A NOVEL APPROACH TO THE CRYOPRESERVATION OF EQUINE AND PORCINE EMBRYOS BY VITRIFICATION AFTER BLASTOCOELIC MICROMANIPULATION

by

LUCY ELEANOR RAY

(Under the direction of Gary Heusner)

ABSTRACT

Vitrification of equine embryos shows fairly consistent success rates when they are frozen at ≤ 300µm or about 6.5 d after ovulation. In this study, the effects of the reduction of blastocoelic fluid and the microinfusion of a cryoprotectant prior to vitrification were examined on 8 d embryos. The equine embryos used for this project were 805µm (1), 820µm (2), 1120µm (3), 1286µm (4), and 979µm (5). All embryos were graded excellent (1) according to IETS guidelines. The embryos were either assigned to a control group in which no micromanipulation occurred (1-3) or to the experimental group which entailed microinfusion of VS1 (1.4 M glycerol in PBS; 4-5) after aspiration of blastocoelic fluid before microinfusion of VS1 (5). During the following vitrification procedure, the embryos were exposed to VS1 and 2 (1.4M glycerol, 3.6M ethylene glycol in PBS) for 5 minutes, VS3 (3.4M glycerol, 6.6M ethylene glycol in PBS) for 1 minute. The embryo in VS3 was then loaded into a 0.25 ml straw, separated by two air bubbles from columns of 0.5 M galactose. The straws were placed in a cooled plastic goblet surrounded by liquid nitrogen vapors for 1 minute and

then finally immersed into the liquid nitrogen. Digital pictures were taken throughout the process. The equine embryos failed to yield a pregnancy with the exception of one (5) which formed an embryonic vesicle at d 15 after ovulation. Ultrasonography at d 28 revealed resorption of the embryo, probably due to heat stress. Due to the low number of equine embryos recovered, porcine embryos were used as a model in another trial. Vitrification of 44 porcine embryos was attempted using identical procedures to those described above. The porcine embryos were cultured for a period of 24 hrs and digital images were also taken. Twenty-six embryos were frozen using the control method and 20 were assigned to the experimental group and treated as described earlier. Initial reexpansion was complete in 16 of the treated and 7 of the control embryos. This showed a significant difference in treatment and control at thaw ($p<.0001$). All embryos were completely dead at 24 hrs.

INDEX WORDS: embryo transfer, vitrification, cryopreservation, equine embryo, porcine embryo

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DEDICATION

I dedicate this work to my family. To Alice and Ann Ray, for never letting me forget where I came from and encouraging me whenever things got tough. To Sam and Suzanne Hinton for teaching me to respect myself, for letting me drive the tractor and work the squeeze chute. Thank you for never letting me settle for what was easiest. To Dwight and Eloise Williams for teaching me about land, animals and life. You have all taught me so much and instilled in me a profound appreciation for farming and respect for the land. You also taught me the value of an education and that nothing is more rewarding than working on land. By watching your example, I learned to work hard and to never give up on my dreams. Thank you so much. I love you and dedicate this work to you all.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Chapter 1

Introduction

As the equine industry expands and grows, there is a greater demand to preserve the genetic material of outstanding performance horses. The ability to successfully store genetics from a stallion is now possible with the cryopreservation of semen. Embryo transfer (ET) allows us to take embryo(s) from a mare so that she can have more than one offspring a year. The success of ET in horses is limited because of problems with superovulation and embryo cryopreservation by vitrification or slow cooling methods (Squires, *et al*., 2003).

Successful embryo cryopreservation would greatly benefit producers by allowing the embryo to be stored until such time as it was possible to transfer it to a recipient mare. This would save the horse industry a tremendous expense by decreasing the number of recipient mares needed for each donor mare since estrous synchronization, one of the major obstacles and expenses in ET, would no longer be an issue. However, expense is not the only reason that successful embryo cryopreservation would benefit the horse industry. Just as deceased stallions can currently still perpetuate their genetics through frozen semen, deceased mares or those who are physically unable to carry a live foal to term would still be able to make a genetic impact on the equine industry.

Chapter 2

Review of Literature

The following review of literature covers the most important aspects of embryo transfer and vitrification in the mare. Information on the factors involved in a successful embryo transfer as well as cryopreservation techniques and the problems associated with them will be presented.

Equine Embryo Transfer

Many factors can affect a successful embryo transfer program. Artificial insemination, embryo flushing and recovery techniques, as well as the problems associated with estrus synchronization and superovulation are a few of the issues that are faced by the horse industry.

Estrus Synchronization and Superovulation in the Mare. Perhaps one of the most common problems in the equine species is the ability to synchronize the recipient mares properly with the donor mare. Because of individual fluctuations in the hormones which affect a mare's estrous cycle, it is exceedingly difficult to exactly coordinate one mare's cycle with another. Another problem associated with the equine species is the difficulty involved in superovulation. Superovulation is difficult in horses due to the fact that ovulation can only occur from the ovulation fossa. If a solution could be found to

one or both of these issues, the cost of embryo transfer could be greatly reduced due to the decreased amount of recipient mares that would need to be maintained per every donor mare. Also, if a large number of embryos could be collected from one mare, then the genetic influence of a mare would be greatly increased similar to the way that the genetic influence of the stallion has increased with the success of artificial insemination and cooled shipped semen.

Recipient mares can be synchronized through intramuscular injection of prostaglandin F2ά. Both mares must be known to be between 6 and 14 days of diestrous and have an absence of pre-ovulatory follicles that could ovulate quickly (Allen, 2005). The injection is given to the donor mare 1-2 days before the recipient mares. However, differences in a donor and recipient mare's cycles provide a need for more than one recipient mare per donor mare in order to ensure that a recipient is in the proper place in her cycle (she should be a day or so behind in the cycle in order to give the embryo its best chance of survival). Because of this, human chorionic gonadotropin (hCG) is commonly given to induce ovulation in an attempt to synchronize ovulation in donor and recipient mares (Hudson *et al.,* 2006*)*. Research has shown that a 4 day spread in cycle difference can be achieved without a decrease in pregnancy rates in horses (Allen and Rowson, 1975). In spite of this, cycles that are as identical as possible in the donor and recipient are desirable.

The mare and other equids fail to undergo genuine superovulation in response to exogenous gonadotrophic hormones. As mentioned before, this is a limiting factor in the ability to create a commercial embryo transfer industry. In cattle, sheep and swine, as many as 20-30 viable embryos can sometimes be recovered from a single flush following

treatment with a hormone to induce superovulation. Similar work in the mare yields 1-2 viable embryos (Squires and McCue, 2006). Single ovulating mares have about a 50% embryo recovery rate per estrous cycle. Some mares (such as Thoroughbreds, draft breeds, and warmbloods) are unique in that they spontaneously double ovulate. They therefore have a greater embryo recovery rate per estrous cycle and are more productive in an embryo transfer program (Squires and McCue, 2006). The problem with induction of superovulation in the mare is primarily related to two factors. The first of these is that equine gonadal tissues exhibit lower concentrations of receptors of eCG, which would respond to the administration of eCG and therefore superovulate, than other species. Secondly, the anatomy of the mare is such that the follicles can only ovulate and release the oocyte through the ovulation fossa because of the existence of the tough and fibrous tunica albuginea which covers the external surface of the ovary (Allen, 2005).

Superovulation could be a valuable tool to increase the percentage of embryos recovered and make equine embryo transfer more financially feasible and affordable to average breeders. Potential benefits of superovulation include increased oocyte collection, increased embryo recovery, and the availability of embryos for freezing just to name a few. Unfortunately, none of the hormone therapies currently used have been successful at inducing superovulation to raise the average ovulation rate of a mare to above 1.2-1.7 per cycle (Squires, 2003). Many approaches to superovulation in the mare have been tried. These include the injection of porcine FSH, inhibin vaccines, EPE (equine pituitary extract), and eFSH (equine FSH).

Porcine FSH has been examined in horses in several studies. In one particular study, mares were given 159 mg of porcine FSH and only obtained 1.6 ovulations

compared to the 2.2 ovulations for mares being given EPE in the same study. Also, the variations in concentrations of porcine FSH were extreme in horses and therefore greatly increased the expense (Squires and McCue, 2007).

Secretion of inhibin from the dominant ovarian follicle suppresses FSH secretion. Therefore inhibin antibodies have been used to decrease inhibin concentrations and increase concentrations of FSH in the mare. However, adverse reactions at the site of injection have prevented the commercial use of inhibin vaccines for superovulation (Squires and McCue, 2007).

A great deal of research has been done with EPE as a means of superovulation. Numerous studies at Colorado State University showed that ovulations per cycle after treatment with EPE ranged from a 2.3 to a maximum of 3.9. An average of 1.96 embryos per cycle was obtained compared to 0.65 embryos per cycle with untreated mares. This data does indicate that the mare is responsive to EPE. However, several factors do tend to play into the degree of responsiveness. These include the day of initial treatment and the frequency of injection. GnRH treatment prior to EPE treatment was also studied but the response to EPE was not enhanced in these trials (Squires and McCue, 2007).

Alvarenga et al. (2001) is perhaps most notable for attempting to improve the superovulatory response to EPE by giving the hormone twice daily. Mares were assigned to one of two treatment groups 6-8 days after ovulation. Prostaglandin was given once and EPE was given once or twice daily for one week. The number of ovulations in mares treated twice daily was greater (7.1 vs. 2.4). The number of embryos recovered was 3.5 for the mares injected twice daily and 1.6 for the mares injected once daily. Unfortunately, in this study it was not possible to determine whether this response was

due to twice daily injections or the fact that these mares got 50 mg of EPE vs. the 25mg of EPE that the once daily mares received.

Equine FSH seems to give a similar result as EPE when used as a superovulatory compound. A recent study found an average of 3.5 ovulations per cycle and 2 embryos per flush on mares who were treated twice daily with 12.5 mg of eFSH (Hudson *et al*., 2006). A commercially available form of eFSH is now available from Bioniche Animal Health (Athens, GA). eFSH has the potential to increase embryo recovery four-fold and can be used effectively in an embryo transfer program. Although the mare's ovary does have the ability to respond to eFSH, her response is much lower than cattle. However, in spite of decreased embryonic numbers, there is still the potential for the collection of multiple embryos each cycle (Allen, 2005).

Artificial Insemination in the Mare. According to legend the first successful artificial insemination (AI) was actually practiced on a horse. In 1322, an Arab chieftain is reputed to have sent a spy to a neighboring village to procure a breeding to a coveted stallion. The spy reportedly stole the semen out of a recently mated mare's vagina and diluted it with camel's milk in a goatskin bag and returned to his master's camp. We are not told how the semen was deposited into the chieftain's mare but are assured that a healthy foal was born as a result (Pickett, 2000). After this, equine AI was not practiced again until Sir Walter Heape successfully collected a stallion and inseminated mares in 1898 (Heape, 1898). Sir John Hammond was the next to use AI in his well known trial that crossed Shetland ponies with Shire horses to determine the effect of uterine size on the size of offspring in 1938 (Walton and Hammond, 1938). Colorado State University became the largest resource for equine reproductive research during the last half of the

century. This is where a majority of our knowledge on the subject of AI in the equine comes from today (Allen, 2005).

There are many types of artificial vaginas (AV) used today. However, the two most commonly used are the Colorado AV and the Missouri AV. The Colorado is very large and heavy and requires filling with as much as 4.5 L of water to create the desired internal pressure and temperature of 46° C to stimulate ejaculation in most stallions. It does retain heat well and is a successful model in most stallions. However, it is heavy and cumbersome to work with. The Missouri model is much lighter and easier to handle. However, it does tend to lose heat rapidly on cold days (Allen, 2005).

Artificial insemination is currently used worldwide as a way to increase to the gene pool and make breedings to stallions available that would otherwise be impossible due to distance. AI can be performed with fresh, cooled or frozen semen. However, insemination with frozen semen has a comparatively low success rate and is not used as frequently. Fresh extended semen can be used same day and is normally used when the stallion and mare are on the same farm. The gel component of the ejaculate which is produced by the seminal vesicles and the bulbourethral gland, is filtered out and the remaining volume is diluted at a 2:1 ratio with an extender that typically consists of a glucose/skim milk base. The dilution ratio will vary according to initial semen concentration and can range from 1:1 to 7:1 (Allen, 2005). Mares are inseminated with a dose of 500 million progressively motile spermatozoa (PMS) initially after collection or 1 billion PMS that have been cooled and stored for 24 hrs at 5° C (Squires, 2005). Insemination is performed when the mare has a dominant follicle measuring at least

35mm. Inseminations can be accompanied by an intravenous or intramuscular injection of human chorionic gonadotropin (Allen, 2005).

Embryo Recovery and Transfer. Although the horse was probably the first domestic species to experience artificial insemination, it is one of the last to undergo embryo transfer. As mentioned before, this is partly because of the difficulty in achieving superovulation in the mare thus decreasing the number of embryos that can be obtained per cycle. In addition, there has been quite a bit of protest to embryo transfer from breed associations in the equine industry which will be discussed later.

Currently the leading countries involved in equine embryo transfer are the United States, Argentina and Brazil. The latter exhibited interest in the technique primarily because of its effect on the polo industry (Allen, 2005). Factors that affect embryo recovery include the number of ovulations, the day of recovery, the age of the donor mare and the quality of the semen. The average recovery rate per cycle of single ovulating mares is about 50% (Squires, 2003).

Embryo recovery is fairly simple and straightforward. A flexible two-way flushing catheter is passed through the mare's cervix and into the body of the uterus where a circular cuff is inflated with air or sterile saline solution in order to occlude the internal os of the cervix and allow the uterus to be filled with the flushing media (Allen, 2005). The uterus was infused with 1-2L of media at a time and this was then drained out through an embryo filter. This is typically repeated about three times for a total of 3-6 L used. The flushing media should contain some fetal calf serum to prevent the embryo from sticking to the sides of the filter dish (Squires, 2003). Oxytocin is often administered during the final flush to aid in completely evacuating all medium from the

uterus and increasing the rate or embryo recovery. A flush takes place on either day 7 or 8 after ovulation when the embryo is already at the expanded blastocyst stage. The equine embryo takes from 144-168 hrs to enter the uterus from the oviduct. Thus flushing at d 6 dramatically decreases the chances of embryo recovery (Allen, 2005).

Embryos can be transferred into the mare either surgically or non-surgically. Surgical transfer is carried out by exteriorizing one uterine horn. This is accomplished by a ventral mid-line laparotomy done under general anesthesia or a flank laparotomy done under local anesthesia while standing in stocks (Allen, 2005). While there is a slightly higher success rate with surgical transfers, the impracticality of the process coupled with increased costs and tremendous discomfort suffered by the donor mare makes these procedures only rarely used on a commercial basis.

Non-surgical transfer is most commonly performed by loading the embryos into a 0.25 or 0.5 ml straw and insertion into the recipient mare's uterus via an embryo transfer gun. An embryo can also be loaded into a flexible AI rod and placed trough the cervix and into the body of the uterus (Squires, 2003). A recently developed non-surgical technique uses a Polansky's duck-billed vaginoscope to enable visualization of the external os of the cervix. The os is then grasped by modified Velsellum forceps which, when pulled backwards, straighten the cervix and uterine horns. This manipulation allows the easy passage of the transfer pipette through the cervix and up into one of the uterine horns. This procedure has the advantage of increased sterility. However, it is labor intensive in that it requires 3 operators to complete the procedure (Allen, 2005).

Poor quality embryos (≥ Grade 3 according to IETS guidelines) have a much lower conception rate than those of good quality (Grade 1 or 2). Embryos that are

abnormally small for their age or have abnormal morphology result in decreased pregnancy rates. Early embryonic death is also seen to be higher in older donor mares. This can be a problem since this is the largest group of donor mares in most embryo transfer programs. However, based upon data gathered at Colorado Stated University, a 70-75% conception rate can be obtained with non-surgical transfer of Grade 1 embryos at day 8. This conception rate only drops to 65% at day 50 examination (Squires, 2003). These numbers provide an optimistic outlook for the future of equine embryo transfer.

Economics and Breed Association Rules. The potential benefits of equine embryo transfer will be quite extensive. Through this process, the genetic effect of mares on different breeds and disciplines can be extended, just as artificial insemination extended the gene pool by allowing more widespread access to the genetics of genetically superior stallions. There is also an added benefit of being able to obtain foals from mares that are not able to carry a foal to term and in some cases mares that are too old to be allowed to carry a foal safely. However, there are distinct economic setbacks with embryo transfer. The most prevalent of these is the significant expense that this procedure costs the average producer. The need for multiple recipient mares to be kept for each donor mare further increases the cost of feed, labor, etc. on a breeding farm.

In addition to economic issues, there are certain biases of some breed associations to be overcome as well. Some researchers blame the refusal of the Thoroughbred industry to partake in any modern reproductive technologies for the slow pace of equine reproductive research (Allen, 2005). This worldwide ban on breeding technologies continues today. The Jockey Club (2007) defines breeding as "the physical mounting of a broodmare by a stallion with intromission of the penis and ejaculation of semen into the

reproductive tract." In addition, "any foal resulting from or produced by the processes of Artificial Insemination, Embryo Transfer or Transplant, Cloning or any other form of genetic manipulation not herein specified, shall not be eligible for registration." In 2002, the American Quarter Horse Association, which is the largest breed registry in the United States, finally approved unlimited registration of foals from a single mare during a given year using embryo transfer (Squires, 2003). Currently, AQHA allows unlimited transfers, as long as the donor mare has been annually enrolled in the embryo transfer program and the proper fees are paid. Within the last six months, AQHA has changed its policy and now accepts transfers from frozen embryos. The fact that a foal is produced through embryo transfer will be listed on its registration certificate (AQHA, 2007).

Other major breed organizations in America have followed the example of the Quarter Horse Association. The American Warmblood Registry requires donor mares to be registered, allows an unlimited number of embryos, but also requires registration of embryos at the time of harvesting in addition to notification at birth (AWR, 2007). Embryos must be implanted within three days of collection in Arabian registry. Enrollment of the donor mare is also required (AHA, 2007). The American Morgan Horse Association allows both frozen and non-frozen embryo transfer. Mares are not required to be enrolled in a specific program as long as their DNA is on file with AMHA. Signatures of both the sire and dam owners are required and transfer papers are required if the ownership of the embryo changes hands (AMHA, 2007).

Equine Embryo Freezing

Chronological Progression of Techniques. Much of the research in cryopreservation of equine embryos has occurred in the last twenty-five years with the advent of successful freezing of both semen and embryos in the bovine species. Originally bovine media and freezing protocol was used in horse embryos (Allen, 2005). As mentioned earlier, the size of an equine embryo when it enters the uterus presents difficulty in freezing due to intracellular ice crystal formation. A study done at Texas A&M University in 1987 specifically studied the morphological changes that occur in equine embryos when exposed to cryopreservation and cryoprotectants. Day 6 embryos from 15 Quarter-type mares were used in the experiment. Electron microscopy was used to determine damage to intracellular organelles of the embryos. The results showed that complete re-expansion of the embryo was not observed in the freezing medium or the holding medium and changes in lipid droplets and mitochondrial degeneration was observed in the inner cell mass of the glycerol-treated embryos (Wilson, 1987). This study is an example of work during this time period that merely continued to prove that higher success rates were achieved in morulae or early blastocysts that were less than 300 µm in size (Allen, 2005). Since then a popular hypothesis is that the difficulty in freezing equine embryos is due to the inability of the cryoprotectant to diffuse into the blastocoelic cavity because of the embryonic capsule which surrounds the embryo. LeGrand used trypsin to partially digest the embryonic capsule to allow diffusion of a cryoprotectant (LeGrand, 2002). In 1994, a study was done to determine the difference of viability of

equine embryos under two different freezing protocols. They found no difference in embryos plunged into liquid nitrogen at -30° or -33°C. However, analysis of morphology of the embryos showed that morula and early blastocyst stages of development showed better quality and more potential to survive (Poitras, 1994). Stout et al. (2005) showed that the formation of the acellular glycoprotein embryonic capsule in horses is instrumental to the survival of an embryo *in vivo*. None of the treatment embryos that had the capsule removed survived into a pregnancy where as four out of six control embryos in which no alteration was made to the embryonic capsule lived (Stout, 2005).

More recently there has been significant work to pinpoint the exact cellular problems with cryopreservation. In 2005, Tharasanit concentrated his research on larger embryos that were more likely to sustain damage during the freezing process. He found that cell damage was predominantly the effect of freezing and thawing rather than the cryoprotectant used and those blastocysts with thicker capsules were less likely to experience this trauma (Tharasanit, 2005). In addition to these findings, more evidence has been gathered to support the theory that smaller equine embryos survive vitrification and cryopreservation better than larger embryos (Eldridge-Panuska, 2005).

Freezing Media and Cryoprotectants. The use of different cryoprotectants has a definite effect on the viability of equine embryos. Ethylene glycol and sucrose were examined in a Japanese study in 1996. The results of this trial indicated that the combined use of ethylene glycol and sucrose in a 2-step freezing regimen yielded an acceptable pregnancy rate when frozen-thawed embryos were directly transferred to recipient mares (Hochi, 1996). Vitrification of embryos is much more difficult to achieve successfully than slow-cooling them. One study (Lagneaux, 1997) compared embryos

that were cooled slowly to those that were vitrified and stored in liquid nitrogen. Embryos were frozen with anti-freeze protein alone or with glycerol combined in a solution with the anti-freeze protein. No significant difference was found in any of the cryoprotectants used in the study. However, there was a significant trend of increasing dead cell area in the freezing group when compared to the cooling group (Lagneaux, 1997). Colorado State University researched the possibility of using methanol as a cryoprotectant. Although this study showed more re-expansion of the embryo than when glycerol was used as a cryoprotectant, the survival of embryos past Day 16 of pregnancy was not enough to be feasible in an industry setting (Bass, 2000). Colorado State University also researched the viability of vitrified embryos following an open pulled straw, cryoloop, or conventional slow cooling methods. No significant difference was found between the three methods (Oberstein, 2000).

Success of Embryo Freezing and Current Technology. Current techniques in equine embryo freezing continue to struggle against the difficulties that come with freezing large embryos (those that are greater than 300µm in size). The greatest success has been achieved with embryos less than 300 µm in size (Allen, 2005). Vitrification procedures have also not been successful in protecting large equine embryos from the intracellular damage associated with freezing and thawing (Squires, 2003). The embryonic capsule is necessary for the survival of the embryo and is thought to have an effect on when the embryo sheds the zona pellucida (Stout, 2005). A recent study (Hudson *et al.*, 2006) from Colorado State University compared the differences between immediate vitrification of embryos to holding the embryos in a cooling device (5-8°C) before vitrification. There were no significant differences in the pregnancy rates of

embryos cooled before vitrification and those which were directly vitrified. However, all of these embryos were classified as small because of previously mentioned complications associated with large equine embryos (Hudson, 2006). Cryopreservation of equine embryos continues to be the subject of many current studies. Although, as mentioned earlier, there is some reluctance on the part of major breed organizations to accept foals who are a product of frozen embryos for registration.

Porcine Embryo Freezing

Many of the same problems that are associated with freezing equine embryos are also problems when freezing porcine embryos. The difficulties associated with pig embryos include sensitivity to hypothermic conditions and cellular disruption in response to freezing. It is for these reasons that pigs were used as a model for equine embryos in this experiment. The following portion of this review of literature will include a brief history of porcine vitrification including the techniques, problems, and successes associated with it.

Chronological Progression of Techniques. Cryopreservation and hypothermic storage of porcine embryos was first studied in the 1970s. However, it has not been until the last 10 years or so that any effective methods of freezing porcine embryos have been found (Dobrinsky, 2002). The reduced viability of pig embryos during culture, and the inability, until recently, to preserve them through cryopreservation has limited technologies available to pigs compared to other livestock species such as cattle, sheep, and goats (Polge, 1985). Cryopreservation offers significant opportunities to swine producers such as preservation of maternal genetic material, global genetic transport,

breeding line regeneration, and genetic resource rescue. There is also the valuable asset of bringing new genetic material into a herd without the health risks associated with live animals (Dobrinsky, 2001). Pig embryos are very sensitive to cold conditions obviously found in cryopreservation which seems to be related to damage in the embryonic cytoskeleton (Dobrinsky, 1997).

Early embryo freezing in pigs showed an interesting trend. Development to the expanded blastocyst stage was essential for survival and live births. Initial research in freezing porcine embryos showed limited live births. However, this established the fact that the process could be successful in spite of the difficulties associated with cryopreservation (Dobrinsky, 2001). The high lipid component of swine embryos compared to those of other livestock species was first hypothesized as a hindrance to cryopreservation by Polge et al in the mid 1970s (Polge, 1974). It was not until 20 years later that the delipation of porcine embryos proved to be beneficial to freezing (Nagashima, 1994). The intolerance of boar semen to freezing also led some to believe that difficulties surrounding this process might be species specific (Dobrinsky, 2002). Vitrification was also suggested as an alternative for freezing pig embryos with the theory that rapid cooling would not allow the intracellular ice crystal formation that disrupts the integrity of the embryo. Cryoprotectants were also introduced but many that were acceptable in other species were found to be toxic to swine embryos (Dobrinsky, 2001). The most recent advances in the embryo cryopreservation of swine include success in freezing pig morulae and development of open pulled straw (OPS) techniques that increase the rate at which vitrification can occur by increasing the surface area and cooling rate, thus increasing survival rate (Dobrinsky, 2002).

Problems Associated with Vitrification in Swine Embryos. As mentioned previously there are several main problems associated with the vitrification of porcine embryos. These include the high lipid content of the embryo, embryonic cytoskeleton disruption, and the stage of the embryo at time of freezing which will be covered in a subsequent section. Pig embryos exhibit an extremely high sensitivity to hypothermic conditions which was verified by early research. Embryos that were cooled to 15 or 20°C established pregnancy while those that were cooled to 5 or 10°C did not. In subsequent research 15°C was identified as the temperature at which damage occurred (Dobrinsky, 2002). The high lipid content of porcine embryos has been hypothesized as part of the reason for the sensitivity of the embryos to hypothermic conditions. In theory, this slows the rate of cooling which enhances the formation of intracellular ice crystals and prevents the penetration of cryoprotectants. Cryoprotectants may not protect embryonic lipids or prevent the phase changes that render these lipids unusable or toxic to porcine embryos. Delipation of the embryos has been very successful in increasing the success rate of pig embryo cryopreservation. Nagashima (1994) proved that delipation was beneficial to the freezing process. He hypothesized that removal of the lipid content resulted in changes in the lipid composition of the plasma membranes present in the embryos and thus delipation limited the phase separation that was present in the control embryos (Dobrinsky, 2001). The cytoskeleton is extremely important to the structural integrity of an embryo. As mentioned previously, cryopreservation is an extremely harsh process on embryos. Formation of ice crystals lyses cell membranes and the process of freezing in liquid nitrogen can denature intracellular function and organelles. The cytoskeleton of an embryo is a network of protein constituents that is distributed

throughout the cytoplasm of the cell. The function of this structure is to form an internal framework that impacts cell shape, cell movement, and cell division. Unfortunately, cryopreservation and cryoprotectants have an adverse effect on the cytoskeleton and can cause disruption of the microfilaments. Therefore, they can be lethal to embryos through disruption of plasma membranes and a lack of the blastocoelic cavity reformation (Dobrinsky, 2001). Vitrification was developed as a technique to rapidly freeze mammalian embryos in order to prevent ice crystal formation. A 1994 study suggested that porcine embryos could be successfully vitrified and found that there was reexpansion of the blastocoelic cavity in vitrified porcine embryos although this was dependent on the stage of the embryo at vitrification (Dobrinsky, 1994). Open pulled straw (OPS) vitrification was developed as a way to vitrify swine embryos by decreasing the diameter in the OPS and increasing the surface area which is then exposed to the liquid nitrogen and thus increasing the rate at which vitrification occurs. There has been great success with that method and, while further research is needed, this should make cryopreservation of porcine embryos much easier (Dobrinsky, 2001).

Stage of Embryo at Vitrification. One universal trend in cryopreservation in swine is that the stage of development of the embryo has a definite effect on its successful cryopreservation. A fairly recent study compared cooling rate on the viability of porcine embryos of several different stages. Vitrified expanded blastocysts showed a survival and hatching rate similar to fresh expanded blastocysts. However, fresh morulae and early blastocysts showed better survival and hatching rates than any of the other groups (Cuello, 2004). Another study suggested that the inner cell mass (ICM) damage in porcine embryos was much lower at the hatched stage than at the expanded stage

(Iwaski, 1994). Advanced stage hatched blastocysts (≥300µm) have also been identified as more difficult to freeze (Nagashima, 1992). One of the most recent studies in porcine embryo cryopreservation investigated the effect of the batch, which is the group of embryos obtained from one donor at a time, on cryotolerance. The viability of hatched blastocysts did not differ among batches but there was a correlation between the overall grade of the batch and the survivability of the embryos in expanded blastocysts (Fujino, 2006).

Future of Porcine Embryo Cryopreservation. Over the last 10- 20 years great strides have been made towards successful, affordable cryopreservation of pig embryos. Hopefully in the future the most successful stage of embryonic development for cryopreservation can be identified and more methods to vitrify embryos can be accomplished. Many barriers must be overcome in order for cryopreservation of swine embryos to be successful in the future. Late morula and early blastocysts are the most successful stages of embryos for freezing. However, these are the stages with which the most difficulties are associated. Also, a dependable form of nonsurgical transfer is also needed in order for embryo transfer to be affordable to the producer (Dobrinsky, 2001). There is an undeniable need for the preservation of genetic material through embryonic cryopreservation. Although pigs are behind other livestock species in this department, great strides have been made recently and will continue to improve in the future.

Conclusion

Of all livestock species, horses and pigs share the dubious honor of being the most difficult when it comes to preserving embryos especially when compared to cattle. While there are differences associated with each species, there are many similarities that

are shared, such as problems with intracellular ice crystal formation that hinder dependable successful vitrification procedures. It is for this reason that swine served as a model for horses as part of this study.

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Chapter 3

A NOVEL APPROACH TO CRYOPRESERVATION OF EQUINE AND PORCINE EMBRYOS BY VITRIFICATION AFTER BLASTOCOELIC MICROMANIPULATION

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Abstract

Vitrification of equine embryos shows fairly consistent success rates when they are frozen at ≤ 300µm or about 6.5 d after ovulation. In this study, the effects of the reduction of blastocoelic fluid and the microinfusion of a cryoprotectant prior to vitrification were examined on 8 d embryos. The equine embryos used for this project were 805µm (1), 820µm (2), 1120µm (3), 1286µm (4), and 979µm (5). All embryos were graded excellent (1) according to IETS guidelines. The embryos were either assigned to a control group in which no micromanipulation occurred (1-3) or to the experimental group which entailed microinfusion of VS1 (1.4 M glycerol in PBS; 4-5) after aspiration of blastocoelic fluid before microinfusion of VS1 (5). During the following vitrification procedure, the embryos were exposed to VS1 and 2 (1.4M glycerol, 3.6M ethylene glycol in PBS) for 5 minutes, VS3 (3.4M glycerol, 6.6M ethylene glycol in PBS) for 1 minute. The embryo in VS3 was then loaded into a 0.25 ml straw, separated by two air bubbles from columns of 0.5 M galactose. The straws were placed in a cooled plastic goblet surrounded by liquid nitrogen vapors for 1 minute and then finally immersed into the liquid nitrogen. Digital pictures were taken throughout the process. The equine embryos failed to yield a pregnancy with the exception of one (#5) which formed an embryonic vesicle at d 15 after ovulation. Ultrasonography at d 28 revealed resorption of the embryo, probably due to heat stress. Due to the low number of equine embryos recovered, porcine embryos were used as a model in another trial. Vitrification of 44 porcine embryos was attempted using identical procedures to those described above. The porcine embryos were cultured for a period of 24 hrs and digital images were also taken. Twenty-six embryos were frozen using the control method and

20 were assigned to the experimental group and treated as described earlier. Initial re-expansion was complete in 16 of the treated and 7 of the control embryos. This showed a significant difference in treatment and control at thaw ($p<.0004$). All embryos were completely dead at 24 hrs

Introduction

Vitrification of equine embryos show fairly consistent success rates when they are frozen at ≤ 300µm or about 7 d after ovulation. However, since an equine embryo does not enter the uterus until 6.5 days after ovulation, flushing an embryo of this size is not always possible. Vitrification of large equine embryos typical of those recovered at d 8 after ovulation is not consistently successful [1]. In this study, the effects of the reduction of blastocoelic fluid and the passive diffusion of a cryoprotectant prior to vitrification were examined. Transfer done with fresh embryos at the expanded blastocyst stage is fairly successful [2]. However, suitable recipients are not always available which is why a successful method of cryopreservation is needed for these embryos. Because of the difficulty in obtaining large numbers of equine embryos, this study was repeated using porcine embryos as a model.

Materials and Methods

The University of Georgia Animal and Use Committee approved all procedures used in these experiments (A2004-10167-c1).

Trial 1

Embryo Recovery. Quarter-type mares (n=10) from 5-20 years of age were used as embryo donors during 23 estrous cycles. During estrus, mares were examined daily through rectal ultrasonography. When a follicle ≥35mm was detected, mares were artificially inseminated every 24-48 hr until ovulation was observed. Rectal ultrasonography was performed daily during the estrous cycle to determine ovulation (d 0 = day of ovulation). Motility of the cooled semen was checked again prior to artificial insemination. On d 8 post ovulation, embryo recovery was performed by uterine flush with 6-8 L of Equipro Recovery Medium (Minitube, Verona, WI). 500 mg of Flunixin Meglumine® (Banamine, Omaha, NE) were given intravenously 1 hr before flushing began. A 100 cc minitube embryo catheter and an EZ Way embryo filter were used to completely extract the embryo form the uterus. Oxytocin (0.2 ml) (Oxytocin Injectable Solution) was administered subcutaneously to stimulate uterine contractions towards the end of the flush. After flushing a stereomicroscope (10-15 xs) was used to find the embryo. An average of 2.6 flushes were performed on each mare.

Following embryo recovery, the embryos were washed using 6 drops of holding medium (Vigro Holding Plus). They were then morphologically graded from 1-4 (Table 1) and randomly assigned to either the control or treatment groups. Digital images (Moticam 2000, Micorscope Depot, Tracy, CA) of all embryos were taken for analysis.

Freezing Methods. Upon recovery, the control embryos were subjected to 5 min in Vitrification Solution 1 (VS 1- 1.4 M glycerol in PBS), followed by 5 minutes in VS 2 (1.4 M glycerol + 3.6 M ethylene glycol) and transferred into VS 3 (3.4 M glycerol + 6.6 M ethylene glycol) before being loaded into a 0.25 ml straw (Equine Vitrification Kit, Bioniche, Athens). Individual embryos were loaded into the center of 0.25 ml straws separated by two air bubbles from columns of 90 µl 0.5 M galactose at the straw ends (Diluent Solution Equine Vitrification Kit, Bioniche, Athens, GA). Total time from the transfer into Vitrification Solution (VS 3) until the embryos were loaded into the straw did not exceed one min. The straws were then placed in a cooled plastic goblet (10 mm x 120 mm, Minitube, Verona, WI) surrounded by liquid nitrogen vapors for 1 min. Finally the goblet was submerged in liquid nitrogen and remained there until transfer to the recipients.

Prior to vitrification procedures described for control embryos, treatment embryos had their embryonic capsule penetrated by a laser to allow aspiration of the blastocoelic fluid. They were then placed in VS 1 where the hole in the capsule allowed passive diffusion of VS 1 into the cavity of the embryo. A standard manipulator (Eppendorf TransferMan NK) was attached to an inverted microscope (Zeiss Axiovert 10) and pre-manufactured microcapillaries were used for holding and microinjecting the embryos (Eppendorf VacuTips, Embryo Transfer Tips). Following micromanipulation of the embryo, freezing procedures of treatment embryos were identical to the control procedures. Of the 5 embryos, 3 (#1-3) were frozen according to control procedures, one (#4) was frozen according to experimental procedures without the aspiration of

blastocoelic fluid being accomplished and one (#5) was frozen exactly according to experimental procedures.

Thawing and Transfer of Embryos. Quarter-type mares (n=4) recipients which had ovulated within 8 days previously were used for transfers. A synthetic progestin (Regumate) was administered orally from 1 d prior to transfer until 21 d after transfer. Intravenous administration of 500 mg of Flunixin Meglumine® (Banamine, Omaha, NE) was accomplished 1 hour prior to transfer.

Straws containing the embryos were warmed in air at room temperature (22-24°C) for 5 s and then immersed in a waterbath at 30°C for 15 s. Straws were then dried with a paper towel and contents were emptied into a 35 ml Petri dish (Minitube, Verona, WI) and mixed. After five minutes embryos were transferred to a 35 ml Petri dish (Minitube, Verona, WI) containing holding medium (Vigro Holding Plus). The embryos were then loaded into an AI pipette (Animal Reproduction Systems, Chino, CA) and single embryos were non-surgically transferred into the uterus of the recipients.

Pregnancy Determination. Recipient mares were examined by ultrasonography (Aloka 500, Choice Medical Systems, St. Petersburg, FL) one week after transfer (d15 after ovulation) to visualize embryonic vesicles. Positive pregnancy diagnosis was then confirmed by repeated scanning at day 28.

Trial 2

Embryo Recovery. Cull sows (n = 2) were serviced naturally twice in 48 hr period. Eight days after the first breeding (d 0), they were transported from the farm to the University of Georgia Meat Laboratory and harvested. At harvest the reproductive

tract was immediately removed and transported to the laboratory for recovery. Embryos were flushed with 30-60 ml of Equipro recovery medium from the oviduct and uterine horn into an EZ Way embryo filter and a sterile, gridlined Petri dish (Minitube, Verona, WI). Embryos were located under a microscope and washed in the recovery medium before transfer to the micromanipulation lab for grading and micromanipulation. Embryos were graded using the IETS guidelines from 1-4. Digital pictures (Moticam 2000, Microscope Depot, Tracy, CA) were taken of the embryos for analysis. Sow A yielded 22 embryo. Sow B yielded 4 embryos. Sow C yielded 6 embryos and Sow D yielded 16 embryos.

Freezing Methods. Upon recovery, the control embryos were subjected to 5 min in Vitrification Solution 1 (VS 1- 1.4 M glycerol in PBS), followed by 5 min in VS 2 (1.4 M glycerol + 3.6 M ethylene glycol) and transferred into VS 3 (3.4 M glycerol + 6.6 M ethylene glycol) before being loaded into a 0.25 ml straw (Equine Vitrification Kit, Bioniche, Athens). Individual embryos were loaded into the center of 0.25 ml straws separated by two air bubbles from columns of 90 µl 0.5 M galactose at the straw ends (Diluent Solution). Total time from the transfer into VS 3 until the embryos were loaded into the straw did not exceed 1 min. The straws were then placed in a cooled plastic goblet (10 mm x 120 mm, Minitube, Verona, WI) surrounded by liquid nitrogen vapors for 1 min. Finally the goblet was submerged in liquid nitrogen and remained there until transfer to the recipients.

Prior to the procedures described for control embryos, treatment embryos had their zona pellucida first penetrated by a laser to allow aspiration of the blastocoelic fluid. Passive diffusion of VS 1 into the cavity of the embryo took place when the embryo was

placed into VS1. A standard manipulator (Eppendorf TransferMan NK) was attached to an inverted microscope (Zeiss, Axiovert 10) and pre-manufactured microcapillaries were used for holding and microinjecting the embryos (Eppendorf VacuTips, Embryo Transfer Tips). Following micromanipulation of the embryo, freezing procedures of treatment embryos were identical to the control procedures.

Embryo Thawing and Culturing. Porcine embryos were thawed and then cultured in a modified NCSU media (Table 1). Straws containing the embryos were warmed in air at room temperature (22-24°C) for 5 s and then immersed in a waterbath at 30°C for 15 s. Straws were then dried with a paper towel and straw contents were emptied into a Petri dish and mixed. After swine embryos were transferred to the media and held in an incubator until further observation. Re-expansion of the blastocoelic cavity was used to evaluate the viability of embryos. Embryos were examined under a stereomicroscope (Wesco) at 40x magnification at thaw, 6 hrs post thaw, and 24 hrs post thaw.

Table 3.1
Embryo Grading Scale
(Callesen, *et al.*, 1995)

1-Excellent. Spherical appearance and uniform cell size, color and texture.
2-Good. Minor imperfections. Trophoblastic separation, irregular shape.
3-Fair. Extruded blastomeres, degenerate cells, and collapsed blastocoele.
4-Poor. Collapsed blastocoele, many extruded blastomeres and degenerate cells.

Table 3.2 NCSU Culture Media
(Long *et al*., 1998)

	mM	M	FW	g/L	250 ml
NaCl	108.73	0.10873	58.44	6.3541812	1.5885 g
KCl	4.78	0.00478	74.55	0.356349	89.0873mg
CaCl2	1.7	0.0017	147	0.2499	62.4750 mg
KH2PO4	1.19	0.00119	136.13	0.1619947	40.4987 mg
MgSO4	1.19	0.00119	120.4	0.143276	35.8190 mg
NaHCo3	25.07	0.02507	84.01	2.1061307	0.5265g
Glucose	5.55	0.00555	180.16	0.999888	0.2500 g
Sodium Lactate	2.73	0.00273	112.1	0.306033	76.5083 mg
Sodium Pyruvate	0.17	0.00017	100 mM	1.7 ml	0.4250 ml
Glutamine	1	0.001	146	0.1461	36.5250 mg
BSA	4 mg/ml			4	1.0000 g

Statistical Analysis. Data was analyzed using statistical procedures from SAS V 9.1 (Cary, NC). Proc GLM was used to determine the effect of date, size, and treatment on the embryos and interactions between size and treatment, size and date, and date and treatment were also taken into consideration. Equine embryo data was not analyzed due to insufficient data. Statistical significance was declared at P value less than or equal to 0.05 and a trend towards significance was declared at a P value less than or equal to 0.10.

Results and Discussion

Equine Results. Four out of the five equine embryos that were recovered were transferred into recipient mares and examined by rectal ultrasonography at d 15. None of the control embryos (#2 and #3) resulted in a pregnancy. The treatment embryo (#4) which underwent injection of the cryoprotectant also did not result in a vesicle at d 15. However, the treatment embryo (#5) which had the blastocoelic fluid aspirated from it before microinjection of the cryoprotectant did result in a vesicle which was diagnosed by ultrasound at d 15. Unfortunately, when the mare was examined again at 28 days, she had absorbed the vesicle. The loss of this pregnancy was thought to be due to heat stress since the transfer took place in late May and summers in Georgia can be quite hot. The highest mean max temperature for May is 86.8 according to data collected from the Athens airport. The data from this trial was not analyzed with SAS due to the extremely small number of embryos obtained.

Figure 3.1 – Equine Control Embryo #2

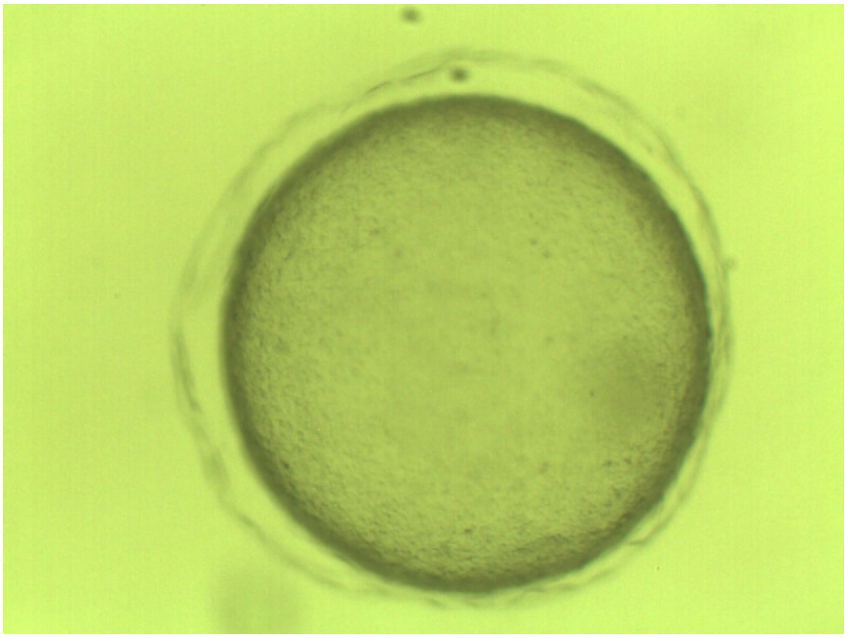


Figure 3.2-Equine Control Embryo #2 Post Thaw

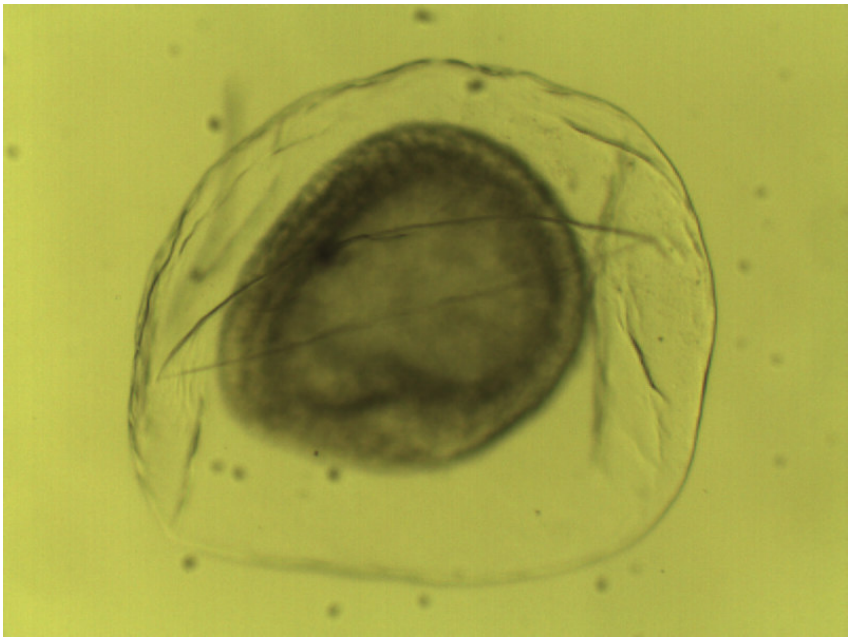


Figure 3.3-Equine Control Embryo #3

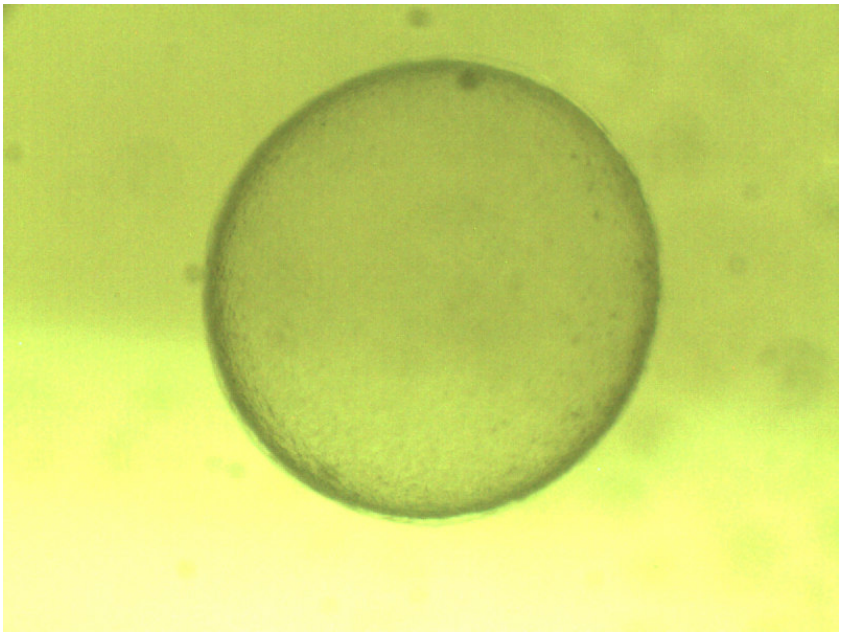


Figure 3.4-Equine Control Embryo #3 Post Thaw

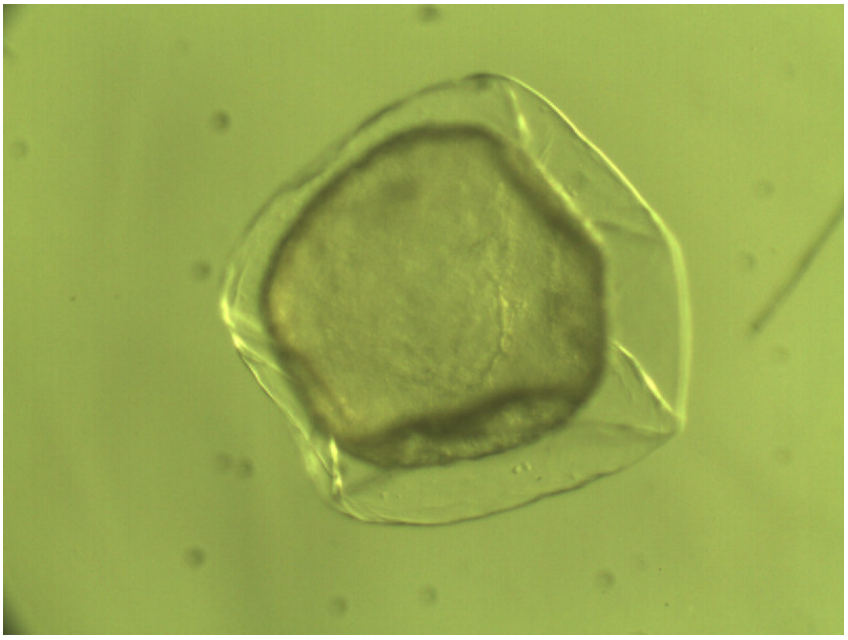


Figure 3.5-Equine Embryo #4

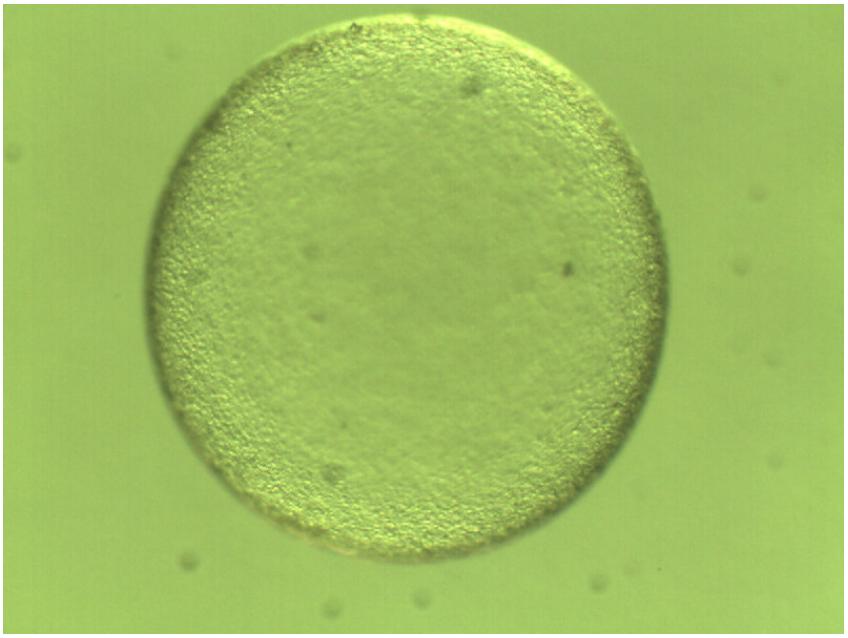


Figure 3.6- Equine Embryo #4 Post Thaw

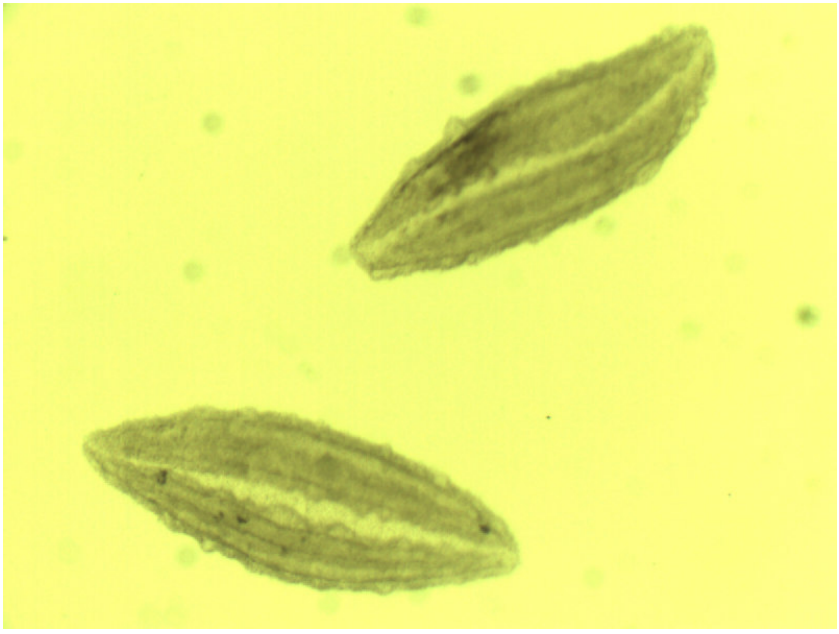


Figure 3.7-Equine Embryo #5

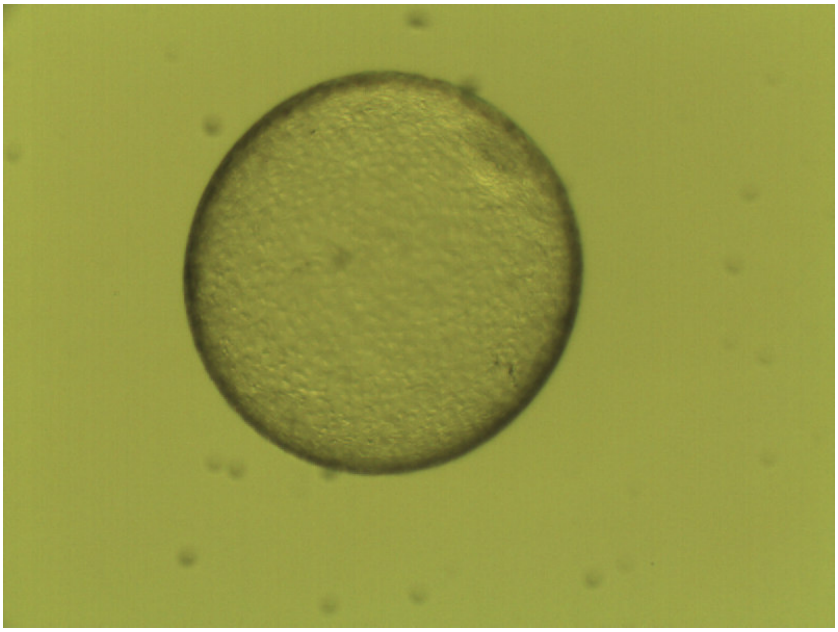
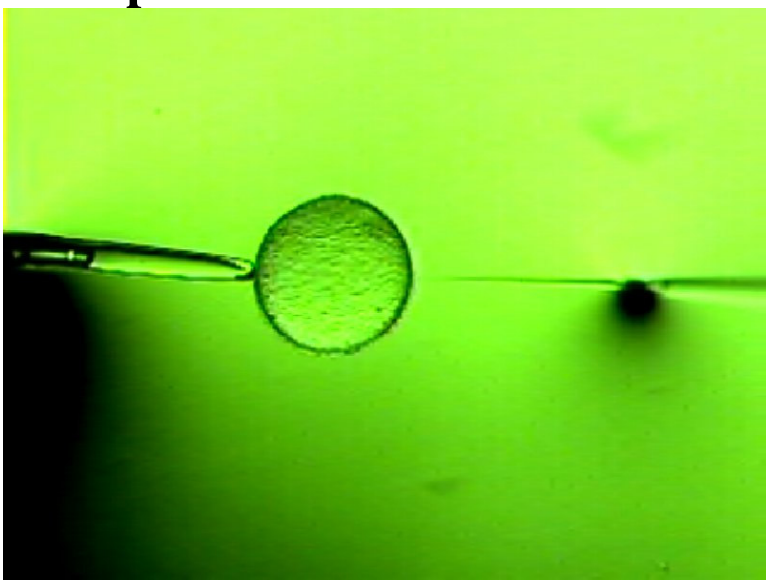


Figure 3.8-Equine Embryo #5 During Manipulation



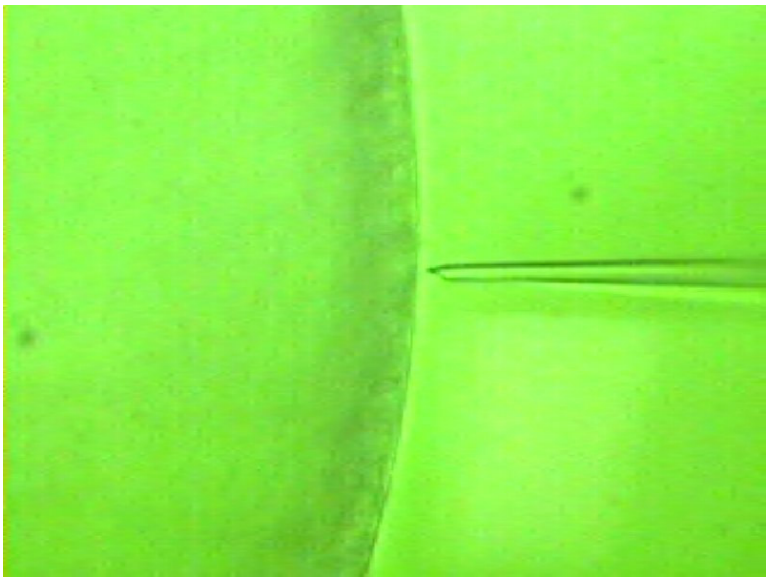


Figure 3.9-Equine Embryo #5 After Aspiration of Fluid

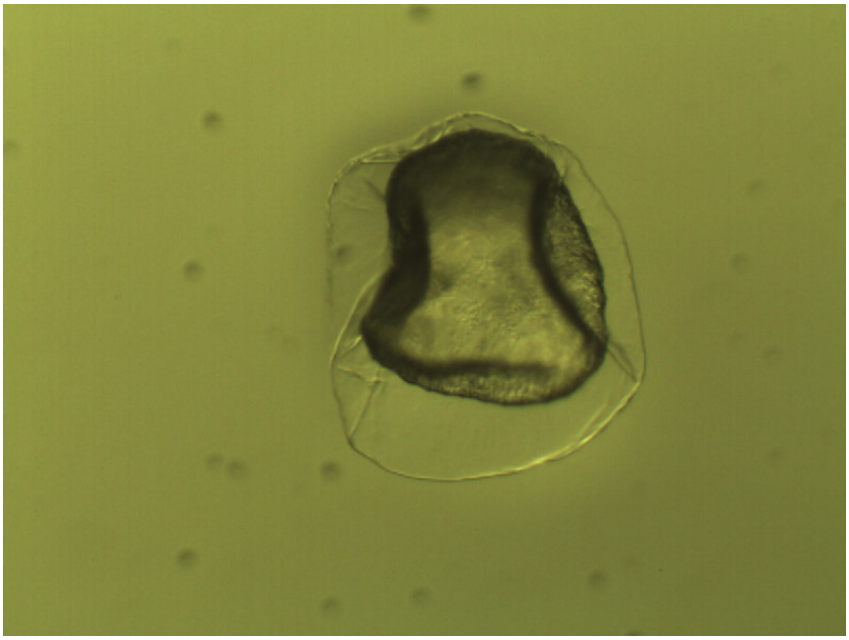
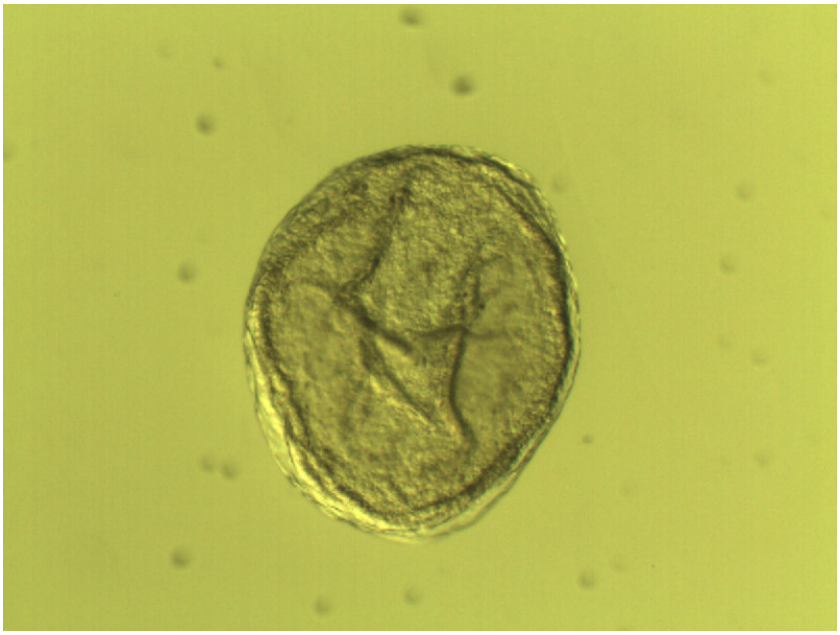
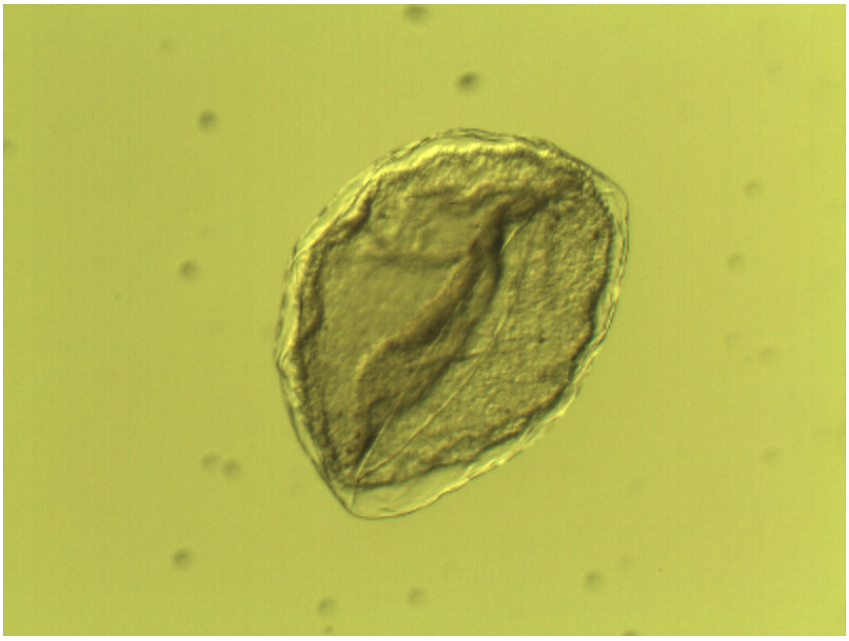


Figure 3.10 Equine Embryo #5 Post Thaw





Porcine Results. Of the original 48 porcine embryos, 2 did not survive the freezing process. Twenty-six embryos were frozen using the control method and 20 were assigned to the experimental group and treated as described earlier. Initial reexpansion at the thaw was complete in 7, partial in 12 and nonexistent in 5 of the control embryos. Two control embryos were lost due to a straw that cracked in the liquid nitrogen tank. Initial reexpansion was complete in 16 and partial in 4 of the 20 treated embryos. Examination at 6 hrs revealed complete degeneration of 10, partial degeneration of 10 and no signs of degeneration in 4 of the control embryos. The treatment group exhibited 3 embryos with no sign of degeneration, 9 embryos with partial degeneration, and 8 with embryos which were completely degenerated at 6 hrs. All embryos were completely degenerated at 24 hrs.

There was a difference ($P<0.0001$) between the treatment and control groups at thaw (Table 3.3). The treatment groups exhibited better blasotcoelic re-expansion. Size had an effect ($P<0.0017$) on the initial re-expansion of the embryos at thaw. Small embryos (<300µm) re-expanded better than the large ones (>300 µm). Date of harvest influenced ($P<0.0017$) the re-expansion at thaw. Embryos harvested on the second day showed better re-expansion than those harvested on the first day. There was no interaction between date and treatment or treatment and size at initial reexpansion. However, there was interaction between the size of the embryos and the date on which they were collected ($P<0.029$) possibly because of reproductive problems discovered in Sow B.

When analyzing the data concerning the status of the embryos at 6 hrs after thawing, there was no significant difference between the control and treatment groups.

Size was again taken into effect and had no significant effect on the status (measured by the stage of degeneration) of the embryos 6 hrs after thawing. The date on which the embryos were harvested had no effect on status at 6 hrs. However there were interactions between the date and treatment, the treatment and size, and the size and date of the embryos after 6 hrs in the culture media ($P < 0.0018$, $P < .0018$, and $P < .0030$ respectively).

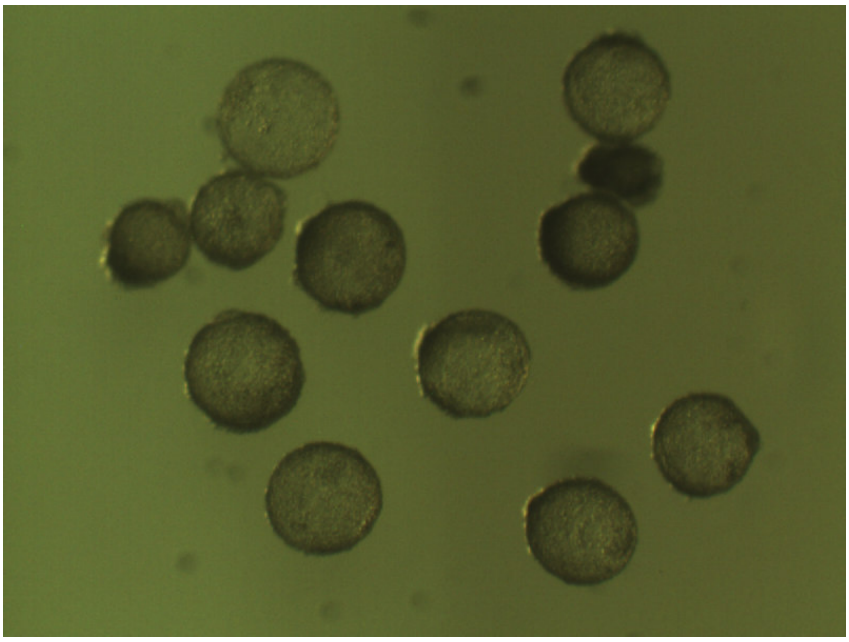
Table 3.3 P-Values of Variables in Swine Data

	Re-expansion P-value	6 Hr P-value
Treatment	<0.0001	0.8976
Size	<0.0017	0.7578
Date	<0.0017	0.0644
Trt*Size	0.1983	0.0018
Trt*Date	0.1983	0.0018
Size*Date	0.0290	0.0030

Table 3.4 LS Means of Variables in Swine Data

	Re-expansion LSMeans	6 Hr LSMeans
Treatment 1	2.124	2.158
Treatment 2	1.105	1.621
Size 1	1.797	1.916
Size 2	1.431	1.863
Date 1	1.369	2.488
Date 2	1.860	1.291
Date 1 Size 1	1.228	1.228
Date 1 Size 2	1.509	1.509
Date 2 Size 1	2.367	2.367
Date 2 Size 2	1.353	0.133
Treatment 1 Size 1	2.304	0.192
Treatment 1 Size 2	1.296	0.192
Treatment 2 Size 1	1.238	0.118
Treatment 2 Size 2	0.698	0.232
Date 1 Treatment 1	1.523	2.229
Date 1 Treatment 2	0.464	1.248
Date 2 Treatment 1	2.070	0.471
Date 2 Treatment 2	1.47	1.526

Figure 3.11 Day 1 Porcine Embryos



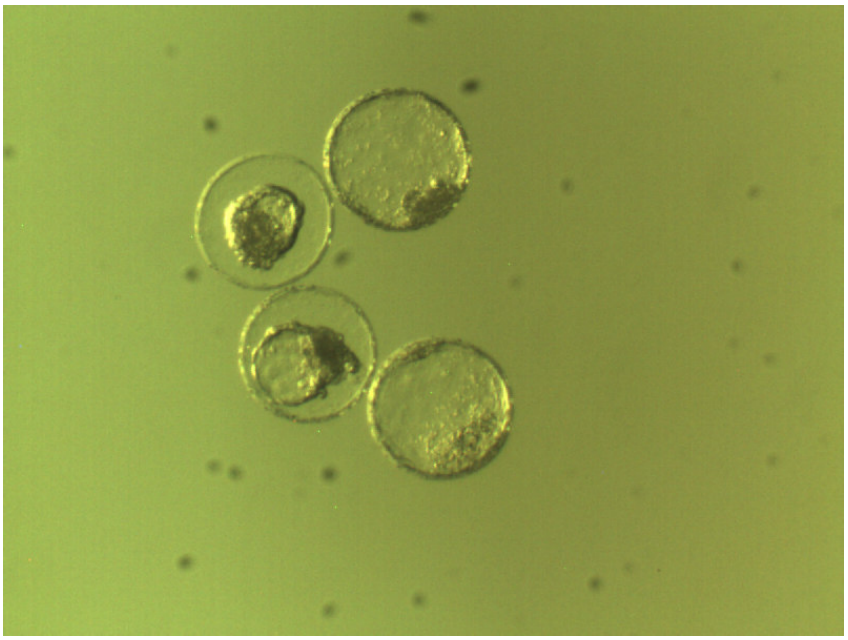


Figure 3.12 Day 1 Control Porcine Embryos in VS1

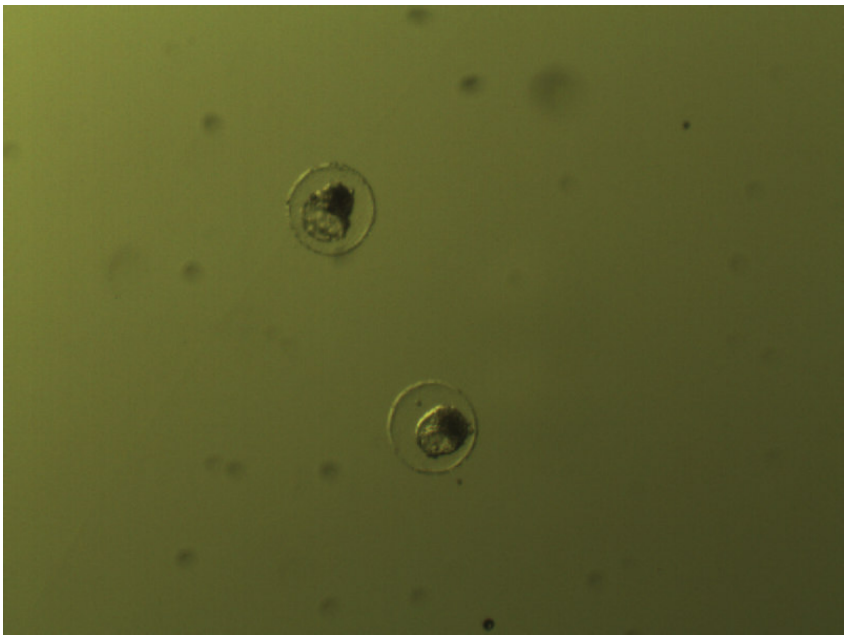


Figure 3.13-Day 1 Control Porcine Embryos in VS2

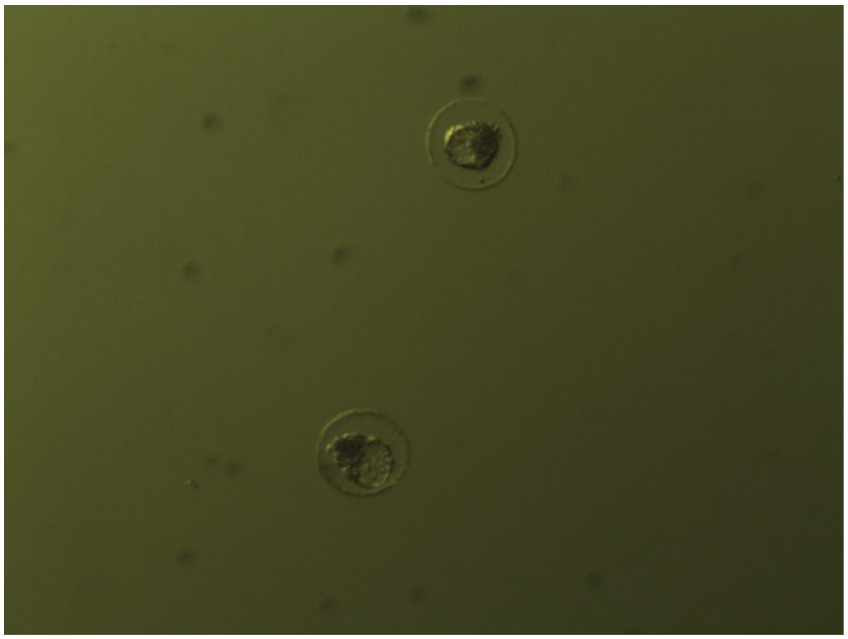


Figure 3.14 Day 2 Porcine Embryos

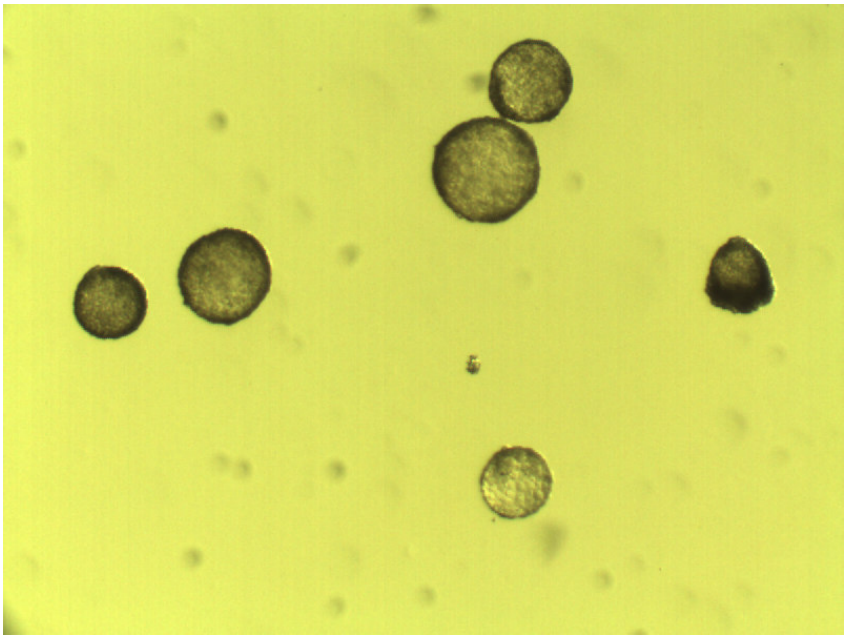
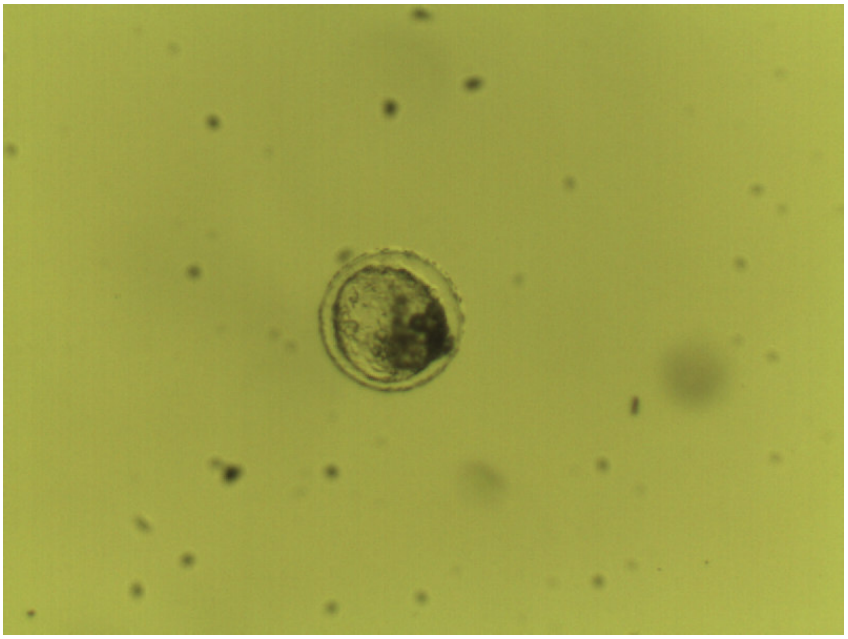


Figure 3.15-Porcine Control Embryo Post Thaw



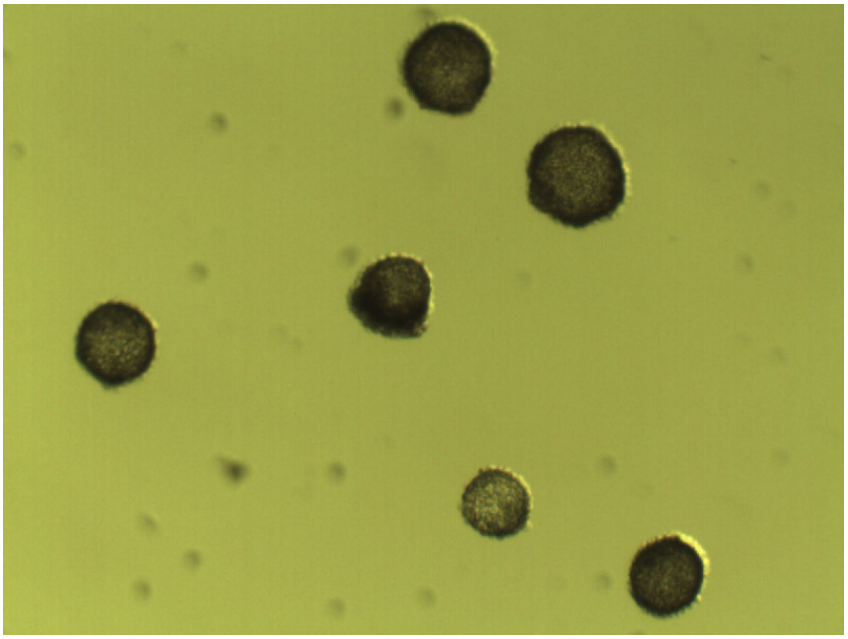
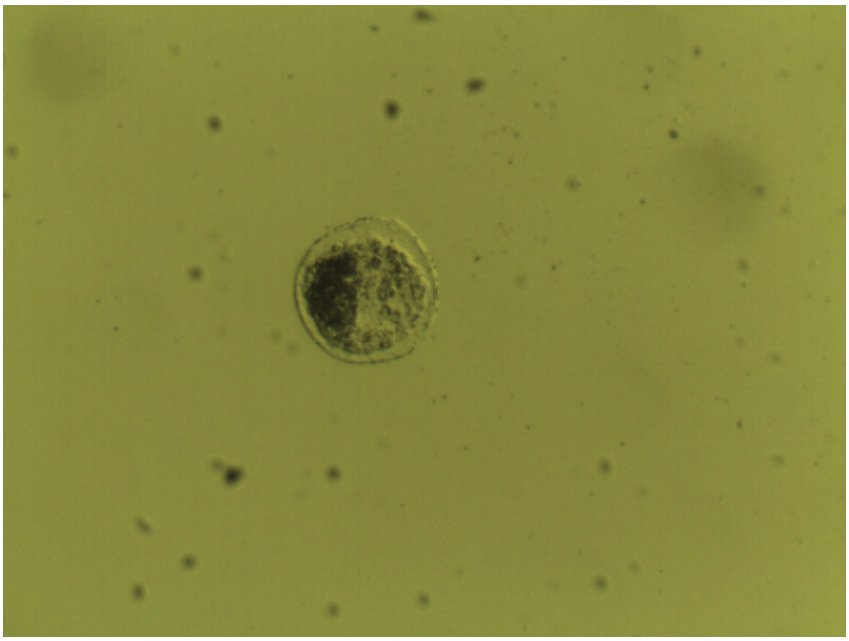


Figure 3.16 Porcine Treatment Embryo Post Thaw



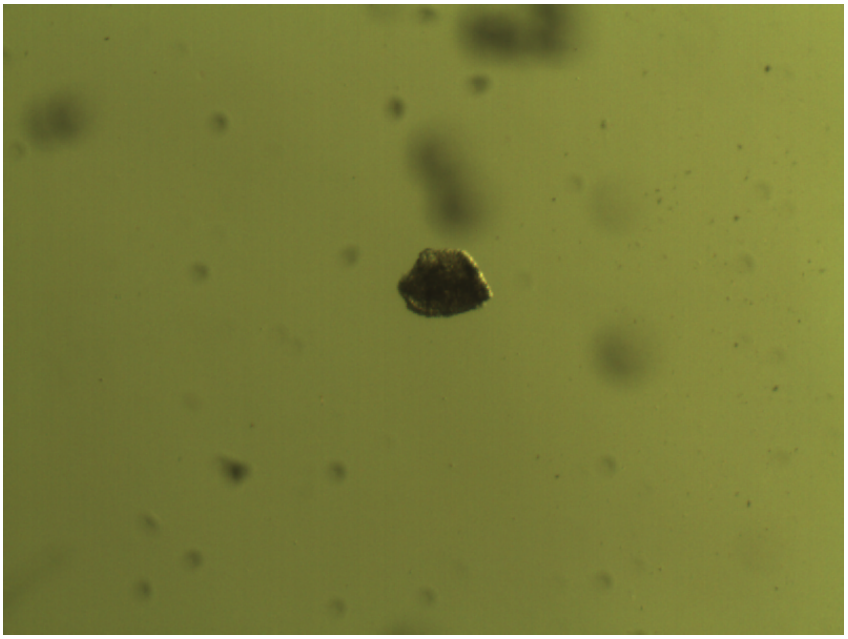
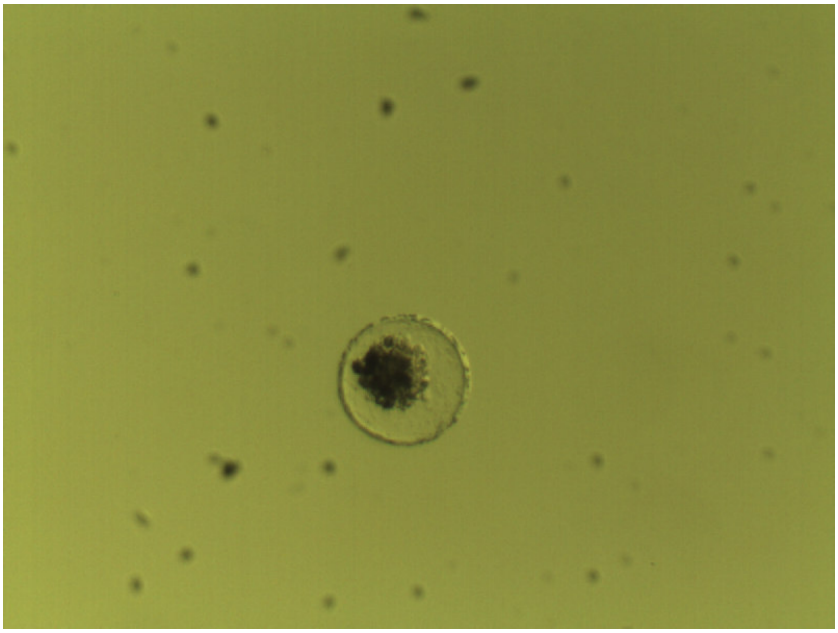


Figure 3.17 Porcine Control Embryos 6 Hours Post Thaw



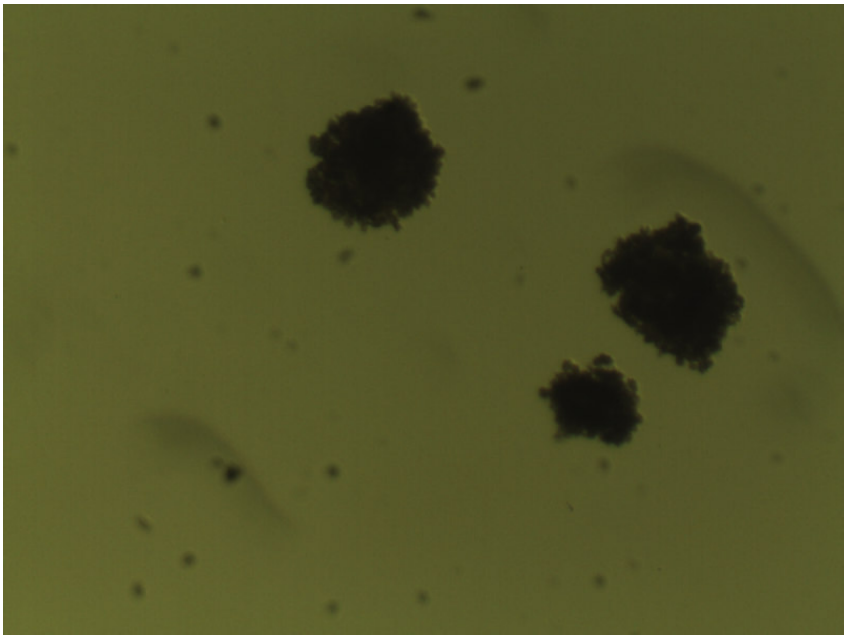
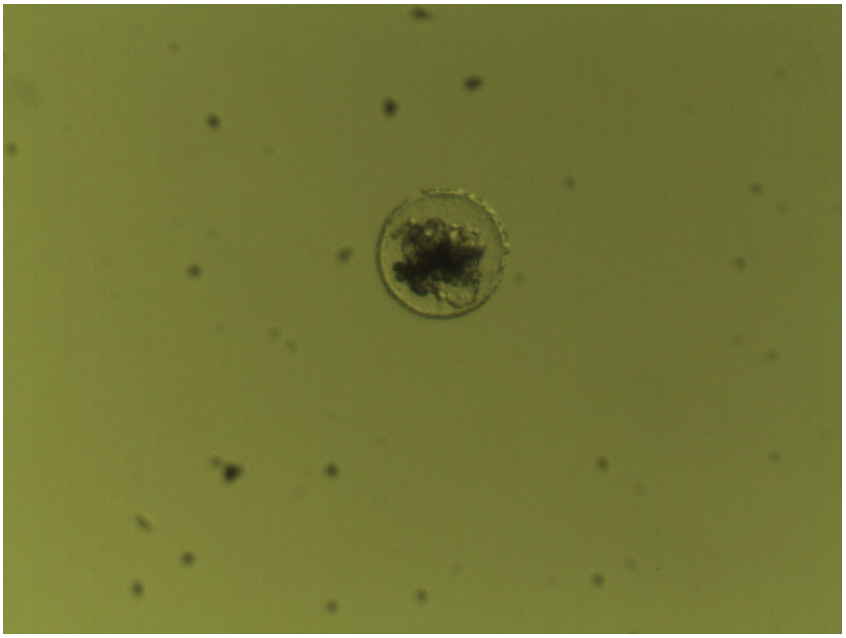


Figure 3.18 Treatment Porcine Embryos 6 Hours Post Thaw



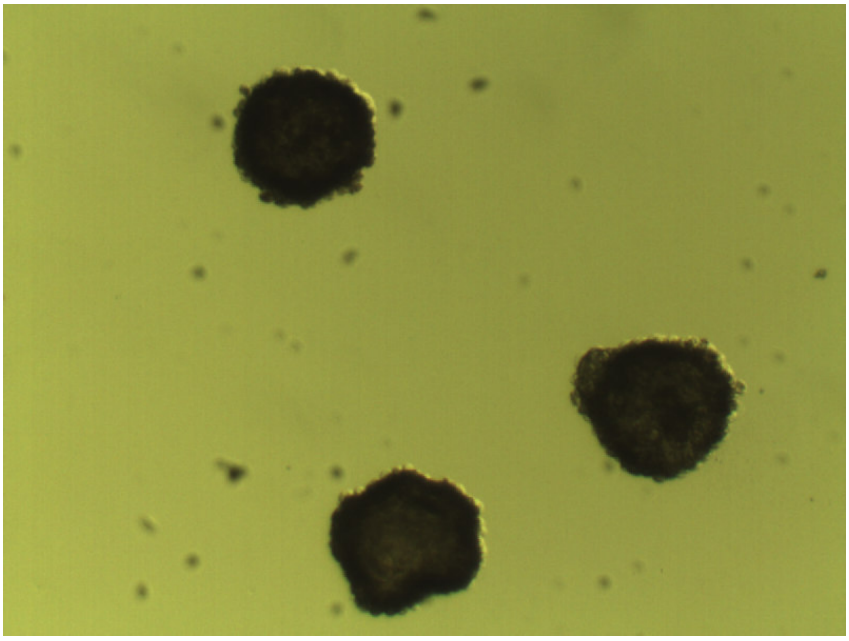


Figure 3.19 Treatment Porcine Embryo at 24 Hours

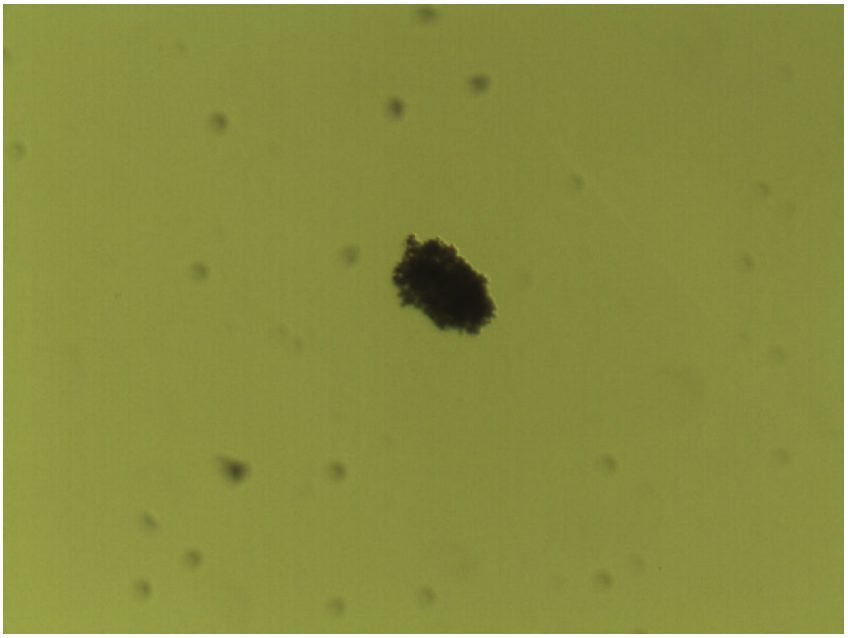
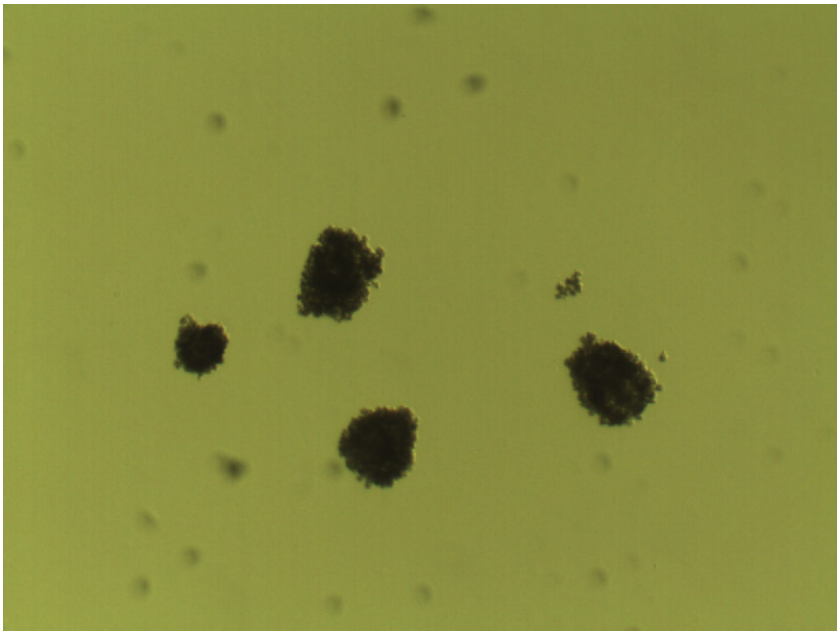


Figure 3.20 Control Porcine Embryos at 24 Hours



Equine Discussion. Unfortunately, there were not enough embryos collected from the mares that were flushed to produce any statistically relevant results about the treatment of the embryos. Normal embryo recovery rates are about 50% (Tharasanit, 2005; Hudson *et al.,* 2006). This study experienced embryo recovery rates significantly below the normal range. Superovulation was not attempted in this study. The number of ovulations resulting from a superovulatory agent, are not enough to be economical in an industry setting. (Squires and McCue, 2007; Alvarenga, 2001)). There are several factors that could have caused this result. The cooled semen that was used for each insemination was evaluated prior to and after insemination for motility and no problems were detected with quality that would inhibit pregnancy although this was this stallion's first year standing at stud. However, the best proof of a stallion's fertility is pregnancy rate in the mares that were covered and due to the fact that 2007 was the first year that he serviced mares this information was nonexistent (Pickett, 2000). Also, the number of services per mare ranged from 1-3 depending on the particular cycle. The average number of services per

cycle was 1.5. Any time foreign material is introduced into the reproductive tract there is the possibility of irritation or infection.

Research has not been done at this time to determine the effect of multiple flushes on a mare's uterus and reproductive tract. It is possible that irritation caused by this process had a detrimental effect on obtaining a pregnancy. Of the mares used in this study, only two contributed embryos. These mares were flushed 6-7 times apiece so inflammation and trauma is very possible. The average number of flushes throughout the entire group of mares used on this project was 3. However, the fact that 5 of the mares were obtained from the UGA College of Veterinary Medicine and so were only used for 1 flush should be taken into account. Other mares that were used included one which had consistent problems with cysts throughout that particular breeding season. The other two mares that were used from the Animal Science unit foaled during the study. One of these mares had a retained placenta and the other foaled too late in the season to attempt any more than one flush. The other five mares that were included in the study belonged to the UGA College of Veterinary Medicine and were bred and flushed only once.

The role of the equine embryonic capsule has been hypothesized to have an effect on maternal recognition (Stout, 2005). However, it is also thought to impede the diffusion of cryoprotectants into the blastocoelic cavity (Allen, 2001; LeGrand, 2002). Therefore, penetrating the capsule and allowing diffusion of the cryoprotectant is a logical next step. The recipient mare that carried a vesicle for 15d and subsequently lost it could have lost the embryo due to heat stress or other factors. This mare had only recently been donated to the program and we were unaware of her reproductive history although she showed no signs of any problems through out the season. Of course, there

is also the strong possibility that the embryos which were treated with cryoprotectant will not result in a long term successful pregnancy. Wilson (1987) found that mitochondrial degeneration was found in equine embryos when 10% glycerol was used as a cryoprotectant. Tharasanit (2005) studied the effect of cryopreservation on the cellular integrity of equine embryos and found that significantly fewer cells died in small equine embryos than in the large embryos. In addition, a negative correlation between capsule thickness and freezability was found to impede the access of the cryoprotectants into the interior portion of the embryo (LeGrand, 2002). However, there is sufficient evidence to warrant another attempt on this study with a greater number of embryos to make its conclusions statistically relevant.

Porcine Discussion. Porcine embryos undergo the same difficulties in cellular ultrastructure when faced with cryopreservation that equine embryos do. They are notorious for their extreme sensitivity to cooling (Dobrinsky, 2001). The difficulties faced include the formation of intracellular ice crystal and degeneration of certain organelles (Iwaski, 1994; Cuello 2004). Vitrification, particularly by the OPS method, has been the most successful. This is presumably because of the increased surface area and thus faster cooling rate present in such methods. This leaves less time for condensation and the formation of intracellular ice crystals in the blastoceolic cavity of the embryo (Varga, 2006). According to Nagashima (1992), the stage of hatching has shown to have no effect on the cryotolerance of the embryo. However, their was some difference in the different gentic groups of the embryos (Nagashima, 1992). However, genetic diversity was not shown to have an effect on cryotolerance in subsequent studies (Fujino, 2007). True results for the viability of porcine embryos post thaw would best be

evaluated by transferring both the control and the treatment embryos into a recipient sow. Obviously this procedure is the most practical way of evaluating viability of the embryos and the usefulness of frozen embryos in a commercial operation. However, pregnancy rates for sows using non-surgical transfer are much lower than those using surgical transfer due to the uterine conformation of the species (Dobrinsky, 1994; Dobrinsky, 1997; Dobrinsky, 2001; Dobrinsky 2002). Due to the numerous difficulties involved in surgical transfer we chose to evaluate the porcine embryos in a culture media and evaluated viability by the degree of reexpansion exhibited by the blastocoelic cavity.

When the data was analyzed, the date on which the embryos were harvested showed a significant effect on their initial reexpansion at thaw. This can be accounted for since it took more time to manipulate and freeze the embryos on the first harvest than the second one. Therefore, initial handling of embryos influences their success in vitrification. Treatment was shown to influence re-expansion at thaw which supports our hypothesis that replacing blastocoelic fluid with a commercial cryoprotectant increases the survivability of embryos. The initial size of the embryo when first flushed had an impact on its initial reexpansion. This is consistent with the research in horses that shows a difference in the size of successfully vitrified embryos. In horses, larger embryos are more difficult to freeze successfully, which is thought to be due to the embryonic capsule of large embryos ($\geq$300 μm) which may prevent penetration of cryoprotectant into the blastocoelic cavity of the embryo (LeGrand, 2002). The lipid layer in procine embryos is also hypothesized to impede the diffusion of cryoportectants. Delipation of porcine embryos resulted in higher success in freezing protocols (Nagashima, 1994). As expected, there was no date/treatment interaction and no size/treatment interaction.

There was an interaction between date and size which could be due to a particular sow which was harvested on the first day and exhibited smaller than expected embryos. In this sow, several cysts were found on both ovaries.

At the six hour interval, neither date, size, nor treatment influenced the status of the embryos. However, interactions between date/size and date/treatment and size/treatment were all significant. A possible explanation for these results is that porcine embryos are notoriously hard to culture and die extremely easily (Long *et al.*, 1999). Therefore the initial reexpansion is probably the best indicator of successful treatment for this particular experiment. However, transferring the embryos back into a recipient sow is undeniably the ultimate test of successful treatment.

Implications

Equine embryo cryopreservation and transfer are a part of the equine industry that is becoming more and more familiar to the producer every day. This new technology allows mares to have a greater genetic impact just as artificial insemination did for stallion. It also allows for mares that are unable to carry a foal full term to still produce viable offspring (Allen, 2005). Past research has shown that small embryos can be successfully frozen and transferred. However, because of their small size and the timetable on which the embryo enters the uterus, large embryos would be more efficient for the industry to freeze. Unfortunately, these embryos are the hardest to freeze because of the embryonic capsule which impedes diffusion of the cryoprotectant into the blastocoelic cavity (LeGrand, 2002). This study showed that embryos treated in this manner can result in a pregnancy. Swine embryos face similar problems as equine

embryos because of the lipid layer that surrounds the embryo. The results of this study showed that passive diffusion of cryoprotectant through the lipid layer and into the blastocoelic cavity results in higher re-expansion of that cavity at thaw. However, other factors, such as reproductive history/soundness of the donor sow or mare, fertility of the boar/stallion can have an effect on the results. Additionally, further research is needed with larger number of equine embryos and with surgical transfer of swine embryos back into a sow.

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Chapter 4

Conclusions

In order for embryo transfer to become economically feasible and practical for both the average equine and swine producer, many changes still need to be accomplished. In the equine species, superovulation is a problem that the industry has not made significant strides in overcoming. Because of the anatomy of the mare's ovary, multiple ovulations are extremely difficult and recovery rates from superovulated mares are only slightly higher than those who have not been superovulated. Estrus synchronization also plays a huge role in the equine embryo industry. By successfully freezing equine embryos, particularly the d 8 embryos that are easiest to flush, the labor and cost associated with keeping large numbers of recipient mares could be greatly reduced. The role of the equine embryonic capsule has been hypothesized to have an effect on maternal recognition. However, it is also thought to impede the diffusion of cryoprotectants into the blastocoelic cavity. Therefore, penetrating the capsule and allowing diffusion of the cryoprotectant is a logical next step.

The conclusions of this study are that more research is needed to completely determine the effect of diffusing a cryoprotectant directly into the blastocoelic cavity of an equine embryo. Large numbers are essential to truly determining this effect. In addition, more research needs to be done to determine the effect of multiple flushes and artificial insemination on the uterine environment. Irritation and infection could easily set in during these procedures. As technology in embryo transfer progresses, so must procedures to determine the effects of this technology on the animals themselves.

Porcine embryos are similar to equine embryos in that they also have problems with a cryoprotectant diffusing into the blastocoelic cavity of the embryo. However, in swine, this is due to the large lipid component of the embryo which acts in the same manner as the embryonic capsule does in horses. Therefore, the same procedure was performed in swine that was performed in horses. In the swine portion of this study, significant positive correlation was shown between the treatment and re-expansion scores. However, it is hard to measure the viability of embryos without returning them to the uterine environment. In future research, swine embryos need to be returned to a sow through surgical transfer in order to best evaluate these results.