C-1000

Effect of Hydroxyapatite Nanoparticles on Subzero Water Transport of HeLa Cells. J. YI and G. Zhao. Department of Information Science and Technology, University of Science and Technology of China, No.96, JinZhai Road Baohe District, Hefei, Anhui, 230026, P. R. CHINA. Email: yijingru@mail.ustc.edu.cn

Nano-cryopreservation is a newly emerged field which is promising to deal with the major challenges in typical cryopreservation methods. Although addition of nanoparticles is found to significantly affect the behavior of devitrification, recrystallization, and thermal transfer properties of cryopreservation solutions, investigation of its effect on freezing response of living cells is scarce in literature. However, there leaves many intriguing questions. For example, as pointed in literature, adding nanoparticles to cell suspension can enhance extracellular ice formation. With decreasing of the temperature, the increased water chemical potential difference across the cell membrane may induce more water flux out of the cells. Nevertheless, adding of nanoparticles may also increase the viscosity of the extracellular solution, which can impede such water transport process. In this study, subzero water transport of HeLa cells with different concentration of hydroxyapatite (HA) nanoparticles under incubation and no-incubation was quantitatively investigated. Hydroxyapatite (HA) was utilized as the nanoparticles material considering its good biocompatibility with cell membrane since unsuitable nanoparticles can be toxic to cells and bring additional cell injury. By obtaining the water permeability parameters, we found the subzero water transport was facilitated by addition of HA nanoparticles. And such influence turned out to be enhanced after incubation. What's more, the higher the concentration of HA, the higher the water permeability. Possible influence mechanisms will be further discussed.

C-1001

Small Molecule Inhibitors of Ice Recrystallization – from Design to Mechanism of Action and Applications. R. N. BEN[1], C. Capicciotti[1], A. Balcerzak[1], H. Thompson[2], A. Hansen[2], and T. Turner[2]. [1]University of Ottawa, Dept. of Chemistry, D'Iorio Hall, 10 Marie Curie St., Univ. of Ottawa, Ottawa, ON K1N 6N5, CANADA and [2]Canadian Blood Services, Edmonton. Email: rben@uottawa.ca

Ice recrystallization has been identified as a significant contributor to cellular injury and death during cryopreservation. Unfortunately, many cryopreservation protocols are suboptimal. Thus, interest has arisen in designing novel ice recrystallization inhibitors and using these moleclues as cryoprotectants. Recently, our laboratory has reported several classes of small molecules that have the ability to inhibit ice recrystallization. These classes include amino acid-based organogelators, non-ionic carbohydrate-based amphiphiles (aryl and alkyl alditols) and *O*-linked alkyl and aryl glycoside derivatives. We have conducted comprehensive structure function studies to optimize the ability of these molecules to inhibit ice recrystallization and also facilitate a more thorough understanding of their mechanism of action. These studies have revealed that long alkyl chains or substituted aryl groups that add "hydrophobicity" to the molecule are essentail for potent inhibition of ice recrystallization and that the relative position of these groups is important. In addition, the choice of counterion can have a dramatic effect on the ability of these compounds to inhibit ice recrystallization. Collectively, our results suggest that a high degree of hydration of these small molecules (and their counterions where appropriate) is a prerequisite for a potent inhibitor of ice recrystallization. A number of these small molecules described above have been investigated as cryoprotectants and have been found to freeze human red blood cells (RBCs) using low amounts of glycerol. Deglycerolization studies and analysis of RBC recoveries have revealed important insights in the mechanism of action of these small molecule ice recrystallization inhibitors.

C-1002

Survivals Approach 100% for Mouse Oocytes Suspended in One-third Concentration of Vitrification Media, Vitrified, and Warmed Ultra-rapidly by an IR Laser Pulse. P. MAZUR[1], B. Jin[1], and F. Kleinhans[2]. [1]The University of Tennessee, Department of Biochemistry and Cellular and Molecular Biology, Room 191N Hoskins Library, White Street, Knoxville, TN 37996-0840 and [2]Indiana University-Purdue University at Indianapolis, Department of Physics, Indianapolis, IN 46202. Email: pmazur@utk.edu

Vitrification is the most sought after route to the cryopreservation of animal oocytes and embryos and other cells of medical, genetic, and agricultural importance. Standard vitrification has been based on two firmly held premises. One is that the vitrification solution in which the cells are suspended must have a very high concentration of a mixture of non-electrolytic solutes, some of which can permeate the cell and some of which can not. We have used EAFS10/10, a solution developed by Pedro *et al*., where E, A, F, and S refer to ethylene glycol (EG), acetamide, Ficoll, and sucrose. The total molality is 7.38 molal, of which 6.5 molal is permeating (EG and acetamide), and the remainder are non-permeating. The second premise is that avoiding ice formation in cells and obtaining high survivals demands the highest of cooling rates. Consequently, a series of devices have been developed over the past decades that achieve cooling rates of $\geq$10,000^{o}C/min by using very small volumes of cell suspensions. One example is the Cryotop. So, current thinking is that successful vitrification requires that cells be suspended in high concentrations of protective solutes and that they be cooled at very high rates to below -100^{o}C. We report here that neither of

these beliefs holds for mouse oocytes. Rather, we find that if mouse oocytes are suspended in media that produce considerable osmotic dehydration before vitrification and are subsequently warmed at ultrarapid rates (10,000,000°C/min) achieved by a laser pulse, nearly 100% will survival even when cooled rather slowly and when the concentration of solutes in the medium is only 1/3rd of standard. Full paper published on-line 3/22/14 at DOI 10.1016/j.cryobiol.2014.02.005 Research supported by NIH grant R01OD011201

C-1003

Cryopreservation of Rat Hepatocytes with Wheat Proteins: Role in Oxidative Stress Protection. D. A. AVERILL-BATES, M. Chow-Shi Yée, M. Grondin, F. Sarhan, and F. Ouellet. Département Des Sciences Biologiques, Université du Québec à Montréal, 141 Président Kennedey, Montréal, QB H2X 1Y4, CANADA. Email: averill.diana@uqam.ca

Hepatocytes are an essential model for the pharmaceutical industry, as they are used for drug toxicity testing. Their availability is dependent on the surgical isolation of fresh cells from animal liver, and cryoconservation allows for the storage of isolated cells for future use. However, the freeze/thaw step associated with this procedure leads to structural damage to the cells, which lowers post-thaw viability and metabolic functions. To circumvent such damage, cryoprotective agents such as dimethylsulfoxide (DMSO) are used, but these are often toxic for cells. The development of alternative cryoprotectants that are less toxic would therefore be beneficial. We have previously shown that wheat protein extracts are efficient for cryopreservation of hepatocytes and other cell types. To identify cryoactive proteins in these extracts, fractions from chromatographic separation were analysed by tandem mass spectrometry. Data analysis allowed us to determine that enolase is a good candidate as cryoprotector, revealing a novel function for this glycolysis-associated protein. The cDNA was cloned and the protein was produced in a bacterial system. Viability tests confirmed that the recombinant enolase is more efficient than DMSO for cryopreservation of rat hepatocytes and that it causes no cellular toxicity. As a first step to elucidate its mechanism of action in the protection of cells, we have determined that the protein decreases the oxidative stress resulting from cryopreservation. Levels of peroxides and nitric oxide after thawing of cells frozen with enolase are lower than those in cells frozen with DMSO. These results show that this plant protein has tremendous potential as a cryoprotective agent for hepatocytes, and possibly other cell types. The use of non-toxic agents that preserve viability and metabolic functions of mammalian cells would represent a major breakthrough in the field. It would allow increasing the availability of functional cells used for toxicity testing required for medical and pharmaceutical advances. FRQNT équipe, Québec, Canada

C-1004

Molecular Modelling of the Dynamic and Thermodynamic Characteristics Associated with the Glass Transition of Amorphous Trehalose/Water Mixtures. L. WENG and G. D. Elliott. University of North Carolina at Charlotte, 2515 Rosy Billed Dr., Apt. 304, Charlotte, NC 28262. Email: lweng@uncc.edu

The glass transition temperature T_g of biopreservative formulations is recognized as a significant parameter for predicting the long-term storage of biologics. As a complementary tool to thermal analysis techniques, molecular modelling has been successfully applied to predict the T_g of several cryoprotectants and their mixtures with water. These molecular analyses, however, have rarely focused on the glass transition behavior of aqueous trehalose solutions. Information about the diffusivity, specific heat capacity, H-bonding dynamics in trehalose/water at sub-T_g temperatures are largely still unavailable in the literature. And the self-association characteristics of sugar solutions in the glassy state are still relatively unknown. Using molecular modelling of several dynamic and thermodynamic properties, this study reproduced the supplemented phase diagram of trehalose/water mixtures, yielding good agreement with experimental values. The structure and dynamics of the H-bonding network in the mixtures were analyzed. The H-bonding lifetime was determined to be an order of magnitude higher in the glassy state than in the liquid state, while the constitution of the H-bonding network exhibited no noticeable change through the glass transition. It is speculated that the extended H-bond lifetime in the glassy state could reflect the slower secondary relaxation dynamics, both of which are primarily related to the local reorientation of –OH groups in the trehalose molecule. It was also found that trehalose molecules preferred to form small, scattered clusters above T_g, but self-aggregation was substantially increased below T_g. This aggregation phenomenon could help to shield preserved biologics from dynamically decoupled water in the vicinity of T_g. Our findings demonstrated the feasibility of probing vitrification phenomena by molecular modelling based on multiple dynamic and thermodynamic properties and provided insights into the glass transition characteristics of aqueous trehalose, enabling a consideration of the implications for biopreservation.

C-1005

A Novel and Highly Efficient Method for Intracellular Delivery and Accumulation of Trehalose. ALIREZA ABAZARI[1], Labros Meimetis[2], Daniel Moore[3], Steven Hand[3], Ralph Weissleder[2], and Mehmet Toner[1]. [1]The Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School, and Shriners Hospital for Children, Boston, MA; [2]Center for Systems Biology, Massachusetts General Hospital and Harvard Medical School, Boston, MA;

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The presence of trehalose on both sides of the cell membrane contributes to protection against desiccation damage. Since mammalian cell membranes are naturally impermeable to trehalose, invention of novel methods for efficient intracellular trehalose delivery has been an ongoing investigation. It has been established that increasing small molecule lipophilicity enhances cellular uptake. Using this concept as a starting point, we designed a series of acetylated trehalose derivatives, with the 6-acetylated analogue (6-Ac-Tre) displaying the most promising properties in rat hepatocytes, among other cells. Once 6-Ac-Tre penetrated the cell membrane, endogenous nonspecific esterases cleave acetyl groups, resulting in release of de-acetylated trehalose in the cytoplasm. The total intracellular concentration of trehalose and its non-fully-de-acetylated variants was 5-10 fold higher than the extracellular concentration of 6-Ac-Tre, reaching concentrations as high as 300mM within 6 h of incubation. Using a diffusion-reaction model, the permeability and reaction kinetics of 6-Ac-Tre conversion to trehalose was calculated and optimum incubation concentration and time were obtained. Further studies on cell metabolism suggest that there is only a minimal impact of intracellular trehalose on cellular function. This study demonstrates a novel and robust method for loading trehalose in primary mammalian cells. Despite the somewhat longer loading time compared to some alternative methods, it is an easy-to-perform method for trehalose loading in cells with unprecedented efficiency. The described method is especially suitable for biopreservation studies on primary cells and in cells which may not be synthetically/genetically manipulated for further therapeutic applications. This approach has been carried forward with comprehensive biopreservation studies in progress.

C-1006

Exploring the Improvement of Human Cell Cryopreservation. T. MORRIS, K. Coopman, C. Hewitt, and A. Picken. Loughborough University, 64 Portland Road, West Bridgford, Nottingham NG2 6DL, UNITED KINGDOM. Email: t.j.morris@lboro.ac.uk

Regenerative medicine is an emerging technology and with over 1000 cell therapies currently in clinical trials there is a need to expand the limited knowledge related to the storage, shipment and preservation of cell therapies. An accepted gold standard of human cell cryopreservation is 10%(v/v) Dimethyl Sulfoxide (Me_2SO) in serum. Concerns mean safer and optimized protocols are needed, including Me_2SO toxicity and thawing. Given its potential toxicity, Me_2SO usage is a key issue in cryopreservation. Methods specify the need to reduce cell exposure time to Me2SO above 0°C as much as possible. The maximum amount of time cells can be exposed to Me_2SO to prevent a detrimental effect needs to be clarified. Thawing is poorly understood in cryopreservation. It is as vital as freezing to prevent injury to cells. Protocols are currently too vague for cell therapy regulation, and need improvement. The time dependent Me_2SO cytotoxicity was evaluated by overexposing human mesenchymal stem cells (hMSCs) to Me_2SO during cryopreservation. The effect on several metrics has been investigated and it has been determined that after 1 hour overexposure after thawing viability is reduced from 96.3±0.6% to 89.1±2.6%, the co-expression of four characteristic markers is changed from 91.1±1.7% to 82.1±0.6%. This significant change could cause adverse effects on product efficacy and patient health, to prevent this, Me_2SO exposure must be kept to below 1 hour. A heat transfer model has been developed and determined that 655J is the required energy to thaw a vial, associated heat transfer rates have also been calculated. Using these values, and additional factors such as pre-thaw stabilization, and fresh-media composition, a multi-parameter experiment will help determine an improved thawing process.

C-1007

The Design of Small Molecule Ice Recrystallization Inhibitors for Use as Cryopreservatives. J. BRIARD[1], R. N. Ben[1], M. Afagh[2], and E. Gardiner[1]. [1]University of Ottawa, 5 Promenade Ave, Nepean, ON K2E5X4, CANADA and [2]Western University, 1151 Richmond St, London, ON N6A 3K7, CANADA. Email: briard.jennie@gmail.com

Ice recrystallization inhibition (IRI) activity is a desirable property for a cryoprotectant. Recently, small molecules having potent IRI activity have been reported by our laboratory (*Chem. Sci.*, 2012**,** 3, 1408). These small molecules are amphiphilic and can form micelles due to their polar carbohydrate-based head and aliphatic tail (*Chem. Sci.*, 2012**,** 3, 1408). We have previously described a correlation between monosaccharide hydrate and IRI activity (*J. Am. Chem. Soc.*, 2008, 130 (10), 2928) and therefore the effect of adding hydrophobic atoms or functional groups to small molecules on IRI activity was investigated. Fluorine is of particular interest because it possesses what's known as the 'hydrophobic hydration effect', in which water molecules tend to order around fluorine atoms in solution (*ChemBioChem*, 2004, 5, 622). Interestingly, the location of a fluorine atom or other hydrophobic functional groups on the aliphatic tail greatly affects the IRI activity of the molecule. During the course of this investigation, several very potent inhibitors of ice recrystallization were discovered and their use as cryoprotectants for Tf-1α cells, used as a model for stem cells derived from human umbilical cord blood, was explored. Post-thaw cell viability and apoptosis of Tf-1α cells cryopreserved with these IRI active molecules will be presented.

C-1008

Compositional and Functional Analyses of Glycosylphosphatidylinositol-anchored Protein (GPI-AP) During Plant Cold Acclimation. DAISUKE TAKAHASHI[1], Yoko Tominaga[2], Yukio Kawamura[1,2], and Matsuo Uemura[1,2]. [1]United Graduate School of Agricultural Sciences, Iwate University, Morioka 020-8550, JAPAN and [2]Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka, 020-8550, JAPAN. Email: u0412002@iwate-u.ac.jp

Cold acclimation (CA) in plants results in alterations of plasma membrane (PM) protein composition, which is critical to increase freezing tolerance. Although, many studies have reported CA-responsive PM integral and peripheral proteins, PM lipid-associated proteins including glycosylphosphatidylinositol-anchored protein (GPI-AP) have not yet been characterized because of their low abundance in the PM. GPI-APs are considered to localize in the PM, apoplast and microdomains enriched in specific lipids and proteins in the PM (PM-MD). Classical gel-based proteomics revealed only 30-44 GPI-APs in *Arabidopsis* PM, while *in silico* analyses predicted that *Arabidopsis* genome encodes hundreds of GPI-APs. Functions of GPI-APs in plants are largely unknown yet but considered to be involved in many physiological processes including acquisition of freezing tolerance during CA. Therefore, we aimed to investigate responsiveness of GPI-APs during CA in detail. First, PM, apoplast, PM-MD and GPI-AP-enriched fractions were isolated from *Arabidopsis* leaves and all of these fractions were subjected into shotgun proteomic analysis. We successfully identified 163 of potential GPI-APs including 31 CA-induced GPI-APs. Among the GPI-APs identified, we selected At3g04010 protein which was identified with a high confidence score (402.39) and increased 3.53-fold during CA. Transient upregulation of *At3g04010* gene expression up to 70 times during CA was determined by qRT-PCR analysis. GUS-fused promoter assay demonstrated selective expression of *At3g04010* in hypocotyl, roots, veins and petiole. Cold-acclimated *At3g04010*-knockdown mutant revealed impaired freezing tolerance compared to wild type. Based on amino acid sequence, At3g04010 protein has domains that have high homologies to beta-1,3-glucanases which regulate cell-to-cell transport. Thus, a GPI-AP, At3g04010, may be important for proper substance transport during CA and freeze/thaw process. This study was in part supported by Grants-in-Aid for Scientific Research (#247373, #22120003 and #24370018).

C-1009

Cryopreservation of Monkey Mesenchymal Stem Cells Inside Alginate 3D Micro-spheres After a High Voltage Encapsulation. O. GRYSHKOV[1], D. Pogozhykh[1], N. Hofmann[1], T. Mueller[2], and B. Glasmacher[1]. [1]Institute of Multiphase Processes, Leibniz University Hannover, Callinstrasse 36, Hannover 30167, GERMANY and [2]Institute for Transfusion Medicine, Carl-Neuberg Strasse 1, Hannover 30625, GERMANY. Email: gryshkov@imp.uni-hannover.de

Cryopreservation is the only one possible way to store rare cell types for long term. Despite this, there are still challenges to preserve stem cells. In order to improve viability and proliferation of cells after cryopreservation we encapsulated them in small alginate beads. The gel-like structure and mild environment inside alginate beads may protect encapsulated cells from cryo-injury and resist the reorganization of ice crystals during thawing. The existing encapsulation methods do not provide alginate beads with narrow size distribution and are not able to generate small beads in repeatable manner. Here we applied high voltage to encapsulate cells in alginate. Such technology has been shown to be advantageous over the commonly used air flow encapsulation [Gryshkov et. al, Mater Sci Eng C Mater Biol Appl 2014]. Mesenchymal stem cells (MSCs) derived from the Common marmoset *Callithrix jacchus* were encapsulated in 1.6% (w/v) sterile alginate at a concentration $1*10^6$cells/ml using high voltage (15, 20, 25kV). Air flow encapsulation was run as a control. MSCs in alginate beads were frozen either immediately after encapsulation or after 24h of incubation. Cryopreservation was conducted with 1K/min cooling rate down to -80°C with 10% DMSO. After storage beads were thawed at 37°C with further removing of alginate using sodium citrate. The recovered MSCs were seeded for proliferation and metabolic activity assays either immediately after thawing or after 5 days of recovery. High voltage encapsulation method was able to generate alginate beads containing cells with narrow size distribution (3-7%) in repeatable manner. The incubation of encapsulated MSCs slightly reduced the proliferation after thawing. Immediately frozen cells recovered at the same rate as fresh control. Our results show an increased proliferation of MSCs frozen immediately after encapsulation. The viability and proliferation of encapsulated MSCs could be further improved by modifying the alginate to allow mammalian cell types to attach to alginate structure and resist ice crystal formation during freezing

C-1011

Low Temperature Cell Pausing: An Alternative Short-term Preservation Method for Use in Cell Therapies. N. J. ROBINSON, A. Picken, and K. Coopman. Loughborough University, Centre for Biological Engineering, Loughborough LE11 3TU, UNITED KINGDOM. Email: n.robinson@ lboro.ac.uk

Background: Advancements in cell therapies have produced a requirement for an effective short-term cell preservation method, enabling time for quality testing and transport to their clinical destination. We aim to ‘pause’ cells at ambient temperature whilst maintaining cell viability and quality post preservation. Storage at ambient temperature effectively bypasses cell damage from freezing and will greatly reduce

costs and reliability of specialist machinery. Methodology: Initially, a human osteosarcoma cell line (HOS TE85) was paused at ambient temperatures (no atmospheric control) for up to 48 hours. Cell assessments were performed immediately after cell pausing and following 24 hour recovery at 37°C, 5% CO_2, 95% humidity. Results: Viability (propidium iodide) remained above 98% and 91% for cells paused at 24 and 48 hours respectively and significantly lower reductive metabolism (PrestoBlue) was observed during pausing compared with non-paused controls ($p \leq 0.01$). Following recovery, metabolic activity became similar to the control and alkaline phosphatase activity (typically secreted by osteoblasts) remained unchanged. Conclusion**:** HOS TE85 cells effectively recover following 48 hours pausing in standard media and show evidence of 'pausing' with regards to lower reductive metabolism at ambient temperature exposure. Current experiments are investigating the effects of prolonged pausing (144 hours), pausing in suspension and application to mesenchymal stem cells. Successful cell pausing will create a simpler, cost effective short-term preservation method for cell therapies.

C-1012

Viability and Differentiation of Human Blood and Marrow Cells Cryopreserved Since 1972, and Homing Effects of Cryopreserved Cells. S. SUMIDA[1], T. Kitamura[1], N. Motomura[2], A. Saitou[2], E. Hagen[3], E. Woods[3], and A. Rowe[4]. [1]Japan Society for Low Temperature Medicine, Dr. Sajio Sumida Clinic, Takeda Bldg. 203, 2-11-6 Ginza, Chuoku, Tokyo 104-0061, JAPAN; [2]Tokyo University, School of Medicine, Depart. Cardiovas. Surg., 7-3-1 Hongo, Bunky-ku, Tokyo 113-0033, JAPAN; [3]Cook General BioTechnology, LLC 1102 Indiana Avenue, Indianapolis, IN 46202; and [4]New York University, Four Brooks, Four Brooks, CT. Email: cryomedicine@nifty.com

Our frozen allogeneic bone marrow transplantation program was started in 1972, but the initial clinical results were so poor, and we switched to frozen autologous bone marrow transplantation (FABMT), resulted in rescuing 293 advanced cancer patients. The bone marrow cells of those patients who had died due to deterioration of cancer remained in a frozen state until today. Those 200 units of bone marrow cells in cryopreservation bags with their pilot tubes had been stored in a liquid nitrogen stocker for up to 42 years. All cancer patients in this study who had agreed to the informed consent of this treatment expired before 1999. Recently, in 2011-2013, we thawed and cultured those frozen marrow cells to confirm the long term (41 years) viability as elucidated by colony formation of erythroid, monocyte, and mesenchymal stroma cells (MSCs), and as MSCs to form their confluent networks. Finally, we established a novel technique to preserve bone marAs previously reported, the bone marrow cells of patients who had solid cancer tumors formed colonies of erythroids, mononuclear cells (small, large, and adipocyte), and mesenchymal stroma cells after thawing and culture in a 5% CO2 incubator. The frozen bone marrow cells formed splendid colonies, not inferior, when compared with fresh marrow cells which were currently collected for controls, and also differentiated the blood cells including red, mononuclear, and mesenchymal cells. As for the subculture of the colony cells, even passage 5 (P5) cells was possible. P2 cells cryoprotected in a 10~15% Me2SO in IMDM were successfully cryopreserved at -80oC or -196oC again for months, so the cryopreservation of a further long term will be possible.row cells more than 40 years using the repetitive freeze-thaw-in vivo (in the cancer patients) or in vitro subculture method (RFTIVIVSCM) on our clinical experiences of FABMT. As adjuvants/supplements, 1) adult human sera (AHS), 2) Limulus tridentatus (member of Brady-telic lines, a living fossil) fresh blood cell lysate (LL), and 3) feeder layer of human saphenous vein fragment (HVT), HeLa cells, and K 562 cells (myeloid leukemia cell line) were tested. LL slightly suppressed colony formation but promoted mesenchymal cell appearance.

C-1013

Cholesterol-loaded Cyclodextrin Improves Goat Semen Cryosurvival by Enhancing Sperm Cholesterol Content and Osmotic Tolerance. V. SALMON[1], P. Leclerc[2], and J. Bailey[2]. [1]Centre de Recherche en Biologie de la Reproduction (CRBR), 909 Rue Monseigneur-Grandin Appt 201, Quebec G1V3X8, CANADA and [2]Laval University, 2425, Rue de l'agriculture, QB GIV0A6, CANADA. Email: mahutin-vianney.salmon.1 @ulaval.ca

The success of semen cryopreservation mainly resides in maintaining sperm membrane integrity and function after thawing. Cholesterol-loaded cyclodextrin (CLC) is used for *in vitro* incorporation of cholesterol into cells to protect them against cold temperature. We hypothesized that CLC treatment enhances sperm cholesterol content to increase its tolerance to osmotic shock and cryo-resistance, thereby improving fertility. Alpine goat sperm were treated ± 3 mg/ml CLC and lipids extracted by the Folch method, then cholesterol was quantified with Liebermann-Burchard reagent, which confirmed that CLC treatment increases the cholesterol content of fresh sperm in either PBS or skim milk extender. Sperm were then treated ± CLC in skim milk extender and cryopreserved. After thawing, sperm cholesterol dramatically fell, even in the presence of CLC, and likely explains the mechanism of "cryo-capacitation". However, CLC treatment helped maintain a normal pre-freeze cholesterol level in the sperm after cryopreservation. Labelling sperm cholesterol with filipin confirmed this result on sperm membranes. Furthermore, fresh sperm in skim milk extender treated 3 mg/ml CLC and incubated for 20 min in hypo-, iso- and hyperosmotic PBS have increased hypoosmotic tolerance, corresponding to the

beneficial effects of CLC at thawing. As expected, CLC treatment in skim milk extender improved sperm viability, motility and acrosome integrity after thawing. Furthermore, ultrasonography after artificial insemination revealed that sperm treated with CLC are 10% more fertile than control semen without CLC.

C-1014

Cryopreservation of Spermic Milt in the Model Species *Ambystoma tigrinum* (Tiger Salamander) for Application in Endangered Salamanders. R. MARCEC[1], C. Langhorne[1], C. Vance[2], A. Kouba[2], and S. Willard[1]. [1]Mississippi State University, Room 244, Box 9655, Dorman Hall, 32 Creelman St, Mississippi State, MS 39762 and [2]The Memphis Zoo, 2000 Prentiss Place, Memphis, TN 38112. Email: rmm415@ msstate.edu

In the face of the amphibian extinction crisis, captive assurance colonies have been established to preserve genetic diversity. Sadly, many captive assurance colonies fail due to reproductive cues which are not entirely understood. In addition, the majority of amphibian conservation and genome banking research has focused on anurans (frogs/toads), despite the fact that caudates (salamanders/newts) face a larger threat of extinction. Therefore, it is critical that assisted reproductive technologies, such as gamete cryopreservation and artificial fertilization, be developed as conservation tools for application in genetic management of threatened caudates. It was hypothesized that a modified anuran sperm freezing protocol could be successfully used in cryopreservation of *Ambystoma tigrinum* (tiger salamander) sperm as a caudate model. Spermiation in male tiger salamanders was hormonally induced via injection with hCG, LHRH, or a combination of these two hormones. Average spermic milt concentrations were $2.75x10^7(\pm7.2x10^6)$/mL. Average total motility upon collection of fresh milt was 53.6%(±17.6%). After collection and initial analysis, fresh milt samples were diluted 1:1 in chilled cryoprotectant treatment solutions. They were then frozen in 0.25mL French straws using a stepwise slow-rate freezing process. Upon thawing, milt suspensions were diluted 1:10 with a 10% Holtfreter's solution in order to activate the sperm for post thaw analysis. Frozen-thawed sperm in the 5% DMSO treatment group had an average total motility of 19.1% (±15.8%) and sperm in the 5% DMSO with 0.5% BSA treatment group had an average total motility of 21.75%(±14.8%). Early trials showed that caudate sperm are not susceptible to toxicity from several cryoprotective media. Moreover, initial fertilization trials indicate that frozen-thawed caudate sperm are capable of producing early cleavage stage embryos. Optimizing cryopreservation protocols for salamander sperm is an important step in building a global genetic resource bank for endangered caudates.

C-1015

High Survival of Mouse Oocytes and Preimplantation Embryos After Vitrification in the Absence of Permeating Cryoprotectants and Ultra-rapid Warming by an IR Laser Pulse. P. MAZUR and B. Jin. The University of Tennessee, Department of Biochemistry and Cellular and Molecular Biology, Room 191N Hoskins Library, White Street, Knoxville, TN37996-0840. Email: pmazur@utk.edu

In a companion presentation at this meeting and in a full paper published 3/22/14 on line in *Cryobiology*, we have reported that when Metaphase II mouse oocytes or 2-cell mouse embryos are suspended in EAFS vitrification media that contain only one-third the concentration of solutes as standard, over 90% survive when assessed by osmotic/morphological criteria and up to 85% survive when assessed by the functional ability to undergo IVF and/or develop *in vitro* to expanded blastocysts, provided that they are warmed at 10,000,000°C/min by a powerfui pulse from an IR laser. The base 0.3 x EAFS medium that we used contains ethylene glycol (EG) [0.70 molal], acetamide [0.71 molal], 70,000 MW Ficoll [0.0013 molal] , sucrose [0.16 molal], and PBS salts [0.15 molal]. Variants included 0, 0.16, 0.25, 0.5, and 1.0 molal sucrose, and 0.0062 molal Ficoll. Sucrose, Ficoll, and the salts can not penetrate the cells, The EG and acetamide can readily do so, but the 2-min exposure time is too short for this to occur. As a consequence, the cells are strongly dehydrated osmotically when vitrification is initiated. This raises the question of whether permeating solutes are needed to achieve high survivals after vitrification. In the companion presentation, we argued that the most important factor aside from ultra-rapid warming was the total molality (and hence, osmolality) of the medium. In the present presentation we address the matter of survival of not only oocytes, but of 2-cell and 8-cell embryos, and morluae and blastocysts vitrified in the total absence of permeating solutes. The morphological and functional survivals of vitrified 2-cell and 8-cell embryos were 92%; the survivals of vitrified oocytes and blastocysts were above 70%. All four of these survivals were obtained with laser-induced warming at 10,000,000°C/min. If warming was slowed 100-fold to the previous standard maximum of 100,000°C/min, morphological and functional survivals were zero. Since no one has heretofore warmed oocytes and embryos at the ultra-high rates used here, we believe that this is the first time it has been shown that these cells can be cryopreserved in a solution that is free of any permeating compounds.

C-1016

Implications of the Consequences of Laser-induced Ultra-rapid Warming of Mouse Oocytes and Embryos to Vitrification Theory and to the Successful Vitrification of Other Cell Types. P. MAZUR. The University of Tennessee, Department of Biochemistry and Cellular and Molecular Biology, Room

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Jin, Kleinhans, and Mazur have reported in this meeting that up to 100% of oocytes and 2-cell embryos survive vitrification in a 3-fold dilution of EAFS medium if warmed at 10,000,000°C/min by an IR laser pulse. The usual cooling rate was 69,000°C/min, but 95% also survived when cooled at 10,000°C/min. Thus, survival is far more dependent on the rate of warming than on the rate of cooling, the reason being that very high warming rates prevent or impede the recrystallization of intracellular ice. In all our experiments, the oocytes or embryos were held for 2.0 min in 1/3× EAFS before vitrification That time was chosen because Pedro (1997) found it to produce the best survival after vitrification. EAFS media are strongly hyperosmotic so that oocytes/embryos placed in them, undergo an osmotic shrink-swell pattern with time. There is an initial rapid shrinkage to a minimum volume due to water loss, followed by a much slower re-swelling due to the slow influx of the permeating solutes. Based on Pedro's data, that minimum occurs near 2 min, and osmotic theory argues that at that point, the cells have lost 85% of their original water and very little permeating solute has entered them. The strong inference, then, is that the high subsequent survivals are a consequence of the 85% osmotic dehydration and are **not** due to the intracellular presence of permeating solutes. Supporting that conclusion is Jin and Mazur's report that 77% of oocytes and 95% of 2-cell and 8-cell embryos survive vitrification when suspended in media containing only the non-permeating solutes sucrose *provided* they are warmed at 1×10^{7}°C/min by a pulse from our IR laser. None survived when warmed 100× more slowly in the absence of the laser. These findings are at odds with orthodox beliefs about the causes of injury from vitrification. They could possibly open the way to the successful vitrification of important cell types that to date have been partially or fully refractory to this approach.

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Improving Cryoprotectant Tolerance of Zebrafish Oocytes. A. EROGLU, E. Szurek, and D. Miao. Medical College of Georgia/Georgia Regents University, 1120 15th Street, CA-2004, Augusta, GA 30912. Email: aeroglu@gru.edu

Successful cryopreservation of zebrafish oocytes and embryos remains elusive due to several obstacles, among which are their susceptibility to osmotic stresses and toxicity of cryoprotective agents (CPAs). We hypothesized that the tolerance of zebrafish oocytes to CPAs can be improved by lowering the overall salt concentration of the cryopreservation medium while simultaneously increasing CPA concentrations. To test our hypothesis, we selected 90% L-15 as a starting isotonic cryopreservation medium and exposed a total of 522 stage III oocytes to increasing concentrations (i.e., 0.1, 0.3, 0.4, and 0.5M) of trehalose in 90%, 50%, and 10% dilutions of L-15 medium at 25ºC for 30 min. The tolerance of the treated oocytes was determined based on their *in vitro* maturation (IVM) rates with respect to that of untreated controls. While 60% of controls matured in 90% L-15 containing no trehalose, IVM rates rapidly declined by increasing trehalose concentrations in 90% L-15, and only a few (1.2%) stage III oocytes were able to mature in 90% L-15 containing 0.4M trehalose. In contrast, 0.4M trehalose was well tolerated in 50% L-15 and 10% L-15 with IVM rates of 37% and 53%, respectively. Further increase in trehalose concentration (i.e., 0.5M) was tolerated only in 10% L-15 with an IVM rate of 44%. Based on these results, we selected 10% L-15 with 0.3M trehalose as a base cryopreservation medium and then exposed a total of 492 stage III oocytes to 10% concentration of methanol, propylene glycol (PG), and ethylene glycol (EG). In the first two exposure groups (i.e., methanol+trehalose and PG+trehalose), the rates of IVM were similar to that of untreated controls while exposure to EG in combination with trehalose significantly reduced IVM of stage III oocytes. Taken together, our results suggest that zebrafish oocytes can well tolerate molar concentrations of certain CPAs when the overall salt concentration of the suspending medium is appropriately lowered.

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Stabilizing Effect of Liposomes on Rat Red Blood Cell Membrane Quality Is Treatment-time Dependent. L. DA SILVEIRA CAVALCANTE, J. P Acker, and J. Holovati. University of Alberta and Canadian Blood Services, 8249-114 Street, Edmonton, AB T6G2R8, CANADA. Email: luciana@ualberta.ca

Liposome treatment minimizes red blood cell (RBC) membrane damage occurring during 42 day hypothermic storage (HS) in additive solutions. The aim of this study was to examine the effect of time of the liposome treatment on the *in vitro* RBC quality parameters. Leukoreduced, packed rat RBCs were incubated for 30 min at 37 °C in AS3 solution with either HEPES-NaCl solution or unilamellar liposomes (1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC):cholesterol, 7:3 mol%, 2 mM lipid) . RBC membrane quality was evaluated by % hemolysis (Drabkins), deformability (ektacytometry), aggregation (syllectometry), hematological indices (Coulter) and microvesiculation (flow cytometry) immediately following RBC-liposome treatment and following 6 weeks of HS. DOPC liposome treatment resulted in decreased in percent hemolysis (1.4 ± 0.2% vs. 2.1 ± 0.3%, p = 0.03), increased in deformability (0.58 ± 0.01 vs. 0.57 ± 0.00, p = 0.03) and aggregation amplitude (15.4 ± 0.9 au vs. 12.5 ± 1.6 au, p = 0.03). After 6 weeks of HS, liposome-treated RBCs continued to differ from control RBCs by exhibiting increased aggregation amplitude (15.9 ± 3.2 au vs. 14.1 ± 0.7 au, p = 0.04). Flow cytometry analysis also showed increased

microparticle (MPs) concentration in liposome-treated RBCs (812,113 ± 322,762 MPs/μL vs. 598,073 ± 132,869 MPs/μL, p = 0.03) and increased phosphatidylserine (PS) exposure in both RBCs (1.7 ± 0.6 vs. 1.5 ± 0.4, p = 0.05) and MPs (55.8 ± 6.5 vs. 34.8 ± 8.5, p = 0.03). The stabilizing effect of liposome treatment on rat RBCs is evident immediately after the incubation for parameters such as hemolysis. However, the presence of liposomes throughout the 6 week storage period induces MPs formation and PS exposure. Considering that most storage-induced membrane lesions start to appear after 2 weeks of HS, future research will examine the time-point when the liposome treatment would be most beneficial.