DRY PRESERVATION OF GERMINAL VESICLES: DEVELOPMENT OF A NEW FORMULATION TO PRESERVE DNA INTEGRITY UNDER ADVERSE ENVIRONMENTAL CONDITIONS

by

Joseph Andrew Orozco Cabral

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Approved by:

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Dr. Gloria Elliott

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Dr. Paola Lopez-Duarte

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Dr. Didier Dréau

ABSTRACT

J. ANDREW OROZCO CABRAL. Dry Preservation of Germinal Vesicles: Development of A New Formulation to Preserve DNA Integrity Under Adverse Environmental Conditions. (Under the direction of DR. GLORIA ELLIOTT)

Advances in the cryopreservation of genetic material has revolutionized human reproductive medicine and animal conservation efforts. Despite many successes, oocytes have been more challenging to cryopreserve than sperm. Their large size makes it difficult to load cryoprotective agents (CPAs) into the cell without toxicity issues, and the high water content can cause damage from formation of intracellular ice crystals during cooling and rewarming. Research has shown that preservation of the genetic material can be achieved with fewer preservation challenges if the germinal vesicles (GVs) are targeted for preservation instead of the whole oocyte. The gamete can then later be reconstituted by depositing the preserved DNA into an enucleated cell. The current study evaluated a novel preservation composition to enable dry preservation of these GVs, which eliminates the need for cryogens, refrigerated transport and storage, and the use of toxic cryoprotectants. The compositions evaluated were based on the naturally occurring protectant trehalose. Trehalose is a sugar that is naturally occurring in animal species, called anhydrobiotes, that can survive extreme dehydration and other adverse conditions. Trehalose is also used in the pharmaceutical industry for its ability to form a glass under appropriate conditions. The glassy state is a solid with an amorphous conformation that is reached by drying (or cooling) the solution in a manner that prevents molecular ordering and crystal formation. Molecular mobility is practically halted in the glassy state, which prevents degradation of materials that are embedded in such a glass. This glassy state must be stringently

maintained in order to maintain functionality of preserved materials, and in the specific case of trehalose, samples held above 44% Relative humidity (RH) will quickly crystallize. The current study investigated additives that could enable more flexible storage and shipping conditions, especially high humidity environments. Feline oocytes were isolated, denuded to separate the oocyte from surrounding cells, permeabilized to allow for intracellular loading of the preservation solution, then immediately dehydrated to an amorphous solid state in an 11% RH environment using microwave-assisted drying to reach this glassy state. These preservation solutions included the disaccharide sugar trehalose, along with salt additives of the choline family, specifically choline citrate, choline acetate, and choline chloride. Choline salts are naturally occurring electrolytes that have been observed to delay or prevent crystallization in trehalose glass systems in previous work. The dried, porated oocytes were subjected to storage conditions simulating ideal (11% RH) and adverse (76% RH) conditions, then rehydrated and analyzed for recovery fraction and DNA fragmentation by TUNEL analysis. High RH is known to cause crystallization in trehalose solutions and can possibly contribute to cellular damage. The formation of crystals in preserved cells can also potentially cause DNA fragmentation. Compositions with salt additives were shown to moderately increase the recovery fraction of GVs from the storage substrate after exposure to adverse conditions but did not have any affect ($p>0.05$), positive or negative, on DNA integrity. A second phase of experiments was performed, narrowing the testing materials to a single additive, choline acetate, and increasing the sample size to improve statistical power. The addition of choline acetate in Phase 1 moderately improved recovery ($p=0.055$), while also improving handling procedures, observed as quick release and glass dissolution from the preservation material and facile collection. In Phase 2, the addition of choline acetate was demonstrated to statistically increase ($p<0.05$) GV recovery from

substrates after exposure to adverse conditions when compared to trehalose alone and yielded equal recovery ($p>0.05$) under ideal conditions. Raman spectroscopy was used to confirm the state of the materials before and after humidity treatments. The control preservation composition of 20% weight/volume trehalose crystallized under adverse conditions, while the trehalose with the choline acetate additive 20% weight/volume remained amorphous. This study found that combining trehalose with a choline acetate salt additive enables good preservation outcomes even under transient high humidity conditions. This may enable more flexible transportation and storage of germinal vesicles that are to be used for reproduction purposes and potentially an overall reduction in the cost and complexity of genetic resource management.

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CHAPTER 1: INTRODUCTION

1.1: BIO-PRESERVATION OF GENETIC RESOURCES FOR REPRODUCTIVE MEDICINE

The successful preservation of gametes has revolutionized the reproductive biology and fertility fields. The preservation of gametes has many far-reaching benefits, enabling women to have children despite fertility problems, and allowing resurgence of animal populations following adverse events and global climate change, preventing total loss of species. While gametes from certain species have been successfully preserved, such as semen from cattle that can be preserved indefinitely and produce offspring long after the sire's death, oocytes in general have been more challenging [1, 2].

Cryogenic preservation is the most dominant technology in the reproductive preservation field, involving the reduction of the temperature of a tissue or cell down to an extremely low level to halt biological processes and essentially stop molecular mobility [3]. To prevent damaging ice crystals from forming inside cells during cooling, often an additional substance is added, known as a cryoprotective agent [4]. While small cells can be cryopreserved quite effectively using moderate levels of cryoprotectants, the high ratio of volume to surface area in large cells, such as oocytes, makes it difficult to load adequate amounts of cryoprotectant without toxicity concerns. Also, large cells often do not dehydrate adequately during slow cooling, resulting in trapped water that can form damaging intracellular ice. Fast cooling ice-free vitrification methods have been employed, but these methods require higher levels of cryoprotectants, and can only be applied to very small numbers of cells at a time to attain the rapid cooling rates required. Overall, the use of cryogenic freezing is costly and technically intense [5, 6]. It requires cryogens, skilled labor, and well-resourced conditions to employ these techniques.

Dry preservation, which is based on creating a meta-stable glass that can be stored without refrigeration, can circumvent issues that arise in typical cryopreservation to better enable field work and facilitate easier global transportation and storage of genetic resources. A glass is formed when a substance is brought below its glass transition temperature (T_g), either by cooling or dehydration, without allowing crystallization to occur. A system below this temperature exhibits solid-like behavior but remains amorphous like its liquid form [7, 8]. In this state, molecular mobility is minimal, and molecules cannot easily align to form crystals. This state can be more easily obtained by using additives, as the glass transition temperature of water alone is extremely low, and requires an almost instantaneous temperature depression [7, 9].

1.2: DRY PRESERVATION IN NATURE

Dry preservation involves removing water from cells to reduce molecular mobility and to decrease the amount of water available for biological processes [10]. Creatures in nature such as tardigrades and some nematodes, collectively called anhydrobiotes, use this strategy to survive harsh conditions [11, 12, 13]. Anhydrobiotes produce molecular protectants in response to dehydration cues, which provide protection against harsh conditions such as extreme dehydration in their environment. Their subsequent rehydration, when conditions improve, can result in their continued survival [11]. The glass-forming disaccharide trehalose is commonly generated in many of these anhydrobiotes and will be utilized in this work. Trehalose is not naturally present in non-anhydrobiotic species and must be added exogenously to preservation formulations.

1.3: TREHALOSE AS A BIOPROTECTANT

Glass-forming protectants are used in preservation formulations to enable formation of a ‘vitrified’ solid-like matrix upon removal of water. ‘Glassy’ or ‘vitrified’ refers to the state in

which a substance exhibits solid-like behavior, but with an amorphous configuration wherein the molecules are disordered rather than crystalline. Because this is a meta-stable state, samples must be maintained at temperatures below the T_g to prevent formation of the more energetically favored crystal form [7, 14, 15]. Any change in the moisture content will change the T_g of the composition, thus slight excursions in environmental conditions of RH and temperature can lead to crystallization of the preservation solution and loss of function of the preserved material [5].

Trehalose is also utilized in the pharmaceutical industry as a preservative for active drug ingredients and other sensitive molecules and biomolecules [16]. This disaccharide is considered non-reducing, without a free aldehyde or ketone, so it cannot reduce other molecules [17, 18]. This facilitates use as a preservative, as it does not change the structure of any other molecules with which it is stored. Trehalose additives have been used effectively to extend shelf-life.

Studies have also shown effectiveness in the use of trehalose as an additive to preserve entire cells. Yeast cells have been studied as models of preservation and have been preserved with greater survivability with trehalose than without the sugar [19]. Red blood cells have also been preserved using trehalose as an additive, in an attempt to replace glycerol, a cryoprotectant with known cell toxicity [20].

1.4: DRY STORAGE CONDITIONS

There are two main requirements for dry preservation with trehalose. The first is reaching the glassy, amorphous state by dehydration, and the second is the maintenance of this state with storage at a controlled humidity and temperature [5, 15]. Dry preservation helps to overcome the logistical challenges of standard cryopreservation by allowing samples to be stored without refrigeration. This eliminates the need for ultra-low temperature storage options, such as liquid

nitrogen or cryogenic freezers, reducing costs and negating logistical challenges, such as refrigerated transportation. The nature of glasses requires maintenance of sufficiently low temperature and moisture content, controlled by environmental relative humidity, to keep samples below the T_g of the matrix. At excursions above these conditions, the glass enters a supersaturated phase that is prone to crystallization, which is theorized to be a reason for cell damage during dry preservation [21]. This excursion of higher humidity may also allow the water content of the system to become high enough to allow processes to resume in the cells, causing damage by natural metabolic activity [2]. Previous work has found storage conditions that are conducive to the maintenance of these amorphous states [5].

1.5: PRESERVING GERMINAL VESICLES

The current work focuses on the preservation of the DNA stored within the nucleoid of a cell instead of preserving the whole cell [5, 22, 23]. Recent research has shown the effectiveness of isolating a nucleoid organelle from an oocyte, transferring it into an enucleated cell, and allowing it to recover cell functions, and even to be fertilized by a spermatozoon. This can be accomplished by harvesting oocytes that have not matured to the point of ovulation. In this state, they have not developed the organelles of a fully mature cell and have just started the selection that is a part of folliculogenesis, brought about by the excretion of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) [24]. The immature oocytes that are initially stimulated by FSH are considered selected, and they will mature more on the path to ovulation. This selection is the result of estrogen release by the oocytes during the follicular phase, which acts as negative feedback to reduce the stimulation of additional oocytes [24].

For the purposes of this study, oocytes that are in this immature stage were selected, as there are no mitotic spindles yet present, which have been shown to be negatively affected by preservation processing [25]. Inside the immature oocytes is a nucleoid organelle, called a Germinal Vesicle (GV), that has tightly bundle DNA in a haploid form ready for further maturation to an ovulated oocyte to be later fertilized by a spermatozoon [26]. This germinal vesicle is the portion of the cell that is the focus of the current preservation study because this is the location of all the genetic material in the cell at this point since the cell has not matured enough to develop other organelles in its membrane. Observing previous studies, the current study is aimed at developing and optimizing protocols to preserve this nucleoid factor, and all but disregarding the integrity of the rest of the cell [11, 23]. The cell membrane can be used as a storage container, after it is porated to allow for faster diffusion of preservation materials to the GV. While no longer providing a membrane barrier, it still adds a layer of physical protection to the GV during the processing and preservation procedure and handling. After this preservation and recovery process, the GVs can then be removed from the cells, and placed into fresh enucleated cells to help propagate the genes that are present.

1.6: EFFECTS OF SALTS ON TREHALOSE

While the proof of concept for dry preservation of germinal vesicles has already been established, there is still considerable room for optimizing the existing baseline protocols [5, 22, 23]. As described previously, the T_g of trehalose delineates the transition between the meta-stable glassy state and the supersaturated liquid state which is highly prone to potentially damaging crystallization [14]. The careful maintenance of storage conditions can keep the material in this amorphous glassy state, but excursion outside of this temperature and relative humidity range can result in crystallization, given sufficient time. The crystallization process of

amorphous glasses is based on temperature, water content, as maintained by environmental relative humidity, and the duration of exposure to these conditions. While trehalose is a very effective bioprotectant under ideal storage conditions, excursions in either temperature or relative humidity for even a short time and can result in crystallization and the loss of the preserved biologics [15].

Although ideal storage methods can be maintained in environmental chambers with fixed relative humidity and temperature, during shipping these conditions are difficult to maintain. There is always the risk of damage to packaging, shipping delays, and temperature and relative humidity excursions. The development of more robust solutions that do not crystallize in response to these conditions would provide protection against unexpected excursions that occur as a part of damage or human error. In recent work, it has been shown that the use of salt additives in trehalose solutions can delay, or entirely prevent crystallization in high relative humidity environments [15]. These studies yielded promising candidates that will be further investigated in the current study. The use of selected salts can delay the onset of crystallization of the glassy trehalose material anywhere from a few hours to several weeks, potentially allowing for excursions outside of ideal storage conditions while maintaining the integrity of the preserved biologics [15]. The present study builds upon this previous research, to investigate the effectiveness of organic salts in improving the formulation used in the dry preservation of germinal vesicles within oocytes [5, 22, 23].

CHAPTER 2: SPECIFIC AIMS

The present study is broken up into two primary aims, the first involving methodology development, and the second to test a hypothesis.

2.1: AIM I: DETERMINE DEHYDRATION PROCESSING PARAMETERS

2.1.1: ESTABLISH MICROWAVE ASSISTED DEHYDRATION PROTOCOL

The use of microwave assisted drying has been previously developed as a method of rapid dehydration of biologics. The process was optimized to maximize the rate and extent of dehydration while limiting exposure to microwave radiation [22]. These previous studies have shown that using short pulses of low powered microwaves can agitate the water molecules within a sample, enhancing diffusion and release from the surface and thus speeding dehydration. An observation in previous dehydration procedures, which included equilibration with a controlled low humidity environment, was that certain dehydration conditions can limit the evaporation of water from a droplet. In some processing conditions, the outer layer of the droplet forms a skin, which then blocks the evaporation of water from deeper within the droplet. This contributes to unequal dispersion of solute during dehydration [27, 28]. In previous work by Deegan et al, capillary flow was observed to form a ring of solute on the outer border of the dried droplet, which would result in a non-homogeneous environment for samples in a droplet of preservation material. The use of a fiber glass filter paper, which has been shown to have a high wettability, was observed to help disperse droplets of preservation material, yielding a thin layer of the preservation solution over the fibers and the biologics within the solution. This further helped the evaporation kinetics of the solution, resulting in more even and efficient drying of cells [29].

Wang et al previously developed drying curves for microwave assisted evaporation of 1.1 M trehalose in buffer [5] and these methods form the basis for developing drying curves for the experimental compositions that will be implemented in the current work. Using a highly wettable glass fiber as a substrate and low powered microwaves to aid in evaporation, data collected will be used to determine the drying kinetics of samples processed at 20% power in an analytical microwave positioned within an 11% RH environmental chamber. Drying curves will be developed for trehalose alone (control) and trehalose with 3 different salt additives prepared in a 2:1 molar ratio, all dissolved in buffer at a consistent total concentration of 20% weight by volume. These drying curves will be established to prescribe processing times for all materials to reach an equilibrium moisture content sufficient for storage.

2.1.2: VALIDATE THE STATE OF PRESERVATION COMPOSITIONS AFTER STORAGE

To confirm that the glassy state is achieved following microwave processing, evaluation by Raman Spectroscopy will be performed. Raman spectroscopy is a non-destructive analysis technique that uses a high energy excitation laser to probe the vibrational modes of the molecules of a substance [30, 31]. When the molecules are in an amorphous state and there is little to no order in the system, the movement of the molecules is slow and large, with the entire molecule vibrating. When in a crystalline conformation, and the molecules are stacked and ordered, the vibrations are small and minor. This allows the spectrometer to pick up the vibrations of individual atoms within the molecule, rather than those of the entire molecule. In this way, Raman spectrum differences between amorphous and crystalline structures can be observed, thereby confirming the expected state of the materials following dehydration and storage [32].

2.2: AIM II: EVALUATE EFFECTS OF CHOLINE SALT ADDITIVES ON STORAGE OUTCOMES

2.2.1: EVALUATE EFFECTS OF SALT ADDITIVES ON RECOVERY FRACTIONS

In Aim II it will be determined if the presence of a salt additive will increase the recovery of preserved GVs in samples exposed to adverse environmental conditions as compared to the control trehalose. Samples will be prepared and dried according to the drying times determined in Aim I, stored in an 11% RH environment for 2 weeks, and then subjected to treatments considered ideal or adverse. The treatment labeled ideal will be the maintenance of the 11% RH environment, while the adverse treatment will be subjected to 76% RH, which is significantly higher than ideal storage, and has been shown to result in trehalose crystallization within a short time. It is intended to simulate the loss of container integrity during shipping and storage. The ease of handling samples preserved in each material subjected to each treatment condition will be examined. The release of the oocyte from the substrate and material and the integrity of recovered product will be quantified as the fraction of cell recovery from the substrate. The effectiveness of the preservation material in terms of the ability to recover the cells in the condition that they were prepared will be evaluated.

2.2.2: EVALUATE EFFECTS OF SALT ADDITIVES ON DNA FRAGMENTATION

The final observation of DNA fragmentation evaluates the quality of DNA in the germinal vesicle, and therefore the likely success of GV transfer into a fresh cell with retention of fertilization ability [18]. The presence of DNA fragmentation in the samples would be considered a failed preservation outcome [5, 33]. The fragmentation analysis will be performed by the TUNEL assay, which will indicate a positive or negative result for DNA fragmentation. Combined, these results will allow us to determine an ideal solution for preservation.

Preservation of DNA integrity in recovered cells, but with low overall recovery rates, would be an acceptable outcome in terms of quality of preserved specimens if many gametes are available for processing, but this would be an inefficient process. For rare gametes this would be unacceptable. High recoverability but with high rates of DNA fragmentation will result in the loss of the genetic material and inability to use the DNA for further fertilization. Therefore, a combination of both factors is of clinical importance and thus both will be taken into consideration to determine an optimal composition.

CHAPTER 3: MATERIALS AND METHODS

3.1: SELECTION OF MATERIALS

The additives that were chosen for use in the present study have been shown by previous research to delay crystallization a glassy preservation solution made up of Trehalose (Pfanstiehl, Inc., USA) in Tris–EDTA (TE; Sigma-Aldrich, MO) buffer by several hours, and even several weeks [15]. Salts were chosen as additives to the preservation solution as their large size provides steric hindrance to the system, preventing crystal formation by molecule alignment, and the ionic interactions between salts and water left water unavailable for trehalose crystallization [15]. The group of salts that was specifically chosen, the choline salts, were selected due to their natural occurrence in cells and animals. Choline is a necessary nutrient in the body, as it helps in the process of forming the lipid bilayer of cell membranes [34]. This made the choline salts an appropriate additive to the trehalose preservation solution, as their potential damage to the cell should be minimal due to their natural occurrence. The specific choline salts that were chosen were selected from previous research, and their properties in solution. Ziaei demonstrated that the salts that performed the best in delaying crystallization were choline citrate, choline acetate, and choline hydrogen phosphate [15]. Choline hydrogen phosphate was eliminated from the list of potential additives due to its acidification of solution when dissociated [17]. This acidification was expected to be damaging to the cells, so the next best performing salt, choline chloride, was selected. Ziaei also evaluated different molar ratios of salt to trehalose to find an optimal mixture of the materials in buffer. Ratios as low as 1:48 were tested, and a 1:2 ratio of salt to trehalose was determined to delay crystallization best. These three salts were then combined with trehalose in a 20% weight by volume concentration to be studied [15]. This concentration was below the saturation point of the solute, which would prevent spontaneous crystallization.

Previous studies have evaluated trehalose as a preservation material at a concentration of 1.1 and 1.5 molar, but for the current study, as seen in Table 1, the use of the 20% weight by volume concentration, was selected [5, 22, 23]. The lower viscosity made the solutions easier to handle during the study and this concentration was also well below the saturation point, thus avoiding inadvertent crystallization. The effects of the higher starting water content in these solutions are not highly significant, as the samples are ultimately being dried to an equilibrium moisture content. The osmotic pressure that these solutions would exert on intact cell membranes is not relevant here, because the oocyte membranes are porated as part of the preservation process [22, 35].

Trehalose/Salt	Molar mass (g/mol)	Trehalose (mol)	**Trehalose (g)**	Salt (mol)	**Salt (g)**	**Wt%**
Trehalose (Prior study)	378.33	0.025	**9.4583**	0.0	**0.0**	**34.25**
Trehalose (Control)	378.33	0.014599	**5.5231**	0.0	**0.0**	**20.00**
Choline chloride composition	139.62	0.0121269	**4.5880**	0.0060634	**0.8466**	**20.00**
Choline acetate composition	163.21	0.0117	**4.4604**	0.0059	**0.9629**	**20.00**
Choline citrate composition	501.61	0.0084	**3.1883**	0.0042	**2.1136 3.2518 (65% aqueous)**	**20.00**

Table 1. Trehalose and trehalose/salt solutions made by combining calculated amounts of solutes and adding tris-EDTA buffer to make 25 mL stock solutions of 20% (w/v) concentration. Trehalose was calculated as an anhydrous molecule (342.496 g/mol) and purchased from supplier as a trehalose dihydrate (378.33 g/mol). Choline Citrate was calculated as an anhydrous molecule and purchased from supplier as 65% aqueous solution.

3.2: DRYING PARAMETERS

Individual drying curves that were used to prescribe microwave processing times were developed for each solution that was used in the study. Following a protocol used by Wang et al, samples were prepared using each solution, with 4 replicates of each solution [5]. Specifically, fiberglass filter paper was cut into 0.5-inch rounds. Droplets of 40µL of each solution were placed onto

each filter paper round. These samples were then placed into specific locations on a carousel, custom manufactured to hold 8 samples on polyethylene syringe filter holders (Millipore, Billerica, MA), and allowing air circulation around the filter (Figure 1a). The carousel was placed in a microwave (CEM SAM 255, Matthews, NC) operating at 20% microwave power. All procedures related to drying, whether they were for drying curve development or for assessment of DNA integrity after drying, were performed in a controlled humidity chamber that was held at 11+/- 1% relative humidity. This relative humidity was achieved by introducing desiccated air to the box in varying increments to maintain the environment [5]. The equipment contained in the chamber included the microwave for drying samples, the Karl Fischer Volumetric Titrator with attached balance, and the vial capping implement used to seal the storage containers. Access was controlled using rubber gloves that were sealed to the acrylic wall of the chamber (Figure 1).

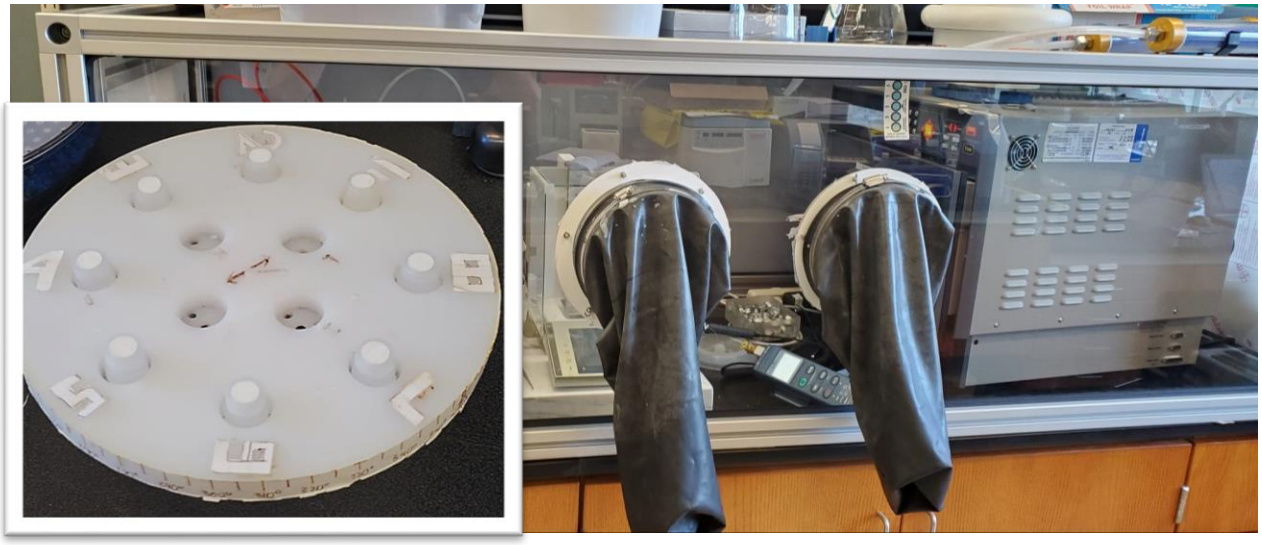


Figure 1. Controlled humidity chamber and microwave setup. The microwave turntable set up to hold 8 samples atop syringe filter holders (inset). Controlled environment chamber with attached access gloves, containing microwave, balance, and Karl Fischer Titrator (background).

The end moisture content was determined at 5 min intervals from 0 to 40 minutes. The moisture contents were evaluated by a Karl Fischer Volumetric Titrator (Mettler Toledo), and the results are obtained as water content in micrograms. In a separate procedure, dry weights of each of the

preservation solutions were obtained [18]. A glass petri dish and 1- inch square piece of glass fiber filter paper (Whatman, Maidstone, UK) were baked in an oven at 120°C to drive off water, then allowed to cool in a desiccant box to prepare for solution. Once cooled, 400 µL of each preservation solution was placed on the filter paper in the petri dish and weighed. The dishes were then baked at 125°C for 96 hours, in replicates of 4 to determine the solids dry weight of the solutions. Using the dry weights of the solutions, along with the water content as determined by the Karl Fischer titration, water content was reported as grams of water per grams of dry weight (gH_2O/gDW).

3.3: OOCYTE PREPARATION

Feline reproductive tracts, including the uterine horns, ovaries, and peripheral fascial tissues, were obtained from a local veterinary clinic, removed during routine ovariohysterectomies, or spay, procedures. These reproductive tracts were placed into a solution of phosphate buffered saline (PBS) augmented with 100 IU/mL of penicillin and 100 IU/mL of streptomycin (Mediatech, Inc., Manassas, VA) to maintain cell viability during processing. The reproductive tracts were then placed on ice and transported to the laboratory to be processed within 2 hours [22]. Once the tissues arrived at the lab, they were immediately processed.

To process the tissues, the ovaries were first excised from the reproductive tract, as shown in Figure 2. The excised ovaries were held in a handling medium on ice while the remaining ovaries were processed. This handling medium was used for tissue holding, and the handling and rinsing of oocytes between the processing steps. It was comprised of minimum essential medium and Hepes Buffer, with 100 IU/mL of penicillin and 100 IU/mL of streptomycin (Mediatech, Inc., Manassas, VA), sodium pyruvate, and bovine serum albumin. The medium provides

essential amino acids to maintain cell growth, while the buffer maintains a biological pH during handling. The addition of penicillin/streptomycin is a standard procedure to discourage growth of bacteria and fungi in the media, while sodium pyruvate acts as a glycolysis intermediate to promote cellular respiration and maintain cell viability while in holding. Lastly, the bovine serum albumin provides another source of protein in the media to encourage cell stability and maintain metabolism.

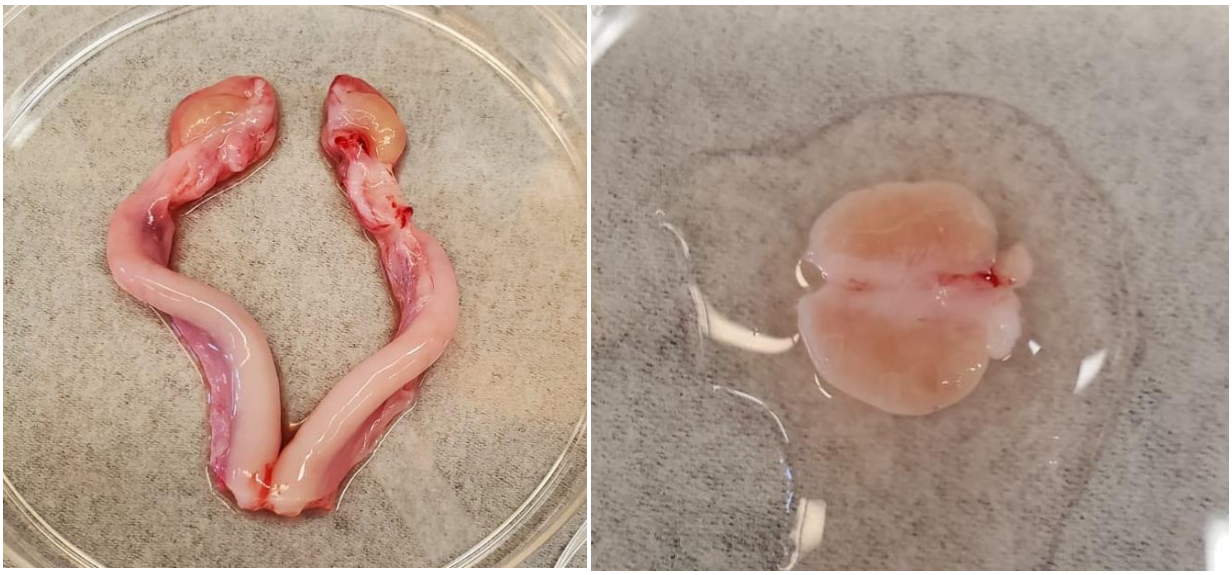


Figure 2. Reproductive tract and ovary. The reproductive tracts are received intact, with the uterine horns, fascial tissue, and ovaries (left). Once excised, the ovaries are bifurcated to expose more tissue (right).

Once the ovaries are excised, each one is placed into a petri dish, bifurcated longitudinally, then followed by various cuts across the ovarian cortex and medulla to open the various follicles that contain the maturing oocytes. Once the tissue has been sufficiently sliced and exposed, it is agitated in approximately 500µL of the handling media to release the oocytes from the tissue. The tissue is pulled aside, and the petri dish is placed under a stereomicroscope for viewing. Using a 10µL pipette, oocytes were located in the media droplet, then gently suctioned and transferred into a fresh drop of media for holding. This process is repeated for all ovaries that

were collected that day. All oocytes were pooled to reduce bias that could potentially be introduced by organ-to-organ differences.

3.4: OOCYTE GRADING

Following the guidelines set by Wood and Wildt, only oocytes that are considered grade one or grade two were selected for study [36]. Grade one oocytes are defined as having a round, intact membrane with a dark center indicating a high lipid content, surrounded by 5 or more layers of cumulus cells, as seen in Figure 3, while grade two are similar except they have less than 5 layers of cumulus cells surrounding the oocyte. Together, the oocyte and the cumulus cells make up what is known as the cumulus oocyte complex (COC), and this is what is observed under the microscope during the selection process.

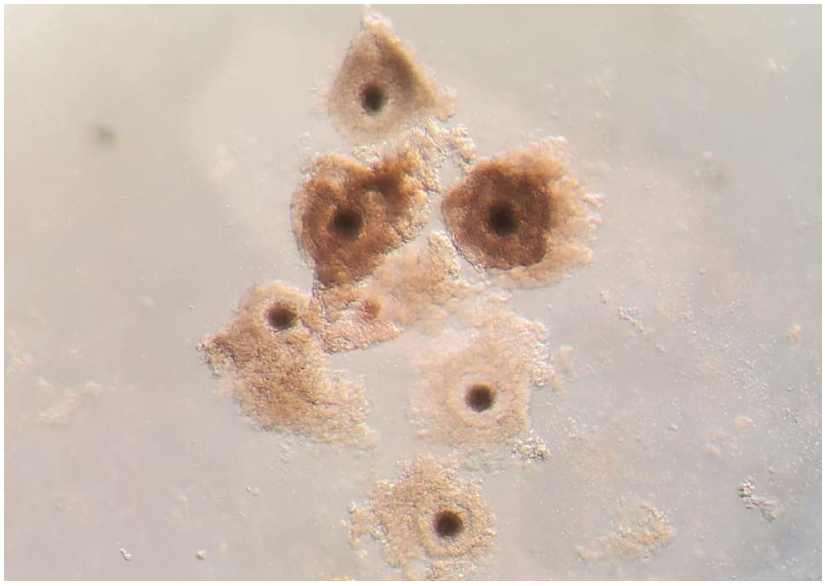


Figure 3. Isolated oocytes. Oocytes were classified as grade 1 based on presence of a solid lipid droplet (black spot) and several layers of cumulus cells surrounding the membrane [36].

In the media after ovary dissection were polar bodies, the non-viable cells that are a result of oogenesis and meiosis, many oocytes that were of a lower grade, therefore not considered high

enough quality to resist the stresses that are applied during processing, and many that were immature and had not yet developed the membrane, known as the zona pellucida. Another type of oocyte that was found was a partially denuded oocyte, lacking at least some of its cumulus cells. This can be a result of under- or over-matured oocytes. As felines are known as induced ovulators, oocytes are not released until the animal takes part in sexual intercourse, and the physical act of mating causes a surge of luteinizing hormone to be released from the anterior pituitary gland which in turn causes ovulation [24]. Because these oocytes are not released until the cat mates, some oocytes have been held in the ovary for sometimes long periods of time, which causes some degradation of the COC.

3.5: SAMPLE PREPARATION

Once the oocytes are isolated, graded, and rinsed in a clean droplet of handling medium, they are prepared for loading of the preservation solutions. First, the oocytes are partially denuded using a Stripper (Cooper Surgical, Malov, Den). The small pipette diameter allows the oocyte to be aspirated by the syringe while removing layers of the cumulus cells that cannot pass through the tip. The oocytes are then placed in a new drop of handling media to remove some of the suspended cumulus cells and rinse them. Then they are placed into a 200 µL centrifuge tube with approximately 100 µL of a 0.2% solution of hyaluronidase (Sigma-Aldrich) and incubated at 37°C for 15 minutes. Following the incubation, the tube is vortexed to agitate the remaining cumulus cells and cause them to be released from the oocyte membrane. The oocytes are then rinsed again in handling media to remove the suspended cumulus cells.

After denudation of the oocytes, they are incubated in 10µg/mL hemolysin (Sigma-Aldrich), to permeabilize the membranes. Since the goal of the preservation is to preserve the GV, the

viability of the cell is not important [22]. Permeabilization of the membrane makes it easier for the preservation solution to diffuse into the cell and surround the GV, but leaves the cellular components intact, providing an extra layer of physical protection during the oocyte handling.

To load the preservation solutions, the oocytes are first rinsed in handling media following the poration process. The GVs with their membranes are then divided into groups and assigned treatments of different preservation compositions, including trehalose control, trehalose with choline citrate, trehalose with choline acetate, and trehalose with choline chloride. Sample distribution into the different treatment groups followed a randomization table, to reduce potential bias from batch-to-batch variation on different collection days. Each treatment group was divided into two processing replicates. One of each paired processing replicate was assigned to a different storage condition. The groups of oocytes are then placed into 300µL droplets of their assigned treatment solution and incubated at room temperature for 10 minutes to allow for solution diffusion across the membrane and GV saturation. After the incubation procedure, the oocytes are collected along with 3µL of solution in the Stripper pipette. A second pipette is used to collect an additional amount of fresh solution, to total 40µL to be placed onto the filter papers. A droplet is formed on the end of the larger pipette, and the oocytes and accompanying solution are injected into the droplet suspended above the filter paper substrate. The larger pipette is then used to deposit the oocytes and solution onto the filter paper, in the center of the circle. The filters are then randomly assigned to a carousel position to reduce bias that could potentially arise from positioning within the microwave cavity. Samples were then dried for durations identified from the drying curves.

A filter containing only preservation solution was also included in each batch of samples to validate the drying procedure with the Karl Fischer titration and ensure that the desired moisture content was reached during the dehydration process.

3.6: SAMPLE STORAGE CONDITIONS

After drying, the filters containing GVs were placed into glass vials, which had been preequilibrated to the environmental 11% RH air by placing them in the controlled humidity chamber at least 30 minutes prior to use. The filters were carefully placed to keep the top of the filter containing the GVs facing upwards. This simplified the recovery process after treatment. The glass vials were then sealed with a rubber stopper and banded with an aluminum strip that was crimped to the lip of the vial [5]. The vials were placed into a storage case on the lab bench. Because the in-vial RH was maintained by the seal, external environmental humidity did not need to be controlled, and laboratory temperatures were constantly maintained at or below 23°C. The samples were kept in this storage case for 2 weeks.

3.7: TREATMENTS

The current study aims to evaluate the effect of different compositions on the preservation outcomes following excursions outside the controlled environmental range typically required to maintain an amorphous, glassy state. The storage condition chosen for this challenge is the relative humidity, and therefore the water content of the preserved system. All samples are prepared in a controlled humidity chamber, which is kept at 11 +/- 1% RH. This humidity is also maintained in the glass storage vials, which were pre equilibrated with the chamber air prior to packaging, and throughout the equilibration hold for 2 weeks. After 2 weeks, samples are subjected to two different humidity levels. As a control, half of the samples – one of each

prepared pair – are kept in their glass vials, assumed to be sealed correctly and thus maintained at 11% RH. This is considered ‘ideal conditions’, since other work has shown that under these conditions, preserved GVs dried to the appropriate moisture level, can remain in a glassy state during storage up to 8 weeks [5]. The other half – the second of each pair – are opened and placed in an environmental chamber (Caron, Marietta, OH) set to 76% relative humidity (Figure 4). This level of humidity was selected because previous work has indicated that at this humidity, pure trehalose glass will rapidly crystallize, while other material combinations should resist crystallization [15]. Therefore, this treatment of an open vial at 76% RH is considered 'adverse

Figure 4. Storage vials and environmental chamber. One of each of the paired samples was opened and the other left sealed for treatment (left). Adverse conditions of 76% RH were induced by the environmental chamber (right).

conditions’, subjecting samples to a humidity excursion. This can simulate a container being damaged in transit, or human error in vial closure. The vials were left overnight, for 16 hours under these conditions, before being rehydrated and evaluated.

3.8: REHYDRATION AND RECOVERY

To evaluate DNA integrity within the GVs, they were rehydrated and removed from their treatment substrate, the glass fiber filter, according to the method of Wang et al. [5], with small refinements in rehydration time and substrate agitation. As in previous work, for rehydration, a solution of minimum essential medium and Hepes Buffer, supplemented with 100 IU/mL of penicillin and 100 IU/mL of streptomycin (Mediatech, Inc., Manassas, VA), and sodium pyruvate was prepared, but without bovine serum albumin (BSA). Air bubbles commonly form in BSA solutions during handling, which can be difficult to distinguish from the oocytes on the fiber filter while trying to locate them.

Each filter was immediately rehydrated after removal from the prescribed 16-hour RH treatment condition. The samples were placed in a well of a 6-well culture plate (Corning, Corning, NY), for convenience of organization, as shown in Figure 5. A volume of 500 µL of the rehydration media was slowly dropped onto the top side of the filters. Under the stereomicroscope, the GVs were quickly located and observed throughout a 5-minute rehydration recovery period, which

Figure 5. Filters in well plate during rehydration. Rehydration media is pipetted onto the filters (left), and allowed to hydrate the substrate, preservation material, and GV samples. Filters are viewed under a microscope to locate GVs on substrate (right).

was established to ensure the GVs became sufficiently hydrated to be handled without causing membrane damage.

The observed GVs behaved differently depending on which composition was used and the conditions they were subjected to for treatment. The trehalose control samples tended to adhere to the substrate for a longer period following deposition of the rehydration solution, while the trehalose with choline acetate released almost immediately. The other two salt additives, choline citrate and choline chloride, aided in sample release, but only modestly.

After the 5 minutes had elapsed, GVs were recovered from the filter substrate. Using the fine-tipped Stripper, oocytes that were on the surface of the filter or that had auto-released from the substrate and had been suspended in the rehydration media were removed. The recovered GVs were placed into a second, corresponding 6-well culture plate with a fresh droplet of rehydration solution to continue rehydration. After an initial search, if not all oocytes had been recovered, the filters were left to hydrate for up to 30 minutes. At the end of this 30 minutes, the filters were searched again, then agitated, using a specific procedure of flipping and folding the fiber filter to help release the oocytes with minimal force applied directly to the cells. Any additional oocytes were recovered and placed into the second droplet of media.

After the 30-minute period was completed, the cells were placed in a droplet of 4% paraformaldehyde to fix the cells and the enclosed GV, for further study, as shown in Figure 6 [37]. This fixation process forms bonds between amino acids in the cell, solidifying its structure for transport and further analysis [38]. The cells remain in this fixative solution for approximately 8 hours and are then transferred into individual tubes with 70% ethanol to dehydrate the samples in preparation for shipping to research partners at the Smithsonian Institute in Washington, D.C. [38, 39, 40, 41]. The tubes that were used resulted in adhesion of

cells to the walls, which was an unexpected consequence of using standard tubes for shipment. This problem had not arisen in previous studies, and due to the fixation process, was not expected to occur [5]. For the second phase of the study, lo-bind tubes were purchased for shipment, drastically lowering adhesion of the fixed oocytes to the tube (Corning, Corning, NY).

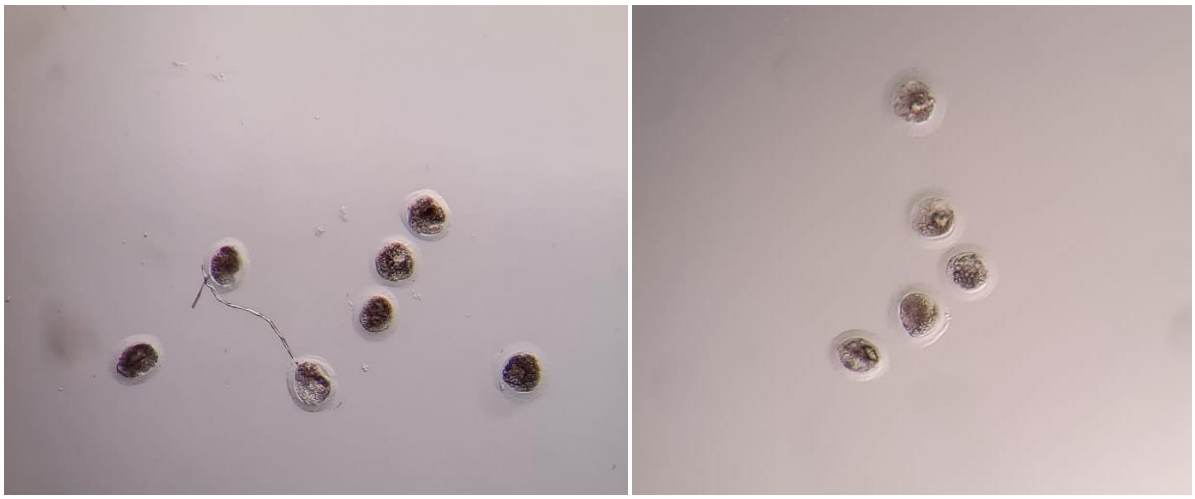


Figure 6. GVs before and after fixation. Immediately following recovery from substrate, the GVs are allowed to rehydrate in media for 30 minutes (left). After all processing is completed, the GVs within the oocyte membranes are fixed with a 4% paraformaldehyde solution (right).

3.9: TUNEL

DNA fragmentation was determined by a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using a cell death detection kit (Roche Applied Science, Indianapolis, IN). After rinsing off the ethanol used for shipping, GVs were permeabilized with 0.5% Triton X100 for 30 min at room temperature before being incubated in TUNEL reaction mixture for 1 h at 38° C. GVs were rinsed with PBS and mounted on slides with Vectashield containing DAPI (Vector Labs, Inc., Burlingame, CA). Each slide was examined with an epifluorescence microscope (Olympus BX41; Olympus Corporation, Melville, NY) using SPOT software 5.0 (Diagnostic Instruments, Inc., Sterling Heights, MI). TUNEL positive GVs, or GVs with identifiable DNA fragmentation, were indicated by the presence of green fluorescence. TUNEL

negative, or cells with no identifiable DNA fragmentation, had no green fluorescence and were instead tagged with the blue counterstain [42].

3.10: RAMAN SPECTROSCOPY

As in previous studies, Raman spectroscopy was used to differentiate between amorphous and crystallized samples [5]. This type of spectroscopy uses an excitation laser to cause vibrations in the molecules of the substance being scanned. Previous spectra were obtained on samples that had been dehydrated from a 1.1 M solution of trehalose in water, but otherwise methodology was similar. The measurements were conducted with a Horiba Xplora confocal Raman microscope with a 1200 g/mm grating. The excitation laser had a wavelength of 785nm. Data were taken using a 40X microscope lens. The laser power was measured at ~15 mW at full power. Hole size was set at 100 µm. All measurements were performed at room temperature. The acquisition time of each Raman spectrum was 10 s.

To create a glassy trehalose matrix, droplets of 40µL were placed on fused quartz microscope cover slides (Alfa-Aesar, Tewksbury, MA) and placed into 120°C oven to completely dry the sample. This resulted in amorphous samples of trehalose on microscope slides that could easily be scanned by the Raman microscope [5]. Standard glass was observed to have a photoluminescence that interfered with acquisition, so the use of fused quartz class was critical for this analysis. The amorphous control sample was transported to the Raman spectroscopy lab in a desiccant box, scanned to provide an amorphous control spectrum, then placed into a 76% RH environmental chamber (Caron, Marietta, OH) for approximately 8 hours until crystals had visibly formed in the material. The sample was again transported to the Raman spectroscopy lab and scanned, yielding the crystallized trehalose control spectra [32].

Using the typical bake-out method to generate a glassy trehalose and choline acetate composition control sample caused a color change of the sample (an orange tint), suggesting some possible transformation of the salt. These baked-out samples were highly photoluminescent, and thus any spectra that may have been present were obscured. Instead, microwave-assisted drying was used to avoid high temperature processing. A droplet of trehalose choline acetate solution was heated on a fused quartz cover slip in 5-minute increments until the sample had become solid, as observed from the outside, and as prodded by a blunt instrument on a parallel sample, yielding an amorphous control. Unlike the trehalose samples, when these compositions were exposed to high humidity, they rapidly absorbed water, moving through the super-saturation state quickly. Conditions that were favorable for crystallization were thus more challenging to obtain. Samples were instead dehydrated into the super-saturated state by heating the sample in 5-minute increments until a viscous skin formed over the droplet. These samples were held at room conditions of approximately 25°C and 55% RH for 60 minutes until crystals formed in the composition. Samples were stored in a desiccant box for transportation to the Raman laboratory for scanning to prevent the transition back to a liquid solution.

Once the spectra of amorphous and crystalline compositions were obtained, amorphous samples were created and exposed to ideal and adverse relative humidity conditions in a manner that paralleled the GV storage experiments. These conditions included storing the fiber filter sample in a sealed glass vial for an equilibration period following the microwave-assisted drying procedure, then exposing one of each of the compositions to the increased humidity environment of 76% RH. Following the humidity exposure period of 16 hours, the samples were placed in a desiccant box and transported for Raman spectroscopy analysis. The goal was to verify the state

of the preservation material in the GV-containing samples after exposure to the same conditions [30].

3.11: STATISTICAL CONTROL AND ANALYSIS

Due to the inherent variation between biological tissues and the possibility for variation between collection days, oocytes obtained from ovaries on any given day were pooled and then randomly assigned to treatments. When collecting the reproductive tracts for testing, all the ovaries were individually excised and pooled for further dissection. While harvesting and grading oocytes from the collected ovaries, all the oocytes from each day were pooled and held for the next step. No oocytes were assigned to treatments upon dissection. A typical collection day varied depending on the season, and phases 1 and 2 took place during the Summer and Fall, respectively. This resulted in an average collection day during phase 1 to yield approximately 10 ovaries, and between 4-6 oocytes per organ. Phase 2 took place during the Fall season, which resulted in a higher number of animals undergoing routine ovariohysterectomies. A typical collection day during phase 2 yielded approximately 20 ovaries, and the same 4-6 oocytes per organ.

Treatment and microwave carousel assignment was performed with the assistance of randomization tables. Table size was determined by the number of selected preservation solutions and the desired sample sizes. Table 2 shows the assignment of random numbers to each entry in the table, which were then ranked to determine assignment order. Each entry represents a replicate for a specific preservation solution group, and the rank order dictates the order that oocytes are assigned to solutions. The number of assignments per day varied, as the number of oocytes collected varied.

Random numbers generated				Rank Order			
0.380865	0.976385	0.643118	0.361219	12	1	7	13
0.017432	0.224637	0.313349	0.204577	24	20	15	21
0.495925	0.121697	0.260765	0.661448	8	22	17	6
0.020983	0.937511	0.410476	0.299924	23	3	11	16
0.42746	0.684529	0.314134	0.445207	10	5	14	9
0.71147	0.254556	0.234934	0.971651	4	18	19	2

Table 2. Random assignment table for preservation solutions. Each cell represents a replicate of a preservation solution during testing. Each column is a solution, and each row is a replicate. The randomly generated numbers were ranked, which gave an order for assignment to preservation solutions.

The randomization on the microwave carousel was performed in the same way, except using a single column for ranking order, shown in Table 3. These ranks were then assigned to preservation solutions in the same order that they were assigned from the previous table. The randomization of the microwave carousel placement aimed to help reduce bias from spatial variation that is inherent in microwave processing.

Random number	Rank Order
0.427700465	7
0.583348627	2
0.862257723	6
0.880053257	3
0.490550329	1
0.178294799	5
0.179228436	8
0.352821899	4

Table 3. Random assignment table for microwave carousel assignment. Each entry represents a position on the turntable. The randomly generated numbers were ranked, which gave an order for assignment to each location on the turntable.

Statistical analysis of each data set was performed using SPSS 27 (IBM, US, 2021) with a standard statistical significance value of $p=0.05$. For Aim I, the determination of drying times for samples, analysis of variance (ANOVA) was used followed by a Tukey-Kramer post hoc test to find differences. For Aim II, ANOVA was again used to find differences between compositions,

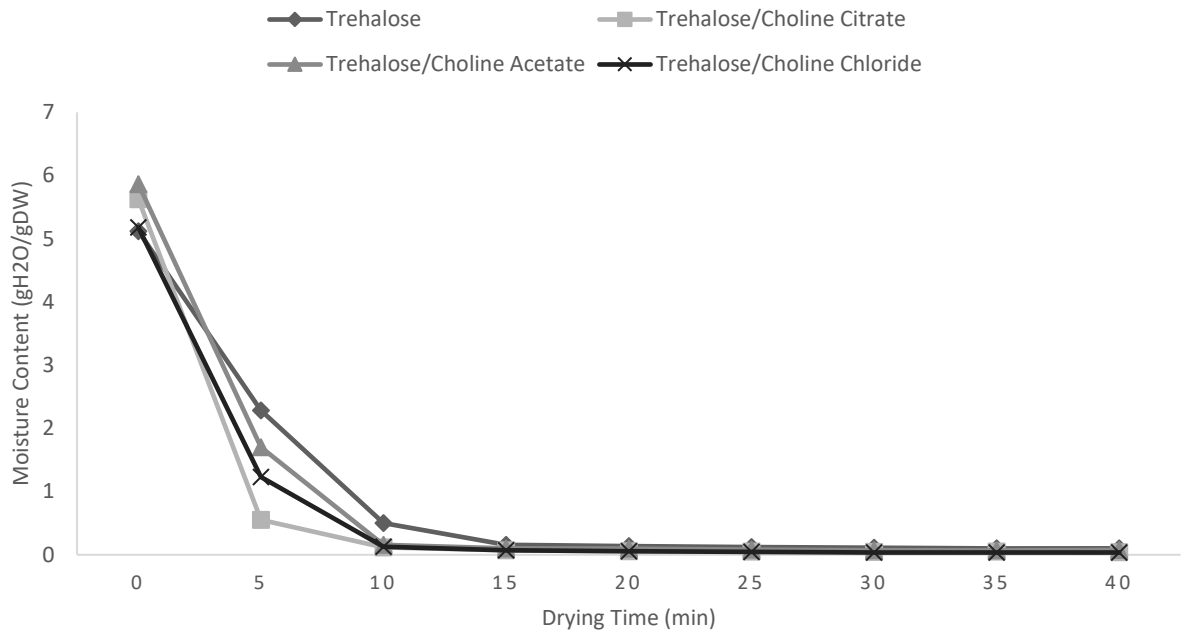
followed by paired t-tests to locate differences between selected pairs. Conclusions were drawn using a standard p-value of $p=0.05$.

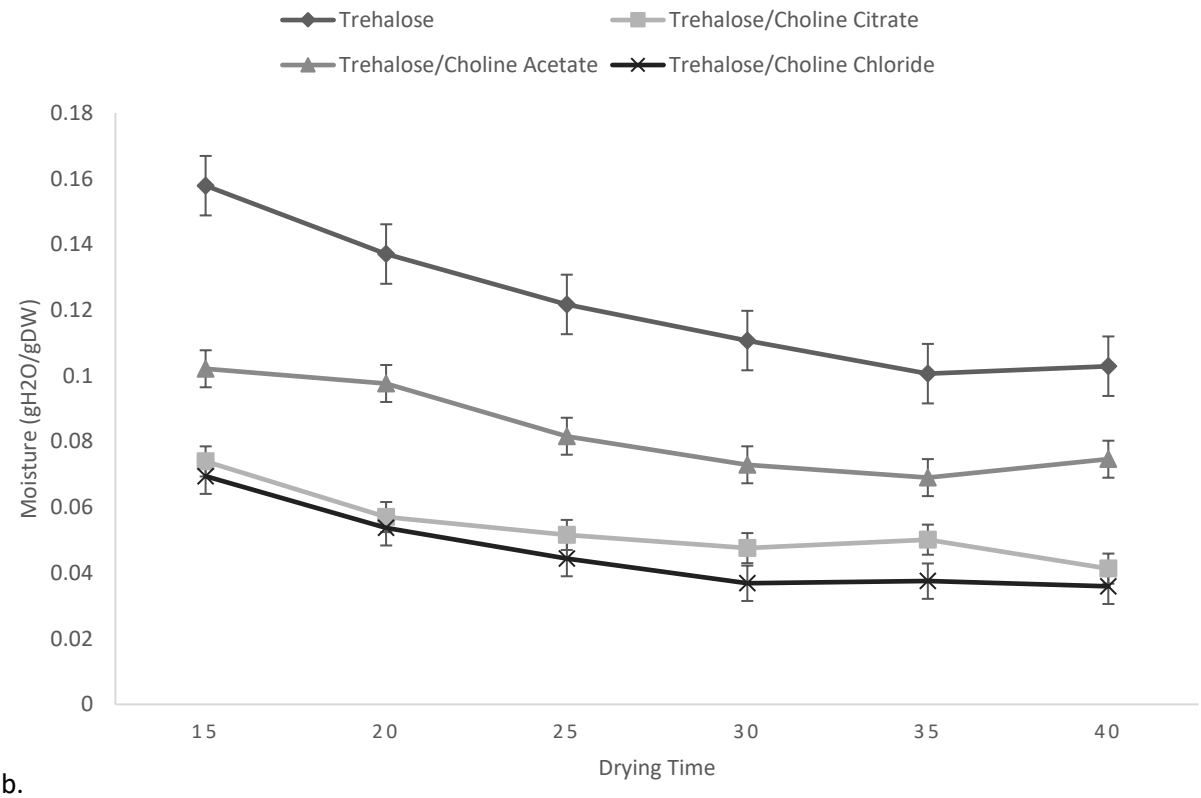
For Phase 2 of the study, one preservation solution was selected to test against the control trehalose material. This decision was made by qualitative analysis, trends, and statistical significance that was found in Phase 1, to increase the statistical power of the findings. Sample sizes for Phase 2 were calculated using a standard power analysis equation based on rehydration recovery data, using a standard p-value of $p=0.05$ and a desired power of 80%. The standard deviations of groups were not equal, so a pooling equation was used.

CHAPTER 4: RESULTS

4.1: THERMAL PROCESSING PARAMETERS

Aim 1 of the current study was to identify the moisture contents of samples dried for different times, establish a drying curve for the materials, then use this curve to prescribe drying schedules for the samples. Moisture content was determined by Karl Fischer titration, and a drying curve was generated for each composition as shown in Figure 7.





b.

Figure 7. Moisture content as a function of drying time with microwave assistance in a 11% RH environment as determined by Karl Fischer Titration. a) entire drying curve from 0 to 40 minutes b) later drying times only

Water content was determined by Karl Fischer titration, which is based on a stoichiometry equation using the mass of the sample, obtained by an attached balance, and the amount of titrant added to the system. Four replicates of each sample were combined and, using an analysis of variance test (ANOVA), the moisture content differences between each time point were compared. The drying timepoint which was significantly different from the last, but not the next, was considered as the ideal drying time, attaining an equilibrium moisture content, while subjecting the samples to the least amount of thermal processing. This point was determined for all materials, and the drying time of 30 minutes was selected as the appropriate drying time for the study. The previous study by Wang et al had determined, by use of one-way temperature sensors, that thermal processing of 30 minutes did not greatly exceed natural physiological temperatures, therefore was unlikely to cause thermal damage to the cells [5]. Throughout experimentation all drying procedures included a separate filter, loaded only with preservation solution, that was analyzed by Karl Fischer Titration to confirm that the expected level of drying was achieved.

4.2: REHYDRATION AND RECOVERY

4.2.1: PHASE 1

For Phase 1 of the current study, 4 different materials were tested, 1 control trehalose solution and 3 compositions with salt additives to the control solution. After the two-week holding period, the samples were subjected to treatments of either ideal (11% RH) or adverse (76% RH) conditions for 16 hours. As shown in Figure 8, it was observed that all preservation materials had a high fraction of oocyte recovery, or removal from their testing substrate, immediately following rehydration from both environmental treatments. In a previous study, oocytes dried in trehalose and stored at 11% yielded a recovery rate of 72.7 ± 12.8% after 8 weeks of storage [5].

In the control trehalose solution in this study, under ideal conditions (11% RH), the recovery rate was 91.6 ± 9.7%. This improvement in recovery rate in this study is attributed to a more thorough and defined protocol for recovery that was developed for the current study.

Even with the higher fractions of recovery across the board, differences could be resolved. For the control solution trehalose, the recovery rate from 76% RH, 70.6 ± 20.9%, was statistically lower than recovery from 11% RH ($p<0.05$). In previous studies it was observed that trehalose crystallizes under these humidity conditions, and it was hypothesized that crystal formation causes damage to the cells and their GVs. In general, in oocytes recovered from the 76% RH treatment, the membranes were less structured and occasionally resulted in separation of the GV from the membrane upon recovery, but the effect was much greater in the control composition. When choline acetate was added to the formulation the recovery was higher, an increase from 87% recovery to 95%, under adverse conditions (76% RH), but significance could not be demonstrated at the 5% confidence level ($p=0.059$). There were no other differences seen ($p>0.05$), either between humidity treatments within each preservation solution group, or between preservation solution groups at the same humidity level.

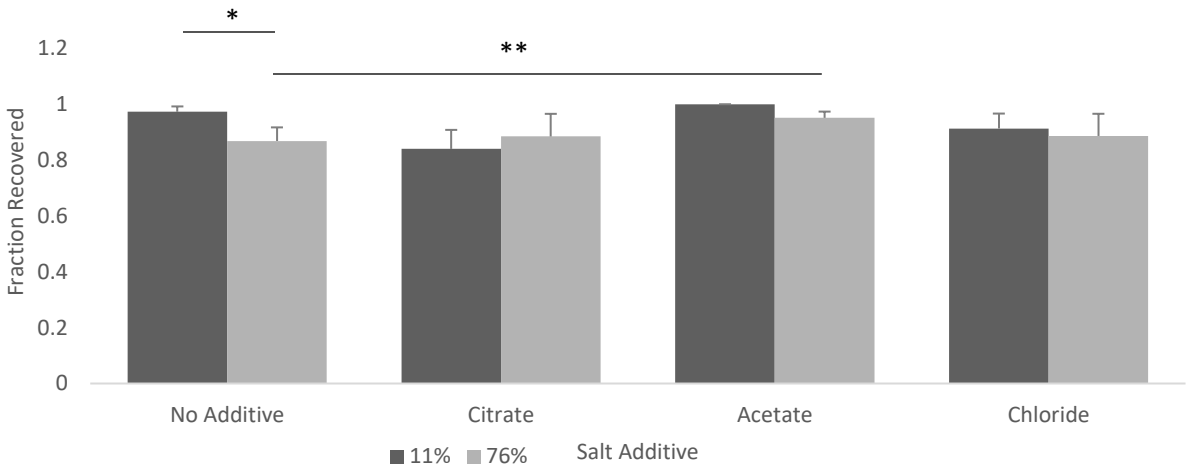


Figure 8. Recovery fractions after rehydration of stored GVs, Phase 1. The recovery fraction of samples in the control trehalose composition after 76% RH treatment was lower (*$p<0.05$) than after storage at 11% RH. The recovery fraction of samples with the choline acetate additive after storage in 76% RH was higher (**$p=0.059$) than those with the control solution under the same conditions, when tested with paired t-tests.

4.2.2: PHASE 2

Phase 2 was performed in the same manner, except with a higher sample size, increasing from six samples in each treatment group to ten, but with only one salt additive – choline acetate. Consistent with Phase 1, as seen in Figure 9, the recovery fraction of samples stored in trehalose in adverse conditions (76% RH) had a lower ($p<0.01$) recovery fraction than those under ideal conditions (11% RH), 70.6% when compared to 91.6%, respectively. Samples in the preservation solution with choline acetate added had higher ($p<0.01$) recovery fractions when compared to the control solution under adverse conditions (76% RH). No difference was observed ($p>0.05$) between the control preservation solution under ideal conditions and the preservation solution that included choline acetate as an additive under ideal conditions. These results are consistent with the results from Phase 1 of the current study, but with higher significance levels.

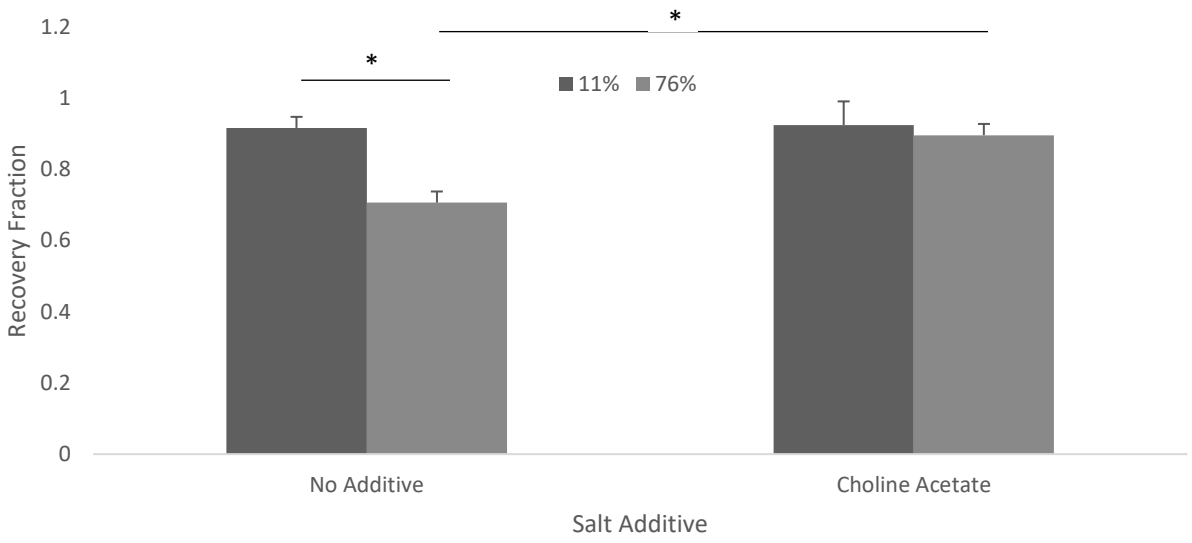


Figure 9. Recovery Fractions of GVs after rehydration, Phase 2. Recovery fractions of samples in the control trehalose composition were lower (*p<0.01) under adverse conditions (76% RH) when compared to ideal conditions (11% RH). Recovery fractions were higher (*p<0.01) for samples in the choline acetate additive composition when compared to the control under adverse conditions, tested with paired t-tests.

4.3: SHIPPING LOSSES

An unexpected challenge to overcome was the adhesion of fixed oocytes to the walls of the tubes used in shipping to the Smithsonian Institute. High losses were observed during shipping for Phase 1 of the study. Although we generally expect little to no losses of GVs during this processing step and thus typically do not look for differences between treatment groups at this stage, in this case the results were interesting and worthy of reporting. As seen in Figure 10, recovery after shipping was as low as 25 ± 7.93%, with the highest recovery of only 68.6 ± 24.6%. This limited the statistical power of the subsequent DNA analysis and warranted additional studies. However, this unexpected ‘adhesion stress’ did indeed stratify the experimental groups in a manner consistent with the hypothesis that the addition of organic salts to the trehalose formulation could improve overall recovery outcome under adverse conditions.

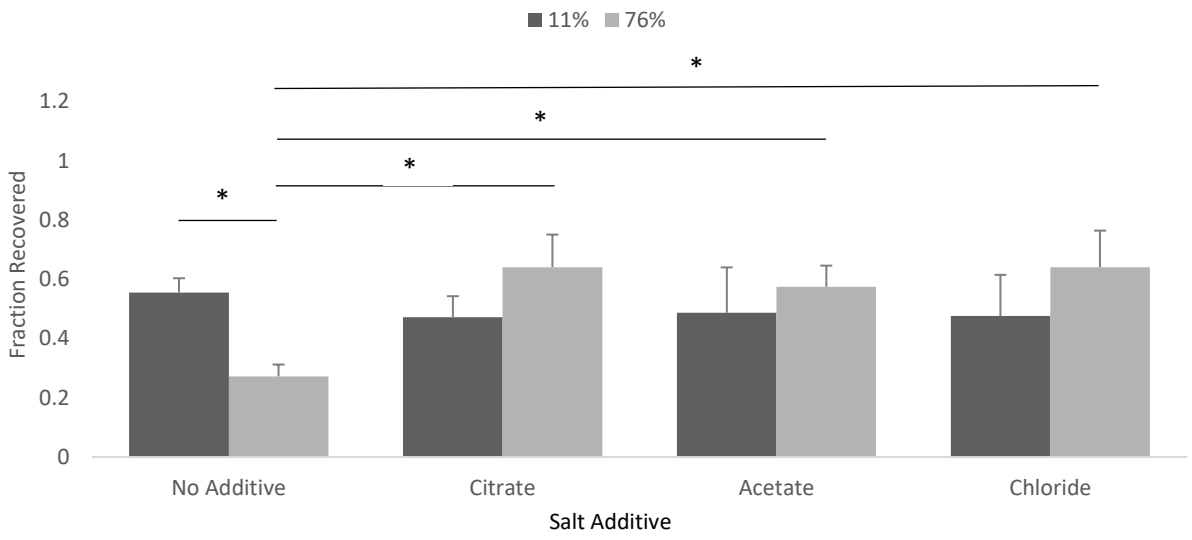


Figure 10. Recovery Fractions of GVs after shipping, Phase 2. Recovery of samples in the control trehalose composition were lower (*p<0.05) under adverse conditions (76% RH) when compared to ideal conditions (11% RH). Recovery fractions were higher (*p<0.05) for samples in all compositions that contained a salt additive when compared to the control under adverse conditions, tested with paired t-tests.

Even though the variation between sample tubes was high, and the average was low, at 52±25% retrieval from sample tubes, increased recovery fractions were observed in all preservation compositions with added salts compared to the control composition under adverse conditions ($p<0.05$). There was also a statistical difference between the treatment groups stored in the control trehalose composition, with the high humidity treatment yielding a lower recovery fraction ($p<0.05$). All compositions stored at 11% RH yielded the same recovery rate ($p>0.05$). Although all samples had been exposed to fixative at this stage, it is possible that the oocytes that experienced adverse humidity conditions during storage were more fragile and thus easily damaged by the adhesion event, contributing to additional losses during the shipping and sample retrieval process. The addition of salts was expected to suppress adverse crystallization, and thus reduce the damage that occurs during storage, a trend that manifested in this data as well, when extended to ‘handling’. This data, while unexpected, continues to support the hypothesis that

preventing crystallization with salt additives can improve the overall outcome of preservation processing.

Phase 2 of the study utilized lo-bind, protein coated tubes, which reduced sample loss to a minimum in all groups, and thus revealed no effects between treatments.

4.4: TUNEL ANALYSIS

4.4.1: PHASE 1

Analysis of DNA fragmentation was performed by a research associate at the Smithsonian National Zoo and Conservation Biology Institute. The samples that were sent overnight to Washington D.C. were tested with a TUNEL assay and were observed for positive or negative TUNEL results. A positive result indicates the presence of DNA fragmentation, at any identifiable level, and negative TUNEL indicates no DNA fragmentation. As observed in Figure 11, all samples exhibited high levels of DNA integrity. Furthermore, no differences ($p>0.05$) were found either between solutions or within solutions under different treatment conditions. These results, representing only the GVs that were recovered, are potentially confounded by the loss of samples during shipping.

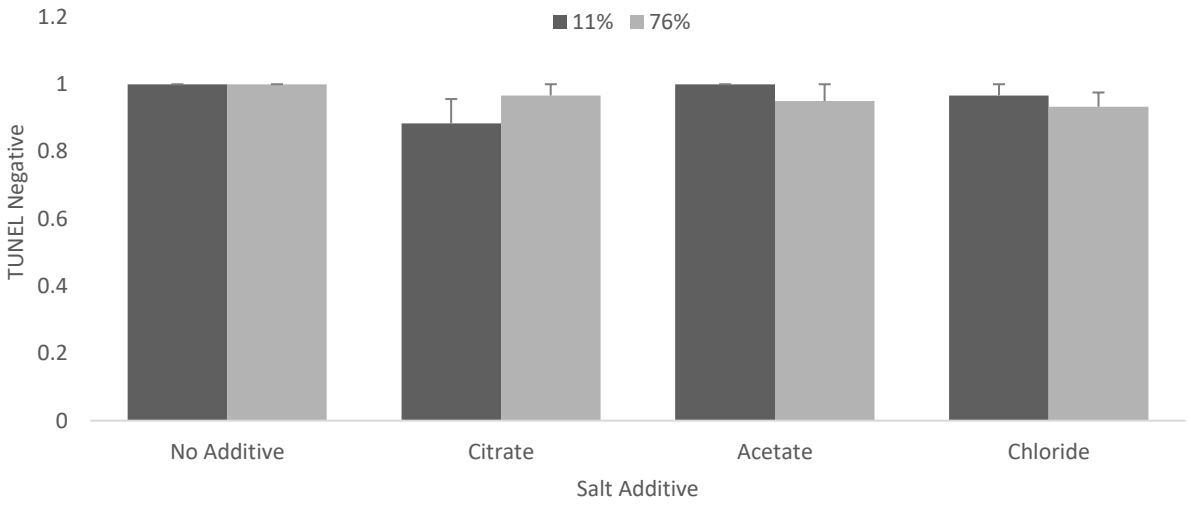


Figure 11. TUNEL Results. TUNEL results displayed as a fraction of TUNEL Negative results. Negative indicates no DNA fragmentation observed. No significant differences ($p>0.05$) were seen between compositions or humidity treatments.

4.4.2: PHASE 2

Samples were again sent overnight to research partners in Washington D.C., and with the use of the low-bind tubes, sample size remained high for testing purposes. As seen in Figure 12, no differences were found between either the compositions or the treatments. After the use of lo-bind tubes that were implemented and tested following Phase 1, the results of the Phase 2 TUNEL assays are more encompassing of the true effects of the treatments and preservation compositions. It can also be noted that while no increases of TUNEL negative results were seen, there were also no decreases in the TUNEL negative fraction of GVs. Consistent with prior research, there is no change in TUNEL negative fractions following dry preservation using trehalose and trehalose-based compositions [5].

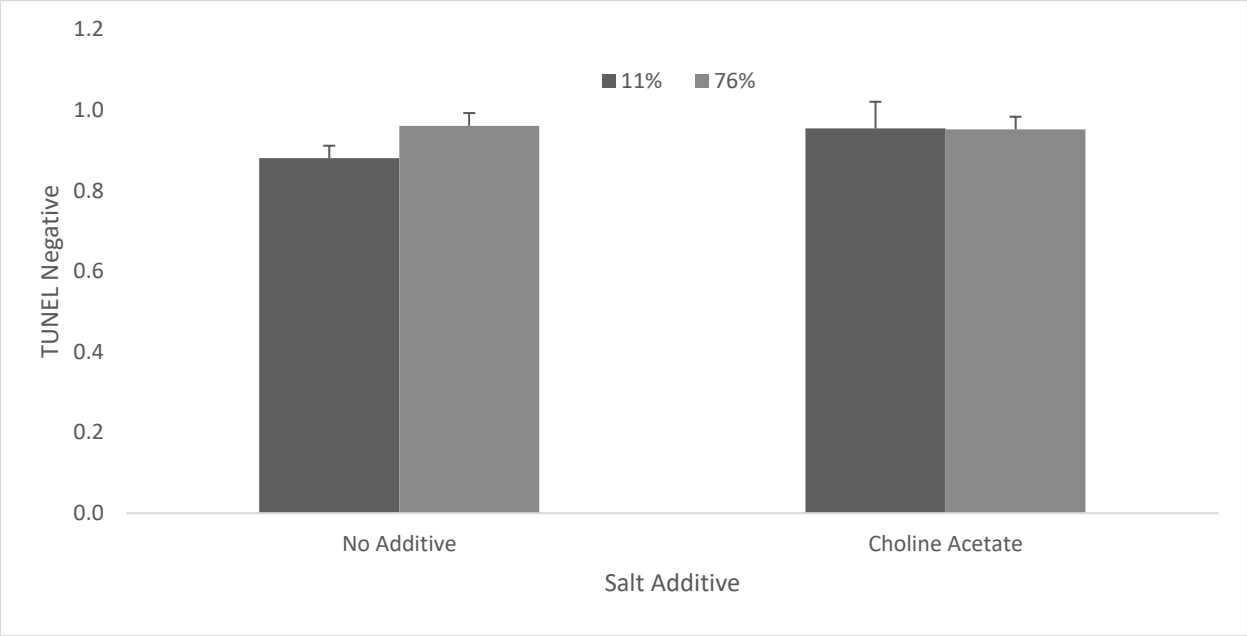


Figure 12. TUNEL results Phase 2. Displayed as a fraction of TUNEL negative results. Negative results indicate no DNA fragmentation seen. No differences ($p>0.05$) were seen between compositions or treatments.

4.5: RAMAN SPECTROSCOPY

Raman spectroscopy was performed in collaboration with a partner lab for Phase 2 of the study. As can be seen in Figure 13, differences between amorphous and crystalline conformations of trehalose can be clearly observed in the shapes of the spectra. The peaks are broadened and less distinct in the amorphous sample (c), compared to the crystalline sample (a). It can be observed that the sample material subjected to the adverse conditions of 76% RH (b) contains the same sharp and distinct peaks as the crystalline control sample, indicating that it is in fact crystalline following an adverse conditions challenge.

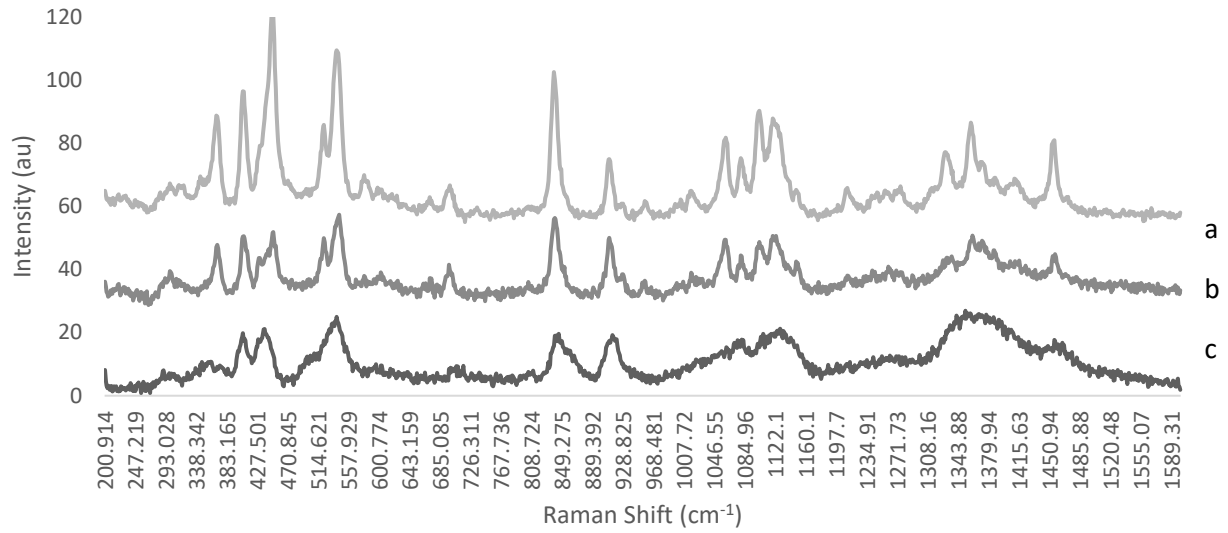


Figure 13. Raman Spectra of pure Trehalose. Sharper and more numerable peaks are associated with a crystalline conformation (a), compared to broadened peaks in the amorphous sample (c). The spectrum for samples held at 76 %RH is given in (b). Samples stored at this condition are consistent with crystalline samples.

The Raman spectra for trehalose-choline acetate compositions are shown in Figure 14. Again, peaks in the crystalline sample are much more intense and sharper than in the amorphous sample. Based on the spectra, the sample that was subjected to the adverse environmental conditions (76% RH) remained in an amorphous state with no evidence that crystals had been formed.

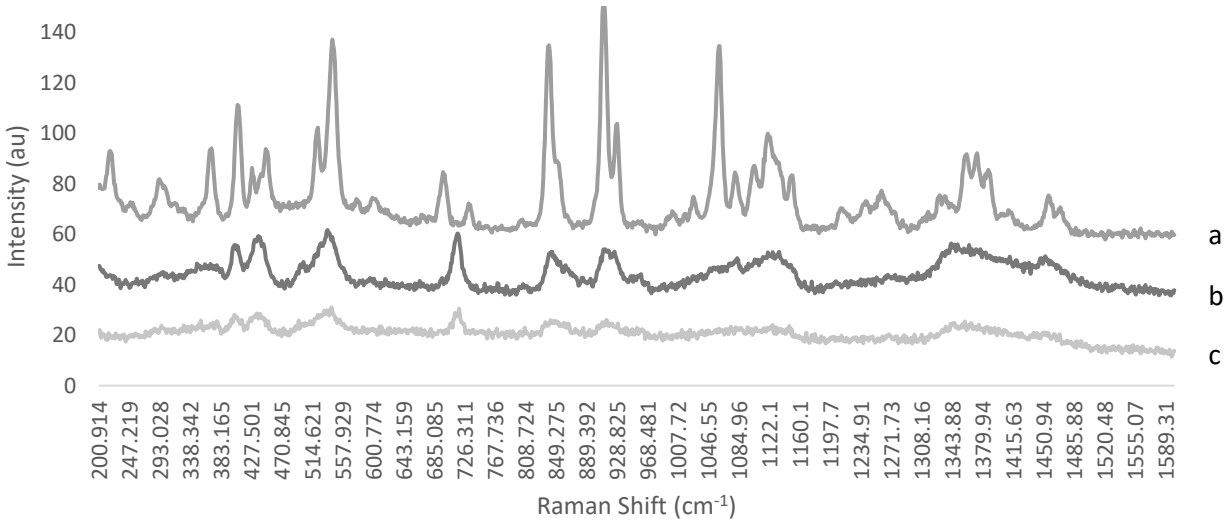


Figure 14. Raman Spectra of Trehalose with choline acetate. Sharper and more numerable peaks are associated with a crystalline conformation (a), compared to broadened peaks in the amorphous sample (c). The spectrum for samples held at 76 %RH is given in (b). Samples stored at this condition are consistent with amorphous samples.

CHAPTER 5: DISCUSSION

The goal of the current study was to explore novel formulations that could potentially outperform the standard trehalose solutions used in previous work, especially under adverse humidity conditions. In testing with multiple salt additives that were previously shown to delay crystallization, the selected salts were narrowed down as studies progressed [15]. The results of GV recovery from substrates and shipping losses that appeared to be affected by treatment illustrate that the current study has yielded an optimized a preservation composition that is more resilient to changes in environmental humidity and does not affect the preservation efficacy under ideal conditions. It is theorized that the salt additive, choline acetate, inhibits trehalose crystallization when exposed to high humidity conditions by ionic forces that interact with available water, and steric hindrance that prevents crystal formation. The absence of crystallization is hypothesized to prevent cell membrane and DNA damage. The salts are also thought to aid in water uptake during the rehydration process, which leads to the increased ease of handling during the rehydration and recovery process.

Using protocols that were established in previous work, water content measurements for samples during every 5 minutes of drying were obtained. Research by Elliott et al had determined that microwave processing for 40 minutes or longer resulted in damaged cell and DNA integrity, possibly induced by the additional microwave energy passing through the cells [22]. The current study established that 30 minutes was sufficient for all compositions. This is in line with previous research, which also utilized 30 minutes of microwave drying time in order to facilitate storage of the GVs in trehalose preservation solution [5]. Although each of the compositions had a different moisture content after drying, they all reached an equilibrium moisture content within 30 minutes, establishing the appropriate drying time.

In the first phase of the current study, 3 different salt additives were tested against a control trehalose preservation solution. All these salts were previously observed to prevent or delay crystallization in high humidity environments and were hypothesized to increase recovery after rehydration of preserved GVs by suppressing adverse crystallization events. In previous research the recovery fraction of samples in control trehalose material was 72.7 ± 12.8% after storage under ideal conditions (11% RH) [5]. Utilizing a redesigned protocol for uniform and thorough rehydration, the overall recovery rate was increased to 91.6 ± 9.7% in Phase 1 of the current study. Even with these high recovery fractions, lower ($p<0.05$) recovery was observed in the control material under adverse conditions (76%) when compared to ideal conditions in samples using the same composition. A modest increase ($p=0.059$) in recovery in the material that contained the choline acetate additive under adverse conditions when compared to the control material under the same conditions. This modest increase was a determining factor in choosing to move forward with additional testing in Phase 2 using only the choline acetate additive. Also noted, no decrease ($p>0.05$) in recovery in any of the salts under ideal conditions was observed when compared to the control material under the same conditions. This is important, as it indicates that addition of the salts to trehalose did not adversely affect GV recovery and integrity under ideal conditions. Although higher recovery under adverse conditions is a good for protecting against temperature and humidity excursions, if there is a decrease under ideal conditions, the material would be detrimental for normal use, making it a poor replacement for the control material.

Qualitatively, there was a handling improvement when using trehalose with a choline acetate additive. The oocyte membranes with GVs tended to release easier from the filter substrate during rehydration. This was observed during the initial 5 minutes of rehydration, before any

agitation was used to dislodge oocytes. Simply using small amounts of water jet pressure from the pipette caused the release of cells from the substrate, enabling them to float in the rehydration media and thus they were easy to locate. This made it easier to find and recover the cells, and because less agitation was needed to dislodge them, it limited mechanical damage. This observation was another determining factor in choosing to move forward with using the choline acetate additive in Phase 2.

With the sample mean and variability determinations from Phase 1, a power analysis was performed. This power analysis indicated that a sample size of 10 GVs per filter, and 10 sets of filters would adequately estimate the population means of the compositions. This new sample size was used to prepare samples during Phase 2, and the data that was obtained during the experiment reinforced previously seen trends. The control material showed lower ($p<0.01$) recovery under adverse conditions when compared to ideal conditions, as in Phase 1. The recovery of GVs in the preservation material that contained choline acetate under both adverse and ideal conditions was significantly higher ($p<0.01$) than the control material under adverse conditions. As in Phase 1, the recovery under ideal conditions was no lower ($p>0.05$) in the preservation material containing choline acetate than it was in the control material. This showed that under standard conditions, the new formulation behaved at least as well as the control material.

After fixation, the GVs were placed into 2mL volume centrifuge tubes with a 70% ethanol solution to dehydrate them. The samples were then sent by overnight delivery on ice. When the sample tubes reached Washington D.C. for the TUNEL analysis, many GVs were not recoverable. Under a microscope, it was observed that the oocyte membranes were adhering to the walls of the plastic tubes and were not releasing, causing a total loss of the GV. There was no

record that lo-bind tubes were used in previous protocols, and this was an unfortunate oversight in Phase 1 studies. Lo-bind tubes are plastic tubes, usually the same material as standard centrifuge tubes, but they are coated with a bovine serum albumin solution prior to packaging. This coating of protein solution helps to seal pores in the plastic and provide a non-stick surface for any samples that are stored within. Lo-bind tubes were utilized in Phase 2.

While this outcome was undesirable, interestingly the extent of sample loss appeared to be affected by the preservation material and treatment in a manner consistent with the hypothesis that the trehalose preservation material is improved by the addition of various salts. The lower ($p<0.05$) recovery of oocytes from samples stored in trehalose under adverse conditions (76% RH) when compared to ideal conditions is consistent with the hypothesis that crystal formation causes loss of oocyte and/or GV integrity. All salts showed increased ($p<0.05$) recovery rates under adverse conditions compared to the control material, and no lower ($p>0.05$) recovery under ideal conditions, which in this case would indicate that the salts helped prevent crystallization and membrane damage, therefore making it easier to recover the GVs together with mostly intact oocyte membranes from the shipping tubes.

TUNEL analysis was performed by research colleagues at the Smithsonian National Zoo and samples were tested for DNA fragmentation by a stain that binds a fluorescent marked molecule to the ends of DNA strands. Theoretically, all samples will have some fluorescence, as even an intact strand of DNA always has 2 ends. This method increases the fluorescence as the number of breaks increases, as more strand ends are created from the same piece. This study tested the presence or absence of above-threshold fluorescence only, and thus was a binary result.

Due to the inadvertent shipping losses, inferences from Phase 1 data were used only to frame the experimental design for Phase 2. However, trends did appear that give additional evidence

pointing the improvement of trehalose preservation solutions with salts. Samples that were tested immediately after hydration were handled very gently, yielding high recoveries across the board. Fixation and shipping may reveal damage and fragility that was not evident in the previous step. The observation that GVs in their oocyte membranes released from the plastic tube at a higher rate in compositions with salt additives is consistent with the hypothesis that the use of salts in the preservation solutions helps to maintain oocyte integrity during adverse conditions. Despite this outcome, because this adhesion problem likely leads to a bias towards collecting only 'good' intact oocytes from the tube, the TUNEL analysis must be interpreted accordingly. For example, it is possible that the samples that would have tested positive for DNA fragmentation were damaged to the point that they could not be removed from the tubes to be tested at all. For this reason, results from the initial Phase 1 TUNEL data must be interpreted in that context. Of samples that were recovered, all compositions performed equally well in preserving DNA integrity and there were no differences between treatments.

Phase 2 of the study was a refined iteration of Phase 1, reducing the salt additives being tested to only one, down from the original three. This allowed an increase the sample size, both in terms of the number of GVs on each filter substrate and the number of filter substrates that were used. Results of the TUNEL assay from the Phase 2 samples showed no differences between the preservation material compositions ($p>0.05$), and no differences between the treatment condition groups ($p>0.05$), with high DNA integrity across all samples. Under these conditions, the addition of the choline acetate additive resulted in a composition that performed as well as the control trehalose composition and did not induce any additional DNA damage.

Raman spectroscopy was performed in partnership with the Zhang Lab in the department of Computer and Electrical Engineering. Scans were taken of dehydrated materials used in Phase 2

of the study, in a manner similar to Raman analysis used by previous work [5]. Fused quartz coverslips were chosen as substrates for their low photoluminescence background. The trehalose samples were prepared by baking 40μL droplets of preservation solution in an oven at 120°C for 48 hours. This resulted in a glassy-state material that could be easily scanned. They were then placed into a high humidity environment to induce crystallization before scanning again. Due to the postulated degradation of the choline acetate that resulted in an orange tint in the dried sample, the preparation process differed for the composition containing the salt additive. Using the same fused quartz coverslips, the samples of the trehalose and choline acetate solution were dried using microwave assisted drying. This enabled drying of the control sample with minimal temperature increase to avoid the theorized salt degradation. These control samples on quartz slides, which could also be visualized by eye, established the amorphous and crystalline control spectra for both compositions.

For the test samples, glass fiber filters were used. The dehydrated filter substrates were treated in the same way as the oocyte samples, to accurately assess the material states in parallel experimentation. This included the drying process, the final equilibration in the sealed containers, and then the use of the environmental chamber to induce the adverse conditions. The spectra obtained confirmed the hypothesis that the salt additive prevented crystallization under 76% RH conditions. As observed in Figure 6, the sample of trehalose preservation material, under adverse conditions, reached a state of crystallization, confirmed by the congruency of the spectrum to the crystalline control spectrum. Conversely, in Figure 7 it is observed that the trehalose material with the choline acetate additive remained in the amorphous state after the induced adverse conditions. Raman spectroscopy is a way to analyze the conformation of a material, by its use of an excitation laser. The Raman microscope detects vibrations in the

molecules after application of the excitation laser. The more amorphous, therefore less ordered, the material is, the broader the spectrum peaks will be, an indication of large movements in the matrix, rather than small vibrations of molecules in a tight configuration. In a crystalline configuration, there is less space between the molecules, and therefore smaller vibrations are recorded. In this situation, the Raman microscope can detect vibrations of individual atoms within the molecules, displaying sharper, more numerous peaks. When observing the control trehalose material spectra (Figure 6), this can be easily observed around 1350 cm^{-1}. In the spectrum of the amorphous control material (c) a large "peak" that is very broad is observed, but when observing the same area in the crystalline control spectrum (a), there are 2 distinct peaks that are both narrower and more intense. These differences coordinate with the vibrations detected at the molecular level (c) that are large and slow – less intense – and the atomic level (a) that are very small and have a higher frequency – higher intensity on the graph. Using these spectra obtained from samples that were run parallel to the cell study, it is confirmed that under adverse conditions, the control material formed crystals while the material with the choline acetate additive did not. This would support the hypothesis that the salt additive prevents crystallization under the adverse experimental conditions.

FUTURE RESEARCH

The effectiveness of novel preservation compositions in germinal vesicle dry preservation were examined in this study, and it was determined that the addition of choline acetate to a trehalose preservation composition improved preservation outcomes. Continued study is necessary to refine methods of drying that can be more flexible depending on the environment. For example, in locations that are not in danger of naturally high temperatures, more gentle drying can be implemented. This can potentially decrease the amount of heat exposure that the GVs would be subjected to, allowing for greater recovery rates and decreased incidence of DNA fragmentation. These lower temperatures would allow sample water content to be slightly higher than our tested conditions, as the environment would prevent the composition exceeding the glass transition temperature at higher water contents. To better understand the state of the materials, and to predict behavior under various conditions, glass transition temperature studies should be undertaken for the studied compositions. These are labor intensive procedures that identify with high accuracy the glass transition temperatures of a composition at different water contents on a curve. Obtaining this data would allow for the prediction of T_g under different conditions, however, glass transition curves are obtained for a specific composition, so compositions using different solute concentrations would be subject to estimation without directly obtaining the T_g data again.

Further study investigating the ability of these materials to not only resist crystallization under high humidity conditions, but also high temperature conditions would be worthy of further study. As can be understood of the glass transition curve, the T_g is dependent on both water content and temperature. While temperature can normally be controlled much more easily than relative humidity in transportation and laboratory environments, it is still a variable that needs to be

controlled, and therefore studies into the resistance to crystallization under varying temperatures would be valuable.

The current study utilized the TUNEL assay to test for DNA fragmentation. This is a very coarse assay, as we are not able to determine the type of DNA damage that has been sustained, nor to what extent. The TUNEL assay allows for a positive/negative result only. The next step of this portion of research would be further analyzing the true extent and type of DNA damage that is sustained during the preservation process. A potential assay to evaluate this in greater detail would be the Comet assay, which can evaluate the relative number of single and double strand breaks in the DNA sample through the visual analysis of an agarose gel-coated slide. This would allow for a better prediction of the viability of the DNA to be used in the GV transplant process to an enucleated cell and further support the concept of dry preservation of genetic material. Also, in the current study, a limited amount of data was obtained pertaining to the chromatin configuration of the preserved GVs. This can also be paired with assays such as Comet to evaluate the efficacy of using the preserved genetic material to transplant to enucleated cells and eventually use for fertilization studies.

REFERENCES

[1] Raheja, N., Choudhary, S., Grewal, S., Sharma, N., & Kumar, N. (2018). A review on semen extenders and additives used in cattle and buffalo bull semen preservation. *Journal of Entomology and Zoology Studies*, *6*(3), 239-245.

[2] Albertini, D. F., & Olsen, R. (2013). Effects of fertility preservation on oocyte genomic integrity. *Oocyte Biology in Fertility Preservation*, 19-27.

[3] Schattman, G. L. (2015). Cryopreservation of oocytes. *New England Journal of Medicine*, *373*(18), 1755-1760.

[4] Meryman, H. T. (1971). Cryoprotective agents. *Cryobiology*, *8*(2), 173-183.

[5] Wang, S., Lee, P. C., Elsayed, A., Zhang, F., Zhang, Y., Comizzoli, P., & Elliott, G. D. (2021). Preserving the Female Genome in Trehalose Glass at Supra-Zero Temperatures: The Relationship Between Moisture Content and DNA Damage in Feline Germinal Vesicles. *Cellular and Molecular Bioengineering*, *14*(1), 101-112.

[6] Leibo, S. P. (2008). Cryopreservation of oocytes and embryos: optimization by theoretical versus empirical analysis. Theriogenology, 69(1), 37-47.

[7] Chen, T., Fowler, A., & Toner, M. (2000). Literature review: supplemented phase diagram of the trehalose–water binary mixture. *Cryobiology*, *40*(3), 277-282.

[8] Moynihan, C. T., Easteal, A. J., Wilder, J., & Tucker, J. (1974). Dependence of the glass transition temperature on heating and cooling rate. *The journal of physical chemistry*, *78*(26), 2673-2677.

[9] Capaccioli, S., & Ngai, K. L. (2011). Resolving the controversy on the glass transition temperature of water?. *The Journal of chemical physics*, *135*(10), 104504.

[10] Mathlouthi, M. (2001). Water content, water activity, water structure and the stability of foodstuffs. *Food control*, *12*(7), 409-417.

[11] Boothby, T. C., Tapia, H., Brozena, A. H., Piszkiewicz, S., Smith, A. E., Giovannini, I., ... & Goldstein, B. (2017). Tardigrades use intrinsically disordered proteins to survive desiccation. *Molecular cell*, *65*(6), 975-984.

[12] Hengherr, S., Heyer, A. G., Köhler, H. R., & Schill, R. O. (2008). Trehalose and anhydrobiosis in tardigrades–evidence for divergence in responses to dehydration. *The FEBS journal*, *275*(2), 281-288.

[13] Crowe, J. H. (2008). Trehalose and anhydrobiosis: the early work of JS Clegg. *Journal of Experimental Biology*, *211*(18), 2899-2900

[14] Simperler, A., Kornherr, A., Chopra, R., Bonnet, P. A., Jones, W., Motherwell, W. S., & Zifferer, G. (2006). Glass transition temperature of glucose, sucrose, and trehalose: an experimental and in silico study. *The Journal of Physical Chemistry B*, *110*(39), 19678-19684.

[15] Ziaei, S. (2018). *Retention of the Amorphous State of Trehalose at High Relative Humidity Using Organic Salt Additives: A Mechanistic Understanding* (Doctoral dissertation, The University of North Carolina at Charlotte).

[16] M.A. Mensink, H.W. Frijlink, K. van der Voort Maarschalk, W.L. Hinrichs, How sugars protect proteins in the solid state and during drying (review): mechanisms of stabilization in relation to stress conditions, European Journal of Pharmaceutics and Biopharmaceutics 114 (2017) 288-295.

[17] Bagheri, B. (2014). *Dynamic water sorption in trehalose-salt mixtures: Effect of composition on retention of the amorphous state* (Doctoral dissertation, The University of North Carolina at Charlotte).

[18] Patrick, J. L. (2016). *Towards the dry preservation of feline sperm: The effect of dehydration on DNA, centrosomal function, and embryo development* (Doctoral dissertation, The University of North Carolina at Charlotte).

[19] Diniz-Mendes, L., Bernardes, E., De Araujo, P. S., Panek, A. D., & Paschoalin, V. M. F. (1999). Preservation of frozen yeast cells by trehalose. *Biotechnology and Bioengineering*, *65*(5), 572-578.

[20] Pellerin-Mendes, C., Million, L., Marchand-Arvier, M., Labrude, P., & Vigneron, C. (1997). In vitrostudy of the protective effect of trehalose and dextran during freezing of human red blood cells in liquid nitrogen. *Cryobiology*, *35*(2), 173-186.

[21] Weng, L., Vijayaraghavan, R., MacFarlane, D. R., & Elliott, G. D. (2014). Application of the Kwei equation to model the Tg behavior of binary blends of sugars and salts. *Cryobiology*, *68*(1), 155-158.

[22] Elliott, G. D., Lee, P. C., Paramore, E., Van Vorst, M., & Comizzoli, P. (2015). Resilience of oocyte germinal vesicles to microwave-assisted drying in the domestic cat model. *Biopreservation and Biobanking*, *13*(3), 164-171.

[23] Lee, P. C., & Comizzoli, P. (2019). Desiccation and supra-zero temperature storage of cat germinal vesicles lead to less structural damage and similar epigenetic alterations compared to cryopreservation. *Molecular reproduction and development*, *86*(12), 1822-1831.

[24] Concannon, P., Hodgson, B., & Lein, D. (1980). Reflex LH release in estrous cats following single and multiple copulations. *Biology of Reproduction*, *23*(1), 111-117.

[25] Comizzoli, P., Wildt, D. E., & Pukazhenthi, B. S. (2008). Impact of anisosmotic conditions on structural and functional integrity of cumulus–oocyte complexes at the germinal vesicle stage in the domestic cat. *Molecular reproduction and development*, *75*(2), 345-354.

[26] Macklon, N. S., & Fauser, B. C. (2001). Follicle-stimulating hormone and advanced follicle development in the human. *Archives of medical research*, *32*(6), 595-600.

[27] Deegan, R. D., Bakajin, O., Dupont, T. F., Huber, G., Nagel, S. R., & Witten, T. A. (1997). Capillary flow as the cause of ring stains from dried liquid drops. *Nature*, *389*(6653), 827-829.

[28] Deegan, R. D., Bakajin, O., Dupont, T. F., Huber, G., Nagel, S. R., & Witten, T. A. (2000). Contact line deposits in an evaporating drop. *Physical review E*, *62*(1), 756.

[29] Weng, L., & Elliott, G. D. (2014). Determination of the relaxation characteristics of sugar glasses embedded in microfiber substrates. *Materials Science and Engineering: C*, *44*, 422-429.

[30] Kotula, A. P., Snyder, C. R., & Migler, K. B. (2017). Determining conformational order and crystallinity in polycaprolactone via Raman spectroscopy. *Polymer*, *117*, 1-10.

[31] Palonpon, A. F., Ando, J., Yamakoshi, H., Dodo, K., Sodeoka, M., Kawata, S., & Fujita, K. (2013). Raman and SERS microscopy for molecular imaging of live cells. *Nature protocols*, *8*(4), 677-692.

[32] Chakravarty, P., Bhardwaj, S. P., King, L., & Suryanarayanan, R. (2009). Monitoring phase transformations in intact tablets of trehalose by FT-Raman spectroscopy. *AAPS PharmSciTech*, *10*(4), 1420-1426.

[33] Klingström, T., Bongcam-Rudloff, E., & Pettersson, O. V. (2018). A comprehensive model of DNA fragmentation for the preservation of High Molecular Weight DNA. *BioRxiv*, 254276.

[34] Zeisel, S. H., & Blusztajn, J. K. (1994). Choline and human nutrition. *Annual review of nutrition*, *14*(1), 269-296.

[35] Lloret, J., Bolanos, L., Lucas, M. M., Peart, J. M., Brewin, N. J., Bonilla, I., & Rivilla, R. (1995). Ionic stress and osmotic pressure induce different alterations in the lipopolysaccharide of a Rhizobium meliloti strain. *Applied and environmental microbiology*, *61*(10), 3701-3704.

[36] Wood, T. C., & Wildt, D. E. (1997). Effect of the quality of the cumulus–oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro. *Reproduction*, *110*(2), 355-360.

[37] Hoetelmans, R. W., Prins, F. A., Cornelese-ten Velde, I., van der Meer, J., van de Velde, C. J., & van Dierendonck, J. H. (2001). Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission and scanning electron microscopy. *Applied Immunohistochemistry & Molecular Morphology*, *9*(4), 346-351.

[38] Eltoum, I., Fredenburgh, J., Myers, R. B., & Grizzle, W. E. (2001). Introduction to the theory and practice of fixation of tissues. *Journal of Histotechnology*, *24*(3), 173-190.

[39] Hopwood, D. (1985). Cell and tissue fixation, 1972–1982. *The Histochemical Journal*, *17*(4), 389-442.

[40] Smit, J. W., Meijer, C. J. L. M., Decary, F., & Feltkamp-Vroom, T. M. (1974). Paraformaldehyde fixation in immunofluorescence and immunoelectron microscopy: Preservation of tissue and cell surface membrane antigens. *Journal of Immunological methods*, *6*(1-2), 93-98.

[41] Hare, D. J., George, J. L., Bray, L., Volitakis, I., Vais, A., Ryan, T. M., ... & Finkelstein, D. I. (2014). The effect of paraformaldehyde fixation and sucrose cryoprotection on metal concentration in murine neurological tissue. *Journal of Analytical Atomic Spectrometry*, *29*(3), 565-570.

[42] Negoescu, A., Lorimier, P., Labat-Moleur, F., Drouet, C., Robert, C., Guillermet, C., ... & Brambilla, E. (1996). In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. *Journal of Histochemistry & Cytochemistry*, *44*(9), 959-968.