

CRYO 2023

JULY 25-27TH



ABSTRACTS

60TH
ANNUAL MEETING



McNamara Alumni Center
University of Minnesota
Minneapolis, MN
United States

ORAL PRESENTATIONS

S1 BIOSTASIS: A TRANSFORMATIVE CAPABILITY FOR HUMAN CIVILIZATION

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Preservation breakthroughs in food and agriculture, cell culture, fertility, conservation, and transplantation have become pillars of modern civilization. In their respective human endeavors, these capabilities have had a transformational impact similar to those of the wheel or steam engine on transportation, and of the printing press on communication. However, given the historical neglect of cryobiology and related orphan fields, much of the potential for biostasis technologies remains untapped. Since its inception 10 years ago, the Organ Preservation Alliance has worked to help the biopreservation research community gain attention and resources commensurate with its true potential. The need for next generation and next level biostasis capabilities have for instance been the focus of roundtables at the White House and on Capitol Hill, new initiatives of the American Society of Transplantation and ISBER, and a seminal consensus article by 40+ leading scientists and physicians, including heads of major medical societies and laureates of the Nobel and Breakthrough Prizes (www.nature.com/articles/nbt.3889.pdf).

Across a National Science Foundation (NSF) funded technology roadmapping process and global Organ Banking Summits at Harvard and Stanford, leading cryobiologists and

other scientist mapped out both (i) the remaining sub-challenges to cryopreserve large biological systems, as well as (ii) specific, converging areas of science and engineering that have advanced exponentially and can be combined to overcome remaining sub-challenges. So far, as a result of this effort, nearly \$300M in new funding has been allocated to new cryopreservation technologies by science agencies, investors, and philanthropists, including but not limited to the Biostasis Research Institute and the flagship multi-university, NSF ATP-Bio Program. This appears to be the beginning of the needed “Biostasis Apollo Program” to transform medicine, biomedical research, and other areas by allowing storage of virtually any living system – from cells, to microphysiological systems and tissues, to organs, to whole organisms – in fully functional states. A biostasis “renaissance” has begun with the potential to again transform human civilization.

Funding: Not applicable

Conflict of Interest: Dr. Giwa is the founder of Ossium Health where he remains a shareholder, is the founder, a shareholder and the CEO of Sylvatica Biotech, is a founder, shareholder and chairperson of Expanse Partners and is a founder and shareholder of Humanity Bio, Inc

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S2 FRONTIERS IN THE PRESERVATION OF BIOLOGICAL SYSTEMS FOR MEDICAL APPLICATIONS

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Recent advances in gene editing, our understanding of the immune system and bioprinting will only accelerate the use of biomolecules, cells, and tissues to treat disease or injury. The clinical use of cells and tissues requires effective methods of preservation for these systems. In its most reductive form, preservation involves three components; water, biological system and cryoprotective agents. This talk will describe approaches (i.e., tools and techniques) to alter the behaviour of water, understand the mechanism of cryoprotection and sensitivity of biological systems to freezing.

Funding: R24OD028444, HL154734, TI2042111

Conflict of Interest: Hubel is founder of Evia Bio

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S3 COMBINING NORMOTHERMIC EX VIVO PERFUSION AND HYPOTHERMIA FOR DIAGNOSIS, TREATMENT AND ENGINEERING OF ORGANS FOR TRANSPLANT

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The field of lung transplantation continues to advance at a rapid pace. In fact, of all organ transplantation, lung transplantation shows the most significant growth worldwide. This is the result of a combination of advances in the field that have improved donor lung utilization and made lung transplantation more possible, combined with increased attention of transplant centers focusing on

increasing numbers of transplants performed per year, resulting in gradually improved outcomes. The development of strategies to repair, immune-modulate or engineer improved organs for transplantation continues to evolve. The future of transplantation will include gene and cell therapeutics enabled by ex vivo lung perfusion and manipulation of organ temperature. The current worldwide standard of lung preservation remains the use of cold flush preservation with a low potassium dextran solution that was developed in Toronto in the late nineteen eighties. This basically capitalizes on the reliable concept of cold (4°C) static aerobic flush preservation that has stood the test of time. However, we in fact have known since then, that 10°C is in fact a superior temperature for storage of lungs. We have recently shown that a combination of 10°C storage and normothermic EVLP can in fact protect and preserve lungs for up to 3 days followed by successful transplant in a large animal transplant model. This will not only enable an increase in the number of lungs transplanted, but also will make transplantation a more attractive career for future generations of surgeons. The science of transplantation continues to evolve and novel practices that are being translated to the bedside are radically changing the opportunities to save lives of patients with end stage organ failure with improved short- and long-term outcomes.

Funding: Not applicable

Conflict of Interest: Author is CMO for Traferox Transplant Technologies, Consultant to United Therapeutics, Lung Bioengineering, Abbott.

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S4 CELLULAR THERAPY AND THE CRITICAL ROLE OF BIOPRESERVATION

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Successful biopreservation is critical to ensure the highest quality of cellular therapies. This presentation will provide an overview of the field of cellular therapy with specific examples highlighting the impact of biopreservation and the need for continued research and optimization.

Funding: Not applicable

Conflict of Interest: None to disclose

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S5 FERTILITY PRESERVATION USING OVARIAN TISSUE FOR WOMEN AND RHINOCEROS

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Fertility preservation by cryopreserving ovarian tissue is required for many prepubertal patient groups, but is also an important method for endangered species. The Northern White rhino is functionally extinct with just two females left. To preserve this iconic species, numerous advanced assisted reproductive technologies and strategies have been utilised around the world. The strategy chosen for the Rhino Fertility Project is to harness the potential of the ovarian primordial follicle reserve by culturing ovarian tissue with the aim of generating eggs in vitro; a process that has been carried out for model species. Through working on the rhino tissues, we have gained insights, identified challenges, and developed

new methods that also benefit our work with human ovarian tissues. Obtaining rhino ovaries to develop such techniques has also proved challenging and this void contributed to the creation of Nature's SAFE (Saving Animals From Extinction); a UK biobank charity.

Funding: Foundation Hoffmann

Conflict of Interest: None to disclose

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S6 CHALLENGES IN DEVELOPING CRYOPRESERVED PFSPZ VACCINES AGAINST MALARIA

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Throughout recorded history, malaria has exacted a huge toll in mortality, morbidity, and disability adjusted life years; currently, annual infections exceed 247 million with 619,000 deaths. Essential for eradicating infectious diseases (e.g. smallpox, polio) is a highly effective vaccine: our goal is a vaccine with >90% protective efficacy. PfSPZ vaccines are cryopreserved: they target *Plasmodium falciparum* (Pf) malaria, and employ metabolically-active sporozoites (SPZ) as the immunogen. PfSPZ are grown, from Pf gametocytes initiated from cryopreserved Pf erythrocytic stage cell banks, in aseptically-reared *Anopheles stephensi* mosquitoes, while cryopreserved eggs provide insurance against mosquito colony collapse. PfSPZ vaccines are attenuated by irradiation, co-administration of an anti-malaria drug or genetically but are all manufactured by an identical process. Specific challenges have included developing a completely aseptic production process, fill-finish and cryopreservation adapted to the time constraints imposed by

cryoprotectant additives, development of a new cryovial with septum that maintains container closure integrity below -150 °C and compatible with high throughput, automated thawing, and use at clinics in remote locations, and vaccine administration route, dose and interval. A cold chain employing liquid nitrogen vapor phase cryoshippers, misperceived as a problem by the conventional vaccine industry, is being aided by the advent of CAR-T and cellular therapies. Our cryoshippers are transported by select couriers and carried the last mile by our personnel. They remain, with weekly LN2 recharging, on site as local storage units, sometimes for several months while vaccine is administered. To date, 38 clinical trials at 19 sites in the US, Europe, sub-Saharan Africa and Indonesia, have demonstrated unprecedented tolerance, and achieved up to 100% protective efficacy. Reverse cryologistics have returned unused vaccine, peripheral blood mononuclear cells and other samples.

Funding: Not applicable

Conflict of Interest: None to disclose

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S7 THE DEVIL'S ADVOCATE REGARDING USE OF ME₂SO FOR CRYOPRESERVATION OF CELL AND TISSUE TRANSPLANT THERAPIES

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Dimethyl sulfoxide (Me₂SO) is a commonly used cryoprotectant in cell and tissue cryopreservation, but its application is not without drawbacks. While Me₂SO has several beneficial properties that aid in preserving cells and tissues, it also presents

challenges and potential adverse effects for clinical cell and tissue therapies. The Good: Me₂SO is an effective cryoprotectant due to its ability to penetrate cell membranes, replacing intracellular water, reducing intracellular ice formation, and preventing cell damage during freezing and thawing processes. Me₂SO has been shown to be compatible with many cell and tissue types without which most clinical cryopreserved cell therapies would not be possible. The Bad: Me₂SO has inherent cytotoxic properties, which can cause damage to cells and tissues. The concentration, temperature, and duration of Me₂SO exposure must be carefully controlled to minimize toxicity. Me₂SO can induce cellular stress responses, affecting cell behavior, metabolism, and gene expression patterns. Me₂SO has been shown to interfere with cell differentiation processes, potentially altering the phenotype and functionality of cryopreserved cells, chromosome damage and mutations. The Ugly: There is an extensive literature on clinical side effects of Me₂SO. Frequently observed acute effects include skin irritation, diarrhea, vomiting, odor, and taste disturbances. Me₂SO is classified as a drug, and the US Food and Drug Administration (FDA) has attempted to ban Me₂SO on more than one occasion, but there is presently no proven replacement. Reports of extreme changes in microRNAs and alterations in the epigenetic landscape suggest that Me₂SO is not inert. Such reports have led to the suggestion that the use of Me₂SO should be reconsidered, especially for cryopreservation of embryos and oocytes, since it may impact embryonic development. In conclusion, Me₂SO offers significant benefits as a cryoprotectant for cell and tissue preservation. However, it is essential to consider its potential drawbacks and limitations. Researchers and clinicians must weigh the benefits against toxicity risks, cellular stresses, and possible alterations in

cell behavior induced by Me₂SO. Continued research and development of alternative cryoprotectants, formulations with reduced concentrations of Me₂SO, and supplements to minimize potential adverse effects is crucial to address the concerns associated with Me₂SO and advance the field of cryopreservation for improved cell and tissue storage and transplantation outcomes.

Funding: Not applicable

Conflict of Interest: None to disclose

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S8 THE VALUE OF DMSO AS A CRYOPROTECTIVE AGENT

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Glycerol was introduced as a cryoprotectant most prominently by Polge, Smith, and Parkes in 1949, and dimethyl sulfoxide (Me₂SO) was introduced by James Lovelock in 1959. Nevertheless, a search of PubMed on 6/19/23 for “dimethyl sulfoxide” or “dimethylsulfoxide” or “Me₂SO” and “freezing” returned 26,663 hits, whereas the same search involving “glycerol,” “propylene glycol,” and “ethylene glycol” returned only 3,386, 1,701, and 976 hits, respectively, attesting to the fact the Me₂SO is the most popular and most successful single cryoprotectant used for most freezing protocols. Me₂SO has the following properties that make it particularly valuable as a cryoprotectant: low viscosity, high membrane permeability, excellent freezing point depression, ability to neutralize the toxicity of amides, and low toxicity for most cell types when used at moderate concentrations even at room temperature. The real or apparent toxicity of Me₂SO is cell type specific, but injury can generally be

avoided by distinguishing between toxic injury and osmotic injury, using a high glucose carrier solution, not exceeding reasonable temperature and concentration limits, using apoptosis inhibitors, and mixing Me₂SO with other agents, including agents whose toxicity can be blocked by Me₂SO, enabling higher total concentrations of cryoprotectant to be used, with greater colligative protection at no greater biological cost. Me₂SO, like all other cryoprotectants, seems to have toxic effects in the frozen state, but unlike other agents, these effects can be mitigated by using Me₂SO in combination with amides. Similarly, Me₂SO has been reported to be toxic to kidneys under some conditions, but not under other conditions, and careful use has allowed it to be a major anchor component of the least toxic vitrification solutions now known for whole kidneys. This valuable cryoprotective agent is not ideal for all cells under all circumstances, and more insight into its toxicity, including cell type specific toxicity, is needed, but the same is true for all known alternatives as well and will likely be true for any newly discovered agents also. In vivo toxicity should be avoided by removing Me₂SO prior to transfusing products cryopreserved with this agent.

Funding: Not applicable

Conflict of Interest: None to disclose

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S9 CRYOPRESERVATION WITH REDUCED ME₂SO AND COLD STORAGE SOLUTIONS FOR CELLS BASED THERAPIES

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Freezing cells is the most common method for long-term preserving of biological materials including cells, organoids, and tissues. For effective freezing, a cryoprotective agents (CPA) is required, mainly for avoiding the osmo-pressure conditions occur during freeze-thaw steps. The most effective and commonly used CPA is Me₂SO, usually at 10% concentration, although it is an irritant with direct correlation to its concentration and exposure time. For therapy approaches and off-the-shelf cell-based products, cryopreservation is a crucial step, however Me₂SO or other permeable CPAs (e.g., Ethylene Glycol), can impact the quality, safety, and efficacy of the cellular products and clinical outcome. For this reason and with the tightening of regulation, cryopreservation solution with reduced concentration of Me₂SO or better without Me₂SO is an advantage. In addition, the possibility of maintaining cells for a short term without the need to freeze them, can be an alternative, that may reduce post-freezing necrosis and apoptosis, eliminate the risk of the exposure to undesired CPAs and enable shipping stability. The present study explores the performance of animal component free (ACF), salt-based cryopreservation solutions with 5% Me₂SO (NutriFreez® D5,) and without Me₂SO as well as NutriStor® a novel defined ACF, salt-based solution, that enables short term storage at 2-8°C, as alternative to the freezing procedure. Results reveal that the cell's viability parameter is not reflecting the growth recovery of the cells. Cryopreservation solutions with reduced Me₂SO and NutriStor® lead to high cells viability and growth recovery, while maintaining cells' identity and features. Short-term cold storage and long-term cryopreservation in reduced concentration of Me₂SO or Me₂SO-free solutions are essential tools that may leverage the use of cell-based

off-the-shelf products. Still, the cells should be carefully examined prior to use, and the methods of testing stored cells should be comprehensive beyond cell viability.

Funding: Not applicable

Conflict of Interest: None to disclose

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S10 WITHDRAWN

S11 CRYOGENIC PRESERVATION OF PERINATAL STEM CELLS IN ME₂SO - SUPPLEMENTED AND ME₂SO -FREE CONDITIONS

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Advanced medical treatments are biological products (viable cells) with a limited shelf life. Ultra-low temperature preservation may circumvent issues. Cryopreservation ensures the immediate availability of the medicinal cells and allows time for quality tests and donor-recipient match. However, new ATMP products such as perinatal stem cells present challenges in relation to viability and stability post-thawing. Our group was the first to report stem cell nature in human amnion epithelial cells (hAEC) isolated from full-term placentae and validated GMP-grade cryopreservation procedure. The exposure to standard-of-care cryoprotective agents, typically Me₂SO, has proven challenging for clinical use and negatively affects cell viability and functions. We performed cryogenic storage of perinatal stem cells, both hAEC and MSC, using commercial GMP-grade solutions. We evaluated both standard cooling rate (1 C/min) and higher or lower freezing rates using a controlled rate

freezer device. The thawing procedure was performed using a warming rate of 60–80 C/min, by immersion in a water bath or a GMP-grade thawing device. We tested 20 different hAEC and WJ-MSC batches using both formulations supplemented with 10% Me₂SO, and new Me₂SO -free cryosolutions. Since detrimental mitochondrial activities (e.g., reduced ATP production) have been reported at low temperatures, we coupled viability and recovery measurements with ATP quantification. We analysed cell adhesion to the substrate upon thawing and proliferation capacity. hAEC presented a superior capacity to survive and perform equally well as freshly isolated cells when exposed to Me₂SO -free cryosolutions. Conversely, MSCs had a batch-to-batch variability in terms of survival and identity in presence of Me₂SO. Cell adhesion and expansion were batch-related with a preference for Me₂SO-free conditions. Controlled-rate freezers or passive freezing devices represent valuable and efficient strategies to control the rate at which cells traverse that temperature difference. Me₂SO-free cryosolutions showed great efficacy in preserving hAEC, without loss of energetic characteristics and efficacy. Such a switch may not serve equally well for MSC. Post-thawing washes may be appropriate for both ATMPs to provide a stable and safe product to infuse in patients.

Funding: Åke Wibergs stiftelse

Conflict of Interest: Founder and stockholder at Tandem Biotherapeutics. Inc.

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S12 BRINGING NEW ORGAN PRESERVATION TECHNOLOGIES TO THE CLINIC

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The development of strategies to repair, immune-modulate or engineer improved organs for transplantation continues to evolve. The future of transplantation will include gene and cell therapeutics enabled by ex vivo lung perfusion and manipulation of organ temperature. This talk will review the commercial and clinical paths machine perfusion has taken, including the advent of organ repair centers to enable dramatically increased utilization and allow engineering of the organs during preservation. Synergies and contrasts for use of advanced preservation technologies in such organ repair centers, and the concept of organ preservation centers, will be discussed.

Funding: Not applicable

Conflict of Interest: SK is CMO of Traferox Technologies and Consultant to Lung Bioengineering and United Therapeutics.

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S13 ORGAN CRYOPRESERVATION BY VITRIFICATION AND RAPID REWARMING: NEW BREAKTHROUGHS IN TRANSPLANTATION

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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. 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. This talk will focus on the two recent breakthroughs from our group over the last year, in successful vitrification, rapid rewarming and transplantation of islets and kidneys.

Funding: Not applicable

Conflict of Interest: None to disclose

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S14 THE VISIBLE HEART® LABORATORIES: FUNCTIONAL ASSESSMENTS OF REANIMATED TISSUES AND ORGANS

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For over 30 years, the University of Minnesota Visible Heart® Laboratories have been reanimating isolated large mammalian tissues and organs, including human, so to perform translational research. We assess the relative viabilities and study the effects of pre- and post-conditioning with pharmacological agents as means to enhance in vitro function. These experiments have also included the study of device-tissue interactions and the biophysical effects of applied ablation. The talk will focus on use of human and porcine organs in a preclinical setting for testing of pharmacological agents and device development, with an emphasis on system perfusion applications in transplant medicine.

Funding: Not applicable

Conflict of Interest: None to disclose

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S15 OPTIMIZING ALKYLATED AMMONIUM-BASED COMPOUNDS FOR ICE RECRYSTALLIZATION INHIBITION (IRI) ACTIVITY

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During cryopreservation, ice recrystallization, defined as the growth of larger ice crystals at the expense of smaller ice crystals, ultimately leads to non-repairable membrane damage and cell death. The development of small molecules as ice recrystallization inhibitors (IRIs) has proven to be an effective strategy to mitigate cryoinjury associated with ice recrystallization in various cell types. Prior work in our laboratory has shown that various ammonium salts exhibit IRI activity. Furthermore, quaternary ammonium salts have been implicated as cryoprotective agents previously. Consequently, we sought to assess and optimize various ammonium-based salts for IRI activity. Preliminary studies focused on modifications made to the quaternary ammonium salt choline chloride (trimethylethanolamine chloride, ChCl). ChCl has been shown to exhibit low cytotoxicity, is a biorelevant molecule, and has been used previously in cryopreservation protocols. Using an SAR-like strategy, derivatives of ChCl were synthesized and tested for IRI activity, including those with modifications to the N-alkyl position(s), the anionic counterion, and the length of the

ethanolic chain. Over 25 derivatives of ChCl were synthesized and tested for IRI activity and all modifications tested improved the IRI activity in comparison to ChCl, with some modifications improving the IRI activity up to 23-fold. Overall, this optimization study represents a promising proof of concept for optimizing ammonium-based compounds for IRI activity using synthetically accessible modifications to either the N-alkyl position(s) or and/or the anionic counterion. These findings support previous work in the Ben laboratory that has shown that a balance between hydrophobicity (i.e., the N-alkyl substituents) and hydrophilicity (i.e., the hydroxyl group) is important for IRI activity. Therefore, the ability to employ alkyl groups as a means of achieving the required balance between hydrophobic and hydrophilic moieties is an important tool for future IRI optimization studies.

Funding: The authors acknowledge the Natural Sciences and Engineering Research Council of Canada (NSERC), Canadian Blood Services (CBS), Canadian Institutes of Health Research (CIHR), and the Canadian Glycomics Network (GlycoNet) for financial support.

Conflict of Interest: None to disclose

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S16 MULTISCALE TRANSPORT AND 4D TIME-LAPSE IMAGING IN PRECISION CUT LIVER SLICES

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Monitoring cellular processes across different levels of complexity, from the cellular to the tissue scale, is important for understanding tissue structure and function.

However, it is challenging to monitor and estimate these structural and dynamic interactions within three-dimensional (3D) tissue model. Therefore, the aim of this study was to design a method for imaging, tracking, and quantifying 3D changes in cell morphology (shape and size) within liver tissue, specifically a precision-cut liver slice (PCLS). A PCLS is a three-dimensional (3D) model of the liver that allows the study of the structure and function of liver cells in their native microenvironment. Here we present a method for imaging liver tissue during anisotonic exposure in a four-dimensional, multispectral manner. Two metrics of tissue morphology were measured to quantify the effects of osmotic stress on liver tissue. We quantified the changes in nuclei position and calculated the changes in volumetric responses of tissue-embedded cells. During equilibration with permeating and non-permeating solutes, the nuclei showed a change in position and directional displacement under osmotic stress. Thus, we demonstrate that nuclei could be used as a probe to measure local osmotic and mechanical stress. Moreover, we demonstrate that cells change their volume within tissue slices as a result of osmotic perturbation and that this change in volume is dependent on the position of the cell within the tissue and the duration of the exposure. The results of this study have implications for a better understanding of multiscale transport, mechanobiology, and triggered biological responses within complex biological structures.

Funding: Not applicable

Conflict of Interest: None to disclose

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S17 DIRECT MEASUREMENT OF RICE (*ORYZA SATIVA*) CALLUS CELL RESPONSES TO PVS2

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Plant vitrification solution 2 (PVS2) is frequently used when cryopreserving plant species that cannot be stored in seed banks. Much of the research with PVS2 has focused on finding adequate cryopreservation protocols to improve the plant survival after thawing. This research can be improved by understanding the permeation rate of PVS2 across plant cell membranes. In previous studies, we have used brightfield microscopy and coherent anti-Stokes Raman scattering (CARS) microscopy to study rice callus cell membrane responses and permeation by three individual cryoprotectants found in PVS2: 15% wt/v Me₂SO, 15% wt/v ethylene glycol, and 15% wt/v glycerol in aqueous solutions. Brightfield studies showed that membrane plasmolysis and deplasmolysis typically occurred between 30-100 s and 100-600 s, respectively after cryoprotectant exposure. Conversely, the CARS microscopy studies showed that the deuterated cryoprotectants permeated the rice callus cell membranes more quickly, between 5-50 s, indicating that membrane response occurs long after cryoprotectant permeation. The work presented here explores cryoprotectant permeation kinetics in complete solutions of PVS2 using our established experimental framework. Brightfield microscopy shows that when cells are exposed to PVS2, cell membrane plasmolysis typically occurs between 30-60 s while any further membrane distortion occurs between 200-700 s. In contrast, CARS microscopy shows that cryoprotectant permeation occurs faster, between 5-20 s, and we observe no statistical differences between the times for different cryoprotectant permeation into cells. These

results highlight that cryoprotectant permeation rates are increased when using complete solutions of PVS2 and occur before cell membrane response.

Funding: USDA and National Science Foundation

Conflict of Interest: None to disclose

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S18 SIMULTANEOUS SCREENING OF MEMBRANE PERMEABILITY AND TOXICITY FOR DISCOVERY OF NEW CRYOPROTECTANTS

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Cryopreservation has the potential to enable long-term storage of organs, tissue, and living cells. However, despite some successes with small samples like embryos, it remains a challenge to cryopreserve larger specimens like tissues and organs. For such specimens, vitrification methods are particularly promising. A major challenge in the design of vitrification methods is selection of cryoprotectants (CPAs) that should ideally permeate cell membranes and promote formation of a glass without causing cytotoxicity. To address this challenge, we have developed a method for the fast measurement of cell membrane permeability and toxicity. Bovine pulmonary artery endothelial cells (BPAEC) were cultured in

96 well plates and stained with calcein AM, which has previously been used as a marker of cell volume for measuring cell membrane permeability. A FlexStation 3 plated reader was used to automate CPA addition and subsequent fluorescence measurements at 1.5 s intervals at 4 °C and 25 °C for 27 different chemicals. The resulting fluorescence data was used to estimate the cell membrane permeability to CPA and water. The same well plate was then used to assess CPA toxicity. Fluorescence was measured after the cells had been exposed to CPA for ~10 min, then again after removing the liquid from the well. Toxicity was estimated from the ratio of these fluorescence values. An entire well plate can be measured in less than an hour, which is nearly 100x faster than our previous microscope-based method for measuring CPA permeability, while also offering a simultaneous estimate of CPA toxicity. With the exception of glycerol (which has a relatively low permeability), most commonly used CPAs have high cell membrane permeability and low toxicity. Our experiments have revealed some other less commonly used chemicals that meet these criteria, including dimethylacetamide, 2-methoxyethanol and diethylene glycol.

Funding: This work was supported by funding from the National Institutes of Health (R01 EB027203).

Conflict of Interest: None to disclose

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S19 DIGITAL HOLOGRAPHY: A TOOL TO MEASURE TEMPERATURE AND CHARACTERIZE PHASE CHANGES DURING VITRIFICATION AND REWARMING

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Biospecimens at various scales in volume such as cells, embryos, and organs can be successfully vitrified at the required critical cooling rates. However, the rewarming stage remains a challenge due to devitrification. Various techniques are under investigation to facilitate ultrarapid and uniform warming, such as laser nanowarming and Joule heating. Laser nanowarming is performed by dispersing plasmonic nanomaterials in cryoprotective agent solutions (CPAs) to rapidly convert optical radiation into heat, supplied by a laser beam. Although vitrification and laser nanowarming has shown promising results, the viability of biological samples remains low. Therefore, the warming process needs to be further enhanced by understanding the local temperature history and phase change kinetics. To address this, we designed a digital holography interferometry (DHI) setup to measure and visualize temperatures in both droplets and large volumes. CPA droplets are vitrified by dunking them into liquid nitrogen and then brought back to the beam path. Large CPA volumes are placed in a cuvette and cooled via a thermoelectric setup. As the CPAs are vitrified and rewarmed the DHI HeNe laser passes through the sample. A CMOS camera records the interference of that beam and the reference beam, resulting in a hologram. Through image processing, the refractive index change is calculated from the phase difference maps and is converted into a temperature profile. The results give accurate measurements of temperature within the sample, which correlate to the cooling and heating rates. Through these results we observe the vitrification temperature history

to determine phase changes in that stage and heating conditions to optimize current warming techniques and improve biospecimen viability.

Funding: National Science Foundation (NSF) under Grant No. EEC 1941543 and NSF Graduate Research Fellowship Program (GRFP) under Grant No. DGE 1746932

Conflict of Interest: None to disclose

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S20 CELL AND TISSUE CRYOPRESERVATION THROUGH PRESSURE ENHANCED SOLID SURFACE ULTRA-RAPID COOLING

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Cooling rate is critical for cell and tissue cryopreservation. By increasing the cooling rate during freezing, it is possible to decrease the cryoprotective agent (CPA) concentration by limiting or even preventing ice growth. One control parameter that can increase the likelihood of avoiding ice growth is hydrostatic pressure. Previous studies have shown that pressure can reduce the melting temperature (T_m) and the homogenous nucleation temperature (T_h), and on the other hand, increase the glass transition temperature (T_g). These alterations are encouraging indicators to improve freezing concerning eliminating ice formation. In this work, first we show that pressure also influences cooling rate outright. Our physical and mathematical modeling in this study show that increasing pressure up to 200 MPa enhances the cooling rate significantly. Second, solid-surface conductive-cooling is another approach to improve the cooling rate

compared to convective cooling methods such as plunging directly into liquid nitrogen, prevalently used in cryobiology. Our numerical results demonstrate that conductive heat transfer can increase the cooling rate by one order of magnitude compared to convective heat transfer. Using this model and experimental data, we designed and manufactured a cryodevice that can apply cooling to both sides of tissue to achieve a substantial survival depth in a hermetically sealed sample container. In conclusion, understanding and insight into details from various disciplines can aid the design and development of effective cryoprotocols.

Funding: National Science and Engineering Research Council

Conflict of Interest: None to disclose

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S21 SMALL MOLECULE ICE RECRYSTALLIZATION INHIBITORS FOR THE CRYOPRESERVATION OF TISSUE COMPONENTS AND TISSUE CONSTRUCTS

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Despite the use of cryoprotective agents (CPAs) such as dimethyl sulfoxide and glycerol, the cryopreservation of these cellular products is suboptimal and new

strategies/protocols are urgently required. This is because during cryopreservation, the majority of cellular injury/death is the direct result of uncontrolled ice growth, a phenomenon known as ice recrystallization. To address this issue, our laboratory has pioneered several different classes of molecules that possess the ability to inhibit ice recrystallization, ultimately increasing post-thaw viability and/or recovery as well as the functional capacity of progenitor cells and tissues. This presentation will demonstrate the ability of IRIs to increase the post-thaw viability/recovery of dermal tissue constructs which are important for wound healing and skin regeneration where readily available tissue constructs are urgently needed to improve the patient prognosis. We hypothesized that combining innovative tissue engineering with ice recrystallization inhibitors (IRIs) would enable the cryopreservation of dermis suitable for autologous epithelial cell seeding. The self-assembled method was used to obtain the dermal constructs. Briefly, fibroblasts were cultured for 28 days to form a sheet where cells were embedded into a self-assembled matrix. The baseline viabilities of cells in suspension and dermal sheets was initially assessed using 1.5% BSA + 10% Me₂SO solution as a control. Post thaw cell viability was determined using TrypanBlue and long-term viability/functionality was observed at 96 hrs in culture after thawing. Tissue viability was assessed using metabolic and DNA-staining assays (AlamarBlue and SytoxGreen). Our results indicate that a number of different classes of IRIs decreased post-thaw fibroblast cell mortality in suspension (38-52%), and increased post-thaw tissue viability (20-27%). Production of a complete skin equivalent using cryopreserved dermis and seeded keratinocytes is currently underway. Collectively, these studies illustrate the tremendous potential of the IRI technology

platform to effectively cryopreserve tissue components and tissue constructs.

Funding: Not applicable

Conflict of Interest: None to disclose

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S22 ISOCHORIC BIO-THERMODYNAMICS: RECENT PROGRESS ON EQUILIBRIUM, METASTABLE, AND NON-EQUILIBRIUM ISOCHORIC BIOPRESERVATION TECHNIQUES

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Isochoric (constant-volume) biopreservation techniques have gained significant interest in the past half-decade, with published work on preservation of human cells, tissues, and organs for medical applications; of whole marine organisms for conservation applications; of fresh food stuffs for agricultural applications; and etc. However, contrary to popular conception, these differing applications do not employ one monolithic technique, but a wide variety of different techniques that are unified only by their use of macroscopic isochoric confinement. In this talk, we will review the three principal thermodynamic variants employed in isochoric biopreservation: equilibrium isochoric freezing, metastable isochoric supercooling, and non-equilibrium isochoric vitrification. For each approach, we will discuss the differing thermodynamic and kinetic considerations at play; appropriate application domains; and specific state-of-the-art protocols. We will conclude by offering high-level perspective on the direction of the field, and identifying the

most significant open questions that may help guide future research.

Funding: Not applicable

Conflict of Interest: MJPP, AC, and BR have filed patents related to isochoric biopreservation, and MJPP and BR have a financial interest in a private company related to these technologies.

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S23 TEMPERATURE-DEPENDENT THERMOPHYSICAL PROPERTIES OF CPAS AND TISSUES RELEVANT TO CRYOBIOLOGY

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The objective of this study is to create a comprehensive living database of thermophysical properties for the benefits of the cryobiology community, in the range of -150°C to 0°C (<https://www.atp-bio.org/>). Thermophysical properties are essential for accurately modeling the behavior of cryoprotective agent (CPA) cocktails and tissues, ultimately improving the design and outcomes of cryopreservation protocols and cryotherapy procedures. The focus of this

study is compiling temperature-dependent thermal conductivity, density and thermal expansion coefficients for various materials, while future efforts will target incorporating specific heat and elastic material properties to this database. The property of thermal conductivity is related to the ability of a material to transfer heat across the domain. Density and thermal expansion also affect the material response to changes in temperature in various ways. In fluids, density differences can drive fluid flow, while in solids, expansion and contraction can lead to mechanical deformation. This study further reviews common measurement techniques, ranging from molecular-scale to cellular-level and macro-scale. In turn, this can inform the user on the appropriate measurement method for a specified sample type, application, and temperature range.

Funding: Authors acknowledge National Heart Lung and Blood Institute (NHLBI) of the National Institutes of Health under award number R01HL127618 for the thermal expansion and density related work.

Conflict of Interest: None to disclose

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S24 DELAYED INJURY AND CELL-TYPE-SPECIFIC RESPONSES DURING CRYOPRESERVATION OF ENDOTHELIAL MONOLAYER

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The cryopreservation of endothelial cell monolayer is an important stage that bridges the cryopreservation of cells in suspension to tissues. Previous studies have identified clear distinctions in freezing mechanisms between monolayers and cells in suspension, as well as developed novel protocols for monolayer cryopreservation. Recently our group has shown that human umbilical vein endothelial cell (HUVEC) and porcine corneal endothelial cell (PCEC) monolayers grown on Rinzl plastic substrate can be cryopreserved in 5% dimethyl sulfoxide, 6% hydroxyethyl starch, and 2% chondroitin sulfate, following a slow cooling protocol with rapid plunge to liquid nitrogen from -40°C . However, viability assessments were done immediately post thaw, which may have resulted in an overestimation of cell viability due to possible delayed injury responses. Here, we show that for the optimal protocol condition of plunge at -40°C , HUVEC and PCEC monolayers exhibited no immediate post-thaw injuries and no delayed injury response after overnight culture. HUVEC monolayers experienced reduced proliferation during the post-thaw culture, while PCEC monolayers maintained a proliferation rate comparable to non-treated controls. The difference in the low-temperature responses between HUVEC and PCEC monolayers is enhanced under high temperature plunge conditions that would result in intracellular ice formation. HUVEC monolayers exhibited intermediate immediate membrane injury but a pronounced delayed injury response, while PCEC monolayers showed significant immediate membrane injury but no delayed injury response. Therefore, we confirm that our previously designed endothelial monolayer cryopreservation protocol is successful for HUVEC and PCEC monolayers, and we identified several cell-

type-specific responses during the freezing process.

Funding: This work was funded by the Canadian Institutes of Health Research (CIHR PS 166125). Janet A.W. Elliott holds a Canada Research Chair in Thermodynamics.

Conflict of Interest: None to disclose

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S25 FREEZE-DRIED DECELLULARIZED HEART VALVES AS STARTER IMPLANTS FOR HEART VALVE REPLACEMENT THERAPIES

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Decellularized heart valves have the potential to replace a malfunctioning valve. Such tissue engineered heart valve scaffolds not only have regenerative capacity, but also exhibit growth capacity when implanted in children. We have shown that heart valves can be freeze-dried after decellularization processing ensuring off-the-shelf availability. Sucrose is needed as lyoprotective agent to mitigate pore formation in the tissue caused by ice crystals and to preserve biomechanical characteristics after freeze-drying. Exposure to highly concentrated sucrose solutions is needed to completely diminish pore formation. In vivo trials in animals have shown that freeze-drying does not alter the long-term durability and repopulation potential of decellularized grafts. Here we aimed to study oxidative

damage in freeze-dried heart valves during storage. Decellularized porcine aortic heart valves were analyzed by nitroblue tetrazolium (NBT) staining and Fourier transform infrared spectroscopy (FTIR) to identify oxidative damage during freeze-drying and subsequent storage as well as after treatment with H₂O₂ and FeCl₃. NBT staining revealed that sucrose at a concentration of at least 40% (w/v) is needed to prevent oxidative damage during freeze-drying. Dried specimens that were stored at 4 °C depict little to no oxidative damage during storage for up to several months. FTIR analysis combined with a feed forward artificial neural network model showed that fresh control, freeze-dried and stored heart valve specimens cannot be distinguished from one another, whereas H₂O₂ and FeCl₃ treated samples can be classified based on differences in characteristic molecular group vibrations. This implies that freeze-drying and storage have little effects on the overall biomolecular fingerprint of the scaffolds. Taken together, we conclude that sucrose can minimize oxidative damage caused by freeze-drying, and that subsequent storage has little effects on the biochemical composition of heart valve scaffolds.

Funding: Not applicable

Conflict of Interest: None to disclose

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S26 COMPRESSION-MEDIATED CRYOPROTECTANT DELIVERY ENABLES CRYOPRESERVATION OF INTACT INTERVERTEBRAL DISC

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In the US, over 1.3M people annually undergo invasive surgery to alleviate back pain associated with intervertebral disc (IVD) degeneration. These surgeries do not treat IVD degeneration, and often lead to further health complications. These problems have driven the research community to seek new solutions, such as IVD allografting, tissue engineering and stem cell therapies. In all of these cases, the lack of readily available human IVDs is a significant barrier. While it is currently possible to obtain fresh IVDs from deceased donors, the IVD cells only remain viable for a short time. This makes it impractical to use fresh IVDs as allografts, and complicates the logistics of IVD research. Cryopreservation of the IVD would solve these problems. However, previous attempts to cryopreserve the IVD have resulted in significant loss of cell viability. The major challenge is that cryoprotectants (CPAs) are required to prevent freezing damage, but most CPAs are toxic within a matter of hours. Because of its large size, CPA diffusion into the center of the IVD would take days. To address this challenge, we examined the potential for accelerating CPA delivery into bovine IVDs by compressing the disc and leveraging the subsequent swelling to draw in CPA. Our results confirm that conventional diffusion-based CPA delivery requires more than 3 days for CPA to fully permeate the IVD. In contrast, we were able to fully equilibrate the IVD in less than 5 h using compression-mediated CPA delivery. When compression and swelling was used to deliver CPA before cryopreservation, cell viability was excellent throughout the IVD, matching the viability observed for fresh controls. This remarkable result was only possible when compression-mediated CPA delivery was used. Less than 10% viability was obtained when CPA was delivered by diffusion alone, without prior compression of the IVD.

Funding: Not applicable

Conflict of Interest: None to disclose

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S27 AN EFFICIENT METAL CONTAINER AND STORAGE METHODS FOR SUCCESSFUL ARTICULAR CARTILAGE VITRIFICATION

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Vitrification as a successful preservation method relies on multiple factors including cryoprotectant agent (CPA) composition, method of CPA loading/unloading and type of storage container. In this study we used the Ovarian Tissue Cryosystem (OTC) as a CPA loading container and a cryo-bag as a storage container to vitrify 7-mm diameter porcine osteochondral dowels. The metal OTC has a much smaller volume of solution requirement (5 ml total), and it is considered “no-touch” and sterile, while the cryo-bag is known as a standard method for tissue storage in tissue banks. We also compared the effects of 2 methods for storing cartilage tissues in the cryo-bag: (a) storage in cryo-bags with a surrounding vitrification solution versus (b)

storage in cryo-bags without a surrounding vitrification solution. Three porcine stifle joints (n=3) from sexually mature pigs were collected from a slaughterhouse and 4 osteochondral dowels (7 mm) were recovered from each joint. Dimethyl sulfoxide, ethylene glycol, and propylene glycol were permeated into porcine articular cartilage using a 430 minute 4-step cryoprotectant permeation protocol. Chondrocyte viability was assessed by a cell membrane integrity stain in 3 different stages: (1) just before CPA loading (Fresh), (2) after CPA loading (CPA Toxicity), and (3) after vitrification-rewarming (Warming). There were no significant differences ($P > 0.05$) in the chondrocyte viability of 7-mm osteochondral dowels after vitrification-rewarming with or without the surrounding vitrification solution (94.63 ± 3.97 vs. 96.17 ± 1.95 , respectively). Moreover, there were also no significant differences in the chondrocyte viability between vitrified groups and fresh group ($P > 0.05$). In conclusion, this study demonstrated that using the metal OTC as a CPA loading container and the cryo-bag as a storage method (with or without surrounding solution) can be used as an efficient and reproducible method for osteochondral dowel vitrification and long-term storage.

Funding: This study was funded by the Edmonton Orthopaedic Research Committee and the University (of Alberta) Hospital Foundation. JAW holds a Canada Research Chair in Thermodynamics.

Conflict of Interest: None to disclose

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S28 THERMOMECHANICAL STRESS ANALYSIS OF POPLITEAL ARTERY IN VARIOUS CONFIGURATIONS DURING VITRIFICATION

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While peripheral arterial disease (PAD) may require replacement of popliteal artery with a suitable vein graft, in roughly 30% cases the saphenous vein is not suitable for vascular reconstruction. As a solution, tissue engineered blood vessels (TEBVs), created ahead of time for the patients at risk of PAD, can be preserved and used when required for transplantation. This study presents thermomechanical stress analysis of popliteal artery during cryopreservation by vitrification, which is the only alternative for ice-free long-term preservation of large - size specimen. Unlike other organs such as heart or kidney, where the shape and size of the organ limits the selection of domain configuration, popliteal artery can be bent into various configuration such that heat transfer to the tissue can be improved. The current study compares the thermal stresses in artery in two different configurations: (i) spiral, and (ii) helical, both inside the cylindrical container placed in a convective environment. The cooling and rewarming protocols have two stages each, with annealing near glass transition temperature of cryoprotective agent (CPA) during cooling as well as rewarming. The thermal stress distribution in spiral configuration shows high stress towards the center of the domain, suggesting that the tissue should be kept closer to the container wall. While the stress in the artery is observed to be less than 1 MPa, the stress in the surrounding CPA can be higher, which can lead to structural damage. The thermal stress in the CPA and artery during slow rewarming stage can be at least a magnitude higher than the stress at the end of cooling, which can be mitigated with volumetric heating methods such as nanowarming. This study demonstrates how

the computational tools can be used for designing the cryopreservation protocol to minimize the structural damage during vitrification.

Funding: This research has been supported by the National Science Foundation (NSF), grant no. EEC 1941543. The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the NSF.

Conflict of Interest: None to disclose

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S29 CRYOPRESERVATION OF LIVER TISSUE SLICES FOR PHARMACOLOGICAL APPLICATIONS

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There is a significant need in pharmacology for better in-vitro drug testing and discovery models given the poor rates of success for translation of drugs from pre-clinical to clinical trials. Liver tissue slices have the potential to serve as simple and versatile models representing aspects of the whole organ architecture, phenotype, and function, allowing for understanding non-hepatocyte cell interactions in drug metabolism and helping mitigate inaccurate drug clearance rates that hepatocyte-only based models suffer from. However, at present, they are not

easily cultured, nor are they “off the shelf” available for testing. One way to address scaling and availability of liver slices is cryopreservation. Cryopreservation can not only be applied to both animal and human tissue, enabling ideal testing, but also allowing for preservation of pooled effects across diverse population groups. In this project we successfully cryopreserved rat liver tissue slices, 250 µm in thickness and 5mm in diameter, prepared using a vibratome after coring. The slices were diffusively loaded with 7.7M Ethylene Glycol and Sucrose based cryoprotective agent in a stepwise manner. The loaded tissue was then vitrified and rewarmed using an ETFE cryomesh. The tissues were then cultured along with controls for a week in 96 well plates with serum-free, chemically defined media in low volumes (100-250 µl). The viability and function of the tissues were evaluated using a variety of assays including live/dead staining, LDH release, urea production and oxygen consumption. Results show that the cryopreserved slices maintained viability and functionality throughout the culture period, with measures comparable to controls. This work demonstrates the feasibility of cryopreservation and banking of liver tissue slices, which could potentially enable a dramatic shift in the effectiveness of pharmacological drug discovery and testing. The simplicity of the system may be used in the future for large animal and human tissues.

Funding: This material is based upon work supported by the National Science Foundation under Grant No. EEC 1941543, NSF EEC-1941543, NIH R01 DK132211 and Biostasis Research Institute.

Conflict of Interest: None to disclose

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S30 OUTCOMES OF NANOWARMED VITRIFIED ARTICULAR CARTILAGE TRANSPLANTATION IN A PORCINE MODEL

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The limited supply and short storage time of fresh cartilage osteochondral allografts present challenges in clinical practice. Vitrification is a promising technique for tissue cryopreservation, however it has limited application to large specimens due to ice crystal formation during rewarming. Our previous study has shown that nanowarming improves the viability of vitrified grafts, particularly in the superficial layer, by reducing rewarming-induced damage (Cryobiology 109 (2022) S133). Building on these findings, we present the first in vivo experiences with nanowarmed articular cartilage specimens in a 4-month transplantation study. Osteochondral specimens from the trochlea groove of mature Yorkshire pigs were harvested and divided into fresh (control) and vitrified nanowarmed groups. After implantation in Hanford miniature pigs, the knees with implants were analyzed by imaging (µCT and MRI), macroscopic scoring and histopathology. The in vivo pig model was successfully developed, with fresh grafts integrating well. The 4-month vitrified grafts showed partial bone loss, connective tissue and cartilage formation at the implant site,

while fresh grafts displayed similar changes at earlier timepoints (1-3 months), suggesting that there is potential for normalization of the vitrified grafts if they had been maintained beyond 4 months. The cartilage of the vitrified grafts was thickened in all cases. Location of vitrified grafts impacted results, with significant differences observed ($p=0.0046$) in proximal and distal placement. However, similar changes were noted in both fresh and vitrified samples (Fig 1). This study demonstrates the successful development of a pig model for assessment of future optimization of vitrification and nanowarming protocols. The vitrified grafts regenerated more slowly than fresh, possibly due to lower cell viability in the middle and deep zones of osteochondral plugs or residual cryoprotectants. The study highlights the potential of nanowarming to improve the viability of vitrified grafts, while emphasizing the importance of graft location in future studies.

Funding: NIH grants R44AR073136, P20GM121342, and R01DE021134

Conflict of Interest: ZC, EDG, LHC, and KGMB are employees of T3LLC

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S31 FIT-FOR-PURPOSE ANALYTICAL METHOD DEVELOPMENT TO SUPPORT THE CHARACTERIZATION AND TESTING OF CELL THERAPY PRODUCTS

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Cryopreservation is an important biomanufacturing process that enables to preservation and delivery of life-saving cell therapy products. The pre-analytical step of

cryopreservation can alter the properties of the cells in ways that effect their downstream analysis. For example fresh cells vs cryopreserved cells may require different approaches for evaluating cell viability based on changes to the cell membrane induced by the cryopreservation process. In this talk, I will describe a new ISO standard for the characterization and testing of cell therapy products (ISO 23033:2021) that describes a fit-for-purpose approach to establishing analytical methods. I will also provide examples of an approach to establish fit-for-purpose cell viability assays that can be extended to the evaluation of cryopreserved cells.

Funding: Not applicable

Conflict of Interest: None to disclose

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S32 ACUTE NEED FOR CRYOPRESERVATION STANDARDS TO FACILITATE THE BIOECONOMY

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There are currently significant global initiatives to expand the bioeconomy, term that generally refers to the share of the economy based on products, services, and processes derived from biological resources. For many of the fields that are included in the general rubric of bioeconomy, such as regenerative medicine and cellular agriculture, cryopreservation is crucial to their existence as a field of study. The role of cryopreservation and cryobiological knowledge will only increase in importance as the fields attempt to develop from solely

research fields into industrial sectors, however the lack of standards relating to cryobiology threatens their ability to make this transition. Needed standards are as simple as standardization of sizes of storage boxes so that equipment from different equipment manufacturers would be interoperable, or as sophisticated as documentary standards that detail optimal processes for cryopreserving samples, retrieving critical samples, and methods for assessing success of those processes that are based on the most up-to-date cryobiological research. There are organizations such as the Standards Coordinating Body for Regenerative Medicine (SCB) that is actively catalyzing development, publication and implementation of consensus standards for these fields, but unfortunately specific efforts in the area of cryobiology are lagging other areas relevant to regenerative medicine. SCB is creating a Focus Area covering Cryobiology with the support of leading firms in the area to facilitate identification of standard gaps, development of standards.

Funding: Not applicable

Conflict of Interest: None to disclose

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S33 ASSESSING CELLULAR AND ORGAN-LEVEL HEALTH, VIABILITY, AND FUNCTION OF VITRIFIED KIDNEYS AND ISLETS FOR TRANSPLANTATION

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Cryopreservation by vitrification is becoming a biological reality. However, a critical problem is how to best assess the

health, viability, and function of biomaterials after rewarming. Here we summarize assessment measures for two model systems (islets and kidneys). Pancreatic islet cryopreservation was done by our cryomesh approach. Viability was assessed by live/dead staining. Cell health by TMRE stain for mitochondrial membrane potential, Seahorse oxygen consumption rate, ATP measurement, TEM for mitochondrial morphology, Annexin translocation for apoptosis, and TUNEL stain for DNA integrity. Architectural integrity by histology and TEM. Function by glucose-stimulated insulin release and transplantation. Vitrified islets (mouse, porcine, non-human primate, human, and stem cell-derived) demonstrated >89% cellular viability with restored ATP content, normal mitochondria and respiration, and regulated insulin secretion in vitro. Vitrified islets durably restored normoglycemia in 9/10 syngeneic transplants. Rat kidney cryopreservation was by vitrification and nanowarming. Kidneys were assessed for morphology (histology and TEM) and vascular integrity (confocal staining and vascular resistance), in vitro function using normothermic oxygenated machine perfusion (NMP), and in vivo by transplantation. During NMP, nanowarmed kidneys made urine, consumed oxygen and glucose, and showed venous and urine electrolyte composition similar to control organs. In syngeneic transplants (nephrectomized recipients), all nanowarmed kidneys (n=7) reperused immediately and uniformly and made urine. Although there was a period of early graft dysfunction lasting 2-3 weeks (peak Cr 12.6 mg/dl), renal function fully normalized after that (Cr 0.4-0.8). Metabolic dysfunction (hyperkalemia, hypervolemia, metabolic acidosis) resolved by POD14. At POD30, serum and urine analysis demonstrated complete normalization of renal function. Microscopy showed focal ATN, hyaline change with

intact basement membrane, near normal architecture, and vascular endothelium. Vitrification and rewarming of islets and kidneys maintained biological integrity and function for successful transplantation. Further in-depth biological characterization for immunological response and long-term graft survival will be critical for clinical translation.

Funding: NIH DK117425, NIH DK13120, NSF EEC-1941543, and Biostasis Research Institute

Conflict of Interest: None to disclose

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S34 CRYOPRESERVATION CONSIDERATIONS FOR PROCESS INTERMEDIATES IN CELL THERAPY

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Cryopreservation is an important tool in cell therapy manufacturing. Cryopreservation of cellular intermediates allows for stable storage, testing, and shipping to manufacturing sites. Optimization of cryopreservation for cellular intermediates may reduce downstream process variability. Variables including cell concentration, formulation technique, freezing protocol, and cold chain handling were evaluated to determine their impact on cryopreservation performance. These results contribute to our understanding of the cryopreservation process.

Funding: Not applicable

Conflict of Interest: Authors are employees of and have stock ownership in Bristol Myers Squibb.

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S35 COMPARING TRYPAN BLUE AND FLUORESCENT SYTO 13/GELRED ASSAYS TO INVESTIGATE THE EFFECTS OF FREEZE/THAW AND DIFFERENT CRYOPROTECTANTS ON MEMBRANE INTEGRITY

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Cells experience different stresses during cryopreservation. The most fatal stresses experienced by cells happen during cooling to sub-zero intermediate temperatures (−15 to −60 °C) and warming from these temperatures, which means that cells endure this situation twice. Therefore, the first step in a cryopreservation study is evaluating cell response to freeze/thaw stress, which is essential in order to design and optimize cryopreservation protocols. The cell response to freeze/thaw stresses depends significantly on the properties of the cell membrane. Furthermore, the cell membrane is thought to be the primary site of freezing injury. Hence, membrane integrity is a fundamental measure of cell response to freeze/thaw stress. Membrane integrity is a measure of the percentage of cells in the population with intact (undamaged) membranes. H9c2 myoblasts were cryopreserved with two different freezing protocols in this study. The cell membrane integrities were measured immediately after thaw as well as after cryoprotectant removal through two different methods. A hemocytometer-based trypan blue dye exclusion assay was compared with a dual fluorometric SYTO13/GelRed assay.

While there have been studies in other fields that compared these two assays, it is important to compare assays specifically in the cryobiological context. In other words, the effects of cooling/warming processes as well as the presence of different cryoprotectants on the differences between these two assays have been studied.

Funding: Canadian Institutes of Health Research (CIHR PS 159672)

Conflict of Interest: None to disclose

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S36 CORAL REEF ECOSYSTEMS ON THE EDGE

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Climate-driven ocean-warming events are threatening coral reefs. With approximately three-quarters of the coral reefs expected to experience annual bleaching by the end of this century resulting in severe extinction pressure, now is the time to develop innovative cryo-conservation tools to preserve and secure coral reef biodiversity. To ensure that coral species can survive, our laboratory, along with national and international colleagues, have cryopreserved both coral sperm, larvae, and symbionts and stored this genetic material in biorepositories around the world. These frozen assets have been used for assisted gene flow for the endangered Elkhorn coral in the Caribbean. However, the increased frequency of coral bleaching is having a negative impact on coral reproduction and much of the technology described thus far is dependent on two nights of spawning a year for most

species. Moreover, previously cryo-preserveable symbionts appear to have changed physiologically after bleaching events necessitating newer cryopreservation protocols to be developed. To increase the pace and expand our ability to preserve coral, we began examining how to cryopreserve thumbnail-sized pieces of coral with isochoric vitrification. If successful, we could preserve coral throughout the year not just within the very limited time-window of spawning. All these cryopreservation efforts could partner with the Coral Biobank Alliance, whose goal is to bring all species of coral into captivity, thus ensuring sufficient biodiversity and genetic diversity for most coral is secured. Our goal is to democratize our cryo-conservation efforts so that they are inexpensive and readily accessible to professional across the globe to rapidly expand our global reach and impact. Although coral reefs may survive in areas on the edge of extinction for decades to come, to ensure healthy specimens capable of surviving the cryopreservation process are collected, these focused, strategic cryo-collections must be accomplished within the next decade.

Funding: This work was generously funded across institutions by grants from the Revive & Restore Catalyst Science Fund, the Paul M. Angell Foundation, the Volgenau Foundation, the Donner Foundation, Paul G. Allen Family Foundation, Taronga Conservation Society Australia, Anela Kolohe Foundation, Cedar Hill Foundation, Smithsonian Institution, Smithsonian Women's Committee and ATP-Bio.

Conflict of Interest: None to disclose

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S37 CONSERVING RARE PLANTS: PAST ASSUMPTIONS, CURRENT

UNDERSTANDINGS, AND FUTURE RESEARCH OPPORTUNITIES

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With two in five plant species at risk of extinction, there is an urgent need to research new techniques to conserve germplasm. Traditionally, storing seeds in freezers at -18C and ~20% relative humidity has been the least expensive way to conserve the greatest genetic diversity of an endangered plant species. Although seeds of most plant species can be stored safely and long-term in freezers, new evidence confirms longevity of seeds held in -18C storage varies by ecology, the latitudinal distribution of species, and the climate at the collection site. Many species have unknown or short-term longevity in freezers. This evidence increases the need for alternative strategies such as cryopreservation, in vitro, and field gene banks to conserve species and begs for a greater understanding of cell characteristics related to longevity in storage. These alternative strategies offer great promise for species that produce few or no seeds, are short-lived in traditional storage, or cannot survive drying and freezing. Yet, each method requires a specialized workforce with expertise, specialized facilities, adequate space, and financial support for the research required for success. Because most of our knowledge about in vitro and cryopreservation is based upon work with commercial crops and wild species have much more variation than commercial crops, data and techniques for wild plant species are needed. Ongoing efforts to identify exceptional species, to determine traits associated with longevity in storage, to refine in vitro and cryopreservation protocols, and to develop a predictive model to guide

collections and storage options are underway by professional plant conservation networks. These collaborations promise significant conservation advances.

Funding: Not applicable

Conflict of Interest: None to disclose

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S38 CRYO-ASTROBIOLOGY: THE SEARCH FOR HABITABLE ENVIRONMENTS ON DISTANT ICY WORLDS

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In 2023, the search for Extraterrestrial life is no longer confined to science fiction. Over the last few decades, cutting-edge research efforts and ambitious space exploration missions have aimed to study both the origins of life on Earth and the possibility of life in our solar system and beyond, giving birth to the burgeoning field of Astrobiology. At its core, astrobiology seeks to answer three fundamental questions: i) under which physical and chemical conditions may life appear and prosper, both on Earth and other worlds, ii) which habitats in our solar system and beyond are the most auspicious for extraterrestrial life to be present today, and iii) how can we detect any such life. Thanks to the past half century of robotic space exploration, we suspect today that the most probable places for life beyond Earth are cold icy worlds in the outer solar system. These ice-covered moons of Jupiter and Saturn (such as Europa, Titan, Enceladus or Ganymede) host cold abyssal oceans up to several hundreds of kilometers deep, which have been present for billions of years and contain most of the liquid water in our solar system. In this talk, I will review the last few

decades of research efforts by NASA and other space agencies to explore these cold exotic worlds, and explain why and how planetary scientists and astrobiologists are investigating the notion that extraterrestrial life might be thriving in cryo-environments. Finally, I will outline current challenges in dissecting the thermodynamics of these icy oceans, and highlight the many bright opportunities for the fields of cryobiology and astrobiology to coalesce in the coming future.

Funding: NASA Solar System Workings Grant 80NSSC17K0775, Icy Worlds and the Titan and Beyond nodes of NASA Astrobiology Institute (08-NA15-0021 and 17-NAI82-17)

Conflict of Interest: None to disclose

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S39 OPTIMIZING OSMOLYTE-BASED HIPSC-DERIVED CARDIOMYOCYTE CRYOPRESERVATION

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Due to the poor proliferation of mature cardiac cells, hiPSC-derived cardiomyocyte therapies have proven effective in cardiac regeneration; in order to increase the reach of application, cryopreservation must be optimized. This study aims to increase recovery of hiPSC-derived cardiomyocytes throughout the cryopreservation process by using combinations of osmolytes (sugars, sugar alcohols and amino acids) instead of Me₂SO – maintaining native cell function and expression post-thaw, and eliminating cells loss from washing. Standard preparation of cells, by singularizing and exposing to Me₂SO, risks anoikis apoptosis and

unnecessary cell loss from washing post-thaw to remove Me₂SO. Mature cardiac cells are chemically differentiated from UMN PCBC-16iPS via Wnt pathway inhibition and non-differentiated cells are purified using a sodium l-lactate solution. Purified cardiomyocytes are singularized and resuspended in an optimized combination of osmolytes, then frozen in a controlled rate freezer at a rate of -1°C per minute and a manual nucleation when vials read -4°C, stored in vapor phase liquid nitrogen, and thawed and replated. A differential evolution algorithm is used to optimize the levels of a trehalose, glycerol, and isoleucine selected to function as a non-Me₂SO CPA. Upon thaw, cells are replated and assessed for recovery and reattachment at 2 and 24 hours post-thaw. At 24 hours, seven solutions compositions had an averaged recovery of 86.67%±22.36 outperforming Me₂SO at 51.65%±32.6 post thaw. Looking at the five best performers overall, osmolyte solutions averaged a recovery of 1.55±0.219 at 24 hours post thaw normalized to Me₂SO. Preliminary results indicate that osmolyte solutions result in increased attachment at 24 hours post-thaw compared to Me₂SO at the same freezing conditions, without the need for additional washing steps. In conclusion, non-Me₂SO freezing solutions are a comparable CPA for cardiomyocytes to reduce cell loss and handling steps for infusions, research, and banking.

Funding: Not applicable

Conflict of Interest: None to disclose

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S40 COMPARISON BETWEEN DIMETHYL SULFOXIDE AND GLYCEROL FOR CRYOPRESERVATION OF SWINE-COLOSTRUM-DERIVED CELLS

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Mesenchymal stromal cell (MSC)-based therapy is envisaged to be beneficial in viral infections such as COVID-19 due to the ability of MSCs to modulate the immune system and fight infection and inflammation. MSCs require cryopreservation in order to make them available for clinical and biotechnological applications. MSCs have been isolated from various sources, and recently, MSC-like cells were isolated from colostrum. We used interrupted slow cooling (graded freezing) to examine cryoinjury experienced by MSC-like cells from swine colostrum. This method allowed us to identify and mitigate cell damage from increased solute concentration during slow cooling, and from intracellular ice formation during rapid cooling. Cells were loaded with either dimethyl sulfoxide (Me₂SO) or glycerol, cooled and ice-nucleated at -5 °C, then further cooled at 1 °C/min to -40 °C. Cells at interrupt temperatures along the cooling path were either thawed directly, or plunged into liquid nitrogen for storage, and later thawed. Post-thaw viability was assessed by flow cytometric analysis of cell membrane integrity. We found that both 5 % (wt/wt) Me₂SO (loaded for 15 min on ice) or 5% (wt/wt) glycerol (loaded for 1 h at room temperature) yielded cells with high post-thaw membrane integrity (~86%) when cells were cooled to at least -30 °C before being plunged into, and stored in, liquid nitrogen. Cells cultured post thaw displayed MSC-characteristic osteogenic differentiation similar to fresh unfrozen control. Fresh and cryopreserved MSC-like cells exhibited antimicrobial activity against the pathogenic

bacteria *Staphylococcus aureus*. Medium conditioned by fresh or cryopreserved MSC-like cells that were pre-exposed to *S. aureus* also demonstrated antimicrobial activity. Thus, we show that swine-colostrum-derived MSC-like cells may be cryopreserved using Me₂SO or glycerol, yielding highly viable cells with osteogenic potential; however, cells cryopreserved with glycerol retained higher antimicrobial activity post thaw. This work was published in *Cryobiology* 97, 168–178 (2020).

Funding: This work was funded by the Natural Sciences and Engineering Research Council of Canada (EGP 521869-17) and by Alberta Innovates (CASBE G2018000082). JAW Elliott holds a Canada Research Chair in Thermodynamics.

Conflict of Interest: None to disclose

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S41 BRAIN ORGANIDS ON DEMAND: CRYOPRESERVATION AS A SOLUTION

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Brain organoids, a human 3D brain model, derived from induced Pluripotent Stem Cells (iPSCs). This model contains different types of neurons, astrocytes, oligodendrocytes. Brain organoids are electrophysiologically active. The unique feature of our model is high standardization and reproducibility. To meet the demand of scientific interest for such a model around the world in academia and industry, we developed a brain organoid cryopreservation protocol and establishing a bank of organoids on demand. A series of endpoints were measured to make sure that

cryopreservation doesn't perturb main functionality of the organoids. To generate brain organoids, we differentiated human iPSCs, to neural progenitor cells (NPCs) and brought NPC to form 3D brain organoids under constant gyratory shaking for up to 8 weeks. For cryopreservation, we used three different ages of brain organoid – 2,4 and 6 weeks of differentiation. We started with seven different freezing media. Organoid viability and morphology was assessed after defrosting at 24h, 2 week and 4 week later. Four out of 7 media were discontinued due to low viability after defrosting. Functional end points immediately after defrosting and recovery period were assessed in remaining 3 media. Neurite outgrowth, resazurin reduction assays were performed to test functionality and viability of the recovering organoids. Immunohistochemistry was performed to assess the morphology of different cell types. As ultimate functional endpoint electrophysiological activity after freezing was assessed. The results showed that cryopreservation of rather immature brain organoids is more feasible than older cultures and it can serve as a time saving opportunity to optimize toxicity studies on demand. Further optimization steps are necessary to cryopreserve more mature cultures.

Funding: Not applicable

Conflict of Interest: None to disclose

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S42 IMPACT OF POST-THAW STRESS MODULATION ON HEMATOPOIETIC PROGENITOR CELL SURVIVAL

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Cryopreservation plays a critical role serving as an enabling process providing on-demand access to biological material facilitating large scale production, storage and distribution of living materials. Despite serving a critical role and substantial improvements over the last several decades, cryopreservation often remains a bottleneck impacting numerous areas including cell therapy, tissue engineering and tissue banking. Studies have illustrated the impact and benefit of controlling cryopreservation-induced delayed-onset cell death (CIDOCD) through various “front end” strategies, such as specialized media, new cryoprotective agents and molecular control during cryopreservation. While proving highly successful, a substantial level of cell death and loss of cell function remains associated with cryopreservation. To this end, in this study we investigated the impact of modulating cell stress response during the post-thaw recovery phase in an effort to improve recovery of previously cryopreserved samples. Investigations focused on the impact of modulating apoptotic caspase activation, oxidative stress, unfolded protein response and free radical damage in the initial 24 hours post-thaw on overall cell survival. Human hematopoietic progenitor cells (hHPC) cryopreserved in both traditional extracellular-type and intracellular-type cryopreservation freeze media were utilized as a model cell system to assess impact on survival. Our findings demonstrate that through the modulation of several of these pathways, improvements in hHPC recovery was obtained regardless of the freeze media and dimethyl sulfoxide concentration utilized. Specifically, through

the use of oxidative stress inhibitors, an average 20% increase in overall viability was observed. Further, the results demonstrated that using the post-thaw recovery strategy on samples cryopreserved in intracellular-type media (Unisol™), improvements in overall cell survival approaching 80% of non-frozen controls were attained. While work remains, these results represent an important step forward in the development of improved cryopreservation processes for use in discovery science, commercial and clinical settings.

Funding: NIH NHLBI Grant No. 5R44HL130805-03

Conflict of Interest: None to disclose

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S43 20 YEARS IN ENZYMATIC ISOLATION AND CRYOGENIC PRESERVATION OF HUMAN HEPATOCYTES

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Hepatocyte transplantation is gaining acceptance for the treatment of liver disease. Our laboratory has always been active in the translation of hepatocyte transplant technology from bench to clinic. Our 30 years of experience of more than 2,000 human livers allowed us to standardize reagents and procedures in accordance with Good Manufacturing Practice (GMP). We developed and validated rapid and sensitive functional assays to ensure freshly isolated hepatocytes, as well as cryopreserved products, function adequately and better match the recipient's needs. Commonly human hepatocytes are isolated by a mechano-enzymatic procedure, and several different surgical procedures and enzymatic

solutions have been tested and described. Cryogenic preservation of primary human hepatocytes has been largely reported as ineffective and largely variable among donors. Thirty years of experience in isolating hepatocytes led us to design and optimize a surgical/enzymatic GMP procedure to isolate human cells for clinical applications. We have performed hepatocyte isolation not only on tissues rejected from organ donation, but also on explanted organs, fetal liver tissues, and neonatal donors, recently proposed as alternative cell sources for liver diseases, and tested cryopreservation solutions on several batches. We tested 8 different GMP-grade formulations. A large spectrum of primary cells has been analyzed to determine a normal range of activities to expect from isolated cells. Data collected on recovery, viability, apoptosis, plating adhesion, and multiple hepatic functions including basal and induced Cytochrome P450 (CYP) activities, phase II conjugation, and ammonia metabolism allowed us to determine the normal range of metabolic activities and synthetic capacity for cryopreserved liver cells. Our long-term database can serve as quality control and form the basis for an auxiliary strategy to validate clinical products but also evaluate stem cell sources proposed as hepatocyte replacement.

Funding: Åke Wibergs stiftelse

Conflict of Interest: None to disclose

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S44 CRYOPRESERVATION OF ANOPHELES STEPHENSI (MOSQUITO) EGGS: EFFECTS ON GENOTYPIC AND PHENOTYPIC CHARACTERISTICS

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A method for the cryopreservation of *Anopheles* eggs has been developed that provides long-term, stable storage of anopheline mosquitoes at minimal cost and effort. Batches of *A. stephensi* eggs, stored in liquid nitrogen vapor phase, are an insurance against colony collapse and also, banked under good manufacturing practices, are the seed material for manufacturing lots of PfSPZ vaccines. Here we examine the effects of cryopreservation on population selection and the characteristics of *A. stephensi* grown from thawed eggs. We optimized conditions for the small scale rearing of mosquitoes that matched life history traits of larval growth, pupation, eclosion, wing length, mating success, blood feeding success and fecundity compared to control *A. stephensi* reared in Sanaria's main insectary. Different batches of cryopreserved eggs were thawed, the hatch rates determined and L1 larvae transferred to the optimized rearing conditions side-by-side with L1 mosquitoes from the insectary. Genome-wide SNP analysis revealed no differences in SNP profiles between adults from fresh eggs to adults that emerged from cryopreserved eggs, suggesting no genetic bottleneck selection from egg cryopreservation. Hatching of cryopreserved eggs occurred over a longer period compared to insectary derived eggs, reflected also in a similar timeline spread for pupation. Adult mosquitoes from cryopreserved eggs were able to mate, feed on blood and produce fecund eggs at rates similar to insectary-derived mosquitoes. Rates of infection with *Plasmodium falciparum* were comparable to controls. Our data demonstrate that cryopreservation does not lead to changes in

the genotype or phenotype of *A. stephensi* (aside from the slight delay in larval hatching and pupation), that the process is sufficiently robust to enable resuscitation of the colony when needed, and adds security to Sanaria's vaccine manufacturing process.

Funding: Not applicable

Conflict of interest: None to disclose

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S45 SULFORAPHANE AUGMENTS POST-THAW QUALITY, BIOCHEMICAL FEATURES, AND FERTILITY POTENTIAL OF BUFFALO SPERMATOZOA

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Sulforaphane is a natural and highly effective antioxidant safeguarding the reproductive system, and alleviate oxidative stress. This study was designed in order to elaborate L-sulforaphane effect on semen quality, biochemical parameters, and fertility of buffalo (*Bubalus bubalis*) spermatozoa. Collected semen from five well-restrained buffalo bull with artificial vagina (42 °C), evaluated for volume (ml), consistency (color), motility, and concentrations. After critical examination, semen was diluted (50 x 10⁶, 37 °C) in extenders with (2 µM, 5 µM, 10 µM, and, 20 µM) or without (control) sulforaphane, cooled (from 37 to 4 °C), equilibrated (4 °C), filled (straws, 4 °C), and cryopreserved (LN2, -196 °C). Data analysis exhibited that sulforaphane addition in extender augments total motility (%; 10 µM, and 20 µM than control), progressive motility (%), and rapid velocity (%; 20 µM than control), and velocity parameters (average path velocity, µm/sec, straight line velocity,

µm/sec and curved linear velocity, µm/sec, 20 µM than control, and 2 µM). Moreover, sulforaphane augments functional features (membrane functionality, mitochondrial potential, and acrosome integrity) of buffalo sperm (20µM than control). Sulforaphane improves seminal plasma of buffalo biochemical features i.e., seminal plasma Calcium (µM), and total antioxidant capacity (µM/L), followed by reduction in lactate dehydrogenase (IU/L), reactive oxygen species (104 RLU/20 min/ 25 million), and lipid peroxidation (µM/mL) in 20 µM than control. Lastly, sulforaphane augments fertility rate of buffalo sperm at 20µM than control, and 2 µM. Conclusively the existing study revealed that adding L-sulforaphane (20 µM) in a freezing medium augments motilities, kinematics, functional parameters, and fertility rate of buffalo spermatozoa. Correspondingly, sperm favorable biochemical features were also augmented with sulforaphane followed by reduction in oxidative stress parameters. Further studies are highly recommended to define the particular mechanism of action of sulforaphane in augmenting buffalo post-thawed semen quality, and fertility potential.

Funding: Not applicable

Conflict of Interest: None to disclose

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S46 CRYOPRESERVATION AS A METHOD OF CONSERVATION FOR THE THREATENED MONARCH BUTTERFLY

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Cryopreservation is a process commonly utilized for genetic resource banking for many threatened and endangered organisms including plant, mammal, and insect species. The monarch butterfly, *Danaus plexippus* was recently classified as endangered by the ICUN, which sparked many efforts to protect the species. Our goal was to determine if cryopreservation of Monarch spermatozoa would be a successful tool for conservation of the species. Monarch larvae were collected and reared to adulthood. Male monarchs were dissected, and the reproductive tract was removed. The collection process focused on the collection of eupyrene spermatozoa from the duplex region of the male reproductive system. The spermatozoa were collected in a solution containing cryoprotectants, frozen using a ramp-down freezing protocol, and transferred to liquid nitrogen for long-term storage. To determine the impact of cryopreservation on spermatozoa survival we compared the proportion of surviving spermatozoa in samples that had been cryopreserved to spermatozoa samples that had not been frozen, as determined through a live/dead stain. Our results show cryopreservation had no impact on spermatozoa survival. To date, this is the first successful cryopreservation of monarch spermatozoa which sets the foundation to continue cryopreservation in this species, and as its potential use in conservation.

Funding: Not applicable

Conflict of Interest: None to disclose

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S47 CRYOPRESERVATION AND OPTIMIZATION OF *DROSOPHILA MELANOGASTER* EMBRYONIC NUCLEI

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The fruit fly, *Drosophila melanogaster*, is a foundational model organism for biological research. With over 150,000 live stocks maintained at stock centers, there is still a continual interest in large-scale cryopreservation of specific *Drosophila* to limit costs and genetic drift. There are currently two methods toward freezing *Drosophila* stocks: vitrification of primordial germ cells and vitrification of embryos. These methods show success in cryopreserving lines of *Drosophila* but both methods have shown to be difficult to replicate. We propose a new Me₂SO-free method of cryopreserving isolated embryonic nuclei using a slow-cooling method and combinations of sugars, sugar alcohols, and amino acids. With the use of a differential evolution (DE) algorithm, we find five different formulations that show 80% recovery or greater specifically using sucrose, glycerol, and isoleucine. In addition, we have developed a method of reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using the heat shock gene, *hsp70*, to determine viability of the nuclei after the isolation process and use RT-qPCR to measure viability of thawed nuclei as well. We have currently seen that expression fold change for nuclei immediately after isolation is 33 where nuclei frozen with poor conditions have a much worse expression fold change of 1. We also use Raman spectroscopy to define characteristic peaks of the nucleus at 785cm⁻¹ and 965cm⁻¹ that help identify osmolyte partitioning and intracellular ice formation

under different freezing conditions. As an alternative to the above vitrification methods, this method looks to regenerate stocks by embryonic nuclear transplantation.

Funding: This work is supported by the
National Institute of Health R24OD02844

Conflict of Interest: None to disclose

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S48 BIODEGRADABLE CAPSULES AS AN ALTERNATIVE CONTAINER TO SPERM CRYOPRESERVATION OF AQUACULTURE MEDITERRANEAN FISHES

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We aimed to evaluate the efficiency of hard-gelatine and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative containers in European eel and gilthead seabream sperm cryopreservation. European eel (*Anguilla anguilla*; n = 12) and gilthead seabream (*Sparus aurata*; n = 12) sperm samples with motility over 60% were cryopreserved in plastic straws, hard-gelatine, and HPMC biodegradable capsules. Total motility (MOT

- %), progressive motility (MOTp - %), and the curvilinear (VCL - $\mu\text{m/s}$), straight line (VSL - $\mu\text{m/s}$), and average path (VAP - $\mu\text{m/s}$) velocities were evaluated by CASA-Mot software. Sperm viability was performed using a Live and Dead KIT and an epifluorescence microscope. For quantified DNA damage, the alkaline comet assay was performed. Head-DNA (%), Tail-DNA (%), Tail Moment, and Olive Moment were evaluated by CaspLab software. European eel sperm cryopreserved in all containers reduced the MOT and MOTp. A highest VCL was observed in fresh sperm compared to sperm cryopreserved in both capsules. Fresh and post-thawed sperm stored in plastic straws showed higher VAP than sperm cryopreserved in the HPMC capsules. The cryopreservation using plastic straws and hard-gelatine capsules reduced the cell viability. DNA damage was not different between fresh sperm and thawed samples. In gilthead seabream, post-thawed sperm showed lower kinetic parameters than fresh sperm; however, these parameters did not differ between sperm samples cryopreserved in the different tested containers. Thawed samples showed lower viability than fresh sperm. Fresh sperm and cryopreserved in plastic straws showed lower DNA damages than samples frozen in capsules. The hard-gelatine and HPMC biodegradable capsules can be used as an alternative to straws for European eel and gilthead seabream sperm cryopreservation.

Funding: This study was supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and by Generalitat Valenciana (THINKINAZUL/2021/012; THINKINAZUL/2021/024) to SEASPERM. WAG-L has a Margarita Salas postdoctoral contract (RD 289/2021. UAB) by the Spanish Ministry of Universities. LF has a PhD contract from Generalitat Valenciana

(GRISOLIAP/2020/063). TSF (141717/2019-0 and 200285/2021-1) and MPS (200452/2022-3) have fellowships from Brazilian National Council for Scientific and Technological Development (CNPq).

Conflict of Interest: Patent application on the methodology filed by TSF and DPSJ (September/2022) and is pending.

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S49 MANAGEMENT DECISIONS FOR SEED CRYO-STORAGE AT USDA'S PLANT GENE BANK

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USDA's National Laboratory for Genetic Resources Preservation (NLGRP) preserves plant and animal genetic resources important for global food security. About 98% of one million+ plant accessions are orthodox seeds. Orthodox seeds survive drying and can be successfully stored at a range of low temperatures. The majority of NLGRP's seed collections are stored at -20°C in massive walk-in freezers. However, a portion of orthodox seeds (~50,000 accessions) are stored above LN vapor in 25 x 5000L cryotanks. The choice of freezer vs LN storage was once based on whether seeds suffered mechanical damage upon cooling to LN temperatures, usually at rates of 40°C/min . However, we have shown that slower cooling can mitigate some of the mechanical damage observed, such that cryo-exposure causes no decline in initial viability. Mitigating initial damage and progressive strategies in gene banking has led us to seek new criteria to identify accessions to cryopreserve. New criteria under

consideration are taxon, cost of seed regeneration, cost of LN vs -20°C storage, longevity achieved in the freezer, and impending tank failures. One example of questions under consideration is whether the added expense of LN storage is justified if samples are expected to survive 100 years at -20°C? The average age of NLGRP seed tanks is about 25 years and the life expectancy of tanks is 30-35 years, meaning that NLGRP could face multiple tank failures simultaneously in the next few years. Rice, sorghum, barley, rye and triticale comprise 70% of the volume of seed cryotanks at NLGRP. Those same crops store relatively well at -20°C. We propose a proactive strategy that transfers many of these samples to freezer storage with relatively minor impacts on the longevity, in order to save costs and free up space for more difficult-to-store seeds.

Funding: Not applicable

Conflict of Interest: None to disclose

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S50 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*.

Funding: Not applicable

Conflict of Interest: None to disclose

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S51 SEED LIPID THERMAL BEHAVIOR OF CRYOPRESERVED SEEDS OF SOME *DENDROBIUM* SPECIES NATIVE TO INDONESIA

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Orchidaceae is one of the most threatened plant families in the world, with many species included in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Indonesia has one of the richest orchid biodiversity in the world with approximately 5000 species, including *Dendrobium*. Unfortunately, some Indonesia orchids are also listed as endangered or threatened in the IUCN Red List, thus an effort to increase ex situ conservation options for these species is urgently needed. Orchid seed is tolerant to low moisture content but not all species tolerate long-term storage under conventional seed banking temperature (i.e., -20°C). Orchid seed is known to contain lipid and thus may be relatively susceptible to oxidation during storage and crystallization at low temperature. For example, *Dendrobium strebloceras* (from Maluku) contains 68% of lipid is polyunsaturated, with linoleic acid as the main fatty acid. We aimed to investigate seed lipid thermal behavior in *D. lineale*, *D. wulaiense*, and *D.*

trilamellatum (three *Dendrobium* species from Papua), and *D. capra* (from Java and Lesser Sunda island). We did cryopreservation and analyzed thermal properties of dry seeds using differential scanning calorimetry (DSC). Seeds were pre-dried to c. 40% relative humidity and stored briefly at -20°C, transferred to cryovials and directly cryopreserved. We found no significant difference in germination of seed cryopreserved and non-cryopreserved. The main features of the seed thermal fingerprints for these species were: (i) first onset temperature during melting of -89 to -69°C; (ii) with a main melting peak around -29°C. The thermal fingerprint was similar pre- and post-cryopreservation. We conclude the effectiveness of direct cryopreservation for these *Dendrobium* species but aim to widen our studies to more species in this group so that more general conservation recommendations can be made.

Funding: Research Organization for Life
Science and Environment, NRIA-Indonesia

Conflict of Interest: None to disclose

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S52 AN INTERCALIBRATION EXPERIMENT ON MICROALGAE CRYOPRESERVATION

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Ensuring that experiments can be replicated in different labs is a critical aspect of the scientific method and is essential for maintaining the validity and replicability of scientific results. An intercalibration event involving seven labs in five different countries took place on microalgae cryopreservation within the European Blue Biobank project. The aim of the experiment

was to produce detailed documentation and standardized procedures on microalgae cryopreservation that would ensure the reproduction of the procedures and results. Using the same biological material, *Phaedactylum tricornutum* and *Isochrysis galbana* were both provided in kind by the Roscoff Culture Collection (RCC). All institutions were provided with a template to fill in the data, a document with all the experimental parameters, a detailed cryopreservation protocol and a final questionnaire. The results were anonymized for the analysis given below. The results showed a very different outcome on cell survival and growth among laboratories. Among the participants, 86% had prior experience with cryopreservation, 86% had the equipment needed prior the participation in this experience and coincidentally 86% considered the protocol clear enough that they didn't need any further clarifications. We have identified several sources of variability and pointed out possible ways to reduce them in the future. In any experimental procedure is prone to human errors, although those can't be completely eliminated, personal training and adequate feedback have proven to noticeably reduce them. Another source of variability is insufficiently detailed documentation; this can be improved by holding a prior discussion with the destined users of the protocol, understanding their background and generating best practices and protocols for the target audience in order to reduce personal interpretation and improvisation when following said protocols. Other sources of variability are differences in equipment, reagents, variability in sample conditions and environmental conditions. These are more difficult to control but nonetheless, we should be aware of how important of a role each source of variability can play depending on the cell type we are cryopreserving.

Funding: European Blue Biobank (EBB), EAPA_501/2016, funded by Interreg Atlantic Area (EU).

Conflict of Interest: None to disclose

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S53 CRYOPRESERVATION BY VITRIFICATION OF LEMNACEAE (DUCKWEED): ADJUSTMENT OF ILLUMINATION FOR REGROWTH INDUCTION

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The unique biodiversity of the aquatic Lemnaceae family, which mainly reproduce vegetatively, is so far deposited in vitro, e.g. at the IBBA Duckweed Collection, Rutgers Duckweed Stock Cooperative. Cryopreservation of duckweed genetic resources would save labor to maintain in vitro germplasm collections of these fast-growing plants and avoids somaclonal variation. Cryopreservation by vitrification efficiently eliminates the risks of intracellular ice formation during freezing-thawing and enables a high-throughput processing. However, desiccation of plant tissue, necessary for providing vitrification, may irreversibly affect structure and functionality of biomolecules. The light-induced death of Lemnaceae plants after contact with plant vitrification solution 3 (PVS3) apparently is the key issue that challenges duckweed regrowth after cryopreservation. When thawed duckweed is incubated under conventional diurnal light regime, plants die within a day, after degradation of mature chloroplasts which were irreversibly affected

by previous desiccation. In darkness, thawed plants remained alive during at least 1 month, however no regrowth was observed for most accessions. We hypothesized that although the development of new fronds from meristems containing only proplastids is triggered by light, the initiation of regrowth completely relies on support from other tissues of thawed fronds. Thus, in our work we focused on design of a balanced illumination regime that avoids the death of the thawed plants during the first week of illumination, but induces development of new fronds in all tested accessions. Through precise adjustment of illumination during early regrowth stage, reproducible regrowth for 20 accessions of *Spirodela*, *Landoltia* and *Lemna* genera after cryo-freezing in PVS3 was achieved. The results demonstrate that precise light adjustment could be an efficient tool of regrowth induction after cryopreservation.

Funding: Alexander von Humboldt Foundation, Philipp Schwartz-Initiative

Conflict of Interest: None to disclose

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S54 EFFECT OF DIFFERENT TYPES OF NANOPARTICLES ON DIELECTRIC HEATING OF CRYOPRESERVED BIOMATERIALS

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Dielectric heating is one of the promising methods for rewarming cryopreserved biospecimen due to its potential to achieve very rapid and uniform rewarming. However, the electromagnetic energy absorbability of a large biospecimen (>50 mL) is relatively low to achieve high warming rate, especially at temperatures below -50°C. Previous studies have utilized magnetic nanoparticles (MNPs) to improve the rewarming performance, but MNPs are not ‘catalysts’ for the dielectric heating. The MNPs actually improve hysteresis heating instead of dielectric heating. This research aims to study the effect of different types of nanoparticles on the warming rate and uniformity in large biospecimens using dielectric heating method. Three types of nanoparticles were selected in this study: Fe₃O₄ (MNPs), SiO₂ (dielectric nanoparticles), and CuO (thermal nanoparticles). Firstly, the dielectric properties of these three types of nanoparticles were measured in the temperature range between -150 °C and 5 °C, which were critical factors determining efficiency of energy transition from electromagnetic to internal energy for heating. The nanoparticles were added into three prepared vitrification solutions of DPVP (41% Me₂SO + 6% Polyvinylpyrrolidone), respectfully, and then cooled down to -150 °C. To understand the effects of these nanoparticles on the dielectric heating, the samples with different types of nanoparticles were rewarmed by the single-mode electromagnetic resonant (SMER) system, and the temperature changes at sample’s center and edge locations were recorded during the rewarming. The results showed that the addition of dielectric and thermal nanoparticles could significantly increase sample rewarming rate and uniformity in comparison to the use of MNPs. Then, three DPVP solutions with the different nanoparticles were used as vitrification solutions to make the 50 mL

Jurkat cell suspensions in cooling and SMER rewarming experiments. The viability of the cells that preserved using the new solution after rewarming achieved a significant improvement compared to the DPVP only.

Funding: Murdock Foundation Grant, University of Washington CoMotion Innovation Grant and Royalty Research Fund

Conflict of Interest: None to disclose

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S55 A DEEPER UNDERSTANDING OF THE STRUCTURE ACTIVITY RELATIONSHIP OF O-ARYL-GLUCOPYRANOSIDE ICE RECRYSTALLIZATION INHIBITORS

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The phenomenon of ice recrystallization has detrimental effects on biological materials during cryopreservation, including reduced post-thaw recovery, viability, and functionality. Inhibition of ice recrystallization using small-molecules improves the cryopreservation outcomes of numerous cell types. Several O-aryl-glucopyranoside ice recrystallization inhibitors (IRIs), including (4-methoxyphenyl)- β -D-glucopyranoside (β -PMP-Glc, IC_{50} = 28.8 mM [26.5-31.2]), are effective for cryopreserving red blood cells at reduced glycerol concentrations. However, further development of these IRIs requires a deeper understanding of the relationship between their structural features and inhibitory activity. Effective small-molecule IRIs disrupt the transfer of water molecules between ice crystals, which is related to the formation of a dense hydrogen bond (HB)

network between an IRI and bulk water molecules. Our laboratory has extensively investigated the effect of modifying this HB network on IRI activity with synthetically facile structural changes at the glycosidic bond (C_1) and to the stereochemistry of each carbon bearing a secondary hydroxyl within the pyranose ring (*i.e.*, D-glucose and D-galactose IRIs). However, it is the individual hydroxyls of a carbohydrate-based IRI that act directly as both HB donors and acceptors. Therefore, investigating the role that each hydroxyl plays in the formation and overall integrity of the HB network and its relationship to IRI activity is necessary. Removal of HB donation *via* selective methylation to the hydroxyls at C_2 through C_6 finds that when comparing to the parent compound (β -PMP-Glc), C_2 hydroxyl methylation does not change IRI activity (IC_{50} = 24.8 mM [17.7-33.4]), while C_4 hydroxyl methylation results in dramatically improved IRI activity (IC_{50} = 3.19 mM [3.04-3.37]). Therefore, a HB donor at the C_2 and C_4 positions may not be vital to IRI activity, and these positions may be further substituted without activity loss. This study critically informs the future development of small-molecule glucopyranoside IRIs and provides deeper insight into their mechanism of action.

Funding: Not applicable

Conflict of Interest: None to disclose

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S56 IDENTIFYING THE KEY STRUCTURAL ATTRIBUTES NECESSARY FOR IRI ACTIVITY IN N- FUNCTIONALIZED 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. To help direct future IRI development, a classic synthetic / medicinal approach, where small incremental changes to 2FA are introduced and activity is assessed. For example, 2FA is truncated to *N*-(2-fluorophenyl)-D-xylonomide (2FX) through the removal of C₆ and the IRI activity of 2FX is then compared to that of 2FA. This change specifically had been a key step in identifying the importance of the C₆ position to the observed IRI activity in 2FA. It was also found that the selective dihydroxylation of aryl-gluconamides was found to increase their aqueous solubility; these findings lead to the discovery of *N*-(2-fluorophenyl)-4-deoxy-D-gluconamide (C₄d2FA), an IRI active aryl-gluconamide which possesses comparable activity to 2FA with vastly superior aqueous solubility. This approach has been applied to other IRI active *N*-functionalized gluconamides, most notable *N*-octyl-D-gluconamide (NOG), with the goal

of identifying positions for future functionalization as well as dehydroxylated derivatives which retain IRI activity while improving aqueous solubility. Herein we report that *N*-octyl-6-hydroxyhexylamide (C₆HNOA) has identical IRI activity to NOG and thus has been identified as a next generation IRI.

Funding: Not applicable

Conflict of Interest: None to disclose

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S57 DIAGNOSTIC TOOLS FOR ASSESSING CRYOPROTECTIVE AGENTS DIELECTRIC PROPERTIES OVER BROAD TEMPERATURE/FREQUENCY RANGES

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Cryoprotective agents (CPAs) are essential in biomedical preservation applications. Despite their widespread use, there is often limited data available on their properties. Of note, even with the field's interest in a range of electromagnetic rewarming approaches, there is a lack of critical dielectric property data to better understand these applications. Comprehensive dielectric data across a range of temperatures and frequencies is important to appropriately design approaches for and compare effectiveness of dielectric, RF

nanowarming, high intensity ultrasound, and microwave heating of cryopreserved biomaterials. Two approaches will be presented to investigate CPA dielectric behavior: a probe method and a resonator method. The coaxial probe method is a wide band technique (100 MHz to 3 GHz), but the probe is not compatible with cryogenic temperatures and measurements are limited to suprazero and high subzero temperatures. The split ring resonator method is a narrow band technique (i.e., measures at a specific resonance peak) which can be used for a wide range of temperatures including cryogenic (down to at least -150°C). While each method offers limitations, together they provide a comprehensive measurement approach. Here, we present the first known data on dielectric loss as function of frequency (30 kHz to 4 GHz) for CPAs such as VS55 and M22 from the suprazero to cryogenic temperature range using both methods. Furthermore, we will discuss results from our investigation of the dielectric loss of individual CPA components (e.g., formamide and Me₂SO), their classification as high or low loss, and assessment on overall impact on CPA loss performance. These methods lay the groundwork for a more comprehensive understanding of CPA design based on dielectric performance in biomedical preservation.

Funding: Not applicable

Conflict of Interest: None to disclose

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S58 CHILLING INJURY IN PIG OOCYTES IS CORRELATED WITH LIPID MEDIATORS

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Pig oocytes are sensitive to temperatures below 15°C, which makes cryopreservation difficult. Cytoplasmic lipid droplets may be involved in the sensitivity because droplet removal increases the tolerance to cryopreservation. Previously, we showed that the suppression of the TRPA1 expression, which is a cold-sensitive channel, markedly reduced the chilling injury of pig oocytes at 15°C for 15 min (cold treatment). We also showed that inhibition of cytosolic phospholipase A₂, arachidonate 5-lipoxygenase (5-LOX), and lysophosphatidylcholine acyltransferase 2 (LPCAT2) attenuated the chilling injury in maturing oocytes during cold treatment, whereas a specific inhibitor for cyclooxygenase did not. In the present study, we investigated whether leukotrienes and platelet-activating factor (PAF) are involved in chilling injuries in maturing pig oocytes by injecting double-stranded (ds) RNA of 5-LOX and LPCAT2 into immature oocytes, which can suppress the expressions of the enzymes. Specifically, 2–10 pl of ds-RNA (1 pl/pl) of the enzymes were injected into pig oocytes at the germinal vesicle stage, and the oocytes were cultured for 12 h. Then, the maturing oocytes were subjected to cold treatment and further cultured for 36 h. The mRNA expression levels for both enzymes at 12 h of culture after injection were as low as 20–40%. The suppression of 5-LOX expression markedly increased both the survival and maturation of oocytes after cold treatment, and similar results were observed for the suppression of LPCAT2 expression. These results demonstrate that the chilling injury in maturing pig oocytes is correlated with leukotrienes and PAF through the activation of TRPA1. Further studies are

required to clarify the mechanism by which pig oocytes are injured by leukotrienes and PAF.

Funding: JSPS KAKENHI Grant Numbers JP18H03953, JP22K19250

Conflict of Interest: None to disclose.

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S59 EFFECT OF CRYOPROTECTANT EQUILIBRATION ON TRANSMEMBRANE POTENTIAL OF BOVINE OOCYTES

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The major physiological event during cryopreservation is equilibration to and from high concentration to permeable cryoprotective agents. The focus of this study was to analyze the effect of large and rapid osmotic changes on transmembrane potential of bovine oocyte. To evaluate this, the objectives of the studies were to check the effect of non-permeating (different PBS concentrations) and permeating solution (10% EG) on transmembrane potential of bovine oocyte. For this purpose, oocytes were exposed to alternating sequence of PBS treatments i.e., initial hypotonic followed by hypertonic (iso > 0.5X or 0.3X > iso > 2X) and initial hypertonic followed by hypotonic (iso > 2x > iso > 0.3X or 0.5X). For permeating solution oocytes were exposed to 10% ethylene glycol. The change in membrane potential was observed by electrophysiological technique i.e., intracellular recording and the volume of oocyte was found by image j software. The resting membrane potential was observed before the start of experiment and the change in the voltage was recorded during exposure

to anisomotic condition throughout time. The transmembrane voltage was hyperpolarized in the presence of hypotonic solution and depolarized in the hypertonic solution. The volume vs voltage graphs showed slopes of 9.6 and 43.42 in permeating and non permeating treatments respectively which indicated a negative correlation between volume vs voltage with R2 value of 0.36 and 0.56 in overall permeating and non-permeating treatment groups respectively. The Welch test indicated the significant different between slopes of volume vs voltage in permeating and non-permeating groups (t=2.58 and p=0.02).

Funding: Not applicable

Conflict of Interest: None to disclose

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S60 THE IMPACT OF FOR THE TREATMENT OF PANCREATIC TRANSPORTATION ON PREPUBERTAL OVARIAN TISSUE PRE AND POST CRYO/WARMING

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Ovarian tissue cryopreservation (OTC) is the most preferred fertility preservation method for prepubertal girls undergoing gonadotoxic therapy. However, the handling, transportation and cryopreservation of ovarian tissue during this process needs refinement. Delay in OTC can occur due to non-availability of the facility, hence needing transportation from remote locations. It is

still not clear for how long tissue can be held until cryopreservation, without affecting its integrity and functionality. Here, we studied the impact of holding time on ovarian tissue pre and post cryo-warming. Prepubertal BALB/c mouse ovaries (n=6) were vitrified prior and after hypothermic holding (HH) at 4°C for 48 hours to simulate tissue transportation. Fresh, vitrified and HH+vitrified ovarian tissues (n=6) were heterotopically xeno-transplanted onto previously ovariectomized females. Pre-transplantation analyses included Neutral Red uptake assay and H&E staining followed by follicle staging using Gougeon's criteria and follicle grading analysis. Post transplantation analyses were carried out by estrous cycle assessment, measuring diametric growth of transplanted grafts and H&E staining with staging and grading analyses. OTC impacted the follicle viability as well as follicle health/grade, pre and post hypothermic holding. The different follicle stages/populations were comparable. Transplantation of fresh, vitrified and HH+vitrified ovarian tissue resulted in growth of transplanted grafts and regain of estrous function in ovariectomized infertile females. However, it was observed that females transplanted with vitrified and HH+vitrified tissues, cycled fewer times than fresh tissue transplanted females and adult healthy females. Follicle staging (n=6) revealed notable differences amongst primordial, primary, secondary and antral follicle populations at 2 and 4 weeks post transplantation. An insignificant decrease in the percentage of healthy follicles was observed in vitrified and HH+vitrified grafts as compared to fresh transplanted grafts. These observations suggest that hypothermic holding of mouse prepubertal ovarian tissue until 48 hours has an impact on the outcome of OTC pre and post transplantation.

Funding: Not applicable

Conflict of Interest: None to disclose
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S61 A REVISED 2P MODEL FOR MEMBRANE TRANSPORT; FITTING BOVINE EMBRYO PERMEABILITY COEFFICIENTS

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Modelling membrane transport of CPA and water can help optimize cryopreservation methods. We may assume that the rate of change of intracellular osmolality, dM^i/dt , resulting from transmembrane transport, i.e. the rate of dissipation of the driving force, or the work performed per unit time, is proportional to the magnitude of that driving force. The existing 2-parameter (2P) formalism is not 'symmetrical', as it appears to apply above assumption to the description for the solute flux, but not to that for the water flux. We propose a symmetrical 2P formalism, in which both the change of intracellular osmolality resulting from the flux of solute ($dM^i_{(sol)}/dt \cdot V_w^i(t)$) and the change resulting from the flux of water ($dM^i/dt_{(wtr)}$) are proportional to the magnitude of the respective driving force. Thus, for solute flux we use $dM^i_{(sol)}/dt \cdot V_w^i = -P_s A (M_s^i - M_s^o)$ (superscripts ⁱ and ^o denote intra- and extracellular space), from which we derive the underlying change of moles of solute $dn_s^i/dt = -dm/dM \cdot P_s A (M_s^i - M_s^o)$. Herein, dm/dM is the reciprocal of the derivative of the (polynomial) function that gives M at each value of m . The description of dn_s^i/dt (rather than ' dN_s^i/dt ') allows calculation of dV_s^i/dt by multiplying with the partial molar volume. For water flux,

$dM^i/dt_{(wtr)}$ is also taken proportional to its driving force, i.e. to $-RT(M^i-M^o)$, from which we derive that $dV_w^i/dt = dm/dM \cdot L_p ART(M^i-M^o)$. Here, as an example, we used published values of m and M for binary glycerol/water solutions to fit measured osmotic volume excursions of a bovine morula exposed to 5 vol% glycerol with the two mentioned models, or with a model assuming Raoult's law (i.e. $m = M$). These three models differ only a few percent in the estimates for L_p and P_s but can have appreciable differences in predicting potential osmotic injury in vitrification protocols.

Funding: Funded by the Dutch Ministry of Agriculture, Nature and Food Quality (Project numbers WOT-03-001-069, and PPP reproduction 4400003241)

Conflict of Interest: None to disclose

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S62 LONG-TERM PRESERVATION OF RED BLOOD CELLS THROUGH DEEP SUPERCOOLING

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Red blood cell (RBC) transfusion is a common treatment for various acute and

chronic cases like anemia. RBC transfusions are facilitated by cold storage of RBCs. The conventional RBC preservation is storage at 4 °C for 42 days in additive solutions like saline-adenine-glucose-mannitol (SAGM). However, stored RBCs undergo time-dependent metabolic injuries, negatively impacting their quality during storage. This study aims at improving the quality of stored RBCs through deep supercooling and enabling their preservation beyond 42 days at subzero temperatures. Pooled RBCs from three human donors are stored as 1 ml aqueous suspensions in solutions at +4, -5, -8, and -10 °C for up to 18 weeks. Inhibition of ice nucleation is ensured by sealing the sample surface using mineral oil. Samples are tested for lactate, hemolysis, lipid peroxidation, and osmotic fragility at selected time points. The tested additive solutions are SAGM, CPDA, AS-7, and E-Sol 5. Lowering temperature suppresses metabolic activities, as observed by lactate levels. Compared to the unbuffered, acidic solutions like SAGM, storage in a next-generation RBC alkaline storage solution, E-Sol 5, results in lower hemolysis and lipid peroxidation at subzero temperatures. Although cold storage demonstrates better performance in the short run (<10 weeks) for hemolysis, supercooled storage at -5 °C proves more effective when the storage time is extended beyond. Supplementing E-Sol 5 with the antioxidants Resveratrol and Melatonin further decreases injury levels at subzero temperatures. Storage in alkaline solutions supplemented with antioxidants decreases hemolysis and oxidative stress originating from supercooling. Storage at -5 °C in an appropriate solution allows longer preservation with lower hemolysis than cold storage. Extended storage of RBCs can alleviate global blood shortage.

Funding: Research was supported by the National Institute of Health under award number R01HL145031 and National Science Foundation under award number EEC-1941543. The content is solely the responsibility of the authors and does not necessarily represent the official views of these institutes.

Conflict of Interest: None to disclose

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**S63 INTEGRICELL™:
ACCELERATING CELLULAR
THERAPY, DE-RISKING
LEUKAPHERESIS COLLECTION,
CRYOPRESERVATION, AND
INTEGRATING GLOBAL LOGISTICS**

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Cell Matters, a Cryoport company, supports IntegriCell™ platform with leukapheresis cryopreservation services. Early cell-based therapies used fresh leukapheresis products and were typically manufactured adjacent to the point of collection. As the number of clinical trial sites grow nationally and internationally, the logistics of shipping fresh leukapheresis products became increasingly challenging. Today, as a significant percentage of cell-based therapies are derived from leukapheresis, the industry has turned towards cryopreservation as the solution of choice. This resolves not only the logistical challenge but improves overall quality. Here, we illustrate the benefits of Cell Matters cryopreservation process standardization on leukapheresis quality upon thawing. Fresh leukapheresis from healthy donors were

cryopreserved and thawed using Cell Matters optimized process. Thawing after a short cryopreservation period (i.e., within 12 days after cryo-processing), demonstrates preservation of cell quality as illustrated by high level of Viable Nucleated Cells (VNC, >90%), high total nucleated cell (TNC) recovery (>80%) and preservation of Whole Blood Cell (WBC) sub-populations (including CD3+, CD14+, CD19+, CD56 + among CD45+). Importantly, because IntegriCell™, dedicates its services to provide optimal starting material to Cell Therapy manufacturing, we demonstrate that T cell population expansion upon CD3/CD28 activation was maintained upon Cell Matters cryopreservation process. Furthermore, the quality and consistency of those data were confirmed after long storage periods (i.e., 3, 6 and 12 months) with a post-thaw cell viability of $94,9 \pm 1,5\%$ and post-thaw TNC recoveries maintained higher than 80% for all time points assessed. Finally, no difference in viable T cell population was observed between short and up to 12 months cryopreservation period ($63,6 \pm 9,0\%$ and $68,3 \pm 7,2\%$ respectively). Similarly, expansion upon T cells activation was maintained after long storage. Together, these results highlight that Cell Matters' standardized cryopreservation process, developed for the IntegriCell™ platform, preserves critical attributes of Cell Therapy starting material over prolonged periods of time.

Funding: This work was supported by the SPW grant AR8319

Conflict of Interest: None to disclose

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**S64 POST-HYPERTONIC LYSIS OF
RED BLOOD CELLS AND CELL
VOLUME REGULATION**

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This study has tried to explain the post-hypertonic lysis of human red blood cells (RBC). It has been suggested previously that the nature of post-hypertonic lysis might be osmotic, caused by excessive cell swelling during resuspension to isotonic medium caused by salt loading under hypertonic conditions. However, it was problematic to explain the salt loading due to the assumption of plasma membrane impermeability to cations and a lack of knowledge on plasma membrane ion transport. This work applies an alternative approach based on the current cell physiology, which recognizes the presence of electrochemical gradients for cations and the dynamic nature of normal cell steady state (pump-leak concept). It is emphasized that cells are capable of acute cell volume regulation (CVR) by exploiting these gradients for cations to increase (decrease) the number of intracellular ions and thus upregulate (downregulate) cell volume by passive dissipative leaks, not requiring the input of metabolic energy. Considering the CVR perspective, the experimentally observed RBCs' sodium uptake and swelling are consistent with the acute regulatory volume increase response applied by cells to reduce stress from cell dehydration. It is therefore hypothesized that hypertonicity-induced cation channels (HICCs), a major element of acute CVR also present in the RBC plasma membrane, are responsible for the increased cation permeability in RBCs exposed to hypertonic media. Applying a model based on ion flux equations, accounting for plasma membrane potential, shows that increased cation permeability facilitated by HICCs allows Na⁺ uptake and depolarization of the plasma

membrane while facilitating the entry of Cl⁻ ions resulting in significant NaCl uptake. Besides the RBCs' behavior, the involvement of CVR machinery might also explain the behavior of other cells deviating from the expected osmotic behavior based on the ion impermeability assumption.

Funding: This research was financially supported by the VEGA agency (project no. 2/0134/21)

Conflict of Interest: None to disclose

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S65 AMPHIPHILES PREVENT POSTHYPERTONIC HEMOLYSIS OF ERYTHROCYTES

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The effect of glycerol and amphiphilic compounds of human erythrocytes under posthypertonic shock (PHS) conditions was studied. Erythrocytes were pre-treated with glycerol at a final concentration of 15% (20 min, 37°C) and afterwards exposed to PHS (1.5 mol/L NaCl, 20 min → 0.15 mol/L NaCl, 5 min) at a 37 or 0°C. Amphiphilic compounds were added to the rehydration medium prior to the cell transfer. Trifluoperazine (TFP) and chlorpromazine (CPR) as cationic amphiphiles, sodium decyl sulfate (C10) as anionic, and decyl-β-D-glucopyranoside (DGP) as nonionic amphiphiles were used in this research. The level of erythrocytes hemolysis was determined spectrophotometrically ($\lambda = 543$ nm). All investigated amphiphilic compounds reduce the posthypertonic

hemolysis level of erythrocytes pre-treated with glycerol almost equally (~5 times) at 0°C. At the same time, the antihemolytic activity of amphiphiles was about 80%. At temperature of 37°C, nonionic DGP reduced the level of posthypertonic hemolysis by 1.5 times, anionic C10 did not affect the hemolysis level, and usage of cationic TFP and CPR led to additional damage of erythrocytes. The fact that all amphiphilic compounds exhibit a high antihemolytic activity under PHS of erythrocytes at 0°C indicates that the protective effect depends on the erythrocyte membrane state. At a low temperature, when the mobility of the membrane components decreases, the amphiphilic compound molecules manage to integrate into the bilayer, "fix" the microdefects formed at the dehydration stage and prevent their further increase to the size of hemolytic pore of cell at the rehydration stage.

Funding: Not applicable

Conflict of Interest: None to disclose

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S66 EFFECT OF NONPENETRATING CRYOPROTECTANT AGENTS ON INTRACELLULAR ICE FORMATION IN T CELLS

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To understand the effects of nonpenetrating cryoprotectants on intracellular ice formation in human T cells, rapid-freezing experiments were conducted using Jurkat lymphoma cells suspended in cryoformulations that did not contain any penetrating cryoprotective

agents. Tests were performed using proprietary cryoformulations consisting of an isotonic carrier supplemented with either a non-ionic polymer (total osmolality 311 ± 7 mOsm/kg) or a disaccharide (total osmolality 545 ± 4 mOsm/kg); control groups included isotonic media containing 0-5% (v/v) Me₂SO. Intracellular ice formation was detected by high-speed video cryomicroscopy during rapid cooling (150°C/min) to temperatures of -65°C or lower, following controlled formation of extracellular ice at minimal levels of supercooling (1.0-1.5 K). Surprisingly, the total incidence of intracellular ice formation was low in the absence of penetrating cryoprotectants (14% in isotonic media, 27% in dilute polymer solution, and 30% in disaccharide solution), but much higher in the presence of 5% Me₂SO (85% incidence). Suppressing the formation of extracellular ice in nonpenetrating cryoformulations during the first 12-20 K of rapid cooling caused the cumulative incidence of intracellular ice formation to increase approximately 3-fold, reaching levels comparable to those observed in Me₂SO solutions. This suggests that cell dehydration (driven by freeze-concentration of the extracellular solution) is significant in the absence of penetrating cryoprotectants, but not in the presence of Me₂SO. Preliminary results from morphometric analyses of Jurkat cells frozen without cryoprotectants were consistent with the hypothesis that cell water loss occurs during extracellular ice formation at the initial equilibration temperature and during subsequent rapid cooling. Furthermore, the observation that intracellular ice growth velocities in cells frozen in nonpenetrating cryoformulations were slower than in cells containing Me₂SO also suggested that cell water content was lower in the former case. Therefore, penetrating cryoprotectants like Me₂SO may promote intracellular ice

formation in T cells by inhibiting cell dehydration during freezing.

Funding: This work was funded in part by GlaxoSmithKline and the Villanova University College of Engineering.

Conflict of Interest: None to disclose

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S67 WATER CONFINEMENT EFFECT ON CRITICAL COOLING AND WARMING RATES IN TISSUE-CPA SYSTEM

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Understanding and controlling the phase transition of water is the foundation of cryopreservation. Water is an important constituent of all biological materials and is found naturally in confined spaces inside biological materials. Water in confined domains acts differently than in bulk domain, and has been studied in many porous materials, including biological materials. Physical and calorimetric properties of water in confined spaces deviate largely from in non-confined space. Past studies have shown the effect of confinement on mechanical, physical and chemical properties of a material containing confined water. Here we aim to characterize this effect in tissues loaded with cryoprotective agents (CPAs), which is important for the design of cooling and rewarming protocols for tissue and organ

cryopreservation. Phase diagrams and critical rates for dilutions of cryoprotective agent (CPA) concentrations were measured in solutions and tissue equilibrated with CPA. Specifically, we report on water confinement effects on critical cooling and warming rates inside a biological material (rodent kidney tissue) equilibrated with a common CPA (VS55). Kidney biopsies (3 mm diameter) prepared from tissue slices (1.2 mm thick) are equilibrated (>1 week) in a range of CPA (VS55) concentrations (75- 100%) and then tested for CCR, CWR and transition temperatures (T_m , T_g , T_d & T_h). We found that there is a measurable reduction in CCR (max ~88%) and CWR (max ~27%) inside tissue equilibrated with CPA as compared to bulk CPA solution. This knowledge is critically important for appropriately choosing CPA concentrations and cooling rates in vitrification protocols for tissues and organs. While we currently report the effects for a single model system, these methods can be expanded to a wider range of CPAs and tissues and lay the groundwork for a more mechanistic understanding of confinement.

Funding: The material in this work is supported by NIH R01 DK117425, NSF EEC-1941543, Biostasis Research Institute and Medtronic-Bakken Endowed Chair for Engineering in Medicine, University of Minnesota.

Conflict of Interest: None to disclose

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S68 EXTREME VALUE STATISTICS FOR ESTIMATING THE FREEZING PROBABILITY OF SUPERCOOLED WATER

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The propensity of water to remain in a metastable liquid state at temperatures below its equilibrium melting point holds significant potential for cryopreserving biological material such as tissues and organs. The benefits conferred are a direct result of progressively reducing metabolic expenditure due to colder temperatures while simultaneously avoiding the irreversible damage caused by the crystallization of ice. Unfortunately, the freezing of water in bulk systems of clinical relevance is dominated by random heterogeneous nucleation, and the marked unpredictability of this behavior has prevented implementation of supercooling outside of controlled laboratory settings and in volumes larger than a few milliliters. Here, we develop a statistical model that jointly captures both the inherent stochastic nature of nucleation using conventional Poisson statistics as well as the random variability of heterogeneous nucleation catalysis through bivariate extreme value statistics. By quantifying freezing probability as a function of temperature, supercooled duration, and system volume, while accounting for nucleation site variability, this study also provides a basis for the rational design of stable supercooled biopreservation protocols.

Funding: This research received financial support from the National Science Foundation (NSF) Graduate Research Fellowship under Grant No. DGE 1752814 as well as by the NSF Engineering Research Center for Advanced Technologies for Preservation of Biological Systems (ATP-BIO) under NSF EEC Grant No. 1941543.
Conflict of Interest: None to disclose
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S69 EXTRACTING ICE CRYSTAL NUCLEATION AND GROWTH RATES FROM CALORIMETRY CURVES

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We propose a method for determining the time, and therefore temperature, dependent nucleation and growth rates during crystallization. We do so by linking the partial differential equation governing the time dynamics of the crystal size distribution to kinetic (Avrami) parameters describing heat release. This approach is tested in-silico by nucleating and growing time-dependent diffusion limited aggregates with time varying geometry, growth rates, and nucleation rates. The associated heat release was analyzed, showing that nucleation and growth rates could be extracted with high fidelity.

Funding: This work was supported by the National Science Foundation, Grant No. 1941543, NSF Engineering Research Center for Advanced Technologies for Preservation of Biological Systems (ATP-Bio).

Conflict of Interest: None to disclose

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S70 DEVELOPMENT OF IMPACT-INDUCED DROPLET RELEASING FOR SUPERFLASH THAWING IN CRYOPROTECTANT-FREE CRYOPRESERVATION

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We previously reported a cryoprotectant-free cryopreservation method based on the inkjet technique. In the freezing process, the cell suspension is ejected as microdroplets from the inkjet head; then, the droplets are deposited onto a substrate cooled by liquid nitrogen. The deposited droplets were cooled ultrarapidly and vitrified on the substrate. During the thawing process, the substrate with the droplets was thrown into a prewarmed medium. To obtain high cell viability by avoiding recrystallization, the droplets must be warmed ultrarapidly, which can be achieved by reducing the droplet size and the substrate thickness. However, we already examined 70 pL droplets and a 5 μm thick substrate and it is technically difficult to reduce them further. Meanwhile, the warming rate could be improved by thawing only the droplets in the prewarmed medium. In this study, we developed a method to thaw only droplets by impact-induced droplet release from the substrate. In this method, the substrate is fixed to the rotating mechanism, which rotates the substrate with the frozen droplets and finally strikes the stopper. The impact by striking the stopper causes the droplet to detach from the substrate and fall into the prewarmed medium. Almost all the droplets detached from the substrate and fell into the prewarmed medium. The heat transfer simulation showed that this method could warm twice as fast as the previous method with the substrate. As a result, the cell viability for 200 pL droplets was increased from 59.7 % to 80.0 % while the cell viability for less than 70 pL droplets decreased. The decrease in the smaller droplets should be due to recrystallization by gradual warming during falling. Although this method requires further examinations, the potential of the substrate-free warming was confirmed.

Funding: JSPS KAKENHI Grant Numbers 21J21994 & 21H01783

Conflict of Interest: None to disclose

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S71 THE POLYMER-SIZE OF SUCROSE AFFECTS ITS MATERIAL AND PROTECTIVE PROPERTIES DURING DRYING

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The vitrification hypothesis posits that organisms that can survive desiccation do some by forming non-crystalline amorphous (vitrified) solids, which serve to slow down detrimental processes, such as protein unfolding and aggregation. Vitrification has been shown to be essential for desiccation tolerance however, nearly all sufficiently heterogeneous systems (e.g., a cell) will not form crystals when desiccated and thus will vitrify despite being desiccation sensitive. We hypothesize that there must be some property(s) of vitrified systems that make them more or less protective. Here we generate several different glassy systems and attempt to correlate specific properties with protective capacity. In our vitrifying mixtures we use the disaccharide sucrose, and we add small amounts of glycerol to modulate the properties of these dried mixtures. Using dried sucrose/glycerol mixtures we measure several key properties including the glass transition temperature (T_g), glass fragility (m -index), and water content, as well as enzyme protection capacity. There was a strong correlation between protective capacity and T_g , such that as T_g is lowered (plasticized) by the addition of glycerol protective capacity is diminished. We go on to test how the polymerized state of sucrose affects its glassy properties in relation to protection. We find that while there is a

strong correlation between sucrose T_g and protection ($R^2 = 0.848$) no such relationship exists for either of the polymers of sucrose tested. Our results demonstrate that for vitrified solids composed for different sugars there are diverse physical and material properties that promote protection. Understanding how components promote or impede the protective properties of a vitrified material will help advance technologies for the stabilization of biological material.

Funding: This work was supported by DARPA grant W922NF-20-2-0137 to TCB. This work was partially funded through a fellowship to JFR administered by Wyoming NASA EPSCoR (NASA Grant #80NSSC19M0061). JFR is supported by the USDA National Institute of Food and Agriculture, Hatch project #1012152. The authors gratefully acknowledge financial support from the NSF (CHE 0619920 to NA and IntBio 2128069 to TCB) and the Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institute of Health (Grant #2P20GM103432)

Conflict of Interest: None to disclose

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S72 A CPA TOXICITY SCREENING OF CRYOPROTECTIVE SOLUTIONS IN RAT HEARTS

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In clinical practice, donor hearts are transported on ice prior to transplant and discarded if cold ischemia time exceeds ~5 hours. Methods to extend these preservation times are critically needed, and ideally this storage time would extend indefinitely, enabling among other things donor-to-patient matching and improved immune tolerance induction. Previously, we have demonstrated successful vitrification and rewarming of whole rat hearts without ice formation by perfusion-loading a cryoprotective agent (CPA) solution prior to vitrification. These hearts, however, did not recover any beating during normothermic perfusion assessment (although some electrical activity was measured). This loss of function occurred even in controls with CPA loading/unloading alone (no vitrification) which points to chemical toxicity of the CPA solution (VS55 in Euro-Collins carrier solution) as the likely culprit. To address this, the established CPA cocktail of VEG was compared to VS55 using *ex situ* rat heart perfusion. The CPA exposure time was 150 min and the normothermic assessment time was 60 min. Using Celsior as the carrier, we observed partial recovery of mechanical function (atria-only beating) for both VS55 and VEG. Upon further analysis, we found that the VEG CPA cocktail resulted in 50% lower LDH release as compared to VS55 (N=4, $p=.0175$), suggesting VEG has lower toxicity than VS55. We also tested other established carriers such as University of Wisconsin (UW), though none produced better functional recovery than Celsior. The average time that UW-treated hearts spent in cardiac arrest was 30 min versus 1 min for Celsior-treated hearts (N=4, $p=.0286$). Other carrier solutions (Euro-Collins and LM5) did not produce beating at all. We therefore recommend VEG in Celsior be used for reduced toxicity in future studies, and we identify CPA solution development in a high-throughput cardiac system as a promising

approach to achieve healthy levels of functional recovery in whole hearts.

Funding: NIH R01 HL135046-01A1, NSF EEC-1941543, Medtronic-Bakken Endowed Chair for Engineering in Medicine, and T32-HL139431

Conflict of Interest: None to disclose

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S73 SUCCESSFUL TRANSPLANT OF KIDNEYS AFTER VITRIFICATION AND NANOWARMING

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Organ banking would remove the influence of time from organ transplantation. A promising approach to achieve this is vitrification wherein viable storage is theoretically indefinite. While vitrification of rabbit kidneys was first successfully demonstrated decades ago, the ability to reproducibly rewarm whole organs while preserving viability and function has proven elusive due to ice formation (i.e., devitrification) and/or cracks during rewarming due to insufficient and/or non-uniform rewarming. To overcome these limitations, we first applied an engineering-

based transport model to optimize CPA delivery and achieve reproducible vitrification of VMP-perfused rat kidneys. Next, we used “nanowarming”, employing alternating magnetic fields to remotely couple to and heat iron oxide nanoparticles that are perfused in CPA throughout the organ vasculature prior to cooling to achieve fast and uniform rewarming of vitrified kidneys. After unloading, nanowarmed kidneys were assessed by normothermic perfusion and transplantation. Nanowarmed kidneys (n=4) produced urine and functioned comparable to 24-hour cold-stored or VMP-only treated organs when tested by ex vivo normothermic perfusion. Nanowarmed kidneys (n=5) were transplanted in nephrectomized recipients, and all demonstrated rapid reperfusion and urine production. There was some early dysfunction lasting 2-3 weeks (peak Cr 12.6 mg/dL), but renal function normalized (Cr 0.4 to 0.8 mg/dL) by day 21. All measures of renal function were normal at the end of follow-up (day 30), including serum Cr, protein, and electrolytes; calculated glomerular filtration rate; and absence of proteinuria. Histologic and TEM appearance was comparable to fresh control kidney transplants. Using a rat kidney model, we demonstrate the first reproducibly successful vitrification, long-term storage (up to 100 days), rewarming, and transplantation of organs that restore full renal function and solely sustain the life of nephrectomized transplant recipients. Importantly, since this approach provides uniform rewarming, it is scalable to larger organs and provides a viable pathway to clinical organ banking.

Funding: DK117425, HL135046, DK131209, DK132211, NSF grant EEC-1941543, and a gift from the Biostasis Research Institute funded in part through contributions from LifeGift, Nevada Donor

Network, Lifesource, Donor Network West, and Lifebank.

Conflict of Interest: The authors declare the following intellectual property related to this work: “Cryopreservative compositions and methods” Pending U.S. Patent Applications 14/775,998 and 17/579,369 (M.L.E and J.C.B). All other authors declare no competing interest.

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S74 OPTIMIZATION OF REWARMING PROCESS FOR CRYOPRESERVED ORGANS USING A SINGLE-MODE ELECTROMAGNETIC CAVITY: A NUMERICAL STUDY

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Cryopreservation of large-scale biospecimens, tissues or organs is critical for advancing biomedical research and clinical applications, such as organ transplantation. However, currently used warming methods may result in low rewarming rate and temperature non-uniformity in the cryopreserved organs, which can cause lethal ice-recrystallization/devitrification and thermal-stress induced fracture in organs. Electromagnetic heating offers a volumetric heating approach to achieve optimal rapid and uniform warming. In this study, a biophysical model and computer programs were developed to investigate the temperature changes in multiple types of tissues and organs during the warming processes inside a single-mode electromagnetic resonant (SMER) cavity. The model was first validated by comparing the temperature profiles between experimental data and numerical simulation

results. They agreed well with each other. The validated model and computer simulation was then applied to study key factors and characteristics of organ rewarming processes, including: (1) temperature profiles and rewarming rates in the cryopreserved organs; (2) temperature gradient and thermal stress inside organs; (3) optimization of the cavity design to fit organ variations in various organ biophysical properties and dimensions. From this study, the SMER performance to generate rapid and uniform rewarming in large-scale tissues and organs were simulated and investigated, which provides guidance for the future cavity development and the elimination of the harmful thermal stress. In addition, the findings from this study revealed valuable insights on the cavity design to accommodate different size of organs and its further optimization to ensure the rapid and uniform rewarming under different cryopreservation conditions.

Funding: Not applicable

Conflict of Interest: None to disclose

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S75 VITRIFICATION AND ULTRA-RAPID WARMING OF MOUSE SKIN

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Around 1.1 million burn injuries in the US require medical attention annually. The definitive treatment for severe burns is skin grafting, but many patients are too sick on initial presentation to undergo autologous grafting. Instead, cryopreserved human

allogeneic skin grafts are used for coverage to prevent infection and allow for patient resuscitation. Traditional skin cryopreservation uses slow cooling with 10-20% Me₂SO or glycerol as cryoprotective agents (CPAs) but results in poor cell viability, which is vital for the neovascularization and recovery of wounds. Injury results from ice formation that disrupts cells and the macroscopic skin architecture. Therefore, we developed an alternative skin cryopreservation technique using ice-free vitrification paired with rapid rewarming (VR) using metal form radiofrequency (RF) heating. This study tested VR of full-thickness tail skin grafts in a mouse model. CPA (DP6) was loaded stepwise using a protocol developed by predictive diffusion modeling and verified by micro-CT and empiric testing of viability using AlamarBlue. DP6-loaded skin grafts (~8x6mm) were vitrified and stored at -150°C until needed and rewarmed using our ultra-rapid metal form approach in an RF coil. CPA was unloaded (stepwise), and skin grafts were tested directly for viability or transplanted onto the back of syngeneic recipients. The viability of CPA-only controls was 100% (N=8), VR skin was 100% (N=7), and slow warming was 81% (N=4). The 30-day transplant result shows that the VR skin grafts demonstrated high-level engraftment (consistent with revascularization) and no necrosis (N=10) and were comparable to the control transplant group (N=10). In contrast, the injury control (N=2) and convectively warmed skin grafts (N=4) had reduced viability after rewarming and appeared pale and necrotic after transplant. These positive results show that vitrification can improve the viability, reliability, and function of donor skin for transplant to address burn therapy and other regenerative medicine applications.

Funding: NSF EEC-1941543 and Biostasis Research Institute

Conflict of Interest: None to disclose

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S76 FABRICATING HUMANS: ETHICAL, LEGAL AND SOCIAL ISSUES OF 3D HUMAN ORGAN BIOPRINTING

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Biofabrication also known as bioprinting represents an emerging new science promising astonishing progresses in the research and clinical applications. Biofabrication is “cousin” of tissue engineering and regenerative medicines because they use human biological materials to build constructs. However it raises specific ethical, legal and social issues (ELSI). This abstract outlines the main ethical, legal and social issues related to 3D tissue and organ bioprinting and the ways to explore them further. While 3D tissue and organ bioprinting seem to offer promising solutions to atavic ethical problems by opening viable alternative to animal testing, xenotransplantations, persistent organ demand, at the same time they raise conflicting issues which deserve to be considered deeply. Firstly, 3D organ and tissue biofabrication currently requires expensive technologies. Hence it may create a social discrimination based on the individual richness by leaving alone patients who have limited financial resources. Secondly, 3D bioprinting is still a futuristic approach which needs to be extensively validated. While 2D bioprinting (tissues such as skin) and hollow tube printing (such as blood vessels) are simpler, hollow and solid organ are far more complex and require long-

term development and testing. Therefore, 3D bioprinting raises the need for scientists to carefully handle the expectations of patients and their families. This requires, as first, the need to give loyal, clear information to patients about known benefits and harms of this technology. Furthermore, clear information should be given on the future potential use of bioprinted tissues/organs, especially in case they survive to their donor (for autologous stem cells). Finally, 3D organs bioprinting requires to set out specific regulations. To date, 3D organ bioprinting is not subject to dedicated legislations except for a limited countries. Many ethical and regulatory issues are still open: definition of 3D bioprinting constructs (organs Vs products), legal/regulatory classification (organ, drug, device?), property rights (ownership, patentability, legacy), commercialization (profitable machine or a medical treatment?), human enhancement.

Funding: Not applicable

Conflict of Interest: none

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S77 METRICS OF CORAL MICROFRAGMENT VIABILITY

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Coral reefs are being degraded at unprecedented rates and decisive intervention actions are urgently needed to help them. One such intervention in aid of reefs is coral cryopreservation. Although the cryopreservation of coral sperm and larvae has been achieved, preservation of coral fragments including both its tissue and skeleton, has not. The goal of this paper was to understand and assess the physiological stressors that might underlie coral fragment cryopreservation and the long-term consequences of these physiological exposures to continued growth. Therefore, we assessed small fragments (~0.5 x 0.5 mm²) from the Hawaiian coral, *Porites compressa*, examining: 1) the sensitivity of the fragments and their algal symbionts to chilling temperatures; 2) the sensitivity of the coral to complex cryoprotectants; 3) methods to safely remove the algal symbionts from the coral fragment for cryopreservation, given the two symbiotic partners may require different cryopreservation protocols; 4) continued growth over time of coral fragments once returned to running seawater after treatment exposures; and, 5) assessment of health and viability of microfragments after treatments examining the distribution of green fluorescent protein and fluorescent symbionts. Technological advances in cryotechnology promise to support successful coral fragment cryopreservation soon, and its success could help secure much of the genetic and biodiversity of reefs in the next decade.

Funding: Not applicable

Conflict of Interest: None to disclose

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**S78 CRYOPRESERVATION OF
DIVERSE SYMBIODINIACEAE
SPECIES: FATTY ACID PROFILES,
GROWTH AND PHOTOSYNTHETIC
PERFORMANCE**

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Symbiodiniaceae are a diverse group of dinoflagellates, which can be both free-living and/or associated with a variety of protists and other invertebrate hosts. Maintenance of isolated strains in culture is labour-intensive and expensive. Cryopreservation provides an excellent avenue for their long-term storage. Different cultured isolates from six Symbiodiniaceae genera were cryopreserved using either rapid freezing or controlled rate freezing techniques using dimethyl sulfoxide (Me₂SO) as the cryoprotectant agent (CPA). Symbiodiniaceae isolates from four genera had high cell survival (> 40%) after thawing. The isolates that failed to cryopreserve had very low survival rates (< 15%). These isolates were cryopreserved using [a] a lower final 10% Me₂SO concentration, and [b] grown in media with increased salinity before cryopreservation experiments. For the two isolates that had low survival rates when using a lower concentration of Me₂SO, increased salinity treatments were trialled

before freezing. There were high cell viabilities in *Symbiodinium pilosum* cultures after treatment with increased salinities of 44 parts per thousand (ppt) and 54 ppt culture medium. *Fugacium* sp. cryopreserved after salinity treatments of 54 ppt and 64 ppt. Fatty acid (FA) analyses after the salt pre-treatment experiment showed changes in FA production. FAs production increased in response to high salinity treatments as compared to normal salinity (34 ppt). After thawing and allowing the cultures to recover in their normal salinity media, we investigated the photosynthetic performance and growth rates of an endosymbiotic and free-living isolate using Pulse Amplitude Modulated (PAM) fluorometry. The results showed that cryopreservation does not affect their photosynthetic ability nor growth rates. Molecular investigations are underway to explore whether genes involved in enhancing FA production and overall cryopreservation success can be identified.

Funding: This research was supported by funding from the New Zealand Ministry of Business, Innovation and Employment, Contract number: CAWX0902 and a Cawthron Institute Internal Capability Investment Fund scholarship.

Conflict of Interest: None to disclose

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**S79 THE LAST OF US: SURVIVAL OF
CRYOPRESERVED SALAMANDER
SPERM DEPENDS ON CRYODILUENT**

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Gamete survival through the cryopreservation process can be impacted by a range of factors including diluents and cryoprotectants. This research aimed to test six different cryodiluent treatments and their effects on several sperm parameters in tiger salamanders (n=11). The diluent HAM's F-10 nutrient mix (HAM's), diluted HAM's, or 10% Holtfreter's was added to milt samples to bring the concentration to 1×10^6 sperm/mL. Then, the cryoprotectant dimethylformamide (DMFA) or dimethylsulfoxide (Me₂SO) was mixed 1:1 with the sperm samples and cryopreserved in 200 μ L straws using liquid nitrogen. A total of 87 straws (1-4 straws/treatment/male) were thawed in a 40°C water bath for 5 seconds and assessed for relative total motility and viability. A subset of straws (n=41) was used for *in-vitro* fertilization with fresh eggs from female tiger salamanders (n=8). We found that both cryoprotectant and diluent had a significant effect on post-thaw relative motility, where Me₂SO was better ($p < 0.01$) than DMFA ($38.7 \pm 4.5\%$ vs. $29.6 \pm 5.0\%$) and HAM's was better ($p < 0.01$) than diluted HAM's and 10% Holtfreter's ($45.4 \pm 6.4\%$ vs. $34.1 \pm 4.0\%$ or $23.0 \pm 4.0\%$). Viability was significantly higher ($p < 0.05$) in samples frozen with Me₂SO compared to DMFA ($32.8 \pm 3.9\%$ vs. $27.6 \pm 4.1\%$), while samples with diluted HAM's had higher ($p < 0.01$) viability compared to 10% Holtfreter's ($34.3 \pm 3.9\%$ vs. $26.9 \pm 3.5\%$). DMFA resulted in higher ($p < 0.05$) embryo cleavage rates than Me₂SO regardless of diluent ($29.8 \pm 3.2\%$ vs. $5.7 \pm 1.7\%$). The combination of diluted HAM's and DMFA resulted in the highest ($p < 0.05$) embryo cleavage rates ($40.1 \pm 5.9\%$) overall. These findings support the use of HAM's as a diluent for cryopreserving salamander sperm, however further work is

needed into methodologies for washing salamander sperm cryopreserved with Me₂SO as the chemical appears toxic during the fertilization process. Biobanking cryopreserved sperm is a critical component to genetic management of declining amphibian species. By increasing fertilization capabilities of frozen gametes, both captive and wild populations can avert extinction.

Funding: This work was supported by the Institute of Museum and Library Services (Grant #MG-30-17-0052-17)

Conflict of Interest: None to disclose

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S80 CRYOPRESERVATION OF CORAL MICROFRAGMENTS

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Corals and the reefs they build provide numerous benefits, are culturally relevant to coastal populations, and serve as oceanic nurseries for thousands of marine species. Today their continued persistence is threatened by combinations of local and global stressors. The loss of species and genetic diversity has stimulated an interest in reef conservation, and these intervention strategies include marine protected areas, oceanic coral nurseries, and land-based repositories in aquaria and aquaculture facilities. Additionally, advances in cryopreservation have allowed for the emergence of “cryoconservation” wherein live, cryopreserved cells (coral sperm) from reefs in the Caribbean and Pacific are secured in gene banks as insurance against loss of genetic diversity; this material has also been used for assisted gene flow, and more recently, live coral larvae have been successfully cryopreserved. Although corals can reproduce sexually through spawning, these annual events provided limited opportunities to secure genetic diversity, and reproductive material is increasingly negatively impacted by ecosystem stressors. Coral asexual reproduction via fragmentation allows for cryopreservation of the whole coral organism (and genome) and decouples the process from yearly reproductive events. Here we use a novel technique, isochoric vitrification, to cryopreserve and revive small (1 cm²) coral “microfragments” and use oxygen-uptake respirometry to determine 24-hr post-thaw survival. Immediate next steps will explore techniques to allow for the continued survival of fragments beyond this initial benchmark and, if successful, unlock

the potential for rapid securing of global coral diversity.

Funding: Revive & Restore Catalyst Science Fund

Conflict of Interest: BR filed a 2017 patent application related to isochoric vitrification, which is under review as of the date of submission of this work. MPP and BR have financial stake in a commercial entity that holds the license to said patent application. The other authors declare no conflicts of interest.

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S81 MAXIMIZING THE UTILITY OF CRYOPRESERVATION AS AN ENABLING TECHNOLOGY IN CORAL REEF RECOVERY AND RESILIENCE

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Coral reefs worldwide are facing a crisis as the impacts of ocean warming and acidification increase. The loss of genetic and biodiversity caused by coral bleaching can reduce the ability of coral to adapt to the changing climate, and the loss of coral populations and the reef structure that they provide will severely impact the more than a quarter of all marine life that depends on coral reefs for habitat. Cross-cutting research programs like the Reef Restoration and Adaptation Program in Australia are developing interventions to support cost-

effective, large-scale, reef restoration, built around the generation and deployment of new corals onto reefs at the scale required to effect meaningful reef recovery. Cryopreservation and biobanking can play a crucial role in these large-scale programs by facilitating genetic management of populations through selective breeding, reducing the dependence on synchronous spawning, supporting research, and preventing the extinction of coral species. The integration of sperm cryopreservation into large-scale restoration programs for coral will require upscaling of existing methods for both the cryopreservation process and the use of cryopreserved samples, to meet the requirements of aquaculture production. In collaboration with coral aquaculture scientists, we set out to optimise cryopreservation processing pathways for coral sperm, utilising a semi-automated approach to integrate and streamline existing protocols for the assessment, handling, and cryopreservation of coral sperm. We also undertook a series of experiments to quantify the use of cryopreserved sperm for the production of *Acropora* coral larvae, and showed that cryopreserved sperm can be used to produce coral larvae at a scale suitable for integration with aquaculture. The methodologies described will be applicable for coral cryopreservation and biobanking practitioners on reefs around the world, and provide a case study in the transition of cryopreservation technologies from research laboratory to industry application at scale.

Funding: Reef Restoration and Adaptation Program and Taronga Foundation, Taronga Conservation Society Australia

Conflict of Interest: None to disclose

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S82 THEORETICAL BASIS FOR CRYOPRESERVATION MODE OF SPHEROIDS FROM MESENCHYMAL STROMAL CELLS

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The development of methods for cryopreservation of spheroids is a current problem. Solving this problem will allow more effective use of spheroids in regenerative medicine. The plasma membranes permeability coefficients for water and cryoprotectants determine time characteristics of mass transfer through the cell membranes, and therefore the optimal modes of cells cryopreservation. While freezing more complex multicellular formations rather than cell suspension, it is necessary to take into account their generalised characteristics. Integral characteristics that determine the time parameters of the mass transfer between the spheroid and the environment can be considered as analogues of the membrane's permeability coefficients of the individual cells. We determined the parameters of spheroids from mesenchymal stromal cells: osmotically inactive volume; permeability coefficients for water and Me₂SO molecules and the activation energy of their penetration. We calculated the osmotic behaviour of multicellular spheroids under cooling conditions using a system of differential equations describing the kinetics of changes in the spheroid relative volume and the concentration of the penetrating substance during the crystallization of the medium. The theoretical calculations showed that cryopreservation with cooling rates of 0.75 - 1 degree/min allows to achieve sufficient

dehydration of spheroids, which can reduce the level of damage to spheroids by intracellular ice. On the other hand, this level of dehydration does not lead to damage to cellular structures, since the relative volume does not reach the value of the osmotically inactive volume ($\alpha = 0,684$). The relative volume of the spheroid at such cooling rates reaches values of 0.88 to 0.85 at -40 to -60°C. When these temperatures are reached, further cooling can be carried out by moving to a low-temperature refrigerator (-80°C) or immersing in liquid nitrogen.

Funding: Not applicable

Conflict of Interest: None to disclose

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S83 CRYOPRESERVATION OF STEM CELLS BY ELECTROPORATION-ASSISTED LOADING WITH SUCROSE

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A new method based on the electroporation-assisted introduction of natural cryoprotectants (CPAs) such as sucrose, trehalose or raffinose into human cells to completely eliminate animal-derived components and minimize the concentration of synthetic CPAs used in standard freezing solutions. In this method, the permeability of the cell membranes is briefly increased by short electrical pulses. This creates pores that close again after a short time. As long as the pores remain, the natural CPAs can penetrate the cells despite their molecular size and thus also protect the intracellular space. The aim

of this study is to validate the process parameters of the electroporation process (voltage, sucrose concentration) for cryopreservation of human mesenchymal stem cells using sucrose loading by electroporation. Thereby, the high efficiency during loading and high vitality rates -of over 90%- after electroporation should be achieved in order to keep cell loss before the freezing process as low as possible. The mesenchymal stem cells before and after electroporation by biochemical and imaging methods are analyzed. Subsequently, both the electroporated and non-electroporated cells are cryopreserved using slow-freezing techniques and analyzed based on cell viability after 1, 3 and 7 days of recultivation.

Funding: Leibniz Young Investigator Grants (LYIG) of the Leibniz University Hannover, Germany

Conflict of Interest: None to disclose

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S84 TRACKING THE PERMEATION OF PVS2 COMPONENTS IN LIVING MENTHA X PIPERITA SHOOT TIPS USING OPTICAL MICROSCOPIES

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Cryopreservation of vegetatively propagated crop collections has emerged as a solution to the global plant conservation crisis. The successful cryopreservation of these collections relies upon the exposure of 1-2 mm shoot tips to highly concentrated mixtures of cryoprotective agents (CPAs) to reduce water content and increase the viscosity of cells prior to liquid nitrogen exposure. Despite the successful use of CPA mixtures such as PVS2 (15% w/v dimethyl sulfoxide, 15% w/v ethylene glycol, 30% w/v glycerol and 0.4 M sucrose), they are not universally cryoprotective and very little is understood about how these CPAs protect cells and tissues from freezing damage. Coherent anti-Stokes Raman scattering (CARS) microscopy is a non-invasive vibrational imaging technique that detects a single chemical bond of interest, allowing for direct visualization of CPAs within living plant cells. Previously, we have presented results pairing CARS microscopy with both brightfield and two-photon endogenous fluorescence microscopies to explore the extent of dimethyl sulfoxide permeation within peppermint shoot tips. This presentation extends our exploration to ethylene glycol and glycerol components of PVS2, providing a comprehensive picture of how individual permeating components of PVS2 localize in peppermint shoot tips. Further, we will present the latest results of immunofluorescent tissue staining, providing an even more comprehensive view of tissue development and cellular components.

Funding: We gratefully acknowledge financial support from USDA NIFA Grant Number 12835363. Imaging experiments were performed in the Advanced Light Microscopy Core, part of NeuroTechnology Center at University of Colorado Anschutz Medical Campus, supported in part by Rocky Mountain Neurological Disorders Core Grant Number P30 NS048154 and by

Diabetes Research Center Grant Number P30 DK116073.

Conflict of Interest: None to disclose

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S85 UNDERSTANDING AND MITIGATION OF CRYOPROTECTANT-INDUCED FREE RADICAL DAMAGE FOR SUCCESSFUL TISSUE VITRIFICATION

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Successful tissue vitrification remains challenging due to different damage mechanisms, including free radical damage induced by cryoprotectants (also known as cryoprotective agents, CPAs) and cooling/warming. The objective of the present study was to understand and mitigate CPA-induced free radical damage at the cell level. To this end, human primary fibroblasts and dermal keratinocytes were isolated from our tissue models (human fibroid and skin, respectively) and exposed to increasing concentrations (30%, 40%, and 50% v/v in PBS) of three permeating CPAs (i.e., Me₂SO, ethylene glycol, and propylene glycol) at room temperature for 30 and 45 minutes. The addition and removal of CPAs were done either in one step or in multiple steps to reduce osmotic stresses. Untreated primary cells served as controls. After CPA removal and a recovery period of 2 hours at 37°C,

formation of superoxide that gives rise to different free radicals was monitored using MitoSOX staining. All three CPAs induced increased superoxide formation in both cell types. As the CPA concentrations and exposure durations increased, increased superoxide formation was observed along with shifting MitoSOX fluorescence staining from cytoplasm to nucleus, which suggests increased apoptotic processes. Interestingly, reducing osmotic stresses by adding and removing CPAs in multiple steps, in turn, also reduced superoxide formation. However, this reduction was less pronounced at higher CPA concentrations and longer exposure durations, suggesting the need for development of sophisticated approaches, such as minimizing osmotic stresses along with antioxidant treatments, which are currently under investigation.

Funding: This work was supported by funding from the National Institutes of Health (R01 EB027203).

Conflict of Interest: None to disclose

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S86 AN ASSOCIATION BETWEEN INTRACELLULAR ICE RECRYSTALLIZATION-INDUCED DNA DAMAGE, THE CELL CYCLE, AND CHROMATIN CONDENSATION

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The recrystallization of intracellular ice is a preeminent factor underlying the cell death precipitated by intracellular ice formation

(IIF). However, there has been limited investigation into what, precisely, are the cellular mechanisms through which this recrystallization triggers cell death. We speculated that DNA damage would be one such mechanism, caused by the recrystallization of intracellular ice, rather than IIF itself. Stemming from this, we hypothesized that decondensed euchromatin, a state of DNA that is theoretically more pliable in the dynamically shifting unfrozen fraction which exists during recrystallization, would be rendered less sensitive to this DNA damage. HepG2 cells were cultured to confluency on 12 mm poly-d-lysine-coated glass coverslips. One cohort was subject to serum deprivation for 24 h prior to freezing, rendering the cells in the G0/G1 phase of the cell cycle where chromatin would primarily exist in a condensed state. Nucleation was induced at -10 °C in the absence of any cryoprotectant; a condition confirmed to cause 100% IIF. In one cohort, cells were thawed at 37 °C immediately following nucleation, whereas the other was kept at -10 °C for 30 min to exacerbate recrystallization prior to thawing. All samples were subject to the Comet assay damage immediately after thawing (thus before apoptosis would manifest), with subsequent calculations for the tail moment (tail length x DNA tail %), a robust index of DNA damage, performed. Recrystallization of intracellular ice promoted a large increase the tail moment (+serum: $p = 0.01$; -serum: $p = 0.0005$). Furthermore, serum deprivation did indeed render the cells more susceptible to this recrystallization-induced DNA damage as again indicated by the heightened tail moment ($p = 0.003$). Our results confirm that recrystallization of intracellular ice promotes DNA damage, and that this damage is more profound when a majority of the chromatin exists in a condensed state.

Funding: Not applicable

Conflict of Interest: Jason Acker is a co-founder of PanTHERA CryoSolutions, a company which manufactures ice recrystallization inhibitors.

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S87 DYNAMICS OF PROTEIN HYDRATION WATER AT CRYOGENIC TEMPERATURE REVEALED BY NEUTRON SCATTERING

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Hydration and glass transition vitrification are essential factors determining the physical property and quality of food. It is essential to investigate the molecular dynamics and hydration state of foods over a wide temperature range from cryogenic to room temperature. Incoherent neutron scattering (INS) can measure the molecular thermal fluctuations in-situ at cryogenic temperature as well as at ambient temperature. INS can observe the dynamics of food molecules and hydration water separately by using the isotope effect of hydrogen. Therefore, INS is a promising experimental method for the analysis of water dynamics and glass transition of food materials. Molecular dynamics (MD) simulation gives information on the molecular structure and dynamics with atomic scale. The selective observation of food molecules and hydration water by INS, and the complementary analysis of hydration structure and dynamics with MD simulation are effective methods for studying food hydration, and then food preservation. Hydrated lyophilized proteins exhibit a phenomenon called dynamical transition, similar to the glass transition. In this study, the hydration states of hydrated lyophilized protein of globular enzymes at various water activities from 0.10 to 0.95 were investigated

by INS and MD simulations. To observe the dynamics of protein and water, we used D₂O-hydrated and H₂O-hydrated protein, respectively. It was found that the water activity dependence of the onset of protein glass transition is correlated with the percolation transition of hydration water (network formation of water via hydrogen-bonding). Protein dynamical transition is coupled with the fluctuation of the hydration water network covered over the protein surface. In addition, the hydration water dynamics change significantly at the transition temperature. The percolation of hydration water induces the protein glass transition.

Funding: Not applicable

Conflict of Interest: None to disclose

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S88 TAKING BIOSPECIMENS TO A HIGHER STANDARD: BIOBANKING STANDARDS AND RELATED TOOLS TO ENSURE THAT NEXT-GENERATION BIOTECHNOLOGY PRODUCTS ARE FIT FOR THEIR INTENDED PURPOSE

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Biobanking standards and best practices are critical for ensuring that biospecimens are fit for an intended purpose (FIP) and that the results of studies using biospecimens from biobanks are meaningful and reproducible. This presentation will explore the elements and implementation of ISO 20387 General requirements for biobanks. Examples of related tools, including other standards and best practices, such as the International Society for Biological and Environmental

Repositories (ISBER) Best Practices 5th Edition, will also be introduced as complementary tools to define a biobank's path to FIP. Since FIP will be different for every biobank, biobanks are well served by putting together their own "toolkits." Considerations for the assembly of this toolkit will be explored. One or more case studies will also be described.

Funding: Not applicable

Conflict of Interest: None to disclose

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S89 PREANALYTICAL VARIABLES FROM A MULTI-INSTITUTE STUDY: CONCERNS AND FUTURE DIRECTION

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Proper documentation of preanalytical data is an essential part of biobanking. These data are key indicators of sample quality and integrity and must be meticulously recorded to limit sample to sample variability. Standard operating procedures, study workflows and lab manuals are designed to reduce inconsistency of preanalytical data thus when analyzing samples collected within a study, the number of variations is limited, ultimately producing assay data that is consistent and reproducible. A multi-institute study collected whole blood and processed it for PBMCs over a period of several years and shipped cryopreserved PBMC to a central location for flow cytometric analysis. 544 samples were received and thawed. To assess the quality of the samples, preanalytical collection and processing data were gathered from each site and analyzed for quality indicators. Additionally, thawed PBMCs were assessed

for percent recovery and viability. 213 were processed and stored in <8 hours, 229 between 8 and 24 hours, and 98 were processed and stored after 24 hours, of which, 33 were processed >36 hours from time of collection. Pre-cryopreservation viability was recorded for only 249 samples of which 236 had viabilities of $\geq 90\%$. Viability was not recorded for the remaining samples. After thaw, 42 samples had a percent recovery of <20%, 87 had recoveries at >20% and $\leq 50\%$, 110 >50% and $\leq 75\%$, and 303 were >75% recovery. Post-thaw viabilities were at $\leq 50\%$ for 51 samples and >50% for the remaining 493 samples. Data from the sites varied greatly and brings to light several areas of concern. Data points reported were often estimates or not recorded. Processing times varied greatly, indicating that SOPs were not followed; ultimately causing concern over the validity and reproducibility of assay data resulting from these samples. Future direction supports the need for stronger oversight to ensure consistent data reporting and processing.

Source of Funding: Not applicable

Conflict of Interest: None to disclose

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S90 DEVELOPMENT OF HIGH CONCENTRATION CELL BANKING PROCEDURE

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Mammalian cell culture that can be produced on an industrial scale has become the dominant production system for recombinant proteins. A typical mammalian cell culture includes the steps of thawing a vial from the relevant cell bank, bringing the amount of biomass to a sufficient size for production, and production steps. The seed train is a critical part of the bioprocess, producing enough biomass to inoculate the production bioreactor and start protein production in an optimized manner. In a conventional seed train, this is achieved by passing cells through a working cell bank flask through increasingly larger cultivation systems including shaker flasks, rocking motion bioreactors, and stirred tank bioreactors. Efforts are being made to create a high concentration cell bank to avoid these intermediate culture steps and to allow direct inoculation of a production bioreactor with cells from cryovials or cryobags. The common cell banking concentration for mammalian cells is $10\text{--}15 \times 10^6$ cells/ml. In this study, cell banks with different concentrations up to 200×10^6 cells/mL were created and these cell banks were inoculated directly into production to test the growth profiles of the cells and the amount of monoclonal antibodies produced. The ultimate goal of this study is to reduce time, labor, cost and operational risks by using high concentration cell banks.

Funding: Not applicable

Conflict of Interest: None to disclose

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S91 APPLICATION OF PORTABLE CRYODEVICE AS A SOLUTION FOR CENTRALIZED CRYOBANKING

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Ovarian tissue cryopreservation (OTC) is the best option for fertility preservation (FP) in prepubertal girls and women with cancer. Thus, interest in adopting the OTC has been growing all around the world. However, the cost to establish and maintain one cryopreservation center for thirty years is more than €5 million. Therefore, the establishment of central cryobanks would overcome these difficulties. For such central cryobanks, the ovarian tissue transportation (OTT) network is necessary to increase the patient's access to the OTC technique. However, OTT is considered an empirical procedure and is associated with the negative effect of ischemia. Hence, shortening the pre-cryopreservation time could be a key strategy to reduce ischemia-induced damage and improve graft longevity. An ovarian tissue cryopreservation network (OTCN) would address the shortages of OTT and expand the FP options available to patients who are in geographic areas that don't have an OTC cryobank. OTCN can be called "The patient stays- the staff comes". One approach to achieve this goal is employing portable cryo-devices such as Asymptote and Mr. Frosty. Therefore, we examined the feasibility of portable cryopreservation devices, including a controlled rate asymptote freezer and Mr. Frosty against the programmable slow freezer for the establishment of an OTCN. To determine the application of the different cooling machines, the viability, reactive oxygen species (ROS) levels, total antioxidant capacity (TAC), and morphological characteristics of bovine ovaries were evaluated in frozen groups and results were compared with the fresh group. Our results indicate that Asymptote can freeze the ovarian cortex similar to a programmable slow freezer; however, we observed lower cell viability and TAC and

higher ROS, and degenerative follicle when we used Mr. Frosty device. In conclusion, a portable liquid nitrogen-free device like Asymptote can be considered as a possible solution for the establishment of an OTCN.

Funding: SHRF Establishment Grant and NSERC (RGPIN-2017-06346) to JB

Conflict of Interest: None to disclose.

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S92 THE IMPACT OF CRYOPRESERVATION ON THE FUNCTIONAL RECOVERY OF LUCIFERASE REPORTER CELLS

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Though in vivo animal models are commonly used in cancer biology and drug research, their imprecision and lack of physiological relevance pose a major hurdle for clinical translation. ATCC's luciferase-labeled cell lines provide a simple, sensitive, and versatile means to measure specific biological processes through bioluminescence imaging. Because these engineered cell lines are a powerful tool for oncology and immuno-oncology research, it is important to consider the impact that low-temperature storage will have on the luciferase reporter functionality. Cryopreservation using a controlled-rate freezer followed by storage in the vapor phase of liquid nitrogen is the gold standard for long-term preservation of mammalian cells at ATCC. It is a well-known phenomenon that cryopreservation and subsequent thawing are stressful processes that generally require a recovery period in culture for growth, metabolism, and functionality to return to normal. Because of

this, it is important to understand how these processes impact the resulting recovery time required for our luciferase reporter cells to be able to provide the accurate and repeatable results expected. In this study, we compared the immediate post-thaw viability, growth, and functionality characteristics of THP-1 luciferase reporter cells (ATCC® TIB-202-NFkB-LUC2™) to those of non-frozen controls. As expected, the cells immediately recovering from cryopreservation had different characteristics than those in fresh culture. Specifically, we identified an atypical cellular response to immediate post-thaw lipopolysaccharide (LPS) stimulation. Observing the impact of preservation on the cell characteristics and understanding the mechanisms that lead to these phenotypic changes provide us with an opportunity to optimize our cryopreservation formulations and procedures toward reducing stress and enhancing recovery. Minimizing this recovery time will enable our customers to start their research faster.

Funding: Not applicable

Conflict of Interest: None to disclose

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S93 BANKING CORAL LARVAE AT SCALE WITH CRYOMESH: OPPORTUNITIES AND CHALLENGES

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Coral populations around the world are threatened by human activity and warming oceans. Novel and scalable approaches to cryopreserve coral germplasm, algal symbionts, and adult tissues can improve access to banked material for biological research and reef restoration efforts, thus supporting biodiversity and increasing population resilience to climate change. We recently vitrified and rewarmed larvae of the solitary Hawaiian stony coral species *Lobactis scutaria* (mushroom coral) with one-step loading of a 3.5-M multi-component cryoprotective agent (CPA) solution, removal of excess CPA, liquid nitrogen plunge cooling, warming on a novel cryomesh substrate, and two-step CPA unloading. With optimization of substrate geometry and experimental methods, estimated cooling rate within the larvae was 9.4×10^4 °C/min, warming rate was 1.2×10^5 °C/min, and post-thaw larval recovery reached 85%. This result improves on prior vitrification work with the same species using 1-μL droplet plunge cooling of the same CPA solution on a plastic film followed by nanoparticle-mediated infrared laser pulse warming (plunge cooling rate $< 6.9 \times 10^4$ °C/min, laser warming 4.5×10^6 °C/min, 43% recovery). Here we present our cryomesh results, propose technical directions and collaborative efforts that will allow deployment of this and related technologies at a scale that will support research and restoration activities, and describe the remaining challenges in cryopreservation of larvae of a wider range of coral species.

Funding: Paul M. Angell Family Foundation (CON-Sp22-10), National Science Foundation (EEC 1941543), National Institutes of Health (R01DK131209).

Conflict of Interest: None to disclose

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S94 IMMUNOMODULATING RESPONSE OF AUTOLOGOUS CRYO-HEMATOLOGICAL TREATMENT IN PATIENTS WITH MALIGNANT MANIFESTATIONS

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Cryoinduced antigen is one of the main terms of autologous and allogeneic cryo-immunological medicine. The newly formed cryomodified protein components, coupled with cryocell detritus / cryocell debris formed extracorporeally in living biological tissue—specifically, human blood after exposure to ultralow temperatures—are crucial in cryo-immunology from the perspective of the formation of immunological substances at ultralow temperatures. The aim of the presented study was to evaluate the immunomodulating response of autologous cryo-hematological treatment with the objective of beneficial antitumor effect. In this study, the native blood was frozen to -180°C rapidly, at a temperature stabilization of $\pm 1\%$, to achieve complete blood component desolation. A one-minute rapid freezing followed by complete inactive thawing of the blood sample was used only once as a freeze–thaw cycle. Parameters of immunophenotyping with cellular immune status were laboratory-determined in blood in patients with malignant diseases on pre- and

post-treatment days after extracorporeal blood procedures applying ultralow temperature *in vivo* and *in vitro*. In response to mitogen treatment *in vitro*, the absolute count of CD69+ circulating B-cells progressively increasing during the post-procedure period, indicating the positive effect of subcutaneous injection of extracorporeal freeze-thawed autologous blood on naive B-cell proliferative capacity. A sharp progressive increase of both the absolute and relative number of CD56+CD45+CD69+ lymphocytes in response to the treatment of blood sample with pokeweed mitogen *in vitro*, was registered during the post-procedure period. We hypothesized that the extracorporeal frozen-thawed blood produces new cryoinduced specific immunogenic substances in human blood (e.g. cryomodified protein components, cryoantigen, cryocell detritus) and generates cell-mediated anti-tumor responses (e.g. cryoinduced anticancer antigen), changing of phenotypes in peripheral blood of patients and triggering specific and non-specific cellular immune responses, which are important to initiate tumor rejection. Equally, we assume that the generated after the injection of post-frozen blood antibodies could be an important protagonist in antibody-dependent antitumor immune responses, suggesting a potential systemic benefit to treatment malignant manifestations.

Funding: Not applicable

Conflict of Interest: None to disclose

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S95 NERVE PROTECTION DURING PROSTATE CRYOSURGERY

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Cryosurgery is a minimally invasive approach to the treatment of focal prostate cancer (PCa). A major complication is the cryoinjury to the cavernous nerve in the neurovascular bundle (NVB). This nerve cryoinjury halts conduction of action potentials (APs) and can eventually result in erectile dysfunction and therefore diminished quality of life for the patient. Here, we propose the application of cryoprotective agents (CPA) to the regions of the nerves in the NVB, prior to prostate cryosurgery, to minimize non-recoverable loss of AP conduction. We modeled a cryosurgical procedure based on data taken during a clinical case and applied ex-vivo porcine phrenic nerves and rat sciatic nerve with temperature profile of NVB. The APs were measured before and after the CPA exposures and during 3 h of recovery. Comparisons of AP amplitude recovery with various CPA compositions reveal that certain CPAs (e.g., 5% Me₂SO + 7.5% Trehalose and 5% M22 for porcine and rat nerves, respectively) showed little or no toxicity and effective cryoprotection from freezing (on average 48% and 30% of recovered AP, respectively). In summary, we demonstrate that neural conduction can be preserved after exposure to freezing conditions if CPAs are properly selected and deployed onto the nerve.

Funding: This work is funded by Medical Immersion Technology (MIT) Sàrl.

Conflict of Interest: None to disclose. A patent application is pending related to this work. **Corresponding Author*:** bischof@umn.edu

S96 COLD-RESPONSIVE NANOPARTICLES-POTENTIATED CRYOIMMUNOTHERAPY FOR COMBATING LOCALIZED AND METASTATIC BREAST CANCER

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Cancer immunotherapy is a revolutionary treatment strategy that utilizes the host's immune system to identify and eliminate tumors. However, the effectiveness of this approach is greatly limited by the immunosuppressive (i.e., immunologically cold) tumor microenvironment (TME), which prevents the immune system from properly recognizing and attacking cancer cells. Cryosurgery, a minimally invasive surgical technique that uses low temperatures to kill cancer, has been shown to impact the TME and generate a cryoimmunotherapy effect to improve treatment of localized tumor. Unfortunately, the capability of cryoimmunotherapy against distant/metastatic tumor, the major cause of mortality in cancer, is not shown yet. In this study, we developed an in-situ cryo-immune engineering (ICIE) strategy that can reprogram the immunologically "cold" TME into a "hot" one with increased immunoactive cells and decreased immunosuppressive cells, to potentiate the immune system's attack on cancer cells. Notably, it increases the ratio of CD8⁺ cytotoxic T cells to the immunosuppressive regulatory T cells by

>100 times in both primary and distant tumors. This is achieved by using a combination of cryosurgery and cold-responsive nanoparticles containing a chemotherapeutic drug and an immune checkpoint blockade siRNA. The immunogenic cell death induced by cryosurgery is further enhanced by the cold-responsive nanoparticles, which specifically target the tumor, release the chemotherapeutic drug and immune checkpoint blockade siRNA into the cytosol upon cryosurgery. This promotes the maturation of dendritic cells, which activates both the CD8⁺ cytotoxic and the effector as well as central memory T cells. These immune cells work together to attack not only primary tumors but also distant and metastatic breast tumors in female mice, known as the abscopal and vaccine effects. Collectively, ICIE represents a potentially effective and durable way of leveraging the immune system for fighting against cancer and its metastasis.

Funding: This work was partially supported by a grant from the National Institutes of Health (NIH R01CA243023 to XH).

Conflict of Interest: None to disclose
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S97 CRYOSURGERY FOR FOOT AFFECTIONS IN BALUCHI HORSES (*EQUUS FERUS CABALLUS*)

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Cryoablation works by tissue freezing, injury to cells results in tissue necrosis. Cryosurgery was performed on foot affections of 8 Baluchi horses affected with canker (n=5) and thrush (n=3). To remove the necrotic tissues in the affected areas, wounds were washed and curetted. The lesions were subjected to cryoablation using stainless steel probes having cutting-edge tips for tissue freezing with cryo-necrosis as the outcome. The probe was immersed in liquid nitrogen (-196°C) for about one minute, Cryoablation in 5 Baluchi horses affected with canker showed marked healing and there was reduced lesion size by day 11. The procedure was performed at an interval of 11 days in horses affected with canker, at about day 33 preceding the first treatment, the affected area was completely healed. In 3 Baluchi thrush-affected horses, which were subsequently treated with cryoablation, marked improvement was recorded on day 13. The procedure was performed at an interval of 13 days in thrush-affected horses. At about day 39 preceding the first treatment, the affection was completely healed. All the affected horses healed completely up to 3 treatments.

Funding: Not applicable

Conflict of interest: None to disclose

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S98 INVESTIGATION OF ADJUNCTIVE CRYOABLATION AND CHEMOTHERAPY FOR THE TREATMENT OF BLADDER CANCER

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Due to rising annual incidence of bladder cancer (BC), the need for new strategies to treat BC continues to increase. While various treatment options exist, many if not all remain suboptimal. Several studies have shown cryoablation to be a promising approach yet a lack of basic information pertaining to BC response to freezing has limited clinical use. As such, in this study we investigated the response of two human BC cell lines, SCaBER (muscle invasive BC) and UMUC3 (an intermediate transitional cell carcinoma) response to freezing. The data show that a single 5 minute freeze to -10°C did not affect cell viability, whereas -15°C and -20°C results in a significant reduction in viability 1 day post freeze to <20%. These populations, however, were able to recover in culture. A complete loss of cell viability was found following a single freeze at -25°C. Application of a repeat (double) freeze resulted in complete cell death at -20°C. In vivo porcine studies demonstrated the consistent destruction of bladder tissue following exposure to <-20°C. Studies investigating the impact of adjunctive subclinical cisplatin (1µM) pre-treatment in combination with freezing were then conducted. The combination cisplatin pre-treatment and mild (-15°C) freezing resulted in complete cell death. This suggests that subclinical doses of cisplatin may be synergistically effective when combined with freezing. In summary, these results suggest

that freezing to temperatures in the range of -20 to 25°C results in a high degree of BC destruction. Further, the data suggest that the combination resulted in a shift of the minimum lethal temperature for BC from -25°C to -15°C. These findings, in combination with previous reports, suggest cryoablation alone or in combination with low dose chemotherapy may provide an improved path for the treatment of BC.

Funding: NIH NCI Grant No.
1R43CA210761-01A1

Conflict of Interest: None to disclose

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S99 EVALUATION OF A NOVEL EUS-GUIDED CRYOCATHETER FOR THE TREATMENT OF PANCREATIC CANCER

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There is a pressing need for the development of new devices facilitating advanced minimally invasive approaches for the *in situ* treatment of pancreatic cancer (PaCa). In this study, we evaluated a new endoscopic ultrasound (EUS) compatible cryocatheter (Frostbite) for its potential use in transesophageal *in situ* ablation of PaCa and

liver cancer. The cryocatheter was passed through the working channel of a EUS endoscope. Evaluations included a 37°C ultrasound gel model, tissue engineered PaCa model and an acute porcine study wherein 6 lesions were created within the liver (under IACUC Approval). Assessment included measurement of ice ball size, isotherm profile in real time and destruction area created. A single 5 minute freeze protocol was employed for all evaluations. Bench studies demonstrated the generation of a 2.4cm diameter iceball with a tip temperature of <-170°C and penetration of the -40 and -20°C isotherms to 1.5cm and 2.1cm (respectively) following 5mins. Analysis of tissue destruction using PaCa tumor model revealed the creation of a 2.1cm ablation area 1 day post freeze. The porcine study demonstrated generation of a 2cm x 3.1cm (diameter x length) ablation zone following a single 5 minute freeze protocol. The porcine study also demonstrated the ability to deliver targeted destruction of tissue in close proximity to major vasculature without damage. The results of this study demonstrated that the cryocatheter was able to rapidly and effectively freeze targeted tissue via an EUS-guided approach. The results showed FrostBite consistently ablated a 2cm x 3cm area using a single 5 minute freeze protocol across all models. Analysis of ablation efficacy revealed ~70% destruction within the overall frozen mass compared to <40% attained with current percutaneous based cryodevices. Although further studies are needed, the results demonstrated the potential of this new approach to provide a next-generation strategy for the treatment of PaCa.

Funding: NIH NCI Grant No.
1R44CA183265-03

Conflict of Interest: None to disclose

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S100 OSMOLALITY BASED EFFECTIVE HEAT CAPACITY MODEL FOR CRYOABLATION SCENARIOS

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Simulations are used to predict temperature distributions during cryoablation scenarios, enabling the estimation of the ablation success based on derived thermal histories in the region around the cryocatheter as well as the simulation of dependent measurements. Simplifications in modeling the thermal properties during phase transition can affect the simulation outcome. In this work, an effective heat capacity model (EHCM) is presented for considering the latent heat during phase transitions based on temperature-dependent osmolalities of solutions. The latent heat release/absorption during freezing/thawing was derived from the change in frozen solvent mass (water) based on the freezing point depression of the remaining liquid solvent with temperature-dependent concentrations. The latent heat of eutectic crystallization/melting is considered in the model by distributing it over a temperature window around the eutectic point and applying it to the EHCM. The model was experimentally verified by differential scanning calorimetry measurements on saline solutions considering varying concentrations and masses that were compared with corresponding simulated heat flow profiles in

a temperature range between -40°C and 25°C. These profiles were obtained by developing thermal equivalent circuits of the experimental setup, whose components (combined thermal resistances and heat capacities of the sensor, crucibles, and the sample) were derived from calibration measurements and thermal properties of the samples according to the proposed model. Simulated heat flow profiles show a high level of similarity with corresponding measurements for the latent heat absorption during ice melting. Deviations during eutectic melting can be partially explained by the smoothing step necessary for consideration in numerical simulations. The presented EHCM only depends on a few parameters to describe temperature-dependent latent heat release/absorption of saline solutions. Model extensions and experiments are planned to consider solutions more similar to blood and tissue, providing a basis for EHCMS based on the composition of tissues applicable in cryoablation simulation scenarios.

Funding: Not applicable

Conflict of Interest: Dr. Fischer is a founder and shareholder at AFreeze GmbH.

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S101 WITHDRAWN

S102 SORTING OF LARGE PARTICLES AS A MODEL FOR USE IN ISLET CRYOPRESERVATION

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Pancreatic islet transplantation offers a potential curative approach to diabetes but has been limited in application due to the lack of adequate islet supply needed at the time of transplant (~300,000 islets for a possible reversal of diabetes). Cryopreservation of pancreatic endocrine cells could circumvent supply chain challenges for quality and required islet equivalence (IEQ). Cryopreservation protocols are more effective when tailored to the size and volume of the biological sample (CPA loading protocols, cooling, and warming rates). Human islets vary in size ranging from 50-400 μ m, and our group has recently demonstrated a generalized cryopreservation protocol to preserve them. However, protocols specific to IEQ could further improve outcomes, but this requires a platform for high throughput sorting. Additionally, clinical translation will require purification of the isolated cell products (e.g., removal of acinar tissue). Microfluidic platforms provide a unique opportunity to screen and manipulate islets. Recent work on using surface acoustic waves-based devices to actuate small particles (< 50 μ m) and cells has shown great potential in performing high throughput separation. This work aims to separate large (>50 μ m) biomaterials such as pancreatic islets by size using travelling surface acoustic waves. We demonstrate high throughput label free sorting of polyethylene particles by size. Particles were focused into

narrow streamlines inside the device and were optically interrogated for their size. Particles larger than a set cut-off diameter (150 μ m) were actuated into a separate outlet using acoustic pulses. We tested the effect of control parameters (actuation signal, flowrates, sample dilution) on efficacy of sorting. Our device can sort relevant particle samples at about 25,000-100,000 particle-mixture/hour rate. Translation of this work to islets provides a key technology for improving outcomes in islet cryopreservation and transplant. This sorting technology can also be used for other biological systems such as embryos, and stem cell spheroid clusters.

Funding: National Institutes of Health (Grant R01DK131209)

Conflict of Interest: None to disclose

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S103 ULTRASOUND-BASED GEOMETRIC MODELING OF THE OVARY WITH APPLICATIONS TO CRYOPRESERVATION

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The long-term goal of this study is to develop cryopreservation means for ovarian tissues, for the benefit of women and girls undergoing cancer treatments such as chemotherapy and radiotherapy. These aggressive treatments may lead to premature ovarian failure, while cryopreservation of the ovaries during the cancer treatments may

preserve their reproductive capability. The objective of the current study is threefold: (i) to develop a computation framework to reconstruct a geometric model of an ovary from 3D ultrasound images; (ii) to create a geometric model databank for ovaries; and (iii) to thermally analyze the feasibility of ovary cryopreservation by vitrification using those models. The computation framework was developed using the commercial codes Slicer3D and SimpleITK, while benchmarking it against manual segmentation. Ten geometrical models of the ovary were created in this study, from imaging datasets of healthy women undergoing reproductive treatments. Each model includes the organ contour and the contours of the largest follicles. The pilot thermal analysis of vitrification was conducted assuming thermophysical properties of the cryoprotective agent (CPA) solution DP6 + 0.175M Sucrose, a promising cocktail which we have recently experimented with on various blood vessel models. The thermal analysis was designed to identify the minimum cooling rate required on the ovary container surface, in order to facilitate complete vitrification at the center of the organ. This analysis further explores the thermal effects associated with partial crystallization in the follicles, an effect which can be expected due to CPA loading limitations in these areas.

Funding: This research has been supported in parts by the National Science Foundation (NSF), award no. EEC 1941543; the Department of Academic Family Medicine, College of Medicine, University of Saskatchewan, Saskatchewan, Canada; and the Biothermal Technology Laboratory, Carnegie Mellon University, Pittsburgh, PA. The content of this study is solely the responsibility of the authors and does not necessarily represent the official views of the NSF, the University of Saskatchewan, or

Carnegie Mellon University.

Conflict of Interest: None to disclose

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S104 ROBOTIC PLATFORM FOR AUTOMATED MICROINJECTION FOR CRYOPRESERVATION APPLICATIONS

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Cryopreservation has been widely used to bank gametes and embryos for IVF and other assisted reproductive technologies. However, due to the permeability barrier, cryopreservation of yolk-laden embryos such as zebrafish and golden shiner has not been successful. Recently, researchers have created a proof of concept for cryopreservation of zebrafish embryos by microinjecting cryoprotective agent (CPA) and gold nano-rods (GNR) into the yolk. We have developed a computer vision guided robotic platform that automated the targeted microinjection of model organisms such as zebrafish, *Drosophila melanogaster*, and golden shiner embryos. We used automated microinjection to increase the overall throughput of the cryopreservation of zebrafish embryo and pave the way for commercialization. We demonstrated that automated microinjection of CPA in zebrafish embryos improves vitrification

rates and survivability of cryopreserved embryos post-thaw significantly compared to manual microinjection. In a similar vein, such a platform might successfully cryopreserve and rewarm other aquatic species such as golden shiner. The golden shiner was chosen because its embryo shape is comparable to that of the zebrafish embryo. Furthermore, cryopreservation of golden shiner embryos has the potential to increase the productivity of the Minnesota aquaculture industry. We microinjected golden shiner embryos with different volumes of CPA and GNR using automated microinjection system at high cell stage. We further checked survivability of golden shiner embryos post microinjection. We hypothesise that as microinjectant volume increases, CPA concentration increases, resulting in decreased chilling and warming rates and higher cryopreservation success rates. We discovered that if the volume of CPA as microinjectant is larger than 10 nl, the mean value of survival rate of golden shiner embryos is less than 60%. These findings proves that a survivability study for golden shiner embryos can be performed utilizing robotic microinjection system. Furthermore, the finding sets the way for future cryopreservation research with golden shiner embryos.

Funding: National Institute of Health - 1R21OD028214 & 1R24OD028444, Minnesota Sea Grant, National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce - NA18OAR4170101

UMII MnDRIVE PhD Fellowship

Conflict of Interest: ASJ, ADA, SBK are commercializing automated microinjection system explained in this abstract.

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S105 CONDUCTION-DOMINATED CRYOMESH FOR ORGANISM VITRIFICATION

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Vitrification-based cryopreservation is one of the most promising approaches to achieving long-term storage of biological systems for maintaining biodiversity, healthcare, and sustainable food production. Despite recent success using a “cryomesh” approach to cryopreserve both organisms and organoids, further improvement in cooling rates can enable increased biosystem viability and the ability to cryopreserve new biosystems, which have been previously unsuccessful due to their sensitivity to cryoprotective agents. Here we show such improvements are possible by enabling conductive cooling through the cryomesh based on heat transfer scaling. Conduction-dominated cryomesh can improve cooling rates from 2 to 10 fold (i.e., 0.24 to 1.2×10^5 °C/min) in a variety of biosystems. Higher thermal conductivity and minimizing the nitrogen vapor barrier are key parameters to achieving improved

vitrification. To demonstrate the impact of conduction-dominated cryomesh, we show improvement in vitrification rates over traditional convective cryomesh in three ecologically and biomedically important biosystems encompassing a range of relevant biosystem sizes, including coral larvae (100 μm), *Drosophila* embryos (500 μm) and zebrafish embryos (1.2 mm). Finally, based on the demonstrated ability to load at least 20 to 400 biosystems per 4 cm^2 mesh, a conductive mesh design is necessary to scale mesh area to large sizes while maintaining uniformity of cooling rates (up to at least 20 cm^2 , and likely larger). We demonstrate not only a theoretical foundation for improved vitrification in μm to mm biosystems but also the capability to scale up for biorepositories and/or practical use.

Funding: National Science Foundation (EEC 1941543), National Institutes of Health (R01DK131209) (Z.G., N.Z., K.S., M.L.E., E.B.F., and J.C.B.). Smithsonian Institution, Smithsonian Women's Committee, Hawaii Institute of Marine Biology, Revive and Restore, the Paul M. Angell Foundation, the Volgenau Foundation, Cedar Hill Foundation, and Anele Kolohe Foundation (N.Z., J.B., J.D., and M.H.). UMII MnDRIVE Ph.D. fellowship (A.S.J.). National Institutes of Health (R21OD028214 and R24OD28444) (S.B.K.). National Institutes of Health (R01GM044757), National Institutes of Health (R21OD028758), National Institutes of Health (R24OD034063) (A.L.N. and T.S.H.).

Conflict of Interest: Z.G., N.Z., M.L.E., and J.C.B. are inventors on a pending patent application (US Provisional Patent Application Serial No.: 63/431,241). S.B.K. and A.S.J. are founders of the company Objective Biotechnology. The authors declare that they have no other competing interests.

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S106 EFFECT OF DMA ON CRYOPRESERVATION AND FERTILIZING ABILITY OF ROOSTER SEMEN

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Semen cryopreservation is an important tool for maintaining genetic diversity and for ex situ conservation of worldwide chicken rare breeds. Recently we reported that dimethylacetamide (DMA), as a cryoprotectant, maintains efficiently post-thawing motility and viability of rooster semen after 1h equilibration. DMA concentrations >0.6 M gave higher percentage of motile sperm, but appeared negative for the velocity parameters. Further, it was suggested that wider 0.5-ml straws would induce less supercooling-related damage. In the current study, we further investigated effects of DMA and of straw size in the following experiments. Experiment 1. Pooled semen of 7-10 roosters per replicate ($n=4$) was cryopreserved at $\sim 100^\circ\text{C}/\text{min}$. Motility parameters (CASA) and viability percentage (DAPI) were assessed in fresh (before addition of cryoprotectant) and post-thaw semen. The viability, total motility, progressive motility, and most CASA

velocity parameters were lower in frozen-thawed than in fresh semen. Comparing all post-thawing conditions, there was no effect of the volume of the straws on semen quality. Overall, 0.9M DMA gave significantly higher post-thaw total motility ($p < 0.01$) and progressive motility ($p < 0.05$) than 0.6M DMA, independently of the volume of straw used. Differences were not significant for all other motility parameters. In Experiment 2, 40 hens ($n=8/\text{group}$) were inseminated twice, with an interval of two days (100 million sperm per hen per day), with fresh pooled semen (from 10 roosters) equilibrated for 1h with 0, 0.6, 0.9, 1.2, or 1.5M DMA. Eggs are currently being collected and are incubated to determine the fertility rates. In this study, we confirmed that better post-thaw sperm quality is found after freezing with 0.9M DMA than with 0.6M DMA. The results of experiment 2 will show if holding semen with various DMA concentrations affects fertility.

Funding: Dutch Ministry of Agriculture, Nature and Food Quality (Project number WOT-03-001-069)

Conflict of Interest: None to disclose

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S107 EFFECT OF SURFACE PROFILE OF SUPERHYDROPHOBIC CARBON SOOT COATINGS ON THE VIABILITY OF HUMAN SPERMATOZOA UPON SLOW FREEZING

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Subjecting droplets of human semen to slow freezing via superhydrophobic carbon soot coatings is an innovative cryopreservation technology enabling outward osmosis, balanced dehydration of the cells and mitigated intracellular icing. This leads to higher survival rates and better recovery of the initial sperm motility compared to the cases of freezing in hydrophilic cryovials. Herein, we report pioneering experimental results concerning the effect of surface profile of five non-wettable soot coatings, differing by morphology, chemistry, porosity and roughness, on the viability and motility of human spermatozoa following slow freezing. Initially, 250 μL of liquefied semen of nine patients are mixed in a cryovial with cryoprotectant SAGE in a ratio 1:1 vol %. After 3 min equilibration, the sperm motility and concentration are measured using CASA. Afterwards, 50 μL droplets are carefully dispensed on an uncoated glass slide (control sample) and five soot-coated glass substrates, preliminary mounted in a rectangular Plexiglas cryochamber equipped with a copper-based bottom. To it is soldered a copper rod immersed in liquid nitrogen that cools the entire system with a rate of $\sim 2 \pm 0.2$ $^{\circ}\text{C}/\text{min}$. The samples are cooled to -20 $^{\circ}\text{C}$ and stored in a container with liquid nitrogen prior to being removed one at a time and subjected to thawing at room temperature. Then, the sperm viability and motility are assessed again and the careful analysis of the obtained dataset shows that nearly 100% recovery of the initial kinetic parameters (progressively motility and curvilinear velocity) is achievable with two of five carbon soot coatings – to the best of our knowledge, a result that has not been reported so far. Moreover, correlations among the freezing/thawing rates of semen droplets on different superhydrophobic soot coatings and the post-thaw sperm viability and motility are identified, thus, revealing strong potential for

subtle adjustment of the cryopreservation protocols based on the individual peculiarities of each patient.

Funding: This research was performed thanks to the financial support of Bulgarian National Science Fund under grant № KP-06-H57/1/15.11.2021

Conflict of Interest: None to disclose

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S108 POST-THAW MURRAH BUFFALO (*BUBALUS BUBALIS*) SPERM TRAITS USING ICE RECRYSTALLIZATION INHIBITORS

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Ice recrystallization inhibitors (IRIs): Sodium-dodecyl-sulphate(SDS), n-octyl- β -d-glucopyranoside(NOG) and 4-methoxy-phenyl- β -d-glucopyranoside(4MPG) were added to Tris-Egg-Yolk-Glycerol(TEYG) semen extender for cryopreservation of buffalo bull semen with hypothesis that post-thaw semen kinetic and functional parameters will improve. In pilot study: 6 semen ejaculates (2 per bull); experiment 2: 27 semen ejaculates (9 per bull) and experiment 3: 3 semen ejaculates (one bull) were evaluated. SDS (2,1,0.5,0.25,0.15,0.125,0.0625 and 0.0312%),

(33,22,11,5.5,2.5,0.75,0.5,0.25,0.125,0.0625 ,0.03125 and 0.0156mM), and, 4-MPG(220,165,110,55,50,25,12.5,6.25,3.125 ,1.56 and 0.78mM) in TEGY were used to assess post-thaw Total Motile (TM) and Rapid Progressive (RP) sperm. In experiment 2 best IRI concentrations from pilot study were used to evaluate post-thaw sperm attributes. In experiment 3 best concentration of IRIs along with different freezing protocols was used to assess post-thaw sperm motility and viability traits. Superior concentrations of IRIs in TEGY semen extender were: SDS 0.125, 0.0624, and 0.0312 %; 4-MPG 3.125, 1.56, and 0.78 mM; NOG 0.0624, 0.0312, and 0.0156 mM. Improvement ($p<0.05$) in post-thaw buffalo bull semen seen with SDS 0.125 %, NOG 0.0624 mM and 4-MPG 3.125 mM in TEGY extender. SDS 0.125% in TEGY extender resulted in the best sperm attributes of all the IRIs and concentrations used. Freezing protocols P24 (freezing rate of $-30^{\circ}\text{Cmin}^{-1}$ from 4°C to -15°C ; $-40^{\circ}\text{Cmin}^{-1}$ from -15°C to -60°C ; and $-50^{\circ}\text{Cmin}^{-1}$ from -60°C to -140°C ; plunged in LN2 at -196°C) and P26 (freezing rate of $-30^{\circ}\text{Cmin}^{-1}$ from 4°C to -15°C ; $-60^{\circ}\text{Cmin}^{-1}$ from -15°C to -60°C ; and $-50^{\circ}\text{Cmin}^{-1}$ from -60°C to -140°C ; plunged in LN2 at -196°C) along with SDS 0.125% resulted in similar post-thaw sperm attributes. Buffalo bull semen cryopreserved in SDS 0.125% and P25 (freezing rate of $-30^{\circ}\text{Cmin}^{-1}$ from 4°C to -15°C ; $-50^{\circ}\text{Cmin}^{-1}$ from -15°C to -60°C ; and $-50^{\circ}\text{Cmin}^{-1}$ from -60°C to -140°C ; plunged in LN2 at -196°C) protocol resulted in higher ($p<0.05$) sperm attributes.

Funding: All India Coordinated Research Project (AICRP) on Nutritional and Physiological Interventions for Enhancing Reproductive Performance in Animals- Indian Council of Agricultural Research (ICAR)- National Institute of Animal

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Conflict of Interest: None to disclose

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S109 EXPRESSION AND IMMUNOLOCALIZATION OF AQP3 AND AQP11 IN BOVINE SPERM

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Osmotic imbalance is one of the challenges that a sperm confronts during the process of cryopreservation that contributes to a significant reduction in post-thaw sperm viability. Aquaporins are transmembrane proteins that play a key role in maintaining the osmotic balance by efficient transport of water and solutes across the sperm plasma membrane. The present study is aimed to determine the sperm cell population distribution of the relative abundance of AQP3 and AQP11, and AQP localization patterns in the bovine ejaculates with varying post-thaw viability. Holstein bull ejaculates (n=22) were analyzed through CASA for post-thaw motility and the relative abundance of both aquaporins in individual sperm cells was detected via flow cytometry. A regression analysis indicated a positive relation between motility and the relative abundance of both proteins. The relative abundance of AQP3 ($r^2=0.31$, $F(1,20) = 9.37$, $p=0.006$) was found to be higher as compared to AQP11 ($r^2=0.23$, $F(1,20) = 6.03$, $p=0.023$) in the ejaculates. The immunocytochemistry revealed distinct localization patterns for these proteins where AQP was primarily localized in the acrosomal region and the midpiece in contrast with AQP11 which was stained in the entire tail of the sperm. No difference in localization pattern was

observed in the ejaculates with low or high post-thaw motility. Our findings are important as it is the first study to provide insight into the relative abundance of aquaporins in live bull spermatozoa. Based on these findings we conclude that AQP3 and AQP11 may be used as markers of bull sperm cryotolerance. Further understanding of the underlying mechanisms of aquaporin function in sperm membrane physiology will contribute to optimal media design for improved cryopreservation of bovine semen.

Funding: NSERC, Semex Canada

Conflict of Interest: None to disclose

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S110 DETERMINATION OF THE EFFECT OF EPIGALLOCATECHIN GALLATE ON OXIDATIVE STRESS, APOPTOSIS AND SPERM QUALITY IN RABBIT SEMEN EXTENDER

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The aim of this study was to determine the effect of epigallocatechin gallate (EGCG) on semen quality after freeze-thaw in rabbit sperm cryopreservation. For this purpose, semen was collected from six male New Zealand rabbits. After the quality of the collected semen was checked macroscopically and microscopically, it was diluted 1:1 to be pooled. The semen sample was then divided into 5 equal parts as control, 25 μ M EGCG, 50 μ M EGCG, 100 μ M EGCG and 200 μ M EGCG. After the semen samples were cooled and filled into 0.25 mL straws, they were frozen with a programmable semen freezing device and stored in a liquid nitrogen tank. Frozen straws were thawed at 37° C for 30 seconds. Total motility, progressive motility, and rapid and medium speed spermatozoon rates were significantly higher in the 50 μ M group after freezing and thawing than in the other groups ($p<0.05$). The static sperm ratio in the 50 μ M group was found to be lower than in the other groups ($p<0.05$). When the flow cytometry results were examined, there was no statistical difference between the groups in terms of dead and live sperm ratio. The rate of dead sperm with acrosome damage, was found to be lowest in the 50 μ M group ($p<0.05$). The 50 μ M and 100 μ M groups had a high mitochondrial membrane potential spermatozoon ratio, while the other groups had a statistical difference ($p<0.05$). The lowest rate of apoptosis rate was seen in the 25 μ M and 50 μ M groups, while no statistical difference was found between the other groups. The ROS ratio was the highest in the control group, while it decreased significantly in all EGCG supplement groups. Finally, adding EGCG to rabbit semen extender improved the quality of the semen. In addition, the fact that the moderate concentration of EGCG used in our study gives more positive results than higher or lower concentrations can be basically explained by the toxic or insufficient effect of

high or low doses when the results of other studies with additive substances are evaluated.

Funding: This study was supported by Kahramanmaraş Istiklal University Scientific Research Projects Coordination Unit. Project Number: 2021/2-2

Conflict of Interest: None to disclose

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POSTER PRESENTATION

P1 THE FRAGILITY OF VITRIFIED GLASSES CORRELATES WITH THEIR PROTECTIVE CAPACITY DURING DESICCATION

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How organisms survive extreme water loss is one of the enduring mysteries of organismal physiology. Increases in intracellular viscosity, leading to the formation of a vitrified (glassy) state has been implicated as being necessary, but not sufficient, for conferring desiccation tolerance. This implies that there are properties of vitrified systems that make them more or less protective. What properties of a vitrified system make it desiccation-tolerant or -sensitive are unknown. We begin by examining key properties of in vitro vitrified systems composed of a desiccation protective disaccharide (maltose, sucrose, or trehalose) and glycerol; including micro-crystallization, glass transition temperature, residual water content, and 'glass fragility.' We find that different weight-by-weight mixtures of these two desiccation protective molecules result in vitrified material with distinct material properties. We test the capacity of these mixtures to preserve the function of a desiccation-sensitive enzyme, lactate dehydrogenase, during drying and find that the material properties glass transition temperature and glass fragility correlated best with increased protective capacity. We go on to next assess whether our in vitro observations are corroborated in vivo using

organisms with comparable desiccation tolerant and sensitive life stages. Utilizing desiccation tolerant and sensitive life-stages from a nematode worm, fungus, brine shrimp, and plant, we find that while many properties did not correlate with survival, the glass fragility of each organism was increased in the sensitive life stage relative to the tolerant life stage. These data suggest that the fragility of a vitrified material is a property influencing its ability to preserve biological material during drying both in vitro and in vivo. Understanding the underpinnings of desiccation tolerance not only advances our understanding of organismal physiology but provides avenues for the development of technologies to reduce our dependence on the cold-chain as well as for increasing food security.

Funding: This work was supported by DARPA grant W922NF-20-2-0137 to TCB. This work was partially funded through a fellowship to JFR administered by Wyoming NASA EPSCoR (NASA Grant #80NSSC19M0061). JFR is supported by the USDA National Institute of Food and Agriculture, Hatch project #1012152. The authors gratefully acknowledge financial support from the NSF (CHE 0619920 to NA and IntBio 2128069 to TCB) and the Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health (Grant # 2P20GM103432)

Conflict of Interest: None to disclose

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P2 ANOTHER GIFT FROM NATURE TO HUMAN BEINGS: HIMALAYAN ANTIFREEZE PROTEIN THAT ACTS AS A CRYOPROTECTANT

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Red blood cell (RBC) transfusion is a life-saving strategy. However, the shelf-life of RBCs is 42 days to the maximum. Even after decades of research, the strategies for long-term cryopreservation method of RBCs are not applicable. Since the past, glycerol is used as the cryoprotectant for preserving RBCs but it enters the cell and lead to hemolysis that must be balanced against their cryoprotective effect for clinical applications. Antifreeze proteins are astonishing proteins in nature that provide solution to the cryopreservation problems in food and medical industry. The utilization of AFPs for RBCs cryopreservation is limited because of shaping of ice crystals by hyperactive AFPs. However, plant-AFPs are weak AFPs and they can be utilized for the purpose. Furthermore, the isolation of non cell-penetrating antifreeze peptide can be the solution to the problem and prove to be a boon to mankind. Here, we have purified an AFP from Himalayan barley that survives the cold desert conditions of Himalayas. A protein fraction with ice recrystallization inhibitory properties was isolated by ammonium sulphate precipitation and chromatography. The polypeptides were identified by MALDI-TOF/TOF and the antifreeze protein was cloned, expressed and purified from E.coli cells. The purified AFP was used as a cryoprotectant for RBCs. The RBCs showed 90% cell recovery after cryopreservation with liquid nitrogen and rapid thawing at 42 °C It was comparable to the Me₂SO based cryopreservation and therefore, provides an opportunity to study plant based AFPs for cellular cryopreservation. This also leads to the

gateway to synthesize AFP based biomaterials for cryopreservation.

Funding: Department of Health Research, India

Conflict of Interest: None to disclose

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P3 MODEL-GUIDED DESIGN AND OPTIMIZATION OF CPA PERFUSION PROTOCOLS FOR WHOLE ORGAN CRYOPRESERVATION

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Vitrification preserves organs indefinitely, but only after perfusion loading of high (8-9M) and potentially toxic concentrations of cryoprotective agents (CPAs). Historically, development of CPA perfusion loading protocols that limit toxicity has taken years or decades. Here, we combine a two-compartment Krogh cylinder model with a toxicity cost function to theoretically optimize this process, and we have used loading of VMP in a rat kidney as our system for physical and biological validation. First, we derived the parameters in the transport model and measured their values using perfusion, yielding $V_b = 86.0\%$ ($r_a = 3.86 \mu\text{m}$), $L_p = 1.5 \times 10^{-14} \text{ m}^3/(\text{N}\cdot\text{s})$, $\omega = 7.0 \times 10^{-13} \text{ mol}/(\text{N}\cdot\text{s})$, and $\sigma = 0.10$. Next, we measured

the toxicity cost function model ($J = \int_0^t \beta C^\alpha dt$) where J is the non-dimensional toxicity, C is the concentration (M), and the parameters $\alpha = 3.12$ and $\beta = 9.39 \times 10^{-6}$ [1/(min· M)] are found by fitting. Combining these models, we obtained a kidney loading protocol resulting in equivalent CPA loading (sufficient for vitrification) with shorter overall exposure (25 min less = 19% less) at the same temperatures as a gold standard protocol (Fahy et al. Cryobiology 2004). We tested the new CPA loading protocol alongside the gold standard protocol, with both groups n = 6. Physically, all kidneys from both groups were able to achieve vitrification (verified visually and by micro-CT) after cooling at a mean rate of 20.5 °C/min in a controlled rate freezer. Biological assessments on three CPA loaded and unloaded (not vitrified) kidneys from each group were assessed by transplant and acute recovery. Measures included creatinine, potassium, sodium, and chlorine; all sub-groups were within viable ranges. These physical and biological data validate our model for optimization of CPA loading in the kidney and open the possibility of extending this approach to other organs.

Funding: NIH R01DK117425, NIH R01DK132211, NIH R01HL135046, NSF EEC 1941543, and a generous gift from the Biostasis Research Institute.

Conflict of Interest: None to disclose

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P4 CRYOPRESERVATION PROCESS CONSIDERATIONS FOR ALLOGENEIC CELL THERAPIES

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The production of allogeneic cell therapies for patients has different requirements for product formulation, freezing, storage, and transport when compared to the methods currently employed for autologous products. Changes to automation must occur to accommodate hundreds to thousands of doses of cellular material. Process choices for formulation, including limitations imposed around pre-freeze incubation, will influence the selection of automated filling devices and final product containers. These choices have further downstream impacts to controlled rate freezing configurations and programs to limit within-lot and lot-to-lot variability. Additional considerations for liquid nitrogen storage systems, shipping, packaging, and processes for accessing samples while maintaining appropriate storage temperatures must be assessed and well designed. Strategies being employed to address these process development areas for allogeneic therapies will be discussed.

Funding: Not applicable

Conflict of Interest: Authors are employees of and have stock ownership in Bristol Myers Squibb.

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P5 PROTOCOLS FOR THE SEMINAL CRYOPRESERVATION OF SOUTH AMERICAN CATFISHES – CURRENT STATUS

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A little over forty years ago, investigations with seminal cryopreservation of South American fish began, being the first record published in a scientific journal with the catfish *Rhamdia quelen* in 1981. Even though *R. quelen* is only the fifth species of South American fish with the largest number of published papers on this topic, it currently has the most efficient developed protocol for these fishes. In a recent review, we analyzed over 150 articles that were in the Electronic searches were performed in the CAB Direct (Springer Nature, 1973–2023), PubMed (MEDLINE, 1940–2023), Scopus (Elsevier, 1960–2023) and ISI Web of Science (Thomson Reuters, 1900–2023). We excluded those that were repeated and that were not South American catfish species and that did not include the topic of cryopreservation. In this summary, only 23 articles were considered and we found and we found that only eight species of South American catfishes compose any cryopreservation study, which does not represent 1% of more than 1,100 species cataloged in South American rivers. In all analyzed papers, the main motivation for research was the potential of the species for

Brazilian aquaculture and circumstantially related to the importance of a certain species in the Brazilian scenario of fish production. For example, when we look at the period in which the only two studies with *P. fasciatus* and *P. corruscans* took place, in the early 2000s, these species were used for the production of catfish hybrids in Brazil. From 2010, *L. marmoratus* became the main species used for the production of catfish hybrids in Brazil, and consequently, two articles were recently published, in 2019-20. Regarding the most investigated internal cryoprotectants in protocols with South American catfishes, methanol is present in about 70% of the published articles and same value with powdered milk, when the external cryoprotectant most used was observed. Sperm motility is the main qualitative variable analyzed, sometimes performed by objective analysis (CASA) (52.4%) and by subjective techniques (47.6%). We understand the need to investigate a larger number of South American catfish species, especially those that are more vulnerable in the wild, as well as the need for standardization and the need for precise qualitative validations for the development of efficient and standardized cryopreservation protocols.

Funding: Not applicable

Conflict of Interest: None to disclose

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P6 LOW TOXIC ZWITTERIONS AS EMERGING CRYOPROTECTANTS

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Dimethyl sulfoxide (Me₂SO) is a typical cryoprotectant but has cytotoxicity and causes DNA methylation and protein denaturation. We have proposed a zwitterion (OE₂imC₃C) as an alternative cryoprotectant to Me₂SO. Since zwitterions have both positive and negative charges in the same molecule, they interact strongly with water molecules and inhibit ice crystal formation. They are also less toxic than Me₂SO because they do not penetrate the membrane and thus do not affect the intracellular domain. Therefore, zwitterions have a potential to be better cryoprotectants than Me₂SO. However, we have found some cell lines are difficult to cryopreserve with only OE₂imC₃C. It is known that the excessive osmotic dehydration produced by the concentration of salts during freezing is lethal to cells. After freezing an aqueous solution containing 10 wt% OE₂imC₃C, the solution was concentrated to approximately 70 wt%, corresponding to 3 mol/L. As mentioned above, OE₂imC₃C does not penetrate the cell membrane like salts. Therefore, cells that are difficult to cryopreserve with OE₂imC₃C could not withstand the excessive osmotic dehydration caused by high concentrated OE₂imC₃C. A more suitable zwitterion should have a low molar concentration. We synthesized a zwitterion with a high molecular weight to achieve a lower molar concentration at the same mass concentration. The high molecular weight zwitterion solution was used to cryopreserve

human kidney cells, which are challenging to cryopreserve with the OE₂imC₃C solutions. It gave a higher number of living cells than OE₂imC₃C after cryopreservation. The optimal concentration was 15 wt%.

Funding: This study was also partly supported by KAKENHI (18K14281 from the Japan Society for the Promotion of Science), Leading Initiative for Excellent Young Researchers (for K.K., from Ministry of Education, Culture, Sports, Science and Technology-Japan), ACT-X (for K.K., JPMJAX1915 from Japan Science and Technology Agency), A-STEP (from Japan Science and Technology Agency), Kanazawa University SAKIGAKE project 2020, and NIBB Collaborative Research Program (21-608), WISE Program for Nano-Precision Medicine, Science, and Technology of Kanazawa University by MEXT.

Conflict of Interest: None to disclose

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P7 IMPROVING STORAGE STABILITY OF CRYOPRESERVED STALLION SPERM FOR STORAGE AT ELEVATED SUBZERO TEMPERATURES

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In equine breeding, cryopreserved semen is routinely stored and transported in liquid nitrogen. This requires special containers, involves safety and maintenance issues, and is associated with high costs. Furthermore, liquid nitrogen may not be easily available in remote locations. Ultralow mechanical freezers and Styrofoam boxes with dry ice can be used for storage and transport of cryopreserved biospecimens. However, frozen storage above liquid nitrogen temperatures (LN₂, -196°C) may negatively affect shelf-life and cryosurvival. In this study, we determined critical temperatures for storage of cryopreserved stallion sperm. We evaluated: (1) effects of cooling samples to different subzero temperatures prior to storing in LN₂, (2) stability at different storage temperatures (i.e., in LN₂, dry ice, -80°C and -20°C freezers, 4°C refrigerator after thawing), and (3) sperm cryosurvival during prolonged storage in evaporating dry ice with gradually increasing sample temperatures (-70°C and higher). Furthermore, we determined how added synthetic polymers (PVP40, Ficoll70) modulate physical properties of preservation formulations and if this can be used to improve stability of cryopreserved sperm. This was done through microscopic analysis of ice recrystallization kinetics in thin frozen specimens, determination of phase transition temperatures, and infrared spectral analysis of hydrogen bonding interactions. Sperm motility and membrane intactness were taken as measure of cryosurvival, and an artificial insemination trial was performed to confirm fertilizing capacity. We found that adding PVP40 or Ficoll70 to formulations containing glycerol reduced ice crystal sizes and growth during annealing. Post-thaw sperm viability data indicated that samples need to be cooled below -40°C before they can be safely plunged and stored in LN₂, and for maintaining viability and fertility during

transport in dry ice, samples should not be allowed to warm up above -50°C. Addition of PVP40 and Ficoll70 was found to increase sperm cryosurvival, especially under conditions where ice (re)crystallization may occur.

Funding: Not applicable

Conflict of Interest: None to disclose

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P8 BETAIN-MEDIATED (UN)LOADING OF MEMBRANE IMPERMEABLE TREHALOSE FOR BLOOD PRESERVATION

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Red blood cells (RBCs) obtained from voluntary donors are stored as densely packed cell units in blood banks. Under normal circumstances, blood banks can easily supply local hospitals with sufficient quantities of blood. However, in remote locations or battlefields that lack a proper blood bank infrastructure, blood transfusions are often performed using untested blood donations, which has been identified as a major health issue by military organizations worldwide. In this study, we plan to develop cryopreservation methods for red blood cells that; (1) require minimal washing, (2) allow sample storage at elevated temperatures, and (3) can also be used for subsequent freeze-drying. This can be achieved using non-reducing sugars (trehalose or sucrose) in

combination with polymers. Our approach was to load RBCs with trehalose (or sucrose) using a combination of mild osmotic shock, fluid phase endocytosis, and freezing-induced uptake. Furthermore, it was tested if betaine, which rapidly moves across RBC membranes, can reduce osmotic damage during sugar loading and unloading. Incubating RBCs in sugar solutions resulted in intracytoplasmic sugar concentrations up to 50 mM with less than 20% hemolysis, which coincided with cell loss in a concentration and time dependent manner. Intracellular sugar concentrations post-thaw were found to be much higher and approached those of the extracellular sugar concentration. Freezing disturbs the membrane permeability barrier function of the cells allowing sugars to pass the membrane. Without pretreatment and additional protective compounds, the cell recovery (up to 60 %) was found to be highest when high cooling rates were used, i.e., directly plunging into LN₂. Decreasing the freezing rate leads to reduced cell recovery rates, despite that this results in higher intracytoplasmic sugar concentrations. Addition of betaine was found to reduce hemolysis during sugar unloading after thawing, but did not reduce hemolysis during sugar loading.

Funding: HEMOFORCE

(E/U2ED/MD010/LF551, Deutsche Bundeswehr)

Conflict of Interest: None to disclose.

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P9 NUMERICAL SIMULATIONS OF DROPLETS IMPACTING ON A COLD SOLID SURFACE

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Solid surface vitrification (SSV) is a type of cryopreservation that is done by dropping small droplets of a sample (e.g., cells in a CPA solution) onto a cold metal surface. In this study, numerical simulations of SSV were conducted by modeling the cooling behavior of droplets of CPA solutions. It was assumed that deposited droplets attain a cylindrical bottom part and half-ellipsoidal shaped upper part. The dimensions of the droplets (20 and 50 microliter) were derived from actual measurements where the heights of the droplets were estimated by analyzing side view images and diameters from top view images of droplets deposited on a copper block pre-cooled with liquid nitrogen. The elliptical surface of the droplet was defined using the r- and z-direction of the principal semi-axes of the ellipsoid. Material properties for heat transfer simulations including density, heat capacity and thermal conductivity were obtained from the literature and extrapolated using polynomial fitting. The impact of CPA type (glycerol and Me₂SO), CPA concentration, and droplet size on the cooling rate was simulated at different CPA mass fractions at temperatures ranging from -190 to -25 degrees Celsius. The heat capacity shows a peak during the water to ice phase transition, which gradually diminishes with increasing CPA concentration, whereas the thermal conductivity decreases with increasing CPA concentration. The cooling rate in the center of a droplet slows down by several seconds when the CPA concentration

is increased from 30 to 60% (v/v). This study provides valuable insights into the cooling behavior of droplets of CPA solutions subjected to SSV that can be used to optimize cryopreservation outcome.

Funding: Grant WO1735/6-2 and SI1462/4-2 of the German Research Foundation (DFG: Deutsche Forschungsgemeinschaft)

Conflict of Interest: None to disclose

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P10 EFFECTS OF CRYOPROTECTANT EXPOSURE ON THE MORTALITY AND BEHAVIOR OF ZEBRAFISH EMBRYOS

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Cryopreservation of mammalian embryos is considered a successful tool in assisted reproduction. However, recent concerns address the possible detrimental effects of cryopreservation of oocytes and/or embryos on human offspring. Additional research on the potential toxic effects of cryoprotectants (CPA) is required. We adopted the zebrafish-embryo model to study the direct effects of CPA exposure on embryo survival and development.

Embryos were selected within 1h after fertilization and randomly allocated to 4 groups of 50 embryos: 1) negative control in dilution water, 2) positive control in 4mg/l 3,4-dichloroaniline, 3) 10 min 4M glycerol, and 4) 10 min 4M propylene glycol. Test group embryos were transferred to dilution water following exposure. Mortality was scored daily and at 120hpf the activity of 32 embryos from the negative control and test

groups was analyzed using a Viewpoint ZebraBox activity tracker. Three replicates were performed. Mann Whitney U-tests were performed in Rstudio to detect significant differences in the movement patterns.

No significant differences in mortality were observed between the negative control and both test groups, which all 3 differed significantly from the positive control. Embryos exposed to propylene glycol showed a significantly longer duration of inactivity compared to the negative control. Additionally, they made significantly less turns with an angle between 90° and 180°. Glycerol exposed embryos were inactive for a significantly longer period and counted less slow movements, hence they swam a significantly shorter distance and had a lower overall swimming velocity compared to the negative control. Moreover, the glycerol exposed group counted fewer clockwise turns between 45° and 90° compared to the negative control.

In conclusion, our data suggest that the embryos exposed to propylene glycol and glycerol were significantly less active. Furthermore, the significant alteration in turning patterns might suggest a minor neurotoxic effect of cryoprotectant exposure that warrants additional research.

Funding: Not applicable

Conflict of Interest: None to disclose

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P11 NUTRISTOR, A NOVEL CHEMICALLY DEFINED SOLUTION FOR SHORT-TERM COLD STORAGE OF CELLS

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Cryopreservation is a vital technology, supporting various cell-based applications, that provides stable and safe extended cell storage. Long-term storage of cells usually done by deep-freezing the cells in a suitable cryopreservation solution followed by thawing the cells when required. The freeze-thaw cycles may lead to negative impact on cells as well as yield loss. In addition, most of the cryopreservation solutions are composed of permeable Cryoprotective Agents (CPAs) that are toxic to the cells (e.g., Me₂SO). Hence careful handling should be done during the process of freezing and thawing. For Advance Therapy (AT) applications there is an advantage to avoid the freezing step and to use the cells without exposure to permeable toxic CPAs. Short-term storage of cells at 2-8°C can be utilized for stable shipment of cell-based products (e.g., cells and organs) for therapeutic applications. NutriStor cold storage solution is a unique Animal Component-Free (ACF), Protein-Free (PF), salt-free bio-preservation solution, that enables short-term (several days) preservation of Human Mesenchymal Stem Cells (hMSC) at 2-8°C, as alternative to a freezing process. NutriStor enables the cold storage of hMSC while maintaining high cell's viability and yield as well as preserving cell's identity and characteristics, including the multi lineage differentiation potential, self-renewal, immunophenotyping, proliferation capacity and genomic stability. Preservation of cellular viability, recovery, and characteristics after storage, is critical and essential for quality-assured cells and clinical outcome.

Funding: Not applicable

Conflict of Interest: None to disclose

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P12 A NOVEL ME₂SO FREE CRYOPRESERVATION SOLUTION DESIGNED FOR ADVANCED CELL-BASED THERAPY APPLICATIONS

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The recent surge in cell-based therapeutic approaches has led to the need for safer, more regulated ancillary materials. Cryopreservation is a crucial step for long term storage of cell-based products and "off-the-shelf" therapy approaches. To date, the use of Me₂SO or other toxic permeable cryoprotective agents (CPAs), such as Ethylene Glycol is common practice. These CPAs can impact the quality, safety, and efficacy of the cellular product and clinical outcome. The development of a defined, Me₂SO free cryopreservation solution holds a unique opportunity to advance the widespread implementation of safe and efficient cell-based therapies. The current study presents the development of a novel, chemically defined, Me₂SO free, animal component free (ACF), cryopreservation solution. In this study, human mesenchymal stem cells were tested and evaluated after being frozen in various cryopreservation solutions, with and without Me₂SO. The results of cell viability after thawing, growth recovery and cell characteristics, are presented and demonstrate the feasibility of Me₂SO free cryopreservation. In addition, this study shows the importance of combining different testing methods for the assessment of the cells after freezing and thawing. Rely on the results of cell's viability alone, although this is the common assay

today, is not sufficient since cell's viability is not necessarily correlative to the ability of cells to grow and recover. For cell-based therapies and clinical applications, careful selection of appropriate freezing solution along with representative evaluation assays for the cells before use, are critical phases to ensure product quality and clinical outcome.

Funding: Not applicable

Conflict of Interest: None to disclose

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P13 COMBINING CRYOABLATION AND IMMUNE CHECKPOINT INHIBITOR FOR IMPROVED CANCER CONTROL

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Accumulating evidence suggests that focal ablation therapy combined with immunotherapies (e.g., immune checkpoint inhibitor, ICI) can serve as a potent in situ tumor vaccination approach by generating a systemic anti-cancer immunity that leads to control of local recurrence and metastasis. Here, we examine the augmented effects of cryoablation with anti-PD-1 ICI in a MC-38 murine colon carcinoma model. MC-38 cells were cultured and injected in the flank of C57BL/6 mice. Tumors grew to 6-8 mm in the largest dimension and were treated (on day 0) by a customized Argon Joule-Thomson cryoablation needle (1.2 mm in diameter), followed by IP injection of anti-PD-1 antibodies at 100 µg on day 1, 3 and 5. For primary tumor growth delay, the tumors

were treated with a sublethal dose until the edge of the tumor cooled to 0°C. For secondary tumor challenge, primary tumors were ablated with a lethal dose by freezing the edge of tumor to -20°C. Secondary tumors were inoculated in the contralateral flank on day 13. In both studies, tumor growth was monitored for two months after inoculation. In MC-38 model, tumor presented very limited response to anti-PD-1 monotherapy when tumor was large. However, the combination treatment of cryoablation and anti-PD-1 did slow tumor growth and improve overall survival as compared to both monotherapies (1-month survival rates: Cryo+anti-PD-1: 14.3%; Cryo: 0; Anti-PD-1: 0). This is correlated with higher frequency of CD8+ tumor infiltrating lymphocytes in combinatory therapy group. In the tumor rechallenge assessment, the incorporation of anti-PD-1 into cryoablation therapy increased the proportion of tumor-free mice from 52.2% with cryoablation alone to 57.1% with combination treatment. These preliminary data indicate that the cryoablation combined with ICI may generate durable antitumor response, prolong overall survival and induce long-term immune protection.

Funding: Boston Scientific Corporation

Conflict of Interest: None to disclose

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P14 EFFECTS OF SACCHARIDES ON POST THAW SPERM QUALITY OF PACIFIC ABALONE *HALIOTIS DISCUS* HANNAI

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Pacific abalone *Haliotis discus* Hannai is a high-priced and high-demandable seafood

because it contains bioactive molecules with antioxidant and anticancer activities. The present study aimed to determine whether saccharides could improve the sperm cryopreservation outcomes as assessed by post-thaw motility, acrosome integrity (AI), plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), DNA integrity, ATP content, fertility, and hatchability. Different types of saccharides namely Glucose (G), sucrose (S), trehalose (T) were separately mixed with either 8% dimethyl sulfoxide (Me₂SO), 8% ethylene glycol (EG), 6% propylene glycol (PG), 2% glycerol (GLY), or 2% methanol (MeOH) at a final concentration of 1, 2, 3, 4, or 5%. The present study reports positive influences of saccharides on post-thaw sperm quality including motility, AI, PMI, MMP, ATP content and fertilization potential. Saccharides dramatically improved the DNA integrity and ATP content of post-thaw sperm, leading to improved fertilization and hatching capacity. Specifically, sperm cryopreserved using 3% sucrose combined with 8% dimethyl sulfoxide (Me₂SO) showed improved post-thaw sperm quality (motility: $58.4 \pm 2.9\%$, AI: $57.1 \pm 3.2\%$, PMI: $65.3 \pm 3.3\%$, and MMP: $59.1 \pm 3.2\%$), ATP content (48.4 ± 1.8 nmol/mL) and DNA integrity ($2.09 \pm 0.20\%$) than sperm cryopreserved with other types of cryoprotectant. The present study suggests that 3% S + 8% Me₂SO is a suitable cryoprotectant for sperm cryopreservation and for molecular conservation of this valuable species.

Funding: This research was part of the project entitled “Development of technology for abalone aquaculture using sperm cryopreservation (grant No. 20180375)” funded by the Ministry of Oceans and Fisheries, Korea.

Conflict of Interest: None to disclose

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P15 EFFECT OF MAGNETIC NANOPARTICLES OBTAINED BY GEL-COMBUSTION IN ICE CRYSTALLIZATION AND RECRYSTALLIZATION

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Cryopreservation is a valuable technique for storing biological samples long-term, but the formation and recrystallization of ice can harm the viability of the tissues. To face this issue, researchers have utilized magnetic nanoparticles (MNPs) to perfuse organs such as kidneys and liver. Employing alternating magnetic fields during thawing enables a quicker and more uniform rewarming process, minimizing the damage that slow and nonuniform rewarming cause. This study aimed to synthesize magnetic nanoparticles via the sol-gel combustion method and mix them with four different cryoprotective solutions (CPAs) to analyze their effect on nucleation temperature, ice crystal formation, and recrystallization. Two types of MNPs were obtained by sol-gel combustion method employing iron nitrate as a precursor and 6-aminohexanoic acid as a combustion agent under a controlled and

non-controlled atmosphere. The resulting MNPs were morphological, magnetic, and chemical characterized. After 1 mg/ml of MNPs was mixed with four different CPAs (10% Me₂SO /20% FBS or 10% Ethylene glycol/20% FBS both with and without 0.3M sucrose) and analyzed under differential scanning calorimetry and cryo-microscopy. The nucleation temperature was identified with DSC, and parameters like ice crystal formation and recrystallization with cryomicroscopy. Results showed no significant differences in the nucleation temperature or melting process, as long as heat transfer occurs through conduction (20K/min). In addition, no visible differences in ice crystal formation and recrystallization were observed when comparing CPA solutions with and without MNP. This suggests that MNP can be successfully integrated into CPA solutions without negative effects on cryopreservation, and the same MNPs concentration has been reported as not cytotoxic. Future experiments are necessary where the thawing is performed by means of an alternating magnetic field where warming rates of 200K/min can be reached.

Funding: This work was supported by the German Academic Exchange Service (DAAD, project number 91725466), Caroline Herschel Program Leibniz University Hannover and the German Federal Ministry of Education and Research project with Colombia (BMBF, 01DN21002).

Conflict of Interest: None to disclose.

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P16 CRYOPRESERVATION OF WHOLE BLOOD BY UTILIZING A MICROFLUIDIC SYSTEM

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Blood stored with commercial stabilizers at 4°C has a maximum shelf life of up to 49 days. After that, the blood must be disposed of due to biochemical ageing processes. To ensure the highest possible recovery rate after freezing down to -196°C and thawing, anticoagulant and shelf-life prolonging substances, cryoprotectants, must be added to the blood. Their disadvantages are cytotoxicity at non-cryogenic temperatures and the osmotic stress caused by the subsequent removal of the chemicals from the blood cells. In this study, a concept for the cryopreservation of blood, by utilizing a microfluidic system, without the application of CPAs is demonstrated. The produced microfluidic system uses a channel structure and a carrier fluid (perfluorotributylamine) to promote the formation of blood droplets in small volumes, resulting in an increased surface area while reducing the volume. This is followed by pre-cooling of the droplets to about 4°C using a freezing unit. Afterwards, the separation of the carrier fluid from the blood and the complete freezing of the droplets is carried out in the modified freezing-unit. To achieve the partition, a mesh-sheathed modular chain is constructed. The cooling is provided by nitrogen vapor blown into the chamber from above to temperatures of -40°C. The results show a formation of microblood droplets in the MFS and thus an improved heat transfer. The desired results can be achieved with both freezing units and proven with experimental investigations.

Funding: Not applicable

Conflict of Interest: None to disclose

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P17 EFFECT OF SUPERCOOLING ON INTEGRITY OF ROOSTER SPERM

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Previous studies of our group showed that sub-zero temperatures in supercooled boar semen are a serious detrimental factor during boar sperm cryopreservation. Like boar semen, rooster semen is quite sensitive to freezing and thawing, but the effects of supercooling on rooster sperm cells have not yet been investigated. The present work focused on studying the effect of sub-zero temperatures in supercooled rooster semen. Four pooled semen samples (one pool per week), each from 5 randomly selected roosters (different roosters per pool) were diluted four times with ASG extender. Semen was assessed as described below. The diluted semen was supplemented with the required volume of extender containing 1.8 M DMA to achieve a final concentration of 0.6 M DMA. After one hour of incubation, the semen was packed in 0.25-ml straws and introduced into a freezing chamber at -10 °C. No ice nucleation occurred in the straws. At time points 2, 30 and 60 min, one straw per time point was removed for assessment of motility (CASA system), viability (DAPI staining) and DNA integrity (TUNEL). The results were compared with the ones obtained from the semen before adding DMA and were analyzed using the Wilcoxon's test. The differences between fresh semen versus

-10°C were not significant, but there was a trend towards lower total motility and progressive motility in supercooled semen, as the P values were around 0.07. In conclusion, supercooling did not have a severe effect on viability, motility and DNA integrity of rooster sperm.

Funding: HORIZON-MSCA-2022-PF-01-01, Project 101109983 CRYOCHICK

Conflict of Interest: None to disclose

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P18 POST-THAWING DILUTION PROLONGS FISH SPERM USE IN AQUACULTURE MEDITERRANEAN SPECIES

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Usually, fish cryopreserved sperm is used without dilution after thawing. However, post-thawing sperm dilution with species-specific extenders can potentiate the quality and prolong its use. We aimed to evaluate the quality of thawed sperm from four fish

aquaculture Mediterranean species diluted in different extenders (or not diluted as control) and maintained at 4 °C over time. European eel (*Anguilla anguilla*; n=21), gilthead seabream (*Sparus aurata*; n=12), European sea bass (*Dicentrarchus labrax*; n=12), and Senegalese sole (*Solea senegalensis*; n=13) sperm samples were cryopreserved. Aliquots of post-thawed sperm were diluted: European eel with P1 medium or P1+BSA, gilthead seabream with non-activating medium (NAM) with pH 7 or NAM with pH 7.7, European sea bass with NAM or NAM+BSA, and Senegalese sole with Mounib or NAM. For each species, an aliquot of post-thawed sperm was not diluted as control. Kinetic parameters were evaluated post-thawing: European eel and gilthead seabream (0, 24, 48, 72, and 96 h), European sea bass (0, 2.5, and 6 h), and Senegalese sole (0 and 3 h). Total motility (MOT-%), progressive motility (MOTp-%), curvilinear (VCL- μ m/s), straight line (VSL- μ m/s), and average path (VAP- μ m/s) velocities were evaluated by CASA-Mot software. Post-thawing diluted samples of European eel sperm showed higher MOT (14%) and MOTp (11%) after 96 h compared with control (MOT: 7%; MOTp: 4%), and maintained the VCL (146 μ m/s) up to 72 h. Post-thawed diluted sperm samples of gilthead seabream and European sea bass maintained the sperm quality until 48 h (MOT: 30%) and 2.5 h (MOT: 19%), respectively, having a positive effect on the sperm kinetic parameters. Post-thawing dilution had no positive effect in the case of Senegalese sole sperm. Post-thawing dilution prolonged use thawed sperm use of European eel, gilthead seabream, and European sea bass.

Funding: This study was supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and by Generalitat Valenciana (THINKINAZUL/2021/012;

THINKINAZUL/2021/024; THINKINAZUL/2021/042) to SEASPERM including the contract of FF-G. (406). WAG-L has a Margarita Salas postdoctoral contract (RD 289/2021. UAB) by the Spanish Ministry of Universities. LF has a PhD contract from Generalitat Valenciana (GRISOLIAP/2020/063). TF (141717/2019-0 and 200285/2021-1) and MPS (200452/2022-3) have fellowships from Brazilian National Council for Scientific and Technological Development (CNPq).
Conflict of Interest: None to disclose
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P19 SIMULATIONS OF CRYOBALLOON-BASED PULMONARY VEIN ISOLATIONS ELICIT SUSTAINED LOW PHRENIC NERVE TEMPERATURES

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Phrenic nerve injury is a complication seen in ablation-based pulmonary vein isolation. This susceptibility of the phrenic nerves to collateral damage is due to their anatomical relationships with the pulmonary veins (PVs). The left phrenic nerve runs posterior to the left atrium (LA) and the right phrenic nerve passes between the right PVs and the superior vena cava. Using a geometry of the phrenic nerves and LA generated from a human heart-lung bloc, we investigated how cryoballoon size and location may impact phrenic nerve temperatures, using

commercial CFD software. The LA blood volume and PVs were previously segmented. 3-matic was used to smooth surfaces and position the cryoballoon so that it fully occluded the desired PV. Fusion 360 was used to further prepare the geometry and generate a “tissue box” to surround the LA and PVs. Using Autodesk CFD, a steady-state analysis of flow was performed with boundary conditions of 50 cm/s at the PV inlets and 0 mmHg at the mitral valve annulus. Next, a transient analysis was performed where the given cryoballoon temperature was linearly decreased from 37°C at 0 seconds to -50°C at 60 seconds and maintained at -50°C until 300 seconds. Phrenic nerve temperatures were recorded every 15 seconds. The LA anatomy from this given patient reconstruction was small, allowing for the 28mm to occlude both PVs on the left and right sides. The 23mm balloon was shown to occlude both left PVs. For ablations of the left PVs, a gradual decrease in temperatures was elicited whereas ablations of the right PVs induced rapid decreases before leveling off at subzero temperatures. The results of this computational study show that for this individual’s cardiac anatomy, cryoballoon therapy applied to the right PV induced a greater risk for exposure to low temperatures during treatment than the left PV.

Funding: Not applicable

Conflict of Interest: None to disclose

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P20 OSMOTIC BEHAVIOR OF T CELLS DETERMINED BY FLOW IMAGING MICROSCOPY

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To develop cryopreservation methods for cell-based medicinal products it is of high relevance to understand cell-specific properties such as osmotically inactive cell volume (V_b) and membrane permeability to water (L_p) and cryoprotective agents (P_s). Flow-imaging microscopy (FIM) was used to determine osmotic properties of Jurkat and T cells based on changes in cell size upon addition of cryoprotective agents (CPA). Jurkat cells are a continuous human T cell line used as model for T cells. Sucrose, a cell membrane impermeable CPA, as well as dimethyl sulfoxide (Me_2SO) and glycerol, both permeable CPAs, were used. To differentiate between viable and necrotic cells Trypan blue staining was applied. Cell volume measurements after equilibrium in osmotic solutions of sucrose in phosphate buffered saline (PBS) were performed to obtain V_b . Kinetic volume measurements upon addition of cells to sucrose in PBS and to Me_2SO or glycerol in PBS were used to calculate L_p and P_s . Cell images were selected by applying several morphological filters such as diameter and pixel intensity to differentiate between viable and necrotic cells. Obtained values for V_b of viable Jurkat and viable T cells were comparable. Two subpopulations of cells for both cell types

with a different V_b value were detected. Necrotic cells showed almost no osmotic response. Kinetic volume changes were used to calculate L_p and P_s at room temperature by fitting data via MATLAB. Jurkat and T cells showed similar shrinkage upon addition to sucrose solution as water is moving out of the cells. Upon addition of cells to Me_2SO and glycerol a shrink-swell process can be observed as both CPAs permeate into cells. Permeation of Me_2SO into the cells was faster in comparison to glycerol for both cell types. Thus, FIM proved to be a valuable tool to analyze the osmotic behavior of viable cells.

Funding: Not applicable

Conflict of Interest: None to disclose

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P21 EXCEPTIONAL HIGH SALT SENSITIVITY OF THE SMALL HEAT SHOCK PROTEIN P26

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Methods of preserving biological materials in the frozen state have been well established and are necessary for research applications and pharmaceuticals. However, current long-term storage strategies still face challenges, including the cytotoxic effects of cryoprotective agents and the financial burden of maintaining ultra-low temperatures. Lyophilization presents an opportunity to mitigate obstacles associated with cold-chain logistics by allowing for sample storage at room temperature, safer handling of specimens, and the potential to extend the shelf-life of preserved biomaterials. While dry-state storage has

been implemented for relatively simple biological molecules, preserving complex systems, such as eukaryotic cells, has not been achieved except for an anhydrobiotic cell line (Pv11) derived from the sleeping chironomid (*Polypedilum vanderplanki*). Animal extremophiles able to survive desiccation upregulate repair mechanisms during drying to ameliorate damages incurred during water loss and rehydration. One exceptional repair protein in *Artemia franciscana* is p26. Unfortunately, past studies to evaluate the structure/function of p26 have been limited due to low yields when expressed in *E. coli*. We successfully enhanced the production of this protein by optimizing the gene sequence and bacterial strain used. We found that p26 is extraordinarily salt-sensitive and precipitates from solution even at modest concentrations of NaCl. Adding low KCl concentrations (50-100 mM) to the purification buffer improved soluble protein yield after transgene expression. Furthermore, protein yields after induction can be enhanced by adding sucrose or trehalose during purification. Bioinformatics revealed that p26 is exceptionally likely to undergo liquid-liquid phase separation, which may partially explain its protective mechanism in *A. franciscana* cysts. Establishing an optimal purification method will allow further exploration of the mechanism(s) by which p26 preserves biological materials and future studies will evaluate its protection of water-stress-sensitive biomolecules during lyophilization.

Funding: This work was supported by NSF award PFI-1827521 to MAM.

Conflict of Interest: None to disclose

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P22 THE SUPPLEMENTED PHASE DIAGRAM (SPD) IN

CRYOPRESERVATION: A RELAXATION ANALYSIS

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Aqueous binary solutions of cryoprotective agents (CPAs), particularly sugars, do not present equilibrium eutectic diagrams. As temperature is lowered at a given concentration, generally over 50%, the mixture becomes supersaturated, and finally reaches the glass transition temperature where it vitrifies. The Supplemented Phase Diagram (SPD) was proposed by A. P. MacKenzie in 1977 to represent both the ideally equilibrium liquidus curves, the freezing and solubility equilibrium curves, and the supersaturated regime. Experimentally, very few induced solubility data for this kind of solutions have been reported in the literature in the last 70 years due to the enhancement of the liquid viscosity. Although several authors present possible analytical forms of the solubility curve, the existence of this equilibrium description, from a purely thermodynamic point of view, may be misleading. There is no physical meaning for the existence of a thermodynamic curve that separates a thermodynamic phase from a non-equilibrium system. We propose the determination of a possible region where some of these solubility states might lie in terms of the change of the viscosity of the solution around the area where the regime turns supersaturated. The study of the viscosity in the supersaturated mixture is essential in its application in cryopreservation of biological systems.

Funding: Proyecto PAPIIT-DGAPA
IN107322

Conflict of Interest: None to disclose
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P23 TREHALOSE PREVENTS HEMOGLOBIN OXIDATION DURING LYOPHILIZATION OF RED BLOOD CELLS

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Over 10 million red blood cell (RBC) units are transfused yearly in the United States. Unfortunately, it is challenging to maintain a sufficient supply of RBCs for transfusion medicine. Blood donations must carefully match the demand for transfusions, and a reliable cold chain is required to sustain transfusable units during transport and storage. The lack of transfusable RBCs or alternative oxygen-transporting blood substitutes is the leading cause of preventable deaths in far-forward settings. We utilized lyophilization to increase the shelf-life of RBCs by dry preservation. Previous research has shown that lyophilized RBCs accumulate methemoglobin (MetHb) during lyophilization and storage. MetHb cannot bind and transport oxygen and limits the functionality of RBCs after rehydration. The scope of this study explores the mechanism by which intracellular hemoglobin oxidation occurs during lyophilization and evaluates potential avenues to mitigate the formation of MetHb. Hemoglobin oxidation was undetectable after freezing and thawing porcine RBCs or purified hemoglobin. Purified hemoglobin oxidized to a similar extent as hemoglobin in RBCs during

lyophilization. MetHb formation was detected as early as three hours into the primary-drying stage of the lyophilization process. Trehalose reduced the degree of hemoglobin oxidation in RBCs loaded intracellularly with the sugar and in lysed RBC samples supplemented with 300 mM trehalose. Finally, freeze-dried cells were lysed after rehydration, and the oxygen-binding properties of the rehydrated hemoglobin were measured. No significant difference was detectable between the P_{50} values and Hill coefficients of hemoglobin before and after lyophilization and rehydration ($p > 0.05$). Furthermore, the rehydrated hemoglobin maintained its allosteric regulation by 2,3 bisphosphoglycerate. This research indicates that hemoglobin within freeze-dried preserved RBCs can transport oxygen efficiently in the body. Future studies will focus on the mechanism by which trehalose inhibits hemoglobin oxidation.

Funding: This work was supported by a US Department of Defense CDMRP grant (W81XWH-20-1-0866) to Jonathan A. Kopechek and Michael A. Menze.

Conflict of Interest: Co-authors Michael A. Menze and Jonathan A. Kopechek have a controlling interest in a startup company focused on commercializing freeze-dried red blood cells.

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P24 GERMPLAM CONSERVATION IN INSECTS BY CRYOPRESERVATION OF GERMINAL TISSUE

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For well over 40 years, the standard and the only available germplasm conservation strategy for insects has been to cryopreserve the embryos. Currently, approximately 12-15 reports of embryo cryopreservation in various insects exist. A majority of these procedures are for dipterans or various types of fly species. Apart from the dipterans, in the case of a hymenopteran, the honey bee, the male germplasm in the form of freshly collected semen could be stored and transported at above freezing temperatures as well as could be cryopreserved for long term storage. Honey bee is the only known species of insects that allows for ejaculatory collection of semen. Similarly honey bee is the only species with a highly standardized instrumental in vivo insemination procedure outside of the mammalian species. In a series of studies, on multiple insect species, we assessed the feasibility of conventional cryopreservation of germplasm tissue as well as germ cell derivatives. The insects and tissue source includes the seminal vesicle from male honey bees (*Apis mellifera*) and the bumble bee (*Bombus impatiens*), the male mosquito (*Anopheles gambiae*) testis and seminal vesicle complex, and the seminal duplex and ovarian tissue in the monarch butterfly (*Danaus plexipus*) as well as seminal duplex in the endangered Sacramento mountains checkerspot butterfly (*Euphydryas anicia cloudcroftii*). This presentation summarizes the outcome of these endeavors which aim to broaden the conservation strategies available for insects of economic importance and those that are threatened due to habitat changes.

Funding: Not applicable

Conflict of Interest: None to disclose

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P25 ALGORITHM OPTIMIZATION OF ME₂SO-FREE CRYOPRESERVATION AND SOLUTE PARTITIONING ANALYSIS OF NK CELLS

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Many Natural Killer (NK) cell therapies are currently under investigation worldwide and effective methods of preservation are critical for clinical use of these cells. NK cell cryopreservation methods currently utilize Me₂SO which results in a loss of NK cell recovery and function post thaw. Moreover, patients receiving cells frozen in Me₂SO have reported gastrointestinal, neurological and cardiac side effects. Thus, developing methods of Me₂SO-free cryopreservation of NK cells is of great interest. To that end, a differential evolution (DE) algorithm was used to determine the optimal composition of a disaccharide, sugar alcohol and amino acid to maximize post-thaw cell recovery of NK cells frozen using controlled rate freezing at 7 C/min. The maximum post thaw recovery was 82%, which was greater than that observed with 10% Me₂SO (66%). Additional studies were performed to look at cooling rate and nucleation temperature effects for this cell type. For cells frozen using 10% Me₂SO, we found that higher cooling rates (5 C/min and 7 C/min) led to better recovery while changing the nucleation temperature did not have a significant effect on recovery. Low temperature Raman spectroscopy was used to quantify the concentration of Me₂SO in NK cells during freezing. At a cooling rate of 1 C/min, the ratio of extracellular Me₂SO concentration divided by the intracellular concentration of

Me₂SO was ~0.4 indicating that the concentration of Me₂SO inside the cell is greater than outside. In recent studies with other cell types, this type of solute partitioning was consistent with poor cell recovery. The higher concentration of Me₂SO inside the cell also suggests that a higher cooling rate is needed, which is consistent with the outcome of this investigation.

Funding: NSF TI 2042111, NIH HL154734

Conflict of Interest: None to disclose

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P26 CHARACTERIZING MULTIPHASE EUTECTIC EQUILIBRIA AT LOW-TEMPERATURE AND HIGH-PRESSURE IN SALTY AQUEOUS SYSTEMS

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All understanding of the complex thermophysical behaviors of low-temperature aqueous systems stem first from their equilibrium thermodynamics. However, many fundamental phase equilibria remain uncharacterized for aqueous systems of interest to cryobiology and planetary science. In this work, we use a simple high-throughput isochoric freezing approach to obtain first-ever measurements of the high-pressure binary (three-phase) and ternary (four-phase) eutectic coordinates for a variety of water-salt systems, with particular applications in compositional analysis of icy worlds and isochoric cryobiology. We will

provide rule-of-thumb scaling arguments for estimating the eutectic pressure-dependence of arbitrary salt solutions of different component numbers, explore fundamental limits on liquid-phase stability under different thermodynamic conditions, and discuss the implications this data has on defining high-dimensional aqueous phase diagrams.

Funding: Not applicable

Conflict of Interest: None to disclose

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P27 EFFECTS OF CRYOPRESERVATION ON NATURAL KILLER CELLS WITH AND WITHOUT ME₂SO

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Natural killer cells have potential in targeting hard to treat cancers and can be sourced from an autologous or allogeneic donor. Whether sent to a manufacturing facility for modification or stored for off the shelf use, these cells must be preserved while maintaining viability and efficacy. Me₂SO has typically been used to cryopreserve cell therapies but has multiple side effects from nausea and discomfort to cardiac arrest. Combinations of osmolytes have shown promise for Me₂SO-free cryopreservation of cell therapies. In this work the effects of cryopreserving natural killer cells with and without Me₂SO has been studied to evaluate compatibility with and robustness for manufacturing and clinical use. Specifically, stability of expanded NK-92 (ATCC CRL-

2407) cells in cryoprotective formulations pre-freeze and post-thaw, sensitivity to cooling rate and ice nucleation temperature, post-thaw apoptosis, and expression of cytokine expression (perforin, granzyme) have been evaluated. Natural killer cells did not experience a significant drop in cell count or viability pre-freeze with Me₂SO or osmolyte based formulations but after thawing cells had a significant drop in viability after 1 hour with Me₂SO and a 15% viability decrease after 2 hours. No loss was seen after 4 hours with osmolytes. Various cooling rates from 1 to 7 °C/min and two ice nucleation temperatures (-6, -8 °C) were evaluated. Frozen cells were thawed and enumerated for immediate post-thaw viability and recovery. The optimal cooling rate was not 1 °C/min but rather 5-7 °C/min and cells were not sensitive to undercooling. Flow cytometric analysis showed a significant loss in perforin content post-thaw but not granzyme despite both proteins being contained in the cytolytic granules of these cells. This loss was not due to pre-freeze incubation with differing formulation osmolarity and pH. Formulation osmolarity however did have a negative effect on post-thaw cytokine expression and post-thaw apoptosis.

Funding: US National Science Foundation
PFI-TT 2042111

Conflict of Interest: None to disclose

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P28 CRYOPRESERVATION OF GERMPLASM IN AQUACULTURE CRUSTACEANS FOR BREEDING PROGRAMS

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As one of the significant seafood sectors, crustacean aquaculture has been growing rapidly. The total aquaculture production of crustaceans in 2018 reached 9,386 million tons, about 1.71 times that in 2010. The major farmed species include the white-leg shrimp *Litopenaeus vannamei*, (52.9%), red crawfish *Procambarus clarkia* (18.2%), Chinese mitten crab *Eriocheir sinensis* (8.1%) giant tiger prawn *Penaeus monodon* (8.0%), river prawn *Macrobrachium nipponense* (2.5%), and giant river prawn *Macrobrachium rosenbergii* (2.5%). To mitigate the risk of disease outbreaks, intensified culture systems with high density and biosecurity have become the major farming operation for shrimp aquaculture. Meanwhile, the domestication and breeding of shrimps have been developed and commercial commodities have been formed for global shrimp aquaculture with superior broodstock, especially for disease resistance and fast growth. To preserve the breeding stocks and the established specific strains/lines, cryopreservation of germplasm (eggs, sperm, or nauplius larvae) would be a useful tool and has been reported in a handful of publications. For embryo and larval cryopreservation, a total of 10 publications (8 species) were identified with the majority on studies of cryoprotectant toxicity and cold storage, no or limited post-thaw survivals were reported. For spermatophore cryopreservation, a total of 24 publications were identified with studies of 7 shrimps and 2 crabs, and successful post-thaw survival or fertility was documented in 4 shrimps and 1 crab. Overall, the effective cryoprotectants were concluded as glycerol, dimethyl sulfoxide, methanol, or ethylene glycol at a 5-15% concentration. The equilibration time after mixing the sperm sample and cryomedium was 15-60 min at room

temperature. Effective cooling of samples was concluded as a slow rate at 0.5-2.5°C/min from room temperature to -32 or -80°C before plunging in liquid nitrogen. Thawing of frozen samples was generally conducted by submerging them in a water bath at 25-30°C for about 2 min. Due to the immobility of crustacean sperm, post-thaw survival was majorly estimated by the eosin-nigrosine dye exclusion method. To date, fertility trials of post-thaw sperm were reported in three species including *Penaeus merguensis*, *Macrobrachium rosenbergii*, and *Penaeus monodon*. For the most farmed *Litopenaeus vannamei*, spermatophore cryopreservation has been reported in 5 publications with 30-80% post-thaw survival, but no post-thaw fertility reported yet. Overall, cryopreservation of germplasm is a useful technology to assist commercial-level breeding programs. To apply this technology (especially spermatophore cryopreservation) to the industry, further investigations about protocol streamlining, quality analysis, and post-thaw fertility tests are needed.

Funding: This study was supported by funds from the Gulf States Marine Fisheries Commission (ACQ-210-039-2019-USM, ACQ-210-039-2021-UFL2, and ACQ-210-039-2022-UFL) and the United States Department of Agriculture (Hatch project FLA-FOR-005385).

Conflict of Interest: None to disclose

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P29 CRYOPRESERVATION LABORATORY: BEST PRACTICES IMPLEMENTED IN QATAR BIOBANK

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Qatar biobank (QBB) is Qatar's National Centre for biological samples and associated data. QBB population-based cohort study recruit adults (age ≥ 18 years), Qataris and long-term residents (live in Qatar ≥ 15 years). QBB is accredited with ISO 29387,9001 and 27001. QBB preserves different types of specimens (plasma, serum, erythrocyte, DNA, RNA, buffy coat, viable cells, urine, and saliva) in 3 different locations as Automated Biostore, Cryopreservation lab and Remote location. QBB cryopreservation laboratory is designed for the region's challenging environmental conditions. QBB uses Storage tanks customized for hot weather to safely supplying liquid nitrogen to freezers (BSD) (n=7); monitored by Filling Management Control System. Different measures are adopted such as manual filling instead automatic filling which create high humidity during the peak of summer (Jun-Aug Temp: above 45°C, Room Humidity (RH): 85-90%). Instead of filling 7 BSD tanks altogether, filling divided into 2 groups to reduce the duration of oxygen deficient atmosphere. All biological freezers and environmental lab conditions are monitored with temperature monitoring system 24/7, oxygen sensors, Automated air extraction system, damper system to supply fresh air to lab during oxygen deficiency and Emergency oxygen mask. Cryopreservation lab access is controlled, and personal oxygen level sensors and PPE required working inside lab. As per OSCHA standard, gradual alarm points: 1st at 19.5% and 2nd at 18% are set. Functional checks are carried out daily, monthly, and annually. Sample traceability monitored during storage, retrieval by using in house Lims software. Conducting safety awareness training, competency assessment for lab staff (n=15). QBB selected vapor phase (< -196°C) for long-term storage to minimize cross contamination, facilitate retrieval, providing safe environment. No accidents in

12 years of practice demonstrating the efficiency of lab. Cryopreserved biospecimens without compromising on safety is a great achievement in biobanking industry.

Funding: Not applicable

Conflict of Interest: None to disclose

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P30 ZIZIPHUS NUMMULARIA EXTRACT AS AN ALTERNATIVE TO ANTIBIOTICS FOR CRYOPRESERVATION OF BUFFALO SEMEN

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The *Ziziphus nummularia* (ZN) leaf extract was evaluated as an alternative to antibiotics for cryopreservation of buffalo semen. The methanolic leaf extract of ZN was prepared and screened for the presence of active components. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were isolated from buffalo (n=10) semen samples. For *S. aureus*, highest zone of inhibition was observed with Gentamycin followed by ZN extract at 30%,

60% and 40%; while minimum inhibition was observed with streptomycin, and none with Penicillin. For *P. aeruginosa*, large inhibitory zone was observed with 60% of extract, following 40% and 30% of extract. Gentamycin shown highest activity among antibiotics whereas Streptomycin and Penicillin did not show activity against *P. aeruginosa*. In vitro dose tolerability (motility) using ZN extract in sodium citrate buffer indicated tolerance of buffalo sperm at 0.25%, 0.5%, 0.75%, and 1% of ZN extract. Buffalo semen was cryopreserved in extenders having ZN extract: Z1 (0.25%), Z2 (0.5), Z3 (0.75), Z4 (1%); and Streptomycin-penicillin (SP; control). Post thaw sperm motility, plasma membrane integrity, viability and livability was similar in extenders having ZN extract (0.25%; 0.5%) and that having antibiotics; while sperm acrosomal integrity was higher in extender having ZN (0.5%) extract. The total aerobic bacterial count was lower in extenders having ZN extract compared to that having antibiotics. In conclusion, *Ziziphus nummularia* leaf extract exhibited activity against bacteria isolated from buffalo semen, maintained post thaw quality and reduced the total aerobic bacterial count when used as an alternative to traditional antibiotics in extenders for buffalo semen.

Funding: Not applicable

Conflict of Interest: None to disclose

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P31 TRANSLATING CRYOPROTECTANT AGENTS FROM ZEBRAFISH TO MAMMALS: A TWO- STAGE APPROACH TO ADVANCE CARDIAC CRYOPRESERVATION

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Cryopreservation of organs is an emerging technique to address shortage of donated organs for transplantation. Cryoprotectant agents (CPAs) serve to halt unwanted ice formation, prevent cell damage, and ultimately protect organs from harm during cryopreservation. The challenges in cryopreserving mammalian hearts have highlighted the need for a comprehensive screening of promising CPAs in high throughput and translationally relevant model organisms.

To meet this need, a large-scale CPA screening was first conducted in zebrafish larvae with the goal of determining optimal cocktails of CPAs. With identified cocktails, survival rates and morphological changes were assessed in larvae following cryopreservation. To translate the findings from zebrafish to mammals, the two most successful combinations of CPAs were chosen based on efficacy and survival for further investigation in rodent hearts. To accommodate the complexity of mammalian hearts, the parameters of the preservation protocol have been optimized for rodent hearts, anticipating further optimization for larger mammalian hearts.

To that end, this study focused on optimizing the protocol of loading CPAs into the rodent cardiac tissue - an ongoing challenge in achieving successful cryopreservation of organs. To identify the best way of minimizing cardiac damage caused by sudden changes in osmotic gradient and CPA toxicity while also achieving accurate preservation outcomes, this study investigated several CPA loading protocols based on the status of cardiac graft health.

Using a Langendorff perfusion system, functional and injury markers were collected during a four-hour recovery period, providing information to evaluate the health of the cardiac graft after CPA loading and unloading - all prior to sub-zero preservation. The findings have important implications for the advancement of cryopreservation techniques for cardiac allografts, which may be an important step in tackling the organ shortage crisis. Moving forward, further research should focus on optimizing cryopreservation protocols, exploring species-specific variations, and evaluating CPA performance in both animal and human trials.

Funding: This research was funded by National Science Foundation under Grant No. EEC 1941543, NIH K99/R00 HL1431149; R01HL157803, Claflin Distinguished Scholar Award on behalf of the MGH Executive Committee on Research, Polsky Award on behalf of the MGH Department of Surgery.

Conflict of Interest: None to disclose

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P32 EXPANDING THE HORIZON: USING FATTY ACIDS AND MANNITOL AS ALTERNATIVE FUELS AND ONCOTIC AGENTS FOR NORMOTHERMIC EX VIVO HEART PERFUSIONS IN ADULT RATS

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Normothermic machine perfusion (NMP) is an advanced technique for ex vivo organ perfusion using a nutrient- and oxygen-rich perfusate at 37°C. The main objectives are to reduce injury, increase preservation duration, assess graft viability, and facilitate transplantation. The perfusate, enriched with glucose as a vital energy source, is crucial in meeting the metabolic demands of specialized organs like the heart in rodent models. Additionally, oncotic agents like bovine serum albumin (BSA) help maintain osmotic pressure and reduces fluid build-up (edema). While glucose and BSA are commonly used in rodent heart perfusions, there is growing interest in exploring alternative fuel options such as fatty acids, which are natural fuels for the heart, and alternate oncotic agents like Mannitol which recently have shown to have some cardioprotective properties. This preliminary study used a fatty acid (Oleic acid)/Mannitol perfusate and compared it with Glucose/BSA (control group) in adult rats (n=3) to assess the cardiac physiology using Langendorff perfusion. Major markers for heart injury (Troponin), heart parameters (vascular resistance, edema, oxygen uptake rate (OUR) and heart rate) during perfusion were analysed for each group. Interestingly, in Oleic acid-Mannitol group, OUR rate was lower during 4 hr perfusion compared with control group, which was expected as fatty acids are more energy dense fuel compared to glucose and yield more energy using lower oxygen consumption. Additionally, the addition of Mannitol, which is less viscous compared to BSA, significantly reduced vascular resistance. Moreover, troponin levels were lower in Oleic acid-Mannitol group (although not significantly different), while edema levels were comparable between both groups. These findings suggest the potential for improving traditional cocktails in rodent heart perfusions to enhance preservation and transplantation

outcomes in clinical settings. Further research is needed to comprehend the full benefits and implications of these alternative fuel options and oncotic agents.

Funding: This research was funded by National Science Foundation under Grant No. EEC 1941543, NIH K99/R00 HL1431149; R01HL157803, Claflin Distinguished Scholar Award on behalf of the MGH Executive Committee on Research, Polsky Award on behalf of the MGH Department of Surgery.

Conflict of Interest: None to disclose.

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P33 RESONANCE RAMAN SPECTROSCOPY DETECTS LEAKY MITOCHONDRION IN RAT LIVERS WITH ISCHEMIA-REPERFUSION INJURY

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Ischemia-Reperfusion Injury (IRI) is a critical problem in organ transplantation that hinders clinical translation of novel technologies aiming to extend organ preservation time without continuous oxygenation. Resonance Raman (RR) spectroscopy is an innovative approach to quantitatively assess mitochondrial redox state via rapid organ surface measurements. Since mitochondrial damage contributes greatly to the development of IRI, RR

reduced mitochondrial ratio (3RMR), a readout of RR spectroscopy on percentage of mitochondria in reduced state, can be a powerful parameter in organ assessment predictive of transplant success/outcome. In this study, 3RMR surface readings were performed on rat livers during three hours of subnormothermic machine perfusion (SNMP) after fresh procurement, as well as one hour and three hours of warm ischemic time (storage in 37°C saline solution) to evaluate mitochondria health following ischemia-reperfusion stress. Preliminary data confirmed that rat livers with 3 hours of ischemia exhibited classic injury parameters such as absent bile production, elevated lactate level, excessive tissue edema, and significantly lower oxygen consumption during SNMP in comparison to healthy fresh livers (n=4 per group, p<0.05). Interestingly, RR results showed that injured livers had lower percentage of reduced mitochondria (3RMR < 10%) than healthy livers (3RMR ~ 15-20%), despite complex IV being fully oxidized at maximum capacity of direct oxygen consumption in all study groups. Further RR spectroscopy analysis suggested that complex III of injured livers significantly leaked electrons/protons during reperfusion with less than 8% in a reduced state after 180 minutes of SNMP. Meanwhile, healthy livers had 30% reduced complex III which remained relatively unchanged through SNMP. These observations agreed with published literature that organs which developed IRI post-transplant commonly had leaky mitochondria resulting in ROS production, oxidation stress, and, finally, cell death. In other words, RR spectroscopy enables quantitative mitochondrial assessment beneficial for detection of organ injury following warm ischemic stress.

Funding: This research was funded by National Institute of Health K99/R00 HL1431149, R01HL157803, and

R01HL242990; Claflin Distinguished Scholar Award on behalf of the MGH Executive Committee on Research; as well as Polsky Award on behalf of the MGH Department of Surgery.

Conflict of Interest: SN Tessier a co-inventor on a pending patent with Pendar Technologies.

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P34 DEVELOPMENT OF A LEVAN-BASED FORMULATION FOR EFFICIENT SPERM CRYOPRESERVATION

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Cryopreservation is widely used in scientific, commercial, and therapeutic applications to store cells, tissues, and organs. While sperm cryopreservation is a common practice in assisted reproductive technology (ART), the process can damage the sperm membrane, leading to poor motility and irregular morphology. To address the issue, this study explored the use of Levan polysaccharide to minimize sperm loss before and after preservation. Levan is an uncommon non-structural polysaccharide found in most microorganisms and a few varieties of plants, which consists of residues of β -D-fructofuranose connected by β -2,6 glycosidic bonds and occasional β -(2,1) fructosyl-fructose branching. Levan's biocompatibility, biodegradability, and biological activity make it a good candidate for biomedical use. The study investigated the cryoprotective capabilities of fructan formulations on human sperm samples by firstly evaluating the effect

of four distinct fructan formulations. The fructans were then used to develop new freezing media to cryopreserve the sperm cells at various intervals, and cell survival and motility were assessed following freezing. In addition, the cryoprotective abilities of fructan-containing commercial freezing media at various concentrations were evaluated. The result obtained indicated that hydrolyzed Halomonas levan (hHL) in combination with Human Serum Albumin (HSA) and glycerol was the most effective formulation as it aided in maintaining the sperm cells' viability and motility after freezing and thawing for a period of time. This indicates that fructans, which have no detrimental effects on sperm cell cryopreservation, may be utilized as efficient cryoprotectants in various freezing procedures, depending on their concentration.

Funding: This research was funded by 118M607 Tübitak 1001 research project program of The Scientific and Technological Research Council of Turkey.

Conflict of Interest: None to disclose

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P35 WITHDRAWN

P36 CHOOSING THE BEST STORAGE MEDIA FOR HYPOTHERMIC STORAGE OF PORCINE OSTEOCHONDRAL DOWELS

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Cartilage allograft transplantation is an effective surgical procedure for large articular cartilage defects; however, it is limited by availability of donor cartilage tissue and storage time constraints. Loss of chondrocyte viability during cold storage limits the potential benefits of transferring viable chondrocytes during the osteochondral transplantation. A successful cold storage protocol of osteochondral grafts relies on determining optimal storage conditions and media composition during cold storage. In the current study, we tested the effects of three different cell culture media (X-VIVO 10, DMEM/F12 and RPMI-1640) on chondrocyte viability during cold storage (4 °C) for 4 weeks. Porcine stifle joints were collected from a slaughterhouse within 24 to 36 hours of the death of the animal and 10-mm osteochondral dowels were cored from each condyle. Osteochondral dowels were stored in each media at 4 °C for 4 weeks and chondrocyte viability was assessed by a cell membrane integrity stain every week. We observed a marginal decline in cell viability in all media for the first 2 weeks ($p < 0.05$) followed by a much steeper decline thereafter with no significant difference between the three media ($P > 0.05$) during 4 weeks of hypothermic storage. These results confirm that all tested media in this study provide good biochemical environments in the short term to support chondrocyte viability during cold storage; therefore, we believe it would be more reasonable to use lower cost options

(DMEM/F12 and RPMI-1640) for osteochondral graft cold storage.

Funding: This study was funded by the Edmonton Orthopaedic Research Committee and the University (of Alberta) Hospital Foundation. JAWE holds a Canada Research Chair in Thermodynamics.

Conflict of Interest: None to disclose

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P37 COLD HABITUATION ALTERS RAT BEHAVIOUR IN OPEN FIELD AND FORCED SWIM TESTS

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It is known that prolonged contacts with cold not only lead to the activation of metabolism and peripheral vasoconstriction, but also affect cognitive processes and memory, change emotionality, behavioral and exploratory activity. Repeated short-term cold effects (RSTCE) can result in habituation - a special form of adaptation, but how they affect behaviour have not been determined yet. RSTCE was hypothesized could affect locomotor behaviour and exploratory activity differently, so we aimed to study the effect of different RSTCE modes on behavioral parameters in rats. White outbred rats (male, $n=10$) were RSTCE (+10°C and -12°C) over two days in the light period for 15 min hourly (9 exposures per day). Animal's behaviour was evaluated in tests 1 and 2 (open field and forced swim, respectively). Horizontal and vertical locomotor activity, the number of grooming episodes, the latent period of locomotor activity and grooming, the number of defecation and urination (test 1); the

duration of passive and active swimming, the time of onset of immobility and its duration, the number of climbing episodes and dives (test 2) were measured. The data were statistically analyzed by the Kruskal-Wallis test. It is shown that the number of peripheral squares crossed and rears decreased, the time of onset of immobility and passive swimming increased, and consequently, the duration of immobility and passive swimming decreased in rats after RSTCE +10 °C. The number of central squares crossed and grooming episodes number decreased in rats after RSTCE -12 °C. In addition, the time of passive swimming, the number of climbing episodes and dives increased, with unchanged immobility time. Thus, cold habitation reduces locomotor behaviour but does not affect exploratory activity. An increase in the time of active swimming and reducing the immobility time in the forced swimming test may indicate a shift in behaviour strategy towards active coping with stress.

Funding: Not applicable

Conflict of Interest: None to disclose

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P38 HIFU REWARMING FOR THE RECOVERY OF CRYOPRESERVED MOUSE THORACIC AORTA

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Fresh vascular tissue is of the greatest interest for tackling cardiovascular diseases (CVDs). Arteries from the leg and breast for coronary bypass, and aortic and aortoiliac transplants,

are the most frequent examples. The unavailability of ready-to-use grafts often involves solutions coming from tissue engineering, which are costly, complex, and technically challenging. An alternative is the cryopreservation of donated vessels, especially arteries. Although the cryopreservation of descending aorta is well understood, the protocols are standardized for cooling, and the choice of cryoprotectants is straightforward, the warming process is more challenging. Warming is generally performed in water or saline baths at 37-42 °C. The convective nature of this way of warming generally leads to temperature gradients normal to the surface, especially if the sample is large. This work brings the successful technology of High Intensity Focused Ultrasound to tissue banking, by providing a fast and homogeneous warming of 1 mm thick murine descending aorta rings. A group of 9-month-old CD1 mice (n=24) were subject to aortic cryopreservation following state-of-the-art procedures (slow cooling in 15% dimethyl sulfoxide at -0.6 °C/min to -80 °C) and rewarmed with HIFU operating at 1.224 MHz, 30 Vdc, 1.8 Amps. A second group (n=30) of the same characteristics was used as a control group and the aortas were thawed in a warm bath. Viability was assessed through cell count and H&E staining, obtaining an average 74% viability with HIFU (60 second exposure) and a peak viability of 100%. This is compared to 9% average viability for thawing in a warm bath. Our approach to vascular tissue proves, first, that the post-thaw viability of tissues is possible by means of HIFU, capable of overcoming the historically arduous fast and homogeneous rewarming challenges. Second, it provides a warming method for non-decellularized aortic graft banking.

Funding: Grant from Spanish Ministry of Science and Innovation EQC2019-005949

Conflict of Interest: None to disclose
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P39 ULTRASOUND REWARMING FOR RECOVERY MOUSE HEARTS AFTER HYPOTHERMIA

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Recently, we proved the beneficial of ultrasound rewarming in the context of cryogenic storage. Therefore, this approach can be the definitive solution to the problem of ice recrystallization, opening the door to organ banking. In this work, we explore this technology in the context of high-subzero hypothermia, where it is well known that the shorter the second warm ischemia prior to anastomosis, the healthier the organ to be transplanted. Our aim here is to show the setup and the results of applying ultrasound for the rewarming of a mammalian organ: mouse heart after hypothermia. Evidently, regarding of this small organ, fast rewarming can be achieved by different classical methods (37 °C perfusate, i.e.), but, unfortunately, this is not the case for larger samples. Thus, the aim of this study is many-fold: 1) Exploring the technology in the context of organ rewarming, with its challenges; 2) Prove that it is applicable to mammalian hearts; and 3) Compare the performance of an intentionally slow rewarmed organ, at air (5 °C/min), for which it is known that there is some damage, with an organ rewarmed at 140 °C/min with ultrasound. Ten mouse hearts were taken to

deep hypothermia (- 6 °C) after perfusing them with 5% (v/v) Me₂SO. Afterwards, they were rewarmed using HIFU at 140 °C/min till they surpassed the physiological threshold (29 °C). Finally, microscopy analysis (Hematoxylin-Eosin), physiological performance with Langendorff organ bath system and ECG monitoring were performed. All ten hearts got their pre-cool beating rate back (98% +/- 3%) and the same ECG pattern as the fresh controls. Morphological analysis also did not show any evidence of damage. This was compared with the lack of any signal of recovery when is rewarmed slowly (at air), in the control group.

Funding: Grant from Spanish Ministry of Science and Innovation EQC2019-005949

Conflict of Interest: None to disclose
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P40 EFFECTS OF PRESERVATIVE SOLUTION AND CRYOPROTECTANT ON LIVING RATS

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This study aims to explore the effects of preservative solution and cryoprotectant on the function and structure of tissues (nerve, muscle, and blood vessel) and organs (heart, liver, spleen, lung, kidney, and gastrointestinal tract) in rats. Thirty SD rats were randomly divided into control group, preservative solution group and cryoprotectant group. Each group was injected intravenously with 2ml NS, 2ml

RPMI1640 medium and 2ml cryoprotectant (10% Me₂SO +10% calf serum +80%RPMI1640 medium), respectively. At the second month (±1 month) and the fifth month (±1 month), the indexes of liver and kidney function were detected, and the tissues and organs were observed and photographed. Then the cell structure and apoptosis of each tissue and organ were further observed by HE staining and TUNNEL staining. The results of the second month showed that compared with control group and preservative solution group, the indexes of liver function were increased in the cryoprotectant group, but there was no significant difference in the general and cell structure and apoptosis of tissues and organs in the three groups. These results suggest that within two months, cryoprotectant can affect the liver function of rats without altering the structure of tissues and organs. The results from the second observation point are not yet available, and the experiment is ongoing.

Funding: Natural Science Foundation of Guangdong Province, China

Conflict of Interest: None to disclose

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P41 OPTIMIZING CRYOPRESERVATION OF *STERCULIA LANCEIFOLIA* AND *LEPISANTHES AMOENA*

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The conservation of plant diversity is crucial due to increasing threats from anthropogenic activities and climate change. Traditional seed banking is an important method of ex situ conservation, but it is not suitable for many tropical species with recalcitrant or intermediate seeds. Cryopreservation has

shown promise for some species, but the method needs to be optimized for others. In this study, we aimed to optimize a cryopreservation method for two tropical species, *Lepisanthes amoena* and *Sterculia lanceifolia*, which cannot be stored conventionally due to recalcitrant seeds. Embryonic axes were excised from seeds, pre-cultured for 1 or 2 days, vitrified using Plant Vitrification Solution 2 (PVS2) for 10, 20, or 30 minutes, and then stored in liquid nitrogen for 1 day. The axes were later recovered and observed for root and shoot growth and callus formation. Preliminary findings indicate that embryonic axes from *Sterculia lanceifolia* were amenable to cryopreservation, with a 70.23% viability rate achieved when pre-cultured with 0.4M sucrose for 2 days, followed by incubation in loading buffer for 20 minutes prior to exposure to PVS2 for 20 minutes and liquid nitrogen for 1 day. However, cryopreservation of *Lepisanthes amoena* embryonic axes using a similar vitrification method was unsuccessful. These findings demonstrate the potential for cryopreservation as a method of ex situ conservation for some tropical plant species with recalcitrant seeds. Further studies are required to optimize the cryopreservation method for *Lepisanthes amoena* and other untried species. This research contributes to the development of conservation strategies for tropical plant diversity.

Funding: Not applicable

Conflict of Interest: None to disclose

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P42 THE EFFECT OF EXTRACELLULAR CRYOPROTECTIVE AGENTS AND ME₂SO ON PHASE TRANSITIONS

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Cryoprotective properties of extracellular cryoprotective agents (CPAs) are generally linked with their ability to form multiple H-bonds with water molecules outside cells. On the one hand, this may decrease the fraction of water molecules able to form ice outside of the cells. The effect reduces the “solution effect.” On the other hand, the CPAs promote partial dehydration of cells. However, not all extracellular CPAs are equally effective with respect to their ability to change the size and shape of ice and, correspondently, protect cells during cryopreservation. In this work, cryoprotective media based on polymers such as dextran, hydroxyethyl starch, and polyvinylpyrrolidone polyethylene glycol were investigated by cryomicroscopy. These polymers, with their characteristic molecular weights, are common extracellular CPAs. Their concentrations ranged from 10 to 300 mg/ml in Ham’s F12 media. Some media contained 10% fetal bovine serum instead of polymers. The cryoprotective media could also include 0.7 M or 1.4 M Me₂SO. Physical processes taking place in the media were additionally controlled by thermomechanical analysis. The different ability of the extracellular CPAs to protect testis interstitial cells was documented. The most successful CPA combination was dextran with a molecular mass of 40 kDa supplemented with 0.7 M Me₂SO. This cryoprotective medium, apart from serum-containing media, suppressed recrystallization. The dextran based medium did not have an effect on ice nucleation and did not promote supercooling. Interestingly, the media based on polyethylene glycol, which was the worst in

terms of cell survival, had the tendency to supercool. Hydroxyethyl starch, polyvinylpyrrolidone and polyethylene glycol behaved similarly to dextran, but cell preservation indicators were lower. Additional physical processes taking place in the space between ice crystals were documented. Some of them were recognized as glass transitions, while the nature of others and their impact on cell preservation are yet to be discussed.

Funding: Not applicable

Conflict of Interest: None to disclose

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P43 SLOW-COOLING AND ICE-SEEDING FOR CRYOPRESERVING HUMAN T LYMPHOCYTES WITH LOW-CONCENTRATION EXTRACELLULAR TREHALOSE

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Cryopreservation of human T lymphocytes has become a key strategy for supporting cell-based immunotherapy. However, the effects of ice seeding on the cryopreservation under relatively slow cooling have not been well researched. The cryopreservation strategy with a nontoxic, single-ingredient, and injectable cryoprotective solution remained to be developed. We conducted ice seeding for the cells in the solution of normal saline with 1%(v/v) dimethyl sulfoxide (Me₂SO), 0.1M trehalose, and 4%(w/v)

human serum albumin (HSA) under different slow cooling rates. With the positive results, we further applied seeding in the solution of 0.2M trehalose and 4%(w/v) HSA under the same cooling rates. The optimal concentration of trehalose in the Me₂SO-free solutions was then investigated under the optimized cooling rate with seeding or without seeding, and in a freezing container. In vitro toxicity of the cryoprotective solutions to the cells was also tested. With ice seeding, we found that the relative viability of the cells (1%[v/v] Me₂SO, 0.1M trehalose and 4%[w/v] HSA) was improved significantly from 88.6% to 94.1%, compared with that without seeding ($p < 0.05$). The relative viability of cells (0.2 M trehalose and 4% [w/v] HSA) with seeding was significantly higher than that without seeding, 96.3% and 92.0%, respectively ($p < 0.05$). With no significant difference in relative viability between the solutions of 0.2M trehalose and 0.3M trehalose, with 4%(w/v) HSA (92.4% and 94.6%, respectively, $p > 0.05$), the solution of 0.2 M trehalose and 4% (w/v) HSA was selected as the optimized Me₂SO-free solution. This strategy could cryopreserve human T lymphocytes without any toxic cryoprotectant and boost the application of cell products in humans by intravenous injection, with the osmolality of the cryoprotective solutions close to that of human plasma.

Funding: This work was funded by National Natural Science Foundation of China (Grant No. 51776130), Shanghai Local Capacity-Building Project (Grant No. 19060502400), and Shanghai Collaborative Innovation Center of Energy Therapy for Tumors

Conflict of Interest: None to disclose

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P44 DISACCAHRIDES-BASED CRYO-FORMULANT EFFECT ON MODULATING PHOSPHO/MITOCHONDRIAL LIPIDS AND BIOLOGICAL PROFILES OF HUMAN LEUKAEMIA CELLS

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The use of novel cryo-additive agents to increase cell viability post-cryopreservation is paramount to improve future cell based-therapy treatments. We aimed to establish the Human Leukemia (HL-60) cells lipidomic and biological patterns when cryo-preserved in Me₂SO alone and with 300 µM Nigeroside (Nig), 200 µM Salidroside (Sal) or a combination of Nig (150 µM) and Sal (100 µM). HL-60 cells were pre-incubated with Nig/Sal prior, during and post cryopreservation, and subjected to global lipidomic analysis. Malondialdehyde (MDA), released lactate dehydrogenase (LDH) and reactive oxygen scavenger (ROS) measurements were also carried out to evaluate levels of lipid peroxidation and cytotoxicity. Results: Cryopreserving HL-60 cells in Me₂SO with Nig and Sal provided optimal protection against unsaturated fatty acid oxidation. Post-thaw, cellular phospholipids and mitochondrial cardiolipins were increased by Nig/Sal as the ratio of unsaturated to saturated fatty acids 2.08 +/- 0.03 and 0.95 +/- 0.09 folds respectively in comparison to cells cryopreserved in Me₂SO alone (0.49 +/- 0.05 and 0.86 +/- 0.10 folds). HL-60 lipid peroxidation levels in the

presence of Me₂SO + Nig and Sal combined were significantly reduced relative to cryopreservation levels (10.91 +/- 2.13 nmole) compared to Me₂SO (17.1 +/- 3.96 nmole). Me₂SO + Nig/Sal combined also significantly reduced cell cytotoxicity post-thaw (0.0128 +/- 0.00182 mU/mL) in comparison to Me₂SO (0.0164 +/- 0.00126 mU/mL). The combination of Nig/Sal also reduced significantly ROS levels to the levels of prior cryopreservation of HL-60. Conclusion: Overall, the establishment of the cryopreserved HL-60 cells lipidomic and the corresponding biological profiles showed an improved cryo-formulation in the presence of Me₂SO with the Nig/Sal combination by protecting the, mitochondrial inner membrane, unsaturated fatty acid components (i. e. Cardiolipins) and total phospholipids.

Funding: King AbdulAziz City for Science and Technology

Conflict of Interest: None to disclose

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P45 THE ROLE OF HSP70 IN THE REGULATION OF THE TOLEROGENIC POTENTIAL OF DENDRITIC CELLS DERIVED FROM CRYOPRESERVED PROGENITORS OF BONE MARROW ORIGIN

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Cryopreservation changes the structural characteristics and functional potential of

cells, which affects the effectiveness of their therapeutic use. Changes in the receptor repertoire and functional activity of dendritic cells (DCs), which are formed *in vitro* from mononuclear cells (MNCs) of bone marrow (BM) depend on the cryopreservation conditions. The change in the functional state of cells after cryopreservation may depend on the expression of hsp70 - activator of the anti-inflammatory process. The purpose is to analyze the effect of cryopreservation on the expression level of hsp70 in MNCs and DCs obtained from them *in vitro* and the peculiarities of their tolerogenic activity in adjuvant arthritis (AA). The influence of 2 modes of cryopreservation of MNCs (cryoMNCs) of the mice BM under the protection of 10% Me₂SO up to -196 °C with a cooling rate of 1 degree/min up to -80 °C (M1); to -40°C (M2) on their viability, and hsp70 content immediately after rewarming and in DCs obtained from them *in vitro* was analyzed. The content of hsp70 was evaluated on a FACScalibur™ flow cytometer using FITC-labeled monoclonal antibodies to hsp70. AA was induced by subplantar administration of Freund's complete adjuvant. The content of IL-10 of animals with AA after the introduction of DCs from cryoMNCs was determined using the Kit IL-10 set. The attestation of the tolerogenic properties of DCs was carried out by their ability to induce T-regulatory cells of mice with AA, activation of IL-10 production, and other components of the anti-inflammatory cascade. Cryopreservation, especially under M2, induces an increase in the content of hsp70 in MNCs, which determines the activation of the formation of tolerogenic DCs. This is accompanied by an increase in the content and functional potential of T-regulatory cells, the production of the IL-10, and a decrease in the clinical signs of AA.

Funding: Not applicable

Conflict of Interest: None to disclose
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P46 DIFFERENT FREEZING CONDITIONS DETERMINE SPECIFIC CHANGES IN EHRlich ASCITES CARCINOMA STRUCTURE AND FUNCTION

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Cryoablation is a promising tool for tumor treatment. The development of relapses after such therapy suggests survival after the cryo-influence of certain tumor cells. We are talking about cancer stem cells - CSCs, the preservation of which during freezing determines the development of relapses. The aim was to examine the nature of redistribution of subpopulation composition and proliferative potential of Ehrlich ascites carcinoma (EAC) after freezing at different temperatures. The object of the study were EAC cells, which were frozen in ascitic fluid at a rate of 10 deg/min to -30°C, -60°C, -80°C, -196°C with subsequent heating at 40–41°C. The number of nucleated cells and their viability were immediately determined after thawing, as well as the subsequent cultivation of EAC for 7 days in the peritoneal cavity (PC). The subpopulation composition was assessed using monoclonal antibodies to CD44, and CD24 on a FACS Calibur flow cytometer. The indices of native EAC cells served as a control. A decrease in the number of nucleated and viable cells was established with the temperature reduction from -30°C to -196°C. The content of CD44⁺CD24⁻, CD44⁺CD24⁺, CD44⁻CD24⁺

subpopulations decreased, while the indices of CD44^{high} cells, which are attributed to CSCs, changed to a lesser extent. When freezing to -80°C, the most pronounced redistribution of the subpopulation composition of EAC was observed with a significant decrease in the content of the most carcinogenic subpopulation of CSCs CD44^{high}. Cultivation in PC of EAC cells frozen to different temperatures showed that their decreased accumulation compared to the unfrozen cells was observed in each case and it was maximal with temperature lowering down to -196°C. However, the growth rate of the "destructive" effect of EAC cells was much greater when the temperature decreased from -60 to -80°C, if compared with its lowering from -80 to -196°C.

Funding: Not applicable
Conflict of Interest: None to disclose
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P47 EFFECT OF CORD BLOOD AND CRYOTHERAPY ON MYOCARDIUM OF OBESE RATS

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Obesity is often associated with an increased risk of coronary heart disease and hypertension. The numbers of obese people are still growing in developed countries. The goal of our study was to assess and compare the influences of sole and combined applications of rhythmic extreme whole-body cryotherapy (RE WBC) and cord blood nucleated cells (CBNCs) injection on myocardium structure in obese aged rats.

Experiments were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine. The whole cooling cycle included 9 procedures (3 procedures/day) of cooling (2 min each) for 5 days with a day interval. Immediately after cooling the skin temperature declined within the range of 3–5 °C, the rectal temperature did not change significantly. Thawed CBNCs preparation was intraperitoneally injected in dose of 3×10^5 CD34⁺-cells/kg (after the sixth procedure in RE WBC+CBNCs group). The specimen was made up to 1 ml volume with plasma autologous to the cells. The rats were decapitated 30 days after the last cooling procedures and/or the injection, and the pieces of myocardium tissue were collected for a histological study. Myocardium structure was assessed in histological specimens stained with hematoxylin and eosin, van Gieson's picrofuchsin. The signs of circulatory disorders, myocardium hypertrophy, interstitial and perivascular edema, and focal cardiosclerosis were found in obese rats. After combined application of RE WBC and CBNCs these signs were poorly expressed. The morphological signs of neoangiogenesis were noted. This means improving blood flow and trophism of the heart, hindering the development of ischemia and necrosis, that maintains an increased number of cardiomyocytes, as well as providing their needs for hypertrophy of the myocardium, directly associated with the diet-induced obesity. Thus, the combined application of RE WBC and CBNCs significantly improves the myocardium structure in obese aged rats.

Funding: Not applicable

Conflict of Interest: None to disclose

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P48 MICRORNA REGULATION OF HEPATIC METABOLISM AND ENERGY-EXPENSIVE PROCESSES DURING TORPOR IN THE GARDEN DORMOUSE

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The garden dormouse (*Eliomys quercinus*) is a fat-storing mammal that undergoes annual periods of hibernation to mitigate the effects of food scarcity, low ambient temperatures, and reduced photoperiod that characterize winter. Like other hibernating species, this animal suppresses its metabolic rate by downregulating nonessential genes and processes in order to prolong available energy stores and limit waste accumulation throughout the season. MicroRNAs (miRNAs) are short, single-stranded, noncoding RNAs that bind to mRNA and enact post-transcriptional suppression, making miRNA ideal for modulating widespread changes in gene expression, including global downregulation typified by metabolic rate depression. Using next-generation sequencing, we analyzed an RNA-seq dataset to determine which miRNAs are differentially regulated during hibernation in the dormouse liver. We found that the expression of 19 miRNAs was altered during hibernation; however, by correcting for false discovery rate one major miRNA (miR-34a-5p) remained significantly downregulated. Gene Ontology and KEGG Pathway Analysis predicted that energy metabolism, nuclear-related functions such as histone binding, chromatin- and chromosomal binding, and the cell cycle are

processes that may be differentially regulated during hibernation due to miRNA regulation. Taken together, our data suggest that miRNA influence appears to be strongly directed toward suppressing energy-intensive processes in the nucleus hence contributing to extend the animal's endogenous fuel reserves for the duration of hibernation. Further studies are needed to elucidate the precise number and roles of miRNAs that change during stress in this animal.

Funding: Not applicable

Conflict of Interest: None to disclose

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P49 SUB-ZERO MICRORNA EXPRESSION IN THE LIVER OF FROZEN *CHRYSEMYS PICTA MARGINATA*

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Hatchling Midland painted turtles (*Chrysemys picta marginata*) can survive freezing while overwintering in their underground nests. Many molecular mechanisms contribute to freezing survival and metabolic rate depression (MRD), including microRNAs (miRNAs), which post-transcriptionally regulate gene expression to suppress non-essential genes and upregulate pro-survival pathways. Herein, the liver miRNA expression profile was investigated in response to freezing in hatchling painted turtles. A total of 204 known miRNAs were identified to be expressed in *C. p. marginata*, with 17 being upregulated and 13 being downregulated during freezing compared to control. KEGG and GO analyses suggested that upregulated

miRNAs inhibit genes of cell cycle, and focal adhesion and adherens junctions, suggesting their role in the downregulation of central metabolic processes necessary for MRD and the maintenance of tissue homeostasis. Conversely, downregulated miRNAs resulted in the predicted upregulation of linoleic acid metabolism and multiple signaling pathways. This upregulation would likely promote homeoviscous adaptations and the expression of pro-survival proteins to protect the liver during freezing. Overall, these findings point toward a strong role for miRNA in freezing-survival by enabling hatchling turtles to suppress non-essential energy usage, and protecting basal hepatic function by activating pro-survival processes.

Funding: Natural Sciences and Engineering Research Council of Canada (NSERC)
Grant # RGPIN-2020-04733

Conflict of Interest: None to disclose

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P50 INCORPORATING COLD PRETREATMENT IN CRYOPRESERVING SHOOT TIPS OF SWEET MYRTLE (*Gossia FRAGRANTISSIMA*)

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Gossia is a genus in the Myrtaceae Family native to Australasia. With the introduction of Myrtle rust, a devastating fungal disease

caused by *Austropuccinia psidii*, to Australia in 2010, a broad range of Myrtaceae species have been under threat, including many *Gossia* species. Currently, there are two *Gossia* species (*G. fragrantissima* and *G. gonoclada*) listed as Endangered under the Environment Protection and Biodiversity Conservation Act 1999 (the EPBC Act) and two other species (*G. acmenoides* and *G. inophloia*) listed as threatened under the scope of state legislations. Urgent *ex situ* conservation efforts are required for these species in face of the limited wild populations and many environmental threats other than Myrtle rust, such as habitat loss. Cryopreservation is an *in vitro* method for long-term conservation, and it is more cost-effective and space-efficient than other *ex situ* methods. Therefore, this study aims to develop cryopreservation protocols for the Endangered *G. fragrantissima*. Preliminary studies showed that *G. fragrantissima* shoot tips cannot survive cryogenic storage with a droplet vitrification protocol if they were extracted from fresh untreated *in vitro* cultures, but some regrowth was observed if extracted from cold pretreated cultures (10 °C for two weeks). In order to optimize the droplet vitrification protocol, shoot tip viability was checked after each major step, including dissection, loading, and dehydration, and used as a guide for protocol development. So far, many factors have been found to have impacts on shoot tip viability, including the types of shoot tips, dissection skills, and loading duration. So far, the highest post dehydration regrowth (59.4%) was achieved with 3-day cold pretreated apical shoot tips followed by 15 min loading solution and 20 min Plant Vitrification Solution 2 (PVS2).

Funding: Australian Flora Foundation and the University of Queensland Research Training Program (RTP) scholarship

Conflict of Interest: None to disclose

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P51 CHEMOTHERAPY-INDUCED MURINE OVARIAN FAILURE TREATMENT WITH CRYOPRESERVED PLACENTAL EXPLANTS

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Ovarian failure (OF) often leads to oncology and then correspondingly to the use of chemotherapy. Placenta and its derivatives are characterized with tropism to organs of reproductive system and can be effective in reproductive pathology treatment. Therapy using placenta derivatives requires preservation of their natural composition and activity that is possible via application of cryobiological biotechnologies. In this work, we studied the effectiveness of OF correction by application of cryopreserved placental explants (CPE). Female BALB/c mice with regular estrous cycle (EC) were used, the OF was modelled by intraperitoneal administration of cyclophosphamide/busulfan. Animals were divided into 3 groups: 1) intact; 2) those with OF model; 3) the ones with OF after CPE application. The effect of CPE application on EC, sexual function and fertility of female mice was studied (cytological/histological examination of uterus/ovaries, blood vessels and glands, EC phases, number of matings and pregnancies). In animals with OF, atrophic changes in ovaries and uterus, reduced number of primary/absence of secondary follicles, decreased cell size (with

nuclei hyperchromia), poor differentiation of ovaries/uterus layers, were observed. After CPE application the ovaries and uterus returned to normal sizes, the cells increased in volume (nuclei became heterochromic), in the uterus, endometrial hyperplasia, an increased number of glands, as well as the myometrium thickening were found. EC regularity was disturbed after OF modelling, restoration of regular cycle was observed in 50% of mice, the relative number of effective matings was 30%. After CPE application, the regular EC was restored in most animals, the relative number of effective matings was 60-80%. In intact animals, pregnancy occurred in 90% of cases, the animals with OF didn't become pregnant, regardless of CPE application. Cryopreserved placental explants are considered to be an effective tool of OF correction by restoring the ovarian and uterine structure as well as sexual function.

Funding: Not applicable

Conflict of Interest: None to disclose

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P52 INCLUSION OF NOVEL ADDITIVES ON EXTENDERS TO IMPROVE RABBIT SPERM CRYOPRESERVATION

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Cryopreservation tolerance has a marked species-specific component, being still suboptimal for rabbit sperm among other species. The cryopreservation process leads to severe impairment of sperm quality after thawing, reducing motility and viability, both crucial parameters for its successful use in artificial insemination. Thus, the search for novel components to improve cryopreservation extenders is needed to increase the rabbit sperm capacity to withstand the cryopreservation process. For this purpose, ejaculates from New Zealand White adult rabbit bucks (n=5; 6-7 months old; IRTA-Torre Marimon, Caldes de Montbui, Barcelona, Spain) were collected. Two independent experiments were performed to test novel additives for rabbit sperm cryopreservation extenders: 1. honey (1.25, 2.5, 5, and 10%), and 2. coenzyme Q10 (100 and 200 µM), and β-carotene/α-tocopherol (500 µM/620 µM and 250 µM/310 µM). Post-thawing sperm parameters analyzed included total motility, cell membrane integrity, viability, nuclear membrane integrity, acrosome reaction, mitochondrial activation, and membrane potential. We also analyzed some biochemical and hormonal components of the seminal plasma, and the proAKAP4 levels, a functional marker of post-thawed spermatozoa in several species. Our findings showed that the 2.5% honey supplementation increased the post-thawing sperm motility compared to the higher concentrations. The post-thawing motility was negatively affected by the coenzyme Q10, but not by the β-carotene/α-tocopherol supplementation. Finally, the analysis of proAKAP4 showed a positive correlation with rabbit seminal plasma factors, including zinc, fructose, and

lactate, paving for validation by fertility trials to test if this molecular marker reflects a differential fertility performance. In conclusion, the cryopreservation protocols used in this study failed to improve the sperm quality parameters of rabbit spermatozoa after cryopreservation, requiring further research.

Funding: Supported by PID2019-108320RJ-I00, IJCI-2015-24380 and RYC2020- 028615-I, funded by MCIN/AEI /10.13039/501100011033 (Spain) and FEDER funds (EU).

Conflict of Interest: None to disclose

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P53 PLANT VITRIFICATION SOLUTIONS FOR *CHLOROCOCCUM DISSECTUM* CRYOPRESERVATION

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There have been many studies, showing the commercial applications of *Chlorococcum dissectum*. It is a very interesting microalgae due to its ability to hypersynthesis fatty acids and the possibility of using it as a promising object for biofuel production. During various cataclysms, there is a risk of loss of important microalgae cultures. Cryopreservation is the most reliable tool for a long-term preservation of microalgae cells with unchanged genetic characteristics. The aim of this study was to develop a method for cryopreservation of *Chlorococcum dissectum* microalgae using different concentrations of plant vitrification solutions (PVS). Microalgae were cultivated with BG-11 medium to the stationary phase of growth at 25±2°C without aeration with round-the-

clock white fluorescent illumination of 52,84 µmol photons m⁻² s⁻¹. We used modified PVS 1 (22% glycerol, 13% 1,2-propylene glycol, 13% ethylene glycol, 6% Me₂SO, 0.4 M sucrose) and PVS2 in 50% and 75% final concentrations. Cryopreservation was performed by direct immersion to liquid nitrogen. Warming was done at 40°C in a water bath. The PVS was washed by centrifugation. Cell viability was determined by counting the number of colony-forming units on plates. The results of post-thaw viability showed the best cryoprotective effect of 50% PVSs on *Ch. dissectum* cells. Colony-forming units values were comparable with the control. The use of 75% PVSs showed low cell viability after cryopreservation, colony-forming units values were about 13% of the control. This may be due to the damaging effect of the high concentrations of cryoprotectants in the solution on the cells at the equilibrium stage. The results of this research demonstrated that 50% modified PVS1 and 50% PVS2 can be successfully used for the low-temperature storage of *Ch. dissectum* microalgae.

Funding: Not applicable

Conflict of Interest: None to disclose

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P54 DETERMINING PHASE TRANSITION TEMPERATURES IN NATURAL POLYSACCHARIDE HYDROGELS USED IN TISSUE ENGINEERING

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Hydrogels are widely used in tissue engineering for manufacturing scaffolds, bioprinting, drug delivery, and cell encapsulation. Tasks associated with designing cryopreservation technology for hydrogel-based tissue engineered structures are especially topical. The most common are gels based on alginates, hyaluronic acid, carrageenan, gelatin, chitosan. Some studies showed these substances to have cryoprotective properties, enabling their use as a basis for wash-free cryoprotective media. Findings related to use of cryoprotective media, containing substances from a number of natural polysaccharides, tending to transit to amorphous state at moderately low temperatures (up to -40...-45°C) are of special interest. Natural gel-forming substances such as hyaluronic acid, carrageenan, sodium alginate have been studied. Phase transition temperatures, corresponding to these solutions transition to vitreous state were obtained using thermomechanical analysis. We used 1% aqueous solutions of hyaluronic acid, carrageenan, and sodium alginate. Samples of 0.5 ml volume were cooled with 4 deg/min rate down to -160°C, an external load was applied and using heating mode with 1 deg/min rate, the phase transitions in a sample were recorded on thermomechanical curve. Research showed the phase transition to vitreous state as occurring within temperature range from -35 to -25°C for hyaluronic acid, from -38 to -28°C for carrageenan, and from -40 to -27°C for sodium alginate. Data on phase transitions, proceeding in cryoprotective medium during freeze-thawing should be taken into account when developing efficient protocols for biological object cryopreservation and recommendations for long-term storage temperatures of biological material. Optimal ratio of temperature-time parameters is especially relevant for drugs used in pharmacology and practical medicine.

Higher temperatures of samples' phase transitions to a solid state provide longer storage periods at moderately low temperatures. Gel-forming substances from a number of natural polysaccharides are promising for use as cryoprotectants during biological object cryopreservation and subsequent long-term storage in low-temperature refrigerators at -40 to -80°C.

Funding: Not applicable

Conflict of Interest: None to disclose.

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P55 PHYSICAL, THERMODYNAMIC AND MICROSTRUCTURAL CHARACTERIZATION OF CHITOSAN-ALGINATE MIXED BEADS FOR CRYOPRESERVATION

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Mixed chitosan and calcium alginate beads were prepared for its possible use in the protection of specimens for cryopreservation with improved functionality. An additional layer of chitosan was added to preformed calcium alginate beads. Chitosan is a relatively inexpensive and widely available polysaccharide, obtained as a food byproduct, with interesting properties in alimentary, agricultural, biomedical and chemical fields, and biocompatible, nontoxic and biodegradable properties. The antimicrobial character of this polymer has proved to be of special utility, protecting beads against bacterial and fungal

proliferation, while chitosan antioxidant properties could have a protective roll on cryopreserved specimens against oxidative damage. Chitosan of several molecular weights were studied and tested. The complementary charges of alginate and chitosan polymers favored the interaction and the formation of the external chitosan layer. This interaction between polymers was characterized by Fourier transfer infrared spectroscopy (FTIR), showing how calcium ions interacting with alginate were partially replaced by chitosan. Information on the internal and external mix-bead architecture was obtained by microscopic techniques, such as scanning electron microscopy (SEM) and low-temperature scanning electron microscopy (cryo-SEM). A smooth, crack-free surface was observed. The external additional chitosan layer have only a small influence in the more relevant physical parameters for mixed beads employment for cryopreservation purposes, such as water and solute intake and loss in dehydration. Consequently, the additional chitosan layer does not appear to significantly alter beads water permeation behavior. Additionally, freezing thermodynamic parameters appear also only slightly altered, after differential scanning calorimetry (DSC). The general conclusion is that chitosan-coated calcium alginate beads behave in a similar way to the uncoated ones for most cryopreservation purposes. Consequently, chitosan addition can be used to protect beads used in encapsulation procedures against microbial contamination and oxidative damage, other properties not being significantly altered.

Funding: The authors would like to acknowledge the financial support of project “SUST_CHIT_BIOPR” (TED2021-129288B-C21) of the Spanish Ministry of Science and Innovation (funded by Next Generation EU) and of the Argentinean Agencia Nacional de Promocion Cientifica y

Tecnologica (ANPCyT) PICT 2018-02352.

Conflict of Interest: None to disclose

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P56 PROSPECTS OF USING SURFACTANTS IN CRYOPRESERVATION OF PLANT GENETIC RESOURCES

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Effective saturation with cryoprotectants is extremely important for the preservation of plant germplasm during cryoconservation, especially when using highly concentrated PVS. Reducing the surface tension of solutions by adding surfactants is actively used in agriculture to wet hydrophobic and hard-to-reach plant surfaces covered with a waxy layer and a dense coating and to accelerate the absorption and distribution of the solution in plant tissues. However, there are no data on the use of surfactants to accelerate the saturation of plant genetic resources with cryoprotective solutions. The purpose of the study was to evaluate the effectiveness of the use of surfactants in cryoprotective media to accelerate the saturation of plant explants. In the study, 100, 80, and 60% solutions of PVS2 with the addition of organosilicone surfactant Siliksan106 in concentrations non-toxic to plants (0.025÷0.15%) were investigated. Using stalagmometry, it was found that the addition of surfactants leads to a 3-fold (depending on concentration) decrease in the surface tension of PVS2 solutions. The osmotic reactions of cells after the addition of

PVS2 were studied under a microscope using the *Chlorococcum dissectum* microalgae as an example, and a significant decrease in the time of cell volume recovery in the presence of surfactants was found. The use of surfactant during the saturation of grape cuttings (Frontinak variety) by the vacuum infiltration method reduced the time of PVS2 passing through the capillary system of the cuttings by more than 3 times. DSC study of low-temperature phase transitions of PVS2 solutions did not reveal significant differences when surfactant was added. Thus, the use of surfactant in the composition of cryoprotective solutions accelerates the cellular and capillary permeability of plant explants for cryoprotectants, without reducing their glass-forming ability. The results prove the great potential of surfactants in the plant cryopreservation.

Funding: Not applicable

Conflict of Interest: None to disclose

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P57 INFLUENCE OF VISCOSITY ON DYNAMICS OF MICROALGAE SATURATION WITH PLANT VITRIFICATION SOLUTIONS

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Currently, a number of effective plant vitrification solutions (PVSs) have been developed but they contain high concentrations of cryoprotectants with limited incubation time. Changing the

incubation temperature and PVS concentration makes it possible to regulate not only cytotoxicity, but also the viscosity of solutions, which affects the dynamics of plant saturation with cryoprotectants. The purpose of this work was to determine the influence of viscosity changes on the dynamics of microalgae' saturation with PVSs. Viscosity in PVSs of different concentrations was investigated in the temperature range from 1°C to 30°C by the capillary viscometer method. It has been shown that the viscosity increases in the series PVS1, PVS2, PVS4, PVS3, which correlates with an increase in the concentration of glycerol, as the most viscous component. Due to the high concentrations of glycerol, the viscosity of 88% PVS3 was 35-48 times higher than that of 100% PVS1, PVS2, PVS4, and when the PVS3 concentration decreased from 88% to 60%, the viscosity decreased by more than 50 times at 1°C. An increase in temperature decreased the viscosity in all investigated solutions. For example, when the temperature changed from 1°C to 30°C, the viscosity of 100%, 80%, 60% PVS2 decreased by 4.5, 3.5 and 3 times, respectively. A correlation was found between the viscosity of PVSs and the dynamics of their penetration into microalgae *Chlorococcum dissectum*. It was shown by microscopy that after dehydration in 60% PVS2, the cell volume of 90% cells was recovered, and in 80% and 100% PVS2, the number of recovered cells was 33% and 27% after 5 min of incubation. Thus, by varying the viscosity of PVSs, it is possible to effectively influence the dynamics of their permeability to plant cells.

Funding: Not applicable

Conflict of Interest: None

to disclose

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P58 ENHANCED CRYOPRESERVATION EFFICIENCY OF HIPSC-DERIVED NEURAL ORGANOIDS BASED ON RAMAN STUDIES

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Three-dimensional (3D) cell culture models, like organoids, derived from human induced pluripotent stem cells (hiPSCs) represent a unique preclinical model system with organ-like physiology. These systems provide many opportunities in biomedical research, like in drug development or diagnostics. Therefore, the impact of hiPSCs-derived organoids is steadily increasing. Since, differentiation and cultivation processes of hiPSC-derived organoids are complex and time-consuming, while having a limited application time window, there is a high demand for “ready-to-use” 3D systems. Efficient cryopreservation routines for organoids would enable safe and standardized stock keeping as well as a fast “ready-to-use” supply. Our work provides insights in underlying mechanisms of cryoinjuries in early neural stem cell organoids as homogenous precursor model of midbrain organoids and implement a basis for development of cryopreservation routines for 3D cell systems. While suitable cryopreservation protocols are available for many single cell types in suspension, procedures for 3D cell systems still lack in efficiency. Main obstacles are inhomogeneous penetration of

cryoprotectants and non-uniform heat transfer during freezing and thawing causing gradients throughout the heterogeneous 3D system. Resulting ice crystallization and osmotic cell shrinking can induce cell disruptions, and even apoptosis. In our studies, SEM ultrastructure analysis of cryopreserved organoids indicate dehydration and disruptions of outer cells after thawing. This compromises the structural integrity of organoids leading to reduced intercellular communication and loss of functionality. To minimize these harmful effects, we introduce a workflow for Raman spectroscopy to derive the diffusion rate of Me₂SO into organoids with different parameters (diameters of 300-600 µm). With adapted incubation times, higher cell viabilities were achieved affirming the assumption that the homogenous penetration of cryoprotectants in 3D systems prior freezing improves cell survival while reducing cytotoxicity. For more detailed validation of recovery, further quality controls were carried out concerning morphology, viability as well as gene and protein expressions.

Funding: Parts of this work have received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No. 828837.

Conflict of Interest: None to disclose

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P59 CRYOPRESERVATION OF MESENCHYMAL STROMAL CELLS SPHEROIDS BY VITRIFICATION

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Formation of spheroids from mesenchymal stromal cells (MSCs) allows to retain the unique properties of MSCs, higher paracrine potential and stemness, providing a background for spheroids use in clinical practice, tissue engineering etc. Cryopreservation is an important step for their implementation in practice. However, spheroids are complex for cryopreservation 3D objects with relatively high density and poor permeability to cryoprotectants. Vitrification is an ice-free, potentially beneficial approach for spheroid cryopreservation. Here we aimed to develop the steps of vitrification procedure for cryopreservation of MSCs-based spheroids. Spheroids were formed from human dermal MSCs by “hanging drop” method with cell concentrations of 1000 and 3000 cells per spheroid. Cells were cultured for 2 days before cryopreservation. Vitrification solution (VS) consists of 15% of dimethyl sulfoxide (Me_2SO), 20% of ethylene glycol, 20% of 1,2-propanediol, 1% of polyvinyl alcohol, 0.5 M of sucrose. Several procedures for exposure to VS were developed. Spheroids were also cryopreserved by conventional method ($1^\circ\text{C}/\text{min}$ cooling under the protection of 10% of Me_2SO). Viability (FDA/EB staining), metabolic activity (Alamar Blue assay), adhesion ability were assessed both after exposure with VS and complete vitrification procedure, or conventional cryopreservation. Multistep exposure with VS was more beneficial compared to 2-step procedure. 3000 cells-spheroids were more resistant compared to 1000 cells-spheroids and showed metabolic activity of $65.0 \pm 0.4\%$ from control group index after exposure, $61.3 \pm 0.3\%$ after vitrification, $62 \pm 0.5\%$ after conventional cryopreservation. Ratio of viable cells per preserved spheroid was higher after

conventional cryopreservation, but amount of preserved spheroids able for attachment was higher after vitrification. MSCs-based spheroids were found to be a sensitive object for slow cooling injury factors as well as for vitrification solution toxicity. Both vitrification and conventional protocols should be further improved for better preservation of MSCs-based spheroids.

Funding: This study was supported by National Academy of Sciences of Ukraine as part of scientific work № 0123U102365/№1 23.01.2023, and by National Research Foundation of Ukraine (project № 2021.01/0276)

Conflict of Interest: None to disclose
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P60 EFFECT OF A SYNTHETIC ANALOGUE OF NEUROPEPTIDES WITH OPIOID ACTION ON THE FUNCTIONAL INDICATORS OF L929 CELLS DURING COLD STRESS

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The search for compounds capable of preventing cell damage caused by cold stress and finding out the ways of implementing their protective effect is an urgent issue of cryobiology. It is promising to use neuropeptides with opioid action as such protective compounds, in particular, synthetic leu-enkephalin H-Tyr-D-Ala-Gly-L-Phe-Leu-Arg-OH (dalargin), which has a wide range of regulatory activity in relation to different tissues of the body. The aim of this research was to study the effect of dalargin on the functional activity indicators

of the L929 line fibroblasts exposed to cold stress. The protective effect of dalargin under conditions of cold stress was assessed by adhesive potential (by the number of attached cells), metabolic potential (by MTT assay) and proliferative potential (by the formed monolayer area and by the restored monolayer area with the use of "scratch" model). To study the mechanism of action, a competitive blocker of opioid receptors, naloxone, was used, which was added to the incubation medium of cells at the same time as dalargin before simulating cold stress. It was established that cold stress led to a decrease in the proliferative, adhesive and metabolic potential of cells by 4, 2 and 3 times respectively compared to native cells. Dalargin adding to the culture medium restored the functional indicators of cells to native values. Simultaneous cultivation of the cells exposed to cold stress with naloxone and dalargin led to a decrease in the neuropeptide protective effect that indicates the receptor pathway of its action. The performed research can be used to improve the formulation of solutions for storage of organs and cells under hypothermia conditions by introducing neuropeptides into their composition.

Funding: Not applicable

Conflict of interest: None to disclose

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P61 EFFECT OF DALARGIN OF CARBOHYDRATE METABOLISM IN RATS UNDER ACUTE COLD EXPOSURE

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Acute cold stress (ACS) has extensive and complex effects on humans and animals. It easily induces a series of adaptive changes in the energy homeostasis system that leads to a change in metabolism rate and body temperature. Homeostasis is regulated by the neuroendocrine system under cold exposure, therefore neuropeptides have eminent roles in the cold adaptation. Some neuropeptides were shown to have an apparent influence on energy metabolic rate and can suppress or enhance heat loss. Although many studies of the effects of various neuropeptides on metabolism and body temperature have appeared recently, lots of questions remain concerning the role of opioids in thermoregulation. Therefore, this study aims to establish the effect of synthetic leu-enkephalin (Dalargin) on carbohydrate metabolism in rats under acute cold exposure. The studies were performed in rats after ACS (rectal temperature 20°C) and subsequent warming. Effect of Dalargin on the rate of changes in rat's body temperature during cold exposure and rewarming were assessed. Subsequently, blood samples were used for determination of glucose, lactate and pyruvate content in serum. MTT test was performed with phytohemagglutinin on PBMCs. It was shown that pre-introduction of Dalargin to rats led to acceleration of body temperature recovery after ACS (1.4-fold, $p<0.05$). Dalargin affected the rate and direction of carbohydrate metabolism responses in rats under ACS, that was confirmed by decrease in lactate (1.6-fold, $p<0.05$), increase in pyruvate (1.4-fold, $p<0.05$) as well as by 2-fold reduction in lactate/pyruvate ratio ($p<0.05$) in serum. In addition, increase in metabolic activity of rat's PBMCs according to the MTT test (1.8-fold, $p<0.05$) at use of Dalargin before ACS was observed. Thus, the study results showed a protective Dalargin effect on carbohydrate

metabolism indices and intensity of blood cells metabolism in rats after ACS. The obtained data may indicate a possible protective role of opiates in thermoregulation during cold stress.

Funding: Not applicable

Conflict of interest: None to disclose

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P62 CRYOPRESERVATION OF MOUSE AORTA RINGS BY LIQUIDUS TRACKING

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Liquidus Tracking is a promising approach for the cryopreservation of large samples. This technique is based on the increase in the concentration of cryoprotectant while the temperature decreases. This implies the reduction of cryoprotectant toxicity to cells and tissues. Nowadays, 3D cells cultures and human cartilage had been recovered with standard liquidus tracking, but the cryopreservation of whole organs is still a challenge. In consequence, an approach to the cryopreservation of organs was attempted by our group, using murine aorta rings. The stepwise system developed has shown great success in the cryopreservation of the nematode *Caenorhabditis elegans*, an excellent animal model for memory-related studies. For this work, the setup was adapted for mouse aorta rings. This involved the design and manufacturing of a cryo-reactor specifically for our purpose. The protocol uses Ethylene Glycol (EG) as cryoprotective agent to reach deep hypothermia, with

several final temperatures, up to – 20 °C. Our protocol, with the necessary holding times during cooling, guarantees the equilibration and diffusion of the cryoprotectant solution into the muscular and endothelial tissue layers of the aorta. The ice crystals formation was thoroughly monitored during the cooling process. The samples were rewarmed at room temperature (22 °C). Viability was assessed upon cell count against fresh samples using Trypan Blue. A viability of 45% was achieved with no ice formation inside the cryoreactor. These results are a promising step towards the cryopreservation of tissues and organs.

Funding: Not applicable

Conflict of Interest: None to disclose

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P63 GOLD NANOPARTICLES – ANTIFREEZE PROTEIN COMPLEXES AS A NANOPLATFORM FOR EFFICIENT CRYOPRESERVATION

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Cryopreservation of three-dimensional (3D) tissues and tissue-engineered constructs (TECs) needs optimization of a variety of parameters ensuring successful preservation of cell functionality, tissue integrity, cell-cell as well as cell-matrix interactions during freeze-thaw (slow freezing) and cooling-warming (vitrification) cycles. Here we report on the development of complexes consisting of gold nanoparticles (AuNPs) and type III antifreeze proteins (AFPs III) as a unique nanoplatform for the protection of cells and tissues from cryoinjury. The research is based on the specific process of “protein corona” formation onto the surface of AuNPs ($D \approx 15$ nm, produced by the Turkevich method). Two types of nanoplatforms were obtained in this work, where AFPs III were covalently and non-covalently conjugated with AuNPs. The structural properties of the AuNPs-AFPs III complexes were studied by electron microscopy, dynamic light scattering, optical spectroscopy, and others. *In vitro* studies with NIH 3T3 cells were conducted to reveal cytocompatibility of AuNPs and AuNPs-AFPs III complexes using resazurin reduction assay. Moreover, such complexes were evaluated towards cryopreservation of alginate macrocapsules (volume ≈ 15 μ l) as model 3D TECs. The results indicate that both AuNPs-AFPs III complexes are stable in aqueous solutions providing the interaction between AuNPs and AFPs III. When cultivated with NIH 3T3 cells, both studied complexes (AuNPs concentration of 1 mg/L) showed no apparent acute cytotoxicity over 3 days, as confirmed by the resazurin reduction assay. Finally, application of AuNPs and AFPs III provided positive effect on the structural integrity of 3D alginate hydrogels after cryopreservation. In summary, the developed AuNPs-AFPs complexes could further contribute to the development of efficient cryopreservation protocols for cell and tissue long-term storage.

Funding: Lower Saxony Ministry of Science and Culture (“NanoCelltic”)
Conflict of Interest: None to disclose
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P64 NEPHROPROTECTIVE EFFECT OF LEI-ENKEPHALIN IN DEEP HYPOTHERMIA APPLICATION

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The purpose of this experiment was to study the effect of deep hypothermia on the kidneys of rats with modeled chronic nephropathy as well as to investigate the nephroprotective effect of synthetic leu-enkephalin dalargin. To simulate the chronic nephropathy in rats, they were kept on a diet high in fat and sodium and calcium salts for four weeks. The development of nephropathy was concluded on the basis of data from periodic studies of the lipid profile of the blood plasma and the filtering capacity of the kidneys. In week 4 the rats were induced with systemic inflammation by injection of bacterial lipopolysaccharide. On day 28 of the experiment, a deep hypothermia was induced in the animals by cooling to a rectal temperature of 20 °C. The obtained results showed that hypothermia in rats with a nephropathy model leads to a significant deterioration of the filtration capacity of kidneys. A significant increase in the relative content of fragmented DNA in the homogenate of the cortical layer of kidneys was also found (23% vs. 6%). The administration of the synthetic leu-enkephalin dalargin to rats before the start of cooling helps to preserve the structure and

filtering capacity of the kidneys of experimental rats at the levels of the corresponding indicators of animals in which chronic nephropathy was modeled, but deep hypothermia was not induced (the control group). The simultaneous administration of the opiate receptor antagonist naloxone to animals suppresses the protective effect of dalargin, which indicates the opiate receptor-mediated effect of the latter.

Funding: Not applicable

Conflict of Interest: None to disclose

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P65 APPLICATION OF 4-DIMETHYLAMINOFLAVONOL FOR THE STUDY OF MEMBRANE STRUCTURES IN CRYOPROTECTIVE SOLUTIONS

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The study of the molecular mechanisms by which cryoprotectants (CPs) act on biomembranes is important for understanding their protective effects and for developing effective cryoprotective media for preservation of biological specimens. Valuable information about such mechanisms can be obtained using fluorescent probes. We evaluated the possibility of using the fluorescent probe 4-dimethylaminoflavanol (DMAF) for studies in cryoprotective solutions, and also used this probe to study the interaction of low molecular weight CP belonging to amide and diol classes (glycerol, ethylene

glycol, 1,2-propanediol, butanediol isomers, Me₂SO, dimethylformamide, dimethylacetamide) with artificial and natural membranes. Studies have shown that the features of the chemical structure and spectral properties of DMAF make it an indispensable tool for studying biomembranes and cells in the presence of CP, the main component of cryobiological systems. Due to the high efficiency of probe quenching with water, the DMAF signal bound to lipid bilayer exceeds the probe fluorescence intensity in a solvent (water or aqueous CP solutions) by 10 and more times. This makes it possible to study structural rearrangements of the lipid component of biomembranes directly in the presence of CP. The studied CPs were found to have a modifying effect on model lipid membranes formed from phosphatidyl choline, which depends on the chemical structure of CPs, their hydrophilic-hydrophobic properties and temperature. Of the CPs studied, dimethylformamide, Me₂SO, and 2,3-butanediol had the strongest effect on DMAF fluorescence parameters. These substances competed most strongly for the DMAF binding sites to the lipid bilayer at the beginning of the anisotropic zone. The high affinity of DMAF to the lipid component of biomembranes allows it to be used to study the mechanisms of interaction of CP with the lipid bilayer by the hydrophobic mechanism allows it to be recommended for the primary screening of CP for suitability for cell cryoprotection.

Funding: The National Academy of Sciences of Ukraine

Conflict of Interest: None to disclose

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P66 HIGH RECOVERY RATES OF PRESERVED CAENORHABDITIS

ELEGANS THROUGH OPTIMIZATION OF CRYOPROTECTANT SOLUTION AND IMPROVED REWARMING

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Caenorhabditis elegans is an important animal model in molecular and developmental biology. Its cryostorage is essential to maintain the different lines used for research. The traditional cryopreservation for *C. elegans* described by Brenner is based on slow freezing and the use of 15% glycerol (Gly). After thawing at room temperature, 35% of the L1-L2 larval stages are recovered, but adults are practically impossible. With the ultra-rapid vitrification technique, survival rates above 90% are achieved, but handling is different. In a previous work (CRYO2022), the cryoprotection solution was optimised using ethylene glycol (EG) and EG with trehalose (TRE). The results were compared with the use of Gly. Three groups of cryoprotectants (CPAs) were tested: Group 1: 15% Gly in S. Buffer, Group 2: 15% EG in S. Buffer and Group 3: 7.5% EG + 0.15 M TRE in S. Buffer. Larvae and adults were cryopreserved by slow freezing. The cryovials were rewarmed at room temperature (air) for 30 min and then washed with S. Buffer, obtaining the following results. Group 1: survival rate of heterogeneous population (SRp) was 25% and in adults (SRa) 0%, in Group 2: SRp (60%) and SRa (3%), in Group 3: SRp (50%) and SRa (15%). In this study, using the same experimental groups, the results are improved by modifying the rewarming strategy. In this case, the vial is rewarmed by shaking in a water bath at room temperature

for 3 min achieving the following results. In Group 1: SRp (25%) and SRa (0%), in Group 2: SRp (80%) and SRa (4%) and in Group 3: SRp (69%) and SRa (8%). In addition to using the optimized cryoprotective solutions, the best rewarming protocols for *C. elegans* larvae is in a water bath (80% SRp) and for adults in air with a subsequent washing (SRa of 15%).

Funding: Not applicable

Conflict of Interest: None to disclose

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P67 INNOVATIVE CRYOPRESERVATION TECHNOLOGY FOR AUSTRALIAN MACADAMIA

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Macadamia is an economically important nut crop that is native to Australia and valued at over AU\$3 billion globally. Most commercial trees originated from a very narrow gene pool selected from hybrids of two edible species: 1) *Macadamia tetraphylla* and 2) *Macadamia integrifolia*. The wild germplasm of *macadamia* is extremely valuable for breeding for future climates/consumer needs, however, is suffering from loss due to habitat destruction. Traditional conservation methods such as seed banking are unfavourable for *macadamia* as seeds are cold-sensitive and cannot be stored at -20 °C. Moreover, *macadamia* is highly out-crossing, therefore

seed conservation is of little value in commercial cases as the seeds are not true to type. Cryopreservation of macadamia using true-to-type shoot tips is a potential tool to conserve and effectively maintain *macadamia* germplasm collections in the long term. This study aimed to establish a proof-of-concept cryopreservation protocol for *macadamia* cultivars using shoot tips. *In vitro* donor plants of *macadamia* cv. 'Beaumont' were either pre-treated at 10 °C for 7 days or left at 25 °C (untreated controls). Shoot tips were dissected from donor plants to a size of 1 x 2 mm under the microscope in a laminar flow. For cold-pre-treated plants, a survival of 83.33 % and a regrowth of 66.67 % was achieved after 4- and 8 weeks post cryoprotectant application. This was an increase of 40% relative to untreated controls. This indicates the importance of cold pre-treatment for *macadamia* shoot tips after cryoprotectant treatment. Trials are underway to immerse cold pre-treated shoot tips in Liquid N₂ with the droplet vitrification method. This study provides a foundation for the preservation of diverse *macadamia* germplasm to support the conservation of commercial cultivars and future breeding and advances in the *macadamia* industry.

Funding: This project is being funded by the Australian Research Council Linkage program in partnership with the Department of Agriculture and Fisheries, Royal Botanic Gardens and Domain Trust, MacQ Pty Ltd, Aurora Macadamias Pty Ltd, MACS Network Operations Pty Ltd, Bush Holdings Pty Ltd, Macadamia Conservation Trust and the Australian Macadamia Society Ltd. Xueying Li is supported by an Australian Government Research Training Program Scholarship administered by The University of Queensland.

Conflict of Interest: None to disclose

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P68 EFFECT OF ANTIOXIDANT VITAMIN E AND CANOLA OIL ON STALLION'S SPERM PARAMETERS

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Recently, antioxidants are used to treat seminal oxidative stress and infertility. Vitamin E has been shown to have antioxidant properties which counteract these destructive effects. Canola oil decreased metabolic disorders that may play an important role in sperm metabolism and maturation. The aim of this study was to investigate the antioxidant properties of vitamin E and canola oil on sperm characteristics of stallion. In this study, 12 adult stallions (over three years old) with an average weight of 400±30 kg were treated for each group for 28 days. This experiment consisted of 4 treatments with 3 replications and the treatments were included: vitamin E (900 IU/ day) and canola oil (50 ml/day) and a combination of vitamin E + Canola oil as well as a control group. After semen collection by AV at 14 and 28 days of study, sperm parameters (progressive motility, morphology and viability) were evaluated by using CASA software, Papanicolaou and Eosin - Nigrosin staining, respectively. The results of this study shows that sperm progressive motility was increased in the groups of vitamin E and canola oil compared to the control group but not significant. Sperm morphology was high significant difference in vitamin E group ($p < 0.05$). Sperm viability showed the higher value in treatment groups than the control group but wasn't significant. The results of this study

concluded, antioxidant properties of vitamin E and canola oil increase the sperm motility in stallion. Also, antioxidants keep fats out of the reach of peroxidation, thereby protecting sperm membranes and increasing sperm morphology and viability. Canola oil also plays a role in energy metabolism and caused improvement of sperm motility in stallions.

Funding: Not applicable

Conflict of Interest: None to disclose

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P69 BOAR SEMEN COOLING IN AN EXTENDER WITH LDL, QUERCETIN, OR RESVERATROL

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Low-density lipoproteins (LDL), quercetin (Q) and resveratrol (R) have been used for sperm preservation to improve sperm quality in different species; however, little has been studied for cooling of porcine semen. This study aimed to evaluate the cooling of boar semen in an extender with LDL, Q or R. Fifteen boar semen samples were extended in a BTS extender supplemented with the treatments: LDL at 6%, Q at 10 μ M (Q10), 30 μ M (Q30) and 50 μ M (Q50), or R at 10 μ M (R10), 30 μ M (R30) and 50 μ M. A control without supplementation was included. Semen was stored by cooling at 16°C for 96 h. Every 24 hours, sperm motility and kinetics were evaluated using a computer-assisted sperm analyzer (IVOS). At 24 and 96 h of cooling, functional

membrane integrity and total antioxidant capacity (TAC) of semen were evaluated using hypoosmotic test and FRAP assay, respectively. At the same times, reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\psi$ M), lipid peroxidation, and vitality of sperm were evaluated by flow cytometry with DHR123, JC-1, C11-BODIPY(581/591) and SYBR14/PI probes, respectively. Statistical analysis was performed using linear models and the Tukey's test. LDL improved total and progressive motility at 24 and 48 h of cooling. Q10 and Q50 decreased ROS at 24 h. LDL, Q10, Q30, and Q50 increased TAC at 24 and 96 h. Q10 and R10 increased the live non-peroxidized spermatozoa at 24 and 96 h, respectively. LDL, Q10, Q30, and Q50 modulated mitochondrial activity by reducing high- $\Delta\psi$ M sperm and low- $\Delta\psi$ M sperm, at 24 and 96 h of cooling. It is concluded that LDL and quercetin can improve the motility, vitality, and antioxidant capacity of boar semen, through the reduction of oxidative stress and the modulation of mitochondrial activity during cooling.

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Conflict of Interest: None to disclose

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P70 EVALUATING A CO₂-BASED CRYOABLATION DEVICE FOR CANCER TREATMENT IN LOW RESOURCE SETTINGS: A BENCHTOP COMPARISON AND IN VIVO VALIDATION STUDY IN VETERINARY MODELS

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This study evaluated the efficacy of a novel, low-cost CO₂-based cryoablation device for cancer treatment, particularly breast cancer, in low-resource settings. The device was evaluated in an *in vivo* efficacy validation study in client-owned pets and a bench comparison study to a commercially available Argon-based system. The study's objective was to demonstrate that, despite taking longer time to freeze, equivalent treatment outcomes can be achieved in both fast and slow cooling systems. In the efficacy validation, 7 client-owned dogs with spontaneous occurring masses, including epithelial cysts, lipomas, soft tissue sarcomas, and mammary carcinoma, were treated with the CO₂-based cryotherapy device. Cryoablated tissue was surgically resected between 7-14 days after initial treatment and analyzed histopathologically. Necrosis was evident in all cryoablated tissue with the extent varying by tumor type. The novel device was also compared to a commercially available Argon-based system in an ultrasound gel tissue phantom with a heat sink to investigate the iceball temperature profile at lethal isotherms inside the iceball. Both devices were used to form a typically sized iceball in a heated ultrasound gel tissue phantom under heat sink conditions, and the iceball temperature distribution was measured at 4 anticipated clinically relevant locations within the iceball. The bench results showed that the CO₂-based device achieved clinically established lethal isotherm sizes despite taking 44 minutes, while the Argon-based device took 8.5 minutes. The bench results will be correlated to *in vivo* necrosis outcomes in an ongoing study with 12 veterinary subjects with mammary masses cryoablated with Argon or CO₂ -based devices to further validate the hypothesis. In

conclusion, the low-cost CO₂ based cryotherapy provides a potential for a minimally invasive technique for both veterinary applications and low resource breast cancer treatment.

Funding: NIH/NCI, TEDCO Maryland Innovation Initiative

Conflict of Interest: None to disclose

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P71 A RAPID APPROACH FOR THE SELECTION OF CANDIDATE PROTEINS FOR CELL CRYOPRESERVATION

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Cryopreservation is an essential procedure for the long-term storage of mammalian cells and other cellular structures such as spheroids and organoids. An efficient storage procedure helps maintain an adequate supply of the biological materials that can later be used for various applications such as reproductive, regenerative and transfusion medicine, human transplantation and drug toxicity testing. Efficient cryopreservation requires that specific protocols are established for the various cell types and cellular structures to maintain viability and functions upon thawing after ultra-low temperature storage. These protocols invariably use cryoprotectants added to the freezing medium to minimize freeze-thaw damage. The most widely used cryoprotectant is dimethyl sulfoxide (Me₂SO), but this chemical is suboptimal because it decreases post-thaw viability,

certain metabolic functions, and attachment efficiency of cells. To find more effective and less toxic alternatives for cryopreservation of mammalian cells, we are developing protocols in which recombinant wheat proteins are used instead of Me₂SO in the cryopreservation medium. Since testing with mammalian cells is tedious, we sought to determine if we could use *Escherichia coli* as a model system for preliminary cryoactivity analysis. For this, we used the TaENO enolase protein, which was shown to be an efficient cryoprotective agent for rat hepatocytes and insulin-secreting human cells. Previous analysis showed that TaENO can prevent the growth of ice crystals upon freezing, but the protein does not possess known ice-binding domains. As a first step to identify the cryoactive domains, various fragments were generated and tested for their ability to protect *E. coli* against freeze-induced death. Preliminary results showed that the C-terminal portion of the protein provides survival after thawing, and we are in the process of narrowing down the sub-regions that are responsible for the protection. Our approach should prove useful to accelerate the identification of proteins and proteins fragments that possess cryoprotective activity.

Funding: This work was supported by research grants from the Natural Sciences and Engineering Research Council of Canada, and from Médicament Québec.

Conflict of Interest: None to disclose

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P72 EFFECTS OF DIFFERENT ETHYLENE GLYCOL CONCENTRATIONS ON THE POST-THAWING QUALITY OF CRYOPRESERVED RAM SEMEN

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This study aimed to evaluate the effect of different concentrations of ethylene glycol (EG) in Tris egg yolk extender for frozen-thawed ram semen. Twenty-five ejaculates were collected from five mature Najdi rams using an artificial vagina. The tris egg yolk extender was mixed with three different concentrations of EG (3%, 5%, and 7%). The accepted ejaculates were gradually diluted (1:4) with Tris egg yolk EG diluents (3%, 5%, and 7%), gradually cooled to 5 °C and kept for four hours in a cold cabinet before being loaded into 0.25 mL straws. Straws were then arranged horizontally and suspended in liquid nitrogen vapor within a foam box (45 x 32 x 18 cm) containing liquid nitrogen at a height of 5 centimeters above liquid nitrogen (120 °C) for 20 minutes before being dipped in liquid nitrogen (196°C). Semen motility and kinematic parameters were assessed by Computer Assessment Semen Analyzer (CASA) after thawing. There was a significant ($P<0.05$) difference between the curvilinear velocity (VCL), straight-line velocity (VSL), average pathway velocity (AVP), lateral head displacement (ALH), and beat frequency (BCF) at different concentrations (3% and 5%). In contrast, the parameters were not significantly ($P>0.05$) different when 3% and 7% concentrations of EG were compared. The highest percentage of fast progressive motility was observed at 3%, while the lowest value was observed at 7%. The slow, rapid, medium, and static progressive motilities were least in 3% concentration when compared with others (5% and 7%). Similarly, the total progressive motility was highest at 3% concentration compared to others. The percentage of total motility observed in the 3% concentration was

significantly ($P < 0.05$) higher than the other concentrations (5% and 7%). In conclusion, 3% EG mixed with Tris egg yolk provided the best ram semen quality regarding motility after freezing compared to other concentrations.

Funding: This project was funded by the National Plan for Science, Technology and Innovation (MAARIFAH), King Abdulaziz City for Science and Technology, Kingdom of Saudi Arabia, Award Number (13-BIO2462-02).

Conflict of Interest: None to disclose

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P73 TRANSCRIPTOME CHANGES IN SHOOT MERISTEMS OF *MUSA* DURING DIFFERENT STAGES OF CRYOPRESERVATION

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The molecular and biochemical changes during stages of cryopreservation is not critically investigated especially in *Musa*, a non-model species. In this study, proliferating meristems of *Musa* AAA cv. 'Borjahaji' (in vitro explants) were cryopreserved using droplet-vitrification. According to earlier experiment, the mean shoot regrowth after 60 min PVS2 exposure was 95.0% while in PVS2+LN exposure, it was 66.7%. Suites of transcripts that are up- or downregulated in response to stresses during different cryopreservation stages were investigated from eight cDNA libraries including duplicates for C (stock cultures), T1 (high sucrose pre-cultured), T2

(vitrification solution treated) and T3 (Liquid Nitrogen treated) meristem tissues. Heatmap represent the cluster analysis of 70 commonly expressed genes out of which 34 are up regulated and 36 are down regulated in all the treatments (T1 vs. C, T2 vs. C and T3 vs. C). The up-regulated and down-regulated DEGs at each stage comprised 11 up- and 61 down-regulated DEGs in T1 vs. C, 36 up- and 44 down-regulated DEGs in T2 vs. C and 7 up- and 26 down-regulated DEGs in T3 vs. C. According to Gene Ontology, the largest were found in biological process followed by molecular function and the least in cellular components categories in all the three stages of cryopreservation. KEGG pathways analysis shown that genes responsible for all the three stages of cryopreservation stresses were identified by commonly upregulated in all five pathways – Tyrosine metabolism, α -Linolenic acid metabolism, Fatty acid degradation, Pyruvate metabolism and Glycolysis/Gluconeogenesis. From this study, it was observed that a hub of mechanisms which play a key role in plant responses to stresses: the activation of enzymes for desaturation or hydrolysis of fatty acids, sensing the energy status for optimizing plant metabolism, the activation of transcription factors, stress responsive proteins, and signal transduction are involved in stages of cryopreservation stresses.

Funding: Not applicable

Conflict of Interest: None to disclose

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P74 CHARACTERISTICS OF COOLED EQUINE EPIDIDYMAL SPERMATOZOA RETRIEVED AFTER TESTES-EPIDIDYMIS STORAGE AT 5°C

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The present research investigated the effects of storage of testes-epididymis complex at 5°C for 24 hours on quality of retrieved sperm and after cool storage. Twelve pairs of testicles were collected immediately after orchiectomy of stallion from veterinary polyclinic, Udaipur. One testes-epididymis (F1) of each stallion was immediately processed for sperm retrieval (i.e. 0 h storage at 5°C) and second testes-epididymis (F2) complex of each stallion was stored at 5°C for 24 hours. Spermatozoa were harvested from the epididymis by flotation method. Twelve to fifteen incisions were made along epididymis before placing in a petri dish filled with approximately 20 ml of warmed skim milk glucose semen extender. During ten minutes, spermatozoa are allowed to swim out of the epididymis. Thereafter, the remnants of the epididymis were removed and the extender with the semen were collected for further processing. Semen evaluation of both epididymal suspensions F1 and F2 was done. Progressive motility 24.25±1.13 and 19.92±1.02% ($p \leq 0.01$); Live sperm (%) 85.67±1.69 and 82.75±1.109%; HOST 73.92±1.54 and 70.25±1.59%; acrosomal integrity 72.92±1.23 and 71.83±0.73% and total sperm abnormality 62.17±3.78 and 68.25±1.47% for F1 and F2, respectively. First epididymal sperm suspension F1 after 24 h of storage cooling (5°C) had PM 22.75±0.92%, viability 75±1.53%, HOST 61.5±1.79%, acrosome integrity 70.5±1.23% and total sperm abnormality 73.84±0.82%. Second epididymal sperm suspension F2

after 24 h of storage cooling (5°C) had PM 22.92±1.52%, viability 70.58±1.10%, HOST 61.09±2.49%, acrosome integrity 68±0.78% and total sperm abnormality 77±1.32%. It is concluded that maintenance of sperm quality parameters after epididymis storage at 5°C for 24 hours enables the storage and transportation of the epididymis for a period of 24 hours. Further, maintenance of sperm parameters after cooling stallion epididymal sperm for 24 hours would therefore results in a more reliable number of sperm doses for insemination.

Funding: From RAJUVAS research funds

Conflict of Interest: None to disclose

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P75 SOME FEATURES OF SLEEP IN WINTERERS AT THE ANTARCTIC STATION

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Participants of Antarctic expeditions often experience "Antarctic syndrome", one of the symptoms of which is a violation of the normal rhythm of sleep, which can negatively

affect the efficiency of winterers' work and their health. Temperature plays an important role among the factors influencing sleep homeostasis. Therefore, in our opinion, it is crucial to take into account the exposure to cold on any disturbances in human homeostasis during an extended stay in Antarctica.

Participants of the 21st, 23rd-26th Ukrainian Antarctic expeditions (54 men and 4 women, average age 38) took part in the study. Self-testing of the winterers was carried out quarterly using the IDS-S30 questionnaire and the Munich Chronotype questionnaire. Volunteers also monitored sleep patterns using portable gadgets. The analysis showed that the dynamics of subjective problems of sleep quality were characterized by individual characteristics, but the peak values of its disturbances (speed of falling asleep, duration of sleep, too early awakening, excessive sleep) fell in the period of the middle of the Antarctic winter. It should also be noted that the winterers, regardless of age and professional activity, had quasi-periodic changes in the total duration of daily sleep representation, the cause of which was not clear. Further research may help clarify the problem of treating the manifestations of the "Antarctic syndrome" in winterers, associated, among other things, with the processes of negative changes in the sleep-wake cycle, which can lead to the accumulation of fatigue with a subsequent increase in stress load on the body with all the adverse aspects of this phenomenon. We are very grateful to the National Antarctic Science Center of the Ministry of Education and Science of Ukraine for the opportunity to conduct the research.

Funding: Not applicable

Conflict of Interest: None to disclose

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P76 AI BASED SYSTEM FOR CREATING FREEZING PROTOCOLS FOR CELL CRYOPRESERVATION

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Artificial intelligence (AI) has grown rapidly in recent years, progressing from the experimental to the implementation phase in a variety of sectors, including medicine. Cryopreservation of oocytes and embryos will benefit from mathematical models. Algorithms based on neural networks and ant colonies will aid in understanding the pattern vitrification and behaviour of cryoprotectants of live cells. Chilling cells too quickly results in the production of fatal intracellular ice, whereas chilling too slowly enhances the harmful effects of the cryoprotective agents (CPA) given to slow down ice formation. To interpret and quantify these data, we develop a mathematical model for cell cryopreservation. The system is assumed to have a spherical geometry with three distinct regions: ice, extracellular liquid media, and cell. The two interfacial boundaries that separate the three zones are subject to movement and must be determined as part of the solution. The presence of CPA reduces the system's freezing point, and the cell membrane shifts as a result of the osmotic pressure difference across the membrane. We utilize a combination of numerical and asymptotic approaches to determine how the temperature, CPA concentration, and concentration of an ion species both within and outside the cell evolve during cooling over a variety of cooling rates and time scales. We illustrate how, given cell characteristics and the cryopreservation process's operating procedure, damage measures can be utilized to forecast an ideal cooling rate. This research has the potential

to influence cryopreservation procedures for freezing single cells. Our methodology enables us to account for the spatial variability inherent in the system at quicker cooling rates, as well as to systematically shrink the system over timelines.

Funding: Not applicable

Conflict of Interest: None to disclose

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P77 EFFECTS OF VITRIFICATION ON APOPTOTIC GENES IN CUMULUS OOCYTE COMPLEXES OF SHEEP

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COCs were collected from both vitrified and non-vitrified experimental group, to investigate apoptotic gene expression using qRT-PCR. The cryopreserved Oocytes were thawed directly for gene expression study. We have compared the gene expression in fresh vs. cryopreserved cocs. The real time PCR results for the comparative expression of the apoptotic gene using mRNA abundance showed the down regulation of BAD, BCL2A1 and MCL1 and expression of BCL 2, BAX and BOK are similar in both vitrified and non-vitrified groups. However BID and BAK were up regulated. BAK is not directly related to inducing apoptosis however it help in release of cytochrome C from mitochondria, whereas BID activates BAX, BCL-2 can bind and inhibit BID ability to activate BAX, as a result anti-apoptotic BCL-2 proteins may inhibit apoptosis by sequestering BID leading to reduced BAX activation which nullify the up regulation of BID and BAX to induce apoptosis in sheep oocyte. In addition, P53 was also down regulated proves vitrification safe for

preservation of immature oocytes in ovine. It was observed that vitrification did not affect the morphology and apoptotic genes expression in ovine cumulus oocyte complexes. Vitrification is suitable technique to cryopreserve the ovine oocytes for further use in assisted reproduction technology for production of superior animals in future.

Funding: Not applicable

Conflict of Interest: None to disclose

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P78 FIELD ASSISTED CRYOPRESERVATION (FAC) OF MOUSE GERMINAL VESICLES (GV) REGULATES REACTIVE OXYGEN SPECIES (ROS), H₂O₂, AND O₂ LEVELS IN MATURE OOCYTES DERIVED FROM VITRIFICATION

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One of the highest interesting options to protect endangered species and maintain infertility ability in humans is oocyte vitrification. A set of different chemical and physical stresses during vitrification and warming causes a reduction in the survival and oocyte maturation rates. Researchers have always been staying on the road to recover oocyte vitrification approaches to halve cryoinjuries. Recently, magnetic fields have been applied to cryopreserve different biological samples. Here, we studied the effects of 20 mT static magnetic field (SMF) on reactive oxygen species (ROS), H₂O₂, and

O₂ levels in mouse MII oocytes derived from vitrification. The GV oocytes were collected from 6-8 weeks-old NMRI mice and divided into fresh (nVit), vitrified with (Vit_SMF) or without magnetic field (Vit) groups. Vitrification was performed like Baniasadi et al. procedure. In the group of vitrification under a magnetic field, the oocytes were placed in the center of the field in all vitrification and warming stages. After post-warming recovery, the oocytes were transferred to the in vitro maturation medium (IVM) for the resumption of meiosis. In addition, DCFH_DA, and DHE kits were used to assess reactive oxygen species (ROS), H₂O₂, and O₂ levels of MII oocytes derived from vitrification. The H₂O₂ level in MII oocytes belonging to the vitrified (Vit) group without magnetic field was increased significantly compared to fresh oocytes (nVit). H₂O₂ levels in Vit_SMF were significantly higher than in nVit oocytes and decreased insignificantly compared to Vit oocytes. O₂⁻² level in vitrified MII oocytes under SMF was significantly lower than in Vit oocytes. However, its level was not significantly different between Vit_SMF and nVit groups. Using SMF for the vitrification of mouse GV oocytes reduces cryoinjuries as well as regulates reactive oxygen species (ROS) levels in the produced mature MII oocytes.

Funding: Not applicable

Conflict of Interest: None to disclose

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P79 POSSIBILITIES OF LOW TEMPERATURES APPLICATION ON WOUNDS AFTER BLAST INJURY

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Metabolic disorders accompany blast trauma with a shift in metabolic processes towards catabolism and a decrease in the utilisation of the primary endogenous substrates. We evaluated the application of using cryo-irrigation for wounds treatment immediately after blast injury. Low-temperature impact on the wound during blast injury helped to reduce the imbalance between the processes of catabolism and anabolism, as well as normalise the utilisation of body waste products. In the first hours (1 and 4 hours), there was an increase in the activity of the use of amino acids in the processes of anabolism, which was confirmed by an increased level of urea. On the other hand, a negative nitrogen balance showed an anxiety stage of the general adaptation syndrome. The decrease in lactate level showed a reduction in the severity of acidosis. A day after cryo-irrigation of the wound, the onset of the resistance phase of the general adaptation syndrome was noted, which was confirmed by the level of urea. The predominance of the aerobic way of obtaining energy over the anaerobic was observed, which was confirmed by a decrease in the activities of creatine kinase and lactate dehydrogenase. Cryo-irrigation lasting 60 seconds had a more pronounced effect on metabolism. Cryo-irrigation stimulated the increase in the activity of catabolism due to an increase in the processes of anabolism, which contributed to the onset of the resistance phase of the general adaptation syndrome in a shorter period. Cryo-irrigation of wounds after blast injury reduced the severity of metabolic disorders and increased anabolic activity. The effect depends on duration (30 or 60 seconds), which would make it possible

to develop an appropriate clinical protocol for cooling wounds after blast injury, depending on the wounds' characteristics.

Funding: Not applicable

Conflict of Interest: None to disclose

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P80 INTERFACIAL ADSORPTION MECHANISM OF FFIBP ICE-BINDING PROTEIN WITH GOLD NANOPARTICLE SURFACE (111): INSIGHTS FROM ATOMISTIC SIMULATIONS

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Microbial Antifreeze Proteins (AFPs) are suitable candidates for industrial purposes due to their potential for cost-effective production. Gold nanoparticles (AuNPs) have unique physical and chemical properties that can be used as a suitable substrate to stabilize and protect the activity of proteins, so it is necessary to study the surface adsorption mechanism of AFPs with gold nanoparticles. In this study, we used the molecular dynamics (MD) simulation technique to investigate the adsorption mechanism of FfIBP as a bacterial AFP on the surface of AuNPs. To study the adsorption mechanism and orientation of the three A, B, and C faces of FfIBP, three

simulation boxes were designed, and each face was placed at a distance of 2.5 nm from the AuNP surface. The simulations were performed by GROMACS 2018.4 software for 300 ns with the CHARMM36 force field. Analysis of the distance along the Z-axis between the protein residues and the atoms of the AuNP surface showed that the systems with the A and B faces above the AuNP surface have a similar mechanism for interacting with the AuNP surface. This absorption was from the region between the A and B faces of the FfIBP protein. In the absorption of two systems with A and B faces, T175, S176, A199, and G200 residues were common residues in absorption. In the system where the C face was located above the AuNP surface, absorption was through the C face and showed the lowest diffusion coefficient among the other two faces. Previous studies have reported the B face as the main ice-binding surface for FfIBP. As a result, considering the free B face during adsorption for all simulations, FfIBP can be a very suitable candidate for AFPs adsorption on the surface of AuNPs and can be considered for further studies in industrial applications.

Funding: Not applicable

Conflict of Interest: None to disclose

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P81 INVESTIGATING THE ANTIFREEZE EFFECT OF FFIBP PROTEIN FACES ON PRIMARY PRISMATIC PLANE OF ICE THROUGH MOLECULAR DYNAMICS SIMULATION

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Antifreeze proteins (AFPs) are a diverse subset of ice-binding proteins (IBPs) that are found in some eukaryotes and prokaryotes. FfIBP is a microbial AFP consisting of three faces (A, B, and C), which has been identified from the Antarctic bacterium *Flavobacterium frigoris* PS1. In previous studies, the B face was identified as a major ice-binding surface that showed a 90% reduction in activity due to the T251Y mutation in the B region. Meanwhile, C face with the T211Y mutation in the C region showed a 21% increase in antifreeze activity, the cause of which was not investigated. Therefore, it is important to study the interaction of each face of FfIBP with the surface of ice at the molecular level using molecular dynamics (MD) simulations. Three simulation systems with three FfIBP faces (A, B, and C) in the presence of a primary prismatic plane of ice and a control system without FfIBP, were designed and filled with TIP5P water. All simulations were calculated for 200 ns at 269 K with the CHARMM27 force field. The CHILL+ algorithm was used to analyze the growth of ice. Results indicated that in the absence of FfIBP, hexagonal ice growth reached 84% of the total system volume after 125 seconds. However, after 200 nanoseconds, A face showed only 36% growth while B and C face exhibited only 20% growth. The B and C faces have similar antifreezing inhibitory effects, while due to the presence of α -helix in A face and improper orientation on the ice surface, it could not prevent ice growth. The

percentage of interfacial ice for the three faces was around 5.2%-5.6%, and 3.5% for the control system. In conclusion, the results showed the importance of further studies of the binding mechanism of FfIBP faces on ice to better understand the antifreeze activity.

Funding: Not applicable

Conflict of Interest: None to disclose

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P82 VITRIFICATION EFFECT ON MITOCHONDRIAL FUNCTION OF HUMAN EMBRYOS

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mtDNA content in expanded and hatching blastocysts derived from frozen and fresh embryos may be related with their implantation rate. Previous studies showed that mtDNA number can rise in aneuploid embryos and advanced maternal age. Blastocyst maturation requires increasing of mitochondrial oxidative phosphorylation (OXPHOS) and ATP level. TEAD4 gene (TEA domain transcription factor 4) function is important to both pre-implantation (blastocoel formation and expression of trophoctoderm genes) and post-implantation events (implantation and development of placenta). In this study with IR.ACECR.ROYAN.REC.1401.080 IRB

code, we compared expression of (TEAD4) as energy metabolism regulator and RNA Polymerase (POLRMT) as regulator of electron transport chain (in collaboration with TEAD4) in expanded and hatching blastocysts derived from fresh and vitrified eight-cell human embryos. Frozen and fresh eight-cell embryos were cultured into expanded and hatching blastocyst stages. The expression of TEAD4 and POLRMT genes were evaluated by Real-Time-PCR method. In each group, four expanded and four hatching blastocysts were evaluated. Data were analyzed by Mann-Whitney test. POLRMT Expression was not detected in any of experimental groups. No significant difference was observed in TEAD4 gene expression between fresh and vitrified expanded blastocysts ($P > 0.05$). There was no significant difference in expression of TEAD4 between fresh and frozen hatching blastocysts ($P > 0.05$). It can be assumed that no or less changes in the function of mitochondrial electron transfer chain was happened after vitrification or that was a transient change. The fact that POLRMT was not expressed in any of the experimental groups, even with the expression of TEAD4, could be due to the role of TFAM. It is a transcription factor whose over expression inhibits POLRMT even in the presence of TEAD4. On the other hand, the lack of POLRMT expression in all experimental groups can confirm previous studies about the minimal activity of mitochondria in the blastocyst stage.

Funding: Financial support of this project was by Royan Research Institute

Conflict of Interest: None to disclose

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P83 IMPACT OF CRYOPRESERVATION ON HUMAN SPERM QUALITY: A COMPARATIVE

STUDY BETWEEN SEMEN AND PROCESSED SPERM CRYOPRESERVATION

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Despite of several advantages provided by sperm cryopreservation; the post-thawed sperm quality is a matter of concern for several ART procedures. The presence of seminal plasma has become a matter of concern during cryopreservation since it might interfere in the penetration of cryoprotectants to spermatozoa as well as its components as it can vary from person to person. However, there are limited studies, which compared sperm quality obtained from processed sperm cryopreservation and semen cryopreservation. Hence, this study evaluated the advantages and disadvantages of processed sperm cryopreservation immediately prior to insemination. A total of 100 ejaculates (60 normozoospermic and 40 abnormal) from patients undergoing semen analysis were split into 5 parts and subjected to cryopreservation using following combination:

- i) Neat ejaculate (NE): not subjected to cryopreservation.
- ii) DAFT: neat ejaculate cryopreservation followed by density gradient preparation

- iii) DBFT: density gradient preparation prior to cryopreservation
- iv) SAFT: neat ejaculate cryopreservation followed by swim up preparation
- v) SBFT: swim up preparation prior to cryopreservation

Post-thaw spermatozoa from each fraction were assessed for motility, morphology, vitality, mitochondrial integrity, and genetic integrity. Density gradient preparation after cryopreservation improved total motility in normozoospermic samples ($p < 0.001$). But there was no such observation in abnormal samples. On the other hand, swim up preparation after cryopreservation selects spermatozoa with better motility, vitality, morphology, active mitochondria, and DNA integrity in normozoospermic samples ($p < 0.0001$). Similar observation was seen in progressive motility and active mitochondria from abnormal samples ($p < 0.01$). Hence, semen cryopreservation followed by sperm preparation will be beneficial in routine clinical practice.

Funding: Not applicable

Conflict of Interest: None to disclose

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P84 EFFECT OF EXPOSURE TIMES ON THE VIABILITY OF POST-THAW BOVINE IN VITRO PRODUCED BLASTOCYSTS

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The application of frozen embryo transfer in the bovine industry is not popular globally, because of the low pregnant rate compared with a fresh one. The post-thawed embryos are more apoptosis blastomeres that come from damage and toxicity effects after thawing. To explore the optimal exposure times after thawing, just before culture or embryo transfer, post-thawed embryos were exposed for 5, 8, and 10 min before embryo transfer into a recipient uterus by the non-surgical method. 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 total survival rates of 5, and 8 min exposed times are significantly higher than the 10 min group (93.3 ± 1.9 , 93.5 ± 1.6 vs. 85.4 ± 2.7). It means that the longer exposure time should be a negative effect on the post-thaw survival rate, and so the transfer of frozen embryos needed to carry out within 8 min after the thaw. To apply in the field of frozen embryo transfer.

Funding: RS-2023-00237137

Conflict of Interest: None to disclose

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P85 RECENT ADVANCES IN MODELING WORKS FOR CELL CRYOPRESERVATION

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Cell cryopreservation is a vital technique in modern medicine and biotechnology, enabling long-term storage and preservation of cells. Recent advancements in numerical analysis have significantly enhanced the efficiency and effectiveness of cryopreservation. This abstract provides an overview of recent progress in modelling approaches for cell cryopreservation. One key area of advancement is the development of mathematical models that capture the complex thermal and mechanical dynamics during cryopreservation. These models consider specific characteristics of different cell types and incorporate variables like cooling rates, cryoprotectant concentration, and the effects of ice formation. By simulating the stresses experienced by cells during freezing and thawing, these models optimize cryopreservation protocols, enhancing cell survival rates and functionality upon thawing. Another crucial aspect of recent modelling works is integrating experimental data to validate and refine the models. This combination of experimental and computational approaches enables a more accurate representation of the cryopreservation process, considering cell behaviour and cryoprotectant influence. By comparing model predictions with practical outcomes, researchers identify areas for improvement and make necessary adjustments. Furthermore, recent modelling

efforts have addressed challenges associated with large-scale cell cryopreservation, such as freezing cell suspensions or tissue constructs. These models consider heat transfer, cryoprotectant distribution, sample size, and geometry. Understanding cryopreservation complexities on a larger scale helps researchers to develop uniform cooling strategies and minimize cell damage. In conclusion, recent modelling advancements have significantly improved our understanding of cell cryopreservation processes and contributed to more efficient and reliable protocols. Researchers can optimize cryopreservation procedures by incorporating mathematical models and experimental data, also considering large-scale challenges to improve cell viability and functionality. Future research may focus on developing more accurate mathematical models, integrating multi-scale modelling, optimizing cryopreservation protocols, validating numerical models, and integrating uncertainty quantification techniques. These efforts could enhance the efficiency and effectiveness of cell cryopreservation, facilitating advancements in biomedical research and regenerative medicine.

Funding: This work was supported by the National Natural Science Foundation of China (No. 82172114), the Anhui Provincial Natural Science Foundation for Distinguished Young Scholars (No. 2108085J37), and the National Key Research and Development Program of China (2022YFC2703000).

Conflict of Interest: None to disclose

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P86 RESEARCH OF THE FRAGMENTATION LEVEL IN EPIDIDYMAL SPERMATOOZOA OF BOARS

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The analysis of the sperm quality involves the determination of the concentration, mobility and viability of the spermatozoa. However, the determination of sperm DNA integrity provides more prognostic information. The method of determining the integrity of DNA of spermatozoa is the determination of the level of fragmentation. When using cryopreserved sperm in a limited amount, it is important to predict the result for the highest quality use. Methodical approaches to determine the level of DNA fragmentation of spermatozoa were applied to deconserved epididymal spermatozoa of two Ukrainian meat boars, which are stored in the Bank of Animal Genetic Resources. It is this biological material that has genetic value and, unfortunately, is available in limited quantities. Analysis was performed using a Halotech fragmentation kit (Halotech, Spain). Determination of fragmentation was carried out at a magnification of 400 times in three repetitions. The presence of a large halo around the head of the sperm indicates that the sperm has intact DNA. The absence of such a halo confirms the fragmented sperm DNA. The activity of spermatozoa of the first boar after thawing was on average $25.0 \pm 5.2\%$. The level of DNA fragmentation in such spermatozoa was on average $70.3 \pm 3.7\%$ ($P < 0.01$). In the second boar, these indicators were $26.7 \pm 4.9\%$ and $75.3 \pm 2.3\%$, respectively ($P < 0.01$). In our previous studies, it has been shown that the level of DNA fragmentation of ejaculated boar spermatozoa after cryopreservation is very high. The average DNA fragmentation level of ejaculated boar spermatozoa in our studies was $90.3\% \pm 4.99$ ($P < 0.01$), and that of epididymal boar spermatozoa was

$72.8 \pm 3.0\%$, which is 17.5% lower, compared with ejaculated boar sperm. It should be noted that the spermatozoa of boars of the Ukrainian meat breed are valuable genetic material and are stored in limited quantities. Therefore, the use of additional methods for assessing the quality of spermatozoa will ensure a consistently high percentage of obtaining embryos in vitro, which in turn will expand the prospects of conducting further research in programs for the preservation of pig breeds.

Funding: Not applicable

Conflict of Interest: None to disclose

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P87 PRESERVATION OF RAM SEMEN: EFFECT OF ADDING SEMINAL PLASMA OF DIFFERENT ANIMAL SPECIES TO TRIS-EGG YOLK EXTENDER ON CHILLED SEMEN

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The present study aimed to compare the effects of seminal plasma (SP) from ram, buck, or camel on the chilled preservation of Awassi ram semen when mixed with TRIS egg yolk extender. Pooled collected semen was diluted with one of the following diluents; TRIS egg yolk (T), TRIS egg yolk mixed with ram seminal plasma (4:1 v/v) (R), TRIS egg yolk mixed with buck seminal plasma (4:1 v/v) (B), TRIS egg yolk mixed with camel seminal plasma (4:1 v/v) (C), or without SP as a negative control (W). After dilution, the semen was cooled gradually, stored at 5 °C, and evaluated at 0 h and every

24 h for seven consecutive days. Semen evaluation included sperm motility, abnormality, plasma membrane integrity, and morphometric parameters. Sperm motility was influenced significantly by the extender supplements. Total and progressive motility decreased ($p<0.05$) with increasing storage time. The total motility was lower ($p<0.05$) in W and T diluents than other diluents on days 1, 2, 6 and 7. Progressive motility was higher ($p<0.05$) in R, B and C diluents than other diluents on days 1-7. Conversely, secondary abnormalities increased ($p<0.05$) with increasing storage time, and higher ($p<0.05$) in diluent B than other diluents. Intact plasma membrane percentage was decreased ($p<0.05$) with increasing storage time, with higher percentages in R and B diluents than other diluents. In conclusion, the ram, buck and camel SP have advantageous effects on the chilled preservation of ram semen.

Funding: Not applicable

Conflict of Interest: None to disclose

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P88 DOES CRYOPRESERVATION OF HUMAN OOCYTES AND EMBRYOS AFFECT ABILITY TO HATCH?

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Mammalian oocytes and embryos -at the pre-implantation stages of development are surrounded by a glycoprotein envelope called the Zona Pellucida (ZP). Hatching from the ZP is critical for implantation and further development of the embryo. Cryopreservation can alter the physical and chemical properties of the ZP, ultimately

leading to impaired hatching of the embryo. The aim of the study was to investigate the effect of oocyte and embryo cryopreservation on the hatching ability of human embryos that were obtained in infertility treatment cycles using assisted reproduction methods. A retrospective cohort study of data from single medical centre was performed. We analysed the hatching rate of the blastocysts obtained in different groups: group 1 (control) (n=275) – fresh embryos obtained after fertilisation of fresh oocytes, group 2 (n=106) – fresh embryos obtained from cryopreserved oocytes, group 3 (n=224) – cryopreserved embryos obtained from fresh oocytes, group 4 (n=84) was formed by cryopreserved embryos obtained from cryopreserved oocytes. Reproductive cells were obtained from patients who were undergoing the infertility treatment programme. Oocytes and embryos were cryopreserved by the Cryotop vitrification method. It was found that the hatching rate was highest in group 1. Cryopreservation factors negatively affected the hatching rate of the blastocyst from the ZP, as this indicator significantly decreased in groups 2 and 3 ($p<0.05$). However, there was no significant difference in ZP hatching rates between oocyte or embryo vitrification groups (groups 2 and 3). The lowest ZP hatching rate was found in group 4 ($p<0.05$). Therefore, there are some risks regarding the ability of cryopreserved blastocysts derived from cryopreserved oocytes to hatch. Since changes in the ZP leading to embryo hatching defects also occur after the cryopreservation of mature oocytes, it is advisable to further investigate the possibility of using immature oocytes for cryopreservation.

Funding: Not applicable

Conflict of Interest: None to disclose

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P89 EFFECT OF HYPOTHERMIC STORAGE UPON RED BLOOD CELL MORPHOLOGY AND OSMOTIC STABILITY

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The analysis of vesicle formation and red blood cells (RBCs) injuries during hypothermic storage (HS) is of great importance since vesiculation is a process, related both to the loss of cell viability and their adaptation to the altered environment. Donor RBCs were used as the object of research. Their cell shapes were analyzed using the optical and scanning electron microscopy, while spectrophotometry was applied for studying the level of hemolysis and RBC osmotic resistance during HS (for 21 days in media of varying compositions). The cell glycocalyx was assessed by means of electron microscopy using ruthenium red as a dye. A method of freeze-fracturing was also used for studying fine structures of the cells. The analysis of the RBCs morphological states and osmotic stability at the initial HS stage (2-7 days) showed the echinocytic population of cells to be the most stable, while heterogenous populations (echinocytes and discocytes) were found to be more susceptible to the action of osmotic factors. Loosening, exfoliation and loss of glycocalyx were evident between the HS 10th and 14th days depending on its conditions. Sphere-echinocytic transformation produced about 60 spicules on the cell surfaces, on average. In case of echinocytes of the III and IV degree the spicule diameters were 435 ± 43 nm and 257 ± 37 nm, while the diameters of the segregated vesicles equaled to 495 ± 87 nm

and 348 ± 65 nm, respectively. Vesicle detachment resulted in losing up to 30% of the red blood cells membrane surface. This process is a completing one in the chain of events during transformation of cells, and to a bigger extent is determined by rearrangements in the cytoskeleton-membrane complex. Analysis of the electron microscopic data obtained thereby suggested a sequence of spicule formation and shedding, including the membrane-skeleton rearrangements, to be involved in this process.

Funding: Not applicable

Conflict of Interest: None to disclose

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P90 EXPERIMENTAL STUDY OF THE EFFECT OF ALTERNATING ELECTRIC FIELDS ON THE FREEZE-THAW PROCESS OF SOLUTIONS

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The effect of alternating electric fields on the freeze-thaw process of solutions is a controversial research problem in the field of cryopreservation and food preservation. The dipole moment of water molecules and the motion of ions in solution under electric fields are possible explanations. In the field of food preservation, a growing amount of evidence for the improvement of food preservation by alternating electric fields has accumulated, and commercial products for alternating electric field-assisted food freezing have been introduced. However, few experimental results have been published in the field of cryopreservation. Even, there are conflicting experimental results published on

the effect of alternating electric fields on supercooling. Therefore, there is an urgent need to publicize more experimental observational evidence in order to better understand the mechanism of the effect of alternating electric field on freeze-thaw. We fabricated a MEMS chip that can accurately measure temperature and apply alternating electric fields during the freeze-thaw process of solutions. And we observed the freeze-thaw processes of several solution systems, including pure water, saline, and commonly used cryoprotectants, under different field strengths and frequencies. With the platinum resistor on the chip, we measured the freezing point and supercooling. The ice crystal size of solutions frozen under an alternating electric field was measured using cryostage microscope and ice crystal segmentation algorithm. Splating experiments were conducted to measure the effect of alternating electric fields on the recrystallization process of solutions. Finally, we fabricated gel food models to verify the effects of alternating electric fields at different stages of food preservation. This study provides detailed thermodynamic data to assess the effect of alternating electric fields on the solution freeze-thaw process and contributes to an in-depth understanding of the mechanism.

Funding: This work was supported by the National Natural Science Foundation of China (No. 82172114), the Anhui Provincial Natural Science Foundation for Distinguished Young Scholars (No. 2108085J37), and the National Key Research and Development Program of China (2022YFC2703000).

Conflict of Interest: None to disclose

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P91 CRYOPRESERVATION OF RAT FETAL NEURAL CELLS AS A

COMPONENT OF SUSPENSION AND AGGREGATES

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Neural cells (NCs) were isolated from brain tissues of rat fetuses 15-16 days of gestation. Cell viability (according to the Trypan Blue test) was $62.2 \pm 12.7\%$. During cultivation in DMEM/F12 medium at a concentration of $2-4 \times 10^6$ cells/ml in the presence of 10% blood serum, viable NCs formed multicellular aggregates 50-150 μm in size. After attachment of the aggregates to the substrate, their cells migrated, spread out, and formed a monolayer, on which β -tubulin 3-positive cells with neuroblast morphology and colonies of undifferentiated nestin/vimentin-positive cells appeared. NCs in suspension and aggregates were frozen in DMEM/F12 in the presence of 10% Me_2SO and in the presence or absence of 10% blood serum at a rate of $1^\circ\text{C}/\text{min}$ to -80°C , after which they were immersed in liquid nitrogen. After thawing, Me_2SO was removed by flushing by centrifugation or by dilution with the cultivation medium to a concentration of 0.25%. The results obtained demonstrated that the viability of NCs cryopreserved as part of aggregates does not depend on the presence of serum in the medium, which is mandatory for successful cryopreservation of NCs in suspension. Aggregates cryopreserved both in the presence and in the absence of serum retained their structure and size and attached to the substrate with the same efficiency, followed by migration and differentiation of their cells. For the full functioning of deconserved NCs aggregates, it was important to preserve their structural integrity. Aggregates destroyed in the

process of washing off Me₂SO by centrifugation did not attach to the substrate and their cells died. Cultivation of NCs cryopreserved as a part of suspension demonstrated that one of the indicators of their viability is the ability to form aggregates.

Funding: This study was supported by National Academy of Sciences of Ukraine as part of scientific work № 0123U102365/№1 23.01.2023

Conflict of Interest: None to disclose

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P92 PHASE STATE OF BAY LEAVES (*LAURUS NOBILIS*) LIPID FRACTION AT LOW TEMPERATURES

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Low-temperature extraction of essential oils with liquefied gases has a number of advantages over other extraction methods. The obtained extracts have a high content of biologically active substances, since all fat-soluble vitamins, carotenoids, tocopherols, chlorophylls, phytosterols, coumarins, etc. are extracted together with essential oils during low-temperature extraction. They are not subjected to heat treatment, preserving in full all the biologically active substances characteristic of the plant. Essential oils and extracts of bay leaves (*Laurus nobilis* L.) have antioxidant, antimicrobial and antifungal properties and are widely used in food, perfume and soap industry. The

purpose of this study was to investigate the properties of lipid fraction of bay leaves obtained by the method of low-temperature extraction. Bay leaves collected in Georgia were dried, crushed to a particle size of 200±50 µm and extracted with chlorodifluoromethane in a specially designed installation. The temperature and pressure during the extraction varied in the range from 12 to 32°C and from 0.6 to 1.2 MPa, respectively. The extraction time was 10 hours. The output of lipid fraction was 5% of the initial mass of raw materials. The total phenolic content of the extract was 11.64±0.5 (mg GAE/g). In the obtained lipid fraction, a high level of total antiradical activity (74.38±3.11%) was found, which may be due to the high preservation of biologically active compounds in this method of preparation. Low-temperature phase transitions were investigated by the DSC method after fast preliminary cooling (~200 °C/min) of the sample. At the heating stage (0.5 °C/min) glass transition at -98.4°C, an endothermic peak at -77.2°C and a complex melting peak with at least three maximums (-35.5°C, -21.1°C, -11.2°C) were registered. The complexity of the melting curves is due to the overlap of the melting processes of several crystalline polymorphic forms. Such complex phase behaviour indicates a complicated structure of the bay leaves lipid fraction.

Funding: Not applicable

Conflict of Interest: None to disclose

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P93 ERYTHROCYTE PERMEABILITY TO CRYOPROTECTANTS VIA NOVEL MODEL-BASED ANALYSIS OF OSMOTIC SHOCK

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Effective use and development of cryoprotective agents (CPAs) rests on quantitative determinations of the permeability coefficient characterizing their rates of transfer through the membranes of the cells being frozen, hence the high interest in this membrane property for existing and potential new CPAs. This presentation focuses on such determinations for bovine, equine and canine erythrocytes (RBCs) for veterinary applications. The experimental method involves immersion of RBCs in hyperosmotic CPA solutions for varied prescribed times, over which varying amounts of the CPA penetrate following initial crenation (shrinkage) of the cells, followed by immersion in isotonic saline causing osmotic shock. The fraction of cells hemolyzed ultimately indicates the amount of CPA that entered the cell, and thence the permeability coefficient. Published investigations using this as well as other “shrink-swell” methods have been based on: (i) idealized models of the cell’s osmotic volumetric response using an apparent osmotically inactive volume in the Boyle-van’t Hoff plot; and (ii) experimental characterizations of the cell’s osmotic fragility that does not account for CPA concentration-dependent damage caused by the initial crenation step. We use a highly quantitative erythrocyte model recently developed by our group to remove both weaknesses of the customary data analysis. Although a separate characterization of the increased fragility due to the initial crenation can be used, we also demonstrate a novel fit

of the osmotic shock data in which both the increased fragility due to the initial crenation and the membrane permeability coefficient are simultaneously determined. New data are reported and analyzed for glycerol and dimethyl sulfoxide in bovine, equine and canine erythrocytes. The impact of the aforementioned weaknesses on permeability coefficient determinations is quantitatively assessed. Results are compared with other reported measurements, as well as first-principles predictions of temperature-dependent permeability of fluid- and gel-phase phospholipid membranes.

Funding: Not applicable

Conflict of Interest: None to disclose

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P94 MAGNETIC NANOPARTICLES DECREASED ROS LEVELS IN VITRIFIED-WARMED MOUSE IMMATURE AND MATURE OOCYTES

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The vitrification of mammalian oocytes is a long-term storage technique to preserve fertility ability in women who are at risk of sterility. Various cryoprotective agents (CPAs) or changes in their contents have been considered to reduce cryoinjuries. Recently, magnetic nanomaterials (MNPs) have been added to CPAs to enhance the thermal conductivity of the samples during

cryopreservation. To gain deeper insight into the effect of MNPs on the vitrification of immature Germinal Vesicle (GV) oocytes, here in the current study, Fe₃O₄ MNPs with 5nm size were applied. GV oocytes were taken from fifteen adult female NMRI mice (6-8 weeks) and divided into non-vitrified (nVit), vitrified (Vit), and vitrified plus 0.004 %w/v MNPs (Vit_NPs) groups. For vitrification, the GV oocytes were first exposed to an equilibration solution containing 7.5% v/v Ethylene Glycol (EG) + 7.5% v/v dimethyl sulfoxide (Me₂SO) and base medium (BM: 10% Human Serum Albumin in HamsF10) for 5-8 min. They were then washed in the vitrification solution comprising 15% Me₂SO + 15% EG + 0.5 M sucrose in BM) with or without MNPs for 1 min. Finally, they were loaded on a cryotop and plunged directly into liquid nitrogen. Warming was followed 3-5 days later in W1 (1.0 M sucrose in BM) for less than 1 min at 37°C, W2 (0.5 M sucrose in BM) for 3 min, and W3 (0.25 M sucrose in BM) for 3 min at room temperature. The survival rate, maturation rate, and reactive oxygen species (ROS) were evaluated. The O₂ and H₂O₂ - ROS levels were examined using DHE and DCFH-DA staining respectively in both GV and MII oocytes belonging to all groups. As shown in Fig 1B, for H₂O₂ at the GV stage, a significant increase was observed after vitrification (Vit group) compared to the nVit and Vit_NPs groups. 18 hours later, in MII oocytes a meaningful increase of H₂O₂ was observed in Vit group compared to the nVit (Fig 1B). Using of NPs couldn't return the ROS level (H₂O₂) to the control nVit group (Fig 1B). About O₂, there is a dominant increase in both GV and MII stage oocytes after vitrification. Also, NPs could decrease the O₂ level in GV and MII oocytes after vitrification. According to the present results, using MNPs in vitrification solution has improved on the post-thawing oocyte quality

such as viability rate, maturation rate, and ROS of oocytes.

Funding: Not applicable

Conflict of Interest: None to disclose

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P95 PRETREATMENT STRATEGIES FOR THE LONG-TERM CONSERVATION OF TEA (*CAMELLIA SINENSIS* (L.) O. KUNTZE) GERMPLASM

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Camellia sinensis (L.) O. Kuntze (Tea) genetic resources need attention for long-term conservation, as an alternative to field genebanks. Indian tea germplasm is known for its diversity which not only assists the development of superior planting material for tea breeding but also offers as a genetic resource for other tea producing countries. We developed an improved cryo-protocol for long-term conservation of tea germplasm. Shoot tips of *Camellia sinensis* were cryopreserved using two efficient cryo-techniques vitrification and V cryo-plate techniques. The regrowth rate of shoot tips obtained after vitrification was in 33.2% at 90 min of PVS2 exposure. Thus, to improve recovery rates and tolerance to cryo-injury, various preconditioning treatments were given to shoot tip donor plants with cold hardening, high sucrose, ABA, Proline, ABA+ Proline and ABA + cold hardening prior to cryopreservation. Vitrification technique resulted in increase of regrowth rate of shoot tips to 60.5% after preconditioned of donor plants with 10 mg/l ABA+ 3 mg/l Proline. Regrowth rate further

increased to 75.6% in V cryo-plate technique when shoot tip donor plants were pretreated with the above ABA+ proline concentrations. RAPD analysis was performed to test genetic fidelity after cryopreservation and results showed 100% monomorphism among all the regenerants and mother cultures. This study shows that preconditioning strategies along with V cryo-plate technique influences and improves the cryo-recovery rate of shoot tips of tea and provides a robust protocol for tea cryopreservation.

Funding: Not applicable

Conflict of interest: None to disclose

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P96 HOMOIOTHERMAL AND HETEROOTHERMAL MAMMALIAN ERYTHROCYTES RESPOND TO ARTIFICIAL AND NATURAL HYPOBIOSIS WITH CHANGES IN RELATIVE CONTENT OF MAIN HEMOGLOBIN FORMS

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Hypometabolic state of the mammalian body is of a wide practical interest. A model of artificial hypobiosis (AH) appears to be useful in studying this problem. Clarification of the peculiarities of function performance by erythrocytes under reduced body temperature (T_b) in mammals is a key to solving the problem of introducing them into a hypometabolic state. The models for such studies are heterotherms able to survive near-zero T_b . Homeotherms are able to T_b lowering in AH under the effect of hypothermia-hypoxia-hypercapnia. Relative

content of hemoglobin forms (oxy-, deoxy- and methemoglobin) of rats and hamsters RBCs during animals' staying and leaving the AH and hamsters' natural hibernation was comparatively studied using the differential spectrophotometry. The content of oxy-, deoxy- and methemoglobins for control animals RBCs were: $73.0 \pm 2.92\%$, $16.7 \pm 0.67\%$, $10.0 \pm 0.4\%$ for hamsters and $73.0 \pm 2.92\%$, $25.5 \pm 1.02\%$, $2.0 \pm 0.08\%$ for rats, respectively. The AH was characterized by T_b lowering (down to $+16^\circ\text{C}$), in the same way for both cold-sensitive rats and cold-tolerant hamsters. Both heterotherms' and homiotherms' being in AH resulted to a redistribution of hemoglobin forms. An increase of oxyHb was observed, it was more enhanced for rats. Meanwhile a percent ratio of hemoglobin forms was practically the same (for hamsters: $78.67 \pm 3.15\%$, $17.67 \pm 0.71\%$, $2.33 \pm 0.01\%$; for rats: $80.0 \pm 3.2\%$, $17.0 \pm 0.68\%$, $3.0 \pm 0.12\%$), as well as comparable to that for hibernating hamsters ($77.25 \pm 3.09\%$, $19.0 \pm 0.76\%$, $2.33 \pm 0.01\%$). The enhanced relative content of oxyHb kept changing up to 24 h post-AH for both animals, while their physiological state was similar to the control even in 2 hours. Thus, data indicate the existence of an erythrocyte "aftersensations" to the AH of mammals, consisted in an elevated relative content of oxyhemoglobin and an increased osmotic resistance, previously noted, keeping up to 24 h post-AH. We believe that the post-hypobiotic response of erythrocytes should be considered in the practical application of controlled hypometabolism of mammals.

Funding: Not applicable

Conflict of Interest: None to disclose

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P97 MICROENCAPSULATION ENABLED HIGH-HEMATOCRIT AND GLYCEROL-FREE

CRYOPRESERVATION OF HUMAN RBCS

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Human red blood cell (RBC) transfusion is a crucial component of modern medicine, providing life-saving treatment for patients suffering from acute blood loss, reduced RBC production, or decreased RBC survival. However, blood can only be hypothermic preserved for up to 42 days, which can cause shortages of blood if there is a huge demand for emergency blood transfusions in emergency conditions such as natural disasters or wars. Therefore, cryopreservation of RBCs is of great significance for providing transfusion timely in emergencies. But, traditional methods for RBC cryopreservation are associated with high concentrations of glycerol (20% or 40%), which result in cytotoxicity and osmotic injuries that must be carefully controlled. However, existing studies on the low-glycerol cryopreservation of RBCs, such as pre-dehydration, intracellular delivery of trehalose, application of new biocompatible CPAs, are still challenging due to the too complex operation process (e.g., long-time incubation), severe hemolysis during incubation, not high enough RBC recovery, questionable FDA approval, and most importantly the low hematocrit levels (~10%) that necessitate significant storage space and additional concentration process before transfusion. This study reports a novel approach for the glycerol-free cryopreservation of human RBCs with a high final hematocrit. By using trehalose as the sole CPA and using the encapsulation of core-shell alginate hydrogel microfibers, we achieved a high recovery of up to 95% while

the final RBC hematocrit was up to 40%, five times previous study. Moreover, the RBCs post-cryopreservation were proved to maintain their bi-concave and discoidal shape. Furthermore, the intracellular ROS/NO levels were not significantly different from that of fresh RBCs. Overall, we developed a novel approach for the glycerol-free cryopreservation of human RBCs at a high hematocrit (~40%), it may provide potential clinical transfusion benefits.

Funding: This work was supported by the National Natural Science Foundation of China (No. 82172114), the Anhui Provincial Natural Science Foundation for Distinguished Young Scholars (No. 2108085J37), and the National Key Research and Development Program of China (2022YFC2703000).

Conflict of Interest: None to disclose
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P98 IMPACT OF MANNITOL ON THE INTEGRITY OF OVARIAN TISSUE AFTER EXTENDED HYPOTHERMIC STORAGE AND CRYOPRESERVATION

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In recent years, ovarian tissue (OT) cryopreservation and transplantation have rapidly developed methods for preserving fertility. However, the effectiveness is insufficient as ischemia of OT graft after cryopreservation remains a crucial factor for follicles damaged. The presence of mannitol in preserved media might have led to reduced

interstitial oedema in organs undergoing ischemia. The research aim was to assess the efficiency of the media containing mannitol (MCM) to prevent ischemia of OT graft after hypothermic storage (HS) and cryopreservation. The research object was fragments of rats' OT. HS was conducted in a fridge at 4 °C. Cryopreservation of OT fragments was performed under the protection of Me₂SO and 1,2-PD using slow freezing. The saturation of cryoprotectants (CPAs) was based on DMEM with 200 mM sucrose. Removal of CPAs was held gradually at 22 °C using sucrose and mannitol in the medium. The function of cryopreserved OT was evaluated by the heterotopic allotransplantation method. Applying MCM during HS showed a high level of follicles integrity (85-95%) after 24 hrs and 48 hrs (54-66%). It was found that the OT grafts after 48 hrs of storage in MCM could restore their hormonal. It was revealed that using mannitol as the main component of removal medium after cryopreservation, led to the recovery of OT hormonal function. A graft of cryopreserved OT in this group had follicles of various maturity rates and corpus luteum with follicular density 4.0±0.5 per mm², whereas, in the group using sucrose as a component of removal medium, this rate was 1.1±0.2 per mm². Thus, mannitol as the main component of medium for HS and CPA removal medium after cryopreservation resulted in the best recovery of morphology and function of OT after transplantation.

Funding: Not applicable

Conflict of Interest: None to disclose

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P99 EFFECT OF ICE RECRYSTALLIZATION INHIBITORS ON ICE NUCLEATION TEMPERATURE DURING

CRYOPRESERVATION OF BUFFALO BULL (*BUBALUS BUBALIS*) SEMEN

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Use of ice recrystallization inhibitors have shown to improve post recovery of frozen semen. Present study was conducted to compare the freezing dynamics of various ice recrystallization inhibitors viz. L-Proline (LP; 1200, 30 mmol/L), Sodium dodecyl sulphate (SDS; 0.125, 0.5%), 4-methoxy phenyl β-D glucopyranoside (MPG; 6.25, 3.25 mmol/L) and N-octyl β-D glucopyranoside (NOG; 0.16%, 0.03%) in tris citrate egg yolk extender during cryopreservation of buffalo bull (*Bubalus bubalis*) semen. Ten ejaculates, each from 3 Murrah buffalo (*Bubalus bubalis*) bulls, maintained at bull station, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India (Latitude, Longitude, 30.55°N, 75.54°E) were collected and extended in tris citrate egg yolk extender; 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 (@-30°C/min from 4°C to -15°C; @-50°C/min from -15°C to -140°C) followed by plunging of straws into liquid nitrogen. Ice nucleation temperatures of each freezing were recorded. The ice nucleation temperatures with LP 1200, LP 20, SDS 0.5, SDS 0.125, MPG 6.25, MPG 3.25, NOG 0.15 and NOG 0.03 and were -11.85°C, -19.25°C, -6.8°C, -16.4°C, -7°C, -15.2°C, -16.7°C, -17.35°C. Analysis of sperm motion traits of respective freezing cycles revealed that optimum results were yielded when the ice

nucleation temperature remained around -16°C during semen cryopreservation of buffalo bulls.

Funding: Not applicable

Conflict of Interest: None to disclose

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P100 INFLUENCE OF FECUNDITY ON CRYOGENIC POLLEN STORAGE IN TOMATO (*SOLANUM LYCOPERSICON L*)

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The preservation of viable pollen through ultra-low temperature storage is a reliable and secure method for long-term conservation of plant germplasm resources. However, the impact of storing tomato pollen at -20°C on fruit set and seed production is not well-studied. Tomatoes (*Solanum lycopersicum* L), known for their nutritional value, require high-quality pollen for successful hybridization in breeding programs. This study investigated cryogenic pollen storage effects on tomato fruit set and seed production. Tomato pollen was stored at -20°C with CaCl₂ as a desiccant and at -196°C after adjusting moisture content to 6.5-7% (wet weight basis). After 6, 8, 10, 12, and 18 months, in vitro pollen germination and tube growth were evaluated. Rehydration at 100% humidity and 15°C for 3 hours, followed by incubation on a semisolid

substrate (1% agar, 12% sucrose, and 50 mg L⁻¹ H₃BO₃) for 6 hours, assessed pollen performance. Respiration activity was measured in pollen stored at -20 and -196°C for 6 and 12 months using a liquid substrate (12% sucrose and 50 mg L⁻¹ H₃BO₃). Pollen stored at -20°C maintained high germination for up to 10 months. However, after 6 months, tube length and respiration activity decreased, reducing fertility and seed production during artificial pollination. Pollen germination ability significantly declined after 12 months. In contrast, pollen stored at -196°C for 18 months showed similar germination, tube growth, and respiration activity to fresh pollen. Cryogenically stored pollen resulted in comparable fruit set and quality compared to fresh pollen during artificial pollination. Moreover, pollination with cryogenically stored pollen resulted in comparable fruit set and fruit quality compared to artificial pollination using fresh pollen. These findings suggest that pollen cryopreservation can be successfully employed for tomato breeding and germplasm storage.

Funding: Not applicable

Conflict of Interest: None to disclose

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P101 USING A NO-TOUCH CLOSED METAL CONTAINER FOR ARTICULAR CARTILAGE VITRIFICATION AND STORAGE

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There are many strategies to perform vitrification of tissues, and in all these techniques, the rates of success are variable due to different efficiency of heat exchange and cryoprotectant agent (CPA) exposure and removal methods. Moreover, many established protocols for vitrification use open systems, and exposing the sample to the nitrogen vapour may permit access of pathogens resistant to cryogenic temperatures. Therefore, to improve heat exchange without exposing the osteochondral tissue to contaminants, while maintaining easy cryoprotectant exposure and removal without direct contact of the operator with the vitrification-rewarming solutions, we used a metal Ovarian Tissue Cryosystem (OTC) as a closed vitrification-storage-rewarming container with two different methods of storage (with or without a surrounding vitrification solution). In this study, 7mm diameter osteochondral dowels were harvested from femoral condyles of 3 porcine stifle joints (n=3 joints and 4 dowels per joint). Dowels were exposed to a multi-CPA solution containing dimethyl sulfoxide, ethylene glycol, and propylene glycol prepared in medium. For storage in liquid nitrogen vapour we used two different methods: (a) storage in OTC with a surrounding vitrification solution versus (b) storage in OTC without a surrounding vitrification solution. Cartilage slices were sectioned from dowels (1) just before cryoprotectant agent (CPA) loading (Fresh), (2) after CPA loading (CPA Toxicity), and (3) after vitrification-rewarming (Warming). Chondrocyte viability was quantified with a dual membrane integrity stain. We

demonstrated that there was no significant difference ($P > 0.05$) in cell viability percentages among Fresh, CPA Toxicity, and Warming groups with or without the surrounding vitrification solution groups (94.57; 81.25; 72.11 and 75.27, respectively). Therefore, the Ovarian Tissues Cryosystem (OTC) opens a new possibility for successful vitrification using a no-touch approach facilitating long-term storage and rewarming of osteochondral tissues and can optimize vitrification-storage-rewarming protocols for future use in clinical applications involving vitrified articular cartilage.

Funding: This study was funded by the Edmonton Orthopaedic Research Committee and the University (of Alberta) Hospital Foundation. JAWE holds a Canada Research Chair in Thermodynamics.

Conflict of Interest: None to disclose

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