CRYOPRESERVATION OF *DANIO RERIO*: DETERMINING THE EMBRYONIC STAGE OF DEVELOPMENT AND CRYOPROTECTANT THAT YIELDS THE HIGHEST HATCHING RATE

A Report of a Senior Study

by

Amanda Brooke Cummings

Major: Biology

Maryville College

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Date approved _____, by _____

Faculty Supervisor

Date approved _____, by _____

Division Chair

ABSTRACT

Cryopreservation of human embryos is optimal at the blastocyst stage, but it is unknown which developmental stage and which cryoprotectant promote successful cryopreservation of the zebrafish *Danio rerio*. For this experiment, *Danio rerio* embryos were collected and cryopreserved (n=564) at six different developmental stages in four different cryoprotectants. Stages used were: zygote (n=53), cleavage (n=102), blastula (n=85), gastrula (n=120), segmentation (n=96), and pharyngula (n=108), whereas cryoprotectants used were 1.5M DMSO/.01M sucrose, 10%DMSO, 10%glycerol, and 15% mixture of ethylene-glycol/sucrose/ficoll$_{400.}$ Of the stages, 1.67% of the gastrula stage embryos hatched, 28.70% of the pharyngeal stage hatched, and 0% of the remaining stages hatched. Chi-Square test of independence showed there was a significant difference in the success rates among the developmental stages (P-value < 0.0001), and Tukey analysis from one-way ANOVA showed the only stage significantly different from the others was the pharyngeal stage (P-value < 0.0001). Chi-Square test of independence was also used to determine there was no significant difference in success rates for the different cryoprotectants. The null-hypothesis that there would be no significant difference in the developmental stages success rates was rejected; however, the null-hypothesis that there would be no significant difference in the cryoprotectants success rates was supported. Therefore, the optimal stage to cryopreserve *Danio rerio* embryos is the pharyngeal stage, and any of the cryoprotectants can be used for cryopreservation

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CHAPTER I

INTRODUCTION

Assisted Reproductive Technologies

Assisted reproductive technologies (ART) are methods used to increase an individual's chance of successfully conceiving. Some of these techniques include In-vitro fertilization (IVF), Intracytoplasmic sperm injection (ICSI), intra uterine insemination (IUI), surrogacy, and cryopreservation of sperm, oocytes, and embryos.

Assisted Reproductive Technologies Prevalence

In the United States, 6.7 million women ages fifteen to forty-four have impaired fecundity (inability to conceive or carry a child full-term; this accounts for 10.9% of women in this age group (Thoma et al. 2014). Also, 1.5 million of married women between the ages of fifteen and forty-four are infertile, has not conceived after twelve consecutive months of unprotected sex, accounting for six percent of married women in this age group (Thoma et al. 2014). As a result, 7.4 million women in the United States between the ages of fifteen to forty-four have used assisted reproductive technologies (Thoma et al. 2014). These statistics show a need for efficient ART methods for women. However, women are not the only individuals who struggle with infertility issues. In 2002, a CDC study discovered 7.5% of men younger than forty-five years of age visited a fertility doctor; this is 3.3 to 4.7 million

men. Of these individuals, eighteen percent were diagnosed with a male-related infertility problem (Chandra et al. 2014). In total, twenty-five to thirty-five percent of couples have infertility due to the male; fourteen to twenty-two percent of couples have infertility due to a tubal factor; ten to twenty-seven percent is due to ovulatory dysfunction; ten to seventeen percent is unexplained; and five to six percent is due to endometriosis (see Table 1, Maheshwari et al. 2008).

Table 1: Representation of infertility causes with percentage of occurrences for females and males (Maheshwari et al. 2008).

Gender	Cause	Percentage
Female:	Tubal Factor	14 – 22
	Ovulatory dysfunction	10 – 27
	Unexplained	10 – 17
	Endometriosis	5 – 6
Male:	Male factor	18

Increased Need for Assisted Reproductive Technologies

In developed nations, many women are postponing having children until they are in their thirties and forties (Mills et al. 2011). This is an issue because at the age of twenty-five women's fertility declines, and the decline accelerates during the mid-thirties (Mills et al. 2011). Moreover, sterility increases from one percent at age twenty-five to five percent at age thirty-five, seventeen percent at age forty, and fifty-five percent at age forty-five (Mills et al. 2011). However, older individuals are less likely to have a live birth; so of these percentages, about twenty percent of the women will have a spontaneous abortion (Leridon 2008). It has been suggested that older women may be more likely to have unexplained infertility due to

the negative affect of age on the ovarian reserve (Gleicher et al. 2006). One way in preventing these issues is by using cryopreservation to preserve eggs before the age in which egg viability decreases.

Cryopreservation

Cryopreservation is a technique used to freeze biological material, specifically living cells or living tissues, to below zero temperatures (-196^{o}C) using liquid nitrogen in order to prevent damage caused from chemicals or time (Yang et al. 2016). Because the biological material is at such a low temperature, morphological, physiological, biochemical, and genetic properties remain unchanged because metabolic activities halt. This prevents cell death and deoxyribonucleic acid (DNA) degradation, and allows the biological materials to be preserved and unchanged for long periods of time (Yang et al. 2016). Cryopreservation is used for both clinical and experimental reasons. Clinical applications of cryopreservation include organ transplants, stem cell transplants, and fertility treatment/procedures (Zhang et al. 2011). The organ transplant applications of cryopreservation are used to determine prolonging the amount of time the organ can be out of the body and be viable once transplanted into the recipient is possible; this has been accomplished in smaller mammals (Zhang et al. 2011), whereas stem cell transplant applications of cryopreservation are used to freeze stem cells so they can be transferred in if a disease occurs to repair, or slow damaged/diseased tissue (Hennes et al. 2015).

The majority of medical uses of cryopreservation are cryopreservation of sperm, oocytes, and embryos. A few reasons one would cryopreserve sperm are prior to cancer treatment (chemotherapy and radiotherapy) to store healthy sperm for later use and for sperm donation. A few examples of why one would cryopreserve oocytes are also prior to cancer

treatment, to preserve healthy eggs to use later, to donate oocytes, and if one disagrees with freezing an embryo. Since 1990, cancer survivors in young adults increased from one in one thousand to one in two-hundred fifty; this is increasing the use of cryopreservation to store gametes substantially (Ayensu-Coker et al. 2013). Reasons one would cryopreserve an embryo (fertilized egg) are if embryos are left over after IVF and for embryo donation.

History of Cryopreservation

In the early 1900s, attempts to cryopreserve of human spermatozoa was determined to be almost impossible, this was until finding that dehydrating cells in sugar prior to freeing them had a beneficial affect (Gook 2010). In 1949, it was accidentally discovered that the glycerol had cryoprotective properties for human sperm; this study also identified a balance between protection and toxicity with using glycerol and other cryoprotectants (Gook 2010). Post thaw motility as a marker of sperm function was an advancement in gamete cryobiology, and soon after this discovery the first birth following cryopreserved sperm was accomplished. (Gook 2010). In the 1950s, glycerol was used in attempts to cryopreserve oocytes, little success came from this. In the 1960s, experiments were carried out to determine the physiology of water movement in the cell, this set the foundation for the future cryobiology (Gook 2010). Successful preservation was only reported using fertilized mouse oocytes in 1977 (Moussa et al. 2014). After determining dimethylsulphoxide (DMSO) was a worthy cryoprotectant, mouse, rat, hamster, rabbit, and primate oocytes were successfully cryopreserved (Gook 2010). In 1983, the first live birth following cryopreservation of a blastocyst (a stage of the developing fetus) occurred (Pavone et al. 2011), and in 1986 the first pregnancy following slow freezing and rapid thawing of human oocytes using DMSO as the cryopreservation occurred (Gook 2010).

Limitations of Cryopreservation

Even though cryopreservation is an advancement for many medical purposes, there are some limitations including ethical issues, access to care, cost of services, and ice crystal formation of frozen embryos (Ayensu-Coker et al. 2013). Religious views vary widely, but matters concerning masturbation deter some individuals' ability to use cryopreservation in order to store semen (Ayensu-Coker et al. 2013). Another ethical issue surrounding cryopreservation stems when adolescents are diagnosed with cancer. Failure to communicate the risk of infertility, and providing options to preserve fertility is required according to the Human Rights Act of 1998 (Ayensu-Coker et al. 2013). However, an issue arises when a child has reached puberty and is able to ejaculate; the reasonableness of allowing the minor access to pornography to achieve ejaculation for semen collection has to be carefully accessed and is seen as an ethical issue (Ayensu-Coker et al. 2013). Alternatives such as electrostimulation and mechanical extraction are available, however they are costly, painful, invasive, and sometimes deemed unnecessary when semen can be obtained through masturbation (these measures are taken for pre-pubertal males) (Ayensu-Coker et al. 2013). Cost barriers also limit access to cryopreservation; in the United States, no state laws or regulations require insurance to cover fertility preservation methods for diagnosed cancer patients (Ayensu-Coker et al. 2013). The average cost for oocyte cryopreservation in 2010 was $10,419 (Van Loendersloot et al. 2011).

Obstacles of Cryopreservation

There are many obstacles to overcome for cryopreservation because there is still a reduced pregnancy success rate using cryopreserved gametes and embryos compared to fresh gametes and embryos. Therefore, there are more improvements needed in gamete and

embryo cryopreservation (Moussa et al. 2014). These obstacles arise from cryopreservation techniques and are different types of cryoinjuries, such as chilling injury, ice crystal formation, fracture damage, multiple aster formation, and osmotic stress (Moussa et al. 2014). Chilling injuries are caused from irreversible changes in lipid droplets, lipid-rich membranes, and microtubules of the mitotic or meiotic spindle; these occur most often during slow freezing techniques. Moreover, vitrification is beneficial for embryos and gametes that have excessive amounts of lipid droplets (Moussa et al. 2014). Ice crystal formation occurs in the medium the cells are suspended in, the nucleus, and in the cytoplasm between negative-five degrees Celsius and negative-eighty degrees Celsius. This can be avoided using vitrification, which has cooling rates between two thousand and twenty thousand degrees Celsius (Moussa et al. 2014). Fracture damage is caused by the mechanical effect of solidified solution, especially in larger cells such as oocytes and embryos. Fracture damage occurs between negative-fifty degrees Celsius and negative-one-hundred and fifty degrees Celsius (Moussa et al. 2014). Multiple aster formation occurs during vitrification. Because cells are exposed to highly concentrated cryoprotectant agents and fast cooling rates, formation of asters (has microtubules that orientate the mitotic spindle apparatus) near the pronucleus is induced. This slows the first cleavage event (Moussa et al. 2014). Osmotic stress occurs from cells shrinking when water is removed due to the different osmotic pressure from the intracellular and extracellular solutions. Because oocytes and embryos are more permeable to water, frozen cells will swell if placed directly into a medium without cryoprotectant agents after thawing. These changes cause damage to the cytoskeleton and zona pellucida. However, using cryoprotectant agents reduces the chance of osmotic stress (Moussa et al. 2014). Because cryopreservation is a relatively new technique, studies need to

be continued to increase success rates by determining the complications that arise intracellularly for cryopreserved cells (Smith et al. 2004). Further research should focus on protein structure and function correlation to gene expression, protein translation, intracellular trafficking, epigenetic modifications, and cellular development. This information should be gained using cell lines, somatic cells, gametes, and embryos (Smith et al. 2004).

Methods of Cryopreservation

Two methods have been used to cryopreserve gametes or embryos: slow freezing and vitrification. Vitrification is a faster method of cryopreservation that takes less than ten minutes, whereas on average slow freezing, on average, takes more than three hours. Vitrification is inexpensive and does not require a machine. Slow freezing is expensive and requires a freezer. The sample volume for vitrification is one to two microliters, while slow freezing sample volume is one-hundred to two-hundred and fifty microliters. Vitrification lowers the risk of ice crystal formation, slow freezing does not. Vitrification has less mechanical damage compared to slow freezing. However, slow freezing has less chemical damage than vitrification. Vitrification works on opened and closed systems, while slow freezing only works on closed systems. Vitrification requires a higher cryopreservation agents than slow freezing. Slow freezing result in low survival and low implantation rates. Slow freezing can also cause spindle abnormalities (Moussa et al. 2014). Vitrification was invented by Rall and Fahy, and is now widely used to cryopreserve human gametes, lab and domestic animals, and mammal embryos as it is much more successful than slow freezing (Moussa et al. 2014).

Humans

Long term storage of sperm can be achieved at -196^{o}C because there is no biochemical activity, not enough thermal energy for chemical reactions to take place, and there is no liquid water which is essential for metabolic activities (Moce et al. 2016). Slow freezing of sperm occurs by combining the semen with the cryoprotective agents (CPAs), glycerol and egg yolk, at room temperature to allow the sperm to survive at low temperatures. This solution of sperm are left at room temperature for about thirty minutes to equilibrate with the CPAs. Next, sperm is loaded into cryovials. Different procedures of optimal freezing protocol for human sperm can be accomplished. Freezing is achieved using liquid nitrogen vapor. If sperm are cooled slowly, they will dehydrate and the chance of ice formation is decreased. The cooling rate depends on the CPA concentration. After freezing, the sperm are stored in liquid nitrogen at -196^{0}C. Thawing can be achieved at different rates. However, warming rates depend on the freezing rate, type of container used to load the sperm, and on the CPA concentration; the sperm are usually thawed at thirty-seven degrees Celsius or room temperature. After thawed, the freezing diluent is removed before the sperm is used. To diminish osmotic stress to the sperm, the sperm should be diluted gradually in several steps with enough time allotted for the sperm to equilibrate to each osmotic condition (Moce et al. 2016). For vitrification, water solidifies as a glass-like structure instead of ice. Vitrification process is more efficient because little damage to the sperm, minimal equipment, and little time is needed. Concentrations up to thirty percent to fifty percent of CPAs are used, and sample volumes are small. Because sperm does not tolerate high concentrations of CPA, vitrification has not been accomplished for cryopreserving sperm (Moce et al. 2016).

Cryopreservation of human oocytes can be accomplished using a slow freezing and rapid thawing method. Freezing and thawing solutions that can be used are 1,2-propanediol (PROH) and sucrose as the CPA. The oocytes are washed in one vial at room temperature. Next, the oocytes are equilibrated for ten minutes in another vial. Then, the oocytes are transferred into a third vial for no longer than one minute. One to three oocytes should be loaded into a plastic straw and transferred to a vertical freezer. To start cooling, the chamber temperature should be from twenty degrees Celsius to negative-seven degrees Celsius at a rate of two degrees Celsius per minute. Seeding can be induced at negative-seven degrees. After ten minutes at negative-seven degrees Celsius, the straws should be cooled to negative-thirty degrees Celsius with a rate of 0.3 degrees Celsius per minute, followed by rapid cooling to negative-one-hundred and fifty degrees Celsius with a rate of fifty degrees Celsius per minute. To complete freezing, the straws should be transferred for storage in liquid nitrogen. To thaw, remove the straws from liquid nitrogen maintaining them in the air for thirty seconds. Next, submerge into a water bath at thirty degrees Celsius for forty seconds. Wash the oocytes in a four step dilution procedure at room temperature to remove CPAs. First at five minutes, then five minutes again, followed by ten minutes, and finally twenty minutes. Culture the oocytes at thirty-seven degrees Celsius and five percent carbon dioxide for two hours (Fadini 2009). Cryopreservation of human oocytes can also be accomplished via vitrification. To cryopreserve human oocytes, glycol, PROH, and sucrose can be used as CPAs, with cryoleaf as a carrier. The cooling procedure consists of four steps at room temperature. First, oocytes should be incubated in a drop of equilibration medium with a flushing medium for three minutes. Second, twenty-five microliters of equilibration medium should be added to the first drop with oocytes and left for three minutes. Third, oocytes

should be transferred into a new drop of pure equilibration medium. Exposure time in the equilibration drop should be no more than nine minutes to allow expansion of the cell to its original volume. Fourth, oocytes should be moved to the surface of vitrification medium at room temperature and washed for one minute. Oocytes should then be loaded onto the cryoleaf tip using about two microliters of vitrification medium, then plunged directly into liquid nitrogen. No more than two oocytes should be placed on each cryoleaf. Fix the cap on the cryoleaf and place in liquid nitrogen for prolonged storage. To warm, a vitrification warming kit with stepwise dilution of cryoprotectants can be used. Immerse the cryoleaf into a box filled with liquid nitrogen where the protective cap is removed and it is still submerged in liquid nitrogen. Immerse the cryoleaf into thirty seven degree Celsius warming medium for one minute at room temperature. Wash the oocytes in dilution medium for three minutes, in the second dilution medium for another three minutes. Next, wash twice the washing medium for five minutes at room temperature. To complete the warming process, place them on the warmer plate, set to thirty-seven degrees Celsius, for five minutes (Fadini 2009).

Cryopreservation of human embryos can occur at the pronuclear, cleavage, and blastocyst stages. It is beneficial to cryopreserve at the pronuclear stage because of the absence of a spindle apparatus in the single celled pronucleate embryo; this increases protection from damage occurring at the freezing process (Surrey et al. 2010). Comparative experiments showed higher survival and birth rates after transfer using cryopreserved pronucleate embryos compared to cleavage-stage embryos (Senn et al. 2000). A disadvantage of cryopreserving at the pronuclear stage is the limitation of information on the embryos developmental potential and morphology (Surrey et al. 2010). A study completed in 2004 found that cryopreservation of pronuclear stage using propanediol/sucrose as the

cryoprotectant a seventy-five percent survived thawing which lead to a twenty-six percent pregnancy rate; with a seventy-two percent survival rate for cryopreserved pronucleate embryos which were the result of ICSI, and an eighty-one percent survival rate for cryopreserved pronucleate embryos where oocytes were inseminated using a sterile petri dish (Marrs et al. 2004). It is beneficial to cryopreserve at the blastocyst stage because the cytoplasmic ratio is high with more cells present (Al-Azawi et al. 2013). Another benefit for cryopreserving blastocysts is that embryo selection can occur due to the ability to assess morphology after genomic activation; this shows developmental potential which ultimately results in increased success rates (Surrey et al. 2010). Yet, experiments comparing cryopreserving embryos at the blastocyst stage with other stages only use the embryos that were selected based off of highest quality (Surrey et al. 2010). This skews the data because the embryos more than likely are not chosen at random, excluding the low quality embryos. However, one study that did not discriminate against the different embryos based off of morphology found that cryopreserving embryos at the blastocyst stage lead to higher implantation and live birth rates compared to those cryopreserved at the pronuclear stage; 32.1 percent of individuals who used cryopreserved embryos at the pronuclear stage did not conceive compared to only 6.5 percent of individuals who used an embryo cryopreserved at the blastocyst stage (Surrey et al. 2010). Although this study did support that cryopreserving at the blastocyst stage yields in increased success rates, many individuals prefer cryopreservation at the cleavage stage because of inconsistent results with blastocyst cryopreservation. To slow freeze a human embryo, a programmable controlled rate – freezing machine can be used. The embryos can be cryopreserved using PBS with 20% HFF, 0.2 mol/l sucrose and fifteen mol/l EG or PROH. The embryos should next be exposed to

increasing concentrations of EG or PROH (0.5, 1.0, 1.5 mol/l EG or PROH, and 1.5 mol/l EG + 0.2 mol/l sucrose or 1.5 mol/l PROH + 0.2 mol/l sucrose, five minutes per each step) in the freezing solution. Then, the embryos must be loaded into a 0.25 mL sterile straw, which should be loaded into the cryo – machine which is precooled to twenty degrees Celsius and kept at twenty degrees Celsius for one minute for equilibration. Next, the straw should be cooled to negative-seven degrees Celsius at a rate of two degrees Celsius per minute, held at this temperature for five minutes, and seeded manually. Next, it should be cooled to negative-thirty-nine degrees Celsius at a rate of 0.3 degrees Celsius per minute and plunged into liquid nitrogen. To thaw the frozen human embryos, the straws should be rewarmed in room temperature for fifteen seconds before plunging them into a water bath at thirty-seven degrees Celsius. The cryoprotectant should then be removed by reverse stepwise dilutions with thawing solutions allowing five minutes for each step (Hee-Jun et al 2002).

Model Developmental Biology Organisms

Two model species have been used to examine fertilization: the invertebrate *Strongylocentrotus purpuratus* and the vertebrate *Danio rerio.* These species have the potential to contribute to our understanding of cryopreservation.

Strongylocentrotus purpuratus, commonly known as the purple sea urchin, can be found along the eastern edge of the Pacific Ocean (Heyland 2014). *Strongylocentrotus purpuratus* is a model organism for developmental and ecological studies. It is commonly used because of its widespread distribution, large population, ease of obtaining adults, and ease of obtaining gametes (Jasny et al. 2006). Also, it was the first non-chordate invertebrate with a fully sequenced genome (Sodergren et al. 2006). Prior to sequencing the genome of Strongylocentrotus purpuratus, many of the genes in their genome was thought to be only

from groups excluding deuterostomes. The genome of this sea urchin allows a comparison to humans and other deuterostomes (Sodergren et al. 2006). Strongylocentrotus purpuratus and humans have about seventy-seven-hundred genes in common (Matema et al. 2006) Reproductive technologies have been studied on this organism.

Cryopreservation of Sperm

To cryopreserve sea urchin sperm, after collection sperm should be maintained on ice in separate test tubes. Sperm should be diluted 1:20 with DMSO in 0.2 micrometers filtered seawater to a final DMSO concentration of five percent. Sperm should be loaded into 0.25 microliter straws and frozen at negative-fifty degrees Celsius per minute (Adams et al. 2004).

Cryopreservation of Embryos

To cryopreserve sea urchin embryos, eggs should be placed in filtered sea water from eighteen to twenty degrees Celsius. Allowing the embryos to develop to blastula stage should proceed as normal. To start the freezing process, blastulae should be taken after nine to nine-and-a-half hours following fertilization. Embryos should be centrifuged at 1800 rotations per minute, then equilibrated with a cryoprotectant for twenty minutes at 0+/-2 degrees Celsius. DMSO in a final concentration of one to 1.5 M can be used as the cryoprotectant. Samples should be equilibrated with DMSO and frozen in liquid nitrogen. Next they should be thawed and washed (Naidenko et al. 1991).

Developmental Stages

Strongylocentrotus purpuratus have radial holoblastic cleavage; the first and second cleavages are meridional and perpendicular to each other. The third cleavage is equatorial, perpendicular to the first cleavage planes. The fourth cleavage, the four cells divide meriodionally forming eight blastomeres with equal volumes (mesomeres). After the seventh

division, a 128 cell blastula is formed (Gilbert 2000). Blastula formation begins at the 128 – cell stage and ends at about 1000 cells (Gilbert 2000). The cells form a hollow sphere surrounding the blastocoel. Gastrulation occurs after the blastula is formed, in which the blastomeres have different sizes and properties and the cell fate of each cell is noticeable through its movements during this phase.

Danio rerio, commonly known as zebrafish, are often used as model organisms because they are the simplest vertebrate. Experimental advantages are that the genome analysis is well under way, easy examination of morphological defects, embryological manipulations are possible, organ systems are similar to other vertebrates, and they have rapid vertebrate development (Bier et al. 2004). Some experimental barriers are that zebrafish are not yet trivial to clone genes, cannot make transgenic animals, and have no targeted gene disruption (Bier et al. 2004).

Cryopreservation of Sperm

To cryopreserve *Danio rerio* sperm, using eight percent methanol with a cooling rate of ten degrees Celsius per minute can be used for increased sperm motility (Yang et al. 2007). Using a sperm concentration of 1.08 to 6.30X10^8 sperm/mL with an equal volume of HBSS – methanol and equilibrating for ten minutes, sperm samples (about 240 µL) should be loaded into a 250 µL French straws and cooled from five degrees Celsius to negative-eighty degrees Celsius with a ten degree Celsius per minute rate in a programmable freezer. When negative-eighty degrees Celsius is reached, the straws should be transferred into liquid nitrogen for storage (Yang et al. 2007).

Cryopreservation of embryo

Cryopreservation can be carried out on *Danio rerio* embryos using the controlled slow cooling method. To cryopreserve the embryos, freezing can occur in 0.25 mL straws in a programmable freezer. The cryoprotectants used should be a mixture of 1.5M dimethyl sulfoxide (DMSO) and 0.1 M sucrose. The slow cooling protocol used should be as listed: Five degrees Celsius per minute from twenty-two degrees Celsius to negative-six degrees Celsius, and held at negative-six degrees Celsius for fifteen minutes; 0.3 degrees Celsius per minute from negative-six degrees Celsius to negative-forty degrees Celsius; two degrees Celsius per minute from negative-forty degrees Celsius to negative-eighty degrees Celsius, and held at negative-eighty degrees Celsius for ten minutes (Lin et al. 2009). Next, the embryos should be plunged into liquid nitrogen (Lin et al. 2009). Thawing should occur in a water bath at twenty-eight degrees Celsius for fifteen seconds, followed by four step-wise removal of the cryoprotectant (Lin et al. 2009).

Developmental Stages

Danio rerio have meroblastic discoidal cleavage (Gilbert 2000). For *Danio rerio* development, the zygote period occurs from zero to forty-five minutes, cleavage period occurs from forty-five minutes to two hours and fifteen minutes, the blastula period occurs from two hours and fifteen minutes to five hours and fifteen minutes, the gastrula period occurs from five hours and fifteen minutes to ten hours, the segmentation period occurs from ten to twenty-four hours, the pharyngula period occurs from twenty-four to forty-eight hours, and the hatching period occurs from forty-eight to seventy-two hours (Kimmel et al. 1995).

Experimental Question

Cryopreservation of the sperm and embryos of *Strongylocentrotus purpuratus* and *Danio rerio* has conducted by previous researchers. The present experiment will utilize *Danio rerio. Danio rerio* adults can reproduce multiple times in a lab setting; *Strongylocentrotus purpuratus,* after being injected with potassium chloride to release gametes, often do not survive as well as *Danio rerio*. Also, since *Danio rerio* are vertebrates, they are more closely related to human beings, thus yielding results more similar to what could be seen in human results compared to results from *Strongylocentrotus purpuratus*. Because *Danio rerio* adults reproduce more consistently in a controlled setting than *Strongylocentrotus purpuratus*, have had successful cryopreservation of gametes and embryos, as well as are more closely related to humans, *Danio rerio* will be examined in this experiment. The purpose of this experiment is to determine both the optimal developmental stage and cryopreservative media that yields the highest successful hatching rate after cryopreservation. Based on previous work in humans that support the beneficial characteristics of the blastocyst for cryopreservation such as high cytoplasmic ratio and a larger number of cells in the embryo (Al-Azawi et al. 2013), it is hypothesized that cryopreserving *Danio rerio* embryos at the blastocyst stage will result in higher success rates.

CHAPTER II

METHODS AND MATERIALS

***Danio rerio* Husbandry**

A ten gallon aquatic tank was used to house 20 sexually mature (7 – 18 months of age) *Danio rerio* with approximately a 50/50 male to female ratio. The dechlorinated tank water was maintained 26°C - 28.5°C with a pH between 6.8 to 7.5 maintained using sodium bicarbonate and a heater probe as needed. Water was continually filtered and aerated using a carbon filter, and the filter was changed as needed. The tank was cleaned as needed by transferring the *Danio rerio* into a separate container while the tank was decontaminated with 70% ethanol and rinsed before reuse. *Danio rerio* were kept at a fourteen hour light cycle followed by a ten hour dark cycle (14:10) fed goldfish flakes two times a day during the week and one time a day during the weekends. All animal protocols were approved by the Maryville College IACUC (see Appendix).

In-tank Breeding

An embryo collector was constructed using wide gapped burlap and a metal tray (See Figure 1). The burlap acted as a mesh filter which was large enough for the embryos to fall through, but small enough so that the fish could not enter. Marbles were placed on the sides of the tray to fill the area between the tank and the embryo collector so that maximum collection of embryos could be achieved. To collect embryos, fish were placed into a holding

tank, the embryo collector was removed from the tank, the mesh was removed, and embryos were pipetted from the tray into a petri dish. A large pipette was used to obtain the embryos from the marbles and transfer them into the petri dish. Once the embryos were collected from the embryo collector and the marbles, the marbles were removed from the tank, and the embryo collector was reassembled. The embryo collector was placed back into the tank, marbles were added to the sides, and the fish were returned into the tank.

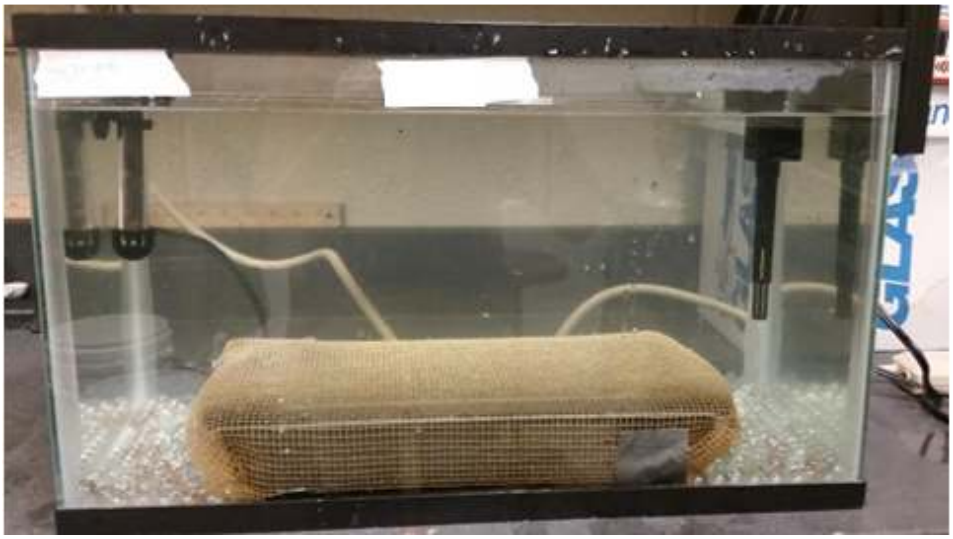

Figure 1: Tank set up showing the embryo collector, marbles, carbon filter, and heater.

Developmental Classification

Embryos were viewed in a petri dish under a microscope. The stage of the embryo was identified as either zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods (Kimmel et al. 1995). Once the developmental stage was determined, the embryos were transferred into a labeled petri dish containing dechlorinated tap water with same stage embryos until all embryos were classified.

Cryoprotectant Determination

Different cryoprotectants were tested to determine which would yield higher embryo hatching rates (See Table 2). The first cryoprotectant tested was a solution of 1.5M DMSO

and 0.1M sucrose. To make this, 117.20 g of DMSO and 34.23 g of sucrose were transferred to a volumetric flask. Dechlorinated water was transferred into the flask to the 1 L line, the solution was mixed and transferred into an amber bottle for later use. The second cryoprotectant tested was a 10% glycerol. To produce this, 10 mL of glycerol and 90 mL of dechlorinated water were measured out using graduated cylinders. These substances were transferred into a beaker for later use. The third cryoprotectant tested was 10% DMSO. To produce this, 10 mL of DMSO and 90 mL of dechlorinated water were measured using graduated cylinders, and transferred into a beaker. The fourth cryoprotectant used was 5% ethylene glycol, 5% sucrose, and 5% $Ficoll_{400}$ with 85% dechlorinated water. To produce this, 4.99 g of sucrose, 5.02 g of $Ficoll_{400}$, 5 mL of ethylene glycol, and 85 mL of dechlorinated water were measured and transferred into a beaker.

Table 2: Representation of the cryoprotectants used indicating the main components and secondary components.

Cryoprotectant	Main Component	Secondary Components
1	1.5 M DMSO	0.1 M Sucrose
2	Dechlorinated water (90%)	Glycerol (10%)
3	Dechlorinated water (90%)	DMSO (10%)
4	Dechlorinated water (85%)	Ethylene glycol, sucrose, $Ficoll_{400}$ (15%)

Embryo Cryopreservation

The embryos of same stages were transferred into 2.0 mL cryovials with the different cryoprotectants. Between 1 and 8 embryos (mean of 7) were transferred into each cryovial. The cryovials were then transferred into a cryovial container that was immersed into liquid nitrogen. The liquid nitrogen tank was sealed and the embryos were removed after one week of the initial time of cryopreservation.

Embryo Thawing

To thaw the embryos, the cryovial containers were removed from the liquid nitrogen chambers and the cryovials were placed into a cooler containing dry ice. After 24 hours, the cryovials were transferred into a refrigerator until the dry ice evaporated and the cryovials reached room temperature. The cryovials were opened and the cryoprotectant was removed in four stepwise dilutions with dechlorinated water. The cryovials were then transferred into a petri dish with same stage/same cryoprotectant embryos for further development.

Hatching Success Determination

The embryos were left in the petri dish labeled stating the developmental stage at the time of cryopreservation, amount of embryos in the petri dish, cryoprotectant used, and date of cryopreservation. Once enough time was allowed for the hatching stage to be achieved (approximately 3 weeks), the total amount of hatched embryos was recorded, as well as the total amount of embryos which did not reach the hatching stage.

Data Analysis

The success rate of each stage of development at the time of cryopreservation was calculated using Excel 2013. The significance of the data and the standard deviation were calculated using SPSS. The graphs were made using Excel 2013 and SPSS.

CHAPTER III

RESULTS

Out of 677 *Danio rerio* embryos cryopreserved, data was analyzed on only 564 of the embryos. *Danio rerio* embryos were separated according to developmental stage. Images of the different developmental stages can be seen in Figure 2. An image of a successful cryopreservation next to a non-successful cryopreservation can be seen in Figure 3. Success rates for hatching after cryopreservation in the four different cryoprotectants are given in Tables 3-6. A Chi-Squared test of independence was carried out using SPSS to determine if there was a significant difference in the cryoprotectants' ability to yield higher success; the results of this test can be seen in Table 7. Also, Chi-Squared test of independence was carried out using SPSS to determine if there was a statistically significant difference in the success rates of the different developmental stages; the results of this test can be seen in Figure 4. To determine the stage(s) that were statistically significantly different from the others, Tukey's post hoc analysis from ANOVA was accomplished via SPSS; analysis for this test can be seen in Table 8. The tables for the statistical analysis produced from SPSS can be viewed in the Appendix.

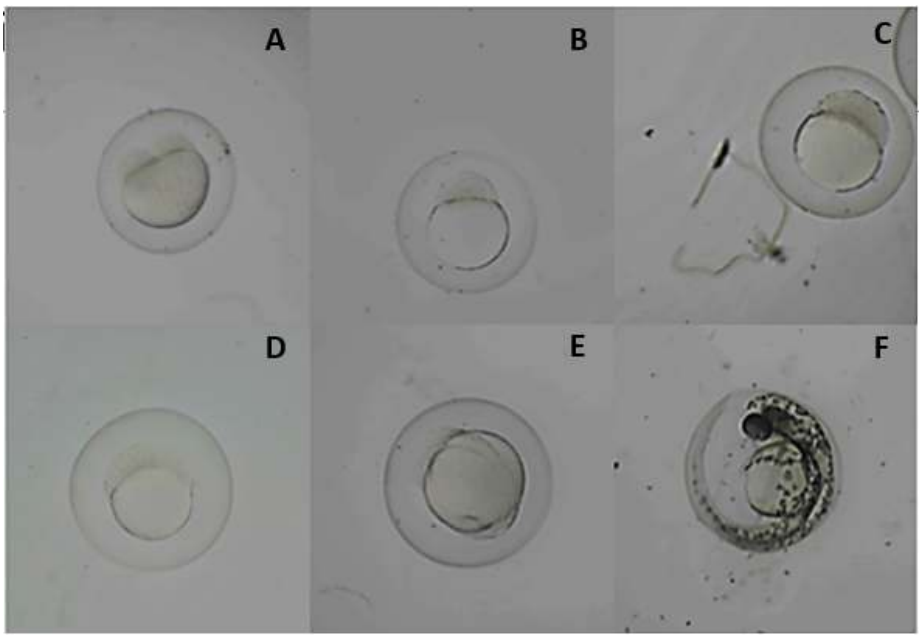

Figure 2: Developmental stages of *Danio rerio* embryos: zygote (A), cleavage period (B) blastula (C), gastrula (D), segmentation period (E), and pharyngula (F).

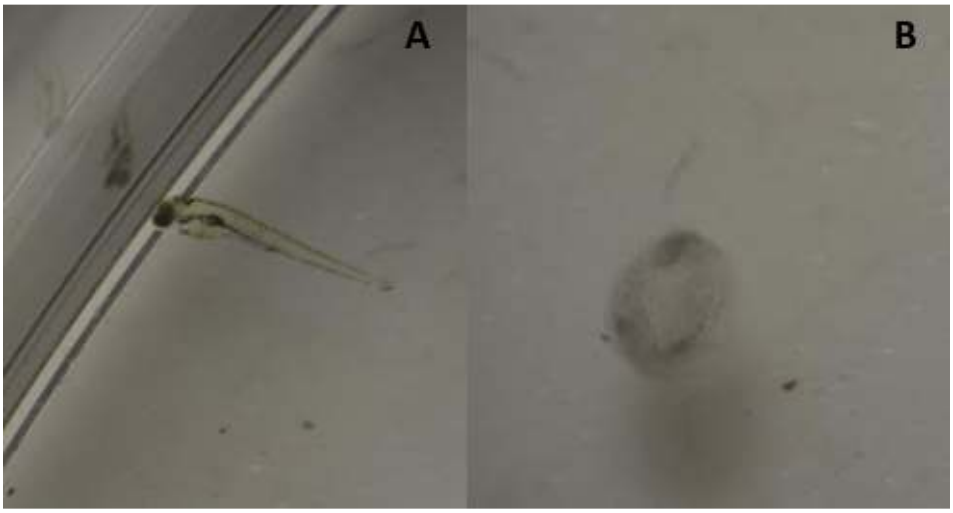

Figure 3: Photograph of a successful cryopreservation (A) next to a photograph of an unsuccessful cryopreservation (B).

Table 3: Success rates for *Danio rerio* embryos cryopreserved in 10% DMSO.

Developmental Stage	Amount Cryopreserved	Amount Success	Success (%)
Zygote	17	0	0
Cleavage	35	0	0
Blastula	26	0	0
Gastrula	32	0	0
Segmentation	21	0	0
Pharyngula	27	10	37.03704
Total	141	5	3.546099

Table 4: Success rates for *Danio rerio* embryos cryopreserved in 10% glycerol.

Developmental Stage	Amount Cryopreserved	Amount Success	Success (%)
Zygote	20	0	0
Cleavage	36	0	0
Blastula	35	0	0
Gastrula	33	0	0
Segmentation	34	0	0
Pharyngula	30	10	33.33333
Total	188	8	4.255319

Table 5: Success rates for *Danio rerio* embryos cryopreserved in 5% ethylene glycol, 5% sucrose, and 5% $Ficoll_{400}$ with 85% dechlorinated water.

Developmental stage	Amount Cryopreserved	Amount Success	Success (%)
Zygote	16	0	0
Cleavage	31	0	0
Blastula	24	0	0
Gastrula	28	0	0
Segmentation	14	0	0
Pharyngula	20	3	15
Total	133	3	2.255639

Table 6: Success rates for *Danio rerio* embryos cryopreserved in 1.5M DMSO and 0.1M sucrose.

Developmental Stage	Amount Cryopreserved	Amount Success	Success (%)
Zygote	0	0	0
Cleavage	0	0	0
Blastula	0	0	0
Gastrula	27	2	7.407407
Segmentation	27	0	0
Pharyngula	31	8	25.80645
Total	85	10	11.76471

Table 7: The results of the Chi-Squared test of independence analysis which compared the different cryoprotectants with the hatching success of pharyngeal stage *D. rerio* embryos. P-value = 0.363.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.193[a]	3	.363
Likelihood Ratio	3.390	3	.335
Linear-by-Linear Association	.028	1	.866
N of Valid Cases	108		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.74.

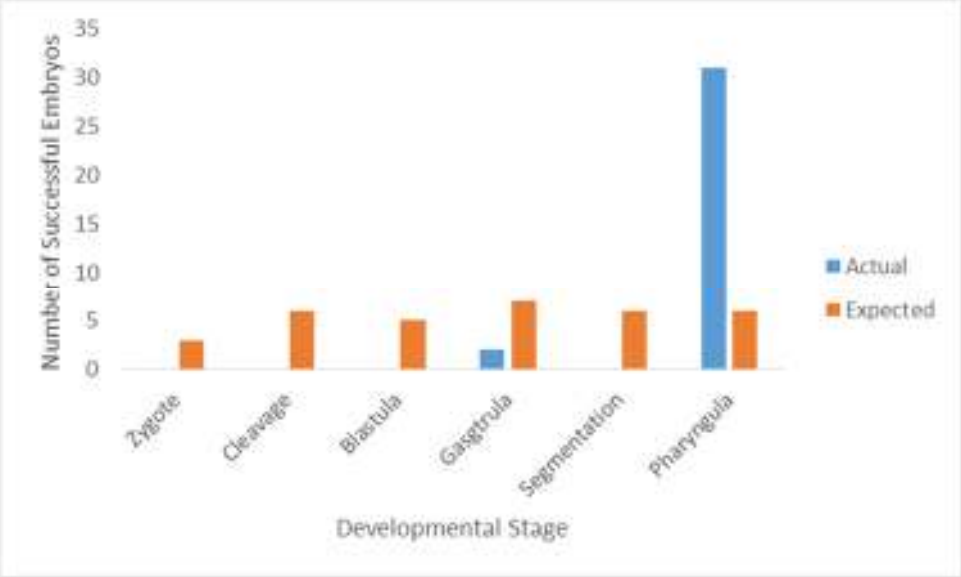

Figure 4: A graph depicting the results from a Chi-Squared test of independence which shows the amount of successful embryos hatched during this experiment (actual) compared to the amount expected to hatch if there was no significant difference among the developmental stages (expected). Each of the actual amounts of successful embryos were significantly different than the expected amounts of successful embryos (P-value < 0.0001)

Table 8: Results from Tukey's post-hoc analysis for the success rates of the developmental stages of *Danio rerio* embryos: zygote (1) cleavage stage (2), blastula (3), gastrula (4), segmentation stage (5), and pharyngula (6).The P-value < 0.0001 for pharyngeal stage when compared to the other stages.

Multiple Comparisons

Dependent Variable: Success

Tukey HSD

(I) DevelopmentalStage	(J) DevelopmentalStage	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	.00000	.03561	1.000	-.1018	.1018
	3.00	.00000	.03081	1.000	-.1053	.1053
	4.00	-.01667	.03469	.997	-.1159	.0825
	5.00	.00000	.03599	1.000	-.1029	.1029
	6.00	-.28319*	.03502	.000	-.3833	-.1831
2.00	1.00	.00000	.03561	1.000	-.1018	.1018
	3.00	.00000	.03099	1.000	-.0883	.0883
	4.00	-.01667	.02833	.992	-.0977	.0643
	5.00	.00000	.02991	1.000	-.0855	.0855
	6.00	-.28319*	.02873	.000	-.3653	-.2010
3.00	1.00	.00000	.03081	1.000	-.1053	.1053
	2.00	.00000	.03099	1.000	-.0883	.0883
	4.00	-.01667	.02982	.994	-.1019	.0686
	5.00	.00000	.03132	1.000	-.0896	.0896
	6.00	-.28319*	.03020	.000	-.3695	-.1969
4.00	1.00	.01667	.03469	.997	-.0825	.1159
	2.00	.01667	.02833	.992	-.0643	.0977
	3.00	.01667	.02982	.994	-.0686	.1019
	5.00	.01667	.02889	.992	-.0657	.0990
	6.00	-.26652*	.02757	.000	-.3454	-.1877
5.00	1.00	.00000	.03599	1.000	-.1029	.1029
	2.00	.00000	.02991	1.000	-.0855	.0855
	3.00	.00000	.03132	1.000	-.0896	.0896
	4.00	-.01667	.02889	.992	-.0990	.0657
	6.00	-.28319*	.02919	.000	-.3667	-.1997
6.00	1.00	.28319*	.03502	.000	.1831	.3833
	2.00	.28319*	.02873	.000	.2010	.3653
	3.00	.28319*	.03020	.000	.1969	.3695
	4.00	.26652*	.02757	.000	.1877	.3454
	5.00	.28319*	.02919	.000	.1997	.3667

CHAPTER IV

DISCUSSION

In previous studies of cryopreservation of embryos, the blastocyst stage was thought to yield the highest success rates because of the high cytoplasmic ratio. However, experiments that compared cryopreservation at the blastocyst stage compared to other stages selected embryos which were of the highest quality based off morphology after genomic activation (Surrey et al. 2010). Since these studies did not randomly select the embryos using bias based off of morphology, it is unknown if the blastocyst is actually the most beneficial embryonic stage for cryopreservation success. To accurately determine which embryonic developmental stage is successful, bias must not be taken when choosing embryos to cryopreserve based off of physiology. For this study, each *Danio rerio* embryo obtained were separated based on their developmental stage and all were cryopreserved to eliminate bias in order to determine the most successful developmental stage for cryopreservation. Also, four different cryopreservatives were used to determine which would yield the highest success rates.

Out of the 677 embryos cryopreserved, data was analyzed on only 564 of the embryos. This is because experimental error occurred from eliminating cryopreserved embryos after being thawed for 4 days. This was initially done because of the 72 hours it takes for *D. rerio* to reach the hatchling stage. Since none had hatched within 72 hours of

being thawed it was thought they did not survive cryopreservation. However, after observing thawed embryos that had been thawed for two weeks, hatchlings were observed. From this, it can be concluded that following cryopreservation, *Danio rerio* embryos become dormant or development is decelerated for a period of time. One study found that *Danio rerio* embryos kept at a lower temperature had slower development than those kept at higher temperatures (Hallare et al. 2005). Therefore, the slowed development of the *Danio rerio* embryos in this experiment could likely be because of the low temperature the embryos are exposed to during cryopreservation. A finding from Moussa et al.'s experiment concluded that cells exposed to high concentrations of cryoprotectants have slower first cleavage event (Moussa et al. 2014). This can lead to the hypothesis that high concentrations of cryoprotectants can slow development or cause embryos to become dormant at later stages. Further experiments should be conducted to research the affect cryoprotectants have on slowing the development of *Danio rerio* embryos following cryopreservation, as well as if the cryopreservation temperature slows development of the *Danio rerio*.

Of the 564 embryos that were observed for more than two weeks after thawing, two of one hundred and twenty gastrula stage embryos reached the hatchling stage, thirty-one of one hundred and thirty-one pharyngeal stage embryos reached the hatchling stage, and none of the other developmental stages were successful. The developmental stages significantly differed in their ability to yield higher success rates. The pharyngeal stage *Danio rerio* embryos significantly differed from the other developmental stages. From the data collected from this experiment, it can be inferred that the developmental stage which yields highest success rates of hatching following cryopreservation is the pharyngeal stage. Previous studies involving cryopreservation of *Danio rerio* embryos involved only blastula stage (Lin et al.

2009). However, since cryopreservation was successful using 1.5 M DMSO with 0.1M sucrose for this developmental stage in Lin's experiment, further analysis should be conducted to determine why the protocol for this experiment did not yield hatching success for blastula staged *Danio rerio*. One study found that none of the embryos (ranging from three hours to forty-nine hours after fertilization) survived storage at zero degrees Celsius for seventy-two hours, nor negative-twenty degrees Celsius for one hour (Zhang et al. 1995); this does not coincide with the findings of this experiment. However, they found that early developmental stages were the most sensitive to chilling; which coincides with the findings of this experiment. They also found that twenty-seven to forty-hour stage embryos (pharyngeal stage) tolerated zero degree Celsius temperature for up to ten hours without adverse effect, but survival did not occur at subzero temperature. This supports that the pharyngeal stage is less sensitive than earlier stages, as supported through this experiment, but does not coincide with this experiment since there was success at subzero temperatures for pharyngeal stage *Danio rerio*. They also found that forty-five to forty-none hour stage embryos showed increased sensitivity (Zhang et al. 1995), which did not correspond to the findings of this experiment.

The four cryoprotectants did not significantly differ in yielding higher success rates for pharyngeal stage *Danio rerio* embryos. However, 1.5 M DMSO with 0.1 M sucrose was the only cryoprotectant that yielded successful hatching following cryopreservation for gastrula stage *Danio rerio* embryos. Further experiments should be conducted to determine why 1.5 M DMSO with 0.1 M sucrose was beneficial in protecting the gastrula stage embryo.

Because human embryos implant to the uterus during the blastula stage (implantation day eight to nine after gamete fusion), transferring a human embryo at the pharyngeal stage

(day thirty-two after gamete fusion) would likely not be a viable option. However, further studies should be conducted without embryo selection based on morphology to determine the developmental stage of development which yields highest success rates following cryopreservation for human embryos.

For *Danio rerio*, there are four areas of future research. First, future studies should be conducted to determine the properties of the pharyngeal stage embryo that is beneficial for cryopreservation to give us a better understanding of cryopreservation and how developmental stage affects success outcomes. Second, further research should determine if development of the *Danio rerio* embryo is being slowed or if the embryo becomes dormant for a period of time following cryopreservation. One study found that developing *Danio rerio* embryos developed at a slower rate in cooler temperatures (Hallare et al. 2005).Third, future research should be conducted to determine the impact 1.5 M DMSO with 0.1 M sucrose has on the gastrula during cryopreservation that increases hatching success of gastrula stage *Danio rerio* embryos; as well as determining if there are specific properties that are more beneficial as a cryoprotectant for the different developmental stages of *Danio rerio* embryos. Fourth, it is unknown if the cryoprotectant has future adverse effects on the offspring; a recent study in mice found that DMSO increased the likelihood of respiratory distress in adults but effects on embryos is unknown (Takeda et al. 2016).

In conclusion, the cryoprotectants used did not differ for the success of pharyngeal staged *Danio rerio* embryos. The developmental stage of *Danio rerio* which yielded higher success rates following cryopreservation was the pharyngeal stage.

APPENDICES

APPENDIX 1

Cryopreservative Analysis

Table 1: Case processing summary of the Chi-Squared test of independence table for comparing the four cryopreservatives used during this experiment.

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
cryoprotectant * outcome	108	100.0%	0	0.0%	108	100.0%
amount of embryos that hatched (count) * outcome	108	100.0%	0	0.0%	108	100.0%

Table 2: Crosstab of the Chi-Squared test of independence table for comparing the four cryopreservatives used during this experiment. Table depicts the amount of experimental unsuccessful and successful as well as what would be the expected amount of unsuccessful and successful if there was no difference among the cryopreservative groups.

Crosstab

			outcome		Total
			unsuccessful	successful	
cryoprotectant	DMSO sucrose	Count	23	8	31
		Expected Count	22.1	8.9	31.0
	10% DMSO	Count	17	10	27
		Expected Count	19.3	7.8	27.0
	15% EGSF	Count	17	3	20
		Expected Count	14.3	5.7	20.0
	10% Glycerol	Count	20	10	30
		Expected Count	21.4	8.6	30.0
Total		Count	77	31	108
		Expected Count	77.0	31.0	108.0

Table 3: Chi-Square test of independence table for comparing the four cryopreservatives used during this table. Table depicts the df value, as well as the P-value.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	3.193[a]	3	.363
Likelihood Ratio	3.390	3	.335
Linear-by-Linear Association	.029	1	.866
N of Valid Cases	108		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.74.

Table 4: Symmetric measures from the Chi-Square test of independence analysis; table showing the Phi and Cramer's V values as well as the approximate significance.

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.172	.363
	Cramer's V	.172	.363
N of Valid Cases		108	

APPENDIX 2

Developmental Stage Analysis

Table 1: Crosstab of the Chi-Squared test of independence table for comparing the developmental stages cryopreserved during this experiment. This information was used to construct the graph in Figure 4.

Crosstab

			outcome		
			did not hatch	hatched	Total
stage	zygote	Count	53	0	53
		Expected Count	49.9	3.1	53.0
	cleavage	Count	102	0	102
		Expected Count	96.0	6.0	102.0
	blastula	Count	85	0	85
		Expected Count	80.0	5.0	85.0
	gastrula	Count	118	2	120
		Expected Count	113.0	7.0	120.0
	segmentation	Count	94	0	94
		Expected Count	88.5	5.5	94.0
	pharyngula	Count	77	31	108
		Expected Count	101.7	6.3	108.0
Total		Count	529	33	562
		Expected Count	529.0	33.0	562.0

Table 2: Chi-Square test of independence table for comparing the four cryopreservatives used during this table. Table depicts the df value, as well as the P-value.

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	126.536[a]	5	.000
Likelihood Ratio	101.302	5	.000
Linear-by-Linear Association	60.705	1	.000
N of Valid Cases	562		

a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is 3.11.

Table 3: Symmetric measures from the Chi-Square test of independence analysis; table showing the Phi and Cramer's V values as well as the approximate significance for the different developmental stages.

Symmetric Measures

		Value	Approx. Sig.
Nominal by Nominal	Phi	.475	.000
	Cramer's V	.475	.000
N of Valid Cases		562	

Appendix 3

IACUC

MARYVILLE COLLEGE INSTITUTIONAL ANIMAL CARE & USE COMMITTEE Application for Use of Vertebrate Animals in Student Research

Provide information after each bold item

Student Name:

Amanda Brooke Cummings

Student Email Address:

amanda.cummings@my.maryvillecollege.edu

Date:

03/02/2016

Senior Study Advisor:

Dr. Drew Crain

Species to be used:

Xenopus laevis, Danio rerio

Age of animals:

adult

Number of animals in study:

Danio rerio: 20 adults, 500+ embryos

Duration of study:

March 15 - December 5

Location of animals during the study (building and room):

Sutton 114

List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Brooke Cummings	865-363-6437	865-363-6437	865-363-6437
Dr. Drew Crain	865-981-8238	865-292-8737	865-292-8737

What will happen to the animals at the end of the study? If euthanasia is required, state the specific methods.

Euthanasia with ms222.

(Do not write below line: For MC IACUC Use)

Maryville College IACUC Approval Number: _____

Date Approved: _____

Signed:

_____

Is it likely that pain/discomfort will be experienced by animals in this protocol?
YES☐ NO☒ If "YES", describe:

Pain or Distress Category:B - see additional document for explanation See listing of Pain or Distress Categories below)

For categories C,D, or E, USDA regulations require that the investigator consider alternative procedures. Please provide a narrative (for instance the end of Chapter 1) describing the methods and sources used to determine that alternatives are not available. If a computer assisted literature search was conducted, provide the names of the database(s) and date(s) of the search.

Pain or Distress Categories

A. ACUTE STUDIES
Studies performed under anesthesia from which the animals are not permitted to regain consciousness, or performed on excised animal tissues collected under anesthesia or following euthanasia.

B. PAIN OR DISTRESS - NONE OR MINOR
Chronic studies that DO NOT involve survival surgery, induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (so long as the tumors do not cause pain and the animals are terminated prior to becoming seriously ill), administration of mildly toxic substances or drugs that cause no significant disease or distress, and antibody production as long as significant disease does not result and antigen booster doses do not include Complete Freund's Adjuvant (CFA).

C. PAINFUL PROCEDURES WITH ANESTHESIA/ANALGESIA
a. Survival surgical procedures.
b. Painful or potentially painful non-surgical procedures; e.g. bone marrow taps, injections into particularly sensitive areas such as foot pads, cardiac punctures, or traumatic procedures such as burns (burns may be category D, depending on severity).

D. MODERATE DISTRESS OR PAIN GENERALLY WITHOUT ANESTHESIA/ ANALGESIA/ TRANQUILIZERS
Induction of moderately distressful or painful disease conditions (examples: arthritis, administration of toxic chemicals, infectious challenges, immunosuppression resulting in infectious disease, peritonitis, severe inflammation, especially of weight bearing surfaces or resulting in external sores), whole body irradiation, stress models, septic shock, hypotensive shock, moderate painful stimuli (examples: low level electrical shock or heat), survival surgical procedures that have the potential to result in long term distressful illness (organ transplants, for example), induction of cardiac ischemia, booster immunizations with CFA, tumor induction or animal cultures that cause significant distress or pain, sight deprivation, restraint for periods longer than 12 hours.

E. INTENSE SUSTAINED OR REPEATED PAIN WITHOUT ANESTHESIA/ANALGESIA
Direct stimulation of CNS pain tracts, nociceptor stimulation by physical or chemical means that causes severe pain (e.g., corneal abrasions), or any category C (see above) procedure if performed without chemical relief of pain.

Investigator Assurance

Check all boxes that apply.

☒ The information provided in this protocol form accurately reflects the intended use of animals for this research activity. Significant changes in procedures will not be undertaken without prior notification and approval of the Maryville College IACUC.

☒ All persons involved in the use of animals on this protocol have been informed of the experimental objectives and methods. Each has received training in the execution of animal-related procedures he/she will perform prior to participation in the protocol, and will participate in any educational or training programs deemed appropriate or necessary by the Maryville College IACUC.

☒ I agree to follow the provisions of the Animal Welfare Act and the guidelines of the National Institutes of Health on the care and use of laboratory animals.

☒ I agree to use anesthesia, analgesia and tranquilization to relieve pain or distress whenever use of these agents will not jeopardize the scientific validity of the data. I have specifically consulted with the Maryville College IACUC regarding any experiments that are classified in pain/distress categories C, D, or E.

☒ I will take appropriate steps to avoid exposure of persons working with these animals to any biohazard agents used in the study.

For any unchecked box above, explain the reason it does not apply.

Purpose of the Study: Briefly describe your proposed research project (or attach a research proposal). Be sure to include a justification for the species and number.

The purpose of this study is to determine which stage of development yields highest success rates, and to determine which cryopreservative yields highest success rates.

Animal Husbandry: Briefly describe the basic animal husbandry requirements for the animals.

For Danio rerio, care will include feeding them, insuring tanks are well maintained, and that the water remains oxygenated.

Potential Scientific Benefits: State potential value of study with respect to human or animal health, advancement of knowledge, or good of society.

The importance of this experiment is if developmental time/cryopreservative for cryopreservation does effect an organisms development. experiments can be conducted to determine if these results parallel with human embryos, and thus increase cryopreservation success. .

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