DEVELOPMENT OF A MULTIFUNCTIONAL INSTRUMENT AND AUTO-GENERATED PROTOCOLS TO MINIMIZE CELL OSMOTIC INJURY DURING CPA REMOVAL

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Introduction

- The CPA removal from cells after thawing is one of essential steps in cell cryopreservation, as any improper cell washing procedure might cause damage to cells.
- A multifunctional cell processing instrument is developed to improve the CPA removal process and the cell preparation process with the minimum processing time and osmotic injury to cells.
- Compared with the traditional centrifuge method, this multifunctional cell processing system can reduce cell clumping and loss as well as potential cell contamination risk. Another significant advantage of the system is that all cell processing procedures can be automatically performed by the instrument.

Simulation Model

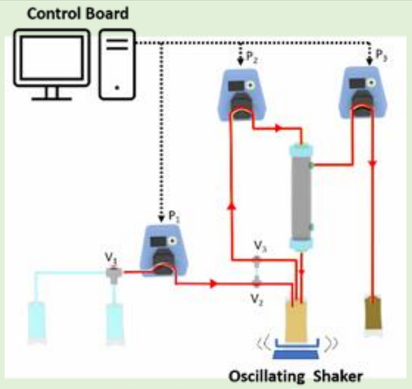
$$\frac{dV_c(t)}{dt} = \frac{dV_s(t)}{dt} + L_p \cdot A \cdot (C^i - C^e) \cdot R \cdot T$$

$$\frac{dM_s(t)}{dt} = P_s \cdot A \cdot (C_s^e - C_s^i)$$

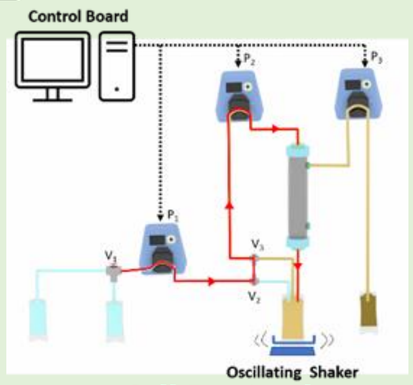
$$M_s(t) = V_s(t)/v_s$$

$V_c(t), V_s(t)$:cell volume and intracellular CPA volume at time t
L_p: Cell membrane permeability coefficient to water
P_s : Cell membrane permeability coefficient to CPA
A : Cell surface area
C^e, C^i: *Extra* and I*ntracellular osmolarity* including CPA and saline
C_s^e, C_s^i: *Extra* and I*ntracellular CPA osmolarity*
$M_s(t)$: Mole of intracellular CPA at time t
V_s : partial volume of CPA(mole/L)

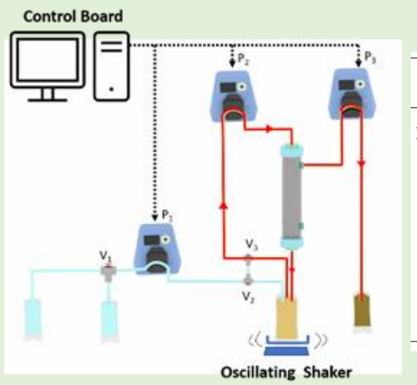
Experiment Setup



CPA Removal



Cell Retrieval

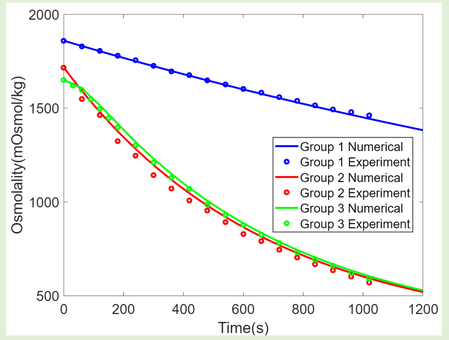


Cell Concentration

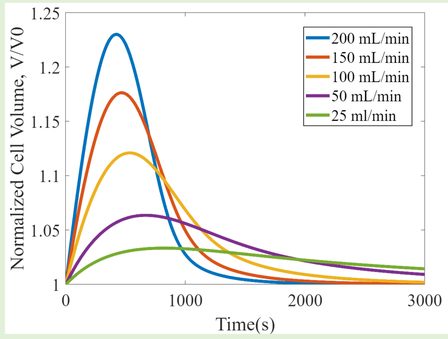
Group	Group 1	Group 2	Group 3
Dilution/Filtration Rate	20mL/min	100mL/min	100mL/min
Total Volume	1110mL	1110mL	1113mL
Osmolality of Dilution Fluid	292	292	1153 (0-60s) 549(61-120s) 376(121-180s) 292(181s-)

Setup of CPA Removal Experiments

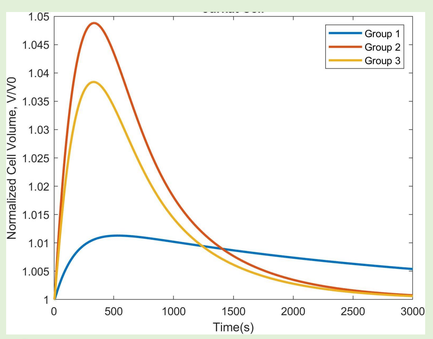
Results and Discussion



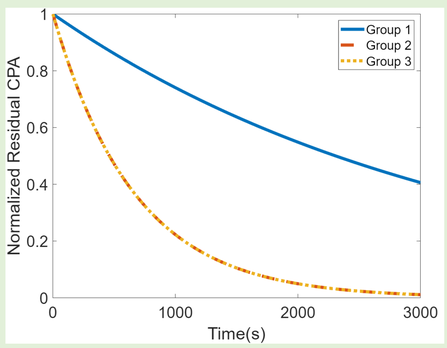
1 Simulation and experiment results of CPA removal experiments



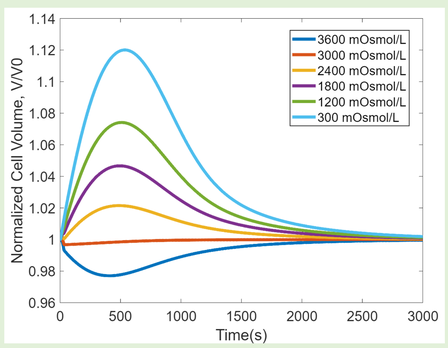
3 Jurkat cell volume change with different CPA removal approaches



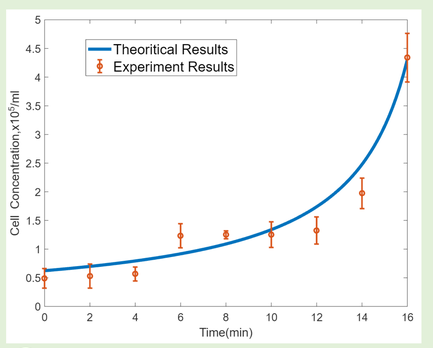
5 Jurkat cell volume change with different CPA removal approaches



2 Normalized Residual CPA with different removal approaches



4 Normalized cell volume change with the different hypertonic solution in a first-half minute on the hypertonic mode



6 Cell concentration function of the multifunctional cell processing system

- CPA removal model is validated by experiment results. (Figure 1)
- The isotonic mode pursuits simpler and faster removal under given cell volume tolerance limits, while the hypertonic mode is superior for minimal osmotic injury.(Figure 2)
- Three types of human cells, Jurkat cells (Figure 3-5), vaginal T cells, and vaginal macrophage, were investigated for their cell volume reactions with different CPA removal approaches.
- With the given cell membrane transport properties, specific optimal CPA removal protocol or treatment can be generated by the algorithm derived from mass transfer equations. (Figure 4)
- The final Recovery rate of Jurkat cells is 86.0% (Figure 6).

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A New Approach for DMSO-Free Cell Cryopreservation

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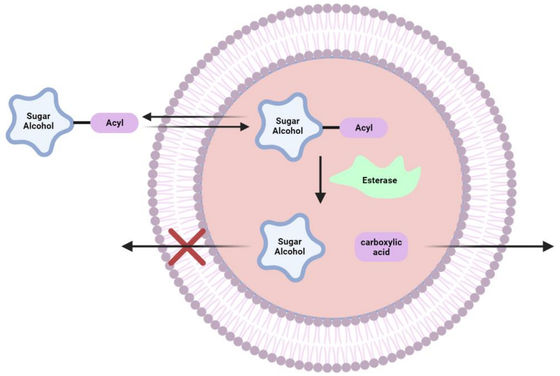
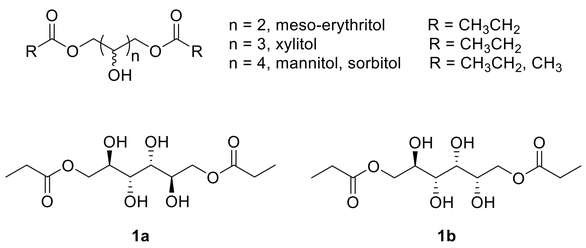
Introduction

Cryoprotectants (CPAs) are substances that protect biological samples from damage from freezing. CPAs could work either extracellularly or intracellularly depending on their membrane permeability. Extracellular CPAs, which are much more common than intracellular CPAs, act outside cells and usually have limited preservative capability. On the other hand, intracellular CPAs could be more effective but their number is limited. DMSO, the most used membrane-permeable, is very effective in the cryopreservation of cells and some tissues. However, DMSO is limited by its cytotoxicity at elevated temperatures and at the high applied concentrations that lead to protective effect. These limitations hamper the direct use of DMSO, especially in clinical applications.

Methods

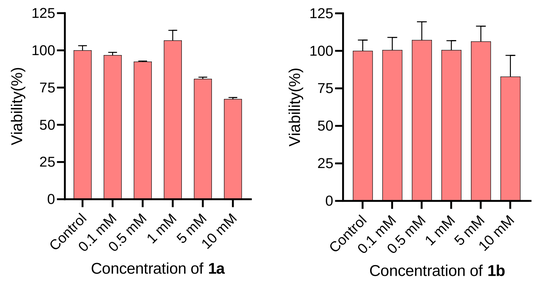




1a 1b

Results

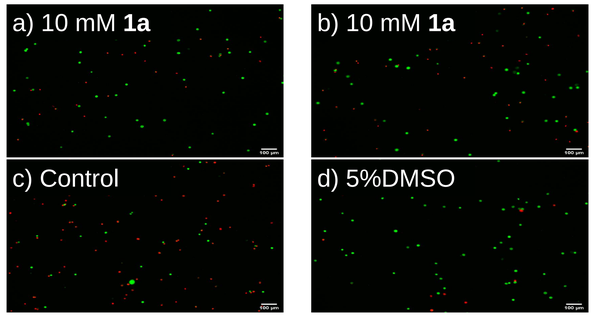
1. Cytotoxicity



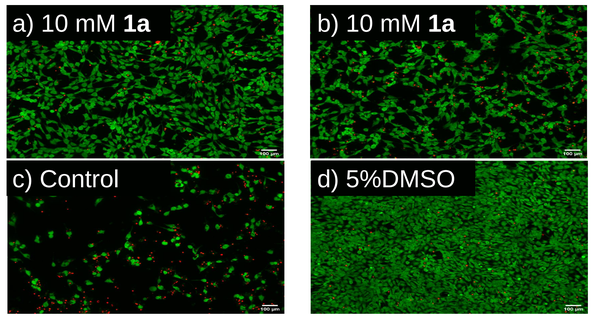
After incubating for 48 hours with 3T3 cells, Compounds **1a** and **1b** do not show cytotoxicity. (Only 10 mM 1a causes a modest reduction in 3T3 cells proliferation after 48 h.)

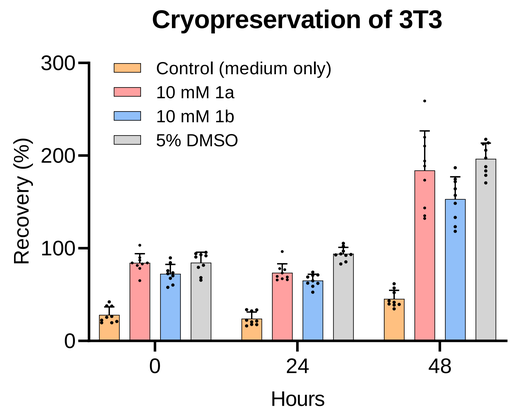
2. Cryopreservation of 3T3

0-hr Post-Thaw



24-hr Post-Thaw





Compounds **1a** and **1b** exhibit similar cryoprotective as that shown by DMSO.

Conclusion

A strategy is developed for developing a new class of CPAs derived from readily available sugar alcohols including sorbitol, mannitol, xylitol, and erythritol. Esters obtained from the partial acylation of these sugar alcohols are water-soluble, non-cytotoxic, and biocompatible at concentrations up to 10 mM. Initial studies indicate that some of these esters are membrane-permeable and exert their cryoprotective effect intracellularly. NIH-3T3 cells treated with some of the acylated sugar alcohols exhibited high post-thaw recovery rates and excellent post-thaw viability which are comparable to the effect of DMSO.

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Acknowledgement

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2. SKM Pharmatech is the Commerical Partner for this project (Kris Huang, kris.huang@skmpharmatech.com)

Pre-activated freezing nucleation close to 0°C

Gabor Vali, University of Wyoming, Laramie, WY, USA

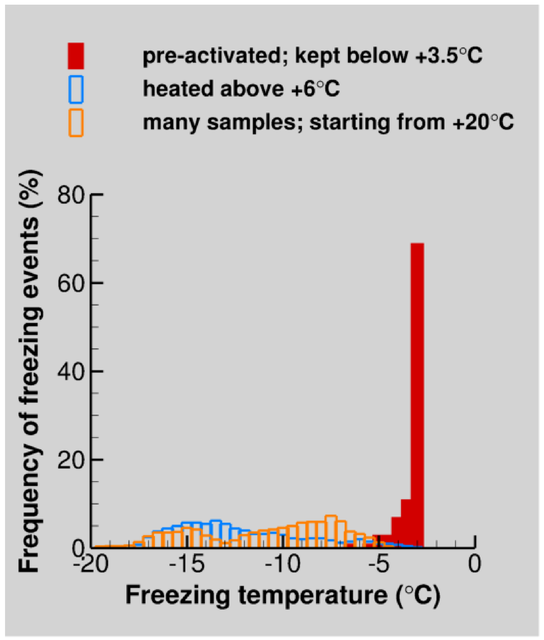
The abilities of different materials to promote freezing at temperatures above homogeneous freezing has been determined for many naturally occurring and synthesized substances. Minerals are the most common inorganic materials that form ice nucleating particles (INPs). Entities of biological origins (certain bacteria, macromolecules from vegetation) are less common but are more effective. Silver iodide and cultures of *Ps. syr.* bacteria are the most effective artificial sources of INPs.

In addition to the basic process of nucleation with INPs that are relatively stable and are exposed to a gradual sequence of lowering temperatures until nucleation occurs, there are a number of ways that the previous history of exposure to different temperature and humidity conditions can influence heterogeneous ice nucleation. Pre-activation is one of these; it is of particular interest because it holds promise in various applications for achieving freezing with very small supercooling below 0°C.

The purpose of this presentation is to call attention to this highly effective ice nucleation process. Pre-activation, has been known to exist for about 70 years but has been little studied and nearly forgotten. Current knowledge is still limited, but recent experiments conducted with mercuric iodide demonstrate consistency with the early results which did involve a larger range of substances..

Main point: Combined with earlier results, the new experiments demonstrate the potential for pre-activated freezing nucleation (PFN) with various substances within a few degrees of 0°C.

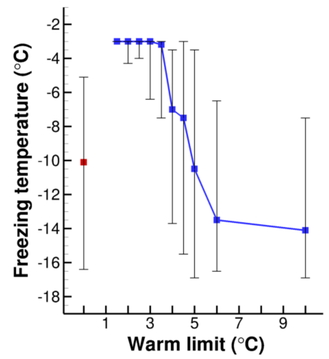
History: Edwards, Evans and Zipper (1970) showed pre-activation on mercuric iodide, lead iodide, cadmium iodide, gypsum, muscovite, L-asparagine, p-benzyl phenol and L-aspartic acid. No clarification emerged as to what common property leads to pre-activation potential on these substances. In contrast, graphite, chlorite, silica gel and a few other substances resisted pre-activation. No follow-up research is known, except for a repetition of the tests with mercuric iodide (HgI_2) by Vali (1992, 2021) and some yet unpublished work by T. Leisner.



The diagram above shows the results of experiments with numerous small drops containing suspended mercuric iodide particles and subjected to continuous cooling cycles.The green histogram shows the freezing temperatures of several different set of drops when first cooled from room temperature. The red histogram shows the freezing temperatures of a set of 99 drops in four cycles of cooling, and with melting at temperatures not exceeding +3.5°C. The blue histogram is for the same drops after four additional cycles with melting at temperatures above +3.5°C. The pre-activated freezing (shown in red) may in fact have been initiated at temperatures closer to 0°C but were not detected earlier due to instrumental limitations.

Recent experiments: The conditions necessary for PFN were delineated in several series of experiments. All were performed with drops placed on a temperature-controlled cold stage. The drops were produced from a suspension of HgI_2 powder at 0.02 to 0,04 g/mL concentration. This amount assured that every drop contained a very large number of particles. Freezing was detected as the drops were cooled by a change in opacity. This method is quite simple by today's standards, and is limited to detecting ice in the drops at -2°C or colder. More complete descriptions of the experiments are given in Vali (2021).

Various tests were directed toward finding the maximum temperature that the drops can be exposed to while melting and still retain the ability for PFN. The results converge on a limit near +3.5°C, but this limit is not completely sharp.The figure below shows the results for a series of freezing cycles with gradual increases of the temperature at which the drops were allowed to melt.



Square symbols indicate the 50th and the bars the 5th and 95th percentiles.

Other experiments with cycling from colder to warmer melting temperatures, or with interleaved ones, show little influence on the upper limit for PFN to be exhibited. These results have been confirmed by recent experiments conducted by T. Leisner at the Karlsruhe Institute of Technology. **Importantly, some pre-activated freezing was detected in his experiments at -1.5°C.**

It has also been shown that the length of time in the melted state has only a minor influence on the subsequent freezing temperatures.

Caveat: The results cited here are taken from larger data sets which also show some unexplained variability of HgI_2. The effects of dissolved substances on PFN is not yet known.

Implications: Results to date are clear on the potential for the PFN process to produce freezing very close to the melting point, but very few substances are known to exhibit PFN. If PFN can be demonstrated for materials that are compatible with tissue preservation, frost damage mitigation, and other applications, it opens the possibility for freezing to be induced at temperatures as close as 2°C or less to the melting point. Clearly, much more research is needed before practical use can be considered.

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Vali, G., 2021: Exploratory experiments on pre-activated freezing nucleation on mercuric iodide. Atmos. Chem. Phys., 21, 2551-256

RAPID AND UNIFORM REMARMING BY SINGLE-MODE ELECTROMAGNETIC RESONANCE CAVITY: EFFECT OF SAMPLE SHAPE

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Introduction

- **Rapid and uniform** warming is needed for the successful cryopreservation of **large biomaterials** (tissue and organ) to avoid ice-recrystallization and thermal-stress-induced fracture.
- **Electromagnetic (EM)** is a promising rewarming method for its fast and volumetric heating principles.
- **Shape** of the sample holder and solution is essential in solving energy conversion and heat transfer analysis.

Objectives

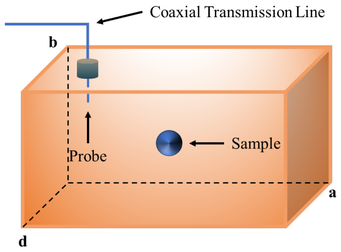
- A numerical simulation model was developed with COMSOL software to determine the **rewarming rate** and **temperature distribution** during the rewarming process in an EM resonant cavity system.
- The numerical simulation model was **validated experimentally** with the heating of 25 mL dimethyl sulfoxide (DMSO) solution.
- Different sample holder shapes including **cube, cylinder, ellipsoid, and sphere** were investigated.

Materials and Methods

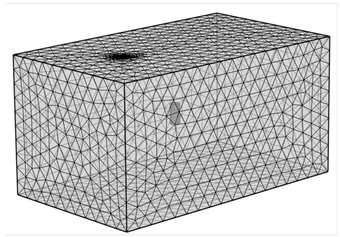
Analytical setup of the EM resonant cavity

Maxwell Equations

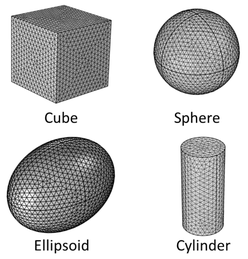
$$\nabla \times \mathbf{H} = -j\omega\varepsilon\mathbf{E} + \mathbf{J}$$
$$\nabla \times \mathbf{E} = -j\omega\mu\mathbf{H}$$
$$\nabla \cdot \varepsilon\mathbf{E} = \rho_e$$
$$\nabla \cdot \mathbf{H} = 0$$



Numerical modeling setup



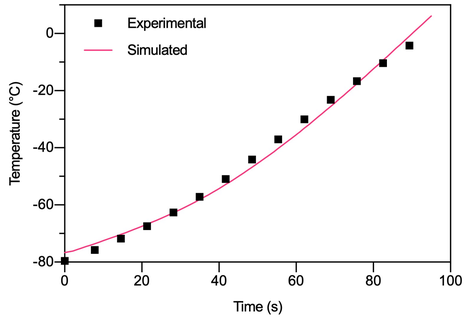
EM Resonant Cavity

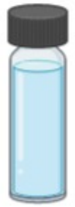


Different Shapes of Sample Holder

The Nyquist criterion was applied to the meshing grid.

Validation of the numerical model

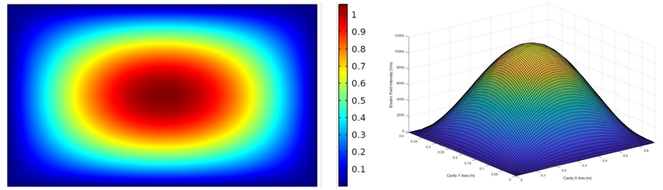




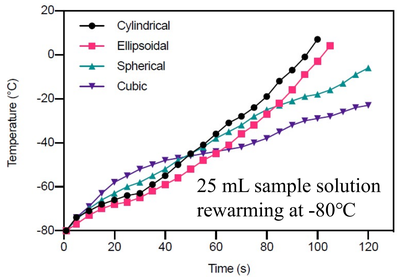
25 mL DMSO Solution

Results

Electric field distribution in the EM resonant cavity

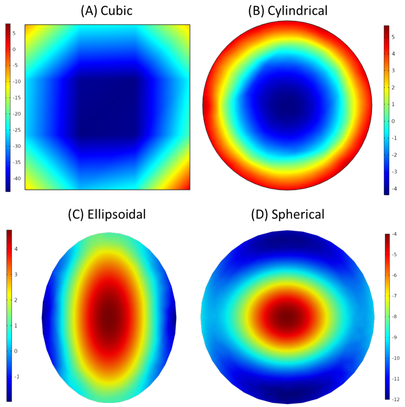


Temperature history during the rewarming process



Shape	Rate (°C/min)
Cylinder	57.47
Ellipsoid	54.36
Sphere	43.15
Cube	31.52

Temperature distribution at the ending of rewarming



Shape	Temperature Gradient (°C/mm)
Cylinder	0.97
Ellipsoid	0.42
Sphere	1.03
Cube	1.84

Conclusion

- Sample holder with sharp surfaces and edges (cubic) are sub-optimal choice for EM cavity heating.
- Cylindrical holder achieved fastest rewarming rate while ellipsoidal holder reached minimum temperature gradient.
- Considering the difficulties in the manufacturing of ellipsoidal holder, **cylindrical** holder is the best shape for **EM resonant cavity heating**.

On-going Work

- Further improvements on the cylindrical sample holder to optimize rewarming uniformity (e.g., **selected CPA solution**, addition of **magnetic nanoparticles**, etc.)

Acknowledgements

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NaCl EXTENDER IMPROVES KINETICS PARAMETERS AND REPRODUCTIVE CAPACITY OF FISH POST-THAW SPERM

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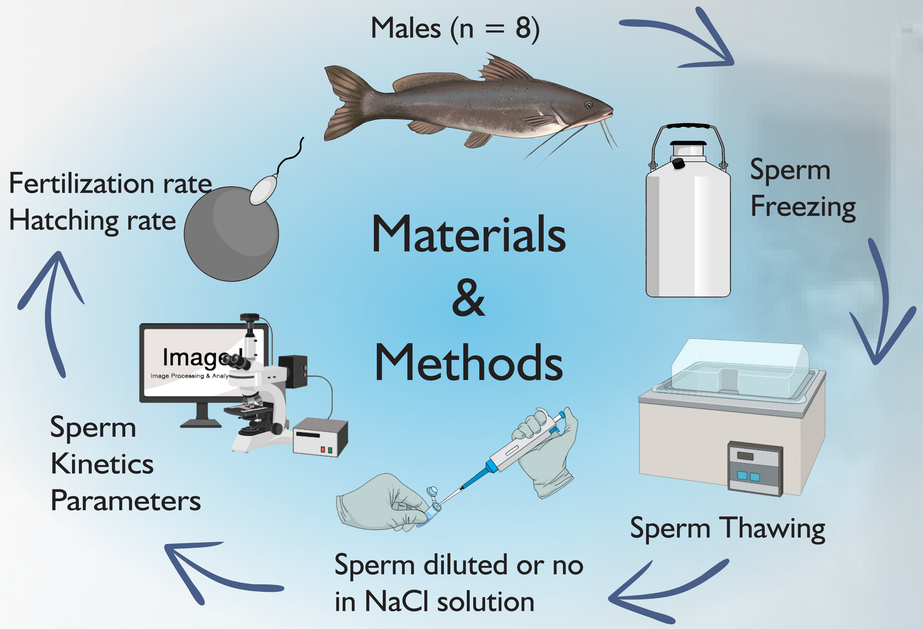
Jhony L. Benato | Lis S. Marques | Eduardo A. Sanches | Danilo P. Streit Jr

Introduction

Sperm cryopreservation is an important technique to scientific and aquaculture purposes. However, researchers try to minimize damage to sperm cells during freezing and thawing, neglecting the period after thawing in which the sperm is in contact with the toxic cryoprotective solution at room temperature.

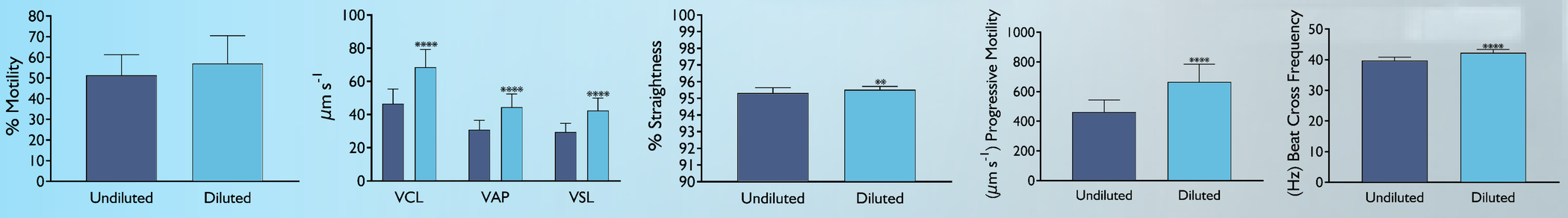
Objective

Thus, the aim was to evaluate the kinetics parameters (CASA) and reproduction capacity of South American Catfish (*Rhamdia quelen*) post-thaw sperm diluted in NaCl solution (325 mOsm kg-1, pH 7.6, 25°C).



Results

** p<0.01;
*** p<0.001,
**** p<0.0001;
Paired T-Test



Fertilization Rate

65% Undiluted

93%**** Diluted

Hatching Rate

55% Undiluted

82%** Diluted

Conclusion

Therefore, the dilution in NaCl extender of South American Catfish *Rhamdia quelen* post-thaw sperm should be used in the protocol for this specie. This methodology should be tested in other.

Cryopreservation of South American Neotropical fish sperm: Current status

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Objective

The aim of the study was to carry out a bibliographic survey on the history of research on sperm cryopreservation of neotropical fish from South America.

Method

Existing scientific studies were searched using the main scientific databases (Scopus, Web of Science, Pub Med and Science Direct)

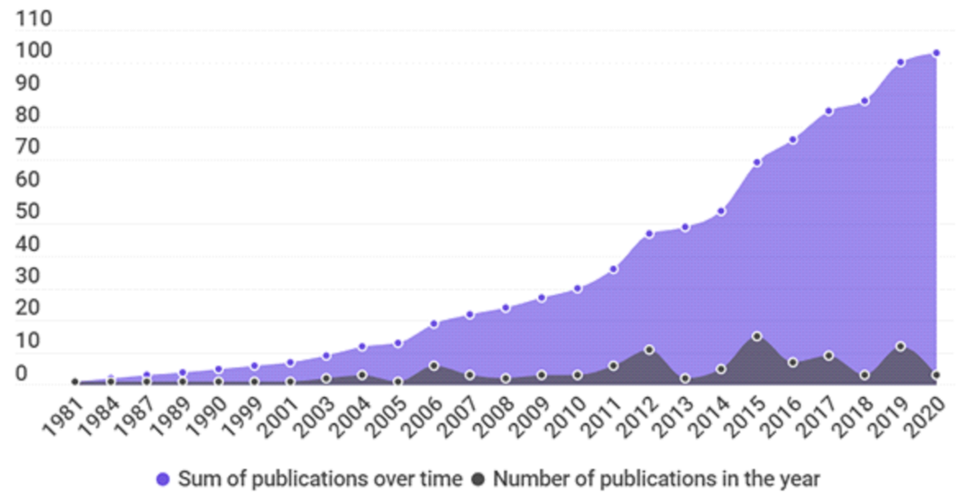
Conclusion

The species P. lineatus, R. quelen and B. orbignyanus have developed protocols with good results and efficiency in the generation of larvae. The use of commercial germplasm banks to produce juveniles destined for fish production farms is not yet a reality in Brazil.

Study location

South America

Publication history



Fish families

SUPPORT:

FERTILIZATION AND HATCHING RATES AFTER CRYOPRESERVATION OF *Rhamdia quelen* MILT CONTAMINATED WITH BLOOD

Raquel S. Santos, Lis S. Marques, Thales S. França, Rômulo B. Rodrigues, Maritza P. Atehortúa, Nathalia S. Teixeira, Thaiza R. de Freitas, Itamar C. Gomes, Jhony L. Benato, Danilo P. Streit Jr

Federal University of Rio Grande do Sul, Brazil

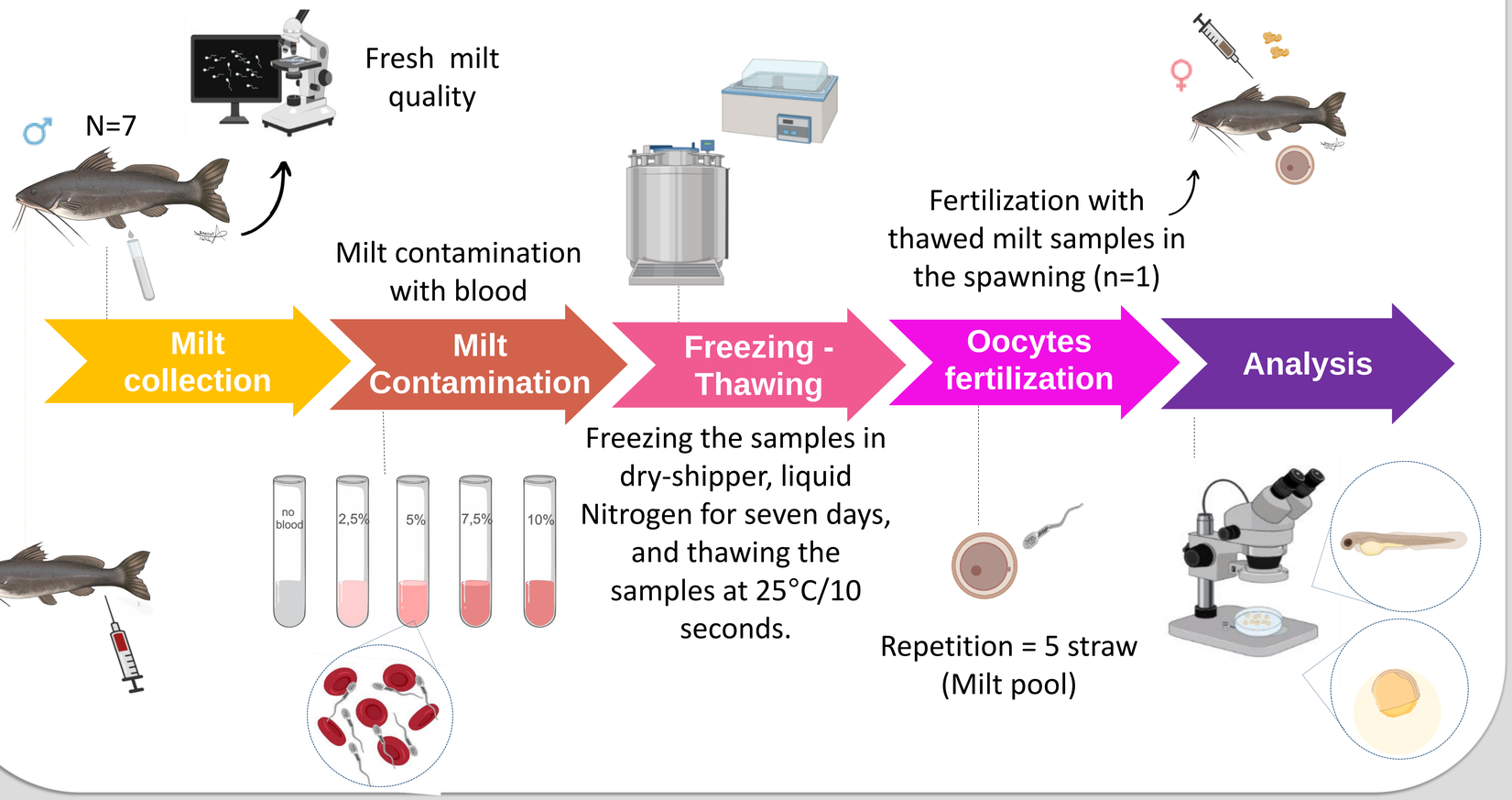
INTRODUCTION

Contamination of fish milt by blood may occur during milt collection and may cause damage to seminal quality, and for this reason the samples are discarded.

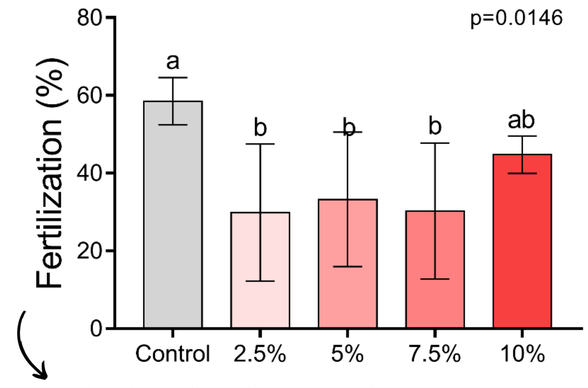
OBJECTIVE

To evaluate fertilization and hatching rates after cryopreservation of *Rhamdia quelen* milt contaminated with blood (2.5, 5, 7.5 and 10%).

MATERIALS AND METHODS

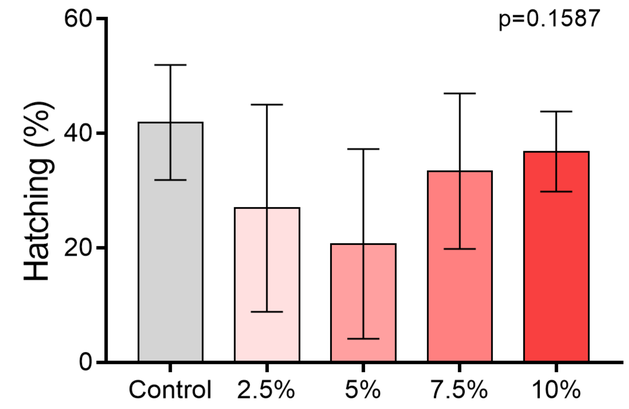


RESULTS AND DISCUSSION



The low fertilization shown can occur due to white blood cells, which affect the fatty acids of the sperm membrane;

Damaged membrane may lose its depolarization capacity, affecting sperm motility.



Acknowledgment

CAPES

CNPq

CONCLUSION

Contamination of milt with up to 7,5%% of blood decreases the rates of sperm fertilization after freezing in *R. quelen,* and do not influence on hatching rate.

EXPECTATION: The analysis of the kinetic parameters of the milt and the characteristics of the offspring generate subsidies for endangered species, in addition to predicting possible financial losses in the cryopreservation efforts.

Vitrification changes the fatty acids profile of zebrafish ovarian follicles at different developmental stages

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[1]University of São Paulo, São Paulo, BR
[2]Federal University of Rio Grande do Sul, Porto Alegre, BR

Introduction

Fatty acids (FA) accumulated during follicular development are essential for oocyte growth and maturation, as well as for the embryo generated. Alterations in the FA profile can compromise the oocyte development viability after cryostorage. Thus, our aim of this study was to analyze the possible changes in the FA profile generated during vitrification.

Methodology

Ovarian follicles at primary growth, previtellogenic and vitellogenesis collected manually in L-15 medium from adult female zebrafish were analyzed under fresh and vitrified conditions. Vitrification was carried out by exposing follicles first to an equilibrium solution (1.5M methanol+2.25M Me_2SO+0.25M sucrose) and then, to vitrification solution (1.5M methanol+5.5M Me_2SO + 0.5M sucrose) and nitrogen storage. FA were extracted using chloroform:methanol:water (2:1:0.5), methylated using acetyl chloride (5% of HCl in methanol), and the FA composition was analyzed as methyl esters (FAME) using a gas chromatograph. FAMEs were identified by comparing their retention times to those obtained from commercial standards.

Results

Initial analysis indicated a difference in FA profile between fresh and vitrified follicles at different stages (Fig. 1). In fresh follicles, there was a higher number of polyunsaturated FA omega-3 and 6 (PUFA n-3 and n-6), contributing to the highest percentage of arachidonic acid (ARA; C20:4n6) and docosahexaenoic acid (DHA; C22:6n3) in the previtellogenic and vitellogenesis follicles. PUFA n-3 and n-6 decreased from 21.27% to 1.93% in previtellogenic follicles and from 16.97% to 1.83% in vitellogenesis follicles after vitrification. Moreover, there was an increase in saturated fatty acids (SFA) after vitrification

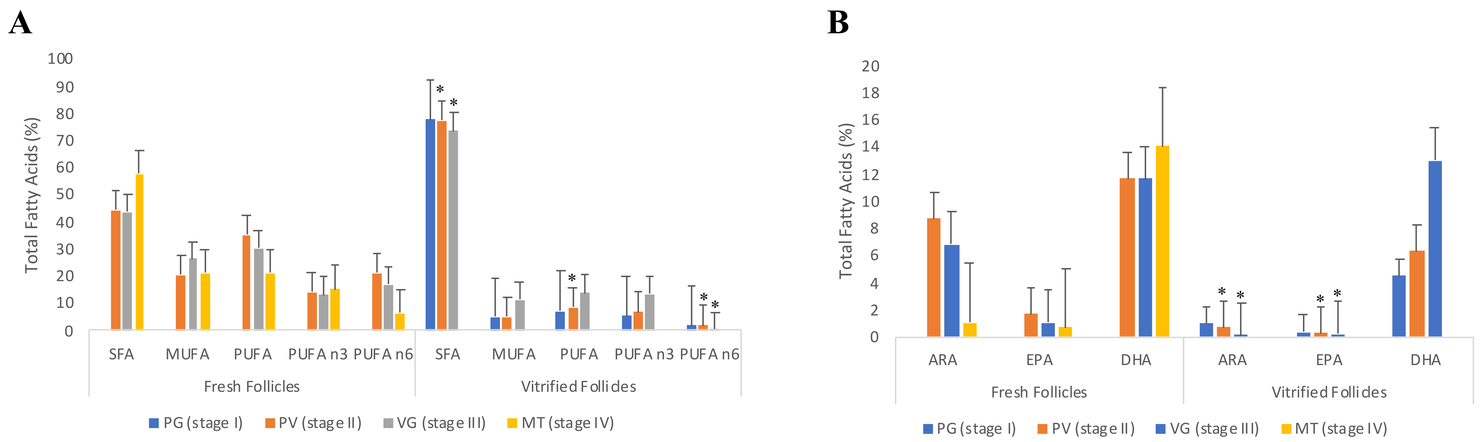

Fig.1. Profile of total fatty acids (A) and polyunsaturated fatty acids (LC-PUFA) (B) in fresh (control) and vitrified ovarian follicles in the following stages of development: primary growth (PG; stage I) in blue, pre-vitellogenic (PV; stage II) in orange, vitellogenic (VG; stage III) in gray and mature (MT; stage IV) in yellow.

Conclusion

Vitrification drastically changed the FA profile in follicles. The increase of SFA and reduction of PUFA suggests a more rigid and less fluid membrane, with less folds, which can impair its functional aspects. Furthermore, the quality of the follicles for future uses could be also impaired, as PUFA like ARA regulates various aspects of fish endocrine function and stimulate follicle maturation.

Comparative Analysis of Cryopreservation of Spermatozoa from *Bombus impatiens* and *Apis mellifera*

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[1]Department of Zoology &Physiology, University of Wyoming, Laramie WY; [2]Edward T. Schafer Agriculture Research Center, USDA-ARS, Fargo, ND 58102

Introduction

Pollinator populations are increasingly under threat from urban and rural landscape change, pesticides, pathogens, and climate change. As more bumble bee species are increasingly at risk of extinction, it is critical to develop methods to preserve their genetic material. Cryopreservation of genetic resources provides insurance against species loss. Cryopreservation techniques have been developed for honey bee spermatozoa but not for bumble bees **(1)**. This protocol will be instrumental for artificial insemination in bumblebees apart from aiding conservation of genetic traits that might otherwise be lost. The purpose of this study was to :

1. Quantify how a simple cryopreservation procedure affects spermatozoa viability using *Bombus impatiens* and *Apis mellifera*

2. Evaluate if the designed procedure could be used for future reproductive protocols for *Bombus* species.

Methods

Spermatozoa from the seminal vesicle of multiple *B. impatiens* and *A. mellifera* were pooled into four samples per treatment for each of the species. Seminal vesicles were dissected in honey bee semen diluent (**2**) and gently crushed to release the spermatozoa. Semen samples were either stained immediately for viability or cryopreserved. The extended semen was loaded in cryo-straws as equal parts of semen & 20% DMSO. The straws were frozen in a KRYO 10 Series 3 freezer (Planer, UK). Spermatozoa was frozen at a rate of 3°C/minute starting at 4°C to -40°C with no seeding.

Cryopreserved straws were stored in liquid nitrogen for two weeks before thawing & viability assessment. Sperm viability was assessed using a sperm live-dead assay kit (Invitrogen/ThermoFisher).

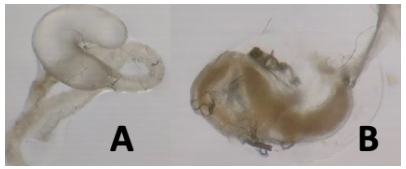

Fig. 1. Male accessory reproductive Structures in *Bombus impatiens*. Seminal vesicle and accessory gland from an immature drone (A) and from a mature drone with abundant spermatozoa (B).

Results

To successfully inseminate queens with cryopreserved semen, the arbitrary acceptable spermatozoa viability has to be ~46% after taking into consideration the dilution factors and the amount required per insemination.

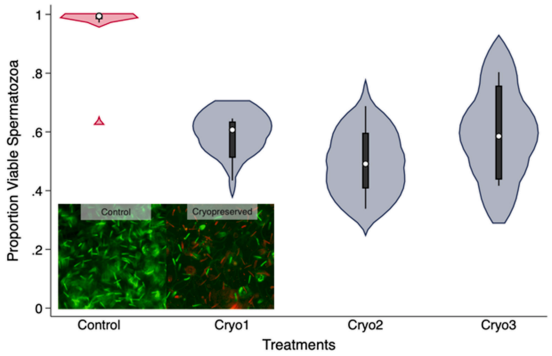

Fig. 2. **Cryopreservation of *Bombus impatiens* spermatozoa derived from seminal vesicles**. Sperm viability decreased significantly after cryopreservation compared to untreated controls. Variation in viability between three different treatment batches and the controls were significant when compared using Dunn's Test ($\chi^2(3) = 15.6$; $p = 0.0093$, 0.0070 and 0.0107; n = 24).a

The results of this study, we believe, are optimal for further insemination studies for *B. impatiens* compared to *A. mellifera*

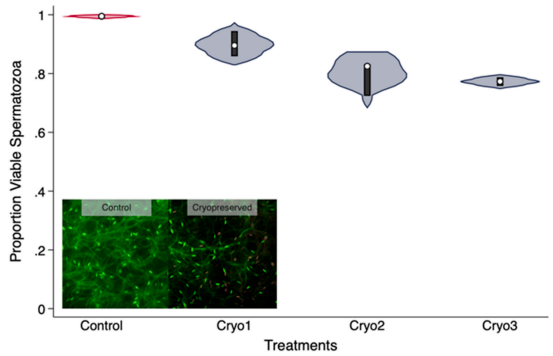

Fig. 3. **Cryopreservation of *Apis mellifera* spermatozoa derived from seminal vesicles**. Sperm viability was significantly lower after cryopreservation compared to untreated controls. Variation in viability between three different treatment batches and the controls were significant when compared using Dunn's Test ($\chi^2(3) = 11.4$; $p = 0.1428$, 0.0132 and 0.0097; n = 14) with the exception of the treatment 'Cryo1'.

Conclusions

This is the first successful attempt to cryopreserve spermatozoa of a *Bombus* species. These results are encouraging as relatively normal spermatozoa and useful viabilities were achieved. This methodology will further our *in vitro* and artificial insemination studies in *Bombus*, as well as provide for long term storage of genetic diversity of *B. impatiens.* Alterations to semen diluent making it more genus specific could also improve sperm viability in *Bombus.* Further research is being conducted to investigate spermatozoa motility and genetic intactness using CASA and TUNEL assays. Adaptation of this methodology to other species of *Bombus* will vastly facilitate conservation efforts for bumble bees. The freezing protocol assessed in this study provides a proof of concept for the genetic preservation of *Bombus* germ cells.

1) Taylor, M. A., Guzman-Novoa, E., Morfin, N., Buhr, M.M. 2009. Improving viability of cryopreserved honey bee (Apis mellifera L.) sperm with selected diluents, cryoprotectants, and semen dilution ratios. **Theriogenology** 72:: 149-159.

(2) Rajamohan, A., Danka, R.G, Rinehart, J.P. 2020. A non-activating diluent to prolong in vitro viability of *Apis mellifera* spermatozoa: Effects on cryopreservation and on egg fertilization. **Cryobiology** 92 (2020) 124-129.

Effect of quercetin on cryopreservation of Japanese Black bulls spermatozoa

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Introduction

- Cryopreservation of sperm and artificial insemination have a significant, positive effect on cattle production.
- During the process of cryopreservation, endogenous antioxidant concentration, semen quality and fertility become compromised due to the generation of reactive oxygen species.

- Quercetin is a component of flavonoids found in the wide range of foods including vegetables and fruits.
- It demonstrates antioxidative, anti-aggregatory, anti-inflammatory and anticarcinogenic features.
- Quercetin has been using as an outstanding antioxidant in terms of potent scavenger of reactive nitrogen species and oxygen species.

Objectives

Aim of this study was to evaluate effect of different concentration of quercetin on cryopreservation of Japanese Black bulls spermatozoa.

Materials and Methods

- semen was collected from four Japanese black bulls using an artificial vagina.
- After initial evaluation diluted with Tris egg yolk diluent, divided into four treatments (0, 50, 100, 200 µM quercetin) and cryopreserved.
- Motility and kinematics of frozen-thawed spermatozoa were objectively evaluated by computer assisted sperm analyzer at 0 and 60 min post-thawing.

Results and Discussion

- After thawing results showed higher percentage of total motility in 100 µM quercetin.
- Also percentage of straight-line velocity is higher in 100 µM quercetin in compare to the other treatments.
- The same results were observed after 60 min incubation of spermatozoa at 37 °C.
- Quercetin could improve motility parameters however high concentration of quercetin might be harmful for sperm cryopreservation.

Conclusion

In conclusion, quercetin improves different motility parameters of frozen thawed bulls spermatozoa, and more investigation for other sperm parameters are ongoing.

THE EFFECT OF MELATONIN ON BOAR SPERM PLASMA MEMBRANE FLUIDITY AND CRYOSURVIVAL

Norma A Ramirez-Campos, Alicia Alcantar-Rodriguez, Oscar Gutierrez-Perez, Alfredo Medrano

Faculty of Superior Studies Cuautitlan, Faculty of Veterinary Medicine and Zootechnic-CEIEPP, National Autonomous University of Mexico.

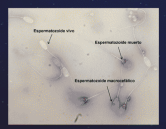
Objective: To assess the effect of melatonin (MLT) as an antioxidant on boar sperm cryosurvival

Material and Methods

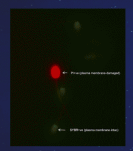
Semen from 6 boars (24 ejaculates) were collected, diluted, and transported to our laboratory

Semen was centrifuged and the cell pellet was resuspended in BF5 freezing medium without glycerol

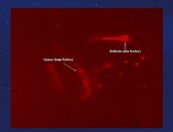
Sperm were assessed



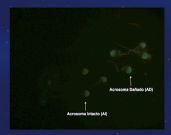
Sperm viability and morphology (Eosin/Nigrosine)



Plasma membrane integrity (SYBR14/PI)



Plasma membrane fluidity (Merocyanine 540)



Acrosome integrity (PSA/FITC)

Diluted sperm were cooled from 23 to 5°C in about 2 h, then more BF5 (with glycerol) was added to reach 200 x 10^6 sperm/ml, and 1% glycerol

Fifteen min later, sperm were packaged in 0.25 ml plastic straws, straws were sealed, and further cooled to -5ºC

Three aliquots of diluted sperm were taken, and MLT i) 0.0, ii) 3.0, and iii) 5.0 mM was added

Then straws were exposed to nitrogen vapors for 15 min and immersed in liquid nitrogen. Straws were thawed at 39°C for 30 s, and sperm were assessed

Results

Effect of Melatonin on boar sperm cryosurvival

Treatment MLT (mM)	Progressive Motility (%)	Plasma membrane integrity SYBR14/PI (%)	Viability Eosin / Nigrosine (%)	Acrosome integrity PSA/FITC (%)	Plasma membrane Hyperfluidity MC540 (%)
0	27.0 ± 2.50	18.1 ± 2.51	35.6 ± 4.15	28.5 ± 5.05	66.0 ± 6.88
3	24.3 ± 2.55	11.3 ± 1.21	22.9 ± 4.08	31.0 ± 5.72	70.9 ± 7.24
5	25.0 ± 2.50	11.7 ± 1.85	24.8 ± 4.14	28.8 ± 4.68	66.2 ± 8.18

Values are Means ± SEM. There were no differences in any variable between treatments

There were no differences (P>0.05) between MLT treatments in any of the variables after thawing. Percentage of sperm showing plasma membrane hyper fluidity before freezing was 7. 2 ± 1.74, but after thawing were 66.0 ± 6.88, 70.9 ± 7.24, and 66.2 ± 8.18 (mean ± s.e.m) for 0, 3, and 5 mM MLT (P>0.05).

Conclusion

MLT in the range of 0.0 to 5.0 mM did not affect boar sperm cryosurvival.

CRYO2021, Virtual Conference, July 20-23, 58th Annual Meeting of the society for Cryobiology.
Supported by UNAM PAPIIT IN220419, IN205421, PIAPI 2030.

OPTIMIZATION OF TURKEY SEMEN DILUTION RATE AND LIQUID STORAGE PERIOD at 4-8^{O}C WITH PLANT BASED EXTENDER FOR OPTIMUM FERTILITY

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[1]Department of Animal Production Technology, Oyo State College of Agriculture and Technology, Igboora.

Introduction

Semen extension and preservation is a very useful technique in realizing the full potential of artificial insemination (AI) in turkey reproduction and breeding

Since commercial turkey breeding are faced with fertility challenges resulting from their inability to naturally mate

Thus, Right choice of appropriate extender is an important prerequisite of processing semen for AI (Ogbu *et al.* 2014).

Materials and Methods

Coconut water Diluents formulation (TCWOE)

Freshly Ejaculated Semen pool of 5 toms

Mass activity & Motility

Semen Dilution (1:3) & (1:6)

Storage at 4-8^{O}C for 0h 12h, 24h, 36h, 48h, 60h & 72h

Microscopic evaluation (progressive motility , viabibility and membrane integrity)

Justification and Objectives

OBJECTIVES

- This experiment aimed at examining the dilution and liquid storage capability of tris coconut-water orange juice extender (TCWO), a plant based extender on turkey semen.

JUSTIFICATION

- Dilution and liquid storage of tom semen remain a reliable approach for rapid genetic improvement, transfer of turkey germplasm within locality and across the globe.
- hence the needs for optimizing the dilution rate and liquid storage duration

Results

Table 1: Percentage motility of TCWO extended tom semen from 0 to 72h storage periods.

Dilution Rate	Preservation periods						
	0h	12h	24h	36h	48h	60h	72h
Neat semen	88.33^{b}	57.00^{c}	19.17^{c}	14.17^{c}	9.17^{c}	2.50^{c}	0.00^{b}
1:3 dilution	88.33^{b}	85.00^{a}	75.83^{a}	65.00^{a}	53.33^{a}	30.83^{a}	4.17^{a}
1:6 dilution	93.33^{a}	73.00^{b}	51.67^{b}	41.67^{b}	25.14^{b}	19.17^{b}	5.00^{a}
SEM	1.00	3.31	6.17	5.15	4.71	3.90	0.92

Table 2: Percentage livability of TCWO extended tom semen from 0 to 72h storage periods.

Dilution Rate	Preservation periods						
	0h	12h	24h	36h	48h	60h	72h
Neat semen	98.00	95.48^{a}	91.97	87.23	79.35^{b}	67.33^{b}	61.83^{b}
1:3 dilution	95.00	95.00^{a}	91.46	90.80	88.63^{a}	84.50^{a}	79.67^{a}
1:6 dilution	97.72	91.70^{b}	91.40	88.15	87.20^{a}	84.83^{a}	81.17^{a}
SEM	0.76	0.78	0.62	1.06	1.55	2.25	2.31

Table 3: Percentage membrane integrity of TCWO extended tom semen from 0 to 72h storage periods.

Dilution Rate	Preservation periods						
	0h	12h	24h	36h	48h	60h	72h
Neat semen	90.79	68.50^{b}	25.17^{c}	22.50^{c}	16.15^{c}	17.27^{b}	1.83
1:3 dilution	91.82	87.91^{a}	78.49^{a}	66.76^{a}	46.16^{a}	30.90^{a}	5.17
1:6 dilution	90.72	76.67^{b}	61.73^{b}	40.53^{b}	35.14^{b}	19.66^{b}	4.17
SEM	1.17	2.74	5.67	4.53	3.57	1.91	0.77

Dilution Rate	Preservation periods						
	0h	12h	24h	36h	48h	60h	72h
Neat semen	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1:3 dilution	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1:6 dilution	100.00	100.00	100.00	100.00	100.00	100.00	100.00
SEM	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Conclusion

Conclusively, dilution of TCWO extender with tom semen at rate of 1:3 resulted in better microscopic semen quality compare to 1:6 dilution rate and undiluted semen.

Clinical Grade Additives Exert Chondroprotective Effects in Porcine Articular Cartilage During Exposure to Cryoprotective Agents

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INTRODUCTION

- Cryopreservation of articular cartilage by vitrification can enable long-term tissue banking of osteochondral allografts[1].
- Supplementation of CPA solutions with ***research*** grade additives improves chondrocyte recovery and metabolic function after exposure to cryoprotective agent (CPA) at hypothermic conditions[2].
- Assessment and incorporation of ***clinical*** grade additives can facilitate the translation of vitrification of cartilage into tissue banks.
  - Chondroitin sulfate (CS), ascorbic acid (AA), and N-acetylcysteine (NAC)
- **OBJECTIVE: 1)** Investigate the chondroprotective effects of ***clinical*** grade additives; **2)** Determine whether a combination of two clinical grade additives would improve cell viability when compared to using only one additive.

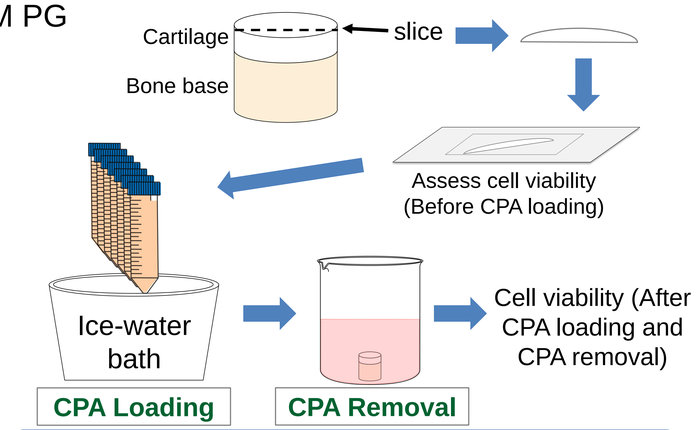
EXPERIMENTAL DESIGN

Sample preparation: Osteochondral dowels (7 mm diameter) were harvested from the condyles of adult porcine stifle joints. Dowels were disinfected under sterile conditions and incubated in DMEM complete at 4˚ C overnight. 85-µm thick cartilage slices were sectioned from the dowels using a vibratome.

CPA Loading: 3M Me_2SO + 3M EG + 3M PG for 180 min at 0 ˚ C.

Experimental Group	Additive Treatment
1	-
2	1 mM NAC
3	0.1 mg/mL CS
4	2000 µM AA
5	1 mM NAC + 0.1 mg/mL CS
6	1 mM NAC + 2000 µM AA
7	0.1 mg/mL CS + 2000 µM AA

Figure 1. Methods flowchart.



AA: ascorbic acid, CPA: cryoprotective agents, CS: chondroitin sulfate, DMEM: Dulbecco's modified eagle medium, M: Molar, Me_2SO: Dimethyl sulfoxide, EG: Ethylene glycol, NAC: N-acetylcysteine, PG: Propylene glycol

Cell viability: Viability was measured with a dual fluorescent membrane integrity stain [6.25 µM Syto 13 + 9.0 µM propidium iodide] and imaged with a fluorescent microscope.

Data analysis: Viability 3.2[3] and one-way ANOVA using SPSS 20.0 software.

RESULTS

ADDITIVE COMBINATIONS

- **AA** significantly improved cell viability compared to no additive (p = 0.0200), NAC (p = 0.044), CS + AA (p = 0.040), and NAC + CS (p = 0.067).
- **CS** showed significantly higher cell viability than the no additive (p = 0.095).
- NAC + AA had significantly higher cell viability compared to no additive (p = 0.037), NAC (p = 0.0770) and CS + AA (p = 0.070).
- NAC, NAC + CS, or CS + AA did not significantly improve percent cell viability compared to no additive treatment (p > 0.10).

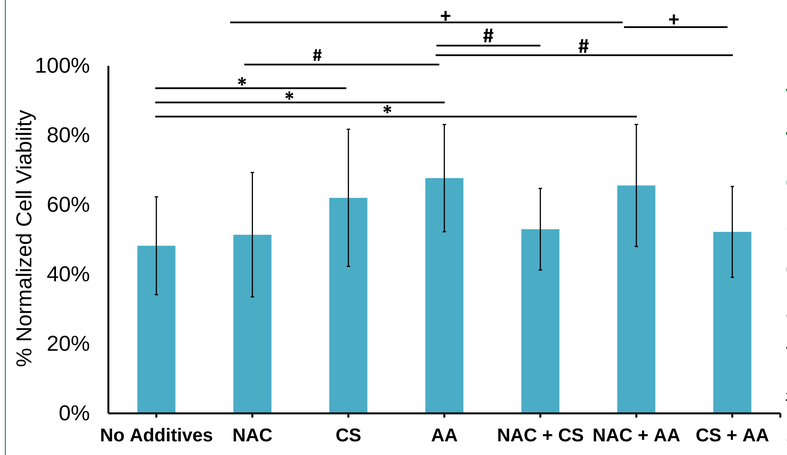


Figure 2. Percent normalized cell viability in additive treatments following substantial CPA exposure and CPA removal (N = 6). Some additive treatments improved cell viability when compared to no additives. Statistical significance:
* p < 0.10 relative to no additive
# p < 0.10 relative to AA
+ p < 0.10 relative to NAC + AA.

CONCLUSION

1. Supplementation with either clinical grade CS or AA improved cell viability.
2. Supplementation with clinical grade NAC did not benefit cell viability.

Overall, supplementation with clinical grade additives in CPA solutions can mitigate CPA toxicity and hence optimize vitrification protocols for future use in tissue banking of vitrified cartilage.

ACKNOWLEDGMENTS

This study was funded by the Edmonton Orthopaedic Research Committee and a Department of Surgery Summer Studentship. Dr. J. A. W. Elliott holds a Canada Research Chair in Thermodynamics.

References:
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IMPROVEMENT OF EQUINE EMBRYO CRYOPRESERVATION VIA LASER ASSISTED MICROMANIPULATION

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Introduction

Equine embryo cryopreservation is affected by three main factors

1. Size
2. blastocoel volume
3. intact capsule

- Group A: ≤300 µm
- Group B: 300 µm<B<700 µm
- Group C: ≥700 µm

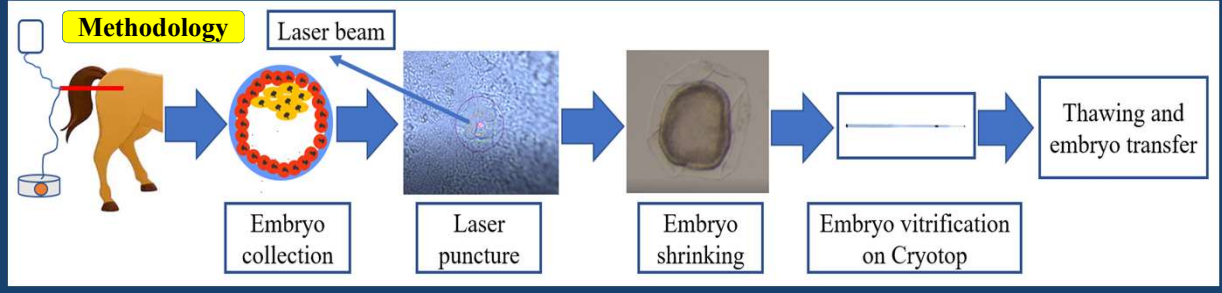
- The capsule presence is crucial to the survival of the early embryo.
- The presence of a capsule makes the embryos less permeable to the cryoprotectants.
- The large blastocoel volume is prone to ice crystal formation.

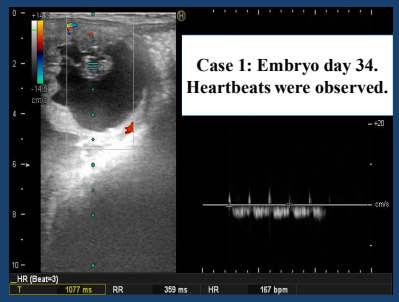
Laser technology

Laser technology is being applied across assisted reproductive technology (ART) to reduce procedure times and increase the consistency and reproducibility of ART techniques such as:

- assisted hatching,
- embryo biopsy,
- Intracytoplasmic sperm injection,
- sperm immobilization/selection,
- and embryo cryopreservation

Methodology

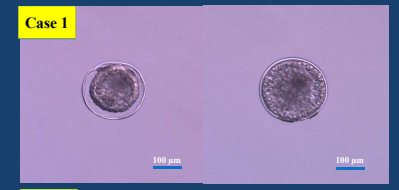


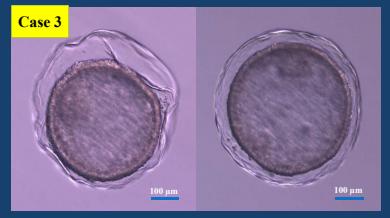


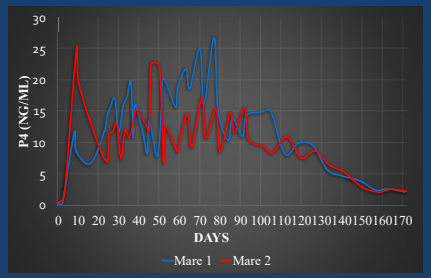
Results and discussion

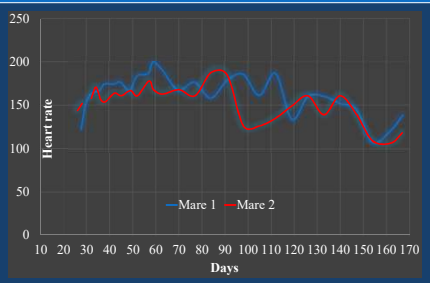
Case number	Group	Embryo grade after collection	Embryo diameter after collection (µm)	Diameter (µm) after thawing		Diameter (µm) after culture		Embryo grade after culture	Method of puncture	Result of pregnancy
				Trophoblast	Capsule	Trophoblast	Capsule			
1	A	1	233	186	241	242	258	1	PMAP	+
2	B	1	684	476	691	453	727	4	PMAP	–
3	B	1	598	414	546	468	548	1	LAP	+
4	B	1	571	431	530	547	558	1	LAP*2, PMAP	–
5	C	1	1235	953	1112	807	1103	3	PMAP*2	–
6	C	1	1360	1153	1244	752	1223	4	LAP, PMAP	–

PMAP: piezo micromanipulator-assisted puncture; LAP: Laser -assisted puncture; *2: 2 times









There was a positive pregnancy in piezo group, case number 1 was categorized into small embryo groups and two others in medium and big size embryos were not showed positive results.

On the other hand, there was a positive pregnancy result in medium size embryo that cryopreserved with LAP technology.

In conclusion, using LAP can be helpful for equine medium size embryos; however, the current methodology is not suitable for big embryos (≥700 µm), and more investigation to improve LAP technology for large equine embryos is on going.

Improvement in the physical properties of freeze-dried soup solid with the addition of gelling agent

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[1] Hiroshima University, JAPAN, [2] Nihon BUCHI K.K.

Fig. Freeze-dried soup products

Introduction

Optimization of both process efficiency and physical quality of freeze-dried soup products is challenging due to large amount of NaCl in the amorphous phase…

NaCl
- strong plasticizer ⇒ easy to collapse during freeze-drying
  high drying temperature : not applicable
- dispersion role ⇒ freeze-dried solid becomes fragile

Proposed approach for improvement

"addition of gel-forming polymers" such as gelatin to soup solution

gelatin-gel ⇒ structural enforcement ⇒ prevent deformation?
during freeze-drying and subsequent processes

Purpose

to investigate the effects of the type and amount of polymer on the physical properties of freeze-dried model soup solid

Materials and Methods

model soup mixture of **NaCl**, **sucrose**, **MSG**

food polymer 1% and 3% (w/v)

gelatin (swelled, melted) | **corn starch** (gelatinized) | **maltodextrin**

samples control | GL | CS | MD

30mL aluminum container

pre-freezing (16 h) -20 °C

differential scanning calorimetry (DSC)
heat scanning : -50 to +20 °C (3 °C /min)

freeze-drying (24 h)
- shelf temperature : 25 or 50 °C
- chamber pressure : 38 Pa

Lyovapor L-200 (BÜCHI Labortechnik AG.)

process efficiency
drying time

quality evaluation
of freeze-dried solid

apparent volume
3D scanning
⇒volume (cm^3)

VL series (Keyence)

dissolution test

mesh box

soup | hot water | 10 sec

weight loss ⇒ dissolution ratio (%)

physical strength

Rheometer | speed: 1mm/s | diameter: 5mm | force | distance | (CR-500DX)

compression ⇒ fracture energy (mJ)

Results and Discussion

Thermal properties (DSC)

eutectic melting

1% addition
⇒no significant difference

3% addition
⇒GL, MD prevented crystallization of NaCl

freeze-concentrated glass transition temperature (T_g')

NaCl reduced T_g' of sucrose. (predicted to be - 62 °C) (Yamamoto et al. 2020)

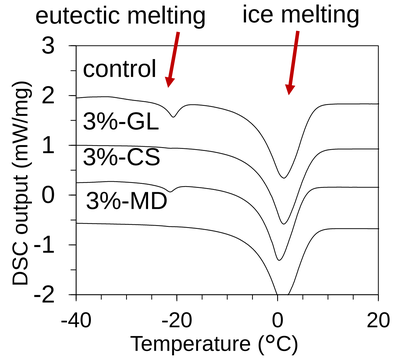

Fig. DSC thermogram

Apparent volume

control ⇒ disrupted

polymer-added solid
prevented structural deformation
no significant effect of drying temperature and polymer concentration

1% GL dried at 50°C

*1% CS was destructed in the measuring process because of the cracks.

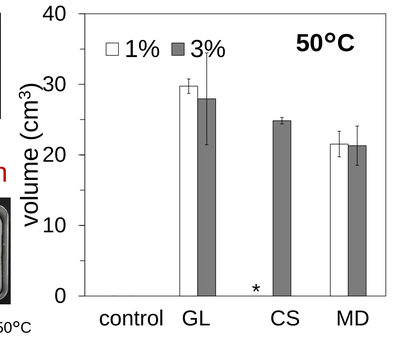

Fig. Apparent volume for the samples freeze-dried at 50 °C

Drying time

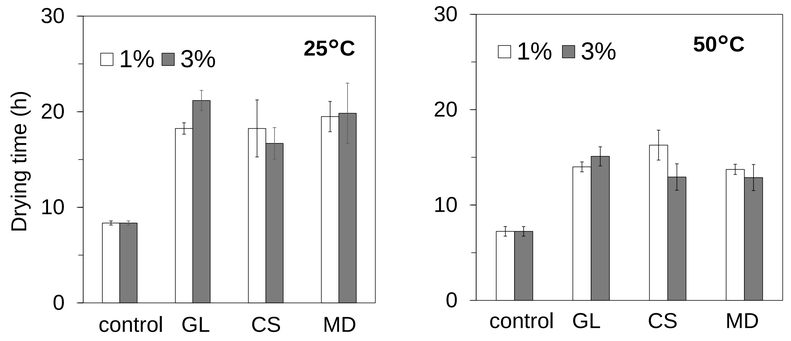

Fig. Drying time for the samples freeze-dried at 25 and 50 °C

control
disrupted structure ⇒ less cake resistance ⇒shorter drying time

polymer addition
no significant difference between 1 and 3% addition

Increase of the drying temperature was applicable in order to improve drying efficiency while maintaining the apparent volume of the freeze-dried solid.

Physical strength

shelf temperature
no significant impact

1% addition
GL : highest breaking energy due to gelation before freezing

3% addition
GL, MD: higher breaking energy than 1% addition
MD: no enhancement effect because MD cannot form a helical structure

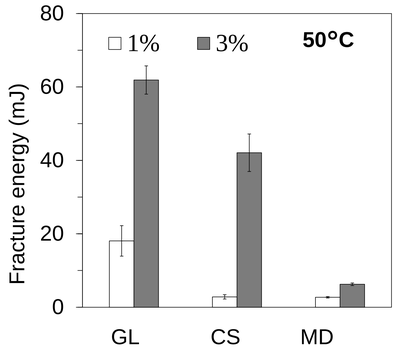

Fig. Breaking energy for the samples freeze-dried at 50 °C

Dissolution property

shelf temperature
no significant impact

1% addition : high dissolution

3% addition:
GL, CS:
dissolution ratio was diminished
⇒ not useful for consumers
MD: high dissolution (>99%) regardless of concentration

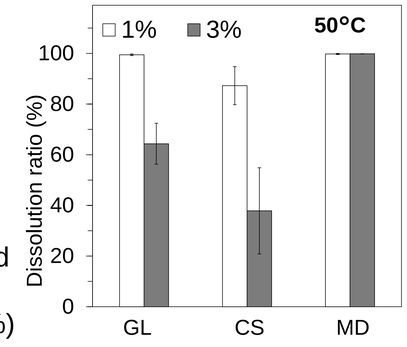

Fig. Dissolution ratio (%) for the samples freeze-dried at 50 °C

Conclusion

optimum condition for the freeze-dried soup
⇒1% GL freeze-dried at 50 °C

gelatin …
- gel-network ⇒ structural enforcement ⇒ no disruption during freeze-drying, distribution process
  dried at high temperature, even if T_g' for the system is low
- high solubility (1% addition) ⇒ convenient for consumer

This approach will be useful for the freeze-drying of sugar-rich foods such as fruits. ⇒ future subject

mango juice

Publication

Sogabe T et al (2021). Effect of polymer addition on the physical properties of freeze-dried soup solid. *Food Sci Technol* 1-10. (in press)

Acknowledgement

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Retention of Hemoglobin by Red Blood Cells After Cryopreservation

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Introduction

One of the most common medical procedures performed in US hospitals is blood transfusions[1]. Unfortunately, red blood cells (RBCs) used for transfusion have a limited shelf life after donation due to the accumulation of storage lesions with detrimental effects on mechanical, morphological, and biochemical properties[2]. Inspired by nature, a biomimetics approach to preserve RBCs for long-term storage is being developed using compounds that occur in animals which have a propensity to survive in a frozen or desiccated state for decades. The most promising compound currently under investigation is trehalose, a non-toxic sugar[3].

Methods

Porcine RBCs obtained from JBS USA slaughterhouse (Louisville, KY) were enumerated using a hematocytometer and prepared for freezing and thawing at concentrations ranging from 50 million to 2.5 billion RBCs/mL. Trehalose from Pfanstiehl Inc. (Waukegan, IL) was employed as a cryoprotective agent when added to the extracellular freezing solution at concentrations ranging from 200 mM to 600 mM. All RBC solutions were frozen rapidly by placing 1 mL of sample into a 1 mL cryovial and dropping it into a dewar filled with liquid N_2. RBC samples were removed from the liquid N_2, and quickly thawed using a water bath set to temperatures ranging from 30 °C to 70 °C. Recovery of cells was determined using a hematocytometer to enumerate RBC concentrations after thawing. Furthermore, RBCs were pelleted at 600 *g* for 10 min after thawing, and the percentage of hemolysis was determined by measuring the amount of hemoglobin in the supernatant versus pellet using a commercially available Hemoglobin Assay Kit (MilliporeSigma, Burlington, MA).

Results

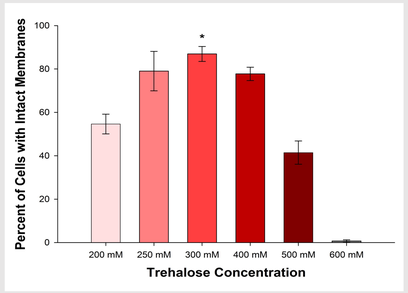

Fig. 1. Percent of RBCs with intact membranes after freezing in a cryoprotective solution containing different concentrations of trehalose and 50 million RBCs/mL, 20 mM HEPES-NaOH, pH 7.1. Thawing was performed at 40 °C. *statistically significant difference from 200 mM ($p<0.05$, $n = 3$, 3 nested replicates).

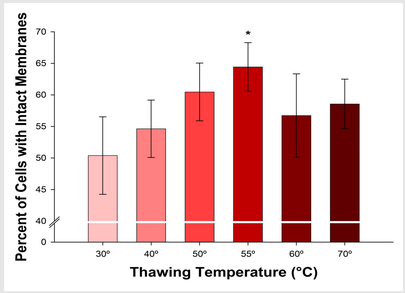

Fig. 2. Percent of RBCs recovered after freezing in 200 mM trehalose, 20 mM HEPES-NaOH, pH 7.1 and 50 million RBCs/mL while thawing at different temperatures. *statistically significant difference from 40 °C ($p<0.05$, $n = 3$, 3 nested replicates).

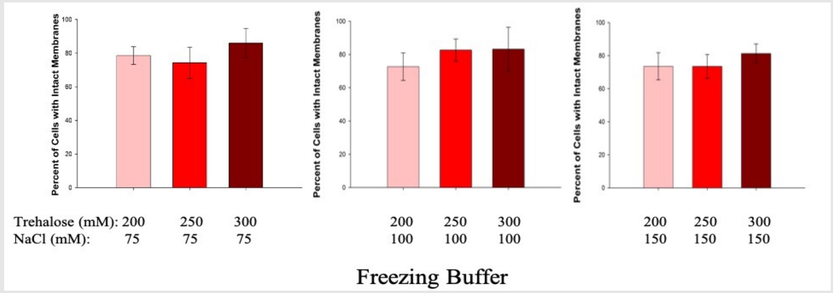

Fig. 3. Percent of RBCs recovered after freezing in a cryoprotective solutions containing different concentrations of trehalose and NaCl, 250 million RBCs/mL, 20 mM HEPES-NaOH, pH 7.1. Thawing was performed at 40 °C ($n = 3$, 3 nested replicates). There was no statistical significance between different NaCl concentrations.

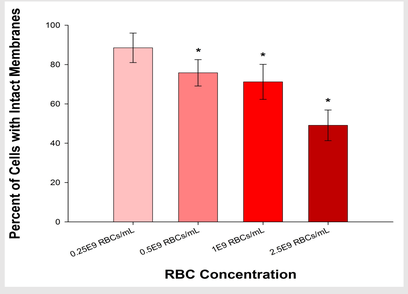

Fig. 4. Percent of RBCs recovered after freezing in a cryoprotective solution containing 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1 and different concentrations of RBCs. Thawing was performed at 55 °C ($n = 3$, 3 nested replicates). *statistically significant difference from 0.25E9 RBCs/mL ($p<0.05$, $n = 3$, 3 nested replicates).

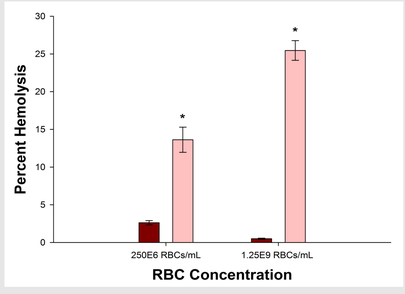

Fig. 5. **Dark Bar**. Percent hemolysis in samples of RBCs that did not undergo freezing and thawing (n = 3, 2 nested replicates). **Light Bar**. Percent Hemolysis of cells after freezing and thawing (n = 3, 3 nested replicates). All samples were placed in a cryoprotective solution composed of, 300 mM trehalose, 100 mM NaCl, 20 mM HEPES-NaOH, pH 7.1 and different concentrations of RBCs. Thawing was performed at 55 °C. * statistically significant difference from control groups ($p<0.05$).

Conclusion

Trehalose was employed as a cryoprotective agent (CPA) to protect RBCs for biopreservation purposes[4]. The effectiveness and feasibility of using trehalose as a CPA was demonstrated and morphological intact RBCs were recovered after freezing and thawing with losses below 15% as judged by cell numbers and hemoglobin retention. Dou et al. (2019) showed similar results using a solution comprised of trehalose and L-proline[5]. Cell concentration significantly impacted recovery after freezing and thawing (Fig. 4). Higher concentrations correlated with lower cell recoveries. A similar finding based on cell drying studies was reported by Wolkers et al. (2002)[6]. According to the cell recovery studies (Fig. 4), it could be determined that the percent hemolysis matches the percent of cells that were not recovered (Fig. 5). This indicates that cells which are not lysed during freezing and thawing were able to maintain their native hemoglobin concentration within the cell, and only lysed RBCs lose their hemoglobin to the supernatant.

References/Acknowledgements

This work was supported by funding from NSF-PFI-1827521. The data that supports the findings of this study are available from the corresponding author upon reasonable request.

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Ice Recrystallization Inhibitors (IRIs) as Novel Cryoprotectants for Human Induced Pluripotent Stem Cells (iPSCs) and iPSC-Derived Neurons (iPSC-Ns)

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Introduction

Human induced pluripotent cells (iPSCs) and their derivatives represent a differentiated modality toward developing novel cell-based therapies for regenerative medicine. However, the challenge lies in optimizing the freezing protocols to ensure that post-thaw iPSCs and iPSC-derived neurons (iPSC-Ns) maintain their viability and functional properties.

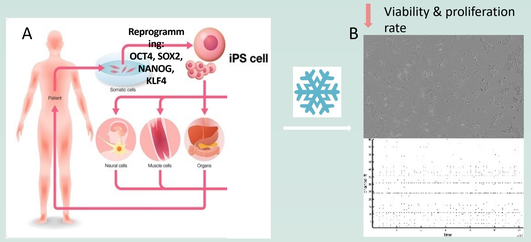


Figure 1. (A) Human induced pluripotent stem cells (iPSCs) and their derivatives. **(B)** Impact of cryopreservation on iPSCs and iPSC-Ns.

Ice recrystallization is a major contributor to cellular injury and death during cryopreservation due to the growth and formation of larger ice crystals at the expense of smaller ones. In this study, we set out to develop a novel cryopreservation strategy to increase viability and functionality of iPSCs and iPSC-Ns exploiting novel ice recrystallization inhibitors (IRIs). We compared a commercially available cryopreservation medium for iPSCs (mFreSR) and for iPSC-Ns (CS10) supplemented with a panel of IRIs.

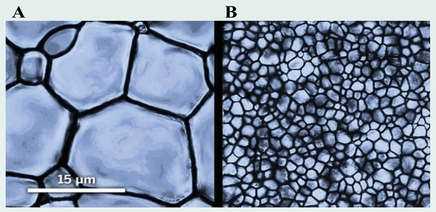
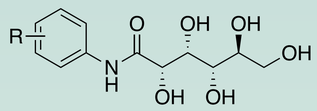


Figure 2. Ice crystal images obtained from the "splat-cooling" assay. **(A)** No inhibition of ice recrystallization (positive control, PBS) and **(B)** inhibition of ice recrystallization.

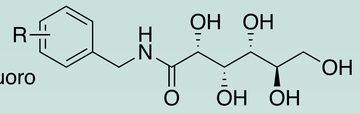
Methods

N-Aryl gluconamide class of IRI

1. R= H
2. R= 2-F
3. R= 4-Cl
4. R= 4-OMe



5. R= H
6. R: 2,6- difluoro



Cell culture and cryopreservation

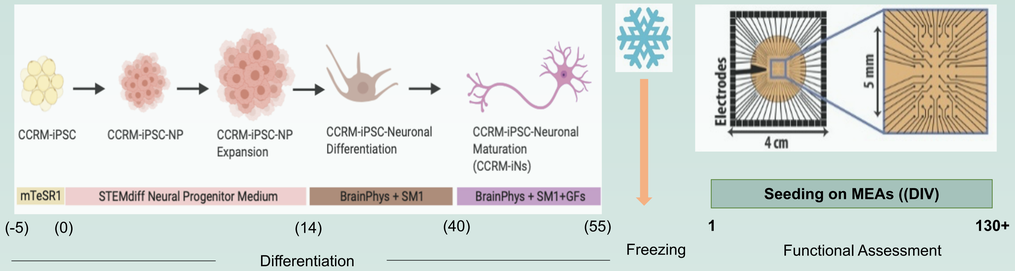


Figure 3. iPSC-derived neurons generated from CCRM-human induced pluripotent stem cells (iPSCs) using the StemDiff Neuron Maturation Kit (Stem Cell Technologies).

MEA and neuropharmacology

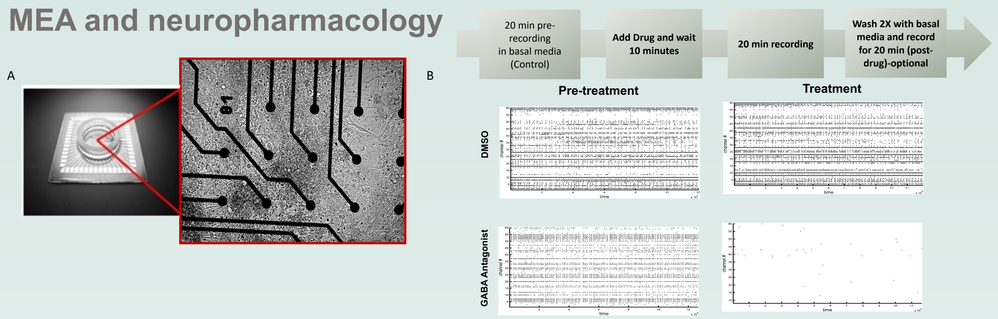


Figure 4. (A) Multi-electrode array system obtained from MultiChannel Systems (Reutlingen, Germany), with the headstage accommodating a 60-electrode MEA dish. Cryopreserved iNs were plated on MEA dishes, allowed to grow for 4 weeks and then recorded. Spike analyses were performed using MATLAB software. **(B)** Treatment regime for the neuropharmacological assessment of cryopreserved iPSC-Ns using MEAs.

Results

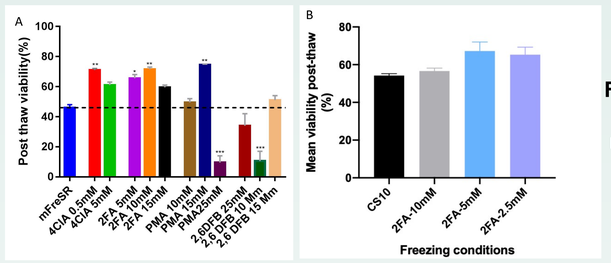


Figure 5. Mean Post-thaw viability of **(A)** HAF-iPSCs using IRIs supplemented in mFreSR containing 10% DMSO and **(B)** iPSC-Ns using IRI supplemented in Cryostor® 10 (CS10)

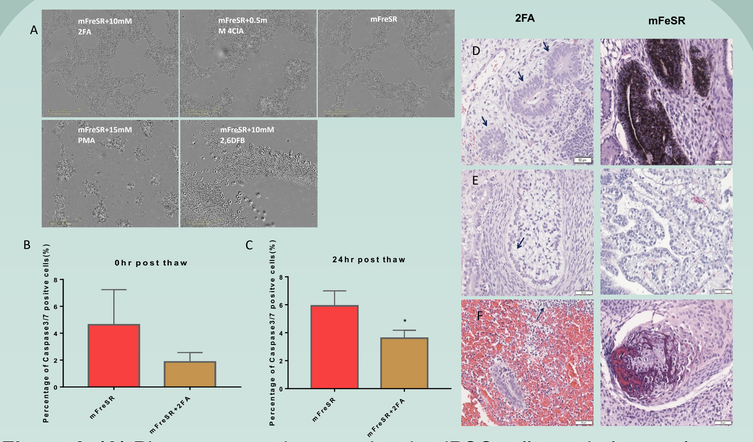


Figure 6. (A) Phase contrast images showing iPSC cell morphology, colony size 48-hr post seeding. Images captured using IncuCyte cell imaging system. **(B-C)** Percentage of Caspase 3/7 positive cells at 0 hr and 24 hr post-thaw, respectively, assessed by the ratio of red fluorescence over confluency. **(D-F)** Histopathologic characterization of human iPSC derived teratomas xenografted into immunodeficient SCID mice. The three germ layers, ectoderm, endoderm, and mesoderm.

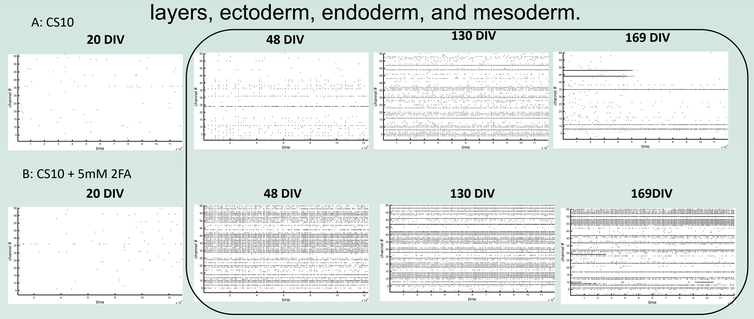
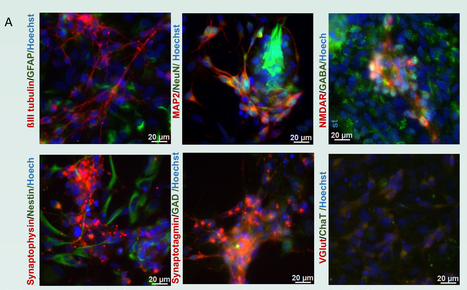


Figure 7. Developmental multi-electrode arrays roster plots of cryopreserved iNs (**A**: CS10, **B:** 2FA). Raster plots showing developmental profile of spontaneous activity in a representative MEA of iPSC-Ns. The y axis in each plot represents electrode number and the x axis represents recording time.



B

Drug	Effect on MFR
TTX	↓↓
Phenytoin	↓↓
Memantine + NBQX	↓
4-amino- pyridine	↑
Verapamil	↓↓
Biccucculine	↓
Muscimol	↓↓
GABA	↓↓
Nicotine	↓

Figure 8. (A) Immunostaining for iPSC-Ns markers at day 21 post-thaw. (B) Neuropharmacological responses of 2FA-iNs to a panel of neuroactive drugs and their effect on the mean firing rate (MFR).

Conclusions

- ✓ **10mM 2FA** supplemented in mFreSR increased post-thaw viability by 20% and enhanced the proliferation of **iPSCs.**
- ✓ **5mM 2FA** supplemented in CS10 significantly decreased the timeframe toward establishing synchronous synaptic activities 48 DIV vs 130 DIV of **iPSC-Ns**.
- ✓ 5mM 2FA-frozen **iPSC-Ns** retained the expression of key neural markers and displayed functional neuropharmacological responses.

Acknowledgment

A BIOCOMPATIBLE ICE NUCLEATING AGENT ELIMINATES SUPERCOOLING AND ENHANCES CELL CRYOPRESERVATION IN 96-WELL PLATES

CRYO2021 VIRTUAL CONFERENCE | JULY 20-23

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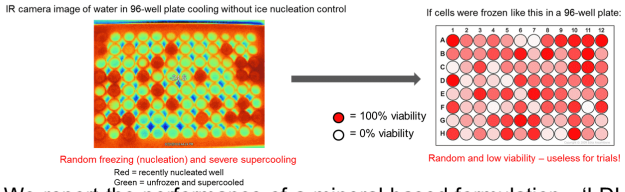
INTRODUCTION

- A reliable method for cryopreserving cells in 96-well plate (96-WP) format would be beneficial for drug screening trials. However, the small (< 1 mL) volumes of cryoprotectant used in wells are prone to variable and often severe supercooling of 20+ °C. Unacceptably low and variable post thaw cell viability results if cells are cryopreserved in 96-well plates without control of ice-nucleation (IN).

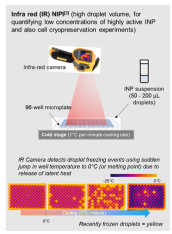


- We report the performance of a mineral-based formulation - 'LDH1' - of ice nucleant delivered via the IceStart™ array system to 96-well plates seeded with immortalised human hepatocyte monolayers (HepG2). The IN potency of LDH1 is comparable to that of existing highly effective ice nucleants, but with greater potential for biocompatibility (low toxicity, higher stability and resistance to sterilisation treatments).

METHODOLOGY

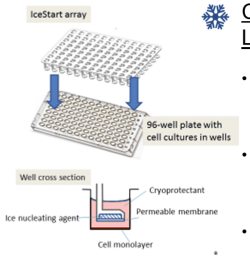
- Detection of IN temperatures by Infra-red (IR) measurement of temperatures of individual wells in cooled 96-WPs

  - IR camera fixed above Asymptote ViaFreeze controlled rate freezer with 96WP mounted on cooling plate.
  - IN temperatures in individual wells derived from lowest temperature reached before sudden release of latent heat associated with nucleation event
  - Temperature calibrated by equilibrium of ice/water during crystallisation after nucleation event.



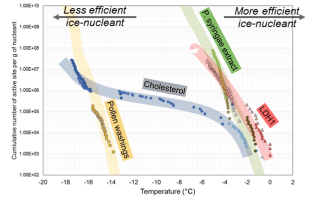
- Cell cryopreservation trials in 96-WPs with LDH1 delivered in IceStart™ arrays

  - Cells were cultured in monolayers for 2 days in polypropylene 96-well plates then cryopreserved with 100 µL per well of CPA (10% Me_2SO and 0.01M trehalose in incubation medium).
  - IceStart™ arrays containing LDH1 powder were inserted into the plates which were then cooled at 1°C min^{-1} using an Asymptote ViaFreeze controlled rate freezer then stored 7 at days at -80°C.
  - Post-thaw viability was assessed with a vital dye (neutral red) and compared with unfrozen control plates to determine survival



RESULTS – Ice-nucleating ability of 'LDH1' compared to other strong ice nucleants

- On a mass by mass basis LDH1 has higher ice nucleating activity than cholesterol, *P. syringae* extract and pollen washing water.



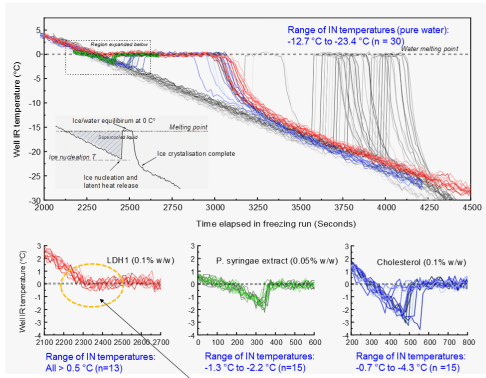


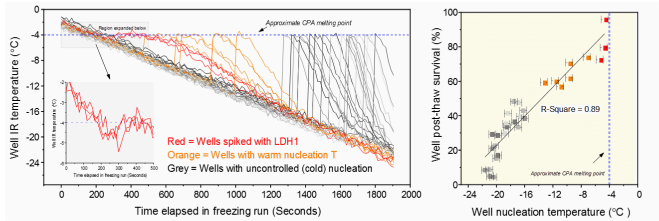
Figure 1 (above): Ice nucleating efficiencies of LDH1 compared to other types of ice nucleating agents normalised to mass. Data from droplet freezing assays supplemented by new IR data of 100 µL aliquots.

Figure 2 (left): IR temperature traces of 96WPs loaded with 100 µL aliquots of deionised sterile filtered water with a variety of ice nucleants added (red = LDH1, green = P. syringae extract, blue = cholesterol) or without any nucleants (grey). Each line represents an individual well

- Supercooling could not be detected (< 0.5° C) when LDH1 is added to 100 µL water aliquots cooled at 1 °C min^{-1} in a 96-WP.

RESULTS – Ice-nucleation temperature dependence on cell survival in 96-WP

Figure 3 (below): **LEFT:** IR temperature traces of a 96-WP with HepG2 cell monolayers undergoing controlled rate freezing showing individual well nucleation events. The wells are divided into three groups: wells were spiked with LDH1 to induce warm IN (red), wells with warm IN which were likely contaminated with LDH1 (orange) and wells with uncontrolled IN (black). **RIGHT**: Scatterplot of the same 96-WP freezing run showing correlation between IN temperature and post-thaw survival compare to an unfrozen control plate with HepG2 cultures. Each point represents one well.

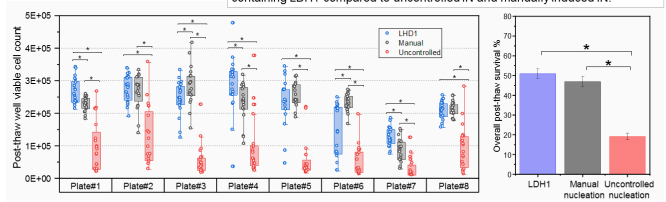


- Post-thaw cell viability and IN temperature are strongly correlated when HepG2 cells are frozen in a 96-WP.

RESULTS – Cryopreservation of HepG2 cultures in 96-WP with LDH1 delivered in IceStart™ arrays

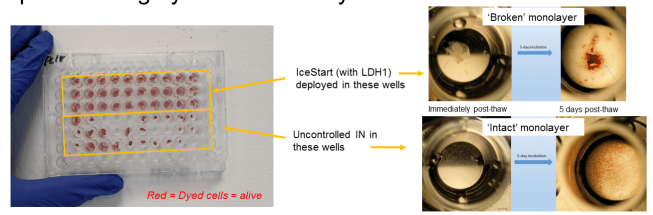
- Plates frozen with IceStart™ arrays containing LDH1 showed significantly higher post-thaw viability compared to uncontrolled nucleation (52.5±2.3% vs. 21.7±1.6%) averaged over 8 replicates of 30 wells for each method, one way ANOVA $p < 0.05$. Plates frozen with manual nucleation at close to melting point were also used as positive control.

Figure 4 Immediate post-thaw viability of HepG2 frozen in 96-WP with IceStart containing LDH1 compared to uncontrolled IN and manually induced IN.



DISCUSSION – Why do high IN temperatures protect cells frozen in 96-WP?

- Controlling IN at close to melting point appears to protect integrity of cell monolayer.



- IN after too much supercooling causes temperature fluctuations in wells that may result in differential contraction between substrate and cell monolayer.

CONCLUSIONS AND FUTURE WORK

- LDH1 is a mineral-based ice-nucleating agent of comparable potency to that of other highly effective non-mineral ice-nucleants but a with greater potential for biocompatibility.
- Using LDH1 via IceStart™ arrays can greatly improve post thaw survival of mammalian cells frozen in 96-well plates. Similar application with smaller well or vessel volumes (eg. 384 well plate) is possible due to small mass needed to control IN.

Oncological and Functional Outcomes after Salvage Prostate Cryotherapy for the Management of Primary Brachytherapy versus Cryotherapy Failures: A Propensity Score Matched Comparison

Hazem Orabi, Ahmed El-Shafei , Ali Aminsharifi, Leah Garber, Thomas Polascik
Duke University, Durham, NC

INTRODUCTION AND OBJECTIVE

To compare the outcome of prostate cryotherapy as a salvage modality for treatment of primary brachytherapy versus cryotherapy failure for localized prostate cancer.

Methods

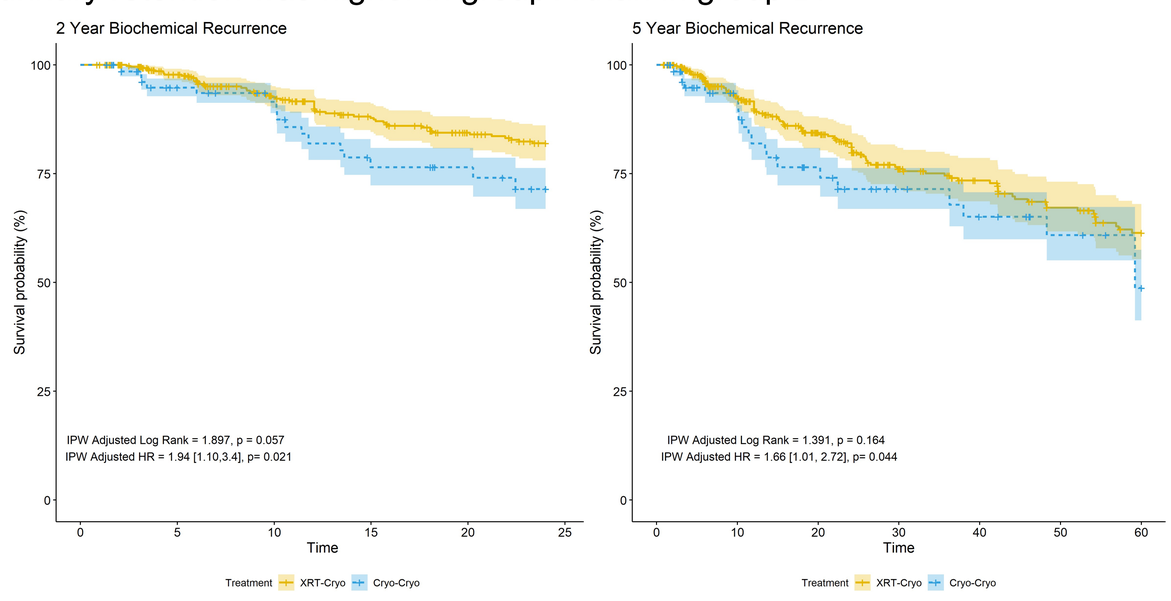
Following IRB approval, we queried the Cryo-On-Line Database (COLD) registry and the Duke prostate cancer database for men treated with salvage cryotherapy (SC) following treatment failure of primary brachytherapy (group A=113) vs primary cryotherapy (group B= 81). Biochemical recurrence (BCR) using Phoenix criteria was the primary endpoint assessed at 2- & 5-years post SC. Secondary endpoints assessed functional outcomes including 12-month urinary incontinence, rate of effective intercourse; recto-urethral fistula and urinary retention. We estimated the association between treatment and biochemical progression-free survival (BPFS) using inverse probability weighted (IPTW) Cox proportional hazards regression. Propensity score analysis, adjusting for Gleason, risk, and PSA, was implemented to account for non-random assignment of primary treatment. To test for differences in the secondary functional outcomes between treatment modalities, we used Pearson's $\chi 2$ test or Fishers exact test, corrected for IPTW.

Conclusions

Salvage cryotherapy after failed primary cryoablation and failed primary brachytherapy has similar oncological and functional outcomes, except salvage cryotherapy after primary cryotherapy had a lower rate of retention and incontinence. This information should be considered when selecting primary insitu organ-preserving therapy for prostate cancer. Additional work with larger numbers of patients is needed to further validate these results with longer follow-up.

Results

194 unweighted subjects were included who had complete data for the primary analysis. There was no statistical difference in 2-year BCR (HR 0.9; 95% CI,0.5–1.7) or 5-year BCR (HR: 0.86; 95% CI, 0.5-1.5) between the 2 groups (Figure 1). There was no statistical difference between the 2 groups regarding the adverse functional outcomes, although the incidence of incontinence and urinary retention was higher in group A than in group B.



Comparative Study Between Salvage Cryoablation of the Prostate After Primary Radiotherapy Failure and After Primary Cryotherapy Failure for Clinically Localized Prostate Cancer

Hazem Orabi, Leah Davis, Yuan Wu, Thomas Polascik
Duke University, Durham, NC

INTRODUCTION AND OBJECTIVE

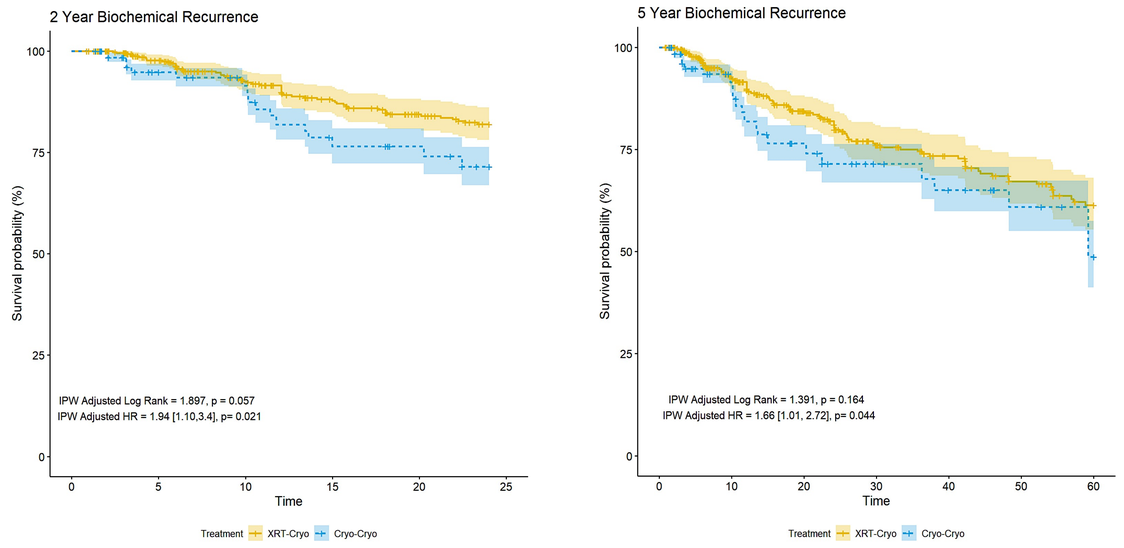
Salvage cryoablative therapy for biopsy-proven recurrence after primary radiation or ablation treatment failure for clinically localized prostate cancer (PCa) is common. In this observational study, we aimed to explore biochemical recurrence-free survival when salvage cryotherapy is performed after primary cryotherapy failure versus primary external beam radiotherapy failure in patients initially presenting with clinically localized prostate cancer.

Methods

After IRB approval, data for patients treated with salvage cryotherapy after primary external beam radiotherapy (group A) or after primary cryotherapy (group B) were collected from The Duke Prostate Cancer database and the Cryo On-Line Data (COLD) registry. Biochemical recurrence (BCR), using Phoenixcriteria, after 2 and 5 years were the primary oncological outcomes. Urinary incontinence, erectile dysfunction, fistula and retention were considered as secondary functional outcomes and were assessed 12 months after salvage cryotherapy. We estimated the association between treatment and biochemical progression-free survival (BPFS) using inverse probability weighted (IPTW) Cox proportional hazards regression. Propensity score analysis, adjusted for Gleason, risk, and PSA, was implemented to account for non-random assignment of primary treatment. To test for differences in the secondary functional outcomes between treatment modalities we used Pearson's $\chi 2$ test or Fishers exact test, corrected for IPTW.

Results

A total of 515 unweighted subjects met inclusion criteria and had complete data for the primary analysis. Those in group B showed an increase in 2-year BCR (HR 1.94; 95% CI, 1.1–1.34) when compared to group A. The same association was seen in 5-year BCR (HR: 1.66; 95% CI,1.01-2.72).There was no statistical difference between the 2 groups regarding the functional outcomes, although incontinence and erectile dysfunction was higher group A than group B.



Conclusions

Risk of biochemical recurrence for salvage cryotherapy after primary cryoablation is higher than following primary external beam radiotherapy. This should be taken in consideration when selecting primary in-situ therapy for PCa. Further work with larger number of patients is needed to assess reasons for failure including whether they are in field or out of field of treatment.

New, LN_2-free solution for cryogenic transport of cell therapies

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Introduction

Timing is critical in the development and delivery of cell therapies, which often involve complex logistics between multiple partners and site. Cryopreservation affords extra time and flexibility, but LN_2-based dry shippers pose challenges. To address these challenges, Cytiva developed the VIA Capsule™ system. This electrically-powered liquid nitrogen (LN_2)-free cryogenic shipping instrument offers an easier-to-handle, more predictable alternative to LN_2-based dry shippers. The system cools and maintains cryogenic temperatures on charge for a short period of time and linearly warms during transit in a passive manner, providing up to 5 days of standby time below -120°C, or the glass transition temperature (Tg') of Me_2SO-containing cell suspensions allowing for safe, long-term cryogenic storage.[1,2,3]

Fig 1. VIA Capsule™ system with cryocooler on and wheels unfolded for charge (left), and transport cap on and wheels folded for transport (right)

We studied the impact of storage periods at temperatures above Tg' to evaluate the effect of transit delays. Here, we include data from transporting cryopreserved samples across the UK by road over 4 days in the VIA Capsule™ system versus a dry shipper.

Methods and results

Transit time and temperature boundaries to maintain sample integrity:

- Storage periods: 5 and 10 days
- Storage temperatures: -60°C, -80°C, -100°C, and -120°C
- Range of cell lines, including an immortalized T-cell line (Jurkat) to emulate the behaviour of some cell therapies (10^6 cells/mL, 5% Me_2SO in cryovials, 1 mL fill volume)
- Analyses post-thaw: viable cell numbers and metabolic activity after 24, 48, and 72 h of re-culture (n = 5)

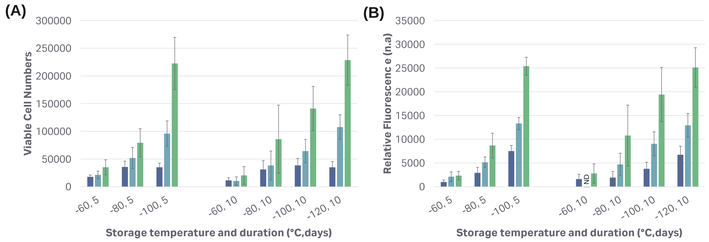
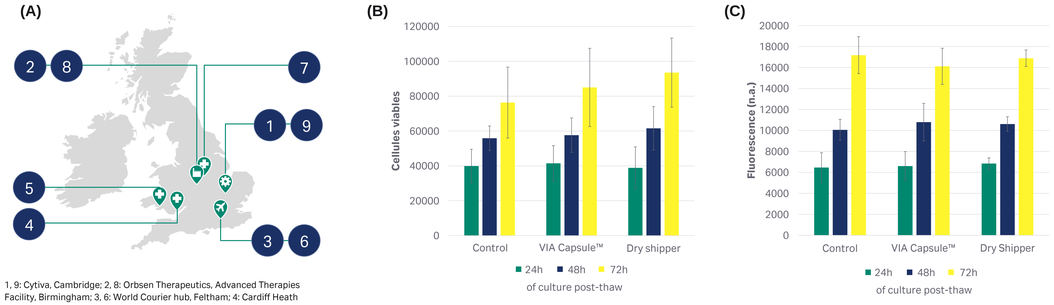


Fig 2. (A) Viable cell number and **(B)** fluorescence redox functional activity indicating active respiratory metabolism in Jurkat cells, 24 h (dark blue), 48 h (blue), and 72 h (green) post-thaw with simulated transport periods and temperatures. (n = 5, error bars indicate ± SD; ND: none detected)

A 5-day transit at -100°C did not significantly impair cell parameters post-thaw compared to a 10-day period at -120°C. However, longer transit periods (10 days) at this temperature or at higher temperatures appeared detrimental, and the extent of the impact increased as the temperature gap to Tg' became more important.

Real-life cryogenic shipping comparison in a VIA Capsule™ system versus a dry shipper:

- Transit time: 4 days via road across various clinical sites (Fig 3A) handled by the specialist courier World Courier
- Samples were loaded from and returned to -150°C cryostorage prior to and after completion of the journey
- Immortalized T cell line (Jurkat) to emulate the behavior of some cell therapies (10^6 cells/mL, 5% Me_2SO in closed system vials, 6 mL fill volume)
- Analyses post-thaw: viable cell numbers and metabolic activity after 24, 48, and 72 h of re-culture (n = 3)



1, 9: Cytiva, Cambridge; 2, 8: Orbsen Therapeutics, Advanced Therapies Facility, Birmingham; 3, 6: World Courier hub, Feltham; 4: Cardiff Heath Hospital; 5: Morriston Hospital, Swansea; 7: University Hospitals Birmingham.

Fig 3. (A) Overview of the multi-leg, multi-party cryogenic journey across the UK, **(B)** post-thaw viability and **(C)** metabolic activity of cryopreserved Jurkat cell samples measured for controls that were maintained in cryogenic storage at the original site (-150°C) versus samples transported in a VIA Capsule™ system or a dry shipper (dry shipper by MVE; n = 3, error bars indicate ± SD, p-values > 0.05, one-way ANOVA; raw data available upon request).

Cell samples transported cryogenically for 4 days in an LN_2-free, electric shipper (VIA Capsule™ system) or in a LN_2 dry shipper presented similar cellular outcomes post-thaw as samples that remained in cryogenic storage at the start site (-150°C).

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Conclusions

Molecular mobility is greatly slowed down in frozen samples, especially below the glass transition temperature (Tg'), which is approx. -120°C in DMSO-containing cell suspensions and theoretically allows for indefinite storage.[1,2,3] Although greatly reduced, some residual molecular mobility still exists above Tg', which may lead to cellular damage over time and impaired cell integrity post-thaw. The extent of this damage depends on the time and temperature gap to Tg' (**Fig 2**).

The VIA Capsule™ system (**Fig 1**) is a predictable LN_2-free cryogenic shipping solution which ensures up to 5-days fully passive shipping window below -120°C (**Fig 3**). Moreover, it is easy to handle and can be recharged anywhere using electricity, mitigating risk from any unforeseen event or delay in transit.[4,5]

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In collaboration with

ATTC Advanced Therapy Treatment Centres | Midlands-Wales Advanced Therapy Treatment Centre

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MVE CRYOSHIPPER CT-50 TILT VALIDATION FOR CRYOGENIC CORD BLOOD SHIPMENTS

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Daniel Gibson, Anthony Nolan, Assistant Director of Cell and Gene Therapy Services, Nottingham, UK

INTRODUCTION

There are over 2500 cord transplants performed worldwide each year[1], all of which require monitored transport of a temperature below -150°C. The process of shipping a cord can have its challenges as there are limitations to vapour based shippers. In the correct orientation a vapour based shipper could maintain a temperature below -150°c for up to 10 days (make and model dependent).

However, if a shipper is tilted during transit, or placed on its side; this can significantly reduce the amount of holding time and can even expose the cord blood unit to a transient warming event or worse, completely thawed.

This validation looks at the worse case scenario for shipment using the MVE CT-50 CryoShipper.

METHOD

The weight of the shipper was recorded prior to priming with nitrogen.

The shipper was filled with liquid nitrogen and allowed to absorb for more than 2 hours.

The excess nitrogen was removed and a recording of the weight of the shipper, post-prime, was measured.

Temperature probes were positioned inside of the shipper and connected to SHIPSLOG temperature loggers.

The MVE CT-50 CryoShipper was tilted and placed onto its side at a 90° angle.

Following this, the CryoShipper was returned to its correct upright position. When the temperature stabilised, the temperature recordings were ended and the weight recorded.

This is used to monitor nitrogen evaporation and extrapolate for total validated duration of the shipper at cryogenic temperatures.

After a delay of 30 minutes, the loggers proceeded to record the temperature reading at 15 minute interval

This procedure was repeated with an MVE Standard CryoShipper and an MVE Qwick 9/500 Cryoshipper. The tilt duration for each was maintained for 48 hours.

RESULTS

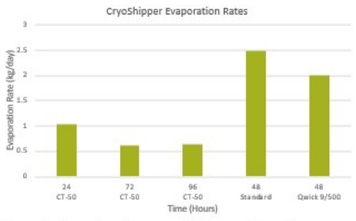
The MVE CT-50 CryoShipper was tilted over three separate durations – 24 hours, 72 hours, and 96 hours. Recordings of the internal temperatures and before and after weights were taken.

The MVE Standard and Qwick 9/500 CryoShippers were each tilted for a 48 hour duration.

	Primed Weight (kg)	Final Weight (kg)	Evaporation Rate (kg/day)	Lowest Temp (°C)	Highest Temp (°C)	Duration below -150°C
CT-50 (24 hrs)	11.42	10.22	1.04	-184.7 -192.2	-182.7 -189.4	>24 hrs
CT-50 (72 hrs)	11.42	9.52	0.62	-188.2 -190.8	-183.4 -187.1	>72 hrs
CT-50 (96 hrs)	11.38	8.34	0.64	-188.2 -190.5	-184.5 -186.8	>96 hrs
Standard (48 hrs)	17.24	11.10	2.49	-196.8 -195.1	-32.9 -30.9	33.5 hrs 34 hrs
Qwick 9/500 (48 hrs)	15.40	11.42	2.00	-193.5	20.9	15 hrs

The CT-50 shipper maintained temperatures below the -150°C adverse warning for the entire duration of each experiment.

The Standard and Qwick shippers warmed past the -150°C adverse warning temperature within 33.5 hours and 15 hours, respectively.

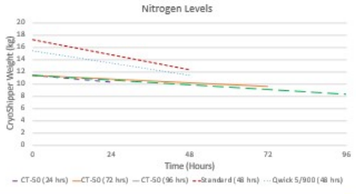


The graph above shows the average daily evaporation rate in kilograms between CryoShippers and tilt periods.

The CT-50 data suggests the initial evaporation rate is higher within the first 24 hours.

Although the MVE Standard CryoShipper maintained cryogenic temperatures for longer than the Qwick 9/500, the evaporation rate was higher. This corresponds to the Standard shipper absorbing a greater amount of nitrogen.

The decline in nitrogen vapour levels are displayed below.



MVE CRYOSHIPPER CT-50 TILT VALIDATION FOR CRYOGENIC CORD BLOOD SHIPMENTS

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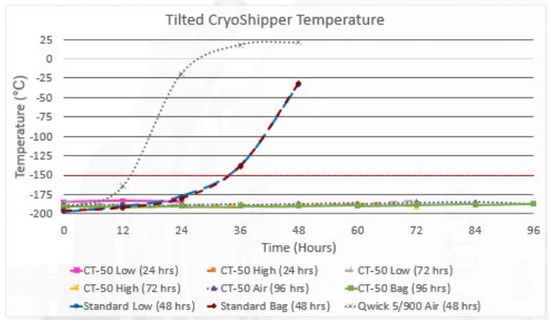
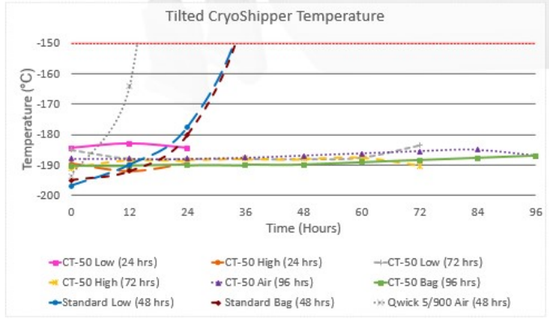
RESULTS CONTINUED





CONCLUSIONS

The MVE CT-50 CryoShipper can withstand being tilted during transit for 96 hours, without causing an adverse warming event, enough time for an international shipment of cord blood, from the UK.

The MVE Standard CryoShipper can withstand being tilted during transit for up to 33.5 hours during shipment of cord blood. Past this time point, an adverse warming event would occur to the sample within.

The MVE Qwick 9/500 CryoShipper can withstand being tilted during transit for up to 15 hours during shipment of cord blood. Past this time point, an adverse warming event would occur to the sample within.

The CT-50 shipper is a more recent design with a felt coated bung which creates a tighter seal. The neck of the shipper is also comparably narrow and as nitrogen vapour is heavier than air, much of the vapour will have remained within the shipper. This can be seen in the temperature readings from the lower probes as when tilted, there was a decrease in temperature for the lower part of the shipper.

The Standard and Qwick 9/500 CryoShippers have wider necks and the bung lid is made of polystyrene. This does not create a tight seal, which was visible after tilting as vapour could be seen escaping from the shipper.

The Qwick 9/500 CryoShipper warmed at a greater rate than the Standard shipper. As the Qwick 9/500 absorbs nitrogen faster than the Standard, the same mechanism allowing for this, may result in an increased release rate of vapour when tilted.

The CT-50 CryoShipper maintained cryogenic temperatures of below -150°C for the entire duration of each analysis.

A maximum temperature of -182.7°C was reached during the 24-hour tilt experiment from the temperature probe positioned, closest to the lid, within the shipper.

The MVE Standard CryoShipper sustained cryogenic temperatures of -150°C and below during a horizontal tilt for up to 33.5 hours.

The MVE Qwick 9/500 CryoShipper internal temperature rose above -150°C in fewer than 15 hours and was into positive temperatures in under 26 hours.

To minimise risk of adverse warming events in the event of a tilt during transit, use of the MVE CT-50 CryoShipper would be advised.

90°
Tilt on MVE CryoShippers was maintained for 24 to 96 hours.

96
Hours after tilting MVE CryoShippers CT-50, internal temperatures were below -180°C, well within the -150°C threshold.

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Words

References

[1] WMDA data

None of the authors have any conflict of interest to declare P071

CONTAINMENT CONSIDERATIONS FOR THE CRYOPRESERVATION OF CELL AND GENE THERAPIES

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Introduction

The commercialization of cells and genes for therapeutic use drives a critical need for the refinement of containment and storage procedures to ensure that the integrity of these sensitive biologics is maintained until administration to the patient. Using a science-based approach to understand and optimize advanced biologic therapies in the context of their containers can help to mitigate risk. Various research studies were conducted to explore containment requirements in the context of cell and gene therapy products and processes. Container performance was evaluated independent of biologic material through analysis of particulate generation and container closure integrity tests measuring both oxygen and carbon dioxide ingress at temperatures down to -180°C. Cryogenic and ultra-low temperature preservation of biologic material was evaluated through examining recovery of therapeutically relevant cells and viral vectors after storage in different container types and sizes. Various effects of vial material and design were observed such as time-to-thaw, where a slower thaw time was driven partly by container shape but resulted in minimal changes to therapeutically relevant primary human cell recovery. Additionally, the consistent and high recovery rates of two viral vector types from cyclic olefin polymer vials was demonstrated by cell-based assays. Together, the results generated provide a strong indication that the storage container and associated process parameters can affect the recovery of cell and gene therapy products.

Materials & Methods

Vials made of cyclic olefin polymer (COP) supplied as Crystal Zenith™ in a Ready-to-Use (RU) format with appropriately sized serum stoppers and corresponding aluminum seals were used as the container closure system for all studies. Relative performance measurements captured the effect of container material and the cryopreservation process on cell and virus recovery and function. Vials were manually filled with appropriate matching volumes of drug product formulation and hand crimped in a biosafety cabinet. Freezing was achieved after the manual fill/finish process as necessary for the drug substance; i.e., an uncontrolled freeze directly into a -80°C freezer for virus and controlled-rate freezing for cell-based therapies.

Table 1: Types of therapies tested in COP vial systems and compared to conventional containers [flexible ethylene-vinyl acetate (EVA), fluorinated ethylene propylene (FEP), and rigid polypropylene (PP)].

Therapy Type	Relevant Drug Substance	Typical Container
Immuno-Oncology	Primary Human CD3+ T Cells	EVA / FEP Blood Bag
Gene Therapy	Adeno-Associated Virus Serotype 2	PP or Glass Vial
Cancer Vaccine	Adenovirus Serotype 5 with Ad35 Fiber Protein	PP Screw-cap Vial
Cell Therapy	Primary Human Mesenchymal Stem Cells	PP Screw-cap Vial
Cell Source Material	Primary Human Peripheral Blood Mononuclear Cells	PP Vial or EVA Bag

To assess relative performance of COP vials with the cryopreservation process, various effects of the polymer material that may impact or influence the functionality of the biological material cryopreserved inside were measured. Performance was defined by fill concentration and fill volume related to surface area, pH following holds on dry ice (solid phase CO_2) and +4°C, Functionality of T cells was assessed by culture outgrowth and population analysis by flow cytometry. Adeno-associated virus (AAV2) and adenovirus (Ad5 and Ad5.F35) were assessed post-thaw by transduction of the HEK-293 cell line followed by flow cytometric detection of enhanced green fluorescent protein (eGFP) fluorescence 48 to 72 hours post-transduction. Recovery was measured in both volumetric and functional terms. The recovery data for hMSCs and PBMCs can be found on our website[2].

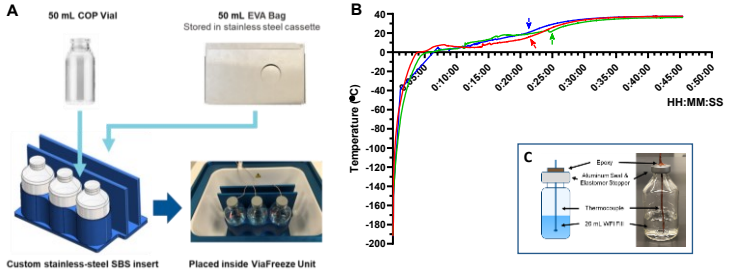

Figure 1: Controlled-Rate Freezing Configuration Using the Custom SBS Insert for the VIA Freeze™ Controlled-Rate Freezer Commonly Used to Preserve Cell Therapies.

A) The vial versus cryobag freezing set-up for cell therapies included a rigid, bare vial with no outer wrap or secondary package and a flexible bag cryo-cassette made of stainless steel. The containers were placed directly into a custom standard size (SBS) aluminum insert used in a VIA Freeze™ controlled-rate freezer. This allowed for vials and bags to be frozen in the same batch. **B)** Typical time-to-temperature measurements of a 20 mL fill in a COP vial rated for 50 mL capacity. Three separate vials were removed from vapor phase LN_2 storage, allowed to thaw in a water bath set at 37°C while capturing data with an electronic datalogger. Arrows denote time vial reached RT. **C)** Thermocouple is placed through the stopper to the center of the fill.

Results

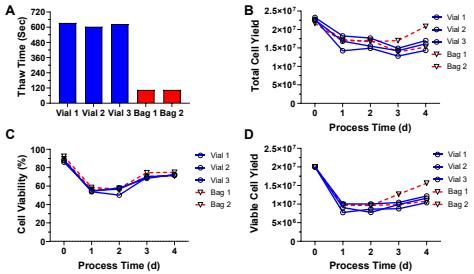

Figure 2: Similar Recovery Profiles of Primary Human CD3+ T cells from Rigid COP Containers Despite a Slower Time to Thaw Compared to Flexible EVA Cryobag.

Controlled-rate freezing is critical to cell health upon thaw; however, thaw time may also present a challenge to cell health. **A)** A 37°C water bath was used to measure thaw times of a 50 mL COP vial filled with 20 mL water (40% rated fill) from cryogenic vapor phase temperatures to 37°C. Observed thaw time between a 50 mL vial and a flexible EVA cryobag is increased ~6x likely due to the increased cross-sectional cylindrical area of the vial compared to the irregular shape of the flexible cryobag. **B)** Cell counts from recovered cell material stored in COP vials or EVA cryobags indicate similar recoveries at Day 0 but diverge at Day 4 of outgrowth in one bag sample (out of a total of 5 containers). COP vials demonstrate consistent recovery, which could be advantageous for manufacturing. **C)** Cell viability was assessed using an automated AO/DAPI dual discrimination method. The post-thaw viabilities were both similar and acceptable for outgrowth culture. **D)** Despite the initial high and consistent cell recovery (yield and viability), viable cell yield from the two bags grew to near 50% different over 4 days. N = 2-3 per container type for all tests.

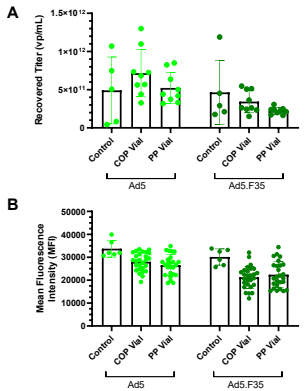

Figure 4: Vial Material did not Affect Ad5 or Ad5.F35 Recovery.

A) Recovery of Ad viral material was measured using OD260 readings of dissociated viral particles, then back-calculated. Both viral types recovered equally as much viral particles after one week of ultra-cold storage. **B)** Transduction activity (MFI) of the tested viral material showed equivalent recovery of the samples tested in A. Control is fresh virus. N=5-24 tests, means ± SD by Two-Way ANOVA with Tukey's MCT displayed.

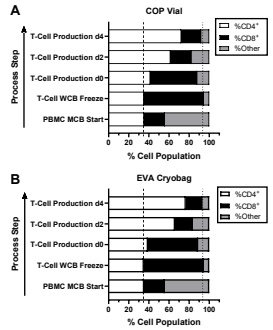

Figure 3: T Cell Population Tracking During Primary and Secondary T Cell Expansion Phases Reveals No Significant Effect of Container Material Between EVA Cryobags and COP Vials.

Phenotypic analysis of cells using a standard flow cytometry analysis panel for the COP vial **(A)** and cryobag **(B)** study conditions. MCB, master cell bank; WCB, working cell bank; CD4+, helper T cells; CD8+, cytotoxic T cells; d0, d2 or d4, cell culture day. Dashed lines indicate starting (left) and ending % T cell across various stages of production.

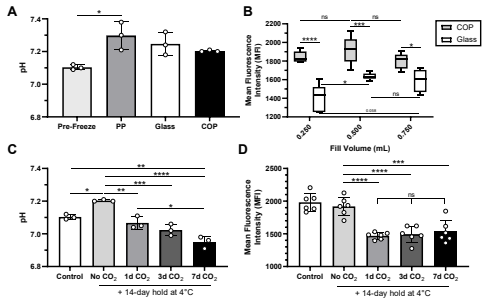

Figure 5: Cryopreservation of AAV2 in COP and Glass Containers on pH of AAV2 in Ultra-Cold Storage.

Changes in pH are known to affect biological material functional stability. One freeze/thaw cycle did not appear to affect MFI despite a slight pH change occurring in the same vials.
A) The pH of 500 µL fills with formulated AAV2 in various vial materials is maintained after one freeze/thaw cycle. **B)** MFI is interpreted as a measure of AAV2 transduction functional activity. COP appears to favor more AAV2 recovery when compared to glass vials of the same rated size and fill volume. **C)** Measured pH of COP vials after either pre-freeze, no dry ice exposure (no CO_2), 1-day, 3-day, or 7-day holds in dry ice followed by a 14-day hold at 4°C. A pH drop of ~0.15 units was observed after 7-days of exposure to dry ice. Although the pH shifted downward in response to increasing dry ice exposure time, the impact of CO_2 over time on AAV2 MFI was minimal. **D)** MFI as a readout of activity of AAV2 as in C. The drop in MFI appeared stable after the initial exposure to dry ice.
Axis labels: CO_2 indicates dry ice storage condition; d indicates time in days. ; n=3 for each group except for MFI data where n=6 (3 trials, 2 replicates each). Data displayed as mean ± SD with *p ≤ 0.1000, **p ≤ 0.0100, and ns = p ≥ 0.5000 by Two-Way ANOVA with Tukey's MCT.

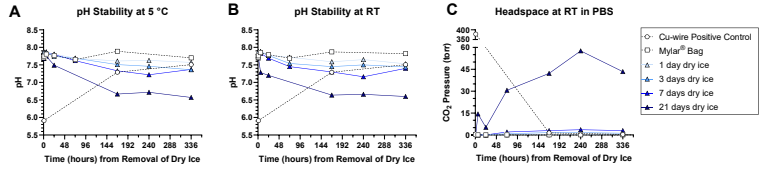

Figure 6: pH Changes From CO_2 Permeability of COP Vials Can Be Mitigated By Using a Mylar® Wrap.

COP vials filled with PBS were subjected to either 1-, 3-, 7-, or 21-days of storage on dry ice then the pH was measured at the times noted. **A & B)** Measured pH of PBS in COP vials after CO_2 storage followed by a 14-day hold between 2 to 8°C. pH changed, but remained acceptable, up to 7 days of exposure to dry ice. **C)** Headspace gas analysis indicated an increase of CO_2 amounts over 21 days time on dry ice, which indicates a possible accumulation coming from the container polymer itself.

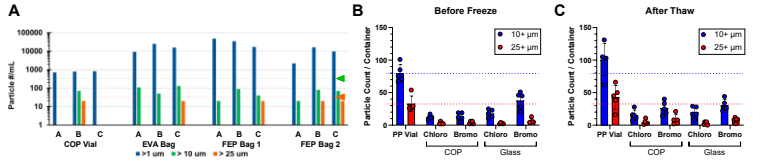

Figure 7: Lower Levels of Particulates in Ready-to-Use COP Vials Compared to PP, EVA, and FEP Containers.

Compared to glass and COP vials, PP vials and flexible FEP and EVA cryobags were found to have more particles overall and before freezing and after thaw. **A)** Particles from containers filled with water for injection were collected by using a standardized washing procedure to collect the intrinsic particulates within the container. Particles were enumerated using a fluid imaging light obscuration instrument and reported as particles/mL wash solution. Individual absolute particle counts per mL shown, n=3 (A, B, C) per container type. Arrows correspond to the particle size limits described in Test 1.B of USP <788> for light obscuration with a container size of 20 mL. This translates to 300 and 30 particle counts per mL for ≥ 10- (green) and ≥ 25-micron (orange) sizes, respectively. **B & C)** Particulate level is a matter of patient safety, and the choice of a container material has implication on particulate level before and after use in cryopreservation processes. Using methods described in USP<788>, we evaluated the PP screw-cap vials commonly used in research against clinical grade COP and glass vials. PP vials shed more particles after a single freeze-thaw cycle while COP remained clean with both bromobutyl and chlorobutyl elastomeric stopper types.

Conclusions & Considerations

The results provide baseline proof-of-concept data that indicate the compatibility of traditionally used storage containers with various cell and gene therapies can affect product quality and that COP container systems may serve as a preferred container for commercial products due to both maintenance of drug potency and consistent recoveries achieved after frozen storage.

More information can be found at our Knowledge Center by scanning the QR code above.

Acknowledgements

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