes in a patient's contract to preserve the biomaterial regarding the possible evacuation of samples during military operations and other emergencies,
6. establishing contact between cryobanks within and outside the country, in order to precisely choose a safe place of evacuation within or outside the country,
7. prior to the biobank evacuation, preparation of documents with a regard to current legislation in the country to where, in extreme circumstances, the samples will be evacuated.
We believe that due to our experience, the documents regulating the cryobanks' management should be supplemented with the above items for proper and safe storage of specimens.

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S32 THE GENERATION OF THERMAL TRANSIENTS DURING ROUTINE ACCESS TO CRYOVIALS STORED IN VAPOUR-PHASE LIQUID NITROGEN – IMPLICATIONS FOR BIOBANKS

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Long-term storage of cells generally takes the form of storage in the vapour phase above liquid nitrogen. The thermal gradients seen in older liquid-nitrogen freezers have largely been eliminated through replacement with high efficiency, narrow-necked liquid nitrogen refrigerators or their more recent cryocooler-powered siblings.
However, storage refrigerators have to be accessed routinely to remove samples for a variety of purposes such as cell production, quality control and cell supply. In removing a sample, the thermal equilibrium of not only that vial but also other vials in the cryo-box and other boxes in the racking tower will be disturbed leading to thermal cycling as the tower is returned to the liquid nitrogen refrigerator.
Recent studies on a number of cells and tissues have indicated that thermal cycling, in which cells are exposed to repeated thermal transients, leads to a reduction in viability after as few as 50 cycles. We therefore investigated the extent of the thermal transients imposed on cryovials under conditions that mimicked normal biobank conditions, using specially adapted cryovials containing thin-wire thermocouples, in order to see how they compared with the transients reported in the literature.
During sample removal, significant thermal events were experienced not only to adjacent vials within the cryo-box but also to cryo-vials in other boxes in the same tower and to vials in adjacent towers once the “warm” tower was returned to the liquid nitrogen refrigerator. Within the opened cryo-box, the degree of warming was dependent on position within the box and the extent to which it was full. Cryovials in unopened boxes within the removed tower behaved similarly, warming to above -100°C within 7 minutes.

The results suggest that significant thermal cycling occurs during routine access and points to the need for controls, mechanical or operational, to reduce these thermal transients.

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S33 MICROBIAL CONTAMINATION OF BIO-STORAGE TANKS CAN RISK REPRODUCIBILITY OF DATA AND ENGENDER PATHOGEN TRANSFER

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The possibility of microbial contamination of LN-stored materials should be a concern for cryo-facilities, of whatever size, as it can generate significant negative impacts, including lack of reproducibility in research outputs and loss or alteration of biological materials. It is clear that contamination builds with time as, consequently, does the risk of contamination-related injury or damage to stored biomaterials. Such contamination may also represent health risks to laboratory workers, the environment and recipients of medical and veterinary therapeutic products. Evidence of such risks in medicine and agriculture have been widely reported. Inevitably, active control of microbial contamination during cryostorage adds to the cost of both research and bioprocessing and this has been discussed, with some control measures being introduced, in critical and high value areas such as medicine. However, in smaller and financially limited facilities there may be a lack of awareness of the potential sources and means of transmission of contaminant organisms. Control measures can also be difficult to address effectively where there are resource constraints, potentially placing the facility at risk in terms of the quality of scientific output. Regrettably, the broader risks to research output and quality that can be posed by microbial contamination have been given relatively little consideration with events generally not being reported. This presentation will briefly review what is known about the sources and frequency of contamination, its likely persistence, accumulation, and transmission. It will consider the gaps in relevant international biobanking standards relating to this issue and identify the challenges that storage facilities must face in order to manage microbial contamination effectively. Key steps that storage facilities might adopt to protect their research outputs, including the supply of vital germplasm, against the potential negative impact of microbial contamination in their LN-storage systems, will be suggested.

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S34 ENHANCING QUALITY BIOBANKING THROUGH EDUCATION

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The importance of co-ordinated, robust sample collection for medical research has been highlighted during the COVID pandemic with the need for rapid, well curated samples and data. The knowledge and skills present within biobanks expedited such collections and the value of expert repository staff was recognised.
Investing in staff is one of the most important activities a biobank can undertake as part of social sustainability.

Biobank personnel are core to the success of the repository and a valued and engaged team is key to provide a solid foundation for the biobank to evolve and flourish. Supporting education gives the individual the opportunity for career development, the biobank a more stable workforce and has impact and value to both the biobank and host institution.
Personnel come to biobanking via many varied routes, and it can be difficult when recruiting to benchmark an individual's knowledge of biobanking and associated processes. The Qualification in Biorepository Science (QBRS) is a new education tool that was jointly developed by the International Society for Biological and Environmental Repositories (ISBER) and the American Society of Clinical Pathology's Board of Certification (ASCP BOC). It provides a biobanking specific, international exam that 'professionalises' biobanking and allows remote learning to fit around daily tasks. All aspects of the biobanking pathway are covered in the syllabus and the exam is taken online, giving wide accessibility. The Wales Cancer Biobank sponsored a laboratory technician to take the test in 2021 and we present the impact and value of the QBRS qualification from a biobank's perspective.

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S35 A CLOSER LOOK ON TIGHTNESS ISSUES OF CRYOVIALS AND STRAWS IN BIOBANKING

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Cryogenic long-term storage of biological samples is a common procedure for sensitive biological samples. It significantly reduces degradation and preserves sample quality over a long period of time. This is essential for comparative medical studies, e.g. cohort studies on population health trends. Neglected for many years, also the integrity of the sample containers is critical for the preservation of sample quality. They must stay tight down to the storage temperature to avoid chemical and biological contaminations. At the same time, they should be easy to handle for automated sample processing.
Plastic vials with screw caps as well as sealable straws have become widely accepted for cryobanking applications. Usually, they are stored in the gas phase over liquid nitrogen, which results in a temperature difference of more than 200K between laboratory handling and storage conditions. Thus, cooling down and warming up means stress to the sample containers. It can cause microcracks or lead to a temporary or permanent failure of the seal between the lid and vial. This leads to contamination or sample loss as well as to chemical alterations, like pH drops and oxidation.
Different methods for the quantification of tightness are presented. Our CO_2 leak test quantifies the risk of sample degradation during the transportation on dry ice by the determination of the carbon dioxide accumulation. The LN_2 leak test is used for work and sample safety. It differentiates between temporary and permanent leakage after LN_2 contact. The transport safety test simulates the pressure and temperature conditions during air transport in order to reveal the risk of sample leakage. Results are shown for various products in order to compare different types of cryovials and to show different tightness problems. To characterize storage stability, tensile tests and DSC measurements of fresh, real-time

stored and cyclically thermal stressed packaging materials are compared.

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S36 IS THE OCCURRENCE OF MICROORGANISM LIQUID NITROGEN STORAGE TANKS A CHALLENGE FOR CRYOBANKING?

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Modern biobanks need to guarantee the high-quality long-term storage of valuable living materials for medical diagnostics, reproduction medicine and conservation purposes. Therefore, cryostorage is often conducted in the vapour or liquid nitrogen phase of liquid nitrogen storage tanks at temperatures below -150 °C. We recently reviewed the literature data to evaluate the potential risk of microbial cross-contaminations during cryostorage. By identifying potential contaminants, their sources in liquid nitrogen storage tanks and assessing their potential harm, we have learned that the samples themselves, the liquid nitrogen, the human microbiome and the surrounding environment are possible routes of contamination. Here, liquid nitrogen is typically not the source of major contaminations and only few studies provided evidence for a risk of microbial cross-contamination. We conducted a short survey among different culture collections demonstrating the awareness for microbial contaminations of storage containers. Most institutions/collections/biobanks prevent potential contaminations by using sealed devices and/or – 150 °C freezers. To further minimize the potential risk of microbial cross-contaminations in cryotanks we recommend reducing the formation of ice crystals that can entrap environmental microorganisms. Based on our results we developed a risk assessment spread sheet which can support biobanking quality management.
Ethical approval: This study does not contain any studies with human participants or animals performed by any of the authors. A questionnaire was distributed among institutions. Before starting the questionnaire, respondents were made fully aware that procedures to ensure anonymity were in place and were informed about the service's Data Protection Terms. Respondents were made aware that submission of completed questionnaires implied their consent that results will be evaluated and published anonymously.

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S37 THE EFFECTS OF ERYTHROPOIETIN ON ISCHEMIA-REPERFUSION WHEN ADMINISTERED BEFORE AND AFTER OVARIAN TISSUE TRANSPLANTATION IN MICE

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In recent years, the demand for methods that ensure the preservation of female fertility has increased. Despite advances in cancer treatment and prognosis, the drugs used continue to cause ovarian function failure. One of the emerging techniques to preserve the reproductive potential of these patients is cryopreservation of ovarian tissue, followed by transplantation. However, a major obstacle to graft survival are ischemia-reperfusion lesions. Thus, the aim of this study was to evaluate the best moment for the administration of Erythropoietin (Epo): before the removal of ovaries for cryopreservation or after receiving transplants, analysing its effect on follicular survival and tissue reperfusion. Thirty Swiss mice were divided into three groups (n=10 each): Cryo Group, Epo Group Before and Epo Group Later. The animals underwent to bilateral ovariohysterectomy and the hemiovaries were cryopreserved by slow freezing. At this time, they also received ovarian tissue transplantation in the dorsal subcutaneous region. The doses of Epo (250 IU/Kg/dose) and sterile saline solution 0.9% were administered every 12/12h, during five consecutive days. A total of 7734 follicles were analysed. From these, 88.3% were classified as morphologically normal (MN). The Epo Group Later showed a better proportion of MN follicles compared to degenerated follicles at D14 post-transplantation ($p < 0.05$), in addition also have the highest percentage of proliferative follicles ($p < 0.05$), higher number of blood vessels ($p < 0.05$), higher mean percentage of tissue area occupied by vessels ($p < 0.05$) and lower incidence of fibrosis ($p < 0.05$). In conclusion, the present study demonstrated that treatment with Epo after transplant recipients reduced ischemic damage in transplanted ovarian tissue, increasing angiogenesis, which resulted in a greater number of growing MN follicles 14 days after transplantation, maintenance of proliferation and survival of ovarian follicles, and reduced areas of fibrosis in the grafted tissue.

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S38 EXOGENOUS MELATONIN AMELIORATES THE NEGATIVE EFFECT OF OSMOTIC STRESS IN HUMAN AND BOVINE OVARIAN STROMAL CELLS

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Ovarian tissue cryopreservation (OTC) and transplantation is a promising method to preserve fertility in reproductive age females with cancer. However, post-thaw follicle loss and impaired tissue function is still a major concern. Cryopreservation induced damage could be in part due to CPA toxicity and osmotic shock. In fact, CPA addition and removal is one of the main challenges associated with osmotic shock-induced damage. Therefore, one way to avoid this damage is to maintain the cell volume within osmotic tolerance limits (OTLs). Ovarian stromal cells (OSCs) are a major population of ovarian structure and play key roles in ovarian function. These cells are sensitive to a deleterious impact of cryopreservation especially during slow freezing methods. Here, we aimed to determine for the first time the OTLs of OSCs and its relationship with ROS levels and mitochondrial respiratory chain (MRC) activity of OSCs. We evaluated the effect of an optimal dose of melatonin as an

antioxidant on OTLs, viability, MRC, ROS levels and total antioxidant capacity (TAC) of both human and bovine OSCs in plated and suspended cells. The OTLs of OSCs were approximately between 200 and 375 mOsm in bovine and between 150 and 500 mOsmol in human. OTLs showed a positive correlation with ROS levels and negative correlation with MRC activity. Our finding demonstrated that melatonin expands OTLs of bovine and human OSCs from 150-500 mOsm/kg to 25-1000mOsm/kg, respectively. Furthermore, melatonin significantly reduced osmotic stress-induced ROS generation and improved TAC, MRC activity and viability in OSCs. Due to the narrow osmotic window of OSCs, it is important to optimize the current protocols of OTC to maintain enough stromal cells alive which are necessary for post grafts follicles development and a longer longevity of grafted tissue. Moreover, addition of melatonin to cryopreservation media may be a beneficial option in clinical applications.

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S39 VITRIFICATION OF BOVINE OVARIAN TISSUE AS A MODEL FOR HUMAN: THE EFFICIENCY OF NON-PERMEATING CPAS AND CLOSED SYSTEM

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Ovarian tissue cryopreservation (OTC) is the only current available approaches to preserve fertility in women who require immediate cancer therapy as well as single women with cancer, and particularly in children. Slow freezing is the standard method for OTC in clinics; however, the vitrification method has gained popularity recently, owing to good results obtained with vitrification of gametes and embryos. However, most protocols for ovarian tissue vitrification (OTV) are adapted from those of gametes and embryos. Hence, OTV has specific challenges. For example, the likelihood of a successful vitrification outcome is highly dependent on the type and concentration of cryoprotective agents (CPAs) as well as the carrier system. Moreover, the possibility of potential exposure to pathogens from other samples during the vitrification and storage procedures is still a concern. Therefore, the aim of this study was to evaluate the efficiency of the vitrification approach with the vitrification solution containing permeating CPAs and non permeating polymers in a closed system. Bovine ovarian tissues were divided into fresh and three vitrified groups (54%, 57%, and 60% CPAs). Vitrification groups were loaded into a high security cryotube, cooled in LN2 vapor, plunged into LN2 for storage, and warmed in two-steps. All groups were examined for viability, reactive oxygen species levels (ROS), antioxidant capacity, and histology. Our results showed no evidence of ice crystal formation in 57% and 60%. Morphologically, a lower percentage of abnormal follicles was observed in the fresh and 57% groups compared to other groups. Furthermore, the fresh, 57%, and 60% groups revealed a higher dense stroma compared to the 54% group. A higher total tissue viability and a lower ROS levels were observed in fresh and 57% groups. In conclusion, the combination of a closed vitrification system with an optimized concentration of CPAs could be useful to improve vitrification outcomes.

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S40 INCREASE IN-VITRO MATURATION RATE OF VITRIFIED MOUSE GV OOCYTES VIA NOVEL XYLOMANNAN COATED IRON OXIDE NANOCOMPOSITE

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The importance of storing gametes increases every day because it allows couples to maintain their fertility, especially in acute conditions in which their reproductive system may be affected. Vitrification could be applied to preserve gametes for a long-time. However, there are some issues with this method. In recent decades, several studies show that using nanoparticles in the vitrification process of oocytes can overcome freezing problems and increase its viability rate and subsequent developments. In this study, purified powder of xylomannan was extracted from Enoki mushroom by the modified Smiderle method. Then, it was added to superparamagnetic iron oxide nanoparticles (SPIONs) while they were in the synthesis system to fabricate the Xylomannan Coated Iron Oxide Nanocomposite (XCIONc). This study aimed to investigate whether use of mentioned nanocomposite (with the concentration of 0.006% (w/v) determined via toxicity test) in vitrification solution, increases post-warming survival and in-vitro maturation (IVM) rates of 6-8 weeks-old NMRI mouse GV oocytes. About twenty immature oocytes in five technical repetitions were vitrified in both (VitX: vitrification solution contained 0.006% (w/v) XCIONc and Vit: vitrification solution without XCIONc) groups using the Kuwayama method. After warming, oocytes were cultured for 20 hours in IVM medium and were stained with Hoechst dye to assess the real nuclear maturation. Obtained data were analyzed by one-way ANOVA analysis using SPSS software ($P<0.05$). Characterization tests such as DLS and Z-potential were performed to determine nanocomposites' hydrodynamic size and stability, respectively. Also, for assurance about the correct formation of nanocomposites, the FT-IR test was applied. Results indicate that vitrified-warmed oocytes in the medium containing XCIONc have a significant increase in IVM rate (non-vitrified (Control): 56.05%, Vitrified: 67.18%, and XCIONc-Vitrified: 89.17%). It is concluded that using the novel XCIONc in the vitrification solution of mouse GV oocytes improves cryopreservation outcomes and enhances their next IVM rate.

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S41 CRYOPRESERVATION OF HUMAN CUMULUS CELLS BY A 3-STEPS SLOW FREEZING METHOD FOR OOCYTE *IN VITRO* MATURATION SYSTEM

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Despite vitrification has a positive effect on oocyte cryopreservation, its use for CCs does not have such positive effects and leads to a decrease in viability and a change in the cell ultrastructure. The aim of this

work was to evaluate the effect of cryopreservation of human CCs by a 3-steps slow method using various carriers on CC survival, and *in vitro* maturation effects on GV oocytes.

Oocytes and cumulus cells were obtained during women infertility treatment by assisted reproductive technologies. CCs were obtained from oocyte-cumulus complexes after denudation. A cryoprotectant solution of 20% ethylene glycol (EG) and 40% human serum albumin was added to the resulting CCs suspension (15 µl) in a ratio of 1:1. The suspension was divided into two aliquots, one was frozen in a microstraw straw (group 1). The second aliquot was frozen on a plastic scapula (group 2). The samples were incubated for 30 min at a temperature of +4 ℃ and then placed 10 cm above a liquid nitrogen mirror for 15 minutes and plunged into it. The microstraw was warmed up in a water bath at 37 ℃, and the plastic spatula was immersed in a heated medium up to 37 ℃. CCs were washed with the culture medium. As a negative control, the CCs were vitrified in a medium containing 15% EG and 15% Me_2SO (group 3). Fresh CCs served as a positive control (group 4).

Cell viability was 43±7.4, 60±6.5 and 25±4.3% for groups 1-3, respectively. After 24h culturing CCs group 2 formed tighter contacts with oocytes than in the other groups, but less than in group 4. Oocyte maturation rates were 23%, 45%, and 10% and 60% for groups 1-4, respectively. Our study has shown that the use of a slow 3-step with 10% EG is the most successful when using an open carrier. Most likely, this is due to the warming stage when cells are significantly damaged and their adhesive ability and maturation influence on oocytes are changed.

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S42 A MICROFLUIDIC APPROACH FOR SIMULTANEOUS AND NONDESTRUCTIVE INVESTIGATION OF OOCYTE MEMBRANE PERMEABILITY

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Oocyte membrane permeability mainly refers to the water conductivity (hydraulic conductivity) and cryoprotectant (CPA) permeability. It plays an important role in oocyte cryopreservation, especially for optimizing the addition/removal of CPA and the cooling/rewarming procedures. Therefore, the study of oocyte membrane permeability is of great significance to fertility preservation. But, traditional manual methods for investigating the permeability are associated with uncontrolled operators and run-to-run variability. Existing efforts are mainly focused on exploring microfluidic-based methods, such as microscope diffusion chamber, polydimethylsiloxane (PDMS) microfluidic chip, etc. However, the commonly used microfluidic methods have shortcomings such as time-consuming, low efficiency, cumbersome data processing, and lack of verification of development potential. This study reports a new microfluidic chip dedicated to oocyte permeability investigation. The newly designed multichannel microfluidic chip with micropillars provides feasible and non-missing oocytes capture. Due to the special structure designation, it can achieve permeability measurement of three oocytes simultaneously at different or the same CPA concentrations. Moreover, a neural network algorithm was employed to process huge experimental data. The above

improvements have greatly improved the measurement efficiency. The results indicate that the permeability coefficients (Lp, Ps) of the oocyte membrane are consistent with those reported in previous works. It was also revealed that the oocyte membrane permeability parameters were related to the CPA concentration. Furthermore, developmental potential of measured oocytes was analyzed by in vitro fertilization and embryo transfer to verify that the study is non-destructive. Overall, we proposed a new microfluidic approach that allows on-chip synchronous and nondestructive study of the permeability of multiple oocytes, it may have great application prospects in the field of fertility preservation and assisted reproductive medicine.

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S43 IMPROVEMENT OF RECOVERY CYCLE OF CRYOPRESERVED POTATO SHOOT TIPS – SMALL CHANGES, BUT HUGE IMPACT

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Improvement and optimization of cryopreservation protocols for its reliable large-scale application is a time-consuming process. The development of robust and efficient protocols implicates the assessment of a high number of diverse genotypes before results can be extrapolated on complete collections. To simplify and improve the recovery cycle of potato shoot tips cryopreserved with the droplet-vitrification method, it was assessed if placing shoot tips after thawing for 9 days on culture medium with normal and stable sucrose concentration of 0.073 M (in darkness), can increase the viability rate, compared to a stepwise decrease of the sucrose concentration from 0.3 M to 0.2 M to 0.1M and 0.073 M during the initial phase of the recovery cycle (control treatment). The experiment was performed with 68 diverse potato landraces which showed a significantly higher recovery rate of 69.8 % when shoot tips were recovered on culture medium with a stable sucrose concentration of 0.073 M, compared to the control treatment (57.9 %). The difference in recovery rates was confirmed during routine cryopreservation: a set of 806 accessions recovered with the control treatment showed an average recovery rate of 60.9 % (years 2018-19), while 1158 accessions processed with the new protocol had an average recovery rate of 75.1 % (2019-now). Currently, CIP conserves 4017 virus-free potato accessions in its cryobank, which represent about 83% of the *in vitro* collection (4851 accessions). Based on a high experimental sample size of 68 tested accessions (1.4 % of population) robust and reliable results about the cryo-response of the whole collection were obtained.

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S44 COLD PRETREATMENT FOR CRYOPRESERVATION OF AN ENDANGERED AUSTRALIAN NATIVE PLANT SPECIES *GOSSIA FRAGRANTISSIMA*

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The Myrtaceae family of plants dominates Australian plant biomass, with many both ecologically and economically important species. Within this Family, *Gossia fragrantissima* (sweet myrtle) is an endangered small rainforest tree endemic to Eastern Australia. A rare trait of the species is that it can hyperaccumulate Mn, Zn, Co, and Ni, providing potential interest for phytomining and phytoremediation. However, this species is at significant risk of local population extinction due to habitat degradation. The Myrtaceae family is also susceptible to the devastating invasive fungal disease 'Myrtle rust', which arrived in Australia in 2010. Urgent conservation and germplasm capture efforts are needed. However, seeds of *G. fragrantissima*, while tolerant desiccation, are unlikely to withstand long-term seed-banking (-20 °C). Cryopreservation in this case could offer a potentially safe, cost-effective and space-efficient solution for long-term preservation of *G. fragrantissima.*

In this study, cold pre-treatment of donor plants and use of droplet vitrification with Plant Vitrification Solution 2 (PVS2) and Vitrification Solution L (VSL) were explored as a first attempt to develop a cryopreservation protocol for *G. fragrantissima*. In addition, cold pre-treatment of donor plants in tissue culture, involving doing cryo-treatment using donor plants pre-treated for different time periods, was explored to pre-condition shoots for improved outcomes. Survival (2 weeks after cryoprotectant treatment) and regrowth (4 weeks after cryoprotectant treatment) of shoot tips exposed to PVS2 were 76.7% and 23.3%, respectively, compared to 63.3 % and 20.0 % for VSL. Shoot tips did not survive after Liquid Nitrogen (LN) regardless of treatment with cryoprotectants. This indicated that PVS2 supported healthier shoot tips with better pre-cryopreservation viability compared with VSL, but was not sufficient for cryopreservation success. Cold pre-treatment was found to be essential for survival of cryoprotected shoot tips following LN incubation. Three weeks after cryo-treatment, highest survival of 16.67% was observed for shoot tips dissected from a donor plant that was preincubated at 15°C for 13 days. This provides a foundation to preserve *G. fragrantissima* through ongoing optimization of culture and media components and provides opportunity for future translation to other endangered *Gossia* species and wider Myrtaceae under threat.

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S45 CRYOBIOTECHNOLOGICAL APPROACHES TO ELIMINATE *SUGARCANE MOSAIC VIRUS* (SCMV) IN SUGARCANE (*SACCHARUM* SPP. L.) INFECTED PLANTS

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We present here the results of a collection of investigations on the use of cryogenic procedures, specifically Droplet-Vitrification (D-V) and V-Cryoplate (V-Cp) for eliminating Sugarcane mosaic virus (SCMV) in sugarcane explants. Using RT-qPCR, the presence of SCMV was verified in both field materials and in *in vitro* donor shoots of cultivars N19 and NCo376 (at SASRI, South Africa). Shoot-tips (3 mm) excised from *in vitro*-grown shoots were subjected to both cryopreservation methods using plant vitrification solution 2 (PVS2) for 30 min at room temperature for osmotic dehydration before cooling in liquid nitrogen.
Plantlets of cultivar N19 recovered from the cryoprotective treatments following D-V and V-Cp protocols, contained virus, but after cooling, no virus was detected. Therefore, viral particles could be eliminated only after cooling (cryotherapy). Plantlets of cultivar NCo376 derived from shoot-tips subjected to V-Cp, with and without cooling, contained virus, whereas those exposed to D-V, with and without cooling, were virus-free. Exposure to PVS2 without encapsulating shoot-tips of NCo376 cultivar was effective in eliminating SCMV (osmotherapy), as well as cooling in liquid nitrogen (cryotherapy). Additional studies were carried out following a D-V protocol and 15 min-exposure to PVS2 at room temperature to investigate the effect of the osmoprotective treatments and of cryopreservation on SCMV elimination using shoot-tips of cultivar CP 72-2086 (at Veracruz, Mexico). The presence of virus was confirmed by DAS-ELISA immunoenzymatic test. No virus was detected in the regenerated plantlets from shoot-tips subjected to cryopreservation. The progressive decrease in ELISA plate absorbances occurred from the first preculture treatment and no significant differences were found in samples from the subsequent steps of the D-V protocol. These results support the growing body of evidence that osmotic dehydration treatments (osmotherapy) and cryopreservation (cryotherapy) may be potentially effective strategies for eliminating the SCMV from infected sugarcane plants.

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S46 A MULTI-MODAL ANALYSIS OF MENTHA X PIPERITA SHOOT TIP RESPONSE TO CRYOPROTECTIVE AGENTS

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Preserving global plant genetic diversity has never been more important as the number of endangered or at-risk plant species continues to rise worldwide. Vegetatively propagated crop collections

have been successfully cryopreserved as 1-2 mm shoot tips treated with highly concentrated mixtures of cryoprotective agents (CPAs) such as PVS2 (15% *w/v* dimethyl sulfoxide, 15% *w/v* ethylene glycol, 30% *w/v* glycerol and 0.4 M sucrose) prior to plunging them into liquid nitrogen. Despite the successful use of these CPAs, little is understood as to how they protect plant cells and tissues from freezing damage during the cryopreservation process. Coherent anti-Stokes Raman scattering (CARS) microscopy is a vibrational imaging technique that allows for the direct visualization of CPAs within living plant cells and tissues by probing specific chemical bonds. By pairing CARS with brightfield and two-photon endogenous fluorescence microscopies, a comprehensive view of the CPA localization within complex plant tissue systems can be explored. We describe a multi-modal approach to investigate how *Mentha* × *piperita* (peppermint) shoot tips respond to dimethyl sulfoxide.

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S47 CRYOPRESERVATION OF CUCURBITACEAE SPECIES POLLEN FOR EXPLORING BREEDING POSSIBILITIES

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Cryopreservation is helpful in the consolidation of quality pollen of wild species and cross compatible species for the execution of timely breeding programme under adverse situation. The supply of viable pollen from pollen banks overcomes the seasonal, geographical or physiological barriers in natural hybridizations and facilitates hybrid development between genera and species. Pollen collection and cryopreservation protocols have been optimised for the following cucurbitaceous crops bitter gourd (Momordica charantia) Spine gourd Momordica dioica. teasel gourd Momordica subangulata subsp. Renigera Bottle gourd (Lagenaria siceraria,) and ash gourd (Benincasa hispida),water melon (Citrullus lanatus), musk melon (Cucumis melo) . Pollen was collected during peak anthesis and moisture content was brought down to less than 10 % in each crop and viability of the same assessed through in vitro germination using hanging drop method. Medium for in vitro germination was optimised for each species. The pollen sample packed in butter paper and in turn put in laminated aluminium foil and sealed air-tightly on all sides transferred to the canister which were directly plunged in to the liquid nitrogen in the cryobiological systems (MACH SM 43). The fertility of stored pollen is tested under field conditions through controlled pollination. The pollen cryobank is managed by periodic replenishment of the cryogen. With the recent advances in allied bio-scientific areas in which cryopreserved pollen could be used in general crosses, shuttle breeding maintenance of B line in male sterile based hybrid seed production. A Pollen cryobank for cucurbitaceous crop will help in breeding and conservation.

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S48 EFFECT OF UNFROZEN WATER CONTENT ON THE PHYSICAL QUALITY OF FREEZE-DRIED FOOD MODEL

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Freeze-drying is widely used to produce high-quality dry food products. However, some food products still face challenging problems; structural deterioration during freeze-drying causes undesirable appearance, prolonged drying time, and poor rehydration. Sugar-rich foods (e.g., fruits) readily collapse because of low freeze-concentrated glass transition temperature (Tg'). By adding a gelling agent, such as gelatin or agar, the severe collapse could be prevented; however, structural deterioration still occurs as shrinkage in the finished product. Although Tg' is concentration-independent, the degree of structural deterioration increases with the solute content. The potential cause of that is assumed to be the increased evaporation resistance related to unfrozen water content induced by the solute fraction. However, there are no quantitative data on the relationship between structural deterioration and unfrozen water. This study aimed to clarify the effect of unfrozen water content on the physical quality of the freeze-dried food model.

The drying temperature, volume changes, and sublimation behavior of the food model were investigated. Sucrose, trehalose, glucose, and maltodextrin solutions (10~40%) were entrapped in 1% agar gel, then freeze-dried at 0.38 hPa with a shelf temperature of 25˚C for 16 hours. The solute-induced unfrozen water and freezing point depression were evaluated using differential scanning calorimetry (DSC).

The primary drying duration and residual water of freeze-dried solids were characterized quantitatively by the unfrozen water ratio of the samples. The primary drying ended readily in the higher concentration matrix due to less frozen water, leaving the works to unfrozen water evaporation. Early finished primary drying is also associated with an early increase in sample temperature, increasing the evaporation resistance. The excess residual water results in undesirable product quality; the sample remains in the jelly-like form, reflecting as the drying process is not completed.

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S49 PROBING INTERACTIONS BETWEEN SMALL-MOLECULE ICE RECRYSTALLIZATION INHIBITORS (IRIS) AND WATER MOLECULES USING ^{1}H NMR

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The phenomenon of ice recrystallization has detrimental effects on biological materials during cryopreservation, and its inhibition using small-molecules has been shown to improve the cryopreservation outcomes of numerous cell types. While recrystallization has been extensively studied in the metallurgical/geological sciences, its inhibition is significantly less studied with ice in cellular applications and the mode of action (MOA) for small-molecules remains largely unknown. To facilitate the discovery of novel small-molecule ice recrystallization inhibitors (IRIs), it is imperative to gain further understanding of their MOA.

Our laboratory has demonstrated that small-molecule IRIs are unable to bind ice surfaces, therefore we hypothesize that they inhibit ice recrystallization by

disrupting the transition of water molecules between ice crystals through bulk water. The behaviour of water and ice using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has been extensively established in the food sciences, comparing pure ice/water to those of compound-supplemented ice/water. Therefore, in this study we aim to use ^{1}H NMR and proton relaxation times to investigate the interaction between water molecules and *N*-2-fluorophenyl-D-gluconamide (2FA), an effective small-molecule IRI.

2FA is frozen in pure water at four concentrations ranging in IRI activity as determined by a modified splat-cooling assay. It is found that the spin-lattice (T_1) relaxation times and calculated activation energy of the IRI-supplemented samples do not differ as compared to pure water ($p > 0.05$). This confirms there is no difference in water molecule organization within the ice-lattice in the presence or absence of 2FA and suggests that 2FA is excluded from the ice lattice during freezing. Additionally, spin-spin (T_2) relaxation times suggest 2FA disrupts water molecules in the bulk water phase in order to reduce the rate of ice crystal growth. These findings have significant implications on the understanding of small-molecule carbohydrate-based IRIs and their MOA to develop more effective compounds for wide-scale clinical, research, and industrial applications.

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S50 IMPACT OF FREEZING METHOD ON PRESERVATION OUTCOMES

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It is well established that the characteristics of ice formation during cryopreservation can impact preservation outcomes in eukaryotic cells. Ice formation can be modulated by various chemical approaches, such as anti-freeze proteins or reagents, and physical means, such as high temperature ice nucleation and controlled rate freezing. While it is a common practice to use passive freezing containers in small scale freezing situations, the limited scalability and lack of control over the freezing process make these methods unsuited for production environments.

In this study, we compared preservation outcomes from multiple controlled rate freezing (CRF) temperature profiles using an identical cryopreservation medium. Cell recovery was measured using standard viability assays and metabolic activity was characterized using extracellular flux analysis at multiple time points following removal from vapor phase nitrogen storage. Additionally, the thermal characteristics of each CRF profile were analyzed using differential scanning calorimetry.

We observed that there was a significant reduction in the variability between CRF profiles, with the experimental CRF profile exhibiting a 76% reduction in the standard deviation of the viability post thaw. Metabolic analysis data showed no significant difference between cells frozen using different CRF profiles when normalized on a per cell basis. Taken together, these data suggest that CRF profile optimization can help to minimize batch-to-batch and intra-batch variability while having little to no impact on cell performance.

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S51 HOW DOES DIMETHYL SULFOXIDE MODULATE GLYCOLYSIS AND GLYCONEOGENESIS PATHWAYS?

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Dimethyl sulfoxide (Me_2SO) is the major component of many vitrification solutions due to its ideal property of eliminating ice formation; however, it is also known to be toxic to cells. Thus, assessing the precise mechanism of the damage that Me_2SO exerts is essential for developing better cryopreservation protocols. Early studies presented evidence that part of the toxic effect of Me_2SO on cells was caused by damage to Fructose 1,6-bisphosphatase (FBP1), a key rate-controlling enzyme in the glycolysis and gluconeogenesis pathways. Therefore, in this work, we proposed to expose purified FBP1 to Me_2SO in series of simplified experiments and assess properties of the enzyme, such as conformation modulations and number of free sulfhydryl groups, post Me_2SO treatment. According to the obtained data, the number of identified sulfhydryl groups were reduced significantly with Me_2SO exposure which suggests that the thiol groups of FBP1 may have been oxidized by Me_2SO. Then, samples were characterized upon simultaneous treatment with Me_2SO and Coenzyme A (CoA) to simulate native cellular response to oxidative stress and protect the enzyme from oxidation. Interestingly, FBP1 conformation was altered in the simultaneous presence of CoA and Me_2SO compared to the Me_2SO alone group suggesting CoA's attempt to rescue FBP1 under the oxidative treatment. To better understand the impact of Me_2SO on reactive oxygen species (ROS) in a native context, we quantitatively assessed the level of ROS in human renal proximal tubule epithelial cells post Me_2SO treatment. Excessive ROS was generated in Me2SO exposed samples compared to the untreated group despite CoA's role to protect the intercellular contents under the exerted oxidative stress (based on the cell-free results). This might be an indication of Me_2SO's cytotoxicity source as the oxidative injury induced by Me_2SO appears to irreversibly damage cellular contents disturbing major oxidative signaling pathways during organ cryopreservation for transplantation purposes.

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S52 SCREENING AND OPTIMIZATION OF CRYOPROTECTIVE AGENTS (CPAS) FOR ELECTROMAGNETIC HEATING OF CRYOPRESERVED BIOMATERIALS

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Rapid and uniform rewarming has been proved essential for the survival of cryopreserved biological samples, inhibiting lethal ice-recrystallization-devitrification and thermal stress-induced fracture (especially in large samples). Electromagnetic (EM) heating is a promising rewarming approach due to its high power and volumetric heating

mechanism. To design and develop such an EM rewarming system, knowledge of the interaction between applied EM field and biological samples is needed. In addition to the basic requirements for CPAs in cryopreservation, such as elimination of ice formation and low toxicity, effective energy conversion and heat dissipation in the biosamples during EM rewarming should be considered, which depend on the selected cryoprotectant agents (CPAs). In this study, methods for screening and optimization of CPAs for EM heating technology are proposed. The dielectric and thermal properties are considered as key factors of the CPAs in the rewarming process, where dielectric properties characterize the absorption of EM power and thermal properties determine the efficiency of heat dissipation. The cavity perturbation method was implemented to measure dielectric constant and dielectric loss. The thermal conductivity was measured with a thermal-needle approach using a microfabricated thermal conductivity sensor. A differential scanning calorimetry (DSC) was used to determine the specific heats of the CPA solutions. These properties of five commonly used vitrification solutions (DPVP, EPVP, PVP, VS55, and VS83) from -120 °C to 0 °C were measured and analyzed. The results indicate that DPVP has the highest dielectric properties and relatively higher thermal properties. Additionally, during the rewarming, the dielectric loss of DPVP increases as temperature rises to about -40 °C, and then drops as the temperature continues to grow. Such an inverted U shape of the dielectric loss trend in the subzero temperature range could reduce the nonuniformity during EM heating, and avoid the so-called "thermal runaway" phenomenon.

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S53 CLINICAL AND POLICY IMPLICATIONS OF ORGAN PRESERVATION

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Transplantation is the best option for most patients with progressive end-stage organ disease, resulting in a significant increase in life expectancy and improved quality of life compared to dialysis. The potential U.S. deceased donor organ supply is estimated to exceed the current number of organs transplanted by 4- to 5-fold. A significant limitation to the number of acceptable organs for transplant is the ischemic injury sustained between recovery and implantation. A method to cryopreserve or "bank" organs prior to transplant would effectively remove the influence of time from the supply chain of organ distribution. This would allow a new paradigm for transplantation that would improve donor/recipient matching, allow for better patient preparation, facilitate tolerance induction protocols, and increase organ utilization while improving graft and patient survival. Many promising technologies are being developed that have the potential to extend preservation times by days to years. These technologies have the potential for significant impact and disruption of the current systems for organ allocation. Here we consider some of the existing and emerging technologies and how they may improve organ allocation and utilization.

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S54 *EX VIVO* AND *IN SITU* OXYGENATED CADAVER ORGAN CARE IN MURINE TEMPERATURE-

CONTROLLED ORGAN-PRESERVING MACHINE AND ECMO

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The lack of strategies capable of prolonging an organ's shelf life has become a bottleneck in transplantation and causes many patients on a waiting list to die. A tolerable ischaemic period for an isolated human heart stored *ex vivo* at 4 °C does not exceed 4-6 hours. Clinically, extracorporeal membrane oxygenation (ECMO) presents a bridge to organ donation after brain and circulatory death as a feasible strategy to increase the donor pool.
For the first time, we present the application of our miniaturized murine organ-preserving machine (OPM) and ECMO to experimentally address cadaver organ preservation.
Non-heart-beating mouse cadavers were subjected to ECMO or oxygenated perfusion whereas isolated hearts from another non-heart-beating group were connected to OPM starting, on average, from 1 h post-mortem. All experiments were performed under normothermia (the perfusion solution and body temperature were kept at 37 °C, respectively).
Calcium-enriched Krebs-Hanseleit solution was used for circuit priming (ECMO), *ex vivo* and *in situ* oxygenated perfusion (in the latter case supplemented with hydroxyethyl starch).
Tissue oxygen tension was real-time monitored using a novel fiber-optic measurement technology.
In an OPM group, despite the prolonged ischemic time, an active heart beating was resumed immediately after primary oxygenated reperfusion. Moreover, following a cold cardioplegia and the second reperfusion in an OPM, stable cardiac activity revival was observed for several hours after explantation.
Similarly, hearts supported both by an ECMO and *in situ* oxygenated perfusion exhibited the heart rate of 150-300 bpm up to 3-hour observation post-mortem. Significantly higher tissue oxygen tension values were documented in animals that underwent oxygenated organ care compared to control mice.
Collectively, our murine temperature-controlled OPM and ECMO represent an indispensable tool for the studying cadaver organ pre-treatment and development of multiorgan preservation protocols. A series of studies on using hypothermia conditions within our murine models is underway.

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S55 CURRENT PERSPECTIVE AND ROLE OF BIOLOGICAL ANTIFREEZE PROTEINS (AFP) IN SUCCESSFUL ORGAN CRYOPRESERVATION

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Progress in the field of tissue as well as organ cryobiology gained popularity with the commercialization of cryopreservation agents like liquid nitrogen, glycerol and other synthetic cryopreservatives. The problems like sub-zero injury after re-crystallization in thawing process ultimately leads to tissue destruction. It also hinders the application of organ banking. With the recent improvements it has made possible to perform transplantation after freezing the tissue and organs. Different studies shows that response of organs vitrification by biological source antifreeze-proteins (AFP) are comparatively safer than the synthetic cryopreserving agents. In current review the different aspects of used AFP and their native peptides in cryopreservation labs has been discussed. The role of AFPs as cryo-protectant for cryopreservation and transplantation of different organs have also been discussed in this review. This article would be also useful comprehension for the scientists working on the role of AFP in tissue cryopreservation all over the world. To promote the clinical application of cryobiology, it is important to know about the process of organ freezing by of biological isolated AFP or their native peptides in organ transplantation after successful cryopreservation and thawing without subzero tissue injuries.

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S56 INCREASED VENOUS PRESSURE: ANOTHER BEAUTIFUL IDEA BITES THE DUST

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In 2020, 21CM reported that kidneys transplanted after vitrification required a urine concentration averaging 96.7% of full M22 concentration to escape signs of ice crystal damage. A follow-up vitrified kidney showed putative inner medullary ice damage 9 days after transplantation despite a urine concentration meeting our previous standard of 94.4% M22. To meet this new standard, a radical new idea was explored: increased venous pressure (IVP). Renal equilibration can be achieved by either perfusion or filtration. Both can be enhanced by higher arterial perfusion pressures, but this is often insufficient. We reasoned that filtration depends on the glomerular capillary pressure, which is increased by arterial inflow but decreased by venous outflow. On that basis, we investigated IVP as a means of increasing glomerular pressure and hence driving filtration and with it, urinary and inner medullary equilibration. To increase venous pressure, we instituted venous cannulation using cannulae of varying lengths. As cannula length increased from 5 to 26 mm, we initially observed a smooth increase in urinary M22 concentration, from about 90% at 5 mm to about 97% of full M22 at 26 mm. Strikingly, in no case did arterial flow require reduction below our preset floor of 0.22 (ml/min)/g owing to increases in arterial pressure, because in no case did arterial pressure reach our upper limit of 80 mmHg. Thus, arterial perfusion

pressure at constant flow depends strongly on renal venous pressure, which was not previously regulated. However, continued investigation of IVP indicated that, overall, it surprisingly provided no net advantage in terms of net urine output or net urine concentration, and renal function was not improved at a given level of equilibration either. IVP shifts arterial flow from vascular perfusion to filtration, but also reduces total perfusion rate, which may negate any advantage. Therefore, new approaches are still desirable.

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S57 SMALL MOLECULE ICE RECRYSTALLIZATION INHIBITORS DRAMATICALLY IMPROVE THE CRYOPRESERVATION OF CELLULAR THERAPY PRODUCTS

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While cell-based therapies are rapidly emerging as a critical aspect of modern health care full deployment of these therapies will entail cryopreservation during manufacture and/or storage. Unfortunately, current cryopreservation protocols are suboptimal. The Ben laboratory has discovered several classes of ice recrystallization inhibitors (IRIs) that control ice growth during cryopreservation and mitigate cellular injury thus increasing the post-thaw viability and functional capacity of Human induced pluripotent stem cells (iPSCs).
Given the current relevance of modelling neurological disease to facilitate the development of new therapeutics for treatment, we have assessed whether aldonamide IRIs are capable of cryopreserving human neurons derived from iPSCs. Cryopreservation of these neurons using 5 mM 2FA (an aryl aldonamide IRI) results in significant increases in post-thaw viability and approximately 2-fold increase in recovery relative to commercially available cryopreservation solutions such as CryoStor 10. In addition, subsequent culturing and analysis using multi-electrode arrays (MEAs) show robust synchronous electrical activity and re-establishment of the neuronal network at day 27 versus day 130 when IRIs are employed. Neurons cryopreserved with 5mM 2FA retained the expression of key neuronal specific/terminally differentiated markers and displayed expected functional neuropharmacological responses following treatment with a panel of neuroactive drugs.
Immunotherapies such as adoptive T-cell therapy are promising strategies to treat various malignancies and consequently we assessed the ability of 2FA to increase post-thaw viability and recovery of T-cell and CAR-T cell products. Our data indicate that 5 mM 2FA increases post-thaw viability and recovery of primary T-cells by more than 2-fold. Similar results were obtained with CD19 CAR-T cells. In vitro targeting and cytotoxic killing assays using EGFR CAR-T and MOCK T-cells indicate that when these cells are cryopreserved with 2FA they are fully functional. These studies illustrate the tremendous potential of the IRI technology to effectively cryopreserve cellular therapy products.

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S58 EXPLORING HIGH CELL CONCENTRATION CRYOPRESERVATION FOR DELIVERY OF ENGINEERED T CELL PRODUCTS

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Currently, many commercially available CAR-T cell therapy products in the US are cryopreserved at cell concentrations of ≤ 70 x 10^6 cells/mL and often favour lower concentration ranges. Next generation engineered T cell products that require significantly higher cell dosages would result in an increased product volume if current cell concentration targets were maintained. Possible downstream impacts of this strategy include the use of larger product containers, additional dosing containers per patient, a larger LN_2 freezer footprint, changes to shipping configurations, longer product infusion time, and increased cryopreservative dosage delivered to patients. Increased cryopreserved cell concentration was evaluated as a potential solution to reduce the total volume required for high dose products. Engineered T cell formulations with a range of 70 x 10^6/mL to 230 x 10^6/mL viable cells, prepared to a final Me_2SO concentration of 7.5 % were assessed for cell health characteristics after freezing and thawing in a small-scale model. Samples were aliquoted into cryovials that underwent slow controlled rate freezing and subsequent LN_2 storage for a minimum of 7 days. Frozen samples were thawed and tested for cell health indicators. No notable differences were detected in cell viability or cytokine secretion between cell concentrations. A slight increase in apoptosis marker expression was correlated with increasing cell concentration. Cryopreservation with T cell concentrations up to 230 x 10^6 cells/mL shows potential to mitigate downstream impact including total product formulation volume, storage and shipping complexity, and changes to clinical administration strategies for high dose products.

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S59 DROPLET VITRIFICATION OF GRANULOCYTES: A PROOF-OF-CONCEPT STUDY

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Granulocyte transfusion therapy is an effective method of restoring innate immunity in neutropenic patients; however, the 24-h shelf-life of granulocytes imposes logistic limitations on access to this therapy. Efforts to cryopreserve granulocytes using traditional freezing regimens have been largely unsuccessful as

granulocytes are uncharacteristically sensitive to ice-induced damage. Thus, we aimed to assess whether a droplet vitrification approach could be leveraged as a strategy to preserve this cell type at low temperatures in a high-throughput manner. Granulocytes isolated from buffy coats were incubated in a "low-CPA solution" for 5 min, transferred to a syringe, and then briefly combined with a "high-CPA solution" in a 0.5 mm mixing needle at a flow rate of 1 mL/min, and vitrified on a copper plate. Two final solution concentrations were tested: 2 M Me_2SO/2 M EG/30% sucrose and 2.5 M Me_2SO/2.5 M EG/30% sucrose. For each solution, we evaluate two separate low- and high-CPA solution combinations, one of which would minimize toxicity during the initial 5 min incubation, and the other minimizing osmotic damage during exposure to the "high-CPA solution". Membrane integrity was evaluated using SYTO13 and GelRed staining. Combinations that favoured higher initial toxicity and minimized osmotic damage performed significantly better for the two tested solutions. The 2.5 M Me_2SO/2.5 M EG/30% sucrose solution did not promote any additional damage following CPA exposure relative to the 2 M Me_2SO/2 M EG/30% sucrose solution. However, it did result in significantly higher membrane integrity. The most optimal solution tested (low-CPA: 2 M Me_2SO/2 M EG/20% sucrose; high-CPA: 3 M Me_2SO/3 M EG/40% sucrose; final: 2.5 M Me_2SO/2.5 M EG/30% sucrose) resulted in post-vitrification membrane integrity of 69.5% ± 0.3 % (post-isolation viability: 82.3% ± 0.5%). This initial effort to apply droplet vitrification techniques to granulocyte preservation has yielded promising results that warrant further pursuit of this approach.

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S60 CRYOPRESERVATION TECHNIQUES IN MANUFACTURING, TRANSPORT AND STORAGE OF CAR-T THERAPY PRODUCTS – INITIAL EXPERIENCE AND PROPOSALS FOR FUTURE IMPROVEMENTS

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Several clinical trials have proved the efficacy and safety of genetically modified autologous advanced therapy medicinal products (CAR-T cells) in treatment of malignant lymphoma and the first products were registered in the European Union in 2018. Direct shipment of the CAR-T product to the clinical department is feasible, nevertheless, the ability to store

cryopreserved product gives significant advantages in better planning of clinical application. Some manufacturers prefer cryopreservation of the starting material, concentrate of peripheral blood mononuclear cells (PBMC) at the collection site. The authors describe the cold chain used by the TE CZ000426 for cryopreservation of starting material and storage of final CAR-T product cryopreserved by the manufacturer. The respective cryopreservation protocol used for starting material is based on a combination of Me_2SO with hydroxyethyl starch supplemented by human serum albumin and slow controlled cooling rate in cryobags housed in metal cassettes which achieves the PBMC post-thaw viability of 98.8, SD 0.5 % and MNC recovery of 72.8, SD 10.2 %. The final product is stored in a „dry-storage“ liquid nitrogen container (dry shipper) used exclusively for CAR-T products. The storage high temperature alarm is adjusted to -160 °C. Transport to the clinical department is performed in the dry shipper at a temperature below -150 °C. After product removal from the metal cassette it is inserted into a sterile plastic bag, thawed in a 37 °C water bath and immediately infused. This cold chain fully meets manufacturer´s requirements and has been used till now in two patients. Nevertheless, we have identified at least two weak points in the technology: 1) cryopreservation protocols are based on use of Me_2SO even though delayed onset cell death can be induced and 2) single bagging of the final product can result in leakage and contamination in the event of inappropriate handling.

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S61 CRYOPRESERVATION OF MESENCHYMAL STEM CELL-BASED 3D CONSTRUCTS

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The use of stem cell-based 3D in vitro models becomes a mainstream for biological researches and drug screening that highly increases a demand in their steady supply. Large size and complexity of 3D constructs intricate approaches used for their long term preservation and establishment of cryobanks compared to isolated cells in suspension. The present study aimed to evaluate the effect of slow freezing on mesenchymal stem cells viability and metabolic activity in spheroids and scaffolds. Human mesenchymal stem cells (MSCs) were used for spheroid formation using hanging drop method and seeded into collagen scaffolds prepared by cryotropic gelation technique. The 3D constructs were cryopreserved using a conventional slow-freezing protocol with 10% Me_2SO with or without additional sucrose based pretreatment procedure. Cell morphology and viability within constructs were assessed by fluorescein diacetate and propidium iodide staining. The metabolic activity of MSCs was estimated by Alamar blue test. The seeding concentration of MSCs and the culture period affected the efficiency of spheroid formation. Initial concentration of 5 thousand cells per drop and 3 days of culture were chosen as optimal ones after preliminary screening. MSCs cultured in spheroids had higher proliferative activity and colony forming ability compared to

cells in monolayer. Collagen scaffolds supported MSCs attachment, proliferation and induced differentiation into mesenchymal lineages. Cryopreservation of spheroids using standard slow freezing approach resulted in partial loss of their integrity and decrease in cell viability to 60%. MSCs in scaffolds were more sensitive to cryodamage, the viability rate immediately after thawing did not exceed 50%. Sucrose pretreatment improved cell survival and renewal of metabolic activity in both scaffolds and spheroids by 10-15%. To sum up, cryopreservation of stem cells-based 3D constructs using standard protocols remains challenging and should be optimized. Sucrose pretreatment can serve as a feasible approach to improve the cryopreservation outcome.

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S62 EFFECT OF COOLING RATE ON THE INCIDENCE OF APOPTOSIS IN CRYOPRESERVED T CELLS

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Understanding whether post-thaw cell loss is caused by necrosis or apoptosis can inform strategies for improving the quality of cryopreserved cell therapy products. While the effect of cooling rate on post-thaw necrosis is well documented, past studies on apoptosis in frozen-thawed cells have been limited to relatively low rates of cooling. Thus, we have investigated the mechanism of cell death after cooling at rates ranging from 0.2°C/min to 50°C/min, using Jurkat cells as a T lymphocyte model. Cryovials of cells in 5% Me_2SO were seeded at -6°C, then cooled at a constant rate to -80°C before plunging into liquid nitrogen. After thawing and cryoprotectant dilution, cells were stained with Annexin-PE and RedDot 2 for detection of necrosis and apoptosis by flow cytometry. As expected, intermediate cooling rates (0.5-4.0°C/min) yielded optimal viability (with means in the range 88-93%), while viability was reduced (to 30-62%) at suboptimal and supraoptimal cooling rates. Consistent with these results, the incidence of necrosis was low (4-6%) after freezing in the optimal cooling rate regime, but significantly higher (14-22%) at suboptimal and supraoptimal cooling rates (p<0.05). However, when compared to cooling rates in the optimal regime (which yielded 3-6% apoptosis), the incidence of apoptosis was significantly elevated (p<0.05) not only at suboptimal rates of cooling (22.8±5.2% apoptosis at 0.2°C/min), but also at supraoptimal cooling rates (23.7±3.8% and 48.1±3.6% apoptosis at 15°C/min and 50°C/min, respectively). To test whether apoptosis was associated with mitochondrial damage, disruption of mitochondrial membrane potential (MMP) was assayed using JC-1 staining in cells that had been frozen at 0.2°C/min, 1°C/min, and 50°C/min, revealing a correlation between MMP disruption and apoptosis (R^2=0.97). Our results suggest that rapid-cooling cryoinjury, which is typically associated with intracellular ice formation and loss of membrane integrity, may also result in cell death by causing apoptosis triggered by mitochondrial damage.

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S63 ESTABLISHING AN OPTIMISED PROTOCOL FOR CRYOPRESERVATION OF HEPATOCYTE MICROBEADS FOR CLINICAL USE

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Acute liver failure (ALF) carries a high mortality, especially for neonates and young children, mainly because of the scarcity of timely suitable donor organs. At King's College Hospital, human hepatocyte transplantation have been used as an alternative technique to liver transplantation to treat children with ALF, where human hepatocytes isolated from unused or rejected donor livers (fresh or cryopreserved) become encapsulated in immunologically inert alginate forming hepatocyte microbeads (HMBs). The HMBs are transplanted intraperitoneally, where the encapsulated-hepatocytes support the failing liver by providing the missing liver- specific functions. King's College Hospital established an optimised protocol for the production of clinical grade hepatocyte microbeads. More recently, Dhawan and colleagues (2020) reported safety and feasibility of intraperitoneal transplantation of clinical grade HMBs in children with ALF without the need for immunosuppression, where some patients recovered without the need for liver transplant. Alginate encapsulation allows nutrients, and oxygen diffuse in, and vital proteins and waste products diffuse out. The alginate coating does not allow antibodies, complement or immune cells to enter the microbeads, thus protecting the liver cells from the host immune response without the need for immunosuppression.

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S64 24-HOUR SUPERCOOLING PRESERVATION OF VASCULARIZED COMPOSITE ALLOGRAFTS IN RATS: A PROOF-OF-CONCEPT STUDY

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Vascularized composite allotransplantation (VCA) remains the only alternative for patients with severe disfigurements nonaddressable by conventional reconstructive surgery. However, ischemia time is a major limiting factor considering irreversible muscular injury occurs after 6 hours of static cold storage (SCS). In this study, we aimed to optimize an extended subzero non-freezing preservation protocol for rodent VCAs.
Ten partial hindlimbs were procured from Lewis rats and perfused with subnormothermic machine perfusion (SNMP) with a modified Steen solution mixed with either 300 mM (*n=5*) or 100 mM (*n=5*) of 3-O'Methylglucose (3-OMG). Limbs were then cooled down to +4°C, flushed with and submerged in a preservation solution (HTK + Trehalose 50mM + Glycerol 5% + PEG 5%). VCAs were supercooled for 24 hours at -4°C, then rewarmed and recovered for 2 hours using SNMP. As controls, five limbs were cold stored at +4°C for 24 hours and subjected to 2-hr SNMP recovery. Perfusion parameters, electrolytes and blood gas were monitored. Muscle biopsies were collected for histological analysis.

Both 300 and 100 mM groups displayed similar perfusion parameters at the end of loading SNMP. All limbs were successfully supercooled for 24 hours without ice nucleation. During recovery SNMP, the supercooling groups showed higher glucose consumption and resistances decreased faster compared to controls. Lactate release overtime was lower in the 100 3-OMG group, and potassium levels drastically decreased in supercooled limbs while it remained high in the control group. The 300 mM group showed more edema than 100 mM and SCS groups after recovery (+34.59% ±21.67, +24.76% ±10.81, and +17.80% ±8.2, respectively). Histology showed limited cell injury after recovery in all groups, but notable interstitial edema in both experimental groups.
This study is a proof-of-concept that supercooling preservation of VCA is feasible in a rodent model with a three-phase protocol including CPA loading, subzero storage in a preservation solution and limb recovery with SNMP.

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S65 TOOLS FOR HEART PRESERVATION RESEARCH

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Transplantation is the standard of care for end-stage organ failure as it increases quality of life and life expectancy of patients with chronic end-stage diseases. Although it is a successful treatment, its impact has been significantly hindered by a very rapid rise in organ demand with relatively stable supply, resulting in over 100,000 people on the transplant waiting list in the United States alone. The heart, in particular, remains one of the most challenging organs to allocate for transplantation due to rapid graft degradation that leads to short ex vivo graft viability (4-6 hours). Partial freezing, a new method of high-subzero storage inspired by freeze-tolerant animals in nature, aims to achieve longer preservation times (up to weeks) by coupling cold storage temperatures (-10°C) with cellular dehydration to reduce organ metabolic rate. This new method combines knowledge into cryoprotective agents (CPAs) with advances in machine perfusion to yield promising results in whole organ preservation. However, similar to most

cryopreservation protocols, partial freezing protocols are very complex as they include a wide array of protocol settings that need to be tailored to meet the specific needs of each individual organ. Therefore, the design of whole organ preservation protocols, is a highly complex problem that would greatly benefit from screening of thousands of combinations of candidate CPAs, supplements, and protocol parameters. However, the research tools to efficiently and effectively screen through thousands of possible compound combinations are currently lacking. We present a novel model organism for organ preservation and transplantation research capable of providing the desired balance between translation potential and high throughput experimentation, as well as significant advancements in methodologies for heart handling and preservation.

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S66 BIOLOGICAL CHARACTERIZATION OF VITRIFIED PANCREATIC ISLETS AND TRANSPLANTATION

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Pancreatic islet transplantation can cure diabetes but requires accessible, high-quality islets in sufficient quantities. Cryopreservation could solve islet supply chain challenges by enabling quality-controlled banking and pooling of donor islets. Unfortunately, cryopreservation has not succeeded in this objective, as it must simultaneously provide high recovery, viability, function and scalability. Here, we achieve this goal in mouse, porcine, human and human stem cell (SC)-derived beta cell (SC-beta) islets by comprehensive optimization of cryoprotectant agent (CPA) composition, CPA loading and unloading conditions and methods for vitrification and rewarming (VR). Post-VR islet viability, relative to control, was 90.5% for mouse, 92.1% for SC-beta, 87.2% for porcine and 87.4% for human islets, and it remained unchanged for at least 9 months of cryogenic storage. VR islets had normal macroscopic, microscopic, and ultrastructural morphology. Mitochondrial membrane potential and adenosine triphosphate (ATP) levels were slightly reduced, but all other measures of cellular respiration, including oxygen consumption rate (OCR) to produce ATP, were unchanged. VR islets had normal glucose-stimulated insulin secretion (GSIS) function in vitro and in vivo. Porcine and SC-beta islets made insulin in xenotransplant models, and mouse islets tested in a marginal mass syngeneic transplant model cured diabetes in 92% of recipients within 24–48 h after transplant. Excellent glycemic control was seen for 150 days. Finally, our approach processed 2,500 islets with >95% islets recovery at >89% post-thaw viability and can readily be scaled up for higher throughput. These results suggest that cryopreservation can now be used to supply needed islets for improved transplantation outcomes that cure diabetes.

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S67 VITRIFICATION AND NANOWARMING OF KIDNEYS

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Vitrification, as a promising long-term preservation approach, can increase the storage time to years. Unfortunately, vitrified systems of large tissues and organs cannot currently be rewarmed sufficiently rapidly or uniformly by convective approaches to avoid ice crystallization or cracking failures. We used the volumetric rewarming technology entitled "nanowarming" to address this problem by using radiofrequency excited iron oxide nanoparticles to rewarm vitrified systems rapidly and uniformly. Here, for the first time, successful recovery of a rat kidney from the vitrified state using nanowarming, is shown. First, kidneys are perfused via the renal artery with CPA and silica-coated iron oxide nanoparticles (sIONPs). After cooling at −40 °C min−1 in a controlled rate freezer, microcomputed tomography (μCT) imaging is used to verify the distribution of the sIONPs and the vitrified state of the kidneys. By applying a radiofrequency field to excite the distributed sIONPs, the vitrified kidneys are nanowarmed at a mean rate of 63.7 °C/min. Experiments and modeling show the avoidance of both ice crystallization and cracking during these processes. Histology and confocal imaging show that nanowarmed kidneys are dramatically better than convective rewarming controls. This work suggests that kidney nanowarming holds tremendous promise for transplantation.

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S68 VITRIFICATION AND REWARMING OF MAGNETIC NANOPARTICLE-LOADED RAT HEARTS

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Heart transplantation is lifesaving treatment of choice for patients with end-stage heart failure. However, the scarcity of donor organ is the biggest impediment due to shorter ischemic time of the heart (4-6 hours). Therefore, developing an advanced storage method to extend the current static cold storage time (<6 hours) is crucial. We propose heart cryopreservation by vitrification-cryogenic storage in a glass-like state. In order to vitrify successfully, we perfused the rat heart with cryoprotective agent (CPA) that inhibits ice during cooling. We developed "nanowarming" wherein and silica coated iron oxide nanoparticles (sIONPs) are loaded throughout the organ vasculature, and radiofrequency (RF) coil is used to rewarm the organ fast and uniformly. We optimized cannulation methods to load CPA and sIONPs into the heart and studied distribution of CPA and sIONPs via μCT imaging and ICP-OES. The CPA and sIONP loaded hearts were then vitrified in a control-rate freezer and were rewarmed by either convective warming or nanowarming. Both experimental and modelling data showed the convective warming causes larger temperature difference compared to the temperature difference in nanowarmed hearts. The huge thermal stress resulted cracks in the convectively warmed hearts. Further, nanowarmed hearts were shown to be largely equivalent in tissue integrity and morphology to sIONP and CPA loaded and unloaded hearts: they retained some electrical activity and were clearly superior to convective controls. This study demonstrates that a whole rat heart can be physically vitrified and rewarmed and that biological outcomes can be expected to improve by reducing or eliminating CPA toxicity during loading and unloading.

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S69 ADVANCING ORGAN CRYOPRESERVATION THROUGH SCALABLE POLYMER COATING OF IRON OXIDE NANOPARTICLES

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Using radiofrequency excited iron oxide nanoparticles (IONPs) dispersed in cryoprotective agents (CPAs), we have successfully rewarmed vitrified, cryopreserved (-150oC) biological samples. Previous IONPs, our silica coated sIONP, have limited Fe saturation concentration in CPA. We present a small molecule phosphonate linker (PLink) IONP coating method that is CPA stable, improves saturation concentration, and inexpensive for scale-up (> 1g).
PLink contains a phosphonate "anchor" for high irreversible IONP core binding and a carboxyl "handle" for ligand attachment. PLink displaces initial coatings on commercial IONPs (Ferrotec Inc.): EMG-1200 (hydrophobic) and EMG-308 (hydrophilic) and allows attachment of polymers of interest, through a simple ligand exchange. PLink-polyethylene glycol (PEG) increased colloidal stability, decreasing aggregation, in water and CPAs from minutes (uncoated) to up to 14 days. Heating properties of EMG-1200 in water,

measured at 360 kHz and 20 kA/m, increased from 20 to 150 W/g Fe by increasing PLink-PEG5000 as aggregation decreased. Additionally, PLink-PEG did not decrease the 400 W/g Fe heating of water stable EMG308, indicating PLink preserves the core. Further, we successfully nanowarmed cryopreserved HDF cells in VS55 (common CPA) using both 308-PEG5000 and 1200-PEG5000, viability comparable to fresh. The concentrations of IONP in VS55 reached 25 mg Fe/mL of 308-PEG5000 and 60 mg Fe/mL of 1200-PEG5000, which is above our previous sIONP at 10 mg Fe/mL. The heating rate reached 200 °C/min, 2.5 times faster than our sIONPs tests. PLink coated IONPs have been scaled to over 10 g synthesis and used to nanowarm rat kidneys at and above these rates.
The PLink coating allows for facile, inexpensive, and scalable synthesis of PEG-functionalized IONPs for, as needed for human scale organ cryopreservation. In future experiments, PLink IONPs will be tested at higher Fe concentration in various CPAs, maximizing the heating rates with EMG308 IONPs and translating nanowarming to transplantation.

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S70 COMPUTATIONAL MODELLING AND OPTIMISATION OF SLOW COOLING PROFILES FOR THE CRYOPRESERVATION OF CELLS IN SUSPENSION.

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The cryopreservation of biological materials is a highly complex process, as it involves numerous factors such as the cooling and thawing procedures, the administration of cryoprotective agents (CPAs), as well as the type and composition of cells. While theoretical work has yielded a better understanding of the processes occurring during cryopreservation, the design of cryopreservation protocols and their parameters is currently based on heuristic optimization. This means that for each individual cell extensive trial and error wet lab work is required to optimise each combination of factors for experimental protocols.
Thus, we have developed the software package CryoDynaMo, an extension of the BioDynaMo platform. BioDynaMo allows for the modelling of single cells and tissue via creating a base cell object which contains the general mechanics for each cell, such as inter-cellular forces and osmotic pressure, chemical diffusion, and intercellular forces. BioDynaMo is used to simulate various biological systems, such as developing neural tissue and cancer dynamics. CryoDynaMo builds on this to model the cryopreservation process for cells in suspension via modelling cellular membrane and osmotic properties, statistics for intracellular and extracellular ice formation, and finally heat transfer in 3D space.
Based on our computational approach, we present post-thaw survival predictions for three cell types: (1) Jurkat T lymphocyte cells, (2) Yeast cells and (3) Human induced pluripotent stem cells. We asses these computational predictions with data

from the literature, and additionally compare with novel experimental results.

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S71 SUPERCOOLED BIOPRESERVATION PROTOCOL DESIGN USING PROBABILISTIC SAFETY ANALYSIS

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Aqueous supercooling has shown tremendous promise as a method for low temperature biopreservation, but the omnipresent risk of ice formation has largely hindered its translation outside the laboratory. In this work, we present a framework for the safety-informed design of preservation protocols that utilizes nucleation rate data to predict the probability for nucleation as a function of temperature and time. The analysis leverages the fact that, though random, nucleation kinetics can be described by Poisson statistics. Furthermore, acknowledging that system-to-system variability poses a significant challenge for the clinical translatability of supercooling technology, the analysis framework rigorously incorporates experimental uncertainty and variability into the safety predictions. Finally, we demonstrate how this temperature-dependent probabilistic safety analysis can then be married temperature-dependent biophysical relations to quantitatively incorporate biological aspects of biopreservation into the protocol design and optimization pipeline. In total, this work provides a method by which to engineer supercooled preservation protocols for safety and efficacy in the face of both kinetic and conditional uncertainty.

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S72 hMSCS IN CONTACT WITH ME_2SO FOR CRYOPRESERVATION: EXPERIMENTS AND MODELLING OF OSMOTIC INJURY AND CYTOTOXIC EFFECT

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In this work a combined analysis of osmotic injury and cytotoxic effect useful for the optimization of the cryopreservation process of a cell suspension is carried out. The case of human Mesenchymal Stem Cells from Umbilical Cord Blood in contact with Me_2SO acting as CPA is investigated from the experimental as well as the theoretical perspective. The experimental runs are conducted by suspending the cells in hypertonic solutions of Me_2SO at varying osmolality, system temperature and contact times; then, at

room temperature, cells are pelleted by centrifugation and suspended back to isotonic conditions. Eventually cell count and viability are measured by means of a Coulter counter and flow-cytometer, respectively. Overall, a decrease of cell count and viability results when Me_2SO concentration, temperature and contact time increase.
A novel model is developed and proposed to interpret measurements by dividing the cell population between viable and non-viable cells. The decrease of cell count is ascribed exclusively to the osmotic injury caused by expansion lysis: excessive swelling causes the burst of both viable as well as non-viable cells. On the other hand, the reduction of cell viability is ascribed only to cytotoxicity which gradually transforms viable cells into non-viable ones. The use of the Surface Area Regulation model recently proposed by the authors allows one to avoid the setting in advance of fixed cell Osmotic Tolerance Limits, as traditionally done in cryopreservation literature to circumvent the mathematical simulation of osmotic injury. Comparisons between experimental data and theoretical simulations are provided: first, a non-linear regression analysis is performed to evaluate unknown model parameters through a best-fitting procedure carried out in a sequential fashion; then, the proposed model is validated by full predictions of system behavior measured at operating conditions different from those used during the best-fit procedure.

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S73 OBTAINING TEMPERATURE DEPENDENCE OF CELL MEMBRANE PERMEABILITY PARAMETERS USING NON-IDEAL THERMODYNAMIC ASSUMPTIONS TO MATHEMATICALLY MODEL REAL CRYOPRESERVATION PROTOCOLS

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One of the most important applications of cryobiology is preserving biological matter at very low temperatures (e.g., −196 °C) for long-term storage. Cells and tissues are far from their natural physiological environment at very low temperatures, bringing us many challenges. Mathematical modeling of the cryopreservation process using thermodynamic equations is a beneficial tool for investigating the temperature dependence of cell membrane permeability to water and CPA in order to optimize this process. Because cell membrane permeability to water and CPA can directly affect cell volume via shrinking or swelling, permeability parameters can be obtained by modeling the kinetic cell volume during the cryopreservation process. Previously in this group, cell-specific permeability parameters consistent with non-ideal solution thermodynamics, namely, the permeability of the cell membrane to water (L_P^*), the permeability of the cell membrane to CPA (P_S^*), and the native intracellular volume fraction that cannot leave the cells (b^*), were obtained at room temperature. In this work, we expand the measurement technique to 0 °C to obtain the temperature dependence of the non-ideal permeability parameters for several cell types. The Arrhenius equation is used to theoretically model the temperature dependence of permeability parameters with the room temperature parameters as the reference

points. The experimental data is obtained by subjecting cell suspensions to various solutions with and without a permeating CPA at two different temperatures and recording their kinetic and equilibrium volume separately using a Coulter Counter. The temperature-dependent permeability parameters are ultimately obtained by fitting the experimental data to the theoretical kinetic and equilibrium models and Arrhenius equation using 'MATLAB' programming and a new iterative fitting method. The results of this work will be an excellent assistance for cryobiologists in optimizing the cooling rate, the amount and type of CPA, and how it is added or removed.

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S74 AN AGENT BASED MODEL OF INTRACELLULAR ICE FORMATION ANDPROPAGATION IN SMALL TISSUES

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Successful cryopreservation of tissues and organs would be a critical tool to accelerate drug discovery and facilitate myriad life saving and quality of life improving medical interventions. Unfortunately, success in tissue cryopreservation is quite limited, and there have been no reports of successful organ cryopreservation. One principal challenge of tissue and organ cryopreservation is the propagation of damaging intracellular ice. Understanding the probability that cells in tissues form ice under a given cryopreservation protocol would greatly accelerate protocol design, enabling rational model-based decisions of all aspects of the cryopreservation procedure. Established models of intracellular ice formation (IIF) in individual cells have previously been extended to small linear (one-cell-wide) arrays to establish the theory of intercellular ice propagation in tissues. However, these small-scale lattice-based tissue ice propagation models have not been extended to more realistic tissue structures, and do not account for intercellular forces that arise from the expansion water into ice that may cause mechanical disruption of tissue structures during freezing. To address these shortcomings, here we present the development and validation of a lattice-free agent-based stochastic model of ice formation and propagation in small tissues. We validate our Monte Carlo model against Markov chain models in the linear two-cell and four-cell arrays presented in the literature, as well as against new Markov chain results for 2 × 2 arrays. Moreover, we expand the existing model to account for the solidification of water into ice in cells. We then use literature data to inform a model of ice propagation in hepatocyte disks, spheroids, and tissue slabs. Our model aligns well with previously reported experiments, and demonstrates that the mechanical effects of individual cells freezing can be captured.

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S75 WITHDRAWN

S76 ELECTROMAGNETIC REWARMING FOR CRYOPRESERVATION: A

NUMERICAL COMPARISON BETWEEN MULTI-MODE AND SINGLE-MODE ELECTROMAGNETIC CAVITY

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With the increased demand for long-term preservation of large biospecimen, rapid and uniform rewarming is critical to avoid devitrification, intercellular ice recrystallization, and thermal stress-induced fracture during the rewarming process. By taking advantage of the volumetric heating mechanism of electromagnetic (EM) waves, heating in the EM cavities present a promising solution to the rapid and uniform rewarming for the large volume of biomaterials. In this study, a numerical simulation model was developed to investigate the heating performance for both single-mode and multi-mode cavities under the same dimension and power feeding conditions. The model was first validated by comparing the rewarming rates and temperature profiles between experimental and simulation results in a single-mode resonance cavity. The validated model was further used for the following studies: 1) the electrical and magnetic field distribution in both cavities without biosamples; 2) the electrical and magnetic field distribution in both cavities loaded with biosamples; 3) the rewarming rates in the 25 mL loaded cell suspension; 4) the temperature gradient in the samples during rewarming; 5) the energy conversion efficiency and power utilization rate for both cavities. The results provide guidance for cavity design, mode selection, and further optimization, which demonstrated the single-mode electromagnetic resonance cavity might result in rapid and uniform rewarming for large biosamples with much simpler control mechanisms.

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S77 A MATERIALS SCIENCE VIEW OF CRYOPRESERVATION VIA GLASS FORMATION

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Cryopreservation requires knowledge of the ternary water-salt-cryoprotective agent (CPA) equilibrium phase diagram. For a given composition, the liquidus temperature depression and the level of microsegregation of salt and CPA in the extracellular liquid as a result of extracellular ice formation can be followed. For cells to survive low temperatures, intracellular ice formation must be avoided. Confronted by high salt and CPA concentrations in the extracellular liquid, the cells perfuse water out of their intracellular liquid. The CPA enriched and salt enriched intracellular liquid avoids intracellular ice better and eventually can form a glass. The kinetics of ice crystallization via nucleation and growth are represented by a Time-Temperature-Transformation plot. For a given composition, such a plot shows the cooling and rewarming protocols to avoid ice. It also shows the best hold temperature to intentionally form ice, as is the case for cryosurgery. Heat transfer during solidification of a liquid in a mold is described by the Nusselt Number. Metallic glasses with high Nusselt Numbers are formed at cooling rates of ten million C/s. Cell suspensions have low Nusselt Numbers and can only experience cooling rates of 1,000 C/s, not fast enough to avoid

extracellular ice at nontoxic CPA levels. For individual cells to experience high solute extracellular liquid, those cells must not be encapsulated by the growing ice dendrites. Porcine spermatozoa have been shown to be encapsulated in ice during plane front freezing. Other processing routes can yield glasses in systems that are not easy glass formers. Glassy pure water can be produced by vapor deposition on a very cold substrate. Metallic glasses can be formed by extensive comminution of crystalline powders.

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S78 VITRIFICATION PROCESS CONTROL BY DSC

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Cryopreservation by the vitrification method is based on the prevention of water crystallization and the formation of a glassy state due to treatment with concentrated cryoprotective agents. Both thermal processes, water crystallization / melting and the glass transition can be identified by differential scanning calorimetry (DSC). The optimal composition of the cryoprotectant and the treatment time can be set by measuring the thermal characteristics of the cryopreserved material. DSC measurements can reveal the critical cooling / heating rates or evaluate whether the selected cooling / heating rate can guarantee proper vitrification without harmful ice formation. Diluted plant vitrification solutions PVS2 and PVS3 and their components were measured with TA2920 and Q2000 differential scanning calorimeters (TA Instruments, USA) to identify the safe concentration of the cryoprotectants for their vitrification. The lowest solute concentration suitable for its vitrification started at 0.5 g in 1 g of solution, but the optimal solute concentration approached 0.7 g in 1 g of solution. The "stable" vitrification concentration appeared to be close to 0.8 g in 1 g of solution, when the glass transition temperature corresponded to the Tg′, which characterized to the maximally freeze concentrated solution. Conversely, the presence of Tg′ together with the freezing / thawing peak in DSC measurements always reveals ice damage to the sample using the vitrification method. The optimal cryoprotectant composition can be designed to prevent ice crystallization or cryoprotectant toxicity. The thermal characteristics of selected cryoprotectants and explants subjected to the vitrification method measured by DSC will be demonstrated.

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S79 ASSESSMENT OF VITRIFICATION STATE OF SUPERFLASH FROZEN DROPLETS BASED ON FLUORESCENCE SELF-QUENCHING

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In general, at least one cryoprotectant agent (CPA) is necessary for all the cryopreservation methods to suppress the

generation of the ice crystals and achieve vitrification. We previously reported a CPA-free cryopreservation method based on inkjet technology (Y. Akiyama et al., PNAS (2019) 116, 7738). In the method, the cells are inkjet printed as tiny droplets on a glass substrate cooled with liquid nitrogen. The droplets were vitrified instantaneously over the critical cooling rate known as the minimum cooling rate for vitrification. The study also confirmed that the droplets were vitrified based on a peak shift of OH vibration by microscopic Raman spectroscopy and flashing of the droplet surface by an ultrahigh-speed camera. However, the evaluations require special measurement equipment and expert operations.

In this study, we propose a simple vitrification assessment method based on fluorescence self-quenching. Fluorescence self-quenching occurs by increasing the fluorescence concentration including enrichment by crystallization. Meanwhile, the fluorescence intensity in the vitrified droplets does not decrease as vitrification can suppress self-quenching. So, we defined the degree of vitrification as a ratio of fluorescence intensity relative to a completely vitrified droplet by adding enough amount of CPA. Since the cooling rate increases by reducing the size of the droplet, we analyzed the fluorescence intensity in various sizes of droplets. In the case of a 70 pL droplet, the cooling rate was over the critical cooling rate and the cell viability was as high as the conventional method with CPA, which strongly suggests the droplet was vitrified. As expected, the fluorescent intensity ratio for a 70 pL droplet was significantly high compared to large droplets 200 pL and 450 pL. The result shows that our proposed method has a high potential as a simple and easy method to evaluate the vitrification state in tiny droplets.

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S80 VITRIFICATION OF BIO-ENGINEERED EPITHELIAL CONSTRUCTS

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The demand for human bioengineered tissue constructs is growing in response to the world-wide movement away from the use of animals for testing of new chemicals, drug screening and house hold products. Presently constructs are manufactured and delivered just in time resulting in delays and high costs of manufacturing. Cryopreservation and banking would speed up delivery times and permit cost reduction due to larger scale manufacturing. Our objective in these studies was development of ice-free vitrification formulations and protocols using human bioengineered epithelial constructs that could be scaled up from individual constructs to 24 well plates. Constructs were vitrified using solutions with either 55% (VS55) or 70% (VS70) cryoprotectants then stored at <-135°C for 7 days. After rewarming, construct viability was assessed using alamarBlue and MTT to measure metabolic activity and IL-1α to evaluate the inflammatory response. Initial experiments using single EpiDerm constructs in vials demonstrated viability >80% of untreated control; significantly higher than our best freezing strategy. Further studies transitioned from using glass vials with one construct to deep well plates holding up to

24 individual constructs. Construct viability was maintained at >80% post warming viability and >70% viability on days 1-3 in vitro. Similar viability was demonstrated for other related tissue constructs with demonstration of maintenance of viability after 2-7 months of storage below -135°C.

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S81 VITRIFICATION OF TISSUE ENGINEERED CONSTRUCTS BASED ON MESENCHYMAL STROMAL CELLS

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Tissue engineered constructs (TECs) with mesenchymal stromal cells (MSCs) may be applied in biomedical research, drug development, regenerative medicine. Long-term storage of ready-to-use TECs is essential for their implementation in practice. Conventional cryopreservation protocols accompanied with ice crystals formation may lead to cell death and carrier destruction. Vitrification is achieved without ice formation and considered as an encouraging approach for 3D construct cryopreservation.
Here we aimed to develop the composition of vitrification solution and steps of vitrification procedure to prevent the development of crystallization, vitreous phase cracking and preserve cell viability for two types of TECs: alginate microspheres (AMS), and macroporous scaffolds based ones.
Compositions of dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), 1.2-propanediol (1.2-PD), sucrose were used to design vitrification solution. Polyvinyl alcohol (PVA), polyethylene glycols (PEG-400, PEG-8000) were used as ice blockers. Glass-forming properties were studied by differential scanning calorimetry (DSC). Human dermal MSCs (derived after adult donors' informed consent) in suspension, entrapped in AMS, and seeded into alginate-gelatin macroporous scaffolds were used. Viability (trypan blue, FDA/ethidium bromide dual staining), metabolic activity (Alamar Blue assay, MTT-test), adhesion and differential potential were assessed both after exposure with solutions and vitrification procedure.
Solution comprised 10 % Me_2SO, 20 % EG, 20 % 1.2-PD and 0.5 M sucrose prevented ice formation during rapid cooling and rewarming in cryovials. Vitrification in this solution maintained the viability, metabolic activity and capacity to multilineage differentiation in MSCs within alginate microspheres. This solution did not suppress the ice formation during cooling of the scaffolds. Solution modification via increasing the concentration of Me_2SO to 15% and adding 1% PVA inhibited crystallization in scaffolds. Two-step cooling and rewarming modes were developed based on DSC data to prevent vitreous phase cracking. The modified vitrification protocol provided high viability indexes of MSCs in suspension, AMS, but should be further improved for MSCs in macroporous scaffolds.

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S82 EXPERIMENTAL PROOF OF ICE RECRYSTALLIZATION INHIBITION ON THE C. ELEGANS

MODEL BASED ON A SCALABLE WARMING APPROACH BY MEANS OF HIGH INTENSITY FOCUSED ULTRASOUND

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One of the key challenges in cryobiology is achieving a rewarming that guarantees the successful recovery of living cells and tissues. The growth of ice nuclei during rewarming, or recrystallization, is an important issue as it promotes damage to the cryopreserved samples. To overcome this problem, the rewarming of the sample must be as fast and homogeneous as possible.
We recently theoretically proved that the use of High Intensity Focused Ultrasound (HIFU) can be a very efficient approach for rapid and uniform rewarming of vitrified organs. Here, a HIFU device composed of a wave generator, a power source, and a piezoelectric transducer, is used to retrieve living organisms that otherwise would have died due to ice injury during rewarming. This work presents the application of HIFU to recover cryopreserved nematodes, with a survival rate of up to 90% of individuals of all stages of growth, including adults. Adult *C. elegans* rarely survive through conventional cryopreservation techniques. The nematodes were cooled to -80 ℃ in a 15% v. / v. of glycerol in S Buffer through slow freezing, at a $-1℃/min$ max cooling rate. The HIFU device offers a rewarming rate greater than $200℃/min$ when operating at 60VDC and 2,26A in the interval $[-70\ ℃, -50\ ℃]$. This approach proves the effectiveness of HIFU rewarming of cryopreserved biological samples and opens new possibilities for research, regarding: i) it is a scalable technology in respect to the size of the sample, and ii) it can be easily monitored by MRI-thermography.

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S83 CRYOPRESERVATION OF IPSCS DERIVED NEURAL 3D CELL MODELS AS A FUNDAMENTAL KEY TECHNOLOGY IN BIOTECHNOLOGY AND BIOMEDICINE

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Three-dimensional (3D) organoid cultures derived from human induced pluripotent stem cells (hiPSCs) represent relevant preclinical model systems for human physiology in biomedical research. They provide enhanced possibilities for diagnostics, drug screening or disease modelling, since the expansion capability and the sources of human primary cells are often limited. Thus their impact has been steadily increasing in the recent decade leading to a high demand for ready-to-use 3D models. However, the generation of organoids is time-consuming and the differentiation protocols can take up to several months. Additionally, matured organoids have a limited shelf life with a short time span in which organoids can be used for appropriate down streaming experiments. So far, the long-time storage of ready-to-use 3D cell models is not satisfactorily feasible. Hence, the aim is to enable stock keeping and improve the

constant supply of diagnostically relevant 3D cell systems by cryopreservation. Main hurdles for organoid cryopreservation are the inhomogeneous penetration of cryoprotectants as well as the heat transfer during freezing and thawing, causing critical mass and heat gradients throughout the 3D system. These endanger the structural integrity of the complex organoid, which is essential for the maintenance of their organotypic functions. In this work, early neural stem cell organoids serve as a homogenous precursor model of fully matured midbrain organoids to investigate the mechanisms of cryo injuries in 3D constructs after cryopreservation. Appropriate quality controls are carried out for 3D cell systems after thawing. In this context, both cryopreservation regimes, the conventional slow-rate freezing and the ice-free vitrification were comparatively analysed. The presented studies provide insights into cryo-induced damage processes in early neural organoids and thus implement the basis for the establishment of living biobanks of 3D cell structures, which are an important key resource in biotechnology and biomedicine.

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S84 COMPREHENSIVE LIPIDOMICS USING TARGETED MRM LCMS METHOD IN BOVINE SPERM

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The lipidome is directly associated with the physiological characteristics of the bovine sperm that hold a pivotal position in predicting the reproductive success. Variation in post thaw fertility of cryopreserved sperm among different bulls has been linked to the differences in the lipid profile of the bull sperm plasma membrane. Post-thaw sperm viability has been reported to decrease by approximately 50% during the process of cryopreservation. The current research is aimed to study the variability of bull sperm lipid composition by employing a novel targeted-multiple reaction monitoring (MRM) LC-MS method using QTRAP 6500 mass spectrometer coupled with Agilent 1290 UPLC. Dichloromethane-Methanol lipid extraction from snap-frozen Holstein bull semen samples with varying post thaw viability (good and poor, n=4) was carried out and the lipidomics data analysis was performed using Analyst 1.7 (AB Sciex), MultiQuant 3.0.3 (AB Sciex) and GraphPad Prism 9 software. A total of 184 lipid species were identified representing 11 lipid classes. The sperm lipidome was dominated by phosphatidylcholine (PC) (n=42) and phosphatidylethanolamine (PE) (n=43) classes covering nearly half of the total identified lipid species, whereas, SM (d18:1-16:0) and PE (18:1-18:1) were the most abundant lipids in the semen samples. Among the esterified fatty acids, oleic acid (18:1) was predominant with its presence in 53 of the identified lipid species whereas stearic acid (18:0) and palmitic acid (16:0) were equally present in 41 lipid species. Mean lipid peak area value for the poor freezers was found to be higher in comparison with the good freezers. The findings of this study are important as it identifies for the first time the presence such a large number of lipid species that could ultimately help in understanding the variation in freezability based on the differences in plasma membrane lipid composition. This lipidomic analysis will also help in the identification of potential lipid biomarkers for cryotolerance in bovine sperm.

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S85 DIMETHYL SULFOXIDE AND QUERCETIN PROLONGED STORAGE PERIOD OF COLD-STORED RAT SPERM

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Recently, cold-transport of sperm has been widely used for shipment of genetically engineered mice. Previously, we reported dimethyl sulfoxide (Me_2SO) and quercetin had the ability to protect cold-stored mouse sperm. However, the effects of Me_2SO and quercetin on sperm of other species are not clear. In this study, we examined the motility and fertilization rate of rat sperm stored in cold-storage solution containing Me_2SO and quercetin at refrigerated temperatures. First, rat sperm were stored in a cold-storage solution without Me_2SO and quercetin. As a result, the motility of cold-stored rat sperm decreased at 1 day. Next, the effects of Me_2SO and quercetin were examined based on the motility and fertilization rate of cold-stored rat sperm. Me_2SO at 15% and quercetin at 200 mg/mL were the most effective in maintaining the motility and fertility of cold-stored rat sperm. The cold-stored sperm with Me_2SO and quercetin efficiently fertilized for 3 days. And then, we demonstrated the cold-transport of rat sperm, IVF using the cold-transported sperm, and embryo transfer using the fertilized oocytes derived from the cold-transported sperm. Using cold-transported sperm, we successfully obtained fertilized oocytes by IVF. The fertilized oocytes developed into live pups by embryo transfer. In summary, these results suggest that Me_2SO and quercetin is beneficial to prolong the storage period of rat sperm for 3 days. The storage period of the cold-stored sperm will be practical to transport genetically engineered rats between different institutes.

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S86 CREATING MULTIPLE GENERATIONS OF A THREATENED AMPHIBIAN SPECIES USING ASSISTED REPRODUCTIVE TECHNOLOGIES

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Application of assisted reproductive technologies (ART) in captive breeding programs has led to an increase in reproductive output for numerous amphibian species, leading to higher population numbers, genetic variation, and reintroduction opportunities for imperiled species. However, one major concern of integrating ART, specifically cryopreservation, in breeding programs is the uncertainty of the reproductive viability of offspring. This case study follows the production of the world's first *Anaxyrus houstonensis* toadlets using frozen-thawed sperm through in-vitro fertilization and the subsequent breeding of these individuals. *A. houstonensis* males (n=20) housed at Fort Worth Zoo were administered gonadotropin releasing hormone-analog (GnRHa) or human chorionic gonadotropin (hCG) to stimulate spermiation. Spermic urine was collected up to seven hours post-hormone administration, cryopreserved with 10% N,N-dimethylformamide + 10% trehalose + 0.25% bovine serum albumin, and stored in liquid nitrogen. Female *A. houstonensis* (n=3) were administered a cocktail of GnRHa and hCG to induce oviposition, and eggs from two females were fertilized using fresh and frozen-thawed sperm. Approximately 61 individuals hatched from these two females, and 17 are still housed at Fort Worth Zoo. Out of these 61, 13 were produced using cryopreserved sperm, and two survived into adulthood. One year later, the two cryo-produced offspring, determined to be females via ultrasonography, were administered hormones to induce oviposition and paired with males from the captive colony at Fort Worth Zoo for facilitated breeding. As a result of this breeding event, 11,660 eggs were laid and 9,154 tadpoles were produced, for a 79% hatch rate. Most of these offspring were then released into the wild, with 10 from each pairing held back to monitor development post-hatching. This case study serves as a proof-of-concept that highlights the value of incorporating ART into breeding programs for better population and genetic management of at-risk species by producing reproductively viable offspring.

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S87 THE PHENOMENON OF ZERO SURVIVAL OF SPERM CELLS CRYOPRESERVED IN NANOLITER VOLUMES

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The need for means for cryopreservation of preselected individual sperm cells is well recognized and needs no unique justifications. Obviously, this calls for the development of nanoliter volume chambers for comfortably arresting an individual sperm cell, and consequent protocells. However, while developing such nanoliter chambers and experiencing them, we noticed two particular effects (a) a sudden leap of cell mortality after thawing small droplets of frozen cell suspension below ~ 10 nanoliter, where cells showed zero survival, (b) although there was no significant reduction in living cell count after 30 minutes of soaking in pre-freezing media, it was evident that the smaller the suspending volume, the lesser the sperm swimming velocity is.
The reason for these occurrences turned out to be diffusion of water out of the watery droplet into the surrounding oil phase, which consequently elevates salts concentration within the droplets.

Finally, and interestingly, analytical calculation teaches that the rate of solute concentration within the droplet tends to infinity for volumes of 20 nanoliter and less. This correlates with the finding that sperm cells dead in such volumes. Understanding the problem allowed us to find a solution to the diffusion problem, and reach a good percentage of post-thaw survival, even in volumes smaller than one nanoliter.

In addition, research and development of nanoliter-volume doughnut-like arrayed for cryopreservation of rare sperm cells at individual-cell resolution has been accomplished. Micro-arrayed donut-shaped chambers (DSCs) are miniature vessels which were developed in the frame of this work. Each chamber is designed to act as an individual isolated reaction compartment, which creates an in-vitro assay, mimicking biology environments. Such a device enables individual live cell treatment and analysis, with the assistance of a designated image processing algorithm.

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S88 HYDROPHOBIC SOOT NANOPARTICLES APPLICABLE TO CRYOBIOLOGY AND REPRODUCTIVE MEDICINE AS A FUNCTIONAL ACTIVATOR OF HUMAN SPERMATOZOA

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Sperm cryopreservation is vital in combating the human infertility, but regrettably, the toxicity of cryoprotectants and the occurrence of intracellular icing, osmotic shocks or shrinkage of the cells below a given threshold volume greatly affect the success rate of this technique. Using the virtue of nanotechnologies and depositing water-repellent soot nanoparticles on the inner walls of cryovials may outline new directions in the development of cryobiology. For instance, minimizing by natural means the solid-liquid contact area (even for liquid columns instead of single droplets) and interfacial heat transfer rate, and thus, reducing the icing probability and hindering the incipiency of ice nuclei without the aid of cryoprotectants. Such an approach allows better recovery of the post-thawed human spermatozoa compared to the conventional cryoprotection methods, but doubts related to the soot's cytotoxicity impede its clinical implementability. In this proceeding, we report results from the intermittent evaluation of sperm motility (native and unwashed ejaculates, collected from 20 patients with deteriorated semen parameters or from couples with idiopathic infertility) within 270 min incubation in vials containing soot nanoparticles. The computer-assisted sperm analysis shows that the soot enhances the progressive sperm motility (*class a+b*) at ~50-60 % of tested patients due to electrostatic interactions in the seminal fluid and changes in its biochemistry. The as-synthesized carbon nanoparticles reveal strong potential for future replacement of the commercially available phosphodiesterase inhibitors (e.g., pentoxifylline) that improve the sperm functional competence prior to IVF procedures, but at the expense of premature acrosome reactions or negative effects towards the oocyte function and early embryo development.

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S89 GENOME-WIDE ASSOCIATION STUDY OF CRYOPRESERVED SEMEN QUALITY TRAITS IN HOLSTEIN-FRIESIAN BULLS

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In vitro assessment of bull semen quality is used in artificial insemination (AI) centres as quality control in order to ensure that semen destined to be used for insemination have passed minimum post-thaw standards. Despite these stringent quality control checks, individual bulls that pass the post-thaw quality control checks can still vary in field fertility by up to 25%. A genome-wide association study (GWAS) was undertaken to determine genetic biomarkers associated with semen quality traits. Genotypes from the International Irish Dairy and Beef (IDB) genotype panel containing 40,170 single-nucleotide polymorphisms (SNPs) were available on 681 Holstein Friesian bulls used in AI. Genome wide association analysis was performed using a single SNP regression mixed linear models in WOMBAT. Significance ($P \leq 1 \times 10^{-8}$) or suggestive ($P \leq 1 \times 10^{-5}$) SNPs in close proximity (< 500 Kb) were combined into a single quantitative trait locus (QTL). Genes within a 250 Kb span of the start and end of the QTL were identified. A total of 20 SNPs were significantly associated across all semen quality traits ($P \leq 1 \times 10^{-5}$), of which 2 SNPs (rs41630294 and rs109345125) were related to the percentage of ejaculates rejected after freezing and another SNP (rs41747482) was associated with post-thaw sperm motility. By combining our GWA results with the biological functions of these genes, five promising candidate genes were identified. *ATP13A4* gene on BTA1 and *PELI1* on BTA11 were associated with the percentage of ejaculates rejected after freezing. Post-thaw sperm motility was identified to be associated with *ZW10*, *CLDN25* and *USP28* genes. Therefore, the effect of cryopreservation to post-thaw sperm motility is at least partly controlled by genetic factors. This study provides new insights for further research on the genetic mechanism of these semen quality traits.

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S90 EVALUATION OF THE METHLATION PATTERN OF IMPRINTING GENES IN HUMAN SPERM DURING RAPID FREEZING VERSUS VITRIFICATION

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Sperm cryopreservation, an important technique for long-term sperm storage, causes epigenetic modifications, thereby reducing the fertility potential of thawed sperm. Rapid freezing and vitrification are two sperm cryopreservation methods. However, it is still unknown whether one technique is advantageous over the other. The aim of this study was to compare the effects of these methods on the methylation pattern of paternal imprinting genes *PAX8*, *PEG3* and *RTL1* in human sperm. For this purpose, semen samples were collected from 20 normozoospermic men. Washed sperm samples were divided into three aliquots, including, the fresh, frozen by rapid freezing and vitrification according to the standard protocols. Sperm motility and DNA methylation of *PAX8*, *PEG3* and *RTL1* genes were investigated using CASA and methylation-specific PCR methods, respectively. The results showed that rapid freezing was significantly better than vitrification in motility parameters of thawed sperm, ($P < 0.05$). Moreover, a significant increase in the percentage of gene methylation was detected in the frozen groups compared to the fresh group. Also, *PEG3* and *RTL1* methylation in the vitrification group was significantly increased ($P < 0.05$) compared to the rapid group. Since epigenetic modification of imprinting genes is the main reason underlying the decline in sperm motility and fertility during the freeze-thaw process, our findings indicate that the rapid freezing is a better method for sperm protection against cryo-damages compare to the vitrification.

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S91 CRYOPRESERVATION IN BIOMEDICAL ENGINEERING

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In medicine we face the fact of limited organ and blood donations. In both areas long-term storage of living organs, tissues and valuable cell types could help.
One field of research in the field of lacking numbers of donated organs is tissue engineering. Here, off-the-shelf availability of the tissue-engineered constructs and availability of rare cells on demand be an option. The adequate preservation of tissue-engineered constructs (TECs) guarantees their availability for medical application. However, the preservation of the cell viability, cell-cell and cell-scaffold interactions is challenging during thawing. Thus, this talks addresses the challenges of delicate cell-cell and cell-scaffold contacts, complex heat and mass transfer to avoid inhomogeneous CPA and temperature distribution, selection of freezing containers, optimization of the thawing procedure, xeno-free cryopreservation of cells in suspension by applying electroporation, selection of tissue-specific preservation approach, monitoring latent heat in 3D scaffolds using infrared thermography and discussing thawing profiles in cryobags vs. cryovials. Furthermore, challenges in using cryo store robots for optimizing the cryopreservation process will be discussed.
In the blood transfusion field, a continuous, automated sterile freezing process could solve the shortage of blood donations. We

apply a novel continuous float-process for the cryopreservation of red blood cells. Here we increase the surface area to improve heat transfer and decrease the need for cryoprotectants.

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S92 A CRITICAL TRANSFER TEMPERATURE FROM SLOW COOLING TO CRYOGENIC STORAGE FOR OPTIMAL RECOVERY ACROSS A RANGE OF CRYOPRESERVED MAMMALIAN CELLS

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Cryopreservation of mammalian somatic cells must be carefully controlled to ensure maximum, post-thaw recovery using cooling rates slow enough to allow time for cells to cryodehydrate sufficiently thereby avoiding lethal intracellular ice. In this study, we looked at the effect of different transfer temperatures to cryogenic storage after slow, controlled-rate cooling on the post-thaw recovery of a range of cell lines derived from T lymphocyte (Jurkat), liver (HepG2), ovary (CHO) and bone (MG63). We found that slow, controlled-rate cooling should be conducted down to -50˚C to ensure optimal cell recovery and metabolic activity post-thaw across all four cell lines tested, as opposed to -80˚C, -100˚C or even lower temperatures in common practice. We have also demonstrated that this critical temperature is linked to a physical event that cells undergo between -47 and -59˚C in the presence of the cryoprotective agent dimethyl sulfoxide: an intracellular, colloidal glass transition (Tg'i), as detected by differential scanning calorimetry (DSC). This study ultimately shows that shorter, more time- and cost-efficient cryopreservation protocols can therefore safely be applied, and maybe help expand the adoption of cryopreservation in particular in the cell therapy space.

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S93 DEVELOPING LN2-FREE SHIPPING FOR CRYOPRESERVED CELL THERAPIES

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The shipping of cell therapies is a key emerging application of cryobiological science, with its own unique set of challenges, both cryobiological and practical. While some biological material such as some cell lines and mRNA vaccines can be shipped above the Me_2SO glass transition at dry ice temperatures of around -80°C, this is not typically acceptable for cell therapies. Cell therapies are typically thawed and used immediately, and so even minor cell deterioration during -80°C shipment could impact treatment efficacy and result in injection of poor and apoptotic cells. Modern cell therapies also require cold-chain tracking and traceability throughout their multiple shipments at ultra-low temperatures, from the initial cell extraction to a manufacturing site, to the ultra-low shipment of a final cell therapy to the clinic.
In this study, we examined the impact of different shipment temperatures on Jurkat and HepG2 cells, and then used this data to

engineer a shipping system using Stirling Engine cryocoolers to allow for ultra-low shipment of biological samples without the need for LN_2 or other liquid coolant. Practical difficulties such as keeping the system cool without a continual electricity supply and preventing LN_2 condensation from the atmosphere were resolved to give maximum protection to cell therapies during transportation.

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S94 CRYOBIOLOGY CAN SHAPE THE FUTURE OF ENERGY EFFICIENCY

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Energy demand globally has risen dramatically in the last century. Despite the advent of technology, e.g. solar panels and wind turbines, that enables the utilization of sustainable energy resources like the sun and the wind, in the modern world, we still struggle to meet global energy needs. Moreover, due to the climate change we are forced to gradually abandon the use of certain fossil fuels that increase CO2 emissions and replace them with either nuclear power that inherently poses certain risks related to safety and nuclear waste disposal, or sustainable energy solutions, which are not always reliable and efficient. Though there are other solutions for energy production, like hydrogen, it seems that the energy crisis will not be overcome solely by novel energy production solutions. We need to turn our attention both to using energy more efficiently and to saving energy. In both cases we consume less energy; whereas in certain cases we do not sacrifice the quantity or the quality of goods and services being produced (energy efficiency), in other cases we need to abandon certain products or services to save energy (energy conservation). Energy efficiency measures have already been implemented, in countries like the US, Germany, and Australia as a complementary approach to the energy crisis. Cryobiology has much to offer in relation to global energy crisis, since many industrial processes rely on low temperatures, like food production and storage (e.g. frozen food), transportation (e.g. cold chain logistics) and thermal energy storage (e.g. in the form of ice, or ice slurry). A fundamental approach, for all the above applications, is the control of ice nucleation. Natural organisms cope with low temperatures variations, by controlling ice nucleation, usually under slow cooling conditions. At the molecular level, Ice Binding Proteins (IBPs) play a pivotal role in controlling ice nucleation, either by promoting crystallization (ice nucleation proteins) or by inhibiting it (antifreeze proteins). Both kinds of IBPs have been included in various industrial research projects with promising results for energy efficiency, in many cases. Based on the results of pilot studies we will discuss the potential of cryobiology in energy efficiency.

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S95 CURRENT AND FUTURE POTENTIAL OF ELECTRON MICROSCOPY IN THE HIGH-RESOLUTION 3D STUDY OF CELLS, TISSUES AND IN SITU MACROMOLECULES

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Compartmentalization of mutually exclusive reactions in different regions of cells by membrane enclosed organelles or by self-assembling macromolecular complexes is a critical mechanism of life. The fluorescent tags of optogenetics can display proteins confined to compartments in living cells, but gives no glimpse of the underlying ultrastructure. The electron microscope (EM) has the resolution to display cellular ultrastructure, but within very thin slices (100nm depth) from which it is difficult to understand, measure and compare the complex cellular ultrastructure, particularly with elaborately interdigitating cells in vivo such as stem cells or neurons.
Dramatic advances in electron microscopy (EM) have expanded the potential of EM to study cells and tissues in 3D. For example, Serial Block Face Scanning Electron Microscopy (SBFSEM) now provides ultrastructural resolution throughout a much greater (μm to mm) sample depth, enabling quantitative analysis of ultrastructural features throughout the length of complex cells which can be transformative in many basic and clinical science applications. CryoEM, recognized by the award of the Nobel Prize in Chemistry to Henderson, Frank and Dubochet in 2017, has greatly enhanced macromolecular structure determination (<2.5Å) but the current frontier is to understand the machinery of life in native biological context.
Vitrification (without cryoprotectants) allows cells and tissues to be fixed in a state close to their native condition, locking elements and optogenetic tags in physical space for subsequent targeted analysis. Optogenetic tags can be used to track regions of interest, correlating live cell studies with underlying ultrastructure. Vitrified cells and tissues can be sectioned by cryo-ultramicrotomy (CEMOVIS) and 3D-ultrastructure studied by cryo-electron tomography (cryoET) and/ or elemental spectroscopy techniques. Recently, focused ion beam milling in a dual beam scanning EM (cryoFIB) has allowed lamella of controllable thickness to be prepared for cryoET. The cryoFIB can also generate 3D volumes by sequential sectioning, however, has significant challenges in achieving sufficient signal without beam induced damage under cryo-conditions. Current and postulated advances in EM will be discussed with reference to the study of low temperature biology.

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S96 CRYOPRESERVING MOLLUSKS SINCE 1970

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Gamete cryopreservation has been studied throughout the years, but mainly focused on sperm as many favorable results had been achieved. The work of Lanan (1971) on oyster sperm is the first publications on the cryopreservation of marine invertebrates. Over five decades later, this line of research has explored several development stages and cell types from a wide number of different mollusk species- most of them with a high commercial and ecological value- and has led to specific cryopreservation protocols.
There is still limited work on marine invertebrate cryopreservation. Most publications deal with mollusks, and among them, oysters are the best studied due to their global economic importance. *Crassostrea gigas* represents over 50% of the total work published on oysters, followed by *C. virginica* (Paredes 2015).

This work explores the research done with mollusks and analyzes it, in order to point out the strengths and weaknesses, advances and challenges so we can diagnose the current state of mollusk cryopreservation.

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S97 APPLICATION OF GERMPLASM CRYOPRESERVATION TO SHELLFISH BREEDING PROGRAM

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Globally, aquaculture of molluscan shellfish plays a significant role for seafood production and has more than doubled over the past three decades. Along the US east coast and Gulf of Mexico coast, eastern oysters *Crassostrea virginica* and northern quahogs *Mercenaria mercenaria* are the two major shellfish aquaculture species. To support the aquaculture industry, breeding programs were initiated to develop superior strains through traditional and genomic selections. As an integrated component of breeding, cryopreservation of germplasm (gametes and larvae) can be applied to preserve broodstock, offspring, and specific strains/lines. For eastern oyster breeding in the Gulf of Mexico coast, wild populations in the embayment along the Gulf coast were collected and used as broodstock for establishment of F1 populations with maximized genetic diversity, and selection was conducted in the F1 generation targeting on salinity tolerances. A sperm repository of the F0 broodstock was established, including a total of 102 male oysters from the 17 collection sites. Gamete collection was performed by strip spawn, and fertilization was made by crossing broodstock from different collection sites. Fresh sperm production, motility, and fertility were recorded for quality analysis. A home-made portable aerated freezer system was developed for sample cooling in field at a cooling rate of 10-15℃/min. Post-thaw motility (1 - 30%) and plasm membrane integrity (15.34 - 70.36%) were recorded as post-thaw quality parameters. In addition, cryopreservation of D-stage oyster larvae was developed and applied to preserve the families from the breeding crosses. For northern quahogs, cryopreservation of sperm showed limited success and that of trochophore yielded 27% post-thaw survival, but no reports were on breeding project yet. Overall, application of germplasm cryopreservation to oyster breeding was demonstrated, and a data management plan, including sample collection, phenotype, fresh sample, genotype, cryopreservation, and post-thaw quality, for germplasm repository was developed to support breeding.

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S98 CRYOPRESERVATION DAMAGE ON SEA URCHIN OOCYTES ANALYZED BY ELECTRON MICROSCOPY

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Cryopreservation has been applied recently to aquaculture, genetic programs and conservation in marine environment. Gamete cryopreservation has been studied throughout the years, but mainly focused on sperm as many favorable results had been achieved. However, oocytes are still under research as no successful outcome has been accomplished yet. The aim of this study is to examine the damage in *Paracentrotus lividus* oocytes caused by different cryopreservation methods and cryoprotecting agents (CPAs), using electron microscopy techniques (SEM and TEM). Oocytes and sperm were collected from sea urchins reared in captivity. Slow cooling, vitrification by contact on a precooled metal block, droplet vitrification, straw directly into liquid nitrogen and different CPAs concentrations were used to cryopreserve unfertilized and fertilized oocytes. Samples were prepared mixing 1mL of oocytes with 1mL of CPA in one single step and equilibrating for 5 minutes. All samples were fixed with a solution of 1% glutaraldehyde in sea water and analyzed using SEM and TEM. Results have shown that cryopreservation caused an irreparable damage on the oocytes. Membranes appeared broken and cellular material and vesicles have been extruded from the eggs and are visible on the surface. Although damage is observed in all samples, not all methods and CPA concentrations had the same level of damage. For example, when using vitrification by contact with 1.5M of EG, the ultrastructure of the cytoplasm seemed in better conditions but not the egg surface. The observation of the topography of the eggs surface is essential to have information on their actual conditions. Unfortunately, the egg surface is the region of the cell that is more susceptible to treatments and reacts appropriately with the sperm if the eggs are optimum. It seems appropriate to study the damages cryopreservation causes on the egg surface to try and design successful protocols for oocyte cryopreservation.

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S99 MODULATION OF STRESS FACTORS FOR CRYOPRESERVATION OF *P. LIVIDUS* LARVAE

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The sea urchin *P. lividus* is a species of high economic, ecological and scientific value (Rey-Méndez et al., 2015). Currently, there is already a successful protocol for the cryopreservation of embryos (Paredes and Bellas, 2015) but not for pluteus larvae. Obtaining a protocol for the cryopreservation of pluteus larvae will guarantee improvements in their culture or their applications in bioassays (Bellas and Paredes, 2011). In the present work we aim to achieve a successful protocol for the cryopreservation of *P. lividus* pluetus larvae by modulating two stress factors (salinity and temperature) whose ranges have been previously determined by

bioassays using four pre-freezing pre-treatments (18 ºC-29.5 ‰; 18 ºC-35‰ and 20 ºC-29.5 ‰; 20 ºC -35‰). In addition, toxicity tests were performed with different cryoprotectants: methanol (METH), ethylene glycol (EG), propylene glycol (PG), dimethyl sulfoxide (Me_2SO) and glycerol (GLY), in a range of 0.5- 3M, best results pointed out to METH and Me_2SO as those suitable for cryopreservation. Finally, a cryopreservation experiment was performed with both cryoprotectants supplemented with 0.04M trehalose on 4-arm pluetus larvae (48h-old) developed in these pre-treatment conditions, followed by a simpler and shorter protocol than the one used for embryos (Paredes and Bellas, 2015) with a cooling rate of 1 ºC/min to -35ºC, achieving for the first time the successful cryopreservation of *P. lividus* larvae. Contrary to what we expected, no improvements were observed by incubation on those pre-treatment conditions of low salinity or temperature, on the contrary delays in larval development were observed. Me_2SO was the cryoprotectant that showed effective cryoprotection of the larvae similarly to data from Paredes and Bellas, 2009, when dimethyl sulfoxide despite not being the less toxic compound still resulted in the best cryopreservation outcome. Damage of the larval structure ost-thaw was studied with light and fluorescence microscopy.

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S100 MOTILE SPERM FRACTION SEPARATION FROM CRYOPRESERVED SAMPLES IN TAXONOMICALLY DISTANT FISHES

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The freeze-thawing process leads to a varying percentage of cryo-damaged spermatozoa appearing in the suspension. That obscures the understanding of real cryopreservation effects on spermatozoa that survived the freeze-thawing and saved the ability to fertilise. Moreover, the elimination of cryo-damaged spermatozoa can help improve fertilisation outcomes. Therefore, different techniques for separating spermatozoa with high motility and fertilising ability exist and are used in mammals assisted reproduction. Nevertheless, those methods were not intensively tested in fish, in which spermatozoa become motile for an extremely brief period right before fertilisation. That property and small fish spermatozoon dimensions complicate the separation of highly motile sperm fractions after cryopreservation. We present the data on the application of Percoll density gradient centrifugation for obtaining such fraction of post-thaw spermatozoa in taxonomically distant sterlet *Acipenser ruthenus* and European perch *Perca fluviatilis* (species differing in spermatozoa size, motility duration, and mode of motility activation). Sperm samples (n=5) were cryopreserved and thawed using species-specific routine protocols. After applying Percoll density gradient centrifugation to thawed sperm, obtained fractions of spermatozoa were analysed by CASA to evaluate sperm motility percentage and velocities. In European perch, no highly motile post-thaw sperm fraction was collected. However, centrifugation in sterlet led to obtaining a fraction of spermatozoa with motility percentage and velocity which were not statistically different compared to values for control (not frozen) sperm. Fertilising ability of this separated fraction and not

separated thawed sperm was of the same range, being significantly lower than that of control sperm. At the same time, the rate of malformation of embryos obtained using sperm of separated fraction was found to be not statistically different from the control value, while application of not separated thawed sperm resulted in its significant increase.

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S101 PROTOCOL FOR COMMON CARP SPERM CRYOPRESERVATION FOR AQUACULTURE-SCALE ARTIFICIAL REPRODUCTION

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Cryopreservation of genetic material of different strains of common carp is one of the parts of the Czech National Program for Conservation of Farm Animal Genetic Resources. Application of cryopreserved sperm for fertilisation of eggs on an aquaculture scale requires many small cryo-containers (0.5-1.8 ml), complicating the artificial reproduction practice. Optimisation of cryo-protocol for bigger containers is necessary to improve big-scale fertilisation practice. The current study established the protocol for common carp sperm cryopreservation in 4.5 ml cryotubes using the programmable freezer with freezing mode starting from 6°C, cooling down to -20°C at a rate of -2°C/min, further from -20°C to -140°C at a freezing rate of -20°C/min with sperm:extender dilution rate of 1:1. The extender contained 0.1% w/v sucrose, 0.35% w/v NaCl with 22% v/v of methanol and 5% v/v of ethylene glycol. The thawing was done in a water bath at 60°C for 100 s. This protocol provides repeatable results on post-thaw sperm motility (average±SD) of 39.3±16% and sperm initial curvilinear velocity of 96±26 μm/s (n=56 males) measured by CASA. Sperm from one cryotube (4.5 ml, around $50*10^9$ spermatozoa) can be used for artificial fertilisation of 200 g (around $1.6*10^5$) eggs, followed by treatment with the tannic acid solution to avoid the eggs from stickiness, which is very important for their successful incubation and hatching. A hatching rate of more than 80% was achieved in 12 batches of eggs from 8 females fertilised by five males. The hatching results did not differ significantly from the control group fertilised by not frozen sperm (around $40*10^9$ spermatozoa). Compared to the control, 1.25 times more frozen sperm was used to fertilise the eggs. This is the first report demonstrating an effective protocol for cryopreservation of high sperm concentration carp sperm in cryotubes of big volume.

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S102 CELL AND NUCLEAR OSMOTIC CHARACTERISTICS OF ADHERENT HEPG2 CELLS DETECTED BY USING CONFOCAL MICROSCOPY AND 3D RECONSTRUCTION:

IMPLICATIONS FOR CRYOPRESERVATION

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Cryopreserved hepatocytes are in demand for their utilization in bio-artificial liver devices, hepatocyte transplantation, drug screening and gene therapy. Current cryopreservation success rates for primary hepatocytes are low, and even lower for tissue pieces. To optimize the cryopreservation process, this study focuses on the characterization of osmotic properties of adherent human Hepatoma HepG2 cells. We investigated the cell, cytoplasm, and nuclear volume excursions during the shrink-swell process in the presence of hyper and hypotonic solutions, followed by restoration of isotonic conditions. Real-time volumetric measurements were done by using confocal microscopy followed by segmentation and 3D reconstruction. Our results indicate that the cells and nuclear volume were osmotically sensitive and interdependent, but their osmotic behavior was not linear. In particular, the cytoplasm and nucleus have the same osmotically inactive volume fraction for the hypertonic region. Moreover, after osmotic stress, we found that cells return to an equilibrium volume different from the initial isotonic volume and have different osmotically inactive volume fractions. Finally, the membrane permeability of HepG2 monolayers to water (Lp) and CPA (Ps) was inferred during perfusion with anisotonic and CPA solutions. Altogether, evaluation of these fundamental membrane transport characteristics can be applied in the development of more robust mathematical models, as well as in cryopreservation protocols.

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S103 IDENTIFYING THE KEY STRUCTRUAL ATTRIBUTES NESSESSARY FOR IRI ACTIVITY IN ARYL GLUCONAMIDES

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The Ben Lab has synthesized many classes of small molecule carbohydrate derivatives for use as cryoprotective agents in the cryopreservation of cells and tissues. These compounds, known as ice recrystallization inhibitors (IRIs), function through reducing ice crystal growth, ultimately mitigating the cellular damage associated with cryopreservation. Over a decade of work has identified many structurally diverse classes of IRI active molecules making it difficult to definitively identify common structural attributes essential for IRI activity. To date, *N*-(2-fluorophenyl)-D-gluconamide (2FA) has been one of the most successful small molecule IRI compounds generated. 2FA has shown to improve the cryo-outcomes of a variety of functionally relevant cell types and is commonly used in the Ben lab as a benchmark comparison for new IRI candidates.
Identification of the required structural components to achieve the activity observed in 2FA is incredibly valuable to help direct future structure function work towards development of novel aryl gluconamide IRIs. In order to gain this insight, a classic synthetic / medicinal approach has been devised, where small incremental changes to IRI active aryl gluconamides molecules are introduced and activity is assessed. For example, 2FA is truncated to *N*-(2-fluorophenyl)-D-xylonamide (2FX) through the removal of

C_6 carbon / hydroxyl group and the IRI activity of 2FX is then compared to that of 2FA. This process is repeated sequentially such that all truncated derivatives of 2FA are obtained, including the 2-fluoroaniline building block.
To further validate the hypothesis that the removal of a specific carbon / hydroxyl directly modulate IRI activity, deoxy aldonamides were synthesized and their IRI activity compared to the truncated derivatives. These studies indicate that *N*-(2-fluorophenyl)-4-deoxy-D-gluconamide (C_4d2FA) possesses comparable activity to 2FA while also possessing vastly superior solubility in aqueous media.

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S104 CHARACTERIZING A NOVEL ROTATING COPPER PLATE APPARATUS FOR DROPLET VITRIFICATION

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Vitrification is a promising technique to cryopreserve cells by avoiding injurious ice formation during cooling. Recent advances have leveraged conductive cooling to vitrify bulk cell laden droplets on copper plates surrounded by liquid nitrogen (LN). Here, we characterize a novel apparatus for droplet vitrification using a rotating copper plate.
Our apparatus consists of a 3D printed polyethylene terephthalate glycol cube with an open top. Housed within the cube is a rotating 5 mm copper plate that is connected via a shaft to an external motor. LN in the cube is held to a level touching, but not surpassing, the plate. The plate also features a circumference spanning 3 mm indentation where droplets are dropped onto. A stopper is situated on the indentation to redirect vitrified droplets into a LN filled container. An external touchscreen panel controls the plate's rotation and provides a live plate temperature reading through a T-type thermocouple. To evaluate copper plate vitrification, we compared vitrification between dropping common cryoprotectant solutions directly into LN with using our apparatus.
All droplets of tested cryoprotectant solutions from an 18 gauge needle that vitrified upon direct dropping into LN were also vitrified using our apparatus. However, some cryoprotectant concentrations (2M Me_2SO + 2M ethylene glycol + 30% sucrose, 1M Me_2SO + 1M ethylene glycol + 50% sucrose) only vitrified using our apparatus. The plate's temperature averaged -194°C and we also observed that a small amount of LN overflowed and settled into the indentation. The droplets experienced no inverse Leidenfrost effect in the indentation yet were still surrounded by LN allowing for cooling by both the copper plate and directly from LN. Clinical and scaled-up applications of cell therapies require efficient cryopreservation of bulk cell volumes. Our novel apparatus can help facilitate an uninterrupted and automatic vitrification process using a rotating copper plate droplet vitrification device.

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S105 ENHANCED CONTROL OVER ICE NUCLEATION STOCHASTICITY USING A CARBOHYDRATE POLYMER CRYOPROTECTANT

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Metastable supercooling has emerged as a transformative technique for ice-free biopreservation, but issues of stability inherent to the stochastic nature of ice formation have thus far limited its translation out of the laboratory. In this work, we explore the influence of the bio-based carbohydrate polymer FucoPol on aqueous supercooling using an isochoric nucleation detection technique. We show that FucoPol, a high-molecular weight, fucose-rich polysaccharide, which has previously been shown to reduce average ice crystal sizes after nucleation, also induces a concentration-dependent stabilization of metastable supercooled water, as evidenced by both a significant reduction in nucleation stochasticity (*i.e.* the spread in temperatures over which the system will nucleate upon cooling) and a corresponding increase in the predicted induction time of nucleation. FucoPol is found to confine the stochasticity of ice nucleation to a narrow, well-defined band of temperatures roughly one-third as wide as that of pure water under identical conditions. Importantly, this substantial reduction in stochasticity is accompanied by only a minimal (<1 °C) change in the average nucleation temperature, suggesting that this effect is distinct from colligative freezing point depression. Reducing and characterizing the stochasticity of aqueous supercooling is essential to the engineering design of practical biopreservation protocols, and the results reported herein suggest that high-viscosity polymer systems may provide a powerful and largely unexplored lever by which to manipulate metastable equilibrium phase change kinetics at subzero temperatures.

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S106 STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF ANTIFREEZE PROTEIN APAFP752

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Organisms that survive in freezing temperatures have developed a variety of adaptations to deal with extremely cold climates. One of these evolutionary adaptations are a class of ice binding proteins, antifreeze proteins (AFPs). It is evident that AFPs recognize and bind ice and interact with water via an unusual mechanism. We have investigated the antifreeze protein ApAFP752, an insect antifreeze protein found in the desert beetle *Anatolica polita*. The structure of ApAFP752 was determined by NMR spectroscopy, and we have shown that it is a right-handed beta-helical protein with a TCT and TCI ice-binding surface with the cysteine forming a disulfide bond to a cysteine on the opposite side of the helix. Dynamics data shows a high level of rigidity throughout the backbone of the protein which is consistent with the beta-helical AFPs typically found in insects. ApAFP752 subjected to capillary tube ice recrystallization inhibition assay showed ice recrystallization inhibition capability down to low micromolar concentrations, while buffer control exhibited significant ice reorganization in the same time frame. Current work focuses on the application of this protein in cryopreservation protocols, and on engineering a version of ApAFP752 with enhanced activity by enlarging the ice binding surface.

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S107 WITHDRAWN

S108 WHEN DESIRABLE EMBRYOS ARE CRYOPRESERVED LONG AFTER THEY ARE DESIRABLE

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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 14% more muscle mass compared to other cattle, which results in double muscled offspring. The MSTN mutation consists of a two base pair deletion in the third exon; which causes a premature stop codon at amino acid 313. By increasing muscle mass, more beef can be harvested from the herd. Piedmontese cattle originated in Italy and were 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 would help them achieve their desired herd. The embryos have been cryopreserved for nearly 30 years and have not nearly the value they have had in the past; because Piedmontese cattle are very difficult to raise. This cattle breed has a very strict diet and the offspring often have reproductive issues because they are big and bulky. Other breeds of cattle have become far more desirable in the industry of raising live stock. Our biobank now has to make a decision on what to do with all of these piedmontese embryos and semen.

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S109 CRYOPRESERVATION OF *ANOPHELES STEPHENSI* MOSQUITOES

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Mosquitoes are responsible for transmitting some of humanity's most deadly infectious diseases, and considerable research effort is devoted to studying mechanisms to suppress or block their ability to act as vectors. Our research has been aimed at increasing mosquitoes' susceptibility to the malaria parasite, *Plasmodium falciparum*, in order to more efficiently produce the sporozoite immunogen comprising Sanaria®PfSPZ Vaccine now entering phase 3 clinical trials. Research on mosquitoes is constrained by the requirement for continuous life cycle maintenance under precise controlled conditions. This extremely labor intensive, expensive and vulnerable process has a high risk of colony loss. The ability to cryopreserve mosquitoes would revolutionize mosquito research, facilitating work on new isolates, lab-bred strains, and transgenic lines that currently cannot be maintained. We recently reported a method for the cryopreservation of *Anopheles stephensi* embryos that yields hatch rates of ~25%, with >5 years stability. Key components of the method are harvesting eggs at 15-30 min post oviposition, stepped incubation in 100% deuterated methanol at -7 °C and -14.5 °C, rapid cooling, and rapid warming with concomitant dilution. Hatched larvae developed into fertile, fecund adults: females fed, produced fully viable second generation eggs and could be infected with *Plasmodium falciparum* at high intensity. The basic method can be adapted for cryopreserving small numbers of *A. stephensi* eggs from fragile genetically-modified lines as well as very large numbers of eggs for the purpose of providing a repository from which to rapidly establish new colonies. We have used these methods to successfully cryopreserve several genetically modified

A. stephensi strains and are now focused on developing egg banks under good manufacturing practice for use in the manufacture of PfSPZ Vaccine.

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S110 THE EFFECTS OF CRYOPROTECTANT, TEMPERATURE AND LENGHT OF *IN VITRO* CULTURE IN THE PERMEABILITY OF *IN VITRO* PRODUCED BOVINE EMBRYOS: TOWARDS AN *IN SILICO* DESIGNED VITRIFICATION/WARMING PROTOCOL

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Although vitrification techniques are gaining to cryopreserve *in vitro*-produced (IVP) embryos, the lack of a standard protocol limits their use in veterinary reproduction. This study aimed to determine the permeability parameters (L_p and P_s) of IVP blastocysts at the expanded stage in the presence of EG and Me_2SO at different temperatures (25°*C* and 38.5°*C*) and different culture lengths (Day-7 and Day-8). Day-7 (D7) or Day-8 (D8) blastocysts at the expanded stage were placed in a 25 µL-drop of TCM199-Hepes supplemented with 20% (v/v) FBS in a dish covered with mineral oil and held with a holding pipette on an inverted microscope. Each blastocyst was aspirated with an ICSI pipette to shrink its blastocoel. The collapsed blastocyst was covered with another pipette of a larger inner diameter and, by gliding the dish, it was introduced in a 25 µL drop containing 1.55 M Me_2SO or 1.55 M EG at 25°*C* or 38.5°*C* and left for 10 min. The volumetric response of the blastocysts was recorded every 5 s with a time-lapse video recorder. The hydraulic conductivity (L_p) and CPA permeability (P_s) of the blastocyst cell membrane were determined by fitting the experimental data to a two-parameter (2P) transport formalism. The different effects ($P<0.05$) of the temperature of exposure in L_p and P_s depending on the CPA observed in this study suggest that the period of exposure to the cryopreservation solution needs to be limited to prevent damage from the chemical toxicity of the cryoprotectants. The differences ($P<0.05$) between the L_p and P_s observed in D7 *vs.* D8 blastocysts could explain the lower performance of expanded blastocysts when they are vitrified on D8 with the same method used for D7 blastocysts. Future experiments are guaranteed to validate *in silico*-designed CPA loading steps for the optimization of vitrification/warming at each temperature and day of culture.

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S111 VITRIFICATION OF ONE-CELL RAT EMBRYOS IN CRYOTUBES BY SMALL-VOLUME VITRIFICATION AND RAPID WARMING

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To cryopreserve cells, it is essential to avoid intracellular ice formation during cooling and warming. One way to achieve this is to convert the water inside the cells into a non-crystalline glass. It is currently believed that to accomplish this vitrification, the cells must be suspended in a very high concentration (20–40%) of a glass-inducing solute, and subsequently cooled very rapidly. Herein, we report that this belief is erroneous with respect to the vitrification of one-cell rat embryos. In the present study, one-cell rat embryos were vitrified with 5 µl of EFS10 (a mixture of 10% ethylene glycol (EG), 27% Ficoll, and 0.45 M sucrose) in cryotubes at a moderate cooling rate, and warmed at various rates. Survival was assessed according to the ability of the embryos to develop into blastocysts and to develop to term. When embryos were vitrified at a 2,613 °C/min cooling rate and thawed by adding 1 ml of sucrose solution (0.3 M, 50 °C) at a warming rate of 18,467 °C /min, 58.1% of the EFS10-vitrified embryos developed into blastocysts, and 50.0% developed to term. These rates were similar to those of non-treated intact embryos. Using a conventional cryotube, we achieved developmental capabilities in one-cell rat embryos by rapid warming that were comparable to those of intact embryos, even using low concentrations (10%) of cell-permeating cryoprotectant and at low cooling rates.

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S112 RECENT ADVANCES IN 3D CRYOPRINTING

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3D bioprinting is a fabrication method that involves the layer-by-layer deposition biomaterials and cells. 3D bioprinted scaffolds can be used outside of the body, such as for disease models and drug development, or can be implanted into the body to replace damaged organs and or tissue. Advances in 3D bioprinting will bring this promising technique to the clinic and address the growing shortage of donor organs and tissue. Various types of 3D bioprinting exist, including "3D cryoprinting" or "3D cryobioprinting" which involves depositing biomaterial into a cryogenic environment. Since the introduction of 3D cryoprinting in 2015, this technique has gained popularity as a method of fabricating scaffolds out of soft bioinks that are otherwise difficult to print with. This review discusses recent progress in 3D cryoprinting. First, we discuss the advantages of this fabrication method, including the creation of highly porous scaffolds, protection for cells from environmental stressors during printing, and the ability to print anisotropic tissue in the vertical direction. Additionally, recent work demonstrated that 3D cryoprinting is a streamlined approach to printing and cryo-preserving cell-laden scaffolds for long-term storage. Second, we review various bioinks used for 3D cryoprinting and the crosslinking approaches that have been used with them. Third, we review the various applications of 3D cryoprinting, including the printing of scaffolds for bone, muscle, cartilage, and skin tissue regeneration. Fourth and finally we discuss current limitations in 3D cryoprinting and future trends for this field.

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S113 REVOLUTIONISING CELL BANKING WITH MONOLAYER CRYOPRESERVATION: A NEW PLATFORM FOR DRUG SCREENING

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Cryopreservation enables cells and tissues to be preserved for prolonged periods of time, at ultra-low temperatures, removing the need for continuous culture. Mammalian cells are currently preserved as suspensions in a solution containing 5 – 10 wt % dimethyl sulfoxide (Me_2SO), the most widely used cryoprotectant agent (CPA). Although high cell recovery values can be obtained with suspension cell freezing, a large amount of biomedical research is conducted on adherent cells as monolayers. Current cryopreservation techniques fail to freeze cells in this "ready-to-use" monolayer format, with Me_2SO only providing 20 – 35% recovery, which forms a bottleneck in research and laboratory automation. Additional challenges with monolayer cryopreservation include the reproducibility of cell recovery values due to well-to-well and person-to-person variability, extreme low survival of primary cells frozen in monolayer format and CPA toxicity.

Herein, this talk will focus on the use of a cryoprotectant poly(ampholyte) to enable the large-scale cryopreservation of a lung (a549) and liver (HepG2) cell line in monolayer format. Following optimisation of freezing protocols, the poly(ampholyte) significantly enhanced post-thaw cell recovery of both a549 and HepG2 cells at all cell seeding densities tested, superior to many of the previous approaches that require confluent monolayers. The reproducibility of our cryopreservation approach was confirmed by two independent scientists, achieving similar cell recovery values (>80%), minimal well-to-well cell recovery variability and healthy cell morphology. Cells retained near identical proliferative capacity, metabolic activity and cell cycle status compared to non-frozen cells 24 h post-thaw, confirming their "ready-to-use" status. Drug testing was completed on frozen cells, 24 h post-thaw, to generate dose-response curves and demonstrate that cell banking in monolayer format can allow rapid, reproducible, and efficient drug screening, reducing testing time from 1–3 weeks to 1 day. Our findings confirm that poly(ampholyte) has the potential to revolutionise cell banking, especially for areas within drug discovery, offering a platform where cells are preserved in a "ready-to-use" format for multiple applications.

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S114 UPSCALING CRYOPRESERVATION PROTOCOLS FROM MEDIUM TO LARGE SCALE, SUITABLE FOR

CLINICAL CELL THERAPY PRODUCTION

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Patients with liver failure are treated by liver transplantation. However, there is a major donor organ shortage, globally across all nations. As the liver can repair itself after damage, given time, providing temporary support to the failing liver using a cell therapy approach, with functional hepato-mimetic cells would allow the patient's own liver to recover.
Liver failure often occurs unpredictably and with rapidly onset, so any solution must be readily available off the shelf, and delivered via cryopreservation. HepatiCan, our combined ATMP and medical device can deliver this since the biomass, comprised of human liver-derived cell organoids, is cryopreservable. Our previous work has defined parameters/protocols to cool, store and successfully restore function after thawing on the medium scale and kinetics of freezing on large scale. This study seeks to increase successful cryopreservation/recovery to 2-3L scales, suitable for clinical use.
A comparison of data from medium (~0.1 litre) to large-scale indicated slower recovery of cryopreserved biomass at 2-3L clinical scale. Translating protocols from medium to 2-3L involved practical changes to accommodate the large scale biomass, whilst ensuring eventual GMP compliance. We, and others, have noted that short-term exposures to Me_2SO are not damaging to organoid recovery, however, the inevitable decline in warming rates at large scale introduces longer exposures to cryoprotectants.
Developing methodologies to decrease exposure to cryoprotectants at temperatures >0C, improved the full recovery of viable cell number one day earlier than previous procedures, both by improving the initial cell loss by 10% and increasing biomass replication by 9%. These incremental steps enable full recovery quicker than previous protocols – crucial for rapid patient delivery. These data enable novel hardware designs that can be readily translated to GMP facilities, and thus, suitable for clinical cell therapies.

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S115 INVESTIGATION OF CRYOPROTECTANT THERMOPHYSICAL PROPERTIES IN THE FAST COOLING CRYOPRESERVATION BY DSC TECHNIQUE

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Cryoprotective agents (CPAs) are crucial for decreasing cell damage due to ice formation during cryopreservation. However, high concentrations of CPAs are toxic to cells and tissues, though the required concentrations can be reduced by using higher cooling and warming rates. An insight into the thermophysical properties of biological solutions in the vitrification method is essential to develop a practical cryopreservation protocol. Previous studies of the vitrification process have either been visual and qualitative, or quantitative but at slower cooling rates using differential scanning calorimetry (DSC). DSC approaches are ideal because they can facilitate the study of the behavior of

biomaterials in different freezing conditions quantitatively and precisely, but up to now these approaches have been limited to slower cooling rates. Here we establish an ultrarapid cooling method for DSC, with minimal cooling rates exceeding 2000 °C/min to investigate the thermophysical vitrification behavior of ternary solutions of phosphate buffer saline (1X), dimethyl sulfoxide or glycerol (permeating CPA), and ice blocking polymers (X or Z-1000, non-permeating CPA). We quantify the effect of CPA concentration on the amount of ice formation by the rapid freezing. Our results align with expectations that by increasing the CPA concentration, the amount of ice formation decreases including ice recrystallization. Ice recrystallization increases from 0% to 40% CPA, and after that decreases significantly. The relative amounts of ice recrystallization to the total ice formation are 0.3%, 0%, 67%, 0% in 0%, 20%, 40%, 60% Me_2SO, and 0.3%, 2%, 27%, 49% in 0%, 20%, 40%, 60% glycerol. These results indicate that to eliminate the ice recrystallization, it is not necessary to increase the warming rate for concentrations less than 40% Me_2SO or glycerol after ultrarapid freezing. In conclusion, this knowledge and insight in details by DSC technique can facilitate to design and develop the efficiently fast cooling cryopreservation protocol.

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S116 MOLECULAR MOBILITY AND CONCENTRATION OF PLANT CELLS INTRACELLULAR COMPARTMENTS, AS DERIVED FROM ICE CRYSTAL SIZE MEASURED ON CRYO-SEM MICROGRAPHS

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Cryo-SEM (scanning electron) micrographs present black imprints, corresponding to the ice crystals formed during the cooling process of aqueous solutions within cells and tissues. The etching process (partial sublimation of water from ice crystals at vacuum, inside the microscope) is the responsible of the observed black ice crystal imprints. A first sight of a plant tissue micrograph with subcellular resolution shows how the different subcellular compartments contain crystals of different size. Ice crystal size can be related (for similar cooling rate conditions) to the molecular mobility in the solution before freezing: low mobility allows a larger number of nuclei to form, before all available water is frozen (and, hence, forms smaller crystals), while with higher mobilities, a reduced number of initial nuclei grow to include all water molecules. Molecular mobility (and it opposite, viscosity) are depending on solute type and their concentration. For example, for the same solute composition, mobility will decrease with water content reduction. Intracellular compartments are characterized by having different solute/water ratios, which may change with physiological state.
We propose that the different molecular mobilities/viscosities (as derived from different concentrations/water contents) in subcellular compartments of plant tissues, can be derived or estimated from the

corresponding ice crystal sizes, as observed in cryo-SEM images.
Examples for mint tissue equilibrated to different global water contents and incubated with different solutions are presented. The observation of cryo-SEM micrographs allows easy measurement of ice crystal size and its distribution, with the help of image analysis software. Global water content data and composition for incubation solutions allow estimations of concentrations and motilities, and in spite of the lack of independent data of mobility within each compartment, available information allows at least comparative estimations. The possibilities and limitation of this method, as well as its utility for cryopreservation studies are discussed.

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S117 CHANGES OF SAMPLE TEMPERATURE INSIDE A CRYO STORAGE ROBOT

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Biobanks have become an important infrastructure in the support of scientific research regarding the provision of biospecimens, e.g. blood or tissue. In view of the sample quality, the requirements are constantly increasing in order to guarantee valuable study results. Increasing numbers of biobanks are investing into temperature control systems and fully automated storage robots like the Azenta BioStore™ II. The storage temperature in this system is -80°C but components like the scanner unit and the sample movement system are limited to a minimum temperature of -20°C. To measure the influence of these warmer sections on the samples situated in different positions in the SBS rack during the storage process, we developed a temperature measurement system within cryovials filled with sodium chloride solution. The thus prepared rack with an initial temperature of -80°C was placed in the input-output-module of the robot and automatically transported into a -80°C zone for acclimatization before the tray was moved into different zones of the cryo system.
We measured a temperature increase of up to 20 K during the transfer into the robot. Inside the robot, the maximum temperature increase was below 10 K but during a simulated delay of over 3 minutes inside the scanner the temperature increase was higher (heating rate: ca. 3 K/min). The exposed samples located in the corner of the rack suffered the highest temperature increase.
The results show the importance to reduce the exposure time of the frozen samples in warmer areas. A fast transfer from and into the transport containers with sufficiently low temperatures is crucial to keep the samples at storage temperature. Storage systems with sufficiently low temperatures in every area are therefore recommended.

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S118 CRYOPROTECTIVE ABILITIES OF BARLEY SEED PROTEINS

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In Nature, extremophiles survive harsh environments by employing specialized adaptive strategies. Psychrophilic species

across the biological kingdom produce ice binding macromolecules (proteins and polysaccharides) that allow them to survive at sub-zero temperatures. These ice binding proteins (IBPs) have attracted interest for many years because they possess a remarkable diversity of structures that facilitate ice recognition. These proteins having ice structuring and recrystallization inhibition properties, are known as ice structuring proteins (ISPs). Himalayan region experiences extreme climatic conditions including low temperature and are considered as a 'third pole'. As a result, the flora of Himalayas is expected to be the home of these ISPs as cryoprotective agents. Himalayan wheat and barley provide an edible source of ISPs with recrystallization inhibition properties.

The aim this study is to identify and isolate cryoprotective ISPs from barley. As the protein extract of barley exhibits this property, various purification methods were employed for the isolation of barley AFPs. The sequential extraction for the isolation of seed abundant proteins was performed. The albumins, globulins and prolamin fractions were isolated and subjected to LC-MS/MS for the identification. These fractions were analyzed for their ice recrystallization inhibition (IRI) property using splat assay. Albumins and prolamins did not show any IRI activity. Interestingly, the salt soluble fraction namely globulins of barley when concentrated by lyophilization with high amount of salt (NaCl) showed vitrification during splat assay. The bulk amount of NaCl is not known for its glass transition abilities and it vitrifies only at high pressure. However, it does vitrify under proper confining conditions. Till now, honey is known as a natural vitrifying agent. In the present study, barley globulins are presented as a natural vitrifying agent at a high salt concentration, which will open the prospects of future investigations in this area.

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S119 AN INSECT ANTIFREEZE PROTEIN FROM *ANATOLICA POLITA* ENHANCES THE CRYOPROTECTION OF MAMMALIAN CELLS

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Effective cryopreservation and long-term storage are essential requirements to the commercial and clinical applications of cell-based therapies. Most cryopreservation procedures utilize dimethyl sulfoxide (Me_2SO) as the cryoprotectant, however high concentration of Me_2SO can have harmful effects. Nature employs a variety of compounds and strategies for freeze avoidance and freeze tolerance that enhance the survival of organisms in extreme cold environments. Antifreeze proteins have evolved in numerous species to protect from cellular damage at temperatures below freezing and demonstrated the ability to decrease cellular damage beyond the effects of traditionally used cryoprotectants. Here we present our work on the impact of an insect antifreeze protein from *Anatolica polita* (ApAFP752) on mammalian cell cryopreservation using the human embryonic kidney cell line HEK 293T. The antifreeze protein was evaluated for its

cryoprotective effects intra- and extracellulary with or without additional Me_2SO in the freezing medium. Intracellular AFP was introduced to the HEK 293T cells by transfecting them with a plasmid containing the gene for the enhanced green fluorescent protein – antifreeze fusion protein (EGFP-ApAFP752). Cells were grown for 48 h, then they were frozen in a medium supplemented with various concentrations of Me_2SO (0-20%) and cryopreserved at -196 °C for at least 4 weeks. Control experiments included untransfected cells and cells transfected with plasmid containing the EGFP gene only. In another experiment, recombinantly expressed and purified AFP was also added to the freezing medium. Cellular viability, cellular damage, and cellular metabolism were assessed upon thawing. Our results indicate that both intra- and extracellular ApAFP752 significantly improved cell survival after freeze/thaw, especially at low concentrations of Me_2SO, and a combined intra- and extracellular AFP approach provided the most significant enhancement in cryoprotection. These findings present a potential method to improve the viability of cryopreserved mammalian cells.

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S120 IDENTIFICATION OF COLD SHOCK PROTEINS IN HELIX POMATIA SNAILS: THE PUTATIVE ROLE IN OVERWINTERING

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Helix pomatia is considered as a partially freeze-tolerating/avoiding species, but its cold stress response mechanism is still a mystery. Cold Shock Proteins (CSPs), which have a highly conserved nucleic acid-binding domain, play an essential role in developing freeze tolerance in plants and bacteria. As a result, it is reasonable to speculate that, as in the case of heat-shock responses, there are mechanisms for adaptation to cold conserved throughout phylogeny. Given the lack of data on the proteins involved in freeze tolerance/avoidance in snails, we attempted for the first time to determine CSPs expression during freezing exposure in *H. pomatia* using RT qPCR analysis. Total RNA was isolated from hepatopancreas using Trizol reagent, and RNA quantity and quality were determined using a spectrophotometer. The first-strand cDNA was synthesized from 1µg of total RNA using Transcriptor High Fidelity cDNA Synthesis Kit, with gene-specific primers designed using PrimerPlus3 software. To ensure the correctness of the quantification, the expression was normalized to the reference genes. Standard curves for both target and control genes were obtained using a series of cDNA dilutions as a template to determine PCR efficiencies. RT qPCR was performed at least in five biological replicates using Fast Start SYBR Green Master following the standard PCR program. The relative level of gene expression was calculated according to the Pfaffl method. There are clear seasonal differences between CPSs' expression levels in *H. pomatia*. The highest level was recorded in the summer, while the lowest was identified in winter. Interestingly, there are significant differences in CPSs expression between snails with and without operculum during winter. We suppose that

CSPs in snails, similar to those in other organisms, may be responsible for membrane fluidity changes and function as RNA-chaperones during cold stress, but further research is needed in this field.

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S121 CRYOPRESERVATION OF DIVERSE SYMBIODINIACEAE DINOFLAGELLATES: ASSESSMENT OF THEIR FATTY ACID PROFILES IN RESPONSE TO INCREASED SALINITY TREATMENTS

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Symbiodiniaceae are a diverse group of dinoflagellates with endosymbiotic associations with corals, other invertebrates, and protists. They help in the physiological adaptation of corals and influence their ability to resist and recover from stress. Cells of Symbiodiniaceae species are often isolated and grown in the laboratory. However, maintaining these cultures is labor-intensive, expensive and there is constant risk of contamination. Cryopreservation has become an essential tool for the long-term preservation of microalgal cultures. In this study, we attempted to cryopreserve 15 putative species from six Symbiodiniaceae genera using dimethyl sulfoxide (Me_2SO) as the cryoprotectant agent (CPA). Under 15% Me_2SO treatment, nine Symbiodiniaceae culture isolates were successfully cryopreserved. The culture isolates that failed to cryopreserve or had the lowest post thaw viabilities (<15%) in either of the freezing methods were subjected to two different tests: reducing Me_2SO to 10% or increasing medium salinity before freezing. At 10% Me_2SO, *Symbiodinium necroappetens*, *S. microadriaticum*, *S. pilosum* and *Fugacium* sp. were successfully cryopreserved. Successful cryopreservation with high survival rates post-thawing was observed in *S. pilosum* after increased salinity of 44 parts per thousand (ppt) and 54 ppt, while *Fugacium* sp. cryopreserved after 54 ppt and 64 ppt salinity treatments. Fatty acids (FA) analysis of the 54 ppt salinity-treated *S. pilosum* culture isolate showed an increase of 18% in the amount of saturated fatty acids (SFAs) while *Fugacium* sp. had a large decrease of 32% in the amount of poly-unsaturated fatty acids (PUFAs) produced in comparison to normal salinity. Exploring the effects of increased salinity treatment and the role of FAs in successful cryopreservation will help in developing procedures for different Symbiodiniaceae for these ecologically important taxa.

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S122 ACTIVATION OF THE HIPPO PATHWAY IN *RANA SYLVATICA:*

YAPPING STOPS IN RESPONSE TO ANOXIA

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Wood frogs (*Rana sylvatica*) display well-developed anoxia tolerance as one component of their capacity to endure prolonged whole-body freezing during the winter months. Under anoxic conditions, multiple cellular responses are triggered to efficiently cope with stress by suppressing gene transcription and promoting activation of mechanisms that support cell survival. Activation of the Hippo signaling pathway initiates a cascade of protein kinase reactions that end with phosphorylation of YAP protein. Multiple pathway components of the Hippo pathway were analyzed via immunoblotting, qPCR or DNA-binding ELISAs to assess the effects of 24 h anoxia and 4 h aerobic recovery, compared with controls, on liver and heart metabolism of wood frogs. Immunoblot results showed significant increases in the relative levels of multiple proteins of the Hippo pathway representing an overall activation of the pathway in both organs under anoxia stress. Upregulation of transcript levels further confirmed this. A decrease in YAP and TEAD protein levels in the nuclear fraction also indicated reduced translocation of these proteins. Decreased DNA-binding activity of TEAD at the promoter region also suggested repression of gene transcription of its downstream targets such as SOX2 and OCT4. Furthermore, changes in the protein levels of two downstream targets of TEAD, OCT4 and SOX2, established regulated transcriptional activity and could possibly be associated with the activation of the Hippo pathway. Increased levels of TAZ in anoxic hearts also suggested its involvement in the repair mechanism for damage caused to cardiac muscles during anoxia. In summary, this study provides the first insights into the role of the Hippo pathway in maintaining cellular homeostasis in response to anoxia in amphibians.

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S123 PSYCHROPHILES AND PSYCHROTOLERANTS: SUSTAINABLE SOURCE OF BIO-BASED ECONOMY

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Psychrophiles/cryophiles and psychrotolerants are to a great extent unexplored group of organisms with the capacity to flourish in extreme cold conditions. The disclosures of new psychrophiles and psychrotolerants and their enzymes along with their natural adaptation at low temperature will offer novel industrially significant potentials. Extremozymes such as cold-active enzymes are the enzymes produced by psychrophilic/psychrotolerant microbes, they are better at functional level over their mesophilic partners for applications at extraordinary industrial conditions. These enzymes have incredible financial potential in numerous modern procedures, including industrial and pharmaceutical applications. Psychrophiles can also synthesize antifreeze proteins with unique properties of thermal hysteresis and ice recrystallization inhibition that have one of the promising tools in industrial applications like cryobiology, food storage,

and others. Cold-active enzymes/proteins along with its producing microbes are sustainable source that might be better exploited in numerous biotechnological areas towards the expansion of a bio-based economy.

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S124 PHYSICAL DEMONSTRATION OF VITRIFICATION OF LITER SCALE CPA SYSTEMS AND CHARACTERIZATION OF 120KW RF COIL FOR NANOWARMING OF LITER SCALE SYSTEMS

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Organ cryopreservation by vitrification and rewarming have been demonstrated at size scales up to 50 mL in varied organ systems from rat organs (kidney, heart, and liver) to rabbit kidneys. The next step towards preservation of human organs requires scale-up of the vitrification and rewarming techniques (e.g. liter scale and greater). Vitrification is generally achieved through convective cooling which is size dependent and therefore cooling rates are lower internally as the size increases, which can be a major challenge for vitrification of clinical scale organs. This study demonstrates the vitrification of litre-scale volumes (0.5L, 1L and higher) of VS55, M22, and 40%EG-0.6M-sucrose. Nanowarming on the other hand is based upon volumetric heating and is size independent due to its ability to generate heat via magnetic material (iron-oxide nanoparticles) perfused throughout the organ vasculature, as opposed to convective warming where the heat flux is fixed at the boundary. The concentration of these magnetic nanoparticles can be adjusted for achieving higher and sufficient heating at large volumes. These liter volumes of CPAs can be rewarmed uniformly & rapidly using a state-of-art 120KW radiofrequency (RF) coil with magnetic field strengths variable from 0 to 34 KA/m at 360 KHz and having the capacity to uniformly heat volumes up to 2.5L. The magnetic field generated inside the coil is investigated for uniformity along the axial and radial directions along with the characterization/calibration of the field strength versus system power. This study demonstrates critical milestones for scale-up (multi-liter) vitrification and rewarming of biological material and hence moving towards preservation of human organs.

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S125 DEEP EUTECTIC SOLVENTS FOR CRYOPRESERVATION

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Cryopreservation has had huge benefits for the world at large, including preservation of blood and stem cells, and assisted reproductive technologies. However, there

are many cell types that cannot be stored using current cryopreservation methods, and no organs. In fact, 60% of all donated hearts and lungs are discarded due to inadequate storage methods, and this waste could be overcome with cryopreservation.
The main limitation in cryopreservation is the ongoing reliance on predominantly just two cryoprotective agents (CPAs), both of which are toxic: dimethylsulfoxide (Me_2SO) and glycerol. The toxicity of existing CPAs means that cells must be frozen immediately after addition of the CPA. These CPAs are inappropriate for tissues and organs because there is insufficient time to penetrate to deeper cell layers, leaving them vulnerable to freezing damage. Thus there is a need for different, non-toxic CPAs with tuneable properties.
Deep eutectic solvents (DESs) are highly tuneable solvents, many of which are non-toxic. To date, only a very few studies have examined the cryoprotective applications of DESs, but these have shown comparable viability of cells stored using DESs compared to those stored using Me_2SO. We have characterised a number of DESs for their thermal properties and interactions with mammalian cells, including toxicity and permeability. We have also investigated the interaction of these solvents with model membranes using x-ray and neutron reflectivity. One DES was then carried forward and tested for its cryoprotective effect on four distinct mammalian cell lines. It was just as effective, and in some cases more effective, than Me_2SO at protecting the cells during cryopreservation.
These results provide new avenues of cryopreservation for cell types which cannot be preserved with existing CPAs. This in turn has wide-ranging benefits, especially in the biomedical field.

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S126 WITHDRAWN

S127 WITHDRAWN

S128 AN AUTOMATIC AND CLOSED SYSTEM FOR RAPID REWARMING OF LARGE VOLUME OF CELL CULTURE MEDIA AND CRYOPRESERVED CELL SUSPENSION

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A rapid rewarming has been shown to be beneficial to prevent potential cell cryoinjury caused by intracellular ice re-crystallization or de-vitrification. While some of the protocols or methods have been established for the rapid rewarming process of small volume of cell suspension, rapid and uniform rewarming of large volume biofluids or cell suspensions remains challenging mainly due to the heat transfer issues. An advanced system on achieving rapid rewarming of the large volume biofluids and cryopreserved cell suspensions with precise temperature control during rewarming process has been developed. To keep at the desired temperature range and precisely control the temperature of these fluids during the rewarming process, an optimal temperature control and monitoring system is required. We have designed and manufactured a system that has a compact design, low overall power consumption while maintaining the efficiency of warming performance for high flow rates. Our preliminary data has confirmed that this system could warm up the cell culture media from 0℃ up to 37℃ with a single pass setup and at the maximum flow rate of 50ml/min. Mathematic modelling and

computer simulations on the same system have also been processed and they matched up with the actual performance of the device. With the promising preliminary data, we are performing the rewarming tests on the large volume of cell suspension after cryopreservation at subzero temperatures. Additionally, the present automatic system utilized an insulated highly efficient heat conduction mechanism combined with specially designed heating tracks for disposable tubing set, which is a closed system preventing potential cell contamination, to integrate the goals of rapid large volume fluids warming, precise temperature control, compact design, and low overall power consumptions.

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S129 VISUALIZING PVS2 COMPONENT PERMEATION INTO ORYZA SATIVA CALLUS CELLS BY COHERENT RAMAN SCATTERING MICROSCOPY

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It is vital to conserve agricultural and endangered plant species and preserve their genetic diversity. Cryopreservation methods have been developed to be an efficient, long-term method to secure crops and/or species that are at risk in field and greenhouse collections. Plant vitrification solutions 2 and 3 (PVS2 and PVS3) have been used since the 1990s to ensure cell survival after liquid nitrogen exposure. However, these solutions are not universally protective, and it can take years to establish a methodology to successfully cryopreserve a new plant species. This work aims to elucidate how the penetrating cryoprotecting components of PVS2 and PVS3 interact with live Oryza sativa (Asian rice) callus cells. Coherent anti-Stokes Raman scattering (CARS) microscopy enables the direct visualization of deuterated dimethyl sulfoxide, ethylene glycol, and glycerol penetrating live cells. Additionally, monitoring the change in CARS intensity within cells as they are exposed to penetrating CPAs gives insight into the rate of penetration of different cryoprotectants.

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S130 DONOR-DEPENDENT VARIABILITY OF OSMOTIC CHARACTERISTICS OF RED BLOOD CELLS OF DIFFERENT SENESCENCE LEVELS

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Cell membrane permeability is critical for red blood cell (RBC) cryopreservation, regulating transport of water and solutes across the cell membrane during osmotic changes. A distribution of RBCs of varying biological age, from less dense/recently matured/young RBCs (Y-RBCs) to dense/senescent/old RBCs (O-RBCs), exists in circulation. This study aimed to compare osmotic characteristics of young and old RBC subpopulations based on membrane water permeability, osmotic fragility, and deformability characteristics. Red cell concentrates (RCCs) from five Caucasian, male donors were separated by Percoll-density centrifugation at 5-6 days post-collection. Unseparated RBCs (U-RBCs) and two subpopulations (32.6%±4.13% fractions) in 0.9% NaCl were exposed to 0.68%, 1.6% and 3.5% NaCl solutions. Hydraulic conductivity (Lp) and changes in RBC volume over time were monitored by measurement of hemoglobin fluorescence intensity using an SX20 stopped-flow reaction analyzer. Additionally, samples were tested for osmotic fragility and deformability under an osmotic gradient (LORRCA).
A clear donor-dependent effect on the membrane permeability of different RBC subpopulations was demonstrated. Lp-values for U-RBCs were nearly all lower than those for Y-RBCs and O-RBCs, with the highest for O-RBCs. Changes in cell volume with NaCl concentration demonstrated the maximal shrinkage for O-RBCs for four out of five tested donors. The same trend was observed for RBC deformability under an osmotic gradient: O-hyper parameter was lowest for O-RBCs, compared to both U-RBCs and Y-RBCs. Additionally, Y-RBCs had consistently higher O-hyper values, compared to U-RBCs, among all donors. Interestingly, mechanical fragility index (MFI), measured by the osmotic fragility test, was the highest for U-RBCs (5.09%±0.15%), while both Y-RBCs and O-RBCs were more resistant to lower solution osmolarity (about 4.7%±0.06%). Thus, donor-dependent variation in RBC hydraulic conductivity and other osmotic characteristics of RBCs of different senescence levels within this donor group, indicate the importance of further investigation on the effect of RBC heterogeneity post-donation on osmotic properties of RCCs.

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S131 DEVELOPING AN OPTIMIZED PRESERVATION SOLUTION FOR THE SUPERCOOLED STORAGE OF RED BLOOD CELLS: A COMPARISON OF TWO EXTRACELLULAR ADDITIVES

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Red cell concentrates (RCCs) stored at 4 °C exhibit a significant decline in quality over the maximum allotted 42-day storage period. To both minimize this quality degradation and extend the allowable storage duration, RCCs could theoretically be stored in a supercooled state. In this proof-of-concept study, we have compared quality characteristics between RCCs stored at 4 °C and – 4 °C, while also evaluating the protection afforded by two extracellular additives known to promote low-temperature membrane stability. 10 CPD/SAGM leukoreduced RCCs (<7 days old) were pooled, split, and resuspended in the novel PAG3M RCC storage solution, or in PAG3M individually supplemented with 27.5 mM to 165 mM of trehalose or PEG400. Percent hemolysis, deformability (EImax), and mean cell volume (MCV) were assessed in samples stored for 42 days at either 4 °C or -4 °C. ATP concentrations were additionally assessed in non-supplemented samples. Improved ATP retention was seen in samples stored at -4 °C following 42 days of storage. Hemolysis in these supercooled samples was however significantly higher, while EImax and MCV were significantly lower. Although trehalose supplementation did not improve outcomes, each tested concentration of PEG400 resulted in higher EI max and MCV relative to the non-supplemented condition. EImax, MCV, and hemolysis of the 110 mM PEG400, -4 °C-stored condition did not differ from that of either the equivalent supplemented condition as well as the non-supplemented condition stored at 4 °C. Although RCC storage at -4 °C would ameliorate aspects of the RBC storage lesion associated with ATP depletion, it is evident that there are other mechanisms of damage at -4 °C that are exacerbated relative to conventional hypothermic storage. The addition of PEG400 alleviates much of this damage, and thus provides both an avenue and justification to further pursue this supercooled RCC storage paradigm.

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S132 PRE-DEHYDRATION BY TREHALOSE ENABLES CRYOPRESERVATION OF HUMAN RBCS WITH UNUSUALLY LOW CONCENTRATIONS OF GLYCEROL

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Cryopreservation of human red blood cells (RBCs) has been used in transfusion medicine for many decades, and so far it remains as the only available method for maintaining an inventory of rare RBC units for unexpected and/or emergency blood transfusion. However, the time-consuming and complex processes for deglycerolization of RBCs after thawing do not permit convenient and rapid transfusion, as make the contemporary cryopreservation techniques unavailable for routine transfusion medicine. This presentation will highlight our step-by-step effort on decreasing the concentration of glycerol required by successful cryopreservation of RBCs. We found that a dehydration process using trehalose (0.4 M) prior to freezing make it possible for high-recovery cryopreservation of RBCs

(freeze-thaw recovery ~95%; freeze-thaw-wash recovery ~84%) by LN2 quenching in EP tubes with only 5% (w/v) of glycerol. We hope that this study will trigger further research on dehydration-driven low-glycerol cryopreservation of RBCs.

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S133 PERIPHERAL BLOOD MONONUCLEAR CELLS: CHALLENGES IN COLLECTION, PROCESSING AND STORAGE OF PBMCS IN A BIOREPOSITORY

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Peripheral blood mononuclear cells (PBMC) are an attractive option in translational science; utilized in biomarker discovery, vaccine development, evaluation of checkpoint inhibitors, mechanism of action studies, tumor antigen and peptide responses, and immune and / or molecular profiling and function assays. Venipuncture and blood collection are standard processes throughout clinical and nonclinical research and separation of PBMC from whole blood may be done in most BSL-2 laboratory settings, yielding samples that can be cryopreserved and batched for future analysis. However, biobanking PBMC are not without their challenges. Every aspect, from collection of whole blood, separation method and storage handling potentially impact the quality and integrity of the cells and ultimately the quality and integrity of downstream assays and data. Age of study subject populations have impacts on cell recovery and influence the frequency of cell subsets. Anticoagulants effect assay performance and cell stability. Separation techniques and media affect the distribution of cell subsets and length of time from collection to cryopreservation influences viability and function. Proper storage is crucial for comparability and reproducibility. Despite these challenges, the demand for PBMC continues to grow and the need for cryopreserved samples with consistent, reproducible quality, is a must. Developing best practices, adherence to standard operating procedures and attention to detail are essential for meeting this undertaking.

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S134 CRYOGENIC ENRICHMENT OF MALARIA PARASITES FROM WHOLE BLOOD

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The development of cryopreservation procedures typically requires optimization of multiple parameters including cryoprotective agent (CPA) identity, concentration, duration of incubation, and cooling and rewarming rates, among others. Optimization of these parameters is especially challenging among heterogeneous specimens, where the unique biochemical and biophysical properties of individual cell types complicate identification of a single protocol that is broadly effective. Here, we have leveraged this confounding principle of cryobiology to develop a protocol that enables selective purification of target cells from whole blood. For proof of concept, spiked *Plasmodium falciparum* parasites, a primary causative agent of human malaria,

were used for this study. Among asymptomatic individuals, malaria diagnosis is complicated by relatively low densities of parasites in blood. Thus, our goal was to selectively preserve *Plasmodium*, while eliminating uninfected blood cells, resulting in enrichment of target cells which may be useful from a diagnostic perspective. To accomplish this goal, we evaluated the effects of CPA toxicity and the rate of heat transfer during cryopreservation. Accordingly, a protocol was developed that leads to recovery of ~5% of human blood cells and ~43% of parasites, an ~20-fold enrichment. This protocol is tunable, where gametocyte enrichment of >100-fold is achievable, though there is an observed tradeoff in overall parasite recovery. While translation of this protocol to point of care presents many challenges, the overall approach of cryogenic purification may prove useful for alternative diagnostic applications.

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S135 BIOBANKING IN CURRENT AND FUTURE CELL THERAPIES

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Cell and tissue therapies have been an essential part of the evidence based clinical practice for decades. From tens of millions blood transfusion events to tens of thousands of allogeneic hematopoietic stem cell transplantations every year, cell therapy saves lives of the patients, virtually without actual therapeutic alternatives. Currently, extensive research and advanced clinical trials continuously go on in the areas of regenerative medicine, immunology, and oncology, broadly exploring the potential of stem cell- and non–stem cell-based approaches, as well as the state of the art gene technologies. From blood and stem cell banks and patient samples biobanks to logistics chains, application of hypothermic temperatures and cryopreservation go hand in hand both with current and with prospective cell therapies. Here we make a brief overview of connections of biobanking with the current and future cell therapies as well as share our own experience in several areas of the field. In particular, we present our data on possibilities of cryopreservation of natural and bioengineered blood components, biobanking of natural and bioengineered multicellular placental constructs, cryopreservation and hypothermic storage of placental derived multipotent stromal cells, as well as application of reprogramming and gene editing in cells derived from the patients with an orphan disease. With this, we highlight the importance and necessity of further development and improvement of existing methods of low temperature preservation for the progress and success of a wide range of cell therapy strategies.

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S136 A MULTINATIONAL TERMIS CONSENSUS ON THE DEVELOPMENT OF PRESERVED CELL-BASED MEDICINES

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Secure cold chains for cell therapeutics is crucial to the effective roll-out of advanced cell-based therapeutics. The expanding array of new technologies and methods under development for preservation of cells and tissues offers great promise for the future. The Tissue Engineering and Regenerative Medicine International Society (TERMIS) has developed a regularly updated resource on Regenerative Medicine and approached the authors to implement new and important chapters on cell and tissue preservation and cold chains. The authors engaged contacts in the Society for Cryobiology, the Society for Low Temperature Biology and the UNESCO Chair at the Institute for Problems of Cryobiology and Cryomedicine. As a result a multi-national and multidisciplinary authorship has been engaged by the TERMIS to consider the methods, challenges and current thinking in delivery of secure cold-chains for cellular therapeutics. Topics covered include fundamental aspects in cell preservation, current preservation methods for different cell types and the requirements for their shipment. The presentation will summarise the group consensus on recovery and viability methods and the potential impact of cryostorage on cellular product function. The work has also addressed what is considered current best practice in each of these areas for a range of cell types, including haematopoietic stem cells, T-cell adoptive therapeutic products, tissue specific progenitor cells (such as MSCs), pluripotent stem cells, whole blood, blood products and tissue scaffolds. The presentation will also consider the development and use of ambient and sub normothermic temperature shipment of unfrozen materials as well as cryopreserved therapeutic product. The translation of new technology into medical practice is a critical step to enable access to reliable and safe cell-based medicines and the presentation will address issues for automated manufacture an assessment of some of the key regulatory issues that need to be addressed specifically for preserved cell therapeutics.

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S137 ANTI-FREEZE PROTEINS IN MESENCHYMAL STEM CELL CRYOPRESERVATION

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This presentation discusses the potential of utilizing naturally occurring anti-freeze proteins(AFPs) as components of cryoprotective solutions cells and tissues. Freshly isolated rat mesenchymal stem cells (MSC) were frozen (controlled rate & flash freeze) in buffers containing AFP from different sources: Lysates of sprout cultures of lolium perenne (grass), secale serale (rye), solanum dulcamara (nightshade) and arachis hypogaea (peanut) were cultivated in bioreactors and AFP expression stimulated by cold exposure or methyl jasmonate. A comparator solution was supplemented with Pseudopleuronectes americanus (arctic fish) derived purified AFP. Overall, we observed that a significant reduction of

Me_2SO content is achievable without loss of post-thaw viability and mesodermal differentiation ability in rat MSC using certain formulations of AFP. I discuss the prospect of sourcing AFP from natural sources for various applications in cryobiology.

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S138 SUSPENDED ANIMATION OF COMPLEX SYSTEMS FOR RESEARCH AND CLINIC

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While we have mastered the preservation of several important cell types, what works for a single cell type in suspension often fails to translate to more complex systems. This is because different cell types have different responses to the same CPA and intact tissue has 3D structural considerations, such as cell-cell and cell-scaffold interactions, that have a profound effect on successful preservation. Yet, cryopreservation of complex systems remains a critical bottleneck in broad areas with vast impacts on research and clinic. For example, organ transplantation is the only curative therapy for end-stage organ failure; however, limited preservation durations have contributed to the global shortage of organs. Other limitations on cryopreservation of complex systems have hampered dissemination of important new technologies that could aid drug discovery, such as organ-on-a-chip, or decrease the costs and risks associated with maintenance of live research models, such as zebrafish. In this capacity, we present progress on a broad range of projects aimed at overcoming challenges in cryopreservation of complex systems, including high subzero preservation of organ-on-a-chip for drug discovery, whole livers and hearts for transplantation, and zebrafish larva to safeguard important research resources. Moreover, we aim to demonstrate how we are leveraging scientific advances in one complex system, such as the zebrafish, and translating them to complementary learnings in seemingly different applications, such as organ transplantation. Finally, in all our cryopreservation efforts, we aim to leverage lessons from organisms in nature that exhibit suspended animation. Inspired by freeze-tolerant animals in nature, partial freezing aims to preserve complex systems in the high subzero frozen state.

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Conflict of Interest: The author has patent applications relevant to this talk and serves on the Scientific Advisory Board for a company focused on developing high subzero organ preservation technology. All competing interests are managed by the MGH and Partners HealthCare in accordance with their conflict-of-interest policies.
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S139 Me_2SO -FREE PRESERVATION OF iPS CELLS AND IPS-DERIVED SENSORY NEURONS

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Induced pluripotent stem (iPS cells) cells are an important cell source for cell therapy and regenerative medicine applications. Effective methods of preservation are needed for this cell type. Current methods using dimethylsulfoxide cannot be used in conjunction with robotic cell culture so new methods are needed. Combinations of sugars, sugar alcohols and amino acids have been shown to be effective in preserving a variety of cell types. A differential evolution algorithm was used to optimize the composition of cryopreservation media for iPS cells. A cooling rate of 1°C/min and a nucleation temperature of -4°C resulted in the greatest post thaw recovery. iPS cells were differentiated to form sensory neurons. The freezing response of the cells was determined as a function of time in culture for Day 5, 7 and 14 using the cryopreservation solution developed for iPS cells. At Day 5, the optimum cooling rate for the cells was 1°C/min but by Day 7 in culture, 3°C/min was the cooling rate associated with optimum post thaw recovery. Changes in membrane fluidity and cell size were monitored as a function of time in culture and the changes in freezing response reflected those parameters. This study demonstrated that cells differentiated from iPS cells exhibited differences in freezing response as a function of differentiation state.

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Conflict of Interest: Hubel has financial interest in BlueCube Bio, a company commercializing cryopreservation solutions.
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S140 ICE CONTROL DURING CRYOPRESERVATION OF HEART VALVES AND MAINTENANCE OF POST-WARMING CELL VIABILITY.

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Vitrification of large tissues has been limited by cryoprotectant cytotoxicity and ice nucleation during convection warming. Investigation of heart valve cryopreservation was employed as a model for development of new methods of tissue preservation based upon vitrification and nanowarming. Porcine pulmonary valves were loaded with cryoprotectant formulations step wise and vitrified in 30 mL cryoprotectant formulation in 3 cm diameter tubes ± 7.6mg/mL Fe nanoparticles ± 0.6M disaccharides, cooled at ~7°C/min to -100°C, and stored at -135°C. Nanowarming was performed in a single ~100 second step by inductive heating within a magnetic field. Controls consisted of fresh and convection warmed vitrified heart valves without nanoparticles. After extensive washing cell viability was assessed by metabolic assay. After loading with DP6 and vitrification in VS55 +Fe + disaccharides the leaflets were well preserved with viability similar to untreated fresh leaflets over several days post-warming in vitro. Convection warmed leaflet viability was not significantly different from nanowarmed leaflets immediately after rewarming, however significantly higher nanowarmed leaflet viability ($p<0.05$) was observed over time in vitro. In contrast the associated artery and fibrous cardiac muscle were at best 75% viable and viability decreased over time in vitro. Supplementation of lower concentration cryoprotectant formulations (VS49 and DP6) with disaccharides for vitrification indicates that reductions in

cryoprotectant concentrations are possible. Furthermore, the best outcomes observed included a post-warming incubation step with an antioxidant, α-tocopherol (vitamin E), and an apoptosis inhibitor, Q-VD-OPH, under physiological conditions. This work demonstrates progress towards control of ice formation and cytotoxicity hurdles by application of nanowarming, disaccharide addition to cryoprotectant formulations, and post-warming supplements. These results also suggest that there is a need for further investigation of vitrification and nanowarming for the thicker heart valve components, where the cryoprotectant concentration achieved is likely less than in thin leaflets and ice induced-damage more likely.

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Conflict of Interest: All authors are employees of the Company
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S141 CRYOPRESERVATION AND POST-THAW CHARACTERIZATION OF DISSOCIATED HUMAN ISLET CELLS

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Cryopreservation will enable access to dissociated human islet cells for investigations to elucidate the mechanisms of diabetes, which affects over half a billion people worldwide. The objective of this study was to optimize the cryopreservation protocol for dissociated islet cells by using an iterative graded freezing approach to examine the effects of cooling profile parameters, cryoprotectant type and concentrations, cooling rate, and the presence of HEPES buffer, as well as the effect of cryoprotectant removal. The procedure that gave the highest membrane integrity post-thaw was used to cryopreserve dissociated islet cells. The thawed cells were compared to their unfrozen counterparts in functional assessments including phenotypic marker analysis by flow cytometry and patch clamp electrophysiology combined with single-cell RNA-sequencing.
We obtained post-thaw cells with high membrane integrity (82.3 ± 1.2%) after cooling them in 10% dimethyl sulfoxide and 6% hydroxyethyl starch at 1˚C/min to –40˚C, storage in liquid nitrogen, rapid thaw, and removal of cryoprotectants by serial dilution. HPi2, an endocrine pan-islet cell marker, was highly expressed in both fresh and cryopreserved epithelial cells, indicating the purity of the islet preparations used and the retention of characteristic surface markers post-thaw.

Although there was substantial variation in total exocytosis, rapid and sustained exocytosis, and ion channel activities in different pancreatic islet cell types, these electrophysiological parameters were not affected by cryopreservation in individual cell types. The overall transcriptome qualities (e.g., number of uniquely mapped reads, and number of detected genes) are comparable between fresh and cryopreserved cells, indicating that the optimized cryopreservation method maintained the quality and stability of the transcriptome in dissociated human islet cells.

The application of cryopreserved individual islet cells extends beyond their use in functional studies and single-cell transcriptomics. Cryopreserved dissociated islet cells may represent another source of viable cells for bioengineering pseudo-islets or islet sheets for disease modeling studies and potential transplant applications.

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S142 FROM CELL TO TISSUE: HYDROGEL ENCAPSULATION BRIDGE THE GAP OF ISLET CRYOPRESERVATION

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Islet transplant has proven to be one promising strategy to achieve insulin independence for type 1 diabetics. However, Islet tissue consisting of multi-layers of islet cells is not conducive to CPA addition/removal and uniform cooling/rewarming, resulting in poor cell viability after cryopreservation. Therefore, effective cryopreservation of islet remains a challenge. To address this problem, we have developed an islet cell cryopreservation strategy based on hydrogel encapsulation and intracellular trehalose delivery. Disperse β cells embedded in alginate hydrogels capsules facilitate heat and mass transfer during slow-freezing cryopreservation, and intracellular trehalose delivery through nano-materials avoid the use of penetrating CPA, providing a non-toxic strategy for islet cryopreservation and obtained high cell viability. From β cells to islet tissues, increased complexity of sample structure brings great difficulties for cryopreservation, just as many other tissues are also hard to preserve while almost all cells can be cryopreserved well. Fortunately, hydrogel encapsulation bridges the gap between the cell cryopreservation and tissue cryopreservation, especially for islet. Functional islet cells like β cells, α cells can be encapsulated into hydrogels beads (with familiar size with islet) for improved cryopreservation and further be transplanted to regulate blood glucose in vivo. The morphology and function of

above cell-hydrogel constructs are close to that of islet tissue, and we believe that by further optimizing the interaction between islet cells and hydrogel to better simulate islet tissue, islet-hydrogel construct could become a preferred option replacing islet tissues for cryopreservation and clinical use.

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S143 CRYOPRESERVATION OF COMPOSITE SCAFFOLDS CONTAINING MAGNETIC NANOPARTICLES FOR TISSUE ENGINEERING

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The adequate preservation of tissue-engineered constructs (TECs) guarantees their availability for medical application. However, the preservation of the cell viability, cell-cell and cell-scaffold interactions is challenging during thawing. In this regard, incorporation of magnetic nanoparticles (MNPs) into a cryoprotective solution or the scaffold with subsequent application of inductive heating (so-called nanowarming approach) could help improving heat transfer and preserving artificial tissue integrity and functionality. This research aims to use engineered scaffolds with MNPs for cryopreservation of TECs based on mesenchymal stem cells. The scaffolds were fabricated by electrospinning from polycaprolactone solutions (140 mg/ml in TFE) and different concentrations (2.5%, 5% (w/v)) of MNPs (magnetite or cobalt ferrite). Human bone marrow mesenchymal stem cells (MSCs) were seeded on the scaffolds at static conditions and cultivated for 14 days before cryopreservation using slow freezing 'in air' approach. For cryopreservation, TECs loading with CPAs containing 10% Me_2SO /20%FBS or 10% Ethylene glycol/20%FBS both supplemented with 0.3M sucrose. Cell metabolic activity was evaluated using a resazurin reduction assay over a period of 7 days after thawing. Cell-cell and cell-scaffold interactions were assessed by confocal laser scanning microscopy and scanning electron microscopy.
The cells seeded on the scaffolds keep their normal morphology, whereas the cell-cell and cell-scaffold interactions were not noticeably affected by cryopreservation procedures. The cell viability was preserved to a higher extent when using Me_2SO rather than ethylene glycol. Scaffolds with magnetite exhibit higher metabolic activity than the cobalt ferrite ones after thawing. The proposed model TECs comprising polymeric scaffolds with incorporated MNPs are promissory for the preservation of MSCs.

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S144 NANOWARMING OF LARGE PORCINE OSTEOCHONDRAL TISSUES: IN VITRO AND SIMULATION STUDIES

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Osteochondral allograft (OCA) transplantation is an effective treatment option for articular cartilage defects. Unfortunately, the limited availability of fresh OCAs prevents the broad implementation of OCA transplantation. Ice-free cryopreservation is a promising strategy to prolong the shelf life of viable osteochondral tissue and therefore help alleviate the OCA shortage. However, ice-free cryopreservation of clinically relevant, large-sized OCAs is challenging. A major hurdle is the difficulty of rewarming cryopreserved OCAs with avoidance of ice crystal formation using conventional convection warming due to the limited warming rate. In this study, we developed a nanowarming and ice-free vitrification method for large OCA preservation (>30ml). Vitrified OCAs were rewarmed either in a warm water bath (convection warming) or preloaded with magnetic iron oxide nanoparticles and warmed in a radio frequency (<1MHz) alternating magnetic field (nanowarming). Our results showed that the heating rate of our method was over one order of magnitude higher than that of the convection rewarming. *In vitro* tissue viability assessment, biomaterial and biomechanical tests were performed to systematically investigate the benefits of nanowarming for OCA preservation. Cell metabolic activity and live/dead staining results demonstrated the superior performances of our method in rescuing live cells and maintaining cell functions, especially at the superficial cartilage layer. Electrical conductivity, confined compression, and microindentation tests further showed that cartilage in the nanowarming group tends to have better material and mechanical properties than the cartilage in the convection warming group, being more like the fresh cartilage. Computational modeling was also utilized to recapitulate the experimental observations, and the simulation results support the depth-dependent preservation observations. This study pioneers the application of ice-free vitrification and nanowarming for large-sized osteochondral grafts. This novel biobanking method for OCA preservation could potentially improve the utilization of the limited supply of grafts and increase the implementation of OCA transplantation.

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S145 CRYOPRESERVATION OF H9c2 CELLS IN SUSPENSION

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Heart disease is one of the major causes of death in Canada and the United States. It is also one of the most expensive diseases to treat. The high frequency of cardiovascular disease—including heart failure—necessitates the availability of a platform as a model (e.g., cardiomyocytes and/or engineered heart tissues) in order to i) study heart disease; ii) investigate the efficacy of heart drugs; and iii) assess drug delivery. Convenient access to this platform helps to improve our understanding of heart disease, reduce the cost of drug development, and decrease mortality due to incorrect medications; and only successful cryopreservation can provide these opportunities. Many researchers have tried to effectively cryopreserve cardiomyocytes due to their importance in clinical applications and research; however, the reports regarding cells' survival and function after-thaw (compared to non-cryopreserved cells) are variable. Here we report optimized protocols to successfully cryopreserve H9c2 (a myoblast cell line derived from embryonic rat heart tissue, which is well-defined and commercially-available; safe in reagents compared to HL-1 that needs norepinephrine in growth media; easy to differentiate into cardiac cells compared to differentiating cardiomyocytes from pluripotent stem cells) as an in-vitro cell line model for cardiomyocytes. We applied an interrupted slow cooling methodology (graded freezing) to investigate the H9c2 response to freeze/thaw processes in the absence of cryoprotectant and/or in the presence of dimethyl sulfoxide (Me_2SO); Me_2SO plus hydroxyethyl starch (HES); or glycerol as cryoprotectants. Our results demonstrate how investigating different cryopreservation parameters such as cooling rate, cryoprotectant type and concentration, procedures for cryoprotectant addition (e.g., cryoprotectant–cell contact time and temperature), and ice nucleation temperature is crucial in maximizing the cell post-thaw viability and reporting an optimized cryopreservation protocol. This study can be a step towards the successful cryopreservation of cardiomyocytes and more complicated cardiac biologics such as engineered tissues; and play an important role in providing accessible, inexpensive cardiomyocytes as a model for heart disease studies, drug efficacy, and drug toxicity.

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S146 USE OF CRYOBIOTECHNOLOGY FOR THE CONSERVATION OF EXCEPTIONAL AUSTRALIAN MYRTACEAE SPECIES

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The Myrtaceae is one of the largest plant families present in Australia and is dominant both structurally and floristically in many diverse ecosystems. Many species are also valuable for the resources they provide, such as timber, oils, and fruits. However, Myrtaceae species in Australia are threatened by Myrtle Rust and in urgent need of conservation. Although many dryland species can be effectively

conserved by seed-banking, woody rainforest species are disproportionally likely to be exceptional species with desiccation- or freezing-sensitive seed that cannot be banked. Species with orthodox seed storage behaviour may also become exceptional species if repeated infection by Myrtle Rust prevents seed set. To date, use of cryobiotechnology for conservation of Myrtaceae in Australia has been limited, particularly for wild species. Six exceptional species were therefore assessed for cryostorage suitability, after successful initiation in vitro. Initial survival and subsequent growth of seeds or embryonic axes was compared after pre-culture on half-strength MS basal medium containing 0.09, 0.4, or 0.8 M sucrose for 48 h and incubation in Plant Vitrification Solution 2 at 0 °C (on ice) for 0-60 min before immersion in liquid nitrogen (LN). Seeds of short-lived *Backhousia citriodora* survived storage in LN but had limited growth. Seeds of freezing-sensitive *Rhodomyrtus psidioides* had very limited survival and embryos of desiccation-sensitive *Syzygium anisatum* and *S. fullagarii* no survival after storage in LN, however all three species produced roots and shoots in all treatments that were not exposed to LN. Embryonic axes of desiccation-sensitive *S. australe* and *S. paniculatum* had some survival after storage in LN but no continued growth. These preliminary results suggest that cryobiotechnology provides a viable alternative for the conservation of exceptional Myrtaceae species, with more research needed to optimise protocols for successful cryostorage.

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S147 CRYOPRESERVATION OF SHOOT TIPS FROM QUERCUS BRANDEGEEI, AN EXCEPTIONAL SPECIES FROM MEXICO USING DROPLET-VITRIFICATION

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Oaks (genus *Quercus*) are trees and shrubs of great importance for human life, and they have a tremendous impact on diverse ecosystems of America, Asia, Europe, and North Africa. However, many oak species are threatened with extinction, and traditional methods, such as seed banks, cannot be used for long-term storage. *Quercus brandegeei* is endangered and endemic to Mexico with limited distribution in southern Baja California, making it urgent to provide long-term ex situ conservation methodologies. Shoot cultures from shoot cuttings of an adult tree were initiated, established, and multiplied before cryopreservation trials. Then, axillary and apical shoot tips from microshoots were subjected to a droplet vitrification method. Shoot tip regrowth was obtained after liquid nitrogen exposure, but our results showed that the axillary meristems are less suitable for the cryoprocedure than the apical shoot tips (12.5 and 62.5 %, respectively). Additional experiments are focused on shoot regeneration. The preculture conditions of donor explants and regeneration media after cryoprocedure, are driven to optimize the cryopreservation protocol and understand this oak species' response to abiotic constraints.

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S148 HARNESSING CRYOPRESERVATION TECHNIQUES AS A PATH FOR CONSERVING ENDANGERED (*ARUM PALAESTINUM*): A WILD MEDICINAL PLANT

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Medicinal plants are the predominant constituents of medicines in most medical traditions. In Jordan, about 485 species belonging to 99 different families were reported as medicinal plants. Unfortunately, a red list has been released recently by the World Conservation Union containing a huge number of Jordan medicinal flora that are currently under threat of extinction. Cryopreservation is one of the most promising ex-situ conservation methods for an unlimited period of time. In this research, cryopreservation techniques were harnessed to conserve an endangered medicinal plant (*Arum palaestinum*) which grows wild in Jordan. *Arum palaestinum* calli were exposed to three cryopreservation techniques (vitrification, encapsulation – dehydration and, encapsulation- vitrification). High levels of calli regrowth (75%) were obtained in the vitrification experiment using one step of desiccation in Plant Vitrification Solution (PVS2) for 20 min. Encapsulation-dehydration experiment revealed that sucrose incubation at low concentration (0.1 M) for 1 day and 1 hour of air dehydration was the most effective in calli preservation. Moreover, the highest survival (90%) and regrowth (78%) were recorded in encapsulation- vitrification experiment when *Arum palaestinum* calli were exposed to 10 minutes of dehydration with PVS2 before cryopreservation with liquid nitrogen. According to our findings, these cryopreservation techniques are highly promising for the long-term conservation of *Arum palaestinum*.

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S149 THERMAL ANALYSIS AIDS IDENTIFICATION OF MORE APPROPRIATE STORAGE TEMPERATURES FOR RAINFOREST SEEDS SHORT-LIVED AT -20°C

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Recent research on Australian temperate and subtropical rainforest plants demonstrated that around 74% of species tested could tolerate the drying necessary for seed banking, but a subset of the desiccation tolerant species were either sensitive to freezing or short-lived in storage at -20°C. We used differential scanning calorimetry to explore seed responses during freezing and thawing that might explain this behaviour and help to identify more appropriate storage temperatures. We used a standard program to reduce seed temperature from 30°C to -160°C, then re-warm to 50°C, at 10°C min^{-1}. We searched for thermal transitions occurring around -20°C that may indicate

instability in storage at that temperature and searched for temperatures outside transition zones that might be more appropriate for storage. We then validated the results for eight species from five families by comparing post-storage germination and survival of seeds dried to equilibration with 15% relative humidity, vacuum sealed in foil packets, and stored at 15, -5, -20 and -192°C for up to 2 years. Analysis of thermal response showed that the storage temperature of -20°C often fell within a range in which frozen seed components began to thaw. Simple cryopreservation of whole dried seeds greatly improved survival compared to storage at -20°C for species such as *Abrophyllum ornans* (Rousseaceae) and *Melastoma affine* (Melastomataceae) while storage at temperatures higher than -20°C was more appropriate for species from several genera in the Myrtaceae.

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S150 RAPID VITRIFICATION STRATEGIES FOR PRESERVATION OF PROTOZOA.

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Protozoan parasite *Cryptosporidium hominis* is a leading cause of child morbidity and diarrhea-associated mortality in developing countries, and a cause of waterborne outbreaks worldwide. Research on *Cryptosporidium* parasites has been impeded by many technical limitations, including lacking culture and cryopreservation methods. Consequently, laboratory strains of *Cryptosporidium* must be maintained by periodical propagation in susceptible animals. This is particularly expensive and time-consuming for *C. hominis*, for which gnotobiotic piglet is the only known model of infection. As a result, only one laboratory isolate of *C. hominis* (Tu502) is maintained worldwide, underscoring its importance for vaccine and drug development. Cryopreservation method is therefore urgent to relief effort of maintenance, prevent catastrophic isolate loss and expedite research. No successful method for cryopreservation of *C. hominis* has ever been reported. It is parasite sensitivity to ice formation and limited permeability to cryoprotectants (CPAs) that precludes utilization of classical methods of slow cooling. To address this gap, a vitrification protocol was considered as an ice-free alternative for cryopreservation. Oocyst permeabilization was achieved either chemically by bleaching or thermally. For proof of concept, we first demonstrated that *C. hominis* is amenable to vitrification by cooling at 250,000 °C/min to -196 °C in a cocktail of 0.5 M trehalose and 30% Me_2SO using 2 µl silica microcapillary. To increase volume of vitrified specimen we developed a 100 µl high-aspect ratio polycarbonate cassette. Its increased thermal mass in comparison to microcapillary was compensated by adjusting CPA cocktail to 50% Me_2SO. Oocysts cryopreserved using cassettes exhibit viability at ~65% and infectivity in gnotobiotic piglets. Patent infection establishes with 1-3 days delay in comparison to fresh oocysts. Vitrification of *C. hominis* in larger volumes will

expedite progress of research and enable standardization of clinical trials. This technology is readily available for translation to other cellular systems.

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S151 COMPLEXITIES IN CRYOPRESERVING THE LATE-STAGE EMBRYOS FROM THREE DIFFERENT ORDERS OF INSECTS

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Insects play a major role in the terrestrial ecosystem and are beneficial to the preservation of ecosystems. Under certain circumstances, insects are inimical to anthropogenic activities while these activities themselves are destabilizing ecosystems leading to loss of both the beneficial and pest insect species. Hence it is vital to preserve the germplasm of both the beneficial and pest insect species. However relatively little progress has been made in the development of easily transferable insect germplasm cryopreservation techniques, especially when compared to the existing procedures for many mammalian and piscine species. Insect embryo cryopreservation has been accomplished until recently only in the late-stage embryos and only in three orders of insects. This is due to classical cryopreservation difficulties such as embryonic water permeability, water content, developmental status, *etc*. However, among the successfully cryopreserved insect embryos, albeit with differing levels of difficulties, there are a lot more commonalities than differences in the vitrification protocol. Most differences are related to the process of permeabilization and circumventing the adaptive mechanisms in the embryo that protects them from environmental assault. This presentation discusses some of the adaptive variations among the insect embryos that either facilitates or disrupts attempts to cryopreserve them. Furthermore, the techniques used to design the cryopreservation procedures for insects belonging to the orders Diptera, Lepidoptera and Hymenoptera are discussed. Finally, the working components of the cryopreservation protocol specifically designed to counter the ecological adaptations of insect embryos will be discussed.

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S152 RAPID JOULE HEATING IMPROVES VITRIFICATION BASED CRYOPRESERVATION

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Cryopreservation by vitrification has far-reaching implications such as banking and supplying "off-the-shelf" biological systems for cell therapies, transplantable tissues, research model organisms, and bioconservation. However, rewarming techniques that are rapid and scalable (both in throughput and biosystem size) for low

cryoprotective agent (CPA) concentrations for reduced toxicity are lacking, limiting the potential for translation. Here, we introduce an innovative joule heating–based platform technology to overcome this obstacle, whereby biological systems are rapidly rewarmed by contact with an electrical conductor that is fed a voltage pulse from a connected generator. We demonstrate successful cryopreservation of three model biosystems with thicknesses across three orders of magnitude, including adherent cells (~4 μm), *Drosophila* embryos (~50 μm) and rat kidney slices (~1.2 mm) using low CPA concentrations in all cases (2–4 M). Using tunable voltage pulse widths from 10 μs to 100 ms, numerical simulation predicts that warming rates from 9×10^4 to 6×10^8 °C/min can be achieved, a range exceeding any rapid warming method in the current literature. In all cases, we show joule heating greatly improves the viability of all three model biosystems at low CPA concentration compared to conventional convective warming methods. By applying sufficiently rapid warming rates, we further provide direct videographic evidence showing that we can "rescue" biosystems that develop some ice during cooling by outracing the lethal ice crystal growth during warming. Altogether, our results present a general solution to the cryopreservation of a broad spectrum of cellular, organismal and tissue-based biosystems using low CPA concentrations in a scalable manner.

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S153 ROBOTIC PLATFORM FOR AUTOMATED MICROINJECTION OF ZEBRAFISH EMBRYOS FOR CRYOPRESERVATION APPLICATION

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Microinjection is a widely used technique employed by biologists with applications in transgenesis, cryopreservation, mutagenesis, labeling/dye injection and in-vitro fertilization. However, microinjection is an extremely laborious manual procedure, which makes it a critical bottleneck in the field and thus ripe for automation. We have recently developed a computer vision-guided robotic platform that automated the targeted microinjection of zebrafish embryos. A robotic platform uses a series of cameras to image a Petri dish containing embryos at multiple magnifications and perspectives. This imaging is combined with a machine learning algorithm and computer vision algorithms to automatically detect 100s of embryos on a Petri dish and pinpoint a location on the embryo for targeted microinjection. Once located, the robot automatically guides each embryo on the Petri dish to the micropipette for microinjection. Preliminary results indicate

that the robotic microinjection has the potential to significantly increase the throughput as compared to manual microinjection. The performance of an automated microinjection robotic platform is validated by microinjecting the cryoprotectants into the yolk of the zebrafish embryos for cryopreservation, which is a safe and non-toxic method. Survivability of zebrafish embryos in cryopreservation experiments is mainly affected by the variability of manual microinjection, physical damage due to microinjection and toxicity of the solution injected. The experiments to manually investigate these parameters to improve the survivability of zebrafish embryos will be time consuming and laborious. Therefore, we are using this robot to study the effects of microinjection on zebrafish embryos and derived fundamental principles that can be generally applied in other contexts and species. Preliminary results indicate that, automated microinjection robot has potential to increase overall success rate of cryopreservation and laser nanowarming process by 2 times and vitrification rate by 10%. In the future, this robotic platform can be used to perform high throughput microinjection experiments and it can be extended to automatically microinject a host of model organisms such as fruit fly (*Drosophila melanogaster*) embryos, roundworms (*Caenorhabditis elegans*), mosquito (*Culicidae*) embryos.

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S154 3D PRINTED ORGANISMS FOR HIGH-THROUGHPUT CRYOPRESERVATION

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Cryopreservation of organisms has widespread benefits in areas such as maintenance of endangered species, livestock breeding, food sustainability, and drug discovery. Cryopreservation processes involve continuously assessing the locations of randomly distributed living and moving organisms so they can be collected and transferred to cryopreservation tools with cryoprotectants. The primary method for handling organisms has been manual manipulation. This has resulted in limitations such as low-throughput processes, human error, chemical safety issues, and slow clinical translation. To overcome these barriers, we devised a multi-nozzle 3D printing system that can visualize living and moving organisms and position them into desired 3D spaces. We first developed vacuum-assisted nozzles for printing organisms, which act as the positioners and provide advancements in controllability. The multi-nozzle 3D printing system was enhanced via the integration of vision and laser systems, which act in a closed-loop fashion to continuously adapt to newly updated location information about organisms and target spaces. We demonstrated that our 3D printing system can position various organisms in target locations with testbeds of zebrafish embryos, shrimp embryos,

shrimp larvae, and beetles. In addition, we demonstrated that 3D printing of organisms with cryoprotectants can enhance cryopreservation throughput with testbeds of zebrafish and shrimp embryos. We envision that our 3D printing method can manipulate and interface organisms with current cryopreservation devices and also inspire new devices for high-throughput cryopreservation.

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S155 IMPROVEMENT OF SLOW FREEZING PROTOCOL FOR ADULT *CAENORHABDITIS ELEGANS*

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Caenorhabditis elegans is an important model for studies of memory mechanisms. Although in some studies larvae and adults are used indiscriminately, there are interesting features of it that are only present in the adult stage. This is due to adults have a complex neural network, allowing the evaluation of short-term and long-term memory assays. Therefore, the possibility of its cryopreservation in this status would be of great impact, especially in neuroscience.
However, when it carries out the standard protocol stablished by Brenner and based on slow freezing and 15% Glycerol the recovery rate is very low, below 3%, making it impractical. On the other hand, ultra-rapid vitrification gives survival rates above 90%, but the handling is completely different from the common procedure. In addition, by means of this last technique can be cryopreserved a small number of nematodes, which makes large-scale studies impossible. These facts are translated into a constant search for new cryopreservation alternatives.
For this reason, in this work we have managed to improve the slow freezing protocol. It has been possible not only to increase the number of recuperated larvae, but also the adults, which have a better appearance and are capable of performing all their vital functions. Two cryoprotectant solutions were tested: one with Ethylene Glycol (EG) and other with this diol and Trehalose (TRE). Compared both results, our final approach is based on cold preincubation at 3-4 °C and a cryoprotectant cocktail with 7'5% Ethylene Glycol and 0'5 M Trehalose. The adult recovery rates achieved are around 15%. With this survival, experiments focused on *C. elegans* adults cryopreserved by slow freezing are now feasible.

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S156 THE ROLE OF LAPAROSCOPIC APPROACH IN CRYOABLATION THERAPY FOR INTRA ABDOMINAL MALIGNANCY

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The first cryosurgery for cancer was performed in 1850 by James Arnott in England . In the past , the only way to apply cryo surgery to organization to the body was to perform the open surgical approach. Improvement of cryoapparatus, introducing the finer diameter of the cryo-probes, better imaging system and especially skill improvement, led to the rapid development of the percutaneous approach the late of 20th century. Meanwhile since early 1990s, laparoscopic procedures have developed revolutionary .The first laparoscopic assisted hepatic cryotherapy for liver tumor reported in 1995 by Cuschery. Today ,three ways are available for performing the cryotherapy for intra abdominal malignancy: Percutaneous, open, and laparoscopic approaches. Open approach is still the regular way for cryoablation of voluminous or complex tumor. Percutaneous cryosurgery is generally accepted as therapy of choice as the most minimally invasive approach ,but a special skill with long learning curve is required. The rationale of using laparoscopy in cryosurgery is due to the advantages of minimally invasive surgery techniques an excellent tool for diagnostic and therapy. This technique allows monitoring the whole process of cryoablation to improve the patient safety in more complicated cases and the possibility to perform other surgical procedure, like colon resection simultaneously. In the recent years, more advanced development in laparoscopic devices has been achieved especially in imaging system for better visualisation, application of indocyanine green fluoroscopy, tele surgery, and robotic surgery. These will support the further increasing role of laparoscopic approach in cryosurgery for intraabdominal malignancy.

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S157 COMPARATIVE ANALYSIS OF CRYOABLATION WITH SINGLEHOLE AND MULTIHOLE NOZZLE IN CRYOSPRAY

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A Multihole nozzle (MHN) with three holes arranged in a triangular pattern is used in the present study to spray the cryogen (liquid nitrogen) from cryogun (CS-1) procured from SMT Praha, Czech Republic. It is an attempt to establish MHN in cryospray for the treatment of larger lesions (greater than 15 mm in diameter). Special emphasis is placed on optimizing the spraying distance for MHN through in-vitro experiments. In this perspective, three spraying distances of z = 13 mm, z = 18 mm, and z = 23 mm are selected to spray the cryogen. A single freeze-thaw cycle with a freezing duration of 120 s and a thawing duration of 130 s is used in the study. The results of cryoablation are compared with the conventional technique of cryoablation for the same spraying distance. It has been observed through the temperature contours of thermal images that the area of necrotic zone with MHN on the surface of gel is 39 % larger than the area of the necrotic zone with single hole nozzle (SHN) for the same spraying distance. It means lesions up to a diameter of 26 mm can be easily treated through the present approach. It is interesting to note that with the increase in spraying distance the spray cone increases but the cooling capacity of cryospray decreases and vice-versa. It causes an increase in the halo diameter which is unfavorable for the successful treatment. Hence, the spraying

distance of 18 mm is providing the most optimized results in terms of cryoablation

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S158 PREVENTION OF THE INSTABILITY OF THE DORMANT STATE OF TUMOR CELLS IN CANCER STANDARD DIAGNOSTICS

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The well-known problem of the stability of the dormant state of tumor cells, which can be activated and begin active reproduction due to a still unknown mechanism, is considered. It has been shown that such a mechanism can be a threshold change in the intensity of the processes of spatial migration of these tumor cells, for example, during invasive diagnostic interventions in clinical oncological practice. Our numerous animal and clinical studies *in vitro* and *in vivo* were previously published in the world scientific professional literature point by point using active-passive freezing-thawing cycles under ultra-low temperatures. Initially the hypothetical prototypical human malignant tumor model was created for this study based on personal long-time fundamental theoretical, experimental, and clinical research on ultra-low temperatures in biomedical science and its practical application within surgical oncology concerning diagnostics and prevention of malignant diseases. A discrete set of ranges of changes in the values of the diffusion coefficients, which determines the intensity of cell migration, in which diffusion instability of tumor cell dormancy occurs, has been established. The experimental and clinical investigations *in vitro* and *in vivo* paved the way for a mathematical estimation of the standard tumor diagnostics and cryodiagnostics in living biological matter. The use of an ultra-low-temperature technique namely cryodiagnistics for carrying out standard diagnostic procedures in surgical oncology is proposed, which can prevent the risk of occurrence of diffusion instability of the tumor dormant state.

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POSTER PRESENTATIONS

P1 EXPOSURE TO FLUCTUATING TEMPERATURES MODULATES TRANSCRIPTS WITH RELEVANCE TO SMALL NON-CODING RNAS IN THE COLORADO POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA*

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Cold-hardy insects, including the potato pest *Leptinotarsa decemlineata*, can cope with sub-zero temperatures during the winter months. Such adaptation is associated with molecular changes that notably require tight regulation of gene expression. Small non-coding RNAs, including microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs), have been characterized to various extent for their impact on protein expression and are modulated in different models of cold adaptation. We thus hypothesize that small non-coding RNAs, and key players involved in their synthesis, could play a role in the overwintering survival of cold-hardy insects. To test this hypothesis, miRNAs were isolated from cold-exposed (-5 °C) and control (15 °C) *L. decemlineata*. High-throughput sequencing was performed to highlight several differentially expressed miRNAs under these conditions such as miR-9a-3p, miR-210-3p, miR-276-5p and miR-277-3p ($P < 0.05$). In addition, transcript levels of Ago1, Ago2, Ago3, Dcr2a, Dcr2b, Expo-5, Siwi-1 and Siwi-2, components of pathways associated with small non-coding RNA production or function, were quantified by qRT-PCR in insects exposed to decreasing temperatures (15 °C, 5 °C and -5 °C) and showed variations, albeit not significant, in the expression of multiple targets assessed. Subsequent RNAi-mediated reduction of Ago2 transcript levels in *L. decemlineata* injected with Ago2-targeting dsRNA, while reducing Ago2 expression in injected insects to levels that were 0.23-fold ($P < 0.05$) the ones observed in control insects, did not impact cold survival. Interestingly, a similar approach revealed changes in mortality in insects exposed to warm temperatures with survival of 70.0 % in control insects compared to 32.4 % in Ago2 dsRNA-injected insects ($P = 0.01$). These results further support the changes in the expression status of small non-coding RNAs in insects exposed to low temperatures and provide preliminary evidence that select targets underlying synthesis of small non-coding RNAs could influence adaptation to temperature changes.

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P2 WITHDRAWN

P3 HYALURONIC ACID AS EFFECTIVE CRYOPROTECTIVE AGENT FOR HMSC CRYOPRESERVATION

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The most widespread cryoprotective agent (CPA) dimethyl sulfoxide (Me_2SO) has a narrow threshold between cytoprotective-to-cytotoxic effect and there are concerns about its biological inertness. Mesenchymal Stem Cells (hMSC) have high therapeutic potential due to their unique attributes - high proliferation activity and pluripotency. The aim is to find a balance between cell cryoprotection and simultaneously mitigate Me_2SO -related adverse effects on hMSCs. Accordingly, we employed the hyaluronic acid (HA), the cytoprotective component of the extracellular hMSC‘s niche, that could provide properties beneficial during stem cell cryopreservation.

To describe HA effect during cryopreservation we compared six different combinations of Me_2SO -reduced cryoprotective media supplemented with pharma-grade high molecular weight HA (HMW-HA) (Contipro a.s.) with the golden standard for hMSCs cryopreservation, 10% of Me_2SO cryomedium. The subtype of hMSC – Adipose-tissue Derived Stem Cells (hADSC) were then cryopreserved via uncontrolled cryopreservation. After storage hADSCs were cultivated for 2 weeks follow-up to characterize the cell count, viability, and surface marker phenotype. Decreased Me_2SO concentration had a consequence of affected survival of hADSCs compared to standard conditions. Enrichment of Me_2SO -reduced cryomedium with HMW-HA led to a rescue effect in cell survival and proliferation. Comparison of surface stem cell phenotype markers prior to and after cryopreservation showed a significant increase of stemness marker CD49f on hADSCs co-cryopreserved with HA in comparison to its controls, while expression of other stem cell markers wasn´t significantly changed.

Our results indicate that HA facilitates a substantial decrease of potential harmful Me_2SO in cryopreservation medium to minimal content. Thus, we conclude that HMW-HA of biotechnology origin represents an effective CPA allowing constitution of animal component-free cryopreservation medium including serum-free requirements. This will be convenient namely for clinical use in human medicine.

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P4 EVALUATING L-PROLINE AS A CRYOPROTECTANT FOR 2 AND 3-D CELL MODELS

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Efficient short- and long-term preservation of cells and tissues is essential for clinical and fundamental biomedical applications. Cryopreservation by slow freezing with 10% dimethyl sulfoxide (Me_2SO) as the cryoprotectant is commonly used for tissues but the post thaw outcomes remain less than ideal as the cryopreservation procedures expose the material to osmotic shock, cell shrinkage, cytotoxicity, membrane rupture, apoptosis, perturbations in cell cycle progression and altered cellular metabolism. In particular, there is a need for efficient banking of 2 and 3-D cell models, such as monolayers and spheroids.

This study used a Human Caucasian lung carcinoma cell (A549) spheroid model to investigate whether exposure to the protective osmolyte, L-proline, could improve post thaw outcomes. We have previously shown that it can aid recovery of monolayers of A549 cells. The spheroids were incubated with 300 mM proline for 24 hours before cryopreservation and then cryopreserved in cryoprotectant without added proline. After thawing the spheroids were cultured in vitro for 24 h to 72 h and then assessed. The spheroids which were incubated with proline for 24 hours before freezing showed enhanced post-thaw viability (252.3 ± 143.6%) and total cell yields 33.3 ± 24.1 % when compared to the non-protected group (100%, 18.2 ± 14.3 %). This suggests that preincubation with the osmolyte L-proline is protective.

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P5 DNA DAMAGE OF ZEBRAFISH SPERM AT DIFFERENT STAGES OF THE CRYOPRESERVATION

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One of the main problems during cryopreservation is oxidative stress, which can lead to possible damage to the DNA. Our objective in this study was to evaluate the DNA damage at different stages of the sperm cryopreservation process, testing different cryoprotectants solutions. Sperm from 100 fish were collected by surgical removal of testis. The sperm was suspended in Hank's solution in a 1:25 ratio (testis:extender). Ten sperm pools (10 males) were diluted in Hank's and distributed among the experimental groups. Four cryoprotectants were tested: Me_2SO (T1), Methanol (T2), Me_2SO +Powdered milk (T3), and Methanol+Powdered milk (T4). Alkaline comet assay and enzymatic comet assay (Formamidopyrimidine glycosylase - FPG and Endonuclease III - ENDO III) were performed on fresh sperm samples, after equilibration (10 min), and after thawing. The samples were stained with ethidium bromide and analyzed the total length (µm) of the comet using Comet Imager 2.2 software. The results obtained demonstrate that the toxicity of cryoprotectants during the equilibrium time increased the DNA damage, especially in the evaluation of the alkaline comet assay. We observed that samples cryopreserved using powdered milk with Methanol (T4: Akaline - 49 ± 6 µm; ENDO III – 62 ± 6 µm; FPG - 73 ± 8 µm) and Me_2SO (T3: Akaline - 65 ± 4 µm; ENDO III – 63 ± 4 µm; FPG - 76 ± 8 µm) showed less DNA damage than Me_2SO (T1: Akaline - 91 ± 7 µm; ENDO III – 82 ± 11µm; FPG - 90 ± 5 µm) and Methanol (T2: Akaline - 85 ± 10 µm; ENDO III – 87 ± 16 µm; FPG – 99 ± 3 µm) when used alone. Thus, the use of powdered milk in the cryoprotective solution of zebrafish sperm reduced DNA fragmentation after the cryopreservation process.

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P6 SPERM MOTILITY AFTER FREEZING STINGLESS BEE SPERM

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Cryopreservation of bee sperm is an alternative for the preservation of these important pollinators that are experiencing a serious population decline. Although there are already protocols for freezing *Apis mellifera* sperm, there are no studies on sperm freezing on stingless bees. Therefore, the present study aimed to test a low-cost, easy-to-perform sperm freezing protocol that maintains stingless bee sperm motility. Semen collection was performed by dissecting the reproductive system of *Scaptotrigona bipunctata*. The vesicles were placed in 20 µL of Hayes solution (0.15 M NaCl, 1.80 mM CaCl2, 2.68 mM KCl, 1.19 mM $NaHCO_3$, pH 8.7, 352 mOsmol/mL, 35°C) and punctured so that the sperm could flow into the medium. From each male (n=10), 40 µL of sperm was homogenized in 200 µL of cryoprotectant solution (0.7 M Me_2SO, 0.9 M ethylene glycol, 0.05 M trehalose, and 10% fetal bovine serum). Samples were packed in 250 µL straws, which were kept for 20 min at 4±0.5°C (cooling rate -0.86°C/min). Then, the straws were exposed to liquid nitrogen vapor for 20 min (freezing rate -4.6°C/min). Samples were thawed at 35°C for 20 seconds. To assess motility, the samples were placed in a Neubauer hemocytometer and observed using a camera (Basler Ace 1440-220 µm) attached to a microscope (100x). Three videos lasting 60 s were recorded from each sample. Motility rate was calculated using the Cell Counter plug-in in the free software Image J. The motility rate was higher ($p<0.0001$) in the fresh sperm (22.83±3.38%) when compared to frozen (7.02±4.54%). Although, the cryopreservation procedure leads to the decrease of sperm motility; this study showed that it allows the preservation of stingless bee sperm with a low-cost and relatively easy-to-perform protocol.

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Conflict of Interest: There are no conflicts of interest
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P7 NEW SOLUTIONS FOR CORD BLOOD UNIT CRYOPRESERVATION

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Cord Blood (CB) is an important source of hematopoietic stem cells (HSC) used in transplantation for patients with rare human leukocyte antigen type. Cryopreservation is used to preserve HSC grafts though freezing and thawing can induce loss of function due to cell lesion. Herein, we compared the capacity of 4 dimethyl sulfoxide (Me_2SO)-free freezing media with the generally used cryoprotectant (CPA), Me_2SO, for HSC cryopreservation. CryoProtectPure STEM 5X (CPP), a new Me_2SO -free CPA, is a novel serum-free cryopreservation medium. Cryosolutions testing results *in vitro* and *in vivo* showed that the best post-thaw cell viability, recovery, and potency of CD45+ and

CD34+ cells were achieved with CPP-STEM followed by CryoScarLess, revealing equal or greater results compared to the control Me_2SO. Conversely, others such as CryoNovo did not provide adequate protection. These findings inspired us to further investigate basic biochemical parameters and post-thaw cell function. Also, to understand why some like CryoProtectPure-STEM (CPP-STEM) do well and others like CryoNovo not, we set to compare the impact of the different freezing media on selected biochemical parameters. The Lactate Dehydrogenase assay revealed that Me_2SO (OD=0.38±0.2) and CPP-STEM (OD=0.35±0.2) affect the cellular membrane integrity and leakiness of KG1 cells in similar fashion (n=3). Surprisingly, the LIPID Peroxidation assay revealed that KG1 cells exposed to Me_2SO had a lower levels of cell membrane oxidative stress than those with CPP-STEM. Preliminary results have detected similar number of autophagic vacuoles/flux in KG1 cells exposed to Me_2SO (MFI=355) or to CPP-STEM (MFI=415) (n=3). The impact of the CPAs on energy metabolism and on autophagy are ongoing. Studying the impact of CPAs on CB cell's biochemical activity will shed new knowledge regarding why some CPAs provide better cryoprotection in CB HSPC than others and perhaps contribute to the development of new alternatives to storage of CB units.

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P8 SPERM CRYOPRESERVATION USING ANTIFREEZE PROTEIN IMPROVED POST-THAW QUALITY AND MRNA STABILITY OF PACIFIC ABALONE *HALIOTIS DISCUS HANNAI*

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Pacific abalone *Haliotis discus Hannai* is a highly commercialized seafood in South-East-Asia and known as soft gold of the Ocean. The present study aimed to determine whether antifreeze protein III (AFPIII) could improve the sperm cryopreservation outcomes as assessed by post-thaw motility, plasma membrane integrity (PMI), acrosome integrity (AI), mitochondrial membrane potential (MMP), DNA integrity, fertility, hatchability, and mRNA abundance level of heat shock protein 90 (HSP90). AFPIII was separately mixed with either 8% dimethyl sulfoxide (Me_2SO), 8% ethylene glycol (EG), 6% propylene glycol (PG), 2% glycerol (GLY), or 2% methanol (MeOH) at a final concentration of 0.1, 1, 10, or 100 µg/ml. Post-thaw motility of sperm cryopreserved with AFPIII at 10 µg/mL was 61.3±2.7% in 8% Me_2SO, 54.3±3.3% in 8% EG, 36.6±2.6% in 6% PG and 51.7±3.0% in 2% GLY which was significantly higher than for sperm cryopreserved without AFPIII (8% Me_2SO: 50.4±3.3%, 8% EG: 44.93±2.9%, 6% PG: 27.7±3.1%, 2% GLY: 43.392±2.93%). Post-thaw motility of sperm cryopreserved using 1 µg/mL of AFPIII with 2% MeOH was also improved relative to that of sperm cryopreserved without AFPIII. The use of 10 µg/mL AFPIII with 8% Me_2SO gave the best overall post-thaw motility, AI (60.1±3.9%), PMI (67.2±4.0%), and MMP (59.1±4.3%). DNA integrity of sperm cryopreserved using 10 µg/mL AFPIII combined with 8% Me_2SO was not significantly ($p > 0.05$) different from that of fresh sperm. Cryopreservation using a combination of AFPIII with 8% Me_2SO improved fertilization and hatching rates as compared to that of cryopreservation without supplementation of 10 µg/mL

AFPIII. Sperm cryopreserved using AFPIII had more HSP90 mRNA than those cryopreserved without AFPIII. Results of the present study suggest that 10 µg/mL AFPIII combined with 8% Me_2SO can be used for large scale cryopreservation of Pacific abalone sperm and for hatchery production.

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P9 CRYOPRESERVATION OF EMBRYOS BSF: COMPARISON OF DIFFERENT FREEZING PARAMETERS

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One of the major challenges in modern industry remains the developing of a widely used biodegradation method for reducing mass of various kinds of organic waste by turning them into valuable protein or oil. Cryopreserved of embryos BSF (Hermetia illucens, Diptera: Stratiomyidae), can be used as technological innovations for long-term stock to produce the larvae for improvement both the biodegradation facilities and preservation of BSF genetic strains for the beneficial insect industry.
Cryopreservation protocols of Drosophila melanogaster embryo and several non-drosophila flies were not directly suitable for use with BSF embryos.
In this work has been compared variety of permeabilization, cryoprotectant agent loading, and rewarming protocol for embryo BSF. It was examined 15-16 stages of developmental after oviposition as the most suitable stage for cryopreservation. Embryos were incubated for 72-74 hours at +25 °C and 120 hours for 21.2 °C. Permeabilization was carried out with mixture of D-limonene, 2-methyltetrahydrofuran and variety of concentration of isopropanol (0.1-0.5 %). It was shown, that using 0.2% isopropanol in this mixture has advantages in the survival of embryos after cryopreservation. High concentration CPA (~52 wt%) was used for dehydration to protect against lethal ice formation during ensuing cooling and rewarming. To achieve the optimal balance of dehydration, we compared the CPA (EG, 1,2-PD) solution containing PVP and different concentration mono- and disaccharides (trehalose, sucrose, sorbitol and mannitol). Under the same weight concentration, EG has proven to have the least CPA toxicity and highest survival post cryopreservation. A combination of permeable and non-permeable CPAs leads to superior post cryopreservation survival, compared to permeable CPAs alone with the same total osmolarity. Using 52 wt% EG + 5 wt% trehalose at +22 °C for 5 min lead to maximum the dehydration and post cryopreservation survival. Replacing trehalose with sorbitol, sucrose or mannitol reduced post cryopreservation survival.

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P10 READY-TO-USE ADHERENT HIPSC-DERIVED NEURAL CO-CULTURES BY VITRIFICATION

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Cell-based assays are becoming increasingly important in the pharmaceutical industry and biomedical research. Especially the demand is increasing for model systems derived from human induced pluripotent stem cells (hiPSCs) used in respective assays (e.g. toxicity screenings). These models offer an improved translatability to the human system compared to currently applied animal (cell) models, which lead to increased risks for patients in clinical studies or even to market withdrawals of drugs in the past. Neural cell types, especially co-cultures of neurons and astrocytes, are in the spotlight of current research as neurological side effects are responsible for numerous exclusions of drug candidates. Even though production pipelines for neural cell systems have been published and their functional properties are constantly being improved, there is still no satisfactory solution for their stock keeping. The models, which are often generated and matured for weeks to months, have to be produced on demand in elaborate workflows and exhibit only a short shelf life. The solution of this logistics issue poses an urgent need. The sticking point for their successful storage is the adherent and networking character of these co-cultures, mimicking human brain physiology in vitro, which cannot be sufficiently preserved by conventional nucleation-based cryopreservation approaches. Therefore, we established a vitrification-based cryopreservation approach in an application-oriented, screening-compliant format of adherent hiPSC-derived neural co-cultures (neurons and astrocytes). To minimize the osmotic shock of the cells to the applied media, two media each were used for vitrification (containing Me_2SO /EG/sucrose) and for thawing. The maintained fate of the cell types has been validated by immunocytochemistry staining, gene expression analysis of neural markers and their functionality by calcium flux imaging upon thawing. The realized vitrification-based process in novel die-casted lab ware provides ready-to-use 96 well format plates of hiPSC-derived cell models e.g. for toxicity screenings in pharmaceutical industry.

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P11 WITHDRAWN

P12 A COMPARISON OF TWO DIFFERENT CRYOPROTECTANTS ON POST-THAW SPERM PARAMETERS AND FERTILIZATION SUCCESS IN TIGER SALAMANDERS (*AMBYSTOMA TIGRINUM*)

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Different cryoprotectants have been found to have species-specific effects on post-thaw sperm parameters such as motility and egg fertilization. This study aimed to compare the effect of dimethyl sulfoxide (Me_2SO) versus dimethylformamide

(DMFA) on tiger salamander (*Ambystoma tigrinum*) post-thaw sperm parameters and fertilization capability. Milt samples from two male *A. tigrinum* were standardized to $1x10^6$ sperm/mL and then mixed 1:1 with stock cryoprotectant for a final concentration of either 10% Me_2SO + 0.5% bovine serum albumin (BSA) or 10% DMFA + 0.5% BSA; samples were frozen in straws (4-5 straws/treatment/animal) for 10 minutes at 10 cm height above liquid nitrogen. Straws were then thawed in a 40°C water bath for five seconds and applied to fresh eggs from three *A. tigrinum* females for *in vitro* fertilization. Post-thaw sperm motility, morphology, and viability as well as fertilization and hatch rates were compared between the two treatments using generalized linear mixed modeling. When comparing the post-thaw parameters, sperm treated with Me_2SO had significantly higher ($p<0.001$) relative post-thaw total motility (40.1 ± 14.3% vs. 20.8 ± 5.0%) compared to DMFA. Additionally, there was no significant difference ($p>0.10$) between Me_2SO and DMFA for relative normal sperm morphology (92.2 ± 9.5% vs. 94.1 ± 8.1%) or viability (12.7 ± 7.6% vs. 9.8 ± 6.0%). Sperm treated with DMFA resulted in 11.6 ± 14.9% fertilization and 7.9 ± 13.8% hatch rates while sperm treated with Me_2SO resulted in 0.01 ± 0.02% fertilization and 0.0% hatch rates. A total of 22 *A. tigrinum* larvae were produced using sperm frozen with DMFA. It is possible that Me_2SO has a negative effect on salamander gametes compared to DMFA thus affecting fertilization potential. Overall, the use of DMFA in combination with BSA is a promising cryoprotectant treatment for salamander sperm to generate new offspring for conservation programs.

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P13 CRYOPRESERVATION OF HUMAN ADIPOSE DERIVED MESENCHYMAL STROMAL CELLS IN VARYING ME2SO CONCENTRATIONS IN A LIQUID NITROGEN-FREE SYSTEM UNDER A QUALITY MANAGEMENT SYSTEM

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Cryopreservation is essential for the long-term storage of cellular products. Typically, cells are cryopreserved in a cryopreservation agent (CPA) containing 10% Dimethyl Sulfoxide (Me_2SO) using liquid nitrogen (LN_2) systems and waterbaths for recovery. Me_2SO is cytotoxic at high concentrations which potentially impacts cells post-cryopreservation. LN_2 can raise issues in terms of contamination, sterility, safety and cost, while also being challenging for laboratory maintenance. Furthermore, there is a need for an alternative to waterbaths due to high variability and risks of contamination. The aim of this study is to investigate the effect of varying concentrations of Me_2SO on the cryopreservation of human adipose derived stromal cells (hADSCs) in LN_2-free systems (LN_2-free Controlled Rate Freezer (CRF) and -150°C Freezer for final storage). Viability, recovery, growth, phenotype and proliferation of the cells was examined. hADSCs were cryopreserved in CPAs at four concentrations (0%, 2%, 5% and 10%). Cells were cryopreserved using a LN_2-free CRF at -2°C per minute and transferred to a -150°C Freezer. hADSCs were recovered with the ThawSTAR CFT2 Cell Thawing System. Viability and recovery were calculated immediately post-thaw. Recovered cells were seeded for

growth, morphology and metabolic activity assessment. Cellular phenotype was assessed via flow cytometry. Viability and growth kinetics showed no significant difference at the 2%, 5% and 10% concentrations. Recovery was variable depending on cell density and Me_2SO concentration. However, metabolic activity evaluation in 5% and 10% CPAs were significantly greater than the 0% and 2% CPAs. This study demonstrates that 5% and 10% Me_2SO are the optimal concentrations to use in CPAs for successful storage of hADSCs. Furthermore, the use of a LN_2-free cryopreservation system with a water-free thawing system is a viable alternative with significantly reduced setup cost, low maintenance requirements, and increased operator safety and control.

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P14 COMPARISON OF THE EFFECT OF FROZEN-THAWED VERSUS FRESH SPERM ON OFFSPRING GROWTH IN TIGER SALAMANDERS (AMBYSTOMA TIGRINUM)

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The integration of sperm cryopreservation into amphibian captive assurance colonies provides a viable method of genetic management for threatened species. However, one fundamental concern with cryoproduced offspring is the rate of offspring development and body condition when compared to individuals produced using fresh sperm. This study analyzes the growth of salamander offspring produced using fresh or frozen-thawed sperm. Male and female A.tigrinum were administered exogenous hormones to elicit gametogenesis. Eggs were collected, fertilized with freshly collected sperm or sperm that had been cryopreserved with 10% N,N-dimethylformamide + 0.5% bovine serum albumin, and monitored through hatching. Once embryos hatched, weekly assessments of total body length were measured to track individual growth. Larvae were maintained in 100 mm x 50 mm glass dishes, with 200 mL of water per dish, and were fed a uniform diet of brine shrimp. ImageJ software was utilized to measure total length of each individual, and an analysis of variance (ANOVA) was used to determine differences in growth between larvae produced using fresh or frozen-thawed sperm. Results showed that there was no significant difference in body length between fresh larvae (n=5) and cryoproduced larvae (n=5) for week 2 ($p=0.55$; fresh: 1.52 ± 0.05 cm; frozen: 1.63 ± 0.05 cm) or week 3 ($p=0.22$, fresh: 2.01 ± 0.09 cm; frozen: 2.25 ± 0.02 cm) post-hatching. However, eggs fertilized with frozen sperm produced significantly larger larvae in week 4 than fresh sperm ($p=0.02$; fresh: 2.38 ± 0.08 cm; frozen: 2.74 ± 0.06 cm). Furthermore, 100% of larvae produced from fresh and frozen-thawed sperm survived through one-month post-hatching and continue to develop. This study indicates that there is minimal difference between initial growth of offspring produced using frozen-thawed or fresh sperm. The lack of deleterious effects on development supports cryopreservation as an effective tool for genetic and population management in captive colonies.

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P15 POST-THAW SPERM MOTILITIES IN SEMI-CAPTIVE VERSUS WILD POPULATIONS OF ENDANGERED CHIRICAHUA LEOPARD FROG (*LITHOBATES CHIRICAHUENSIS)*

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Obtaining high post-thaw sperm motilities following cryopreservation is crucial for achieving optimal fertilization rates for genetic and population management of at-risk species. For cases where sperm is collected and frozen in field settings, it is also critical to account for the environmental conditions of the habitats in which animals reside, as factors such as ambient temperature, seasonality, and proximity to water or food resources may impact important sperm parameters. We cryopreserved sperm from >60 endangered Chiricahua leopard frog (*Lithobates chiricahuensis*) males and thawed a subset of straws belonging to two wild populations (High Lonesome Well, n=3 and Cuchillo, n=6) and one semi-captive population (Ladder Ranch, n=2), in New Mexico to assess the effects of location and management strategy on relative post-thaw sperm motility. Ladder Ranch is considered semi-captive as frogs are housed in outdoor, naturalistic ranariums and receive supplemental food. High Lonesome Well and Cuchillo differ in that they are not under human care and experience different environmental conditions, such as temperature. Reproductive hormones were administered intraperitoneally to induce spermiation and total sperm motility was measured under 40x magnification based on flagellar movement. Sperm samples were frozen in-field using 10% dimethylformamide + 10% trehalose at a freezing rate of approximately -25°C/min, then thawed and remeasured to calculate relative total motility. Generalized linear modeling indicated that post-thaw motilities did not differ between the three populations (p>0.05). However, a trend was observed (p=0.07) between High Lonesome and Cuchillo wild populations, with High Lonesome retaining higher relative post-thaw total motility (M=55.9 ± 14.2%) compared to Cuchillo (M=31.3 ± 26.2%). Given the diverse weather patterns to which wild frog populations are subjected to in the desert southwest, it is possible that differing management practices and geospatial locations influence sperm characteristics for *L. chiricahuensis*, although a larger sample size is needed to confirm this hypothesis.

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P16 CHARACTERIZATION OF THAWING KINETICS IN THREE SAMPLE CONTAINER CONFIGURATIONS

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Engineered T cell drug products are cryopreserved to enable storage and transport and must be thawed for both administration and quality control (QC) testing. This study was designed to characterize temperature for three vial configurations starting from Liquid Nitrogen (LN2) or -80 °C temperatures to determine total thaw time at 37 °C and room temperature (RT). Vial configurations were filled with mock cryopreservation solution, and ranged from 1 mL to 5 mL fill and surface area to volume ratios of 3.9 1/cm to 6.3 1/cm. Filled vials were monitored with thermocouples placed internally in the approximate center of the liquid volume, frozen using a controlled rate slow freezing method, and stored in liquid nitrogen until thawed. Vials were thawed at either 37 °C in a water bath or at RT after being transferred directly from LN2 or after equilibrating to -80 °C. Total thaw time for all vial configurations at 37 °C starting from -80 °C and LN2 occurred in < 2 min and < 5.2 min, respectively. Total thaw time for all vial configurations at RT from -80 °C and LN2 occurred in and < 43.4 min and < 43.6 min, respectively. Statistically significant differences were detected between vial configurations within each thawing condition (RT and 37 °C). This data highlights differences in thawing kinetics that can be used to inform process control and deviation management strategies.

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P17 METABOLOMIC ANALYSIS OF MAIZE POLLEN DURING STORAGE

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Pollen is a defining feature of flowering plants and contributes to the reproductive success of many key agricultural crops. Decades of genetic and molecular research have contributed to our understanding of pollen morphology and development. While pollen samples are prevalent in the fossil record, most pollen types are known to exhibit short timeframes of viability. PowerPollen has recently developed novel proprietary technology to facilitate removing grass pollens from the natural pollination system and keep pollen viable under storage until pollination is needed. An outstanding question in the field is what molecular changes occur within pollen after maturation and before death. To determine if altered metabolic profiles are associated with maize pollen viability we performed quantitative metabolomics of fresh maize pollen at maturity and under storage conditions known to prolong

viability. To assess phenotypic differences in metabolite accumulation patterns associated with maize inbreds we also profiled two unrelated inbred genotypes with three technical replicates. Non-targeted GC-MS based total metabolite profiling identified 969 metabolites in total, including amino acids, fatty acids, sugars, and sterols. To assess whether key metabolite(s) may be associated with mature and/or viable pollen post-storage I have implemented the R-based package MetaboAnalyst 5.0. Key metabolites of interest which are altered in response to pollens storage and/or genotype were identified from univariate and multivariate methods. This study enhances our understanding of pollen biology and will advance our understanding of molecular factors that contribute to reproductive success.

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P18 MEMBRANE PERMEABILITY OF JURKAT CELLS TO CRYOPROTECTIVE AGENTS DETERMINED BY FLOW IMAGING MICROSCOPY

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Long-term storage of cell-based medicinal products is usually done by cryopreservation with cryoprotective agents (CPAs) such as dimethyl sulfoxide (Me_2SO) or glycerol. For successful cryopreservation the membrane permeability of cells to water (L_p) and CPAs (P_s) have to be considered. Both parameters can be calculated based on changes in cell volume upon CPA addition. Commonly used techniques are microscopic set-ups or a Coulter counter system. Both methods have their limitation, e.g., laborious image analysis of a limited number of cells (microscopy) and the inability to discriminate viable cells from other particles (Coulter counter). To circumvent those drawbacks, flow imaging microscopy (FIM) was used and evaluated as novel approach to determine L_p and P_s. The volume change of Jurkat cells upon addition of a CPA solution was captured by FIM. With FIM bright-field images of the sample are captured by a camera while passing by a microscope in a flow-cell. CPA solutions with either 1.4 M Me_2SO or 1.4 M glycerol were prepared in phosphate buffered saline. Jurkat cells were stained with Trypan blue solution to differentiate viable from necrotic cells and images were captured over five minutes. The osmotic response was determined at four temperatures. Viable cell images were selected by applying morphological filters within the instrument software. Changes in cell volume over time were used to calculate L_p and P_s by fitting the data via MATLAB. The permeation into the cells of Me_2SO was faster in comparison to glycerol. A temperature dependence of the membrane permeability could be observed. The determined values can help to define a cell-specific cryopreservation process. The different permeability rates of Me_2SO and glycerol should be considered during loading of cells with CPA. FIM proved to

be a valuable tool to determine the membrane permeability of Jurkat cells to Me_2SO and glycerol.

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P19 A PRELIMINARY STUDY ON OVINE OVARIAN TISSUE DEHYDRATION: EFFECTS OF REVERSIBLE CELL MEMBRANE PERMEABILIZATION AND TREHALOSE EXPOSURE

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Adequate long-term safeguard of reproductive cells and tissues is essential in fertility preservation programs. While this has been achieved using conventional cryopreservation and storage in liquid nitrogen, such a procedure is expensive and leads to cell damage. An alternative could be the desiccation after exposure to trehalose (a sugar known as a xero-protectant) and long-term storage of samples at supra-zero temperatures. The present study aimed therefore to investigate the exposure to a reversible cell membrane permeabilizing agent (digitonin) to incorporate trehalose into follicles before ovine ovarian tissue desiccation procedure. Ovaries from four animals were cut into 52 fragments (0.2 x 0.2 x 0.1 mm). One fragment from each animal was immediately fixed for analysis (fresh control) and the remaining samples were divided into 12 groups according to the digitonin concentrations (0, 1.0, 2.5, 5.0, 7.5 and 10.0 µg/mL) followed or not by incubation in 1.0 M trehalose. After rinsing, the percentage of morphologically normal preantral follicles was evaluated by histology. Preliminary results demonstrated that the percentage of normal primordial, primary and secondary follicles gradually decreased as digitonin concentrations increased (fresh control: 71.4 ± 5.2%; 10.0 µg/mL digitonin: 16.7 ± 7.1%). Exposure of the ovarian tissue fragments to 7.5 to 10.0 µg/mL digitonin, followed or not by trehalose incubation, led to damage in more than 50% of the follicles, which showed abnormal morphology as a result of the treatment. On the other hand, trehalose appeared to preserve follicular morphology in samples exposed to lower digitonin concentrations (2.5 µg/mL digitonin: 58.1 ± 4.2% without trehalose and 63.3 ± 5.9% with trehalose; 5 µg/mL digitonin: 55.2 ± 6.1% without trehalose and 58.3 ± 3.4% with trehalose). In conclusion, our preliminary findings indicate that the optimal digitonin concentrations are below 7.5 µg/mL, and they should be combined with 1.0 M trehalose for better results. Now, we will carry out further analysis of the follicles and ovarian tissue to confirm our findings.

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P20 CRYOPRESERVATION OF RASPBERRY IN VITRO CULTURES BY PVS3 SOLUTION

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The aim of the study was to establish a cryoprotocol for in vitro grown raspberry genotypes without the use of dimethyl sulfoxide (Me_2SO). The exclusion of Me_2SO from the cryopreservation process was due to its potential mutagenic effect, which is contrary to the principle of germplasm preservation. The plant vitrification solution No. 3 (PVS3) was chosen as a vitrifying agent. Two selected raspberry genotypes were tested; ´Tulameen´ and a clone classified as AK. Dissected shoot tips from 14-day-old three-nodal segment cuttings were excised. These were then placed in three sucrose solutions as pre-treatments (24 hours) with concentrations ranging from 0.25, over 0.5 to 0.75 M. They were then placed in loading solution (LS) (20 minutes), and immersed in PVS3 up to 150 minutes at laboratory temperature. Subsequently, the shoot tips were placed on aluminium foil and plunged in liquid nitrogen. Warming was performed by immersion in 1.2 M sucrose solution at 40 °C (20 minutes). Survival and regeneration were optimal in the range of PVS3 solution from 90 to 120 minutes. Regeneration in both genotypes was above 50 % in this time range. The evaluated procedure will be used for the preservation of raspberry genotypes in the national cryobank in Prague Ruzyne and as a cryoknife for virus eradication at selected genotypes stored.

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P21 KRILL OIL MICROENCAPSULATION AT LOW TEMPERATURES

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Changes in lifestyle and eating habits have caused non-communicable chronic diseases to increase considerably. and it is estimated that these represent 66% of diseases worldwide. Krill oil is rich in ω3-fatty acids and astaxanthin and has been shown in various studies to have a variety of functions, including positive effects on cardiovascular diseases, metabolic syndrome, premenstrual syndrome, inflammation, colon cancer, among others. However, it is susceptible to degradation caused by various factors, such as temperature, light, and oxygen, which causes the loss of its functional properties. Due to these, microencapsulation emerges as a suitable technique to protect Krill oil during storage to guarantee its functional properties. In the present investigation, the use of spray-freezing into liquid nitrogen was explored as a new technique for the microencapsulation of Krill oil using whey protein as an encapsulating agent. Different tests were carried out with adaptations made to a mini-Spray Dryer B-290 and an emulsion of krill oil: whey protein (1:4) was spray-freezing into liquid nitrogen (−195.8 °C). Subsequently, the microcapsules formed were lyophilized (process conditions of -80 °C and 0.060 mbar).

An encapsulation efficiency of 96.042% was obtained, which indicates the effectiveness of the encapsulation process. The microcapsules had a particle size of 126.03 ± 4.87 μm and a water activity of 0.108, the particle size was greater than that reported in other methods due to the high adhesion between the microcapsules, however, the low water activity indicates a high stability. On the other hand, the microcapsules presented a content of polyunsaturated fatty acids of 5.430 g/100g (1.33% eicosapentaenoic acid and 1.13% docosahexaenoic acid), an astaxanthin content of 55.004 ± 0.048 μg /g and an antioxidant activity of 0.432+ 0.001 mMTrolox/g, which shows that the use of low temperatures ensures a good conservation of the bioactive compounds during the microencapsulation process.

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P22 THE EFFECT OF FREEZING AND STORAGE TIME ON BACTERIA IN BULL SEMEN

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The ability to survive freezing varies between bacterial species. Semen cryopreservation is optimized for sperm survival rather than bacterial survival; knowledge is lacking regarding how cryopreservation affects bacterial survival. The aim of this study was to determine the influence of cryopreservation and length of storage on bacterial count and survival of different bacterial species. Semen samples were collected from 14 bulls, diluted 1:1 with antibiotic-free semen extender and transported to the laboratory at 5°C overnight. Aliquots of semen were cultured on Plate Count Agar and blood agar plates (5% bovine blood) on arrival approximately 24h after semen collection. The number of bacteria was calculated after incubation for 72h at 30°C. The remaining samples were diluted 1:1 in Brain Heart Infusion (BHI) Broth with 30% glycerol and stored at -70°C. After 6 and 13 days, the samples were thawed and cultured as described for fresh semen. The bacteria were re-cultured on blood agar and incubated for one day at 37°C before identification by Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Data analysis was performed by linear mixed model, repeated measures (IBM Statistic SPSS 26); descriptive statistical analysis was used to evaluate differences between species. There were no differences in total bacterial count between fresh semen and semen frozen for either 6 or 13 days. Thirty-one bacterial species were identified, of which 20 were present on day 1; 15 and 17 bacterial species were identified after 6 and 13 days of storage, respectively. Nine bacterial species were present at every time point; a further 8, 2 and 5 species were identified on only one occasion (day 1, 6 or 13, respectively). In conclusion, cryopreservation of semen does not affect total bacterial count but it does have a variable effect on bacterial survival depending on species.

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P23 EFFECT OF EXTRACELLULAR EXOSOMES ADDITION ON THE POST-THAW VIABILITY OF IN VITRO PRODUCED BOVINE BLASTOCYST

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The development of cryopreservation techniques in bovine embryos has focused on embryo survival and quality, but frozen embryo transfer is not popular globally. The main reason is the low pregnant rate compared with a fresh one. The culture system adapted the bovine oviduct epithelial cell (BOEC)-derived extracellular exosomes (EXOs) in culture media (synthetic oviduct fluid: SOF) that mimicked with in vivo condition of the bovine oviduct. This study used Day-7 expanded blastocysts (EBLs) produced by SOF + EXOs or SOF and compared the cry-tolerance of post-thaw blastocysts. Day-7 EBLs were frozen using a freeze control system (CL-8800i: Cryo-Logic, Australia). The embryos were held at - 7 °C for 5 min, seeded with a cotton-tipped stick dipped in LN2, held for 5 min, and finally cooled down to - 35 °C by - 0.5 °C/min. Subsequently, the straws plunged into LN2. Our study unravels the role of EXOs in improving the re-expansion and hatching potential of post-thawed EBLs. Here, we demonstrated that EBLs cultured in the SOF + EXOs group showed remarkably higher (42.0 ± 1.36%) than in the SOF group (27.9 ± 0.77%). Re-expanded and hatching rate of post-thaw EBLs in the SOF + EXOs group showed significantly higher (97.9 ± 2.4% and 76.0 ± 1.4%) compared to the SOF group (83.9 ± 3.7% and 61.7 ± 1.9%, respectively). The results revealed that ROS fluorescent signal intensities in the SOF + EXOs group EBLs exhibited lower than in the SOF group. The mRNA expression of lipid metabolism genes (PPARα, APOA1, CPT1) in the SOF + EXOs group showed significantly higher compared with the SOF group. Our findings revealed the new function of the EXOs in restoring TE integrity and increasing lipid metabolism that accompanied blastocoel re-expansion of post-thaw EBLs.

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P24 WITHDRAWN

P25 PERMEATION OF INDIVIDUAL CRYOPROTECTANTS AND THEIR DIFFERENT COMBINATIONS INTO MOUSE LIVER TISSUE

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Development of successful tissue cryopreservation methods requires specific knowledge regarding tissue permeation of individual cryoprotective agents (CPAs) and their combinations. The present study was conducted to assess whether the diffusion coefficient of individual CPAs changes when combining two or more CPAs. To this end, mouse liver slices were exposed to 3.5 M concentrations of three permeating CPAs (i.e., dimethyl sulfoxide,

ethylene glycol, and propylene glycol) individually or in combination at room temperature for 15, 30, 45, and 60 minutes. Subsequently, tissue CPA concentrations were determined using a gas chromatography/mass spectrometry (GC/MS) method. Our results show that (1) the GC/MS method allows simultaneous measurement of multiple CPA concentrations in a small tissue sample, (2) dimethyl sulfoxide has a higher diffusion coefficient than ethylene glycol, and propylene glycol, and (3) the CPA diffusivity appears to decrease in mixtures with multiple CPAs. These findings may help development of effective tissue cryopreservation methods.

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P26 VITRIFICATION AND SLOW COOLING CRYOPRESERVATION OF CAT OVARIAN TISSUE: APPROACHES COMPARING DIFFERENT CRYOPROTECTANTS COMBINATIONS

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An alternative to preserve wild feline species threatened by extinction is the cryopreservation of gonadal tissue. Techniques, such as slow cooling and vitrification, allow long-term storage. For the success of both techniques, the choice of cryoprotectants is vital. The present study tested two techniques (vitrification vs. slow cooling) and 3 different combinations of cryoprotectants (V1, V2, V3 [vitrification], or SF1, SF2, SF3 [slow cooling]) to evaluate their potential to improve follicular survival. Ovaries from 10 cats were collected and fragmented (n=7), with one fragment immediately fixed as the fresh control, three vitrified and the other three cryopreserved by slow cooling. For vitrification, solutions consisted of Me_2SO and ethylene glycol (EG) with either trehalose (V1) or sucrose (V2), or Me_2SO with trehalose (V3). For slow cooling, solutions consisted of Me_2SO, EG and fetal bovine serum (FBS) with trehalose (SF1), or Me_2SO and FBS with either sucrose (SF2) or trehalose (SF3). After seven days all fragments were thawed, and chi square tests were used to evaluate differences in histological analysis. Regarding primordial follicles, V1, V3, and SF2 had a higher proportion of morphologically normal (MN) follicles when compared to fresh control. Among treatments, V2 had a higher proportion of MN primordial follicles than V1 and SF1; and V3 was greater than V2 and SF1 ($p<0.05$). For growing follicles, V2 showed a statistically greater proportion of MN follicles than the fresh control, while V3 was lower than the fresh control. Among treatments, V2 had a greater proportion of MN growing follicles than V1 and SF2; and V1 was greater than V3 ($p<0.05$). In conclusion, V2, V3, and SF2 preserved the primordial follicle pool, while V2 and V1 showed benefits for follicular development. Then, V2 seems to be the best protocol to preserve follicles from cat ovarian tissue.

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P27 COMPARISON BETWEEN TWO METHODS OF ERYTHROPOETIN INCUBATION FOLLOWED BY CRYOPRESERVATION/ XENOTRANSPLANTATION OF CAT OVARIAN TISSUE

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In recent years, 25 of the species that belong to the family *Felidae* are threatened by extinction and a creation of germplasm banks represents an option to recover gametes from those species. Thus, it is important to improve ovarian tissue cryopreservation and xenotransplantation techniques to produce embryos *in vitro*. However, there are challenges regarding ischemia/reperfusion injuries promoted by xenotransplantation and Erythropoietin (EPO) has been studied to prevent those injuries and preserve follicles. The present study evaluated the incubation of ovarian tissue immediately placed in EPO before (EPO+CRYO) or after cryopreservation (CRYO+EPO) by slow freezing. The ovaries from 8 healthy cats were fragmented and randomly distributed among three groups: EPO+CRYO, CRYO+EPO and CRYO. In the two groups treated with EPO, the strips were soaked in MEM and EPO (100 IUs) for two hours at 37 °C before (EPO+CRYO) or after cryopreservation (CRYO+EPO), respectively, and in the CRYO group the fragments were only cryopreserved. After one week, the fragments were thawed and xenotransplanted to the subcutaneous dorsal skin of 15 *nude* mice (5 mice/group) who were ovariohysterectomized, and each mouse received 8 ovarian fragments. The grafts were retrieved 7, 14, 21 and 28 days after transplantation. Histological and histochemistry analysis were used for follicle counting, classification, and to assess fibrosis. All the three groups presented fibrosis in all fragments, with no significant differences in the comparisons among them ($p>0,05$). However, the CRYO+EPO group displayed a higher number of primordial and growing follicles at 7, 14 and 21 days after transplantation compared to EPO+CRYO and CRYO groups ($p<0,05$). In conclusion, after cryopreservation EPO promoted follicle survival, preserving the pool of primordial follicles and the maintenance of follicle development into further stages.

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P28 COMPARISON OF METABOLITE SIGNATURES OF SIBLING EMBRYOS INSEMINATED BY CONVENTIONAL IN VITRO FERTILIZATION (IVF) AND INTRACYTOPLASMIC SPERM INJECTION (ICSI) USING SENSITIVITY ENHANCED NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

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There is an increasing trend in ICSI usage irrespective of the etiology demonstrating the overuse of this insemination technique. As ICSI bypasses natural barriers, it is important to understand the embryonic behavior in relation to the mode of insemination in non-male factor infertile patients. Embryo morphology or morphokinetic evaluation give quick but not enough sensitive biomarkers about embryo developmental capacity. Hence, the main objective of this study is to understand metabolic physiology and behavior of sibling human embryos inseminated by IVF and ICSI using metabolomic approach. This prospective study included 19 infertile couples with non-male factor infertility undergoing ART treatment. The sibling matured oocytes from each patient was randomly inseminated by IVF and ICSI. Spent culture media (SCM) collected during 96 hours of culture along with medium control samples were profiled using sensitivity enhanced NMR spectroscopy (800MHz) equipped with cryogenically cooled micro-coil (1.7 mm) probe. A significant reduction in the intensity of pyruvate, citrate, glucose and lysine observed in both IVF and ICSI sibling embryos compared to medium control. Further, histidine and valine level were found lower in ICSI embryos compared to medium control during 96 hours of in vitro culture. Notably, between IVF and ICSI SCM, no significant differences found in metabolite intensities. ICSI in non-male factor does not alter the SCM metabolomic signature during 96 hours of embryonic development.

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P29 CRYOPRESERVATION OF HUMAN KERATINOCYTES BY RAPID FREEZING WITH NON-PERMEANT CRYOPROTECTANT

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Effort was made to design cryopreservation protocol for human keratinocytes (HaCaT) that would rely only upon application of non-permeant extracellular cryoprotectant and simple freezing method without need for a controlled-rate freezer. Since HaCaT cells are subject to intracellular ice formation as well as slow freezing injury, permeant cryoprotectants like Me_2SO are usually needed when conventional slow freezing method (1°C/min) is applied. In our approach, HaCaT cells were cryopreserved in isotonic phosphate buffered saline solution with 10 wt.% polyethylene glycol 400 (PEG 400). Freezing was completed in one step by plunging cells into liquid nitrogen while thawing was performed in 50°C water bath. Post-thaw cell viability assessment by trypan blue exclusion method revealed 75–82% viability, thus comparable result to conventional slow freezing with 10 vol.%

Me_2SO (~80%). Proposed cryopreservation strategy minimizes the slow freezing injury by limited time exposure to freeze-concentrated solution achieved by high cooling rate associated with direct plunge into liquid nitrogen while at the same time the intracellular ice formation is minimized by moderate pre-freeze dehydration. This pre-freeze dehydration is caused by non-permeant nature of PEG 400 which increases only the extracellular solution osmolality. This strategy represents a practical cryopreservation method as there is no need for sophisticated freezing and thawing procedures and PEG 400 can be easily washed out after thawing. Novelty of this approach lies in application of PEG in cryopreservation of HaCaT cells as well as application of non-permeant cryoprotectant of relatively low concentration (10 wt.%) in combination with high cooling rate for this cell line, what represent an alternative to conventional slow freezing protocol or vitrification protocol requiring high (~40 wt.%) concentration of typically permeant cryoprotectant.

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P30 ANALYSIS OF INDEPENDENCE BASED ON ICE NUCLEATION EVENTS AMONG MICROPLATE WELLS

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In an earlier work, it was shown that non-isothermal conditions prevail during multiple freezing processes, e.g freezing in microplate wells. These non-isothermal conditions are due to either systematic or non-systematic temperature variations. The former are due to the position of the samples in the microplate, whereas the latter are due to the release of latent heat of freezing, which diffuses from frozen to adjacent samples changing their cooling rate, a phenomenon we described as neighbouring effect. In order to study the non-systematic temperature variations, we used an approach, adapted from previous studies with animal cells, which was based on the discrimination between independent and dependent nucleation events. In independent nucleations there were not any frozen adjacent wells present, whereas in dependent nucleations, there was at least one adjacent frozen well. The result of the analysis of independence which involved water, *Taxus bacata*, an evergreen shrub and *Hippophae rhamonides*, a deciduous shrub, proved that in the latter case, wells with three or four frozen adjacent wells froze at statistically significant warmer temperatures, than wells with one or two adjacent frozen wells. *Hippophae rhamonides* produces abundant ice nucleators active at warm subzero temperatures, which explains the small variation observed in nucleation points. That small variation together with the large duration of crystallization increases the possibility of neighbouring effect, which was also observed in the two other samples though not so pronounced.

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P31 WITHDRAWN

P32 FOLLICLE ISOLATION FROM HUMAN OVARIAN CRYOPRESERVED TISSUE FOR BIOENGINEERING PURPOSE

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Human ovarian cryopreservation is one of the best techniques for fertility preservation in prepubertal girls and women who do not have enough time to postpone their cancer treatment. Since, there is a risk of malignant cells returning after ovarian transplantation, an alternative approach for follicle isolation from cryopreserved ovarian tissue and applying tissue engineering methods to help this patient group can be important. So, the aim of this study is the isolation of human ovarian small follicles (HOSFs) from human ovarian vitrified strips. Ovarian vitrified strips from 3 transsexual patients were warmed in 4 warming solutions containing HTCM, decreased concentration of sucrose, and 20% human albumin serum (HAS). After chopping the warmed tissues, Collagenase I was used for enzymatical digestion of dense human ovary, and HOSFs were isolated mechanically slowly. Then, after morphological assessment and defined follicles size, Calcein AM and Ethidium homodimer I was utilized as live/dead staining for some follicles randomly to determine the amount of follicle healthy, and their damage rate. Although, the data showed that most isolated follicles depending on their morphological and size were primordial and primary, there were some secondary follicles. live/dead staining confirmed the viability of follicles with green color. Using both enzymatic and mechanical methods together is very crucial for HOSFs isolation from human dense ovary. Collagenase I was shown a suitable enzyme for this purpose and can be used in ovarian bioengineering.

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P33 ASSESSMENT OF MORPHOLOGY ON FROZEN THAWED SEMEN OF KOLBROEK BOARS FOLLOWING VARYING GLUTATHIONE CONCENTRATIONS

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Antioxidants partially improve the negative effects of reactive oxygen species produced during cryopreservation. The aim of the study was to determine the effects of glutathione concentrations (0, 1, 5 and 10 mmol/L) supplementation in boar sperm freezing extender on the morphology of frozen-thawed Kolbroek sperm. Six

ejaculates for each group were collected from three Kolbroek boars, pooled and centrifuged at 800 x g for 10 mins. Sperm pellet were re-extended at a ratio of 1:1 with Fraction A (egg yolk 10 mL + Beltsville Thawing Solution 15 mL) extender. After cooling at 5°C for 60 mins, Fraction B (egg yolk 10 mL + Beltsville Thawing Solution 13 mL + 2% Glycerol) extender at a ratio of 1:1 was added into semen. All straws were cryopreserved in parallel using an automatic controlled-rate freezer at -5ºC/min from +4ºC to – 10ºC; -40ºC/min from -10ºC to -100ºC; -20ºC/min from -100ºC to -140ºC. After thawing (37°C), sperm morphology was determined microscopically on a slide after staining the semen samples with Eosin Nigrosin stain. The data was analysed using one-way analysis of variance. Treatment means were compared using LSD-t test. The results indicated that a difference was recorded in live sperm on fresh/raw semen (82.3±1.6) as compared to the control group (38.8±3.8), 1 mM (28.6±9.8), 5 mM (27.6±7.1) and 10 mM (39.2±6.4). However, dead sperm on fresh semen (12.9±1.7) had lower results compared to the other groups ($P<0.05$). No difference was observed on head, tail, midpiece, proximal droplets and distal droplets in live sperm abnormalities. In conclusion, BTS supplemented with different glutathione concentrations in the freezing extender had reduced live sperm% as compared to fresh semen. However, 1 mM was the optimum concentration of glutathione to be added to the BTS extender for cryopreserving semen from Kolbroek boars.

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P34 THE COMPARISON OF SURVIVAL RATE OF IMMATURE OOCYTES FOLLOWING CRYOPRESERVATION BY SOLID SURFACE VITRIFICATION, CONVENTIONAL STRAW VITRIFICATION OR CONVENTIONAL SLOW FREEZING METHOD

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Cattle oocytes are sensitive to cryopreservation process due to the complex structure and sensitivity to chilling. The objective of the study was to compare survival rate of cattle immature oocytes following cryopreservation by solid surface vitrification (SSV), conventional straw vitrification (CSV) and conventional slow freezing (CSF) methods. Ovaries from cattle were collected from abattoir. The different cryoprotectants (CPAs) concentrations (0, 5, 10, 15% followed by 10, 20 and 30%) of Dimethyl sulfoxide (Me_2SO) + Ethylene glycol (EG) were prepared in Medium 199. During CSF, a programmable freezer was used. For the SSV, the device was placed in the stryfoam box containing liquid nitrogen (LN2) before vitrification of the oocytes. For the CSV method, oocytes were loaded into straws (0.25 mL). Cryopreserved oocytes were then subjected into warming or thawing medium and then incubated in the maturation medium for 22 hours. The oocytes morphology and polar body extrusion was evaluated. Treatment means were compared using LSD-t test. The oocytes with zona cracked (9.0 to 46.1%) were recorded in control groups (cryopreserved without CPA) only ($P <$

0.05). Splitting of oocytes were recorded in EG + Me_2SO groups (ranged from 9.8 to 29.0%) with SSV and control with CSV (7.5%; $P > 0.05$). Changed in oocytes shape was recorded in all the cryopreservation methods and different level of CPA% ($P < 0.05$). Furthermore, the leakage of oocytes contents ranged from 0.0 to 28.9% ($P < 0.05$). The highest oocytes with polar body extrusion was recorded from the CSV (22.5%) method (Me_2SO 15 + 15% EG) and was 0.0% on both CSF and SSV methods ($P < 0.05$). In conclusion, the CSV method was the best method to cryopreserve the cattle immature oocytes and subsequent to polar body extrusion.

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P35 THE UNIVERSAL BEHAVIOR OF VISCOSITY IN SUPERSATURATED AQUEOUS SUGAR SOLUTIONS AND THE CRYOPRESERVATION IMPLICATIONS

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In the last decades, several procedures and techniques have been developed in order to minimize the damage caused by the ice formation in cells and tissues in cryopreservation methods. The use of sugars as cryoprotective agents increases the cell survival because sugar solutions promote natural vitrification. Vitrification is a currently method used for cryopreservation and could be reached either by supercooling or supersaturating. In both cases the viscosity of the solution increases remarkably so is feasible to study this transport coefficient with the theory of simple fragile supercooled liquids. At present there are few equations to describe the temperature dependence of experimental values of viscosity for different sugar concentrations. In this work, we present a physical approach for the description of the viscosity of supersaturated aqueous sugar solutions, i. e., sucrose, trehalose, glucose, lactose and fructose in the glass transition region, in terms of concentration and temperature through a linear equation of reduced variable expressed in terms of the glass transition temperature of the mixture. Our analysis displays a universal behavior for each sugar solution in the supersaturated regime. This result for non-penetrating cryoprotectants could help to pick out the best sugar concentration towards vitrification.

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P36 CRYOPRESERVED SAMPLES OF PERIPHERAL BLOOD MONONUCLEAR CELL (PBMCS) FOR FUTURE RESUSCITATION IN QATAR BIOBANK

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Cell preparation tube (CPT) is used in Qatar Biobank (QBB) to produce viable cells or PBMCs (peripheral blood mononuclear cell) that are being cryopreserved in liquid nitrogen for long-term storage for further research studies. CPT contains an additive (Sodium Citrate) and is an evacuated tube intended for the collection of whole blood to be separated into plasma and the cell layer (buffy coat).

The cell layer goes through several washing cycles that is performed under sterile conditions in the biosafety cabinet, and eventually are cryopreserved using the commercial product that contains Me_2SO at a concentration of 10%. Prior to cryopreservation, samples are introduced to CRF (Controlled rate freezer), where they are gradually cooled in a user pre-defined temperature that is 1°C per 1 minute until -30°C, after that cooling rate is increased up to 5°C per 1 minute until -100°C is achieved, to ensure that the freezing process run gradually to keep the cells, the membranes, and cellular organelles safe and intact. Another method of cooling the cells is by using a pre-cooled cool cell box, in which samples are then transferred to be cryopreserved within 4-24 hours of cool cell use time. This later method has been backed up with a validated method in pararell with CRF, in any contingency situation with the goal to cryopreserve high quality PBMC samples. Finally, viable cells are retrieved upon request by researchers. They are being used to evaluate cell function, conduct immunophenotype analysis by flow cytometry, isolate HIV for subsequent analysis, or for immortalization by Epstein-Barr virus transformation, etc. Additionally, to evaluate the quality of the PBMCs in the repository, a validated pre-thaw and post-thaw viability test is being conducted with 80% viability achieved. This will aid in the planning of future repositories in QBB cryogenic stores, which will ultimately allow for retrieval of best quality cryopreserved samples.

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P37 CRYO-PROTECTIVE ROLE OF GRAPHENE OXIDE ON POST-THAW SPERMATOZOA QUALITY OF CROSS BRED (HF X SAHIWAL) AND MURRAH BUFFALO (*BUBALUS BUBALIS*) BULLS

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Graphene oxide (GO) reduces post thaw growth and recrystallization by curving hexagonal shape of ice crystals during cryofreezing. Present study evaluated GO as cryoprotectant for augmenting sperm viability in dairy bulls. Six ejaculates per bull [three cattle and buffalo each] per treatment were used. Experiment one, semen was cryopreserved in TRIS Egg Yolk Glycerol (TEYG) diluent with GO 0.0125, 0.25, 0.5, 0.1 and 0.2 mg ml^{-1}. Semen freezing was carried out at 30°Cmin^{-1} for temperature drop from 4°C to -15°C and from -15°C to -60°C followed by 50°Cmin^{-1} from -60°C to -140°C, and plunged in liquid nitrogen (-196°C). Experiment two evaluated performance of TEYG semen diluent with combinations of GO (G05 as 0.05; G10 as 0.1 mgml^{-1}) and glycerol (T48 as 4.8; T64 as 6.4%). Experiment three evaluated freezing at 30°Cmin^{-1} [Protocol (PRT)I], 40°Cmin^{-1} (PRTII) and 50°Cmin^{-1} (PRTIII) in the critical temperature fall zone of -15°C to -60°C for glycerol 6.4% and GO 0.05 mgml^{-1}. Post-thaw sperm function and viability traits were assessed by CASA, Hypo-osmotic swelling test (HOST), Eosin-Nigrosin staining and Rose-Bengal staining. Sperm total (TM) and progressive motility (PM), VCL, VSL, VAP, HOST response, and sperm viability increased (p<.05) in semen extender with GO 0.1 and 0.05 mgml^{-1}. Sperm abnormalities were lower (p<.05) in GO

0.025 and 0.0125 $mgml^{-1}$. Sperm viability improved ($p<.05$) in G05T64 and G05T48 than in G10T64. Sperm functional and kinetic traits increased ($p<.05$) in PRTIII as compared to PRTI. Sperm abnormalities were lower ($p<.05$) in PRTII and PRTIII than in PRTI for buffalo, whereas, lower in PRTII than in PRTI for cattle. GO 0.05 and 0.1 $mgml^{-1}$, resulted in better plasma membrane function and viability. Freezing rate of 50°$Cmin^{-1}$ in temperature fall zone of -15 to -60°C was better than 30°$Cmin^{-1}$. TEYG extender with glycerol 6.4% and GO 0.05 $mgml^{-1}$ using PRTII improved semen quality in dairy animals.

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P38 COMPARISON OF ICE NUCLEATION 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 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 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 and extended in Tris citrate egg yolk extender and further equilibrated at 4°C for 4 hours in cold handling cabinet. Filled, sealed and printed straws (0.25 ml) containing 20 million sperms were frozen using programmable freezer by applying freezing rate 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 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 bull (-15.66±0.48°C) as compared to buffalo bulls (-17.58±0.75°C) under similar freezing parameters. Individual motility, viability and membrane integrity was significantly higher in crossbred bulls as compared to buffalo bulls. No significant difference was observed in sperm abnormalities. It was concluded that better post thaw semen quality in crossbred bulls might be due to less period of super cooling during cryopreservation.

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P39 CRYOPRESERVATION HAS DETRIMENTAL EFFECT ON MITOCHONDRIAL MEMBRANE POTENTIAL, DNA INTEGRITY AND OXIDATIVE PARAMETERS OF EQUINE SPERMATOZOA

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The present research work was conducted to investigate the effects of cryopreservation on post-thaw mitochondrial membrane potential, DNA integrity and oxidative stress parameters of stallion semen. Six Marwari breed stallions aged between 4 to 11 years reared at EPC-NRCE, ICAR, Bikaner, India under uniform management conditions were selected. A total of 42 ejaculates from 6 stallions (7 ejaculates from each animal) were collected. Sperm pellets were retrieved after separating seminal plasma by centrifugation with Citrate-EDTA primary extender. The secondary extender Lactose-Glucose-EDTA-egg yolk containing 5% Di-Methyl Formamide was used for deep freezing in liquid nitrogen with final sperm concentration of 150 million spermatozoa/ml.
Mitochondrial membrane potential using JC1 stain, DNA integrity using comet test, reactive oxygen species by high-throughput spectrophotometric assay based on Fenton reaction and lipid peroxidation levels using TCA-TBA reagent were evaluated at pre-freeze and post-thaw stages. The stallion semen cryopreservation is associated with reduced fertility as reflected in present results. The major difficulty in equine semen cryopreservation is individual variability. At fresh stage the percent spermatozoa with high mitochondrial membrane potential were 82.2±1.12%, DNA intact spermatozoa were 94.1±0.36%, ROS levels were 80.75±2.37 hydrogen peroxide units per 2.5 million sperm and MDA levels were 143.57±5.25 nmol/1000 million spermatozoa. At post-thaw stage the percent spermatozoa with high mitochondrial membrane potential were 64.82±1.40%, DNA intact spermatozoa were 91.44±0.37%, ROS levels were 129.8±3.26 hydrogen peroxide units per 2.5 million spermatozoa and MDA levels were 201.13±2.14 nmol/1000 million spermatozoa. Percent reduction in spermatozoa with high mitochondrial membrane potential and DNA intact spermatozoa were 44.34% and 7.32% respectively, from fresh to post-thaw stage. Percent increment in mean ROS and MDA levels were 164.97% and 247.58% from fresh to post thaw stage, respectively. It is concluded that cryopreservation has detrimental effect on mitochondrial membrane potential, DNA integrity and oxidative status parameters of equine spermatozoa.

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P40 INVITRO FOLLICLE MATURATION: INFLUENCE OF FSH SUPPLEMENTATION, TISSUE SIZE AND LENGTH OF CULTURE

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Do tissue size, FSH supplementation and length of culture influence follicle

maturation in vitro culture of bovine ovarian cortical strips (BOCS) in gas-permeable dishes?
What we know:
Oxygen availability inside ovarian tissue has been demonstrated to represent a key factor in follicle health and growth during in vitro culture of bovine and human ovarian cortical strips. Although, strip thickness can limit nutrients and gases diffusion in and out of the innermost tissue zone, the presence of the outer medulla in thick strips could positively affect follicle growth. The role of FSH in progression of primordial to secondary follicles in ovarian culture is still debatable.
Study design:
Bovine ovaries from animals aged 8-24 months were collected at a slaughter house. In each experiment (n=3), BOCS either 0.5mm or 1mm thick were cut with tissue-slicer and chopped into 1x1mm2 strips. BOCS were cultured for 10 or 15 days at the same tissue/medium volume ratio with 0, 1, or 5ng/ml FSH. Follicle stages were assessed by histology and viability was estimated by labelling with live/dead assay.
Result:
Overall, 2314 follicles were analysed (histology, 998; viability, 1316). At day 0 most follicles were primordial (primordial, 89.4%; primary, 8.7%; secondary, 1.9%), and had a high viability (94.69%). The best follicle growth and viability was observed in 0.5mm thin BOCS cultured with 5ng/ml FSH. In particular, when compared to 1mm thick BOCS cultured with 5ng/ml FSH, 0.5mm thin BOCS cultured with 5ng/ml FSH showed a higher and significant proportion of secondary follicles at day 10 (0.5 vs. 1mm, % secondary follicles: 26.5 vs. 10, $P<0.05$) and a significantly higher proportion of viable follicles at day 15 (0.5 vs. 1mm, % viable follicles: 89.4 vs. 60.7, $P<0.01$).
These results demonstrate that smaller BOCS thickness and 5ng/ml FSH supplementation significantly improve the *in vitro* follicle maturation.

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P41 ONE STEP PURIFICATION AND REGULATION OF FRUCTOSE 1,6-BISPHOSPHATASE FROM LIVER OF THE FREEZE TOLERANT WOOD FROG, *RANA SYLVATICA*

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The wood frog (*Rana sylvatica*) undergoes numerous changes to its physiology and metabolic processes to survive the winter months, including adaptations that let them endure whole body freezing. The regulation of key enzymes of central carbohydrate metabolism in liver plays a crucial role in mediating the synthesis and maintenance of high concentrations of glucose as a cryoprotectant during freezing as well as glucose reconversion to glycogen after thawing. The present study characterized the regulation of fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) from wood frog liver during freezing, FBPase being a crucial enzyme regulating gluconeogenesis. Liver FBPase was purified to homogeneity from control and frozen wood frogs by a one-step chromatographic process. Kinetic and regulatory parameters of the enzyme were investigated and demonstrated a significant decrease in sensitivity to its substrate fructose-1,6-bisphosphate (FBP) in liver from frozen frogs, as compared with controls. Immunoblotting also revealed freeze-responsive changes in posttranslational modifications with a significant decrease in serine phosphorylation (by 53%) for FBPase from frozen frogs. Taken together, these results suggest that FBPase is suppressed, and gluconeogenesis is inhibited during

freezing. This response acts as an important component of the metabolic survival strategy of the wood frog. By identifying the strategies of biochemical adaptation that underlie natural freeze tolerance, we can have an accurate and deep understanding of the issues that is necessary in the development of finer cryopreservation technologies, particularly those that could be used for whole organ preservation for transplant medicine and biomedical needs.

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P42 CRYOMICROSCOPIC FEATURES OF DAMAGE TO VARIOUS PLACENTAL TISSUES AND TISSUE ENGINEERING-RELATED STRUCTURES

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Cryopreservation of organs, tissues, and tissue engineering-related structures is of huge importance in modern biology and medicine. Currently, the protocols for cryopreservation of cell suspensions are well developed. Cryopreservation of multicellular structures faces some challenges, including the permeability of cryoprotectants and the preservation of cell-cell contacts and intercellular matrix. To create effective cryopreservation protocols, it is necessary to understand the features of cryodamage to multicellular structures, which depends on their properties. Placental tissues, cells and tissue engineering-related structures are promising objects in regenerative medicine. The aim of this study was to compare the structure-dependent features and mechanisms of cryodamage to multicellular structures of placental derivatives. Various derivatives of human placenta were studied: explants of chorion and amnion (unchanged tissue), placental cell in suspensions, spheroids, alginate-encapsulated. The objects were cryopreserved in DMEM with 10% Me_2SO at 1°C/min. Cryomicroscopic and morphological studies were carried out. Preservation was assessed using MTT assay, resazurin reduction test, fluorescein diacetate (FDA) and ethidium bromide staining analyzed by confocal microscopy. Ruptures of the stroma of the villi during crystal formation with the preservation of the cell viability were reported to be a major type of cryodamage to the placental villi. Minimal damage to fetal membranes consisting of compact connective tissue with a single layer of cells was observed. Spheroids were characterized by alterations of cell-cell contracts during freezing. The most pronounced cryodamage was found during cell cryopreservation in alginate carriers. The ability of Me_2SO to interact with alginate, causing its gelling, was noted. Thus, the features of cryodamage to various multicellular structures of placental origin depend on their structure, features of cell-cell interactions, characteristics of the intercellular matrix and its interaction with a cryoprotectant. These features should be taken into account when developing cryopreservation protocols and creating tissue engineering-related structures with predictable cryosensitivity.

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P43 MODELING THE PROCESS OF MEDAKA FISH EGG DIFFERENTIATION AT LOW TEMPERATURE

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Because about 94% of the world's marine fisheries resources are harvested at the greater rate than they are produced, and further harvesting will deplete these resources, aquafarming is essential for sustainable fisheries. Aquaculture production accounting for more than 55% of the total supply of fishery product, indicates that the share of aquaculture production is expanding, and its demand is growing. However, the amount of fish production largely depends on the hatching season of fish, and the price of fish can fluctuate by a factor of two at a maximum. In response, a method to ensure stable production throughout the year by adjusting the time of hatching is being considered. To achieve this, it is considered useful to store fish eggs in high quality at low temperatures and to shift the hatching point by several months. Many researchers have attempted to preserve fish eggs at low temperatures, but have not been able to do so for long periods of time. This is due to the following reasons. The low temperature may cause mortality due to reduced differentiation ability and toxicity caused by the addition of protective substances. Because fish are ectothermic animals, their differentiation ability and survival rate are affected by low temperatures. In this study, medaka fish eggs (Oryzias latipes) in various developmental stages were stored at various low temperature until they hatched or died to define their hatchability. The developmental stage of each fish egg was observed every day at low temperatures. Using these developmental stage profiles at various temperature, the mathematical model using logistic equation successfully expresses the development of fish egg.

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P44 EXPERIMENTAL SUBSTANTIATION OF THE THEORETICALLY DETERMINED MODE OF 3D CULTURE CRYOPRESERVATION

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There is currently a great need for new models and methods of in vitro analysis on 3D cultures, which can become a "bridge" between 2D monolayer cell cultures and animal models. The topical issue for 3D multicellular objects (spheroids) is the search for methods and approaches to determine their effective cryopreservation. The aim of the work was to study the influence of the cryopreservation mode, calculated on the basis of physical and mathematical modeling, on the functional parameters of spheroids after their defrosting. The study was performed on spheroids obtained under antiadhesive conditions from cells of L 929 line on the 7th day of cultivation. The optimal cryopreservation mode for spheroids was determined with the use of physical and mathematical modeling of permeability processes for water molecules and Me_2SO in spheroids. Standard mode was used as a control. The functional potential of spheroids after thawing was assessed by the migration, proliferative and metabolic activity of cells in the composition of

spheroids. It has been proved that cryopreservation of spheroids according to the mode calculated on the basis of the physical and mathematical model led to an increase in the ability of cells to migrate by 3.3 times, increase proliferation by 38% and led to increase in metabolic activity by 2 times compared to the standard mode.

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P45 SPERMATOGONIAL DENSITY IN IMMATURE TESTICULAR TISSUE OF CANCER-DIAGNOSED PRE-PUBERTAL BOYS UNDERGOING FERTILITY PRESERVATION

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Assessment of pre-freeze spermatogonial quantity (SQ) and its association with age or cancer type can be an important predictor of fertility restoration outcomes, in pediatric males undergoing immature testicular tissue cryopreservation (ITT) for fertility preservation (FP). Cancer-diagnosed pre-pubertal boys (n=14) with risk of infertility were recruited between December 2015-September 2020 at Centre for Fertility Preservation, KMC, Manipal. During ITT cryopreservation, a small part of the pre-freeze tissue fragment was fixed, sectioned to assess the SQ after H&E staining. Spermatogonia in a minimum of 25 seminiferous tubules per section were counted to determine the spermatogonia per tubular cross section (S/T). The median age of the patients was 6 years (4.62-9.38, 95% CI). Patients were either diagnosed with hematological malignancies (64.2%, n=9), or solid tumors (35.7%, n=5). The overall mean±SD of S/T in these patients (2.71±2.58) was comparable with that of normative values (3.26±4.10,p=0.68). However, an age-group comparison showed a significantly reduced S/T in 6-10yrs age range patients (0.70±0.14,n=3;p=0.003); all of these patients were diagnosed with stage II Hodgkin's Lymphoma and one of them expired. The S/T in 3-5yrs (2.4±2.1,n=5;p=0.71) and 11-13yrs (5.1±2.34,n=4;p=1.00) age groups were comparable with the normative values. The ITT appearance was dense and inadequate for spermatogonial quantification in both the patients of ≤1year age. Majority of these patients are alive and in remission (71.4%, n=10) while 2 patients were lost to follow-up (14.2%) and 2 patients have expired (14.2%). This study shows the importance of assessing pre-freeze SQ of pediatric males recruited for ITT-FP, as it can vary with age and cancer type. There was a significant reduction of S/T in boys

of 6-10years age diagnosed with stage II Hodgkin's Lymphoma. These findings, though from a small sample size, can be an important predictor in fertility restoration outcomes. Further studies are required to understand post-thaw SQ and integrity.

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P46 IMMERSION HYPOTHERMIA ACTIVATES PROTEOLITIC REACTIONS IN RAT TISSUES

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Due to the high heat capacity of water, which significantly accelerates the process of heat transfer and causes a rapid decrease in body temperature (Tb), immersion hypothermia (IH) is considered the most dangerous types of natural hypothermia, and its deleterious effect extends to the function of multiple organs, including brain, heart, kidney, and blood, as well systems, including the immune system. Proteinases, whose activity is controlled by specific and nonspecific inhibitors, are involved in the implementation of many cellular processes in normal and pathological conditions, in particular, through the production of active proteins. How IH affects balance between proteinases and their inhibitors still poorly investigated. In 6 months-old male white outbred rats, IH (Tb 27.5±0.5°C) was achieved in the "forced swimming" test (water temperature 0°C, 5 min). In blood serum (BS), cerebral cortex, hypothalamus, lungs, heart, liver, and kidneys, the total proteinase activity (TPA), the activity of nontrypsin-like proteinases (NTLP), and their inhibitors – α-1-inhibitor proteinase (α-1-IP) and α-2-macroglobulin (α-2-MG) were studied by highly sensitive enzymatic methods. The data were statistically processed by the Kruskal-Wallis method. IH led to a sharp activation of TPA in all the tissues studied. In 24 hrs TPA decreased in BS, hypothalamus and lungs, but remained elevated compared to the control levels. NTLP activity increased under IH in the BS, but decreased in the hypothalamus. In 24 hrs the NTLP activity increased even more in the BS and heart. Under IH the α-1-IP activity increased in the lungs, heart, and kidneys. The α-2-MG activity increased in the cerebral cortex, hypothalamus, and heart, and increased even more significantly in all the tissues studied in 24 hrs. Thus, proteinases and their inhibitors are involved in both the short-term and long-term response of the body to IH, mainly due to the activation in the proteinase-α-2-MG system.

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P47 LOW TEMPERATURE PHASE TRANSITIONS IN GREEN MICROALGAE SUSPENSIONS

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Green microalgae Dunaliella salina is a promising source of carotene for the biotechnology industry. Cryopreservation is one of the best ways to preserve important strains of microalgae for a long time without losing their characteristic features. It is known that with a high degree of salinity, Dunaliella salina are capable of glycerol hypersynthesis. Glycerol, which accumulates in Dunaliella salina cells under stress, can act as a natural cryoprotectant. The aim of this research was to study low-temperature phase transitions in suspensions of green microalgae Dunaliella salina, which were cultivated on Ramaraj media with different content of sodium chloride. Phase transitions were studied by the method of differential scanning calorimetry at the heating stage (0.5 deg/min) after rapid cooling (~200 deg/min). Glass transition was registered in microalgae suspensions that were cultured in Ramaraj medium with 1.5 M, 3M and 4M of NaCl. This indicates an effective hypersynthesis of glycerol by Dunaliella salina microalgae. An intense exothermic peak was recorded at the heating stage at NaCl concentrations of 1.5 M and 3M (at -85.1°C and -86.8°C, respectively) almost immediately after completion of glass transition. This indicates low stability of the amorphous state in studied cell suspensions and the need for high heating rates to prevent crystallization processes from the amorphous state. The crystallization peak was not recorded in microalgae suspension with 4M of NaCl. The completion of crystallization of eutectic compositions was registered at the same temperature (≈-54°C) in the cell suspensions at all studied concentrations of NaCl. Two separate melting peaks were registered at 1.5 M of NaCl. They probably correspond to the melting of eutectic compositions and ice melting. An increase in NaCl concentration leads to a decrease in the melting point of ice and a lack of separation of the two melting peaks.

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P48 FROZEN BRAIN EPIGENETICS: REDUCED H3K9 METHYLATION IN WOOD FROG METABOLIC RECOVERY

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Winter survival for wood frogs (Rana sylvatica) necessitates freeze tolerance, which involves strong metabolic rate depression (MRD) and reprioritization of finite anaerobic resources to pro-survival pathways. Suppression of highly energetic processes like transcription and translation are characteristic of hypometabolic states, but specific mechanisms remain elusive. Preliminary studies have highlighted epigenetic controls as critical regulators of extreme stress adaptation, including histone lysine methylation, however investigations into nervous tissues are severely lacking from the current literature. In the present work, we used immunoblotting to examine the relative expression of six lysine methyltransferases (SET1A, SETD7, SETD8, ESET, SMYD2, SUV39H1), two methyltransferase-associated proteins (ASH2L, RBBP5) and one methyl-lysine binding protein (HP1γ) across the freeze-thaw cycle and freeze-associated sub-stresses (anoxia, dehydration) of wood frog brains. Global methyltransferase activities on target histone lysine residues (H3K9, H3K27) were also investigated by enzyme-linked immunosorbent assays. Despite few changes during freezing, our results suggest that hypomethylation of transcriptionally repressive H3K9 may be a key facet of thawed brain metabolic recovery. During thaw, we observed significant ($p < 0.05$) decreases in

trimethylated H3K9 (H3K9me3), H3K9-specific methyltransferase activity and expression (ESET, SUV39H1), as well as levels of H3K9me3-binding co-repressor HP1γ — suggesting coordinated roles at the molecular level. We also note thaw-mediated reductions in SETD7 and SETD8 expression despite unchanged levels of target histone residues, to which we propose non-histone roles. Alleviation of repressive epigenetic controls likely contribute to the resumption of a permissive transcriptional state and may induce necessary repair pathways during thawing.

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P49 HISTONE ARGININE METHYLATION IN THE FREEZE-TOLERANT WOOD FROG, *RANA SYLVATICA*

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The wood frog, *Rana sylvatica*, is a unique animal that garnered attention by biologists because its ability to endure winter under a frozen state. In response to cold, this species switches to a hypometabolic state by a coordinated metabolic rate depression (MRD) that allow the animal to prioritize cellular processes necessary for the frog survival. One of the methods for regulating these processes is through epigenetic mechanisms such as histone modifications. Arginine methylation is catalyzed by a family of enzymes called protein arginine methyltransferases (PRMTs). Methylation of arginine residues on histones has been shown to play a role in the chromatin remodeling and regulates aspects of protein dynamics and functioning in several cellular pathways. Relative protein levels of PRMTs and histone marks were assessed in liver and skeletal muscle of *Rana sylvatica* by western blotting across the freeze-thaw cycle. In liver PRMT1 was found upregulated while PRMT2 and PRMT4 were decreased during freezing. Transcriptionally permissive histone residues (H3R17m2a) were found unchanged during freezing but upregulated during thaw in liver whereas those that silence transcription (H3R8m2s and H3R2m2a) were found unchanged during freeze-thaw cycle. In skeletal muscle, most PRMTs where found unchanged, except PRMT1 and PRMT4 whose protein levels were increased during thaw. All histone marks remained unchanged in this tissue. This preliminary results suggest tissue-specific response of PRMT during freezing and thaw cycle in *Rana sylvatica* liver and muscle.

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P50 PHASE BEHAVIOR OF HYDROGEL BIOINKS FOR CRYOGENIC 3D PRINTING

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Most hydrogel bioinks have not good enough mechanical properties. Scientists investigated various combinations of

hydrogels using both natural and synthetic components to make inks more suitable for extrusion 3D bioprinting. It was found that nanoparticles due to physical and chemical interactions could change the rheological and mechanical properties of hydrogels. Bioprinting on a cooling plate expand the use of soft bioinks. The inks are cooled in two fundamentally different temperature ranges (above or below the crystallization temperature), depending on the tasks. The aim of this research was to study the phase behavior of hydrogel bioinks at temperatures below 0°C. Phase transitions in hydrogels based on low viscosity sodium alginate (2%) and laponite RD nanoparticles (0.3%, 1%, 2%, 2.5% and 5%) without cryoprotectants and with the addition of Me_2SO or glycerol were studied by differential scanning calorimetry. 3D cryoprinter was designed with 30 x 30 x 15 (in mm) printing area. The cryoprinter was used to print multilayer figures of simple geometry with the studied bioinks. At concentrations of laponite in the hydrogel from 2% and higher in the temperature range from approximately -50°C to the onset of ice melting, an exothermic effect was registered. The nature of this phase transition is not completely clear, since the phase behavior of a colloidal system with laponite is very complex and depends on many factors. The addition of Me_2SO to hydrogels based on sodium alginate leads to a decrease in the temperature and enthalpy of melting, however, crystallization (-85.5 °C) and melting of eutectic compositions (-63.4 °C) are developed in the samples. In the presence of both glycerol and Me_2SO at high cooling rates (~200 deg/min), a glass transition was recorded at temperatures of -113.6 and -128.2, respectively.

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P51 BICARBONATE CONCENTRATE STABILITY IN FREEZER

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To reduce waste and enable blinding in randomized controlled trials, it is desirable to store sodium bicarbonate after opening the initial container. Past work has established that diluted sodium bicarbonate solutions are stable at -20 °C for up to three weeks after freezing and thawing. However, trials at room temperature and in refrigerated conditions show that higher concentration correlates with reduced stability. For our research it would be desirable to store dialysis bicarbonate concentrate in its original one gallon container at -20 °C, thaw it as needed, and return it to cold storage afterwards. We have previously had success with this at -80 °C, but the impact of the higher storage temperature is not known for this concentration. We froze a one gallon container of 967.2 mEq/L sodium bicarbonate at -20 °C after opening. Every week, we thawed the closed container overnight by air convection. We then stirred the solution until all precipitates dissolved. We tested bicarbonate concentration using a clinical chemistry analyzer and pH using a dialysis pH meter. According to both metrics, the bicarbonate was stable up to at least seven weeks. We conclude that sodium bicarbonate concentrate can be stored at -20 °C for at least seven weeks in its original one gallon container after the factory seal is broken. Seven freeze/thaw cycles over this period for measurement purposes did not degrade the bicarbonate concentrate. Both pH and bicarbonate concentration do not change significantly.

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P52 THERMAL HYSTERESIS AND ICE RECRYSTALLIZATION INHIBITION ACTIVITIES OF ANTIFREEZE PROTEIN-POLY(VINYL ALCOHOL) CONJUGATES

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Antifreeze proteins (AFPs) can bind onto the surface of ice crystals and arrest their growth. Two parameters to evaluate these functions are thermal hysteresis (TH) and ice recrystallization inhibition (IRI) activity. TH is the difference between the freezing and melting temperatures. In most cases, the melting temperature can be roughly approximated to 0°C, thus TH can be considered a parameter that evaluates the ability to prevent ice crystals growth at lower temperatures. Ice recrystallization is a phenomenon in which large ice crystals grow at the expense of small ones over time at a constant temperature below the melting temperature after crystallization is complete, and IRI is to stop this process. Therefore, IRI activity can be considered a parameter that evaluates the ability to prevent ice crystals growth for longer periods of time. Poly(vinyl alcohol) (PVA) is a water-soluble polymer known for its slight TH and high IRI activity. In this study, AFP-PVA conjugates were prepared by binding several AFP molecules to a single PVA chain. The conjugates can be expected to have high TH and IRI activity due to several expected effects: the simultaneous binding of multiple AFP molecules linked to the PVA chain with the ice crystal surface, the synergistic effects of the antifreeze activity of AFP and PVA, and the interaction of PVA with water molecules according to different degrees of saponification. Therefore, we examine the TH and IRI activities of the conjugates and discuss their potential applications.

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P53 EFFECT OF CRYOPRESERVATION ON PLANT GROWTH AND ROOTS CHARACTERISTICS OF RED BEET (BETA VULGARIS L.) CULTIVAR.

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Our studies of asparagus and tomato plants, grown in field from cryopreserved seeds, have shown an increase in their yield. The aim of this research was to determine the effect of deep freezing on red beet seed germination and yield.
All experiments were carried out in 2021. Beet seeds of the «Diy» cultivar of 2018 reproductions were stored in the dark at

room temperature in a hermetically sealed container. Samples with water content of 0.11 g H2O/g−1 dry mass were put in 15 ml polypropylene centrifuge containers Falcon type (450 seeds per container) and transferred into liquid nitrogen for 14 days. Seeds were heated on air at 22ºC in the darkness and were sown in Petri dish and in soil after 4 days after thawing. Untreated seeds served as control. We determined laboratory parameters such as germination percentage, the time of 50% germination, mean germination time, germination index of seeds; dry mass and length of seedling. Such yield indicators were defining: number of leaves, petiole length, haulm length, leaf width; average length, diameter, weight of roots and some biochemical parameters. The data were analyzed using Past 3 statistical software.
No statistically significant differences in all germination tests, dry mass and length of seedling were observed between controls and cryopreserved seeds. Germination percentage of red beet seeds was near 75%. Analysis of plants grown in the field revealed that no significant difference in number of leaves, petiole length, haulm length, leaf width, average length and diameter of roots between groups. However, the mass of root crops obtained from cryopreserved seeds increased significantly. We observed decreasing in amount of total sugar, vitamin C, dry matter, betanine and increasing in amount of nitrates for root crops obtained from cryopreserved seeds.

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P54 DETERMINATION OF MICROALGAE *CHLOROCOCCUM DISSECTUM* CELLS PERMEABILITY COEFFICIENT FOR CRYOPROTECTANTS

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Despite significant progress in the field of long-term low-temperature storage of microalgae, many species and strains have not yet been cryopreserved.The study of the transport characteristics of cell membranes is important in the development of cryopreservation methods. It is known that cell permeability to water and cryoprotectant substanses affects the rate of water loss and cryoprotectant permeability during cooling; more permeable cells are able to tolerate rapid cooling better than less permeable cells.
In this work we focused on *Chloroccocum dissectum* microalgae cells a potential source for biodiesel production. The culture of *Chloroccocum dissectum* cells cultured on BG-11 nutrient medium until the beginning of the stationary growth phase at a temperature of 25±2°C without aeration, under day-and-night illumination with white fluorescent light 52.84 μmol photons m^{-2} s^{-1}. The determination of cell mass transfer processes was performed by volumetric method at T = 19-21°C, comparing the experimentally obtained dependences of cell volume on the time of their contact with cryoprotectant solutions in the cultivation medium, and curves that are obtained from the theoretical model.
We determined the osmotically inactive volume by incubation *Chlorococcum dissectum* cells in different concentrations of NaCl (1 to 3.4 M). In our experiment, this value was 0.43 V/Vo.
The permeability coefficient of the *Chlorococcum dissectum* cell membrane for water was determined. The average value of the filtration coefficient was $3.90\times10^{-14}\pm2.16$ $Nm^{-3}s^{-1}$. The osmotic behavior of *Chlorococcum dissectum* in various cryoprotective compounds:

glycerol, ethylene glycol, propylene glycol, ethanol, dimethyl sulfoxide, sucrose in different concentrations was defined. It was determined that all studied cryoprotectants penetrate the cell wall of microalgae. The investigated cryoprotectants can be placed by increasing penetration coefficient in the following order: glycerol, sucrose, propylene glycol, dimethyl sulfoxide, ethylene glycol, ethanol.

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P55 SPERM SUBPOPULATIONS IN THE CRYOPRESERVED SEMEN OF *PSEUDOPLATYSTOMA RETICULATUM*

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The theory that sperm samples are not homogeneous and that several subpopulations can coexist in the same sample has been accepted among the scientific community. The present study aims to identify the existence of sperm subpopulations in the cryopreserved semen of *Pseudoplatystoma reticulatum* and to determine the effects of the treatments used during the cryopreservation process on the structure of the identified subpopulations. Adult male specimens were induced with a single dosage of carp pituitary (EBH) 3mg/kg body weight. After, they were cryopreserved as a freezing medium, 5% glucose + 10% methanol was used as a control treatment (T1), 30% of seminal plasma of the species (T2) and 30% were added to this cryoprotectant (T1) of artificial plasma (T3), a solution based on the results of the biochemical components of the plasma determined for the species in a previous experiment. Post-thawed semen sperm velocities were measured using the integrated ISAS® C.A.S.A. To separate the subpopulations, a two-step cluster analysis was performed using a Principal Component Analysis (PCA) instead. Three subpopulations were defined for the cryopreserved semen of the species, SP1 (fast-linear), SP2 (fast-non-linear) and SP3 (slow-linear). The subpopulations in T1 were mainly composed of SP1 and SP3 (45.75% and 50.48%) respectively, the sperm belonging to SP2 had a low value (3.77%). In T2 the percentage of SP3 increases (63.03%) compared to T1. The distribution of subpopulations in T3 was mainly dominated by sperm belonging to SP3 with 83.50% of the total mobile sperm, with low values of SP1 and SP2 (10.75% and 5.75%) respectively. Therefore, we can conclude that three sperm subpopulations were identified in the cryopreserved semen of *P. reticulatum* and the most effective treatment in maintaining fast and linear subpopulations was T1 which can show the high degree of cryoresistance in the cryopreservation process.

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P56 EXPERIMENTAL VALIDATION OF A MULTIPHYSICS MATHEMATICAL MODEL FOR BODY DEFORMATION DUE TO THERMO-MECHANICS EFFECTS

DURING CRYOPRESERVATION BY VITRIFICATION

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Achieving vitrification in large specimens requires relatively high cooling rates, which lead to significant temperature variations across the cryopreserved material. In turn, these temperature variations result in nonuniformly distributed thermal expansion and viscosity across the domain, which drive material flow when it behaves like liquid, or give rise to mechanical stress when it behaves like solid and cannot flow anymore. Excessive thermo-mechanical stress during vitrification of large-size specimens can lead to devastating structural damage. Furthermore, material flow at intermediate temperatures leads to surface deformations, which in turn can create the conditions leading to stress concentrations at lower temperatures. In broad terms, stress concentration is the effect of significantly higher localized stress compared to the stress-field average. Moderating the conditions leading to such stress concentration can potentially lower the risk to structural damage, which motivates the current study. A new multiphysics model has been presented recently to simulate body deformations and stress concentrations during vitrification, where the current study comes to validate this model experimentally. Furthermore, while the recently presented model combines concepts from the disparate fields of heat transfer, fluid mechanics and solid mechanics to accurately account for deformations associated stress, the current study investigates the contribution of the solid mechanics analysis to the evaluation of the overall deformation. The experimental validation is conducted using the scanning cryomacroscope – a proprietary device developed to visualize macroscale physical events during cryopreservation. Results of this study demonstrate good agreement between experimental data and modeling of surface deformations. Furthermore, the accompanied analysis demonstrates that the effect of solid mechanics on the overall deformation is marginal, which suggests that fluid mechanics analysis can be used to approximate the overall body deformations to a high degree of certainty.

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P57 A MOLECULAR DYNAMICS SIMULATION INSIGHT INTO THERMAL STABILITY OF TAKA-AMYLASE ENZYME: APPLICABLE IN FOOD INDUSTRY

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Taka-amylase is an important biocatalyst in the starch industry to catalyze starch demolition accelerate fermentation process, and improve bread quality and durability. Freezing in food supply industry is a preservation method to prevent microorganism growth. However, it can lead to cryoconcentration of solute, and subsequently, establishment of water-ice interface, which undesirably affects the enzyme stability. Here, we provided a molecular insight into the temperature effect (242 and 300 K) on the structural stability of Taka-amylase by molecular dynamics (MD) simulation. For the ice formation system (242 K), one Taka-amylase molecule (crystal structure ID: 2TAA) was placed at the top of ice in the box with TIP4P/Ice water model. 200 ns MD simulation was performed by GROMACS 2018.4 and charmm36-jul2021.ff force field. The partial density of the solvent along the z-axis clarifies the bilateral growth of ice during the MD simulation, in accordance with the solvent density decrease over the simulation time at 242 K. The structural stability of the systems during the MD simulations was proved by the RMSD analysis. The result confirms the stabilizing effect of the lower temperature through reducing the molecular mobility. The RMFS plot indicates the significant decrease in the flexibility of the enzyme residues with reducing the temperature. The gyration radius (Rg) of the enzyme highlights the direct effect of the temperature decrease on reducing the structural fluctuations. Based on the DSSP analysis, the temperature reduction increased the number of β-sheet and α-Helix structures. The solvent-accessible surface area indicates a relatively constant value for the hydrophobic surface of the enzyme during the MD time at 242 K while it increased at 300 K. Consequently, the structural stability of Taka-amylase can be preserved with decreasing temperature.

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P58 THE EFFECT OF LOW TEMPERATURE ON THE STRUCTURAL STABILITY OF AMYLOID-β (1-42) DIMER: AN IN-SILICO STUDY THROUGH MOLECULAR DYNAMICS SIMULATIONS

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Amyloid-β(1-42) (Aβ1-42) peptide is the major agent responsible for Alzheimer's disease. Freezing is extensively used in the medical industry for the long-time preservation of human body members (e.g. brain) for transplant surgery. However, it can lead to establishment of water-ice interface, affecting the proteins' stability. Here, we computationally studied the temperature effect (242 and 300 K) on the structural stability of Aβ1-42 dimer by molecular dynamics (MD) simulation. The MD simulation was carried out by using GROMACS 2018.4 with charmm36-jul2021.ff force field and TIP4P/Ice water molecules. The partial density for the solvent along the z-axis proved the bilateral growth of ice during the simulation. The solvent density plot over the simulation time indicated a constant solvent density at 300 K while its decreasing value at 242 K, another proof for the ice growth. The RMSD distribution proved 190 ns as a

suitable time for the study. The radius of gyration distribution plot indicated higher values for the peptides at 300 K that confirmed opening of the structure of the peptides during the simulation. Based on the RMSF plot, the flexibility of the peptides significantly decreased at 242 K, highlighting the temperature effect on the residues' motion. The distance between the center of mass (COM) of the peptides was high at 242 K, proving the lack of accumulation of peptides at this temperature. The distance between the Ala42 and Lys28 residues of each peptide possessed no considerable changes at 242 K in comparison with the initial crystalline structure. The study proved the structural stability of the Aβ1-42 peptides at low temperature, which was in accordance with the available experimental results.

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P59 EFFECT OF CHOLESTEROL ON PLASMA MEMBRANE AND MOTILITY OF PIAU (*LEPORINUS OBTUSIDENS*) SPERM

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Recent studies demonstrate that lipids play an important role in the fertility and cryotolerance of fish gametes. Addition of cholesterol-loaded cyclodextrin (CLC) into the semen increased stability and rigidity of phospholipid hydrocarbon chains of plasma membrane during sperm cryopreservation process. This study investigated the effect of CLC has been tested successfully as cryoprotectant in fish sperm has not previously been tested. Sexually mature fish were induced to spermiation with carp hypofise. The ejaculates from 8 fish were pooled, diluted to 120 million cells/ml in 300 mM glucose and 10% DMSO diluents and then sub-divided into two treatments: without CLC (control) and with 1.5 mg of CLC. After, the semen was incubated for 15 min at 22°C. Diluted semen packaged into 0.5ml straws were equilibrated at 4°C for 15 min and frozen in liquid nitrogen vapor. Straws were thawed and the sperm motility, duration of motility and membrane plasma integrity. Higher percentages of motile (30.9%) and plasma membrane integrity (135.0%) were maintained after thawing sperm treated with CLC compared control (22.7 and 94.8%). Duration of motility not differ between treatments. It is concluded that treatment of cholesterol-loaded cyclodextrin for Piau sperm cryopreservation improves cell cryosurvival.

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P60 INNOVATIVE CRYOPRESERVATION TECHNOLOGY FOR AUSTRALIAN MACADAMIA

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Macadamia is a high-value nut crop that is endemic to Australia and valued at over AU$3 billion globally. The genetic base of the commercial cultivars is narrow as most of the commercial trees originated from Mooloo in regional Queensland, Australia. The wild germplasm of macadamia remains unexplored for breeding and some of the diversity in the wild is suffering from loss due to the destruction of the macadamia habitat. Traditional conservation methods such as seed banking are unfavourable for macadamia as it belongs to exceptional species and cannot be stored at -20℃ for the long term. Moreover, macadamia displays a high level of out-crossing, therefore seed conservation is of little value in the case of commercial cultivars because the seeds are not true to type. Thus, cryopreservation of macadamia is a potentially significant tool to supplement macadamia conservation efforts and effectively maintain germplasm collections in the long term.
The objective of this study is to establish the first cryopreservation technology for macadamia cultivars using true-to-type shoot tips. A tissue culture regeneration system will also be developed to regrow the preserved plant tissues after Liquid N_2 treatment. Shoot tips were dissected in the size of 1 x 2 mm under the microscope by using untreated *in vitro* macadamia cv. 'Beaumont' plants. Preliminary results of shoot tip dissection achieved an 80.0% of survival (2 weeks post dissection) and 76.6% of regrowth (4 weeks post dissection), optimising the first step in developing the cryopreservation protocol of macadamia. Trials of cryoprotectants to prevent the formation of ice crystals during the cryopreservation process are underway. The droplet vitrification method to cryopreserve the shoot tips in Liquid N_2 will then be tested. This study aimed to provide a foundation for the preservation of diverse macadamia germplasm to ensure the macadamia industry of the future.

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P61 COMPOSITION AND BIOLOGICAL ACTIVITY OF CRYOEXTRACTS FROM FETOPLACENTAL TISSUES UNDER DIFFERENT PREPARATION CONDITIONS

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Substantiation of cryogenic technologies for obtaining biologically active compounds from tissues of fetoplacental origin is an urgent issue of cryobiology.Cryoextracts from homogenates of rat placenta (CP) and fetus internal organs (CF) were obtained after a single (-20°C) – mode-1, double (-20°C; -196°C) – mode-2 and triple (-20°C; -196°C; -196°C) – mode-3, freeze-thaw. The protein-peptide cryoextracts' composition was characterized using size exclusion chromatography. The biological activity of CP and CF was evaluated in vitro by their ability to enhance the phagocytic activity of rat blood neutrophil

granulocytes (NG) incubated with an inactivated culture of Staphylococcus aureus. Total protein concentrations in cryoextracts obtained under various freeze-thaw modes were: CF-1 – 20.16; CF-2 – 21.17; CF-3 – 22.17 mg/ml, and in CP-1, CP-2 and CP-3 were 23.81; 22.92 and 25.01 mg/ml, respectively. The proportion of proteins with a molecular weight from 20 to 150 kDa was: CF-1 – 67.77; CF-2 – 62.93; CF-3 – 62.51%, and in CP-1, CP-2, CP-3 – 80.28; 79.22; 72.78%, respectively. Low molecular weight proteins/peptides (< 20 kDa) accounted for 32.23; 37.12; 37.54% in CF-1, CF-2, CF-3 and 19.60; 20.78; 27.04% in CP-1, CP-2, CP-3, respectively.Investigation of cryoextract biological activity by assessing the phagocytic activity of NG showed that incubation of native leukocytes with CP and CF, regardless of the concentration and mode of extract preparation did not result in a significant increase in the number of phagocytic neutrophils. All studied CPs and CFs were characterized by a dose-dependent increase in the absorption activity of NG. The use of a triple freeze-thawing (mode 3) of placental and fetal homogenates allowed more effective extraction of total protein and greater yield of low molecular weight proteins/peptides. The percentage of low molecular weight fractions was significantly higher in CF compared to CP, regardless of the method of cryoextract preparation.

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P62 BIOPOLYMER GELS FOR VITRIFICATION OF SEMINIFEROUS TUBULES

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Vitrification is increasingly used for cryopreservation of immature testicular tissue. The aim of this study was to experimentally evaluate an effect of application of biopolymer gels for vitrification of fragments of seminiferous tubules of testes (FSTT) of immature rats.
Experimental samples of FSTT (2-3 mm3) was encapsulated to cryomedia based on fibrin or collagen gels (5 mg/mL) with Me_2SO +glycerol+sucrose. Exposition was carried out during 10 min with following vitrification (rapid immersion in liquid nitrogen). The warming was carried out on watter bath at a temperature of 50°C by successive transfer of samples to a sucrose solution. Metabolic (MTT-test) and total antioxidant system (TAS) activity were determined as well as histological examination of the samples was performed. FSTT vitrified under the same conditions in Leibovitz-based cryoprotective medium were assumed as a control. The results were processed with Kruskal-Wallis ANOVA test with multiple comparisons.
Histological study showed the most pronounced protective effect for fibrin gel: the spermatogenic epithelium generally preserved histological structure, with the exception of a slight retraction of cells and the presence of single cells with vacuolated nuclei, which diffusely settled throughout the thickness of epithelial layer. The histological structure of FSTT, vitrified in collagen gel-based cryomedium, was characterized by retraction and desquamation of spermatogenic cells, which were less pronounced compared to the control. The analysis of results of MTT-test shown 1.5- and 1.2-fold higher metabolic activity in the samples vitrified in media based respectively on fibrin or collagen gels versus control. TAS activity in both experimental groups after vitrification was also higher (in 1.7 times) than in the control, without significant

difference between investigated biopolymers.
Results of our investigation can be useful for substantiation and development of effective cryopreservation medium for testicular tissue using biopolymer gels.

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P63 CRYOPRESERVED MESENCHYMAL STROMAL CELLS FOR CORRECTION OF ADJUVANT ARTHRITIS

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Cryopreserved mesenchymal stromal cells (MSCs) can promote regeneration in organism not only by direct cell proliferation and differentiation, but also due to their trophic and immunosuppressive potentials. The aim of this study was to determine the indices of regenerative processes in cartilage tissue and cellular immunity in animals with adjuvant arthritis after therapy with cryopreserved adipose-derived MSCs.
Arthritis in male rats was modeled by subplantar injection of Freund's complete adjuvant. On day 7 experimental animals were administered with saline (control group) or cryopreserved adipose-derived MSCs locally or generalized. On day 28 after therapy a content of Treg cells ($CD4^+CD25^+$) in spleen and histological changes of ankle cartilage were determined. The results were processed with Kruskal-Wallis one-way analysis with multiple comparisons.
Obtained results showed an inflammatory process in the animals of control group, which manifested in decreased number of Treg cells, joint swelling, widespread chondrocyte-free zones, weakening of staining, loss of clarity of cartilage tissue contours. At the same time, the local administration of cryopreserved MSCs from adipose tissue contributed to the normalization of the content of Treg cells and structural organization of articular cartilage. Less pronounced regeneration processes in articular cartilage occurred under generalized administration of cryopreserved MSCs from adipose tissues in comparison with the local method. However, generalized administration of cells had a more pronounced effect on the restoration of Treg cells in spleen compared to the local one.
Cell therapy of adjuvant arthritis with cryopreserved MSCs by both local and generalized administration has the correcting effect on the cellular immunity and the stimulation action on the intensity of regenerative processes in damaged cartilage tissue.

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P64 TEMPERATURE DEPENDENCE OF ERYTHROCYTE OSMOTIC RESPONSE TO AN ARTIFICIAL HYPOBIOSIS STATE OF HOMOIOTHERMAL AND HETEROTHERMAL MAMMALS

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For understanding the integral physiological response to a hypometabolism state studies of cellular responses to it are necessary. Comparative study of osmotic responses of rats and hamsters RBCs to animals' being and leaving the state of artificial hypobiosis under conditions of hypothermia-hypoxia-hypercapnia (AH-state) was carried out. Erythrocyte osmotic fragility (EOF) was determined by the method of small-angle light scattering at 25°C, 20°C, and 15°C. Mean EOF value (% NaCl concentration at 50% hemolysis) and the first derivative of a hemolysis curve were calculated. The latter reflects the density of RBC population distribution for osmotic resistance. For both hamster and rat control RBCs, EOF was temperature dependent. Osmotic resistance decreased as the measurement temperature was lowered for rat RBCs. In contrast, for hamster RBCs, lowering the temperature was accompanied by increased RBC osmotic resistance, and at 15°C, it was close to EOF of winter-hibernating animals. The AH-state was characterized by body temperature decreasing (down to 16°C), in the same way for both cold-sensitive rat and cold-tolerant hamster. Both hibernators' and homoioterms' being in the AH-state resulted in significant decrease of osmotic resistance. However RBC of hibernating hamsters had increased osmotic resistance. Furthermore EOF was independent of the measurement temperature; in addition, there was erythrogram narrowing. This suggested in vivo unification of the osmotic properties of RBCs in response to AH-state. The same was observed for hibernating hamsters. Compared with control animals, EOF was significantly lower at 2 h after the AH-state and kept changing up to 24 h post-hypobiotic-state. The osmotic erythrogram form was practically independent of the temperature at 2 h after the AH-state, but was temperature dependent at 24 h. Temperature study of RBCs osmotic behavior revealed features of erythrocyte responses to the artificial hypobiosis state of homoiothermic and heterothermic mammals, and compared to nature one.

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P65 MASTERING THE ASSESSMENT OF FUNCTIONAL ACTIVITY OF CRYOPRESERVED CELLS

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Cryopreservation is one of the most effective tools of long-term preservation of cells, including tumors, to create their depositories. However, any cryopreservation conditions change their structure and functions. Finding the assessment criteria for tumor cells proliferative capacity after cryopreservation is crucial. One of them is the Ki-67 protein content, as it is found in the cell cycle active phases. The research aim was to determine the Ki-67content in Ehrlich carcinoma (EC) cells immediately after cryopreservation and at different stages of *in vivo* cultivation. EC cells were cryopreserved in ascites fluid by 1 deg / min to -80 ° C followed by immersion in liquid nitrogen. Water-bath warmed cells were administered to Balb/C mice intraperitoneally at $3*10^6$ cells/mouse dose. Immediately after thawing and on days 3 and 7 of tumor growth *in vivo*, the EC cell viability was examined with propidium iodide and Ki-67 protein content by flow cytometry. The tumorigenic potential was determined by absolute number of cells in the PC with regard of the ascites fluid

volume. The "stabilized" three-fold passaged EC culture served as control. Cryopreservation was shown to reduce the number of viable cells by 40% compared to controls. The content of Ki-67^+ cells immediately after thawing was 31.31 ± 0.24%, that was trice lower versus the control. Subsequent re-culturing of cryopreserved EC cells restored their proliferative activity: on day 3 the Ki-67^+ cells content increased to 40.91 ± 0.25%, and on day 7 it was 78.52 ± 0.4%. The restoration of their tumorigenic potential, which on the 3rd day was 1.3 * 10^7 cells, and on day 7 reached 18.99 * 10^7 cells in PC, but remained twice lower than the control. Thus, the Ki-67 protein content assessment is an additional molecular marker of the recovery extent for the tumor cells functional activity after cryopreservation.

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P66 DOES SALT-COLD STRESS INCREASE LIPID PRODUCTION IN *CHLOROCOCCUM DISSECTUM* AND *DUNALIELLA SALINA* MICROALGAE CELLS?

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Algae biofuel have emerged as viable renewable energy sources and are the potential alternatives to fossil-based fuels in recent times. *Chlorococcum dissectum* and *Dunaliella salina* have the potential to generate significant quantities of commercially viable biofuel.
We investigated whether salt and short-term cold stress and their combination affect the intensity of lipid accumulation in the cells of the studied microalgae.
Microalgae were cultivated to the stationary phase of growth at a temperature of 25±2°C without aeration with round-the-clock white fluorescent illumination of 3 kLux. *Ch. dissectum* was grown on BG-11 medium containing: 0; 0.06; 0.2 M NaCl. *D. salina* was on Ramaraj medium containing 1.5 and 4 M NaCl. Cultivation at low temperatures was carried out in the dark at 4°C for 24h.
The effect of low temperatures on *D. salina* led to structural changes: an increase the number and size of lipid globules, and an increase in the fluorescence intensity of the Nile Red (NR) dye compared to the intact culture. Lipid synthesis and NR fluorescence were higher in the medium with high NaCl content.
Contrary to expectations, incubation of *Ch. dissectum* at 4°C for 24h did not lead to changes in the lipid composition, regardless of the content of NaCl in the cultivation medium.
The absence of changes in the lipid composition of *Ch. dissectum* cells may be related to their specific features. In contrast to the halotolerant *D. salina*, which is able to adapt to sudden changes in temperature and salinity, *Ch. dissectum* apparently does not have natural genetically determined rapid adaptation mechanisms.
Thus, salt and short-term cold stress, and a combination of these factors, can be used to increase the yield of a commercially important product in *D. salina*, while this method is not applicable to *Ch. dissectum.*

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P67 CRYOPRESERVATION CAN RESTORE THE FUNCTIONAL STATUS OF BONE MARROW CELLS IN ANIMALS WITH ADJUVANT ARTHRITIS

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One of the methods of treatment of patients with rheumatoid arthritis (RA) is autologous bone marrow (BM) cell transplantation with a balanced ratio of hematopoietic (HSC) and mesenchymal stem cell (MSC) subpopulations. Cryopreservation is an effective way of long-term storage of BM cells, however, the different initial state of BM cells in healthy people and donors with RA determines the need to develop optimal modes of BM cryopreservation.
Experiments were performed in CBA/H mice in which adjuvant arthritis (AA) was initiated by administration of complete Freund's adjuvant. BM cells of healthy animals and on day 14 of AA development at a concentration of 6.0 × 106 cells/ml were frozen at 1 deg/min rate to –40°C with 7 or 10% Me_2SO, followed by immersion in liquid nitrogen. The samples were warmed in a water bath at 40°C for 40 s. The content of HSCs of various differentiation extents was determined by in vivo splenic colony formation on days 8 (CFUs-8) and 14 (CFUs-14) after the BM administration to irradiated animals. The functional activity of MSCs in BM was in vitro studied by the content of fibroblast colony-forming units (CFUf).
It was shown that the freezing mode with 10% Me_2SO ensured the highest preservation of the functional potential of BM HSCs and MSCs in healthy animals. For animals with AA, freezing with 10% Me_2SO provided CFUs and CFUf at a level close to the native BM of animals with AA. A decrease in the Me_2SO concentration to 7% led to the restoration of a balanced ratio of both subpopulations of CFUs in the BM of animals with AA compared with native BM of healthy animals.
The data obtained indicate that cryopreservation can be not only an effective method of long-term storage of biomaterial, but also a factor controlling their internal state.

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P68 THE ROLE OF CRYOPRESERVATION IN THE ACTIVATION OF TOLEROGENIC ACTIVITY OF DENDRITIC CELLS

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Cryopreservation is currently considered an integral part of the biotechnological process of using cellular drugs in clinical practice. Tolerogenic dendritic cells (tolDCs) deserve special attention, providing natural tolerance in humans and animals. TolDCs are able to restore the content and function of T-regulatory (Treg) cells in the organism with autoimmune disease. However, tolDCs are sufficiently cryolability, which necessitates the use of their precursors, above all, bone marrow or peripheral blood mononuclear cells (MNCs), more resistant to low temperature factors. The question of whether cryopreservation conditions of MNCs are able to change the tolerogenic potential of dendritic cells (DCs) obtained from them is a topical and unresolved. The aim of the work was to study the mechanisms of activation of tolerogenic activity of DCs obtained from cryopreserved bone marrow MNCs. The structural and functional characteristics of bone marrow MNCs of mice after cryopreservation under different modes were evaluated; their ability to form

tolDCs in vitro, the content of heat shock protein 70 (hsp70), and the glucocorticoid-induced leucine zipper (GILZ) gene expression, which are involved in the implementation of anti-inflammatory action by stimulating the production of IL-10. Tolerogenic properties of DCs after cryopreservation were studied for their ability to induce Treg (CD4+Foxp3+) cells in vitro with CD4 fraction of spleen lymphocytes of mice with adjuvant arthritis. It was found that the functional potential of tolDCs obtained from cryopreserved MNCs is realized by increasing the content of hsp70, GILZ gene expression and restoring the functional activity of Treg cells. The study at the molecular level of the mechanisms underlying the tolerogenic activity of tolDCs obtained from cryopreserved MNCs will contribute to the development of a pathogenetically substantiated approach to the treatment of autoimmune pathologies and the creation of low-temperature banks of these cells.

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P69 STRUCTURAL STATE OF HUMAN SPERM AT OLIGOASTHENOTERATOZOOSPERMIA AFTER FREEZE-THAWING

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The optimization of freezing methods for oligoasthenoteratozoospermia (OAT) spermatozoa is still relevant. The aim of the study was to compare the effects of cryopreservation with glycerol and polyvinylpyrrolidone 360 kDa (PVP) on the morphology and ultrastructure of human OAT spermatozoa.

The study included the ejaculates of OAT men who underwent infertility treatment at the Reproductive Medicine Center. The spermatozoa were evaluated after isolation of the active motile sperm fraction (group 1 - control) and after freeze-thawing. Then each sample was equilibrate 10 min with different cryoprotectant media contained either 10% glycerol (group 2) or 10% PVP (group 3). Then samples were cryopreserved using fast freezing method: by keeping them for 15 min above the nitrogen mirror (the cooling rate was ~ 150 deg/min) and plunged into liquid nitrogen. After thawing the cryoprotectant (group 2) was removed by centrifugation at 1500 rpm for 5 min.

The number of motile sperm in the control group before cryopreservation was 92.5±2.4%. It is difficult to assess the real state of sperm motility in group 3 due to the viscosity of the PVP solution. The results have shown that the sperm head abnormalities rates were 20.57 ± 1.19%, 25.97 ± 2.67, and 19.21 ± 2.67 for groups 1-3, respectively. There were one large or several small vacuoles in group 2 spermatozoa head. The differences of midpiece and tail abnormality rates were insignificant among the all studied groups. The number of cells with multiple abnormalities (head, midpiece, tail) was significantly lower in group 3 comparing with group 2 and had no significant difference with group 1.

We can conclude that after freeze-thawing with glycerol the morphology and ultrastructural characteristics of spermatozoa were significantly decreased compared to the fresh cells. This can be caused by the processes of dehydration and rehydration of cells and formation of intracellular crystals when using penetrating cryoprotectant. In the case of using PVP as a cryoprotectant, morphology and ultrastructure of spermatozoa could be preserves at the level of fresh control.

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P70 CRYONANOTECHNOLOGIES AS A NEW APPROACH TO IMPROVE ONCOPATOLOGY TREATMENT EFFICIENCY

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Chemo- and radioresistance of cancer stem cells determined the need to find their inactivation approaches. Cryoablation is a promising treatment for neoplasms, but there is a recurrence risk. Increasing the efficiency of cryoablation can be achieved through the use of nanomaterials. Our research was aimed to evaluate the rate of Ehrlich ascites carcinoma (EAC) growth when using the nanocomplexes (NCs) based on rare-earth metal orthovanadates as well as cryoeffect. Experiments were performed in the EAC, induced in Balb/C mice by intraperitoneal inoculation ($3x10^6$/mouse): cryopreserved cells (Group 1); after pre-incubation with NCs (Group 2); after pre-incubation with NCs and following cryopreservation (Group 3); native cells (control). The EAC cells (10^7 cells/ml) were cryopreserved by immersion in liquid nitrogen and with slow warming. On day 7 of EAC, the tumor growth intensity as the difference between the absolute number of cells in animals' peritoneal cavity in the control and experimental groups; subpopulation composition of EAC ($CD44^{hi}$, $CD44^+CD24^-$, $CD44^+CD24^+$, $CD44^-CD24^+$, $CD117^+$) were assessed by flow cytometer. The cryoeffect (group 1) was shown to decrease the relative content of all tumor subpopulations, with the exception of the most carcinogenic $CD44^{hi}$ cells. This resulted in a reduced intensity of EC growth by 89.6% and prolongation of the life of tumor-bearing animals by 38% compared with the control. The NCs (group 2) inhibited the tumor growth by 74.7% and prolonged the life of tumor-bearing animals by 19%, accompanied by elimination of $CD44^{hi}$ cells with a simultaneous increase in the content of accessory $CD117^+$ cells. The combined use of cryoeffect and NCs (group 3) inhibited the growth of EAC at the same level if cryoeffect was used a separate factor (by 88.1% and 89.6%, respectively). However, only in this group, the prolongation of animal's life was 42% of the control, indicating the NCs effectiveness in cryoablation practice.

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P71 CRYOPRESERVATION OF *POGOSTEMON YATABEANUS* SHOOT TIPS USING A DROPLET-VITRIFICATION PROCEDURE: IMPORTANCE OF AMMONIUM-FREE MEDIUM FOR REGENERATION

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Dysophylla yatabeana Makino (Labiatae family) is an endangered wild species in Korea, which limitedly occurs in natural habitats and requires urgent conservation measures.
Node-cutting induced shoot tips of *in vitro* plants were cryopreserved using a droplet-vitrification method following improvement of preculture, osmoprotection, plant vitrification solutions (PVS) and regrowth treatments. The starting protocol included preculture with 10% sucrose for 31 h, followed by

osmoprotection with C4-35% (17.5% glycerol + 17.5% sucrose) for 40 min, and cryoprotection with A3-80% (33.3% glycerol + 13.3% dimethyl sulfoxide + 13.3% ethylene glycol + 20.1% sucrose) for 60 min on ice, cooling and warming using aluminum foil strips, and regrowth MS hormone-free medium.
Shoot tips of *D. yatabeana* were sensitive to the osmotic stress evidenced by low survival after step-wise preculture with 17.5% sucrose and cryopreservation without osmoprotection. Among PVS tested including PVS2, PVS3 and their alternatives, A3-80% on ice for 60 min resulted in highest post-cryopreservation survival (80%) and regeneration (20%). Post-cryopreservation regeneration significantly improved (up to 73%) by incubation of cryopreserved shoot tips on ammonium-free medium followed by GA_3–containing medium and medium without growth regulators. In conclusion, cryopreservation of *in vitro* shoot tips using droplet-vitrification was developed as a complimentary conservation approach for *D. yatabeana*. Adjustment of medium composition during the recovery stage was important for regeneration of healthy plants from both cryoprotected-control and cryopreserved shoot tips.
It may be speculated that ammonium in the regrowth medium aggravates the oxidative stress, which was maximized during vitrification solution treatment, LN exposure and unloading. This stress may reduce metabolic activity of the explants, and the key enzymes of ammonia nitrogen metabolism could be retarded several days after rewarming, leading to the accumulation of the toxic levels of ammonium.

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P72 VIABILITY OF DECONSERVED BOAR SPERM DURING PREPARATION FOR FERTILIZATION

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In order to preserve the existing breeds of pigs and reproduce the extinct ones, it is necessary to resort to the use of cryopreserved material. Despite the fact that the procedure of fertilization with cryopreserved wild boar spermatozoa is extremely necessary, there are many difficulties during such manipulations. One of the disadvantages of wild boar semen is its low resistance to temperature fluctuations.
Washing medium, the method of washing, and the temperature at which the process takes place - all this can affect the fragmentation of spermatozoa DNA, the integrity of the plasma membrane, and affect the fertility of each individual spermatozoon (Flores E., 2011).
We performed flotation in two different media to determine the most optimal conditions: Sp-TALP (Parrish J.J. et al., 1988) and Sperm Preparation Medium, (Origio, Denmark). Spermatozoa suspension of wild boar of Myrhorod breed (Kamysh 253) was washed in two media at + 38.5°C and counted in a Makler chamber. After washing in Sp-TALP medium, the concentration of spermatozoa in 1 ml was 80.1 ± 3.1 million. Mobility was at the level of 11.3 ± 1.72%. During similar manipulations using Sperm Preparation Medium, 21.3 ± 2.27 million spermatozoa in 1 ml with motility of 0.7 ± 0.86% were obtained. Therefore, the concentration of spermatozoa in the use of commercial media was 3.8 times lower than in the use of Sp-TALP.
It is important to know the number of mobile spermatozoa. When Sp-TALP medium is used for flotation, the number of

spermatozoa that can be used for fertilization is 6.74 ± 0.557 million in 1 ml. During flotation in Sperm Preparation Medium, this value was 0.127 ± 0.127 million per ml, which is extremely small for further in vitro fertilization procedures.
Therefore, Sperm Preparation Medium is a ready-made commercial medium and, obviously, could simplify and speed up the preparation of gametes for fertilization, its use is not recommended by us for use in the preparation of wild boar spermatozoa for in vitro fertilization.

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P73 POTENTIATION OF CRYOGENIC DESTRUCTIVE EFFECT ON BIOLOGICAL TISSUES: AN EXPERIMENTAL STUDY

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Our study aimed to assess the role of 10% $CaCl_2$ and distilled H_2O as agents potentiating the destructive effect of ultralow temperatures on the healthy liver parenchyma of Wistar rats, tumor models Walker-256 and Guerin. The study was performed on 151 experimental animals - white Wistar rats. The combined cryochemical destruction of carcinosarcoma Walker-256 in the dynamic was characterized by a significantly larger percentage of necrotic change compared to cryo and chemical ablation alone ($p<0,05$). Eight days after cryogenic exposure to the tumor model, the average percentage of necrotic changes were 61,54±2,41%, necrobiotic - 2,93±0,18%; the intratumoral 10% $CaCl_2$ injection - 48,05±2,09% - necrosis and 11,53±0,50% - necrobiosis; the damaging agents combination - 83,65±1,37% necrosis and 6,41±0,65% necrobiosis. The combined application of 10% $CaCl_2$ and cryodestruction significantly reduced the volume of Walker-256 tumor nodes in dynamics compared to cryo- and chemical destruction (cryo vs. combination $p<0,05$; chemical destruction vs. combination $p<0,01$): the average volumes of Walker-256 on the 8th day in the group of cryodestruction were (250,2±15,39) mm^3, chemical destruction - (276,1±20,29) mm^3, and combined exposure - (68,92±2,69) mm^3. Injection of 10% $CaCl_2$ in combination with cryodestruction contributed to more pronounced necrotic tissue changes compared to isolated cryo influence and was characterized by a combination of both coagulation and colliquative necrosis, pronounced inflammatory perifocal infiltration, and more active growth of connective tissue. The combination of distilled H_2O intratumoral injection and cryoexposure to the Guerin tumor model resulted in total cytodestruction of tumor cells. The necrotic tissue was with pronounced interstitial edema and disorganization of tumor stroma fibers. The cellular cytoclasis and karyolysis were visualized in the background of severe interstitial edema. The obtained results suggest a mutually potentiating cytodestructive effect of intratumoral administration of 10% CaCl2, distilled H2O at ultra-low temperatures, by combining chemical and cryo necrosis of biological tissues.

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P74 COLD AS A STRESSOR AND TISSUE DAMAGE AGENT

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Cold exposure can damage tissues and affect metabolic processes in the body. We evaluated the metabolic response during the first and after four hours cryo-exposure by biochemical parameters. The rats were divided into the control and two experimental groups (EG1h and EG4h). EG1h: CK and ALT increased by 1.2 and 2.2 times, respectively. We observed a decrease in AST (1.4), LDH (2.3), as well as magnesium (1.8), calcium (1.9) and urea (3.1). Creatinine, phosphorus and lactate did not change. EG4h: creatinine remained constant. The following parameters were increased: CK – 2.1 times to the control and 1.7 to EG1h, AST – 1.5 times to the control and 2.0 to EG1h, LDH – 1.7 times to the control and 4.0 to EG1h, urea – 1.3 times to the control and 4.0 to EG1h, lactate – 1.6 times to the control and 1.5 to EG1h, calcium 1.9 times to the control and 2.4 to EG1h. Phosphorus increased 1.9 times to the control and EG1h. ALT was 1.4 times higher than the control and 1.7 lower than EG1h. Magnesium decreased 1.7 times to the control. During the first hour, the revealed changes in biochemical parameters are characteristic of the ebb stage in the metabolic response to injury. Also, it can be assumed that there is a double exposure to local cold; the metabolic response to injury overlaps the body's response to stress. Thus, the body is restructuring to maintain homeostasis. After four hours, it is the first phase of stress – anxiety, characterised by negative nitrogen balances and activation of all catabolic pathways to normalise temperature homeostasis. There is a possibility of activating anabolism processes in the liver – a combination of responses to cryo-destruction – local and general. At the same time, the second stage of the body's response to damage – is the flow.

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P75 SOME FEATURES OF AUTONOMIC REGULATION IN ANTARCTIC WINTERERS

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Last decades there has been increased interest for studying of human in the isolation, confinement, extreme (ICE) environments. A wintering at Antarctic station is one of the best models for studying of the ICE-effect.
We aimed to investigate the change of human vegetative regulation during the wintering at the Ukrainian Antarctic station "Akademik Vernadsky". The study comprised 58 winterers of the 21th, 23rd-26th Ukrainian Antarctic expeditions. These were 54 men and 4 women, aged 22-64 (mean age of 38.6 years).
We evaluated the autonomic regulation by analyzing heart rate variability (HRV). HRV was calculated based on an analysis of 3-minute ECGs recorded in sitting position. The data were statistically analyzed by the Mann-Whitney U test.
By the physiological responses the winterers were divided into 2 groups. In one group we found that the average heart rate (HR) decreased by 8% during the over-

winter and in the other group the average HR increased to nearly 8%. During the year the HRV indices changed differently: in group 1 there were an increase in both total power (TP) and high- and low-frequency activity (HF and LF) and a decrease in the LF/HF ratio; group 2 vice versa demonstrated reduced TP, HF, LF, accompanied with a rise in LF/HF ratio with an increase in HR. In particular, we observed a decrease in individual systolic blood pressure in the first group, while in the second there were no significant differences. The distribution of people by the groups did not depend on age, professional factors and previous wintering experience.

Currently, several researchers described the reduction of sympathetic and growth of parasympathetic activity in winterers after overwintering. We assume that it is the cold that can affect these features of autonomic regulation.

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P76 ADIPOCYTES IN WOUND GRANULATION TISSUE AFTER SKIN CRYOABLATION

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The role of adipocytes in wound healing after skin cryoablation is a debatable issue. Morphofunctional features of adipocytes were studied on days 7, 14 and 21 after skin cryoablation in 30 hairless rats. Histological, immunohistochemical, morphometric and statistical methods were used. In all the experimental periods, single adipocytes or their clusters were found in the granulation tissue located in a wound cavity. Within the microscope field of view ×100, the average value of the adipocytes number increased since days 7 to 21 (day 7 – 29.1 ± 2.3, day 14 – 53.9 ± 2.4, day 21 – 74.4 ± 3.9). On day 7 the adipocytes were characterized with a round or oval shape, and on day 14 and especially on day 21 they were a round-oval, oblong or fibroblast-like shape. Since days 7 to 21, an increase the relative number of adipocytes, which expressed alpha smooth muscle actin (day 7 – (8.6 ± 0.8) %, day 14 – (19.3 ± 1.1) %, day 21 – (32.2 ± 1.3) %) was observed. The qualitative and quantitative changes of adipocytes occurred against the background of the maturation of granulation tissue and its transformation into connective tissue. The research performed by the authors indicates that adipocytes can stimulate the wound healing after skin cryoablation by transforming them into myofibroblasts, as evidenced by the change of adipocytes shape and expression of alpha smooth muscle actin. Myofibroblasts are known to produce connective tissue fibers and to promote the wound contraction.

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P77 THE PREPARATION OF SUBCOOLED LIQUID ARGON

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During cryopreservation of biomaterials with liquid nitrogen (LN), vapor film around the specimen may influence heat transfer greatly. The use of subcooled LN or slush nitrogen can bring high cooling rate and increase the survival rate under most situations. However, the production of subcooled LN or slush nitrogen is not easy. Repeated use of the same LN results in less possibility to get subcooled LN or slush nitrogen in subsequent trials. Here we report a new way to obtain subcooled cryogenic fluid. Liquid argon is filled in a stainless-steel vessel and cooled in liquid nitrogen. The thickness of the interlayer of the vessel is 6mm, and nitrogen gas with pressure of 1bar is sealed in the interlayer. About 300mL of liquid argon was put in the vessel with a polyurethane cap and cooled down to 84.3K in 1 hours. The subcooling degree was 3K. A mathematical model was established to calculate the cooling process. The impacts of the filling gas in the interlayer and the thickness of the interlayer were investigated. The cooling ability of subcooled argon and LN were compared by measuring the cooling rate of a plunged straw which had an inner diameter of 0.5mm and contained 25%(v/v) alcohol solution. A cooling rate of 122K/s was obtained in saturated liquid nitrogen, whereas 166K/s was obtained in supercooled liquid argon with 3K subcooling.

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P78 PROCYANIDINS SUPPLEMENTED SOYBEAN LECITHIN-BASED EXTENDERS IMPROVE POST-THAW QUALITY OF GOAT SPERMATOZOA

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Procyanidin (PC) belongs to the proanthocyanidin class of polyphenols, a subgroup of polyphenols with potent antioxidant properties. This study aimed to investigate the effects of different concentrations of PC (0, 10, 20, 40, and 60 μg/ml) in soybean lecithin-based extenders on the semen quality parameters of post-thawed goat semen. Fifteen ejaculates were collected from six healthy, mature Chongming White goats (3–5 years of age). Each ejaculate was divided into five equal aliquots, and then each pellet was diluted with one of the five soybean lecithin-based extenders containing 0, 10, 20, 40, or 60 μg/ml PC (PC0, PC10, PC20, PC40, and PC60 group). The cooled diluted semen was loaded into 0.5 mL polyvinyl French straws and cryopreserved in liquid nitrogen. Frozen semen samples were thawed at 37 °C and assessed for sperm motility, viability, plasma acrosome integrity, membrane integrity, and mitochondria integrity, and the spermatozoa were assessed for reactive oxygen species (ROS), superoxide dismutase (SOD), and malondialdehyde (MDA). The results showed that the sperm viability, motility, acrosome integrity, mitochondrial activity and plasma membrane integrity in the PC40 group (58.49%, 53.45%, 55.37%, 55.16% and 50.46%, respectively) were significantly higher than the other groups. The ROS and MDA concentrations in the PC40 group were significantly lower than the other groups, and the SOD and GSH-Px levels in the PC40 group were the highest than controls. When the concentration of PC increased to 60 μg/ml, the quality indexes of thawed semen were significantly decreased, and the toxicity of PC was found

in the goat semen freezing. In conclusion, the extenders supplemented with 40 μg/ml PC in the goat semen freezing could reduce sperm oxidative damage, decrease apoptotic level and improve sperm quality.

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P79 CONSERVING YACON (SMALLANTHUS SONCHIFOLIUS) THROUGH CRYOPRESERVATION USING THE PVS2 DROPLET VITRIFICATION METHOD

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The perennial root crop yacon [Smallanthus sonchifolius (Poepp. and Endl.) Robinson], native to the Andean mountain region, is a tuberous crop mainly grown for its edible underground organs rich in inulin-type fructooligosaccharides of low caloric value. Due to habitat destruction, land degradation, and environmental changes, there has been a rapid erosion of its genetic diversity. Such condition, along with the risk of pest and diseases, creates the need to use advanced biotechnological approaches as an alternative to preserving the species' genetic material and its biodiversity. This study aims at using the Plant Vitrification Solution No.2 (PVS2) droplet vitrification method to develop an efficient cryopreservation protocol for the long-term preservation of yacon. To carry out the experiment, apical shoot tips (2-3 mm long) were excised from 3-4 weeks old in vitro cultures of four yacon cultivars (one allooctoploid (2n=8x=58) from Ecuador, two allooctoploids from Bolivia, and two dodecaploids (2n=12x=87) from Peru). After pre-treatment (0.3M SUC+12hrs dark), these were placed in loading solution (20 min at 22°C). Three different time intervals for PVS2 dehydration at 0°C were tested (15, 30, and 60 min). Thereafter, shoot tips were exposed to ultra-rapid cooling in liquid nitrogen (1 hr) and then placed in an unloading solution for thawing (22 °C for 15 min). Next, post-cryo cultures were placed on recovery (MS or MS+1 mg/l BA). Post-thaw survival, regrowth, and quality of shoot tips were evaluated. The results showed that PVS2 is an efficient method for the cryopreservation of all tested cultivars of yacon with MS without 0.1 mg/l BA as regrowth media, and PVS2 60 min treatment duration is the most effective in providing the highest survival (87-90%) and regrowth (62-75%) rates, respectively, with no morphological abnormalities, post cryopreservation. The BOL23 genotype showed the highest shoot tip regrowth percentage (75%) post cryopreservation, followed by ECU41 (73%), PER12 (73%), PER14 (70%), and BOL22 (62%).

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P80 WITHDRAWN

P81 A STUDY OF ANTIULCER ACTIVITY OF CRYOCONSERVED PLACENTA EXTRACT ON THE MODEL OF ALCOHOL /

PREDISOLONE-INDUCED STOMACH LESIONS

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Search the new approaches to the treatment of peptic ulcer disease is an urgent problem of modern medicine. One of the potential antiulcer agents is cryopreserved placenta extract. The study was conducted on 28 male rats weighing 200–220 grams. After 24 hours of fasting, rats were administered intragastrically with prednisolone (20 mg/kg) dissolved in 80.0% ethyl alcohol (0.6 ml/100 grams of animal body weight). Cryopreserved placenta extract was administered intramuscularly at a dose of 0.16 ml/kg body weight in the prophylactic mode – once a day for 5 days before the introduction of alcohol-prednisolone mixture. 24 hours after administration of the alcohol-prednisolone mixture, rats were removed from the experiment and macroscopically assessed the condition of the gastric mucosa according to the following criteria: bloating, edema, redness, hemorrhage and folding disorders. For each group, the percentage of experimental animals was calculated according to the specified characteristics and the average value of their expression, which was evaluated on a scale: 0–3 points. The study showed that in 100.0% of control rats (model pathology without treatment) marked (3 [3; 3] points) hyperemia of the gastric mucosa ($p<0,05$). In addition, the presence of hemorrhage, edema and folding disorders caused by the introduction of alcohol-prednisolone mixture was noted. Prophylactic five-day administration of cryopreserved placenta extract before the introduction of ulcerogenic mixture led to a statistically significant ($p<0.05$) decrease in the severity of damage to the gastric mucosa in rats. Thus, hyperemia, hemorrhage and mild edema of the gastric mucosa were observed in only 28.6% of rats. The obtained data indicate the ability of cryopreserved placenta extract in the prophylactic mode of administration to increase the endurance of the gastric mucosa to the action of alcohol-prednisolone mixture.

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P82 CRYSTALLIZATION IN SERUM CONTAINING AND SERUM-FREE MEDIA BASED ON DEXTRAN

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Using serum-containing media for cryopreservation of testicular cells can cause problems connected with transmission of infections or composition inconsistency. Cryopreserved testicular interstitial cells (ICs) can be used for study of testicular defects and in reproductive technologies. The objective of the research is to investigate crystallization in serum containing and dextran based serum-free media as well as its effect on IC survival.
Our preliminary study has demonstrated that ICs can be cryopreserved either in serum containing media composed of 1.4 M Me_2SO and 10% FBS in Ham's F12 or in serum-free media with 0.7 M Me_2SO and 100 mg/ml Dextran (Dex) (M.m. 40 kDa) in Ham's F12. The present research focuses on crystallization examined by cryomicroscopy. Next media were studied: Ham's F12, the medium supplemented with Me_2SO at concentrations of 0.7 or 1.4 M and/or 10% FBS, or 100 mg/ml Dex. The samples also contained ICs and were cooled to -40°C at a rate of 1°C/min.
Cryopreservation of Ham's F12 containing samples resulted in formation of very large crystals about 30 μM in diameter. They tended to fuse and recrystalize up to 200-250 μM. In between the ice crystals salt eutectic took place. Addition of Me_2SO led to very small crystals to form. Their size was about 5-10 μM. However, they got bigger in the course of cooling to about 40-60 μM. Intracellular ice formation was seen. Addition of 10% FBS and 100 mg/ml Dex with Me_2SO decreased ice crystal enlargement by 30-40%. In the medium containing 0.7 M Me_2SO with 100 mg/ml Dex the process of salt eutectic was less pronounced. This was in good correlation with cell survival: 38±5% for FBS containing media; 59±9% for Dex containing media. Therefore, the data display the ability of serum-free medium based on Dex and Me_2SO to depress recrystallization and salt eutectic.

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P83 CHOLESTEROL INCORPORATION INTO CURIMBA SPERM MEMBRANE IMPROVE CRYOSURVIVAL

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When cholesterol is added to sperm membranes before cryopreservation, higher percentages of motile and viable cells are recovered after thawing. Irreversible damage occurs to spermatozoal membranes, during the phase transition, when spermatozoa are cooled from room temperature to 5°C. Some of this damage can be reduced by adding cholesterol to the membrane, thereby altering membrane lipid composition. Adding cholesterol-loaded cyclodextrins (CLCs) to mammalian spermatozoa before freezing increases cell cryosurvival of the cells. However, the benefit effect using of CLC in curimba spermatozoa is unknown. Sexually mature fish were induced to spermiation. The semen was diluted with 10% Me_2SO and different concentrations of CLC (0, 0.5, 1.0, 2.0, 3.0 and 4.0mg per 120 million spermatozoa) were add and kept for 15min at room temperature and then placed into 0.5ml straws were frozen

in dry shipper (-76°C/24h) and subsequently stored in liquid nitrogen. Semen was thawed in a water bath at 60°C/8s. Addition of CLCs increased the percentages of motility and membrane intact sperm surviving cryopreservation compared to untreated sperm ($P<0.05$). After activation no differences were observed in duration of motility for all treatments. In conclusion, CLCs improved the percentage of post-thaw viability in curimba sperm.

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P84 CHOLESTEROL-LOADED CYCLODEXTRIN PREVENT DAMAGE IN PLASMA MEMBRANE OF *PSEUDOPLATYSTOMA CORUSCANS* SPERM

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The reduced fertilizing ability of frozen-thawed sperm results from alterations in the structure and physiology of the sperm cells occurring during cryopreservation. Addition of cholesterol-loaded cyclodextrin (CLC) to the diluents of mammalian increased stability and rigidity of phospholipid hydrocarbon chains of plasma membrane during sperm cryopreservation process. The objective was to investigate the effect of CLC on membrane integrity of surubim sperm after cryopreservation. Milt was collected from six male by under gentle manual pressure, during the reproductive months of November to January. Milt volume (5.75±0.37 mL), fresh sperm motility rate (92.2±3.78%) and sperm concentration (20.1±5.5 billion spermatozoa/mL) were obtained. The semen was diluted (5% gluctose, 10% egg yolk and 10% DMSO, up to 100% of distilled water) and treated with 2.0mg per 120 million spermatozoa or not (control) and kept for 15min at room temperature and then placed into 0.25ml straws were frozen in dry shipper for 30 min and subsequently stored in liquid nitrogen. Semen was thawed in a water bath at 37°C/10s and 10µl of sample was placed in a tube (0.1 ml) and stained with 2µl of Hoechst 333342 plus 2µl of propidium iodide. After 10 minutes of incubated in water bath at 37°C the samples were analyzed fluorescent microscopic (x400). Higher percentages of membrane integrity sperm were maintained after thawing from surubim sperm treated with CLC compared to control (135.9 vs 78.5%, $P<0.05$). Addition of cholesterol to surubim sperm membranes improved cell cryosurvival.

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P85 PLANT VITRIFICATION SOLUTIONS: OVERVIEW OF CHANGES IN THE COMPOSITION AND CONCENTRATION OF CRYOPROTECTANTS

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Cryoconservation of many plant species is possible after adding of a specific cryoprotectant. Cryoprotectant primarily reduces the volume of water before freezing preventing dangerous ice nucleation and mass growth of ice crystals. The second important role of dehydration is the increased likelihood of glass formation in concentrated protoplasm. The most useful are cryoprotectant mixtures in the vitrification process. The first Plant Vitrification Solution (PVS) was published by Steponkus (1989) and almost simultaneously by Sakai in 1990. Since then, many different recipes have been developed. The most common solutions for vitrification are PVS2 and PVS3. The numbers of modified substances in PVS ranged from 2 to 7. Substances in the order of most used are sucrose, glycerol, ethylene glycol, dimethyl sulfoxide and others. The overview deals with modification in concentration and adding or omitting substances from the first published compositions of PVS. The number of published essential modifications is for PVS1 (8), PVS2 (10), PVS3 (5), PVS4 (3), Steponkus´ (1), and Towill´s (2). When comparing different vitrification solutions and their modifications for individual plant species, the optimal ones are usually found. The orientation in PVS modification is an advantage in improving PVS for cultivars or species achieve a higher regeneration rate after cryopreservation. Moreover, when we are tasked with cryopreserving new species or varieties and proposing cryoprotective preservation for them, this overview can be helpful.

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P86 EFFICACY OF ANTIOXIDANT-CONTAINING MEDIA IN CRYOPRESERVATION OF CORD BLOOD NUCLEATED CELLS

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There is a tendency to widespread use of cord blood nucleated cells (NC) in clinical practice. The growing attention of scientists and physicians to the use of NC has led to the need of creation of banks in which samples are stored in a frozen state at a temperature of -196°C without losing their biological properties. Some authors have shown that the process of cryopreservation both in the stages of equilibration with cryoprotectants and directly during freezing-warming can cause cell death by apoptosis. The key initiators of apoptosis may be reactive oxygen species (ROS), the formation of which is activated during cryopreservation. Addition of substances with antioxidant properties to the cryoprotective medium is an approach to avoid or slow down the development of oxidative stress and thus to improve the results of cryopreservation and increase the clinical effectiveness of drugs. The antioxidants trolox (a water-soluble analogue of vitamin E) and L-carnitine (LC) in various concentrations were used for the study. 7.5% dimethyl sulfoxide (Me_2SO) was used as a cryoprotectant. The number of viable intact cells (annexinV^-/$7AAD^-$) was determined by flow cytofluorometry.
The use of a cryoprotective mixture containing 7.5% Me_2SO and the antioxidant trolox in concentrations of 50-70 μM increased the number of viable intact cord blood nucleated cells (annexinV^-/$7AAD^-$) by 12-16% compared to the control cryopreserved under protection of Me_2SO only. L-carnitine when applied to cryopreservation medium was slightly less effective than trolox.

However, the use of LC at concentrations of 15-20 mM can increase by 10% the yield of viable intact cells compared to the control without antioxidant.
Thus, studies have shown the effectiveness and prospects of the addition of antioxidants to cryoprotective media in cryopreservation and long-term storage of human cord blood cells.

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P87 MORPHOLOGICAL CHARACTERISTICS OF FRESHLY ISOLATED AND CRYOPRESERVED SPERMATOZOA OF SAANEN BUCKS IN DIFFERENT SEASONS

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Background. Goat breeding throughout the year may effectively increase livestock if assisted reproductive technologies and cryopreservation of spermatozoa with the best morphofunctional characteristics are used. Objective. To evaluate the influence of seasons on morphological characteristics of goat sperm before and after cryopreservation. Methods. Ejaculate of 3 sexually mature goats of the Saanen breed were obtained during the breeding (September-December) and non-breeding (March-June) seasons. To assess the morphological characteristics smears of sperm were stained and then were visualized under a light microscope with a magnification of x1000. Cryopreservation of sperm was performed in a medium with 10% glycerol and 20% egg yolk. The samples were thawed on a water bath. The morphological characteristics of the cells after cryopreservation were evaluated. Results. After cryopreservation, the number of sperm with defects in morphological structures was significantly higher compared to freshly isolated, both in the breeding and non-breeding seasons ($p \leq 0.05$). In the non-breeding season after cryopreservation, significantly more spermatozoa have head abnormalities than in the breeding season ($p \leq 0.05$). Analyzing the morphological characteristics of the tail part, we revealed the fact of its reduction after cryopreservation, probably due to twisting, loop formation and detachment of its part. Conclusion. Morphological characteristics of Saanen goat sperm significantly change ($p \leq 0.05$) after cryopreservation, regardless of the season. In the non-breeding season, there is significantly more damage to morphological structures in both freshly isolated and cryopreserved sperm, compared with the breeding season.

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P88 HORMONAL STATUS OF AGED RATS WITH DIET-INDUCED OBESITY AFTER UMBILICAL CORD BLOOD ADMINISTRATION

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The number of obese people steadily increases. Disorders of central mechanisms of regulation, when the eating behavior changes and body's neurohormonal shifts occur, are observed in obesity. The objective herein was to evaluate the impact of administration of cryopreserved

nucleated cord blood cells (cNCBCs) on hormonal status of aged rats with obesity. Experiments were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine. Thawed CBNCs preparation was injected intraperitoneally in dose of 3×10^5 $CD34^+$-cells/kg. The preparation was made up to 1 ml volume with plasma autologous to the cells. The next day, a week and a month after cord blood injection the animals were sacrificed and blood was sampled for further studies. The content of thyroxine (T_4), triiodothyronine (T_3), testosterone (T_s), estradiol (E_S) and dehydroepiandrosterone sulfate (DHEAS) was determined by enzyme-linked immunosorbent assay using the standard ELISA kits. The obesity was accompanied by a significant decrease in thyroid and sex hormone concentration in blood serum of aged rats. The cNCBCs administration the next day, a week and a month later promoted increasing not only the total T_3 level, but also total T_4 one in blood serum of aged obese rats, accompanied by a significant increase in Ts concentration as well. The cNCBCs injection augmented the functional activity of thyroid and sex glands, normalized the Ts level, thus increasing the adaptive and compensatory body potential in aged obese animals. The revealed changes are physiologically significant, since thyroid hormones increase the oxygen absorption by organs and tissues, regulate the metabolism of lipids and carbohydrates, thereby participating in energy metabolism and maintaining the constancy of body weight. If a metabolic rate increases, the proteins and fats are decomposed under thyroid hormone impact, resulting in decreased appetite and body weight loss.

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P89 PHYSICOCHEMICAL AND CRYOPROTECTIVE PROPERTIES OF PVA COMPOSITIONS AND LOW MOLECULAR WEIGHT CRYOPROTECTORS

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Preventing ice crystallization during freeze-thawing is one of the main problems of cell preservation. One highly effective recrystallization inhibitor is polyvinyl alcohol (PVA). According to our data, the combination of PVA and low molecular weight cryoprotectants (CP) can significantly improve the results of cells cryopreservation. However, it is not clear which molecular mechanisms influence the cryoprotective activity of PVA in combined media. Using strain-dilatometry, stalagmometry, optical microscopy, fluorescence probes, flow cytofluorimetry, and computer simulations etc., we performed the study of the physicochemical properties of solutions containing PVA (9 and 31 kDa) as well as glycerol (GL) or 1,2-propanediol (1,2-PD). The cryoprotective activity of the solutions was evaluated on donor erythrocytes. It was found that PVA forms micellar structures in aqueous solutions. In 0.1-5% (w/w) PVA solutions changes in the structural organization of the polymer with the formation of local regions differing in size and degree of hydrophobicity were detected. In solutions containing PVA and GL, hydrophobic interactions are enhanced. In media containing 1,2-PD and PVA, there is a change in the structure of the PVA-1,2-PD complex and the decrease

in the hydrophobicity of the media. It was found that the addition of PVA 1,2-PD and GL to aqueous solutions reduces their glass transition temperatures from (-25...-30) ºC to (-90...-95) ºC. This makes it possible to increase the rate of cooling-heating of cells in the range of (-25...-95) ºC, avoiding their damage due to plastic relaxation of thermoelastic stresses. It is assumed that the formation of strong hydrogen-bonded PVA complexes with the surface of ice crystals modifies the surface, depolarizes the water molecules in their environment and thus inhibits further growth of ice crystals. The results obtained are discussed.

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P90 IS IT POSSIBLE TO CRYOPRESERVE ERYTHROCYTES USING NANOPARTICLES?

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The ideology of our study was to determine whether the addition of nanocrystalline cerium dioxide (NCD) to a cryoprotective medium containing glycerol affects the preservation of erythrocytes after cryopreservation. Our choice of NDC is explained by the ability to influence the physical processes that occur during freeze-warming. Experimental samples of erythrocytes we divided into the following groups:1 cryopreservative containing 30 (15 or 5) % glycerol in a ratio of 1: 1 was added to the erythrocytes; 2 cryopreservative containing 30 (15 or 5) % glycerol and 0.02 g / l NDC was added to the erythrocytes in a ratio of 1: Erythrocytes samples were frozen in 2.0 ml cryotubes by immersion into liquid nitrogen, and then they were warmed in a water bath at a temperature of 37°C. We noted that the addition of NCD to the cryoprotectant based on 30 and 15% glycerol did not affect the number of preserved erythrocytes. The preservation rate for cells in samples of groups 1 and 2 after thawing did not differ significantly. Reduction of the concentration of cryoprotectant down to 5% leads to a complete hemolysis of cells. Our previous studies have shown that NCD is able to influence the phase-structural transformations of combined cryoprotective media, reducing the negative effect of factors of cryopreservation. Our findings indicate that it is not enough just to use nanosized particles jointly with a standard cryopreservation protocol to increase cryoprotective properties. The absence of the expected positive effect of NCD in our research may emphasize the need of correction of both the standard protocol for erythrocyte cryopreservation itself and the search for an effective concentration and step of NCD adding to improve preservation rate.

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P91 DEVELOPING CRYO ON-GRID LAMELLA WORKFLOWS FOR THE JEOL JIB 4700F

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Dramatic advances in cryo electron microscopy (cryoEM) have enhanced

macromolecular structure determination but the current frontier is to understand the machinery of life operating in the native biological environment. We wish to visualise the native structure of cells and tissues by cryo electron tomography (cryoET), requiring the preparation of samples 100-300nm thickness.
Since most cell regions are too thick to allow electron beam penetration, cryo sections or lamellae must be produced from vitrified material. Although vitrified cells and tissues can be sectioned in the vitreous state by cryo ultramicrotomy (CEMOVIS), section thickness is restricted (~50 nm) and quality severely limited by mechanical damage. Damage includes tissue compression, scoring and crevassing of sections, each reducing information attainable by cryoET. The best method available is focussed ion beam milling in a dual beam scanning electron microscope at cryogenic temperatures (cryoFIB). This process allows material to be removed with an ion beam, leaving a lamella of controllable thickness, which is transferred into the transmission electron microscope (TEM) for cryoET. These lamella do not have compression or crevassing artefacts and are prepared to a thickness able to contain biologically relevant structures (e.g., a nucleopore at ~200nm diameter). The cryoFIB can also generate 3D volumes by sequential sectioning, imaging of the exposed face of the tissue then further sectioning and imaging. In principal this approach could also be used to understand the complex balance between vitreous and non-vitreous areas in tissues selected for cryopreservation.
Here we report on adapting a JEOL JIB 4700F FIB for cryoFIB milling of tissues for cryoET and cryo 3D volume imaging. Adaptations to the FIB support 24/7 operation at operating temperatures <-169C and ice contamination rates <6nm.h-1. We have optimised vitrification of a reference sample (Euglena gracilis, CCAP 1224/5Z) for cryo lamella production and are developing cryo-correlative workflows. Transferring frozen lamella without contamination for cryoET is also challenging. Adaptations to the cryo stage fitted to the cryoFIB allow lamella to be prepared for cryoET in all commercially available electron microscopes.

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P92 RAM SEMEN CRYOPRESERVATION: EFFECTS OF EGG YOLKS FROM DIFFERENT AVIAN SPECIES

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The effects of adding egg yolks (EY) of different avian species to the Tris glycerol extender was evaluated for post-thawing quality of Najdi semen. The semen samples collected from seven Najdi rams were pooled and diluted 1:4 with the Tris glycerol diluents containing the egg yolks of chicken (C), pigeon (P), goose (G), Japanese quail (Q), duck (D), or turkey (T). The diluted semen samples were gradually cooled to 5 °C within 2 h, equilibrated at 5 °C for 2 h, There are no significant differences between the different dilutions in the results before-thawing in semen quality, including the total progressive sperm motility parameters, total motility, sperm vitality, plasma membrane intactness, DNA integrity, and sperm abnormalities percentages, in comparison with the EY diluents of the other avian species, and then frozen in liquid nitrogen vapour for 8 min before storing them at −196 °C. The frozen straws were thawed at 37 °C for 30 s and evaluated for sperm

motility, vitality, abnormality, plasma-membrane integrity, and DNA fragmentation. The egg yolk samples of the above-mentioned avian species (n = 7/ species) were analyzed for moisture, ash, protein, fatty acid (FA), and trace element contents. The use of the chicken and quail EY diluents resulted in better post-thawing Najdi semen quality, including the total progressive sperm motility parameters, total motility, sperm vitality, plasma membrane intactness, DNA integrity, and sperm abnormalities percentages, in comparison with the EY diluents of the other avian species. The chicken EY had the highest percentages of margaric and linolenic FAs and the lowest percentages of palmitoleic and myristic FAs. Moreover, the percentage of oleic FA was lower in the chicken EY than in the EYs of other species, except turkey. Additionally, the chicken EY had the significantly lowest concentration of Cu, Zn, Fe, and Mn. In conclusion, the use of chicken egg yolk extender is recommended for Najdi semen cryopreservation. The egg yolks of the other avian species studied cannot substitute the chicken EY as they gave poorer post-thawing semen quality. The EY composition, especially FA profile and trace elements concentration, significantly affected the post-thawing quality of buck semen.

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P93 DECREASED RED BLOOD CELL DEGRADATION DURING FREEZE-DRYING, STORGE, AND REHYDRATION

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One of the most common medical procedures performed in U.S. hospitals is blood transfusions. Unfortunately, transfusable red blood cells (RBCs) have a limited shelf life after donation due to detrimental storage effects on morphological and physiological properties. Although transfusions of RBC are essential parts of hospital-based health care, methods to increase RBC shelf life or allow for long-term storage are either cumbersome, non-practical, or entirely lacking. The scope of this study expands upon previous work that focused on increasing the shelf-life of RBCs by storing the cells in a dry state utilizing trehalose as a lyoprotectant. Methods to reduce hemoglobin oxidation and RBC hemolysis during freeze-drying and storage were tested by manipulating freeze-drying buffer compositions, cooling rates, storage conditions, and rehydration buffer formulations. Hemoglobin oxidation in dried RBCs was shown to occur over time during storage. The total amount of oxidation that accumulated with time was reduced by adding 6 mM ascorbic acid. This antioxidant showed positive effects on methemoglobin reduction within the first 75 minutes after rehydration. Furthermore, in the absence of oxygen during storage (vacuum sealed), time-dependent degradation of hemoglobin was still observed and was not significantly different from storage in the presence of oxygen at 0% humidity. Additionally, RBC hemolysis was reduced when utilizing bulking agents with relatively low molecular weights (Dextran MW. 40,000) compared to larger ones (Ficoll MW. 400,000) in the freeze-drying buffer. The

composition of the rehydration solution also played a vital role in RBC recovery. Adding bulking agents to the rehydration solution at pH 5.3 significantly reduced RBCs hemolysis after freeze-drying and rehydration. Overall, advancements in RBC freeze-drying procedures have been developed to bring society one step closer to ending RBC shortages.

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P94 WATER CONTENT AND CELL BIOCHEMICAL STATE TOWARDS TO GERMINATION AND CRYOPRESERVATION OF *BUTIA ERIOSPATHA* EMBRYOS (ARECACEAE)

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Rain forest in Brazil is home of around 50% of plant biodiversity in the world and many of them shows economical potential. Butia eriospatha is an endemic species of Brazilian Atlantic Forest and due to the anthropic intervention, it is in the vulnerable conservation status. In this species, plants are only propagated sexually by seeds which have dormancy and low germination. To manage the limitation of seedling establishment, we study B. eriospatha embryo desiccation tolerance threshold as well the physiological requirement for in vitro germination and cryopreservation. Mature embryos and desiccated ones were in vitro germinated using a culture medium with hormones and antioxidants. The embryo desiccation tolerance threshold was 0.14 gH2O gDW-1 with 93.33% of germination. During embryos desiccation was observed a significant increase in PUT, which resulted in the ratio decrease [(SPD+SPM)/PUT-1] DW), but the SPD was the most abundant polyamine overall. Increase in GPX and APX activity led us to suggest that they are the main enzymes involved in cellular protection during desiccation. An increase of osmoprotector amino acids content, especially glutamic acid (Glu), leucine (Leu), lysine (Lys), glutamine (Gln) also was observed. A specific embryo desiccation stage (0.14 gH2O gDW-1) associated to its biochemical state were successfully used in the high cooling rate technical and result in more than 90% of recovery and in vitro germination. The physiological and biochemistry approach of this study associated to a cryopreservation protocol can be used for plant genetic resources conservation of other Arecaceae species in the world.

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