12-2008

THE DEVELOPMENT AND ANALYSIS OF A CLOSED SYSTEM OF VITRIFICATION FOR MAMMALIAN EMBRYOS

Jennifer Graves-herring
Clemson University, jgraves@clemson.edu

THE DEVELOPMENT AND ANALYSIS OF A CLOSED SYSTEM OF VITRIFICATION FOR MAMMALIAN EMBRYOS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

---

by
Jennifer Eileen Graves-Herring
December 2008

Accepted by:
Alfred P. “Hap” Wheeler, Ph.D. Committee Chair
William R. Boone, Ph.D.
John P. Wourms, Ph.D.
John R. Gibbons, Ph.D.

ABSTRACT

Embryo cryopreservation is an integral part of assisted reproduction because it allows for future use of these embryos. Cryopreservation occurs when there are supernumerary embryos or when an embryo transfer cannot be performed.

There are two main methods to cryopreserve embryos. The most recent is vitrification, which uses high concentrations of cryoprotectants, a short time to cool and avoids ice crystals. The "gold standard" is the slow-cool method, which uses low concentrations of cryoprotectants, a long time to cool embryos and produces extracellular ice crystals.

Prior to introducing vitrification as part of the human cryopreservation regime, it is important that vitrification be evaluated through a research protocol using a mammalian embryo model. In this research, we used the two-cell, mouse embryo model to develop a closed system for vitrification, observe the toxicity of vitrification solutions, and compare blastocyst rates, pup rates and DNA damage between the vitrification and slow-cool method.

Two commercially available devices were used to vitrify embryos. Both devices were studied as an open-system and a closed-system using two-cell and eight-cell mouse embryos. These devices and systems produced similar blastocyst rates. Two-cell mouse embryos were used for subsequent studies due to the increased potential to observe a detrimental effect with the earlier cell stage.

A toxicity study demonstrated that two-cell mouse embryos, after remaining in vitrification solution for 32 minutes, produced fewer blastocysts than did two-cell mouse

embryos that were removed from the vitrification solution at earlier time intervals (1, 2, 4, 8 and 16 minutes). However, when surviving blastocysts were transferred to recipients, they produced pups.

Comparison studies between the vitrification and slow-cool method demonstrated that there were no significant differences in blastocyst rate, pup rate or percentage of normal embryos (not demonstrating DNA damage).

In conclusion, a closed system was developed to vitrify two-cell mouse embryos. The toxicity of vitrification solutions were observed and the comparison of vitrification and slow-cool methods using blastocyst rate, pup rate and DNA damage as determinants showed that both methods were similar.

DEDICATION

I dedicate this dissertation in memory of both my father, John Graves and my father-in-law, Sammie Herring. My father always made me try harder than I thought I could, work harder than I thought I could and strive to do more than I thought possible. This work is proof that his guidance helped me through the struggles of this project.

My father-in-law was proud of my decision to get my Ph.D. and witnessed my many strives to accomplish this goal. Unfortunately, he was unable to see the completion of this project.

I have tried my best to make both my father and father-in-law proud of me. I hope I have accomplished this and that they are looking down and are proud of my achievement.

ACKNOWLEDGMENTS

I would like to thank my husband, Tony Herring, for being my support system to help me get through the many hardships of this work, and reminding me that sometimes you just have to relax.

I would like to thank my mother, Kathy Graves, for always supporting my decisions and helping me be the person I am today.

My deepest gratitude goes to Dr. Bill Boone for his patience, guidance and expertise throughout the trials of this project, writings of multiple abstracts and writing of this dissertation. I am grateful for his dedication to this project.

I would also like to thank Dr. Hap Wheeler, Dr. John Wourms and Dr. John Gibbons for taking on a non-traditional student, and their continued support and contributions to this project.

I would like to acknowledge Eric Holle at the Greenville Hospital System, Oncology Research Institute for collecting and transferring embryos. I would also like to acknowledge the Oncology Research Institute for care of the animals and use of their equipment.

I would like to express gratitude to Dr. Lee Higdon, Jane Johnson and Patty Ruch at the Greenville Hospital System, Division of Reproductive Endocrinology and Infertility for their support and contributions to this project.

I would like to acknowledge the Greenville Hospital System, Department of Ob-Gyn for funding this project, without their support, this project would not have been possible.

TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

LIST OF TABLES

List of Tables (Continued)

Table Page

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

Embryo cryopreservation is an integral part of assisted reproduction because it allows for future use of these embryos. Cryopreservation occurs when there are supernumerary embryos or when an embryo transfer cannot be performed.

There are two main methods to cryopreserve embryos. The first method, and most recent, is vitrification which uses high concentrations of cryoprotectants, a short time to cool and avoids ice crystals. The second method, the slow-cool method is the "gold standard" and uses low concentrations of cryoprotectants, a lengthy time to slowly cool embryos and produces extracellular ice crystals.

Prior to introducing vitrification as part of the human cryopreservation regime, it is important that vitrification be evaluated through a research protocol using a mammalian embryo model. In this research, we used the two-cell, mouse embryo model to develop a closed system for vitrification, observe the toxicity of vitrification solutions, and compare blastocyst rates, pup rates and DNA damage between the vitrification and slow-cool method. The mouse model was used because this laboratory currently uses two-cell mouse embryos as a model for quality control studies. First, as with any study, it is important to understand what research has already been performed.

The published vitrification research described here includes different species and stages of embryos, live births, devices, open and closed systems and liquid nitrogen contamination. The technique of slow-cooling and its drawbacks then will be discussed, along with cryopreservation solutions and comparison studies of vitrification and slow-

cooled procedures. The information will conclude with the importance of genetic studies using cryopreserved embryos.

VITRIFICATION

Vitrification is a process by which cells can be frozen in such a way that a glass-like or vitrified state is obtained. The process eliminates the formation of intracellular ice crystals which can damage organelles within the cell. While vitrification technology has been in existence for over two decades (Rall and Fahy, 1985), the technology only recently started to replace the slow-cooled "gold standard" that has been in existence since 1972 (Whittingham et al.).

Multi-cell embryos from many different species have been vitrified following the first successful vitrification of mouse embryos (Rall and Fahy, 1985). These species include cattle (Vajta et al., 1997), humans (Mukaida et al., 1998), hamsters (Lane et al., 1999a), horses (Oberstein et al., 2001), goats (Begin et al., 2003), rats (Han et al., 2003) and Mongolian gerbils (Mochida et al., 2005). Vitrification of blastocysts has been reported for sheep (Martinez and Matkovic, 1998b), cattle (Park et al., 1999), mice (Lane et al., 1999b), humans (Yokota et al., 2001), monkeys (Yeoman et al., 2001), rabbits (Lopez-Bejar and Lopez-Gatius, 2002), pigs (Misumi et al., 2003), buffalos (Neglia et al., 2003), European polecats (Piltti et al., 2004) and domestic cats (Tsujioka et al., 2008).

Not only has vitrification of embryos from multiple species been reported, but for many animal species there have been reports of implantation and live births that resulted from vitrified embryos. A study by Uechi et al. (1999) demonstrated implantation of

vitrified two-cell mouse embryos. Misumi et al. (2003) demonstrated that healthy piglets could be produced from vitrified blastocysts.

Successful vitrification of embryos followed by live births also has been reported for humans. Yokota et al. (2001) demonstrated the successful vitrification of human embryos with the birth of a baby. Mukaida et al. (2003) reported a 37% clinical pregnancy rate with the transfer of vitrified blastocysts. At the time the report was published, 23 healthy babies had been born (18 deliveries) while 37 pregnancies were ongoing.

A number of different ways to vitrify mammalian embryos have been reported including open-pulled straw (Vajta et al. 1997), grid (Park et al., 1999), hemi-straw (Vanderzwalmen et al., 2000), cryoloop (Lane et al., 1999a; Yeoman et al., 2001), flexipet-denuding pipet (Liebermann et al., 2002b), solid-surface microdrop (Begin et al., 2003), Stripper Tip® (Walker et al., 2004) and the Cryotop (Kuwayama et al., 2005; Kuwayama, 2007). Each device has its unique way to contain the embryos prior to being vitrified in liquid nitrogen.

Open-pulled straws were first described by Vajta et al. (1997) in which they heated French mini-straws (IMV, L'Aigle, France) and manually pulled the straws until the inner diameter (0.8 mm) was approximately half its original size (1.7 mm). Embryos were drawn into the straws by capillary action and then straws were submerged into liquid nitrogen. Vajta et al. (1998) vitrified bovine embryos (n=763) from Day 1 to Day 7 after fertilization *in vitro* (Day 0) and compared them to control (nonfrozen) embryos (n=719). They demonstrated there were no significant differences in blastocyst rates

between vitrified embryos and control embryos from Day 3 to Day 7; however, there were significant differences demonstrated on Day 1 and Day 2 indicating that some embryo stages may be more susceptible to cryoinjury than others.

Park et al. (1999) used electron microscope grids to vitrify bovine blastocysts. These researchers loaded 8-10 embryos onto the grid and plunged the grid into liquid nitrogen. Blastocysts were thawed and observed 24 and 48 hours later. Embryos that were vitrified (87.8% [79/90]) had survival rates (those that reexpanded or hatched) that were significantly lower at 24 hours than control (nonfrozen) embryos (100% [90/90]).

Vanderzwalmen et al. (2000) developed the hemi-straw for use with supernumerary embryos. The tip of the hemi-straw has a trough in which approximately 0.3 µL of medium with the embryos are transferred prior to being submerged into liquid nitrogen. Once in liquid nitrogen, the hemi-straw was inserted into a larger straw for storage (Vanderzwalmen et al., 2000; Vanderzwalmen et al., 2003). Vanderzwalmen et al. (2000) vitrified 16 human blastocysts; once the embryos were thawed, 88% expanded and 44% hatched.

Cryoloops were adapted from protein crystallography, but were modified to vitrify hamster embryos (Lane et al., 1999a), mouse embryos (Lane et al., 1999b) and monkey blastocysts (Yeoman et al., 2001). The nylon loop was attached to the top of a cryovial and the loop was dipped in vitrification solution. Embryos were pipetted onto the solution film that was created within the nylon loop and plunged into liquid nitrogen. The top of the cryovial was screwed into the bottom portion of the vial under liquid nitrogen. Hamster two-cell embryos were vitrified using the cryoloop and later thawed

and transferred (Lane et al., 1999a). Of the 72 blastocysts transferred to recipients, 39 implanted (54%) which was significantly lower than the controls (nonfrozen; 85% [34/40]).

Vitrification of embryos with the use of the flexipet denuding pipet was first reported by Liebermann et al. (2002b). Approximately 2 µL of vitrification media containing embryos were loaded into the pipet by capillary action and the pipet was submerged into liquid nitrogen. Once in the liquid nitrogen, the pipet was placed in a cryostraw. These researchers used this method to vitrify 1 pronucleus (PN) and 3 PN (or more) abnormally-fertilized human embryos (n=217). After thawing, 190 (88%) survived and 147 (77%) cleaved, which was not significantly different than controls that cleaved (85% [115/136]).

Solid-surface microdrop vitrification utilized a liquid nitrogen-cooled surface (Begin et al., 2003). A drop of vitrification solution containing embryos was dropped onto the cooled surface and droplets were placed into cryovials using nitrogen-cooled forceps. Begin et al. (2003) demonstrated that goat embryos vitrified by the solid-surface microdrop method had a low percentage of embryo survival after thawing with no embryos developing to the morula or blastocyst stage. This is in contrast to a study performed by Bagis et al. (2005) that demonstrated that mouse pronuclear embryos vitrified by the solid-surface microdrop method not only had blastocyst development (results varied from 32-62%) but also produced live-births (30% [34/113]).

Walker et al. (2004) used the Stripper Tip® from MidAtlantic Diagnostics (Marlton, NJ) to vitrify mouse embryos. Embryos were loaded into the tip using the

Stripper® device and then the tip was removed from the device and submerged into super-cooled nitrogen or liquid nitrogen. The tip was placed into a Cryo Bio System High Security Straw (CBS; Cryo Bio Systems, IMV Technologies Group, L'Aigle, France) and the CBS straw heat sealed. Morulas and blastocysts that were vitrified in the Stripper Tip® using super-cooled nitrogen had 81.8% (90/110) survival (continued to develop), while those vitrified using liquid nitrogen had 90.7% (97/107) survival. Both groups were significantly different from the controls (99% [103/104]) which were not exposed to cryopreservation solutions or vitrification.

The Cryotop consisted of a plastic holder with a thin film strip (0.4 mm wide, 20 mm long and 0.1 mm thick; Kuwayama, 2007). The embryos were placed onto the strip with just enough vitrification solution to cover the embryos. The device was submerged into liquid nitrogen and the cap was pulled over the strip to help protect it during storage. Kuwayama et al. (2005) used this device to vitrify human four-cell embryos and blastocysts. After thawing, the four-cell embryos produced a 98% (879/897) survival rate and a 27% (136/504) pregnancy/transfer rate whereas blastocysts produced a 93% (82/88) survival rate and a 51% (42/82) pregnancy/transfer rate.

Each of these devices described above are known as an open system of vitrification. The open system allows exposure of the embryos to liquid nitrogen. This would allow for potential contamination by the liquid nitrogen with such disease as hepatitis (Tedder et al., 1995). Such contamination occurred when Hepatitis B contaminated a liquid nitrogen storage tank. The contamination was from leakage of a cryopreservation bag that contained bone marrow from a Hepatitis B positive patient and

resulted in other patients contracting Hepatitis B after receiving autologous bone marrow transfusions.

Bielanski et al. (2000) demonstrated that embryos in open vials or straws tested positive for bovine viral diarrhea virus and bovine herpesvirus-1 when stored in dewar flasks containing cell culture suspensions of the viruses. In contrast, sealed vials and straws tested negative.

Not only can liquid nitrogen be contaminated with diseases, but it can also be contaminated with bacteria and fungus. Fountain et al. (1997) indicated that bacteria and fungi can contaminate liquid nitrogen freezers and fungi can be found in the vapor phase of liquid nitrogen. Bielanski et al. (2003) also noted that bacterial contamination was demonstrated in liquid nitrogen as well as thawed semen and embryos; however, these authors noted that some of the bacterial contamination may have been due to laboratory processing.

To date, there have not been any reports of any disease transmission due to embryo cryopreservation technologies (Vajta et al., 2007); however, there is still the possibility of liquid nitrogen contamination. A closed system of vitrification would lessen the contamination risk.

Chen et al. (2001) reported a type of closed system that used boluses of media on either side of the medium which contained the embryos; however, upon thawing, these boluses would most likely merge with the one containing the embryos and thus contaminate the specimen.

Lopez-Bejar and Lopez-Gatius (2002) reported using a sealed version of the open-pulled straw. They heated and pulled a standard IMV straw and used the portion that contained the cotton plug. They loaded the straw with medium and rabbit embryos and then sealed the opposite end using polyvinyl-alcohol sealing powder. Live-birth rates for vitrified blastocysts transferred into recipient does yielded 51.7% (123/238), which was not significantly different from live-birth rates from fresh blastocysts (58.5% [131/224]).

Isachenko et al. (2005a) vitrified pronuclear mouse embryos in open-pulled straws (OPS). The OPS were placed into larger straws that were heat-sealed on one end then hermetically closed by a metal ball on the opposite end. Using this straw-in-straw technique, a blastocyst rate of 23% was observed. Isachenko et al. (2005b) also used the same straw-in-straw technique to vitrify human pronuclear embryos. The embryos were placed in OPS that were hermetically closed on both ends and plunged into liquid nitrogen. When the embryos were thawed and cultured, they produced a blastocyst rate of 14%.

The CryoTip® (Irvine Scientific, Irvine, CA) was a straw-like device that was heat-sealed after loading with a minimal amount of vitrification solution that contained the embryos (Kuwayama, 2005). The CryoTip® was compared to the Cryotop; the blastocyst survival, pregnancies and deliveries, were not different between the two devices.

The closed system of vitrification may prevent contamination from liquid nitrogen during the storage phase; however, contamination may arise when removing the embryos

from the straw. Maertens et al. (2004) used ionomeric resin straws for storing Hepatitis C, contaminated semen. Although no cross-contamination was detected from sealing or storing, the heating wire used to open the straw was observed to have contamination. This type of contamination could occur with any straw-system that is opened with a nonsterile, reusable device. When having to open a straw-system, the use of sterilized scissors would reduce or eliminate contamination. This would hold true whether the system is used for vitrification or for a slow-cooled procedure.

In conclusion, embryos from different species have been vitrified using a multitude of devices, which are either a closed-system or an open-system. A closed system of vitrification is preferred over the open system to prevent potential contamination from liquid nitrogen.

SLOW-COOLED

Successful slow-cooled freezing methods have been in existence since 1972 (Whittingham et al.). The slow-cooled method of cryopreservation requires that a mammalian embryo be passed through a series of dehydrating solutions that replaces the water in the cell with a cryoprotectant. The embryo is placed inside a freezing vessel (e.g. straw, vial), which is placed inside a freezing chamber. Once inside, the chamber is slowly cooled to a point a few degrees below 0°C. Ice crystals are manually induced (seeding) to the cryoprotectant to prevent super cooling (spontaneous formation of extracellular and intracellular ice crystals) and then cooled to an appropriate temperature prior to plunging into liquid nitrogen.

Seeding is a process in which ice crystals are induced. Usually the seeding process is performed by first placing forceps into liquid nitrogen and then placing the forceps on the outside of the vessel juxtaposed to the meniscus of the cryoprotectant (inside the straw). The forceps should be some distance from the actual embryos. The area in which the forceps are placed, displays ice crystals. This induction of ice crystals allows an area of extracellular ice formation that will continue to form and will prevent spontaneous ice crystallization (both extracellular and intracellular). In addition, these induced ice crystals will allow further dehydration of the embryo and will allow for a slow release of latent heat (Shaw et al., 2000).

The slow-cooling technique utilizes a programmable freezer that uses liquid nitrogen or a liquid bath to slowly cool the chamber of the freezer. Liquid nitrogen freezers can slowly cool the embryos to -180°C, whereas the liquid bath freezers cool to -40°C or -80°C.

The slow-cooling technique of cryopreserving embryos has its drawbacks when compared to vitrification. Some of these drawbacks include cost, time and reliability. Mechanical freezers required to slow-cool specimens are expensive. This expense does not include the yearly maintenance fee or charges for repairs that may be needed. Vitrification of embryos does not have this cost associated with it since embryos are plunged directly into liquid nitrogen without the aid of a mechanical freezer.

The time that is needed to run the slow-cool cycle is approximately 2 to 3 hours depending on the stage of the embryo being frozen; this does not include the time to move embryos though freezing solutions or loading the embryos into straws. The only

time associated to vitrify embryos is the time to move the embryos though the vitrification media, load the embryos into/onto an apparatus and to plunge the apparatus into liquid nitrogen. The total time for such events is 5 to 10 minutes.

The mechanical freezer produces different post-thaw blastocyst development in the mouse, depending on the position in the freeze chamber (Boone et al., 2004). This study also demonstrates that the freezer positions have a significantly different temperature during freezing. There are no variations in temperature due to mechanical equipment when the vitrification apparatus, which contains the freezing solution and the embryos, is plunged into liquid nitrogen.

In conclusion, the slow-cooled cryopreservation method induces ice crystals in the cryoprotectant solution to avoid intracellular ice crystals. It uses a mechanical freezer that requires an extended time to slowly cool the embryos, has the expense of maintenance fees and repairs, and produces variable temperatures throughout its chamber.

COMPONENTS OF CRYOPRESERVATION SOLUTIONS

Cryoprotectants can be toxic to embryos (Shaw et al., 2000). Although vitrification and slow-cooled procedures use cryoprotectants, vitrification uses them at higher concentrations, thus creating an increase in osmolarity (~6000 mOsm compared with ~2500 mOsm for slow-cooled). There are two types of cryoprotectants: permeating, which include glycerol, ethylene glycol and dimethyl sulphoxide (DMSO) and non-permeating, which include saccharides, protein and polymers (Liebermann et al., 2003).

The components of the cryopreservation solutions are often combined. In 1985, Rall and Fahy used DMSO, acetamide, propylene glycol and ethylene glycol in a modified Dulbecco's saline. Ali and Shelton (1993) used ethylene glycol, glycerol and sucrose to vitrify mouse embryos. Bovine embryos were vitrified in ethylene glycol, DMSO and Tissue Culture Medium with HEPES supplemented with calf serum (Vajta et al., 1998); and human oocytes were vitrified in ethylene glycol and sucrose in Dulbecco's phosphate-buffered saline supplemented with human serum albumin (Kuleshova et al., 1999). Also in 1999, Lane et al. (1999b) reported vitrification of human and mouse blastocysts using DMSO, ethylene glycol and sucrose in HEPES-buffered G2.2 medium.

Solutions can also include cryoprotectants such as Ficoll or polyethylene glycol. In 1999, Park et al., reported vitrification of bovine blastocysts using ethylene glycol, Ficoll, sucrose and serum in Dulbecco's phosphate buffered saline. Monkey blastocysts were vitrified in DMSO, ethylene glycol, Ficoll and sucrose in HEPES-buffered TALP containing serum (Yeoman et al., 2001). Misumi et al. (2003) vitrified pig morulae and early blastocysts in ethylene glycol, sucrose and polyethylene glycol in M2 medium.

There have been multiple studies published on the effects of some of the vitrification components. When vitrification was performed with an ethylene glycol based media and a propylene glycol plus glycerol media, bovine embryos that were vitrified with the ethylene glycol based media had higher development (56.4% vs. 33.3%) and hatching rates (35.4% vs. 13.3%; Martinez et al., 1998a). A study by Titterington et al. (1995) determined that glycerol above a concentration of 50% (v/v) was toxic to eight-cell mouse embryos.

Ethylene glycol has a low toxic effect on mouse embryos (Emiliani et al., 2000; Liebermann et al., 2002a). It also has a rapid diffusion and equilibration into the cell through the zona pellucida and cell membrane (Emiliani et al., 2000). Emiliani et al. (2000) noted that mouse zygotes frozen with propanediol produced a higher survival rate and blastocyst rate when compared with ethylene glycol; however, they indicated that ethylene glycol was a good cryoprotectant when four-cell mouse embryos were frozen.

Other researchers also have demonstrated favorable outcomes using ethylene glycol. For example, Shaw et al. (1995) notes that pronuclear and four-cell mouse embryos can be slow-frozen using ethylene glycol without a significant loss of embryos. Hotamisligil et al. (1996) vitrified mouse oocytes in a range of 4 M to 8 M ethylene glycol supplemented with 0.5 M sucrose. They observed that murine oocytes vitrified in concentrations of ethylene glycol up to 6 M supplemented with 0.5 M sucrose obtained membrane integrity and microfilamentous structure, and noted that there was a minimal toxic effect on fertilization and development.

Use of the cryoprotectant DMSO in vitrification solutions has had mixed outcomes. Bovine morula and blastocysts vitrified in 25% v/v ethylene glycol, 25% v/v DMSO in PBS supplemented with pyruvate, glucose and serum have high survival rates (73 to 90%; Ishimori et al., 1993). Liebermann et al. (2002a) noted that vitrification of pronuclear stage human embryos with ethylene glycol and DMSO was a good alternative to vitrification with 1,2-propanediol and DMSO. Trounson et al. (1988) observed blastocyst rates of 60% and 84% when eight-cell mouse embryos were ultrarapidly frozen with a 2 M and 3.5 M DMSO solution. In contrast, vitrification of eight-cell mouse

embryos with a DMSO based solution had low blastocyst rates (53%). As the concentration was increased from 30 to 40% DMSO, even lower survival (blastocyst) rates (0-33%) were observed (Mukaida et al., 1998).

Macromolecules can also be used in vitrification medium. In vitrification media, macromolecules help stabilize the glycoprotein structure of the zona pellucida (Titterington et al., 1995). This, in turn, reduces osmotic stress and prevents fracture of the zona pellucida. When polyvinylpyrroidone (PVP) in the form of Percoll or human serum albumin (HSA) was added to the vitrification solution, there was a significant reduction ($P = 0.01$) in zona damage (Titterington et al., 1995). These authors also noted that Percoll in the vitrification media produced a reduction ($P = 0.01$) in zona damage when compared to HSA.

Ficoll or Dextran can be used as a macromolecule and may be less detrimental than PVP. Kuleshova et al. (2001) were able to culture two-cell mouse embryos to the blastocyst stage at a rate of 100% when embryos were exposed up to 15 minutes in an ethylene glycol based vitrification media, which contained Ficoll or Dextran. Kuleshova et al. (2001) also froze two-cell mouse embryos after being exposed to vitrification solution supplemented with PVP for 15 minutes. This produced a 0% blastocyst rate; however, when the PVP was dialyzed, the blastocyst rate increased to 77 and 83%.

When antifreeze glycoproteins were used as cryoprotectants, mixed results were demonstrated. Antifreeze glycoproteins reduce ice growth and recrystallization (O'Neil et al., 1998). When O'Neil et al. (1998) added antifreeze glycoproteins to a 6 M DMSO vitrification solution to mouse oocytes, a low in-vitro fertilization rate (53%) and

blastocyst rate (20%) were observed; however, when the vitrification medium was cooled on ice before oocytes were exposed to the medium, a higher *in vitro* fertilization rate (94%) and blastocyst rate (66%) were observed. When antifreeze proteins were added to ethylene glycol and propanediol slow-freeze solutions, survival of pronuclear mouse embryos was significantly reduced (Shaw et al., 1995), possibly due to modification of ice crystal growth patterns.

Sucrose is widely used for cryopreservation of oocytes and embryos because it reduces the amount of cryoprotectant needed by acting as an osmotic buffer and reducing osmotic shock (Liebermann et al., 2003). Bagis et al. (2004) vitrified pronuclear mouse embryos in a solution containing equal amounts of DMSO and propylene glycol. The authors also added either sucrose, trehalose or raffinose. It was determined that raffinose was more efficient for cryprotection than sucrose or trehalose. Furthermore, sucrose was less efficient if there was a very short equilibration time. Kuleshova et al. (1999) observed the effect of different sugars in vitrification solution. Their data indicated monosaccharides were more favorable because of their low toxicity and their ability to form a glass-like substance at a lower concentration.

In conclusion, vitrification has been used for mice, cattle, monkeys, pigs and humans, most of which has been performed at the oocyte, early-embryo or blastocyst stage. As indicated above, there are many types of vitrification procedures that can be used; some of which have conflicting results. Consistent results need to be achieved to establish a vitrification standard. Until then, researchers will continue to study various

protocols to improve vitrification with the hopes that it can be used as the universal standard.

COMPARISON STUDIES OF VITRIFICATION AND SLOW-COOLED

The comparison of vitrification and slow-cooled freezing has been studied since 1995 when Van Wagtendonk-De Leeuw and co-workers (1995) studied cattle morulae and blastocysts. They determined that Day-7 cattle morulae and blastocysts frozen using either the vitrification or slow-cooled method produced similar pregnancy rates. The researchers then refined their study and again compared vitrification and a slow-cooled method in a field trial (Van Wagtendonk-de Leeuw et al. 1997). They again demonstrated that pregnancy rates for vitrified and slow-cooled embryos were similar (44.5% versus 45.1%, respectively).

There have been other articles published that compare a vitrification procedure to a slow-cooled procedure. Moussa et al. (2005) compared slow-cooled and Open-Pulled Straw (OPS) vitrification. They indicated that when equine embryos were frozen using either technique, there was no significant difference in the percentage of dead blastomeres per embryo or the percentage of cells entering S-phase of the cell cycle. Stehlik et al. (2005) froze Day-5 human blastocysts using a slow-cooled or a vitrification method. This study indicated that vitrification produced a 100% (41/41) blastocyst survival rate and a 50% (10/20) pregnancy rate whereas slow-frozen blastocysts had an 83.1% (59/71) survival rate and a 16.7% (4/24) pregnancy rate.

Naik et al. (2005) compared two types of vitrification methods (open-pulled French mini straws [OPS] and French mini straws) and a slow-cool method using morula-stage rabbit embryos. The OPS demonstrated better results for percent blastocyst rate (71% [44/62]) and produced a higher percentage of offspring per number of embryos transferred (29% [7/24]) than the other two methods.

Martinez and Matkovic (1998b) studied different solutions for vitrification and slow-cooling. Although vitrification and slow-cooling were not statistically compared; the results demonstrated that when sheep morulae and blastocysts were frozen using either method, embryo development, hatching, pregnancy and weaning had higher percentages with slow-cooling (75.4%, 52,8%, 51.3% and 47.3%, respectively) than with vitrification (54.0%, 30.0%, 40.0% and 38.4%, respectively).

Other species have been used to compare vitrification and slow-cooled methods to cryopreserve embryos with varying degrees of success. When two-cell mouse embryos were vitrified or slow-cooled and then thawed, cultured and transferred to recipient mice, vitrified embryos (10.2%) had a significantly lower ($P < 0.05$) implantation rate than the slow-cooled mouse embryos (22.1%; Uechi et al. 1999). In contrast to this study, researchers using horse embryos found that vitrification using the cryoloop or OPS had similar grades and percent of live cells when compared to slow-cooled methods (Obserstein et al. 2001). In studies performed with rabbit morulae, OPS vitrification demonstrated a higher percent of blastocysts (71%) and a higher percent of live offspring (29%) than those that were frozen with a programmable freezer (55% versus 19%, respectively; Naik et al. 2005).

In 2005, Stehlik et al. demonstrated that Day-5 and Day-6 human blastocysts that underwent vitrification had a higher survival rate, pregnancy rate and implantation rate than those that were slow cooled. Similar results were obtained by Al-Hasani et al. (2007) that demonstrated a significantly higher pregnancy rate with vitrification (36.9%) than with slow-cooling (10.2%); thus, the researchers discontinued their slow-cooling method.

Studies that compare the vitrification to the slow-cooled method have not only been performed with embryos, but they have also been performed with oocytes. Men et al. (1997) cryopreserved mouse oocytes using both freezing methods. Upon thawing the oocytes, these researchers performed *in vitro* fertilization and observed two-cell embryo development. Oocytes that were vitrified had a survival rate of 55% (355/647) whereas slow-cooled oocytes yield a 66% (210/316) survival rate; these values were not significantly different ($P > 0.05$). Fertilization of oocytes after being frozen/thawed were 74% (155/210) for vitrification and 72% (258/355) for slow-cooled.

In 2005, Valojerdi and Salehnia reported 80% (162/203) of mouse oocytes survived vitrification and 85% of mouse oocytes (250/294) survived slow-cooling. Although the survival rate was not significantly different between the two freezing methods, there was a significant difference ($P < 0.001$) in the number of two-cell embryos produced after *in vitro* fertilization. Eighty-five percent (138/162) of the oocytes (138/162) that developed to two-cell embryos were exposed to vitrification while only 1.2% (3/250) of the oocytes exposed to the slow-cool method produced two-cell embryos. Valojerdi and Salehnia (2005) continued their study past the two-cell stage.

Vitrification produced a blastocyst rate of 27% (44/162) whereas the slow-cooled procedure did not produce any blastocysts (0/250).

In conclusion, due to conflicting data, further studies are needed to determine if vitrification is a safe alternative to slow-cooling for cryopreservation of supernumerary embryos.

IMPORTANCE FOR GENETIC STUDIES

Similar to intracytoplasmic sperm injection (ICSI), the rapid adoption of new assisted reproduction technologies like vitrification, raises concerns about the long-term consequences on offspring generated from such technology. When ICSI was first introduced (Palermo et al., 1992), it was a way that an Assisted Reproductive Technology Center could give hope to couples that had male motility problems. The procedure was quickly used by centers without fully understanding the consequences. Scientists are now questioning if the procedure is indeed safe. Recently, there have been studies that link ICSI with diseases such as Beckwith-Wiedeman Syndrome (Edwards and Ludwig, 2003; Debaun et al., 2003) and Angelman Syndrome (Edwards and Ludwig, 2003; Orstavik et al., 2003) as well as imprinting defects (Cox et al., 2002). This same haste observed with ICSI should not be taken with vitrification.

Several laboratories have described the use of vitrification procedures and their ability to produce viable oocytes, embryos and produce live-births from these embryos; however, genotypic damage should also be studied due to aneuploid zygotes and malformed fetuses that can arise from vitrification (Kola et al., 1988). In contrast,

Yokota et al. (2001) demonstrated vitrification of human embryos with birth of a child with no known anomalies and a normal karyotype. Takahashi et al. (2005) used a questionnaire to perform a retrospective analysis which determined that 1.4% (2/147) of cases from vitrified blastocysts and 2% (4/205) of cases from fresh blastocyst transfer had congenital birth defects.

Vitrification studies should not only evaluate if embryos can survive the process and produce live-births, but also include studies that evaluate potential alteration at the genetic level. Such information may help predict the future of offspring produced using this potentially improved method of preserving oocytes and embryos.

One method that may give some insight into DNA damage due to vitrification is the Comet Assay. The Comet Assay detects single- and double-stranded DNA breaks. This assay has been used to detect DNA damage in hamster (Takahashi et al., 1999), cattle (Takahashi et al., 2000) and mouse (Fabian et al., 2003) embryos. Takahashi et al. (1999) observed DNA damage to hamster embryos by light exposure or oxidative stress. These researchers were able to detect DNA damage in one-cell and two-cell embryos and determine there was more DNA damage in *in vitro* cultured embryos than there was in those embryos that were developed *in vivo*. Takahashi et al. (2000) also used the Comet Assay to demonstrate that bovine embryos that were cultured *in vitro* at a 20% oxygen concentration had an increase in DNA damage when compared to those cultured *in vitro* at 5% oxygen. Fabian et al. (2003) studied apoptosis in mouse embryos by inducing apoptosis and analyzing embryos using DNA staining and the Comet Assay.

A pitfall of the Comet Assay is that it cannot discriminate in the type of DNA damage present. Therefore, if there is damage to individual blastomeres, degeneration of polar bodies or degeneration of individual blastomeres, it will be observed as a comet tail. Since the occurrence of degeneration of polar bodies occurs in all embryos, embryos with normal DNA may show some type of comet tail. Those embryos with induced damage will normally display a longer tail length, indicative of more DNA damage. According to Chan et al. (2001), the Comet Assay still needs to be refined to produce "optimal sensitivity." Although there are some pitfalls to using the Comet Assay, it can give an indication of the presence of damage at the genetic level.

In conclusion, genetic studies are important to perform due to concerns about long-term consequences of offspring. Although the Comet Assay has some pitfalls, other researchers have demonstrated that it can be used to detect DNA damage in embryos.

CHAPTER SUMMARY AND RESEARCH OVERVIEW

Vitrification has been around for over two decades and there are many facilities using this method to cryopreserve embryos. Some facilities are using the open-system method which has the potential for contamination of embryos from liquid nitrogen. Some facilities are using a closed-system method which has become available commercially within the past couple of years. Our studies with a closed system of vitrification started in 2003 at which time commercial vitrification devices were not readily available. Some of the concerns with vitrification are liquid nitrogen

contamination, the toxicity of the cryoprotectants, conflicting data from researchers when comparing vitrification to the slow-cooled method and genetic damage.

The following chapters will describe research that we have performed using the two-cell mouse embryo model. The second chapter will discuss vitrification using two devices, two different stages of development and the open and closed system. The third chapter will discuss time that embryos can remain in vitrification solution without altering blastocyst rate and live birth rate. In addition, comparisons of DNA damage will be made between embryos exposed to vitrification solutions for different intervals of time. For embryos that are damaged by the lengthy stay in vitrification solution, the study will determine if the embryos can still produce live-births. The fourth chapter will compare the vitrification and slow-cooled method on the basis of blastocyst rate and pup rate. The fifth chapter will compare DNA damage between vitrified and slow-cooled two-cell mouse embryos cultured to the blastocyst stage. The sixth chapter also will compare DNA damage as determined by the Comet Assay; in this study, embryos will be cultured to the eight-cell stage.

The outcome of these studies will help to determine if vitrification can replace the slow-cool method of freezing embryos. If the vitrification method provides comparable results to that of the "gold standard" slow-cool method, then it offers the potential to eliminate the cost, time and reliability associated with the mechanical freezer used to slow-cool embryos.

CHAPTER TWO

A COMPARISON BETWEEN AN OPEN AND CLOSED SYSTEM OF VITRIFICATION

INTRODUCTION

Vitrification is a process by which cells can be frozen in such a way that a glass-like or vitrified state is obtained. Vitrification eliminates the formation of intracellular ice crystals. Vitrification of mouse embryos was first reported in 1985 (Rall and Fahy, 1985). Since then, this technique has been extended to vitrification of early-cell embryos in cattle (Vajta et al., 1997) and humans (Mukaida et al., 1998) as well as blastocysts for cattle (Park et al., 1999), mice (Lane et al., 1999b), humans (Yokota et al., 2001), monkeys (Yeoman et al., 2001) and pigs (Misumi et al., 2003).

Not only has vitrification of embryos from multiple species been reported, but for many animal species there are reports of implantation and live births. A study by Uechi et al. (1999) demonstrated a decrease in the implantation rate of vitrified two-cell mouse embryos when compared to the standard slow-freeze or fresh cultured embryos of the same cell stage and species. Misumi et al. (2003) demonstrated that healthy piglets could be produced from vitrified blastocysts.

Live births are also reported for humans. Yokota et al. (2001) demonstrated the successful vitrification of human embryos with the birth of a baby with no known anomalies and a normal karyotype. Mukaida et al. (2003) reported a 37% clinical pregnancy rate with the transfer of vitrified blastocysts. At the time the report was published, 23 healthy babies had been born (18 deliveries), while 37 pregnancies were

ongoing. Takahashi et al. (2005) used a questionnaire to perform a retrospective analysis which determined that 1.4% (2/147) of cases from vitrified blastocysts and 2% (4/205) of cases from fresh blastocyst transfer had congenital birth defects. Although the previous studies have demonstrated live births, due to the infancy of the technique, no long-term follow-up studies are available.

A number of different ways to vitrify embryos have been reported including open-pulled straws (Vajta et al., 1997), grid (Park et al., 1999), hemi-straw (Vanderzwalmen et al., 2000), cryoloop (Yeoman et al., 2001) and Cryotop (Kuwayama et al., 2007). None of these systems are a closed system, thus they allow for exposure of embryos to liquid nitrogen that can harbor hepatitis (Tedder et al., 1995) and possibly other infectious diseases. Chen et. al. (2001) reported a type of closed system using boluses of media on either side of the medium containing the embryos; however, upon thawing, these boluses would most likely merge with the one containing the embryos and thus contaminate the specimen. The CryoTip™ (Irvine Scientific, Irvine, California) is a device, that after loading with a minimal amount of vitrification solution containing the embryos, can be sealed (Kuwayama, 2007). When the CryoTip™ was compared with the Cryotop for blastocyst survival, pregnancies and deliveries, there was no difference observed (Kuwayama, 2005).

In 2003 our laboratory set out to develop a closed system of vitrification using items that could be purchased commercially. The first device used was a Stripper Tip® (MidAtlantic Diagnostics Inc., Marlton, New Jersey) in which mouse embryos were surrounded by 1.0 µL of vitrification solution and then both ends of the tip were sealed

using a heat sealer (Figure 2.1). The second device used was the nozzle from a Cryo Bio Systems (CBS; L'Aigle, France) Straw in which 1.0 µL of vitrification solution containing mouse embryos were drawn into the nozzle (Figure 2.1). The nozzle was then heat sealed on both ends. We compared the Stripper Tip® and the CBS nozzle as open and closed systems using two- and eight-cell mouse embryos (Figure 2.2).

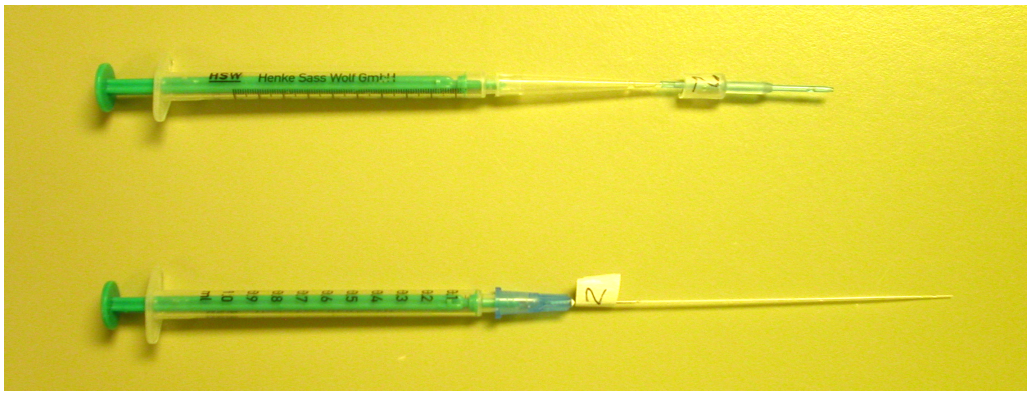


Figure 2.1. Vitrification devices.
The top device is the aspiration nozzle with pipet tip and the bottom device is the Stripper Tip® with needle. Both devices are attached to a 1 mL syringe.

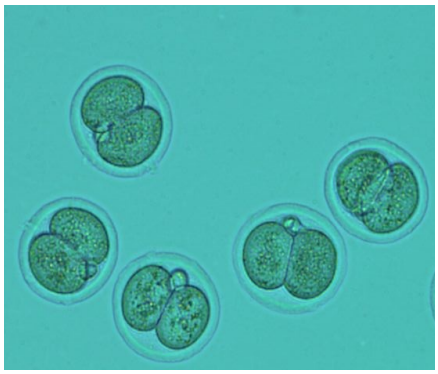
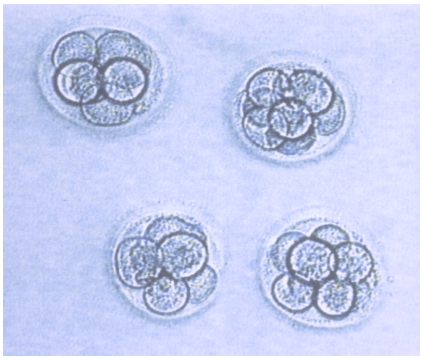
A.  B. 

Figure 2.2. Mouse Embryos.
A) Two-Cell Mouse Embryos. B) Eight-Cell Mouse Embryos.

MATERIALS AND METHODS

Male and female mice (B6C3F1) were purchased from Jackson Laboratories (Bar Harbor, Maine). This strain of mice was chosen because it is the strain that our laboratory uses for the mouse toxicity assay and because we have had good survival results when the embryos were slow-cooled and thawed. All mice were handled according to an Institutional Animal Care and Use Committee protocol for this project. Once the female mice were ready to be bred, they were injected with pregnant mare serum gonadotrophin on Day 1, and then injected with human chorionic gonadotrophin and mated on Day 3. On Day 5, the female mice were euthanized, the oviducts removed and embryos were retrieved at the two-cell stage.

Vitrification of 794 embryos occurred at the two-cell stage or the embryos were cultured overnight to the eight-cell stage and then vitrified. Two-cell and eight-cell mouse embryos were exposed to a medium consisting of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; In Vitro Care, San Diego, CA), 7.5% ethylene glycol (Sigma, St. Louis, MO) and 7.5% dimethyl sulfoxide (DMSO; Sigma) for 3.5 minutes. The embryos then were transferred to a vitrification solution consisting of DPBS with 15% ethylene glycol and 15% DMSO (Boone and Tucker 2003, personnel communications). Embryos were placed in one of four systems (open-150 µm Stripper Tip®, closed-150 µm Stripper Tip®, open-CBS nozzle or closed-CBS nozzle) and plunged into liquid nitrogen for storage.

After at least three days of storage, thawing of the embryos occurred by removing the device from liquid nitrogen and exposing it to air. The area within the straw

containing the embryos was thawed quickly by rubbing the straw between the thumb and index finger for 2-3 seconds. Embryos then were expelled from the system, collected and moved through a series of four solutions at 37°C. The first solution consisted of DPBS with 1 M sucrose and 20% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA). The second solution consisted of DPBS with 0.5 M sucrose and 10% SSS. The third solution consisted of DPBS with 0.25 M sucrose and 5% SSS and the fourth solution consisted of DPBS with 0.125 M sucrose and 2.5% SSS (Testart et al., 1986). The time of embryo exposure to each of the four solutions was 2 minutes, 3 minutes, 5 minutes and 5 minutes, respectively. Following thawing, embryos were cultured and assessed for development.

Culturing of embryos occurred in 50 µL drops of Human Tubal Fluid (Irvine Scientific, Santa Ana, CA) overlaid with oil for 48 or 72 hours. Blastocyst rates then were observed (Figure 2.3). Eight trials were performed. Chi-square analyses were performed to determine which system, cell stage and device combination yielded the most favorable blastocyst rate.

RESULTS

Overall, the eight-cell stage embryos vitrified in either the Stripper Tip® or CBS nozzle provided a higher blastocyst rate than did two-cell stage embryos vitrified in the same manner (84% [337/399] vs. 61% [243/395]; $P < 0.001$; Table 2.1). The open system did not differ from the closed system (76% [291/384] vs. 70% [289/410]; $P = 0.09$). No differences in blastocyst rate were detected between devices (Stripper Tip® or

CBS nozzle) within systems (open or closed; $P > 0.05$) with the exception of the two-cell open-system Stripper Tip® vs. CBS nozzle (74% [73/98] vs. 53% [48/91]; $P = 0.002$; Table 2.1).

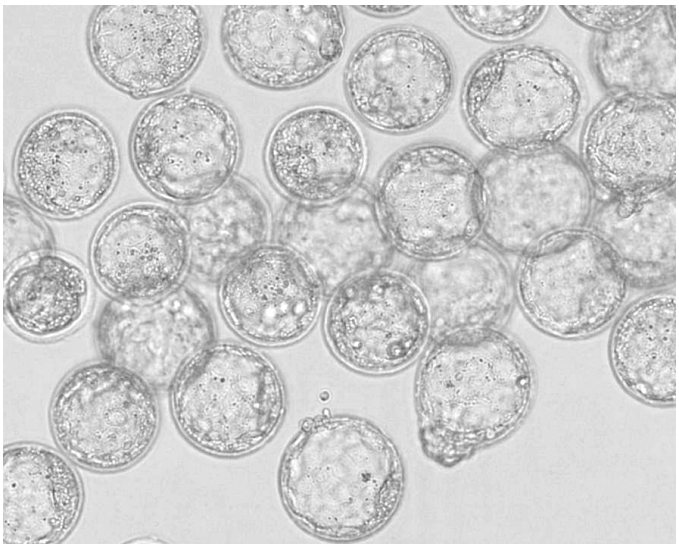
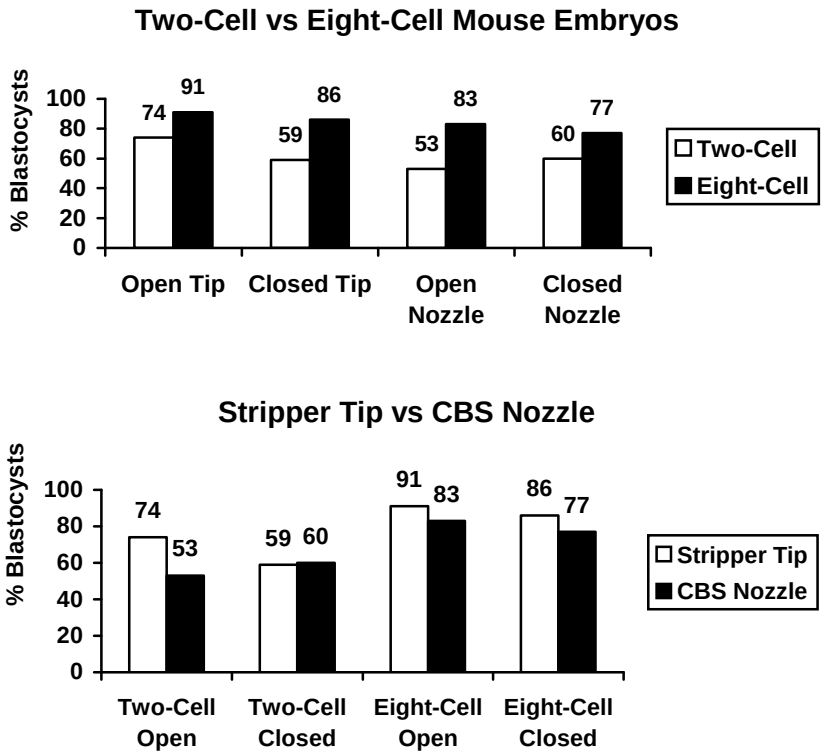


Figure 2.3. Mouse Blastocysts.

Table 2.1. Two-Cell vs. Eight-Cell Mouse Embryos, Stripper Tip vs. CBS Nozzle. Cryopreservation of two- and eight-cell mouse embryos (n=395 and 399, respectively) in either an open- or closed-Stripper Tip® or an open- or closed-CBS nozzle. Numbers above columns represent percent blastocysts.



DISCUSSION

This study describes a closed system that can be used successfully to vitrify mouse embryos, thus minimizing the potential for exposure to contaminants that may be found in liquid nitrogen. Results indicate that there is no significant difference in blastocyst rates between the open and closed systems with the exception of the two-cell, open system. Although the open system produces a slightly higher blastocyst rate, overall, it was not significantly different; therefore, the authors have selected the closed system for future studies to prevent embryo contamination from liquid nitrogen.

In 2004, Walker et al. used the Stripper Tip® to vitrify murine morulae and early hatching blastocysts and observed a 90.1% survival and continued development rate. This is the only other author known to use the Stripper Tip® as a device to vitrify embryos; although, Liebermann et al. (2002b) used a similar device, the Flexipet denuding pipette, to vitrify human 1 pronucleus (PN) and 3PN embryos. The authors then thawed and cultured the vitrified embryos. There was no significant difference in blastocyst formation between the 1PN, 3PN and control embryos. Since this time, other closed-vitrification devices have been discussed in the literature.

Information on the commercially available CryoTip™ was published in 2005 by Kuwayama et al. This group vitrified human blastocysts that produced a 93% survival rate after thawing. (Embryos were considered to have survived if they displayed a "normal response to osmotic changes during the dilution process".) Although Walker et al. (2004) and Kuwayama et al. (2005) had high survival rates for vitrified embryos, they were using embryos of a more advanced cell stage than were used in our study. In our

study, the eight-cell mouse embryos had a higher blastocyst development rate than did the two-cell mouse embryos, indicating that more developed embryos survived vitrification and thawing better than less developed embryos. Cseh et al. (1997) demonstrated mouse embryos frozen at the two-cell, four- to eight-cell, morula to early blastocyst, and expanding and expanded blastocyst stages developed in culture after being thawed at the rate of 51% (43/84), 47% (44/94), 80% (56/70) and 17% (10/59) respectively. Although these researchers determined that the two-cell and four- to eight-cell group were not different ($P > .05$), the morula and early blastocyst group was higher ($P < .05$).

The vitrification system we used does have some drawbacks. One is that we did not have precise control of the thawing temperature since we thawed in the air and then finished the process by rolling the straw between the fingers. Other vitrification systems use a water bath, which could easily be incorporated into the thawing protocol to control the thawing temperature. Another drawback, which is common for any straw system, is the need for the operator to practice with the device to be successful; this also is true of our heat-sealed, closed system of vitrification. Embryos can easily be destroyed if the operator does not pull a complete bolus of medium into the device. The bolus will split if the operator pulls the medium too quickly and thus provide embryos with the potential to stick to the sides of the Stripper Tip®.

Since 2003, many authors have published reports describing various devices and systems to vitrify embryos. Our laboratory was able to successfully vitrify embryos

using two types of closed systems which provide the advantage of alleviating concerns regarding possible liquid nitrogen contamination of embryos.

CHAPTER THREE

THE EFFECT OF TIME IN VITRIFICATION SOLUTION ON EMBRYO DEVELOPMENT, BIRTH RATE AND DNA DAMAGE

INTRODUCTION

Vitrification is a process by which cells can be frozen in such a way that a glass-like or vitrified state is obtained. This process eliminates the formation of intracellular ice crystals which can damage organelles within the cell. Vitrification of mouse embryos was first reported in 1985 (Rall and Fahy). Since then, this technique has been extended to early-cell embryos in cattle (Vajta et al., 1997) and humans (Mukaida et al., 1998) as well as blastocysts for cattle (Park et al., 1999), mice (Lane et al., 1999b), humans (Yokota et al., 2001), monkeys (Yeoman et al., 2001) and pigs (Misumi et al., 2003).

One concern regarding vitrification is the exposure of the embryos to high-osmolarity cryoprotectants because of the detrimental effects that have been observed. Rall (1987) determined that embryos remaining in vitrification solution containing dimethyl sulfoxide, acetamide and propylene glycol at 4°C for 10 to 15 minutes were able to survive, but none were able to survive after remaining in the same solution for 30 minutes. Exposure of Day-4 mouse embryos to glycerol, dimethyl sulfoxide or propylene glycol for 20 minutes, was shown to be toxic (Ali and Shelton, 2007).

To avoid embryo exposure to high concentrations of toxic cryoprotectants for extended periods, vitrification protocols generally require that embryos remain in the vitrification solution for only 1 to 2 minutes (Lieberman et al., 2002b; Kuwayama et al., 2005). This can be difficult, especially since some vitrification devices have an extensive

learning curve before the technique is mastered. Even with practice, one may not be able to quickly pick up embryos and vitrify within the time required; therefore, determining the length of time that embryos can safely reside in vitrification solution is important.

One way to determine the toxicity of a vitrification solution is to analyze embryos using the Comet Assay to detect DNA damage following exposure to these high osmotic solutions. This assay was first performed by Ostling and Johanson (1984) to observe murine lymphoma cells and later modified by Singh et al. (1988) to observe "single-stranded DNA breaks and alkali-labile sites." The premise for the Comet Assay is that damaged DNA strands will migrate out of a cell during electrophoresis to create the tail segment of the comet (the longer the tail, the more damage present); whereas, undamaged DNA will remain in the cell creating the head of the comet. This assay has been used to detect DNA damage in bovine oocytes (Chung et al., 2007) as well as hamster (Takahashi et al., 1999), bovine (Takahashi et al., 2000) and mouse (Fabian et al., 2003) embryos. Stowinska et al. (2008) and Kalthur et al. (2008) used the Comet Assay to analyze DNA damage in cryopreserved sperm. To our knowledge, our study is the first to use the Comet Assay to detect DNA damage in cryopreserved mouse embryos.

The objectives of this study are 1) to determine the length of time in vitrification solution to observe a reduction in blastocyst rate; 2) to determine the effect of time of exposure in vitrification solution on blastocyst rate and pup rate; and 3) to determine the blastocyst rate and percentage of embryos demonstrating DNA damage due to extended exposure in vitrification solution.

MATERIALS AND METHODS

Male and female mice (B6C3F1) were purchased from Jackson Laboratories (Bar Harbor, ME). This strain of mice was chosen because it has demonstrated good survival results when the embryos were slow-cooled and thawed. All mice were handled according to an Institutional Animal Care and Use Committee protocol for this project. Once acclimated, the female mice were injected with pregnant mare serum gonadotrophin on Day 1, and then injected with human chorionic gonadotrophin and mated on Day 3. On Day 5, the female mice were euthanized, the oviducts removed and embryos were retrieved at the two-cell stage. The same procedure to collect two-cell mouse embryos was used in each of the three experiments.

Experiment 1: Determine the length of time in vitrification solution to observe a reduction in blastocyst rate.

The first experiment consisted of three trials. Trial 1 had two-cell mouse embryos remaining in vitrification solution for 1, 2, 4 or 8 minutes. Trial 2 had embryos remaining in vitrification solution for 1, 2, 4, 8, 16 or 32 minutes. Trial 3 had embryos remaining in vitrification solution for 1 or 32 minutes. For all trials, two-cell mouse embryos were exposed to a medium consisting of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; In Vitro Care, San Diego, CA), 7.5% ethylene glycol (Sigma, St. Louis, MO) and 7.5% dimethyl sulfoxide (DMSO; Sigma) for 3.5 minutes. The embryos then were transferred to a vitrification solution consisting of DPBS with 15% ethylene glycol and 15% DMSO (Boone and Tucker, personal

communication). Both media were at 4°C immediately prior to use and remained at room temperature thereafter.

After remaining in vitrification solution for the specified amount of time, the embryos were collected and moved through a series of four thawing solutions at 37°C. The first solution consisted of DPBS with 1 M sucrose (Sigma) and 20% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA). The second solution consisted of DPBS with 0.5 M sucrose and 10% SSS. The third solution consisted of DPBS with 0.25 M sucrose and 5% SSS and the fourth solution consisted of DPBS with 0.125 M sucrose and 2.5% SSS. The amount of time that the embryos remained in each solution was 2 minutes, 3 minutes, 5 minutes and 5 minutes, respectively.

Embryos then were placed into 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific) overlaid with washed mineral oil and cultured in an incubator at 36.7°C with 5% CO_2 and air. After 72 hours, blastocyst rates were determined.

Experiment 2: Determine the effect of vitrification solution on blastocyst rate and pup rate when embryos remain in vitrification solution for 32 minutes.

This experiment used the same media and methods as described for Experiment 1. The difference was that all embryos remained in vitrification solution for 32 minutes prior to their exposure to the thawing solutions. Also, this experiment used control embryos which were collected and placed into HTF without being exposed to any vitrification or thawing solutions. After culturing for 72 hours, the blastocyst rate was determined for the controls and 32-minute exposure embryos.

Cultured embryos were divided into three groups. The first group was control blastocysts. The second group consisted of early and expanded blastocysts from the 32-minute exposure group, and the third group consisted of four-cell to morula-stage embryos from the 32-minute exposure group. Embryos from all three groups were transferred into designated recipient mice.

Experiment 3: Determine the blastocyst rate and percentage of embryos demonstrating DNA damage due to vitrifying embryos that remain in vitrification solution for 32 minutes.

Similar to Experiment 1, embryos were exposed to the vitrification solution; however, for this experiment they remained in vitrification solution for either 1 minute or 32 minutes. The embryos then were placed 10 at a time into a 150 μm Stripper Tips® (MidAtlantic Diagnostics Inc., Marlton, NJ), which were sealed at both ends using a Cryo Bio Systems SYMS Sealing System (Cryo Bio Systems, L'Aigle, France). Within 1 minute of transferring the embryos into the 15% ethylene glycol and 15% DMSO vitrification solution, the sealed Stripper Tips® were placed vertically into a goblet on an aluminum cane, which resided in 4 liters of liquid nitrogen in a Styrofoam container. A second goblet was inverted and attached to the top of the cane to secure the Stripper Tips® within the goblet. The canes were covered with cardboard sleeves and transferred to a storage tank containing liquid nitrogen.

To thaw the vitrified embryos, Stripper Tips® were removed from liquid nitrogen and exposed to air. The area within the Stripper Tip® containing the embryos was

thawed quickly by rubbing this location between the thumb and index finger for 2 to 3 seconds. Both ends of the Stripper Tip® were cut off and the embryos were expelled from the device with the aid of a 0.1 mL bolus of media using a 25 gauge needle attached to a 1 mL syringe. The embryos were collected and moved through the same series of four thawing solutions at 37°C described for Experiment 1.

Once thawed, embryos from the 1-minute and 32-minute exposure groups were placed into designated 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, CA) overlaid with washed mineral oil and cultured in an incubator at 36.7°C with 5% CO_2 and air. On the same days that the embryos were thawed, fresh two-cell mouse embryos were collected for controls (cultured in HTF) and for positive controls (cultured in HTF with 1% hydrogen peroxide to induce DNA damage). Controls and positive controls embryos were cultured in the same environment as described above. After 72 hours, blastocyst rates for controls, positive controls, 1-minute and 32-minute exposure groups were determined. Chi-square analyses were performed on the blastocyst rate for each group.

Once blastocyst rates were determined, the Comet Assay Kit (Trevigen®, Gaithersburg, MD) was used to evaluate the four groups of embryos (controls, positive controls, 1-minute exposure and 32-minute exposure) for the presence of comet tails. Manufacturer's recommendations to perform the assay were optimized for our laboratory. Embryos were placed into 75 µL of melted agarose. The melted agarose containing the embryos then was placed on a Comet Assay slide. The slide was held at 4°C in the dark for 30 minutes and then placed into a 4°C lysis solution for 1 hour. After this incubation,

the slide was placed into an alkaline solution at 4°C for 30 minutes. This was followed by two 5 minute rinses of the slide with Tris-Borate-EDTA (Fisher Scientific, Pittsburg, PA).

Electrophoresis was performed for 20 minutes at 20 volts and 300 amps. The slide was rinsed in alcohol for 5 minutes, allowed to dry and then placed into a desiccator. At the time of analysis, 50 µL of SYBR® Green I was added to the slide and the slide was then observed using fluorescence microscopy with a fluorescein isothiocyanate filter.

Each embryo was observed and an image was captured using Slide Book Software (Intelligent Imaging Innovation, Inc., Denver, Colorado). All embryo images were printed and examined for the presence or absence of a comet tail. Chi-square analyses were performed on the percentage of observed comet tails for all four embryo groups.

RESULTS

Experiment 1: The first trial produced a 100% (10/10) blastocyst rate for each of the 1-, 2- and 8-minute exposure time (in vitrification solution) groups. The 4-minute exposure time group produced a 78% (7/9) blastocyst rate. The second trial produced a 100% blastocyst rate for the 1 (n=9), 2 (n=8), 4 (n=10) and 8 (n=10) minute exposure time groups. The 16-minute exposure time group produced a 95% (19/20) blastocyst rate and the 32-minute exposure time group produced a 44% (8/18) blastocyst rate. The third trial

produced 100% (22/22) blastocyst rate for the 1-minute exposure time group and a 72% (38/53) blastocyst rate for the 32-minute exposure time group.

Experiment 2: The blastocyst rate was significantly different ($P < 0.001$) between the control embryos (95% [61/64]) and the 32-minute exposure time embryos (76% [193/255]). After the embryos were split into three groups (control, 32-minute blastocyst stage and 32-minute multi-cell and morula stage) and transferred to recipient females, there was no significant difference ($P = 0.3$) in pup rates between the control embryos (31% [15/48]) and the second group of embryos, which included early and expanded blastocysts (42% [20/48]). However, there was a significant difference ($P < 0.05$) in pup rates between the second group and third group of embryos, which included four-cell to morula group (2% [1/48]). There was also a significant difference ($P < 0.05$) in pup rates between the control embryos and the third group of embryos.

Experiment 3: There was no significant difference ($P = 0.316$) between the blastocyst rates for the 1-minute (57% [69/120]) and 32-minute (51% [68/133]) exposure groups of vitrified and thawed embryos. In addition, the percentage of embryos that presented comet tails for the controls, positive controls, 1-minute exposure and 32-minute exposure group was 21% (28/134), 41% (25/61), 14% (14/100) and 61% (52/85) respectively. The 1-minute exposure group was not significantly different than the control group ($P = 0.174$) but was significantly different from the 32-minute exposure group ($P < 0.001$) and

the positive controls ($P < 0.001$). The 32-minute exposure group was significantly different from the control group ($P < 0.001$) and the positive control group ($P = 0.016$).

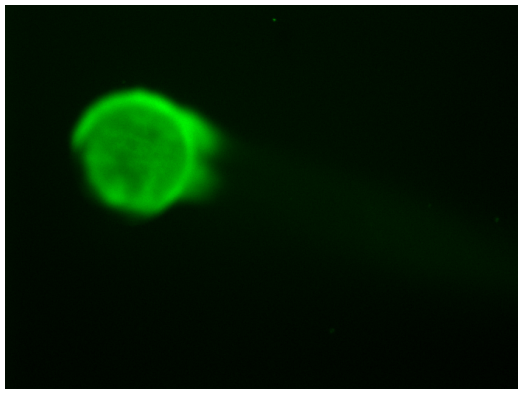


Figure 3.1. Mouse blastocyst with some DNA damage (as indicated by the small comet tail).

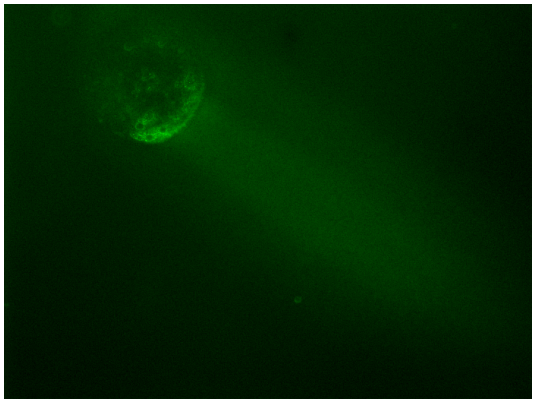


Figure 3.2 Mouse blastocyst with a large amount of DNA damage (as indicated by the large comet tail).

DISCUSSION

Experiment 1 explored the amount of time (1, 2, 4, 8, 16 or 32 minutes) an embryo could be exposed to vitrification solution before a reduction in blastocyst rate was observed. For this study, the blastocyst rate was considered reduced if it was less than or equal to 80%. (A blastocyst rate of 80% is considered normal development for two-cell mouse embryos cultured in our laboratory.) In this experiment embryos were not vitrified, but were instead exposed to vitrification and thawing solutions and then cultured to the blastocyst stage. In Trial 1 a reduced blastocyst rate was observed for the 4-minute exposure group (78%) but not for the 8-minute exposure group (100%). In Trial 2, a slight decrease in blastocyst rate was observed for the 16-minute exposure group (95%), but only the 32-minute exposure group demonstrated the defined reduction in blastocyst rate (44%). In Trial 3, a decrease was observed at the 32-minute exposure group (72%). The reduction in blastocyst rate observed at 32 minutes determined the extended time in vitrification solution for Experiment 2 and 3.

Rall (1987) determined that eight-cell mouse embryos remaining in vitrification solution containing dimethyl sulfoxide, acetamide and propylene glycol at 4°C for 10 to 15 minutes were able to survive, but none were able to survive after remaining in the same solution for 30 minutes. Our study demonstrated a decrease in blastocyst rate at 32 minutes; however, 72% of the embryos in Trial 3 were able to develop.

Ali and Shelton (2007) demonstrated exposure of Day-4 mouse embryos to glycerol, dimethyl sulfoxide or propylene glycol for 20 minutes to be toxic (blastocyst rate of 27.3% [12/44]). In our study the 16-minute exposure group was able to produce a

95% blastocyst rate. Although we did not observe blastocyst rates for times between 17 to 20 minutes, we demonstrated that 44% (Trial 2) and 72% (Trial 3) of embryos exposed to vitrification solution for 32 minutes were able to develop. This is a higher developmental ran than either Rall (1987) and Ali and Shelton (2007) observed, indicating that vitrification solutions may not be as toxic as once thought.

Experiment 2 demonstrated that the blastocyst rate for the 32-minute exposure group was 76% (193/255) which was significantly different ($P < 0.001$) than the control group (95% [61/64]) indicating that the extended time in vitrification solution inhibited blastocyst growth. Although we determined in Experiment 1 the blastocyst rate for 32-minute exposure embryos was 44%, we had a higher blastocyst rate in Experiment 2 (76%). The difference in blastocyst rates could be the result of the low number of embryos (n=18) used in Experiment 1.

In Experiment 2, the number of embryos used in the 32-minute exposure group was four times the amount of embryos used in the control group (255 vs. 64). In order to transfer 16 embryos to each designated recipient mouse (8 to each oviduct), we needed to ensure there would be adequate numbers of embryos available for transfers of the second group (32-minute blastocyst group), and third group (32-minute multi-cell to morula stage group). There was no significant differences ($P = 0.3$) between the pup rates for the controls (31% [15/48]) and the second group (42% [20/48]), indicating that those embryos that remained in vitrification solution for 32 minutes and produced blastocysts could produce pups at the same rate as embryos that were not exposed to vitrification

solution. In contrast, those embryos that remained in vitrification solution for 32 minutes but were unable to produce blastocysts (32-minute multi-cell to morula stage group) were unable to produce pups (2% [1/48]) similar to the controls (31% [15/48]; $P < 0.05$).

Experiment 3 demonstrated that although there was no difference in blastocyst rates between the 1-minute and 32-minute exposure group in blastocyst rate, the 32-minute exposure group embryos had significantly more DNA damage as determined by the Comet Assay. A pitfall of the Comet Assay is its inability to specify the exact type of DNA damage. If there is damage to individual blastomeres, degeneration of blastomeres, or degeneration of polar bodies, the Comet Assay cannot delineate these differences. Even though the Comet Assay does not pin-point the origin of the DNA damage, it can provide useful information as to the extent of the damage present by the length of the comet tail.

In summary, exposure of mouse embryos to vitrification solution for 32 minutes does cause a decrease in blastocyst rate compared to embryos that are not exposed to vitrification solution. However, if the embryos grow to the early or expanded blastocyst stage and are transferred into recipients, these embryos can produce pups at the same rate as those that are not exposed to vitrification solution despite the higher rate of DNA damage demonstrated by the 32-minute exposed embryos.

CHAPTER FOUR

BLASTOCYST RATE AND LIVE BIRTHS FROM VITRIFICATION AND SLOW-COOLED TWO-CELL MOUSE EMBRYOS

INTRODUCTION

Successful slow-cooled freezing methods have been in existence since 1972 (Whittingham et al.) and are considered the "gold standard" for cryopreserving embryos by many cryobiologists. This technique requires that a mammalian embryo be passed through a series of dehydrating solutions that replaces the water in the cell with a cryoprotectant. The embryo is placed inside a freezing vessel (e.g. straw, vial) and placed inside a freezing chamber. Once inside, the chamber is slowly cooled, to a point a few degrees below 0°C. Ice crystals are manually induced (seeding) into the cryoprotectant to prevent super cooling and then the freezing vessel is cooled to an appropriate temperature (-30°C or lower) prior to plunging into liquid nitrogen.

Vitrification is a process by which cells can be frozen in such a way that a glass-like or vitrified state is obtained. Vitrification uses viscosity which eliminates the formation of intracellular ice crystals. While vitrification technology has been in existence for over two decades (Rall and Fahy, 1985), the technology has only begun to replace the slow-cooled method in the last decade. Vitrification was first used in mice embryos (Rall and Fahy, 1985), but has been extended to early-cell embryos in cattle (Vajta et al., 1997) and humans (Mukaida et al., 1998) as well as blastocysts for cattle (Park et al., 1999), mice (Lane et al., 1999b), monkeys (Yeoman et al., 2001), humans (Yokota et al., 2001) and pigs (Misumi et al., 2003).

A number of different devices have been used to vitrify embryos including open-pulled straws (Vajta et al., 1997), grid (Park et al., 1999), hemi-straw (Vanderzwalmen et al., 2000), cryoloop (Yeoman et al., 2001) and Cryotop (Kuwayama et al., 2005). Since none of these systems are a closed system, they allow for potential contamination via liquid nitrogen with such disease as hepatitis (Tedder et al., 1995) or human immunodeficiency virus-1 (Benifla et al., 2000). Chen et al. (2001) reported a type of closed system that uses boluses of media on either side of the embryo-containing medium; however, upon thawing, these boluses could merge with the one containing the embryos resulting in contamination of the specimen. Today, there are heat-sealed, closed-system devices available commercially to prevent this potential contamination; these devices include the CryoTip® (Irvine Scientific, Irvine, California) and the High Security Vitrification Kit (Cryo Bio System, L'Aigle, France).

The slow-cooling technique of cryopreserving embryos has drawbacks compared to vitrification. Some of the drawbacks include cost, time and reliability. The mechanical freezer used to slow-cool specimens is costly and this cost does not include the yearly maintenance fee of the freezer or repairs that may be needed. Vitrification does not incur these costs since embryos are plunged directly into liquid nitrogen, eliminating the need for a mechanical freezer.

The time needed to run the slow-cool cycle is approximately 2 to 3 hours, depending upon the stage of the embryo being frozen and the freezing protocol used; furthermore, this does not include the time to move embryos though freezing solutions or to load the embryos into straws. The time required to vitrify embryos is commonly less

than 15 minutes. This includes the time to move the embryos though the vitrification media, load the embryos into/onto a device and plunge the device into liquid nitrogen. Since most human vitrification protocols do not put more than two embryos per straw, the time it will take to vitrify the embryos is dependent upon the number of straws to be frozen.

The mechanical freezer produces varying post-thaw blastocyst development in the mouse, depending on the position of the embryos in the freezing chamber (Boone et al., 2004). This study also demonstrated that different freezer positions have significantly different temperatures during the freezing process. These variations in temperature due to the mechanical freezer do not occur in vitrification because embryos are directly moved into liquid nitrogen.

One problem with cryoprotectants is that they can be toxic to embryos (Shaw et al., 2000). Although both slow-cooled and vitrification procedures use cryoprotectants, vitrification uses them at a higher concentration, thus creating an increase in osmolarity (~6000 mOsm compared to ~2500 mOsm for slow-cooled). In most vitrification protocols, embryos have a short exposure time to the vitrification media prior to being vitrified (Lieberman et al., 2002b; Kuwayama et al., 2005). When two-cell mouse embryos were exposed to vitrification solution for 32 minutes then cultured to the blastocyst stage and transferred to recipient mice, there was not a significant difference ($P = 0.3$) in pup rate (42% [20/48]) when compared to those not exposed to vitrification solution (31% [15/48]); however, there was a significant decrease ($P < 0.001$) in

blastocyst rate (76% [193/255] vs. 95% [61/64] respectively; Graves-Herring et al., 2005).

A number of studies have been published that compare slow-cooled procedures to vitrification procedures. Moussa et al. (2005) indicated that when equine embryos were frozen using slow-cooled or Open-Pulled Straw (OPS) vitrification, there was no significant difference in the percentage of dead blastomeres per embryo. Stehlik et al. (2005) froze Day-5 human blastocysts using a slow-cooled or a vitrification method. This study indicated that vitrification produced a 100% (41/41) blastocyst survival rate and produced a 50% (10/20) pregnancy rate per embryo transfer whereas slow-frozen blastocysts produced an 83.1% (59/71) survival rate and produced a 16.7% (4/24) pregnancy rate per embryo transfer.

Naik et al. (2005) used morula-stage rabbit embryos and compared two types of vitrification apparatuses (open-pulled French mini straws [OPS] and French mini straws) and slow-cooled freezing. The OPS demonstrated the highest percentage of blastocyst rates (71% [44/62]) and produced the highest percentage of offspring per number of embryos transferred (29% [7/24]) when compared to the embryos frozen in the mini straw method (blastocyst rate 48% [10/21]; offspring rate 4% [1/24]) or the slow-cool method (blastocyst rate 55% [17/31]; offspring rate 19% [5/27]).

While laboratories have had success with open-system vitrification procedures, there is potential exposure to viruses that are harbored in the liquid nitrogen. To prevent this potential, the purpose of this study was to use closed-system vitrification to 1) compare vitrification to a slow-cooled method using two-cell mouse embryos; 2)

compare the pup rate from embryos frozen/thawed via vitrification and slow-cooled methods; and 3) compare weights from pups produced via both methods at 30, 60, and 90 days following birth.

MATERIALS AND METHODS

Male and female mice (B6C3F1) were purchased from Jackson Laboratories (Bar Harbor, Maine). This strain of mice was chosen because it is the strain that our laboratory uses for the mouse toxicity assay and because we have had good survival results when the embryos were slow-cooled and thawed. All mice were handled according to an Institutional Animal Care and Use Committee protocol for this project. Once the female mice were ready to be bred, they were injected with pregnant mare serum gonadotrophin on Day 1, and then injected with human chorionic gonadotrophin and mated on Day 3. On Day 5, the female mice were euthanized, the oviducts removed and embryos were retrieved at the two-cell stage.

Vitrification

Two-cell mouse embryos were exposed to a medium consisting of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; In Vitro Care, San Diego, California), 7.5% ethylene glycol (Sigma, St. Louis, Missouri) and 7.5% dimethyl sulfoxide (DMSO; Sigma) for 3.5 minutes. The embryos were transferred to a vitrification solution consisting of DPBS with 15% ethylene glycol and 15% DMSO (Boone and Tucker, personal communications). Both media were at 4°C immediately

prior to use. Embryos were placed, ten at a time, into a 150 μm Stripper Tip® (MidAtlantic Diagnostics Inc., Marlton, New Jersey) identified with an inch of colored tape containing the date and number of embryos. The Stripper Tip® was then sealed at both ends using a Cryo Bio System SYMS Sealing System (Cryo Bio System, L'Aigle, France). Within one minute of transferring the embryos into the 15% ethylene glycol and 15% DMSO, the sealed Stripper Tip® was placed vertically into a goblet on an aluminum cane residing in 4 liters of liquid nitrogen in a Styrofoam container. Another goblet then was inverted and attached near the top of the cane to secure the Stripper Tip® within the goblets. The cane was covered with a cardboard sleeve and transferred to a storage tank containing liquid nitrogen.

To thaw vitrified embryos, Stripper Tips® were removed from liquid nitrogen and quickly thawed by rubbing this location between the thumb and index finger for 2-3 seconds. Both ends of the Stripper Tip® were cut off and the embryos were expelled from the device with the aid of a 0.1 mL bolus of media using a 25 gauge needle attached to a 1 mL syringe. The embryos were collected and moved through a series of four solutions at 37°C. The first solution consisted of DPBS with 1 M sucrose and 20% synthetic serum substitute (SSS [Irvine Scienific, Santa Ana, California]). The second solution consisted of DPBS with 0.5 M sucrose and 10% SSS. The third solution consisted of DPBS with 0.25 M sucrose and 5% SSS and the fourth solution consisted of DPBS with 0.125 M sucrose and 2.5% SSS. The amount of time that the embryos remained in each solution was 2 minutes, 3 minutes, 5 minutes and 5 minutes, respectively.

Slow-Cooled Procedure

Two-cell mouse embryos were rinsed in Solution A for 15 to 20 seconds at room temperature (23°C). Solution A consisted of Dulbecco's Phosphate Buffer Solution (PBS; Sigma) plus 20% human donor serum (tested negative for Hepatitis B, Hepatitis C, HIV, HTLV1/2 and Rapid Plasma Reagin). Next, embryos were moved into a second rinse of Solution A for 15 to 20 seconds.

Embryos then were moved into room-temperature (23°C) Solution B that consisted of Solution A plus 1.5 M propanediol (PROH). After 15 minutes, embryos were moved into room-temperature Solution C, which consisted of Solution B plus 0.1 M sucrose (Testart et al., 1986). While in Solution C, ten embryos were placed into a 0.5 mL Cryo Bio System (CBS) straw, which was heat-sealed using the Cryo Bio Systems SYMS Sealing System. After a total of 15 minutes exposure to Solution C, the straw containing the embryos was placed into a Kryo 10 Series II Planer Freezer (Sunbury-on-Thames, Middlesex, United Kingdom) and the freezing protocol was initiated. Settings for the freezing protocol were as follows: Start temperature of 23°C with a decrease to -7°C at a rate of -2°C per minute; hold for 15 minutes; -7°C to -30°C at a rate of -0.3°C per minute; hold for 10 minutes; -30°C to -180°C at a rate of -50°C per minute; hold for 10 minutes; and plunge into liquid nitrogen. Manual seeding was performed 5 minutes after the -7.0°C hold had begun. Following the freeze, straws were removed from the programmable freezer and placed into a storage tank containing liquid nitrogen.

To thaw slow-cooled embryos, a straw was removed from liquid nitrogen and placed on the counter at room temperature for 40 seconds; thawing was completed by

rolling the straw between the fingers. Once thawing was complete, the exterior of the straw was dried and both ends were cut. Embryos were expelled from the straw by attaching a 1 mL syringe to one end of the straw and pushing the medium out with a bolus of air.

Embryos were moved through four solutions of decreasing osmolality (Thawing Solutions A, B, C and D) at 5-minute intervals. Solutions A, B, and C were held at room temperature, while Solution D was held at 37^{o}C. Thawing Solution A consisted of PBS, 1.0 M PROH, 0.2 M sucrose and 20% human donor serum. Thawing Solution B consisted of PBS, 0.5 M PROH, 0.2 M sucrose and 20% human donor serum. Thawing Solution C contained PBS, 0.2 M sucrose and 20% human donor serum and Thawing Solution D contained PBS and 20% human donor serum.

Post-Thaw Procedures

Embryos frozen, using either cryopreservation method, were thawed over a period of four trials. Once thawed, embryos were placed in 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, California) overlaid with washed mineral oil and cultured in an incubator with a 36.7°C, 5% CO_2 and air atmosphere. After 72 hours, blastocyst rates for both methods were determined.

For three of the trials, blastocyst transfers were performed. The expanded blastocysts for both cryopreservation methods were collected and placed into separate tubes containing 0.5 mL of HTF supplemented with 10% SSS to prepare for embryo transfer.

Recipient CD1 female mice were mated with vasectomized males and checked for plugs. Blastocysts were transferred 48 hours later to two recipient females (one for each method) for each of the three trials. Blastocysts were transferred, eight at a time, into each uterine horn for a total of sixteen blastocysts per recipient. Recipient females were observed 17 days later for pups. Pups were observed for physical anomalies, weaned and separated by gender on day 25. All pups were weighed on days 30, 60 and 90 after birth.

Statistical Information

Thirty-two straws (sixteen vitrification straws and sixteen slow-cooled straws) were thawed over a period of four trials. For each trial, a randomized set of four straws from each method was thawed. Statistics were performed by chi-square for blastocyst rate comparison and pup rate comparison between the vitrification and the slow-cooled method. Student's t-test was performed for comparison of pup weights (by gender) for the vitrification and the slow-cooled method.

RESULTS

The overall blastocyst rate for the vitrification method was 52% (69/132) versus 58% (92/158) for the slow-cooled method. The overall percentage of pups born for the vitrification method was 31% (15/48) versus 29% (14/48) for the slow-cooled method. Neither the blastocyst rate nor pup rate was significantly different ($P > 0.05$) when the two cryopreservation methods were compared.

Fifteen pups (five males, nine females, and one male that did not survive after birth) developed from vitrified embryos, while fourteen pups (two males, 11 females, and one unknown [cannibalized after birth]) developed from slow-cooled embryos. There was not a significant difference ($P > 0.05$) in gender in the vitrified group; however, the gender was significantly different ($P < 0.05$) in the slow-cooled group. The average weight of the males from slow-cooled embryos was 23.6g, 34.0g, and 41.6g, respectively on days 30, 60 and 90, whereas the average weight of the males from vitrified embryos was 23.5g, 34.1g, and 40.2g, respectively on the same days. Female pup average weights from slow-cooled embryos were 19.4g, 24.9g, and 28.7g, respectively on the three weigh dates, whereas female pup average weight from vitrified embryos were 19.3g, 24.1g, and 28.3g, respectively. There were no significant differences between freezing methods ($P > 0.3$) when comparing males on each day weighed and females on each day weighed. One pup that originated from a vitrified embryo had hydroencephalitis, which is sometimes observed in mouse colonies.

DISCUSSION

This study indicated that, for cryopreserved mouse embryos, there is no significant difference in blastocyst rate or pup rate between the closed-system vitrification and the slow-cooled cryopreservation method. Although one pup in the vitrified group demonstrated a physical abnormality, this is sometimes observed in normal mouse colonies. Furthermore, there was no significant difference between the weights of the offspring following weaning. In contrast, blastocyst development

observed by Uechi et al. (1999) indicated a significant difference between vitrification (22.3%) and slowly frozen-thawed method (32.8%) for two-cell mouse embryos. These researchers also observed a significant difference in implantation rates from blastocysts derived from vitrified-thawed (10.2%) two-cell embryos and slowly frozen-thawed (22.1%) two-cell embryos.

In contrast to Uechi and coworkers (1999), Walker et al. (2004) demonstrated that when two-cell mouse embryos were frozen, thawed and cultured to the blastocyst stage, blastocyst development for programmable rate freezing (80.6%) was significantly lower than vitrification (90.7%). In the Walker (2004) study, vitrification occurred by first plunging a Stripper Tip® into liquid nitrogen and then later sealing the device inside a CBS straw. Our study is similar to Walker et al. (2004) in that we also utilized the Stripper Tip® and compared a vitrification method to a slow-cooled method; however, our study does not expose embryos to potential contaminates from liquid nitrogen. Furthermore, our study compared live birth rates (not just implantation rates) and post weaning weights of pups produced from blastocyst transfers.

Simultaneous to our research, others started using modified sealed systems with vitrification. Kumasako et al. (2005) reported a human pregnancy after vitrification of zygotes using 0.25 mL plastic straws (IMV, France) that were heat sealed and held in liquid nitrogen vapor prior to plunging. Not only have these closed systems produced live births, but successful live births with open-vitrification systems have been demonstrated in pigs (Misumi et al., 2003) and humans (Yokota et al., 2001; Mukaida et al., 2003; Takahashi et al., 2005).

Kuwayama et al. (2005) vitrified human four-cell embryos with a Cryotop (Kitazato Co., Fujinomiya, Japan) in which embryos were placed in a small volume of medium and then placed on a polypropylene strip. The strip was subsequently submerged into liquid nitrogen. A 90% embryo survival rate and a 45% live birth per transfer rate were obtained using this method. These authors also successfully vitrified human blastocysts (93% survival) with the CryoTip® (Irvine Scientific) method, which is a heat-sealed closed system. The CryoTip® method produced a 48% delivery per transfer rate.

Many vitrification methods such as the cryoloop, grid, hemi-straw and open-pulled straws allow embryos to come in contact with potentially contaminated liquid nitrogen. In contrast, we have successfully vitrified embryos in a heat-sealed, closed-system, which prevents liquid nitrogen from contaminating the embryos. Furthermore, as evidence that our system works, we have produced offspring (although the number of transfers per group were low, we would have had to produce over 8,000 offspring to have a statistical power of 0.8) from vitrified and slow-cooled two-cell mouse embryos as well as observed post-weaning pup weights. The combination of blastocyst rate, live births and observation of post-weaning body weight has not been demonstrated in other studies.

In the vitrification and slow-cooled methods, there were more females born than males. Although there is not an explanation for this outcome, Perez-Crespo et al. (2005) demonstrated that male mouse embryos are more vulnerable to stresses than female embryos. These researchers demonstrated that female embryos can survive heat stress better than male embryos in both *in vivo* and *in vitro* conditions. They also suggest that

female embryos can survive better than male embryos under oxidative stress conditions which can be caused by a variety of stresses. Since cryopreservation is a process that causes stress to the embryo (upregulation of stress-related genes [Boonkusol et al., 2006]), this may be one reason for the increase in the number of females observed in our study.

One drawback of a straw system is the need for the operator to practice with the device in order to be successful; this is true of our heat-sealed, closed system of vitrification. Embryos can easily be destroyed if the operator does not pull a complete bolus of medium into the device. If the operator pulls the bolus of medium too quickly, the bolus will split and embryos may potentially stick to the sides of the Stripper Tip®. Although a long learning curve may be necessary for some vitrification devices, this was not observed for this vitrification device. When three embryologists in our laboratory were instructed on how to use the device, they were able to successfully vitrify and retrieve all embryos by the third use of the device (results not published).

Another drawback of this vitrification system is that we do not have a well-controlled temperature thawing step for vitrification. Commercially purchased vitrification systems use a water bath, which we could incorporate into the thawing protocol to control the thawing temperature.

Since our study demonstrated that there were no significant differences in blastocyst rate, pup rate or phenotypic characteristics of the pups produced from slow-cooled versus vitrified embryos, we plan to further investigate the potential effects of vitrification on genotypic damage via the Comet Assay. The impetus for this study is that

aneuploid zygotes and malformed fetuses can arise from vitrification (Kola et al., 1988). If the subsequent study indicates that vitrification does not cause genetic damage, the technique allows the scientist an additional, less time-consuming, method of cryopreserving embryos. The variation associated with mechanical freezers (Boone et al., 2004) would be eliminated and the final outcome would be a more cost-effective system to freeze embryos.

In summary, vitrification yielded similar results as the more labor-intensive, slow-cool method for cryopreserving mouse embryos, as measured by blastocyst rates, pup rates and post weaning weights.

CHAPTER FIVE

DNA DAMAGE IN VITRIFIED AND SLOW-COOLED TWO-CELL MOUSE EMBRYOS

INTRODUCTION

Vitrification is a process by which cells can be frozen in such a way that a glass-like or vitrified state is obtained. By using viscosity, vitrification eliminates the formation of intracellular ice crystals. The use of vitrification of mouse embryos was first reported in 1985 (Rall and Fahy, 1985). Since then, this technique has been extended to early-cell embryos in cattle (Vajta et al., 1997) and humans (Mukaida et al., 1998) as well as blastocysts from cattle (Park et al., 1999), mice (Lane et al., 1999b), monkeys (Yeoman et al., 2001), humans (Yokota et al., 2001) and pigs (Misumi et al., 2003).

The use of vitrification for embryo cryopreservation has sparked a debate regarding the merits of using open- versus closed-vitrification systems. Open systems used to vitrify embryos include open-pulled straws (Vajta et al. 1997), grid (Park et al., 1999), hemi-straw (Vanderzwalmen et al., 2000) and the cryoloop (Yeoman et al., 2001). All of these open systems allow for potential contamination from liquid nitrogen that may harbor infectious diseases such as hepatitis (Tedder et al., 1995).

Many different types of closed-vitrification systems have been researched. Chen et al. (2001) reported a type of closed system that uses boluses of media on either side of the embryo-containing medium; however, upon thawing, these boluses could merge with the one containing the embryos resulting in contamination of the specimen. Today, heat-

sealed, closed-system devices exist to prevent this potential contamination. These devices include the CryoTip® (Irvine Scientific, Irvine, California) and the High Security Vitrification Kit (Cryo Bio System, L'Aigle, France).

Vitrification has quickly become a preferred method of embryo cryopreservation in human Assisted Reproductive Technology laboratories. Several laboratories have described the use of vitrification procedures and their ability to produce viable oocytes and embryos, and have reported live-births from vitrified embryos; however, due to the report of aneuploid zygotes and malformed fetuses that can arise from vitrification (Kola et al., 1988), studies of possible genotypic embryo damage due to vitrification should be undertaken.

One assay designed to detect DNA damage is the Comet Assay. This assay was first performed by Ostling and Johanson (1984) to observe murine lymphoma cells and later modified by Singh et al. (1988) to observe "single-stranded DNA breaks and alkali-labile sites." The premise for the Comet Assay is that damaged DNA strands will migrate out of a cell during electrophoresis to create the tail segment of the comet, whereas, undamaged DNA will remain in the cell creating the head of the comet. This assay has been used to detect DNA damage in bovine oocytes (Chung et al., 2007) as well as hamster (Takahashi et al., 1999), bovine (Takahashi et al., 2000) and mouse (Fabian et al., 2003) embryos.

The objectives of this study are to determine: 1) the blastocyst rate for two-cell mouse embryos frozen via a vitrification or a slow-cooled method and 2) determine

whether either of these two freezing methods causes DNA damage (as indicated by the presence of a comet tail).

MATERIALS AND METHODS

Male and female mice (B6C3F1) were purchased from Jackson Laboratories (Bar Harbor, ME). This strain of mice was chosen because it is the strain that our laboratory uses for mouse embryo-toxicity assays and we have had good survival results when the embryos were slow-cooled and thawed. All mice were handled according to an Institutional Animal Care and Use Committee protocol for this project. Once the female mice were acclimated, they were injected with pregnant mare serum gonadotrophin on Day 1, and then injected with human chorionic gonadotrophin and mated on Day 3. On Day 5, the female mice were euthanized, the oviducts removed, and embryos were retrieved at the two-cell stage.

Two-cell mouse embryos were used for this two-part study. Part I of the study observed the blastocyst rate of two-cell mouse embryos that were frozen with either vitrification or slow-cooling.

Vitrification Procedure

Two-cell mouse embryos were exposed for 3.5 minutes to a medium consisting of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; In Vitro Care, San Diego, CA), 7.5% ethylene glycol (Sigma, St. Louis, MO) and 7.5% dimethyl sulfoxide (DMSO; Sigma). The embryos then were transferred to a vitrification solution

consisting of DPBS with 15% ethylene glycol and 15% DMSO (Boone and Tucker 2003, personal communications). Both media were at 4°C immediately prior to use. Embryos were placed 10 at a time into a 150 μm Stripper Tip® (MidAtlantic Diagnostics Inc., Marlton, NJ) which then was sealed at both ends using a Cryo Bio Systems SYMS Sealing System (Cryo Bio Systems, L'Aigle, France). Within 1 minute of transferring the embryos into the 15% ethylene glycol and 15% DMSO, the sealed Stripper Tip® was placed vertically into a goblet on an aluminum cane which resided in 4 liters of liquid nitrogen in a Styrofoam container. Another goblet then was inverted and attached near the top of the cane to secure the Stripper Tip® within the goblets. The cane was covered with a cardboard sleeve and transferred to a storage tank containing liquid nitrogen.

To thaw vitrified embryos, Stripper Tips® were removed from liquid nitrogen and exposed to air. The area within the Stripper Tip® which contained the embryos was thawed quickly by rubbing this location between the thumb and index finger for 2 to 3 seconds. Both ends of the Stripper Tip® were cut off and the embryos were expelled from the device with the aid of a bolus of 0.1 mL of media using a 25 gauge needle attached to a 1 mL syringe. The embryos were collected and moved through a series of four thawing solutions at 37°C. The first solution consisted of DPBS with 1 M sucrose (Sigma) and 20% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA). The second solution consisted of DPBS with 0.5 M sucrose and 10% SSS. The third solution consisted of DPBS with 0.25 M sucrose and 5% SSS and the fourth solution consisted of DPBS with 0.125 M sucrose and 2.5% SSS. The amount of time that the

embryos remained in each solution was 2 minutes, 3 minutes, 5 minutes and 5 minutes, respectively.

Slow-Cooled Procedure

Two-cell mouse embryos were exposed for 15 to 20 seconds to room temperature (23°C) Solution A which contained Dulbecco's Phosphate Buffer Solution (PBS, Sigma) with 20% human donor serum. Next, embryos were moved into a second rinse of Solution A for 15 to 20 seconds. Embryos then were moved into room temperature Solution B consisting of Solution A plus 1.5 M propanediol (PROH, Sigma). After 15 minutes, embryos were moved into room-temperature Solution C, which consisted of Solution B and 0.1 M sucrose (Testart et al., 1986). While in Solution C, ten embryos were placed into a Cryo Bio System (CBS) straw which was heat-sealed using the CBS SYMS Sealing System.

After a total of 15 minutes of exposure to Solution C, straws containing the embryos were placed into a Kryo 10 Series II Planer Freezer (Sunbury-on-Thames, Middlesex, United Kingdom) and the freezing protocol was initiated. Settings for the freezing protocol were as follows: Start temperature of 23°C with a decrease to -7°C at a rate of -2°C per minute; hold for 15 minutes; -7°C to -30°C at a rate of -0.3°C per minute; hold for 10 minutes; -30°C to -180°C at a rate of -50°C per minute; hold for 10 minutes; and plunge into liquid nitrogen. Manual seeding was performed 5 minutes after the -7.0°C hold had begun. At the conclusion of the freezing protocol, straws were removed from the programmable freezer and placed into a storage tank containing liquid nitrogen.

To thaw slow-cooled embryos, straws were removed from liquid nitrogen and placed on a counter at room temperature for 40 seconds. Thawing then was completed by rolling the straw between the fingers. Once thawing was complete, the exterior of the straw was dried and both ends were cut off. Embryos were expelled from the straw by attaching a 1 mL syringe to one end of the straw and pushing the medium out with a bolus of air. The embryos were moved through four solutions of decreasing osmolality (Thawing Solutions A, B, C and D) at 5-minute intervals. Solutions A, B, and C were held at room temperature, while Solution D was held at 37°C. Thawing Solution A consisted of PBS, 1.0 M PROH, 0.2 M sucrose and 20% donor serum. Thawing Solution B consisted of PBS, 0.5 M PROH, 0.2 M sucrose and 20% donor serum. Thawing Solution C contained of PBS, 0.2 M sucrose and 20% donor serum. Thawing Solution D contained PBS and 20% donor serum.

Culturing Procedure

Once thawed, embryos cryopreserved using either method (vitrification or slow-cooled), were placed into 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, CA) overlaid with washed mineral oil and cultured in an incubator at 36.7°C with 5% CO_2 and air. In addition, controls (cultured in HTF) and positive controls (cultured in HTF with 1% hydrogen peroxide), also were cultured in the same environment as described above. After 72 hours, blastocyst rates for controls, positive controls and both freezing methods were determined. Chi-square analyses were performed on the blastocyst rate for each group.

Comet Assay

Part II of the study used the Comet Assay Kit (Trevigen®, Gaithersburg, MD) to evaluate the four groups of embryos (controls, positive controls, vitrified, slow-cooled) for comet tails. Manufacturer's recommendations to perform the assay have been optimized for our laboratory. Embryos were placed in 75 µL of melted agarose. The melted agarose containing the embryos then was placed on a Comet Assay slide. The slide was held at 4°C in the dark for 30 minutes and placed into a 4°C lysis solution for 1 hour. After this incubation, the slide was placed into an alkaline solution at 4°C for 30 minutes. This was followed by two 5 minute rinses of the slide with Tris-Borate-EDTA (Fisher Scientific, Pittsburg, PA).

Electrophoresis was performed for 20 minutes at 20 volts and 300 amps. The slide was rinsed in alcohol for 5 minutes, allowed to dry and placed into a desiccator. At the time of analysis, 50 µL of SYBR® Green I was added to the slide and observed using fluorescence microscopy with a fluorescein isothiocyanate filter.

Measurements were taken for the length of each embryo's comet tail using Slide Book Software (Intelligent Imaging Innovation, Inc., Denver, Colorado). In order to distinguish normal comet tails from abnormal comet tails, a confidence interval was created for the controls. The upper bound limit for the confidence interval was used as a boundary for undamaged embryos. Any tail length less than or equal to the upper bound limit was considered normal, whereas any tail length that was greater than the upper bound limit was considered abnormal. Chi-square analyses were performed on percentage of normal tail lengths for all embryo groups.

RESULTS

For Part I (observation of blastocyst rates), controls produced a blastocyst rate of 100% (97/97) which was significantly different ($P < 0.001$) from the positive controls (66% [71/108]), vitrified (66% [64/97]) and slow-cooled (66% [66/100]) groups. The positive controls, vitrified and slow-cooled groups were not significantly different ($P > 0.9$) from each other.

For Part II, the Comet Assay was performed and measurements were taken for the length of each embryo's comet tail. The controls were analyzed and the upper bound limit of the 95% confidence interval was determined to be 46 µm; therefore, any tail length longer than this was considered abnormal. There was no significant difference in the percent of normal tail lengths ($P = 0.08$) between controls (78% [67/86]) and positive controls (64% [34/53]). There was a significant difference ($P = 0.02$) in the percentage of normal tail lengths between the control and vitrified (56% [26/46]) groups. There was also a significant difference ($P < 0.05$) between the control and the slow-cooled (40% [17/42]) groups. There was a significant difference ($P = 0.02$) between the positive control group and the slow-cooled group, but no difference ($P = 0.54$) between the positive control group and the vitrified group. There was no significant difference ($P = 0.14$) in the percentage of normal tail lengths between the vitrified and slow-cooled groups.

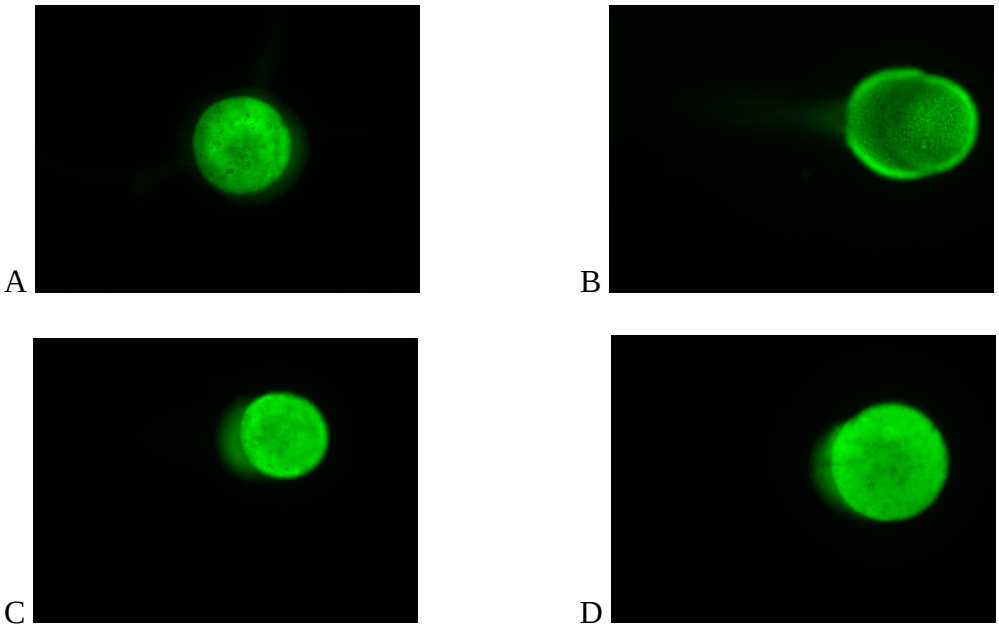


Figure 5.1. Observation of blastocysts for a comet tail.
A) Control blastocyst with no comet tail. B) Positive control blastocyst with comet tail present. C) Blastocyst that was vitrified at the two-cell stage with small comet tail. D) Blastocyst that was slow-cooled at the two-cell stage with small comet tail.

DISCUSSION

Vitrification has been used since 1985 (Rall and Fahy, 1985). Since that time researchers have performed vitrification using open systems (Yeoman et al., 2001; Park et al., 1999; Vajta et al., 1997) and closed systems (Kuwayama, 2007). A drawback of the open systems is the possibility of liquid nitrogen contamination of embryos (Tedder et al., 1995); therefore, a closed system is preferred. Although several laboratories have used vitrification procedures and are able to produce viable oocytes, embryos, and live-births from these embryos, genotypic damage should be a concern due to reports of aneuploid zygotes and malformed fetuses that can arise from vitrified embryos (Kola et al., 1988).

As in the case of intracytoplasmic sperm injection (ICSI), the rapid adoption of new Assisted Reproduction Technologies (ART), such as vitrification raises concerns about the long-term consequences on offspring generated from such technologies. The earliest reported use of ICSI in a human ART facility occurred in 1992 (Palermo et al.). The technique was then rapidly implemented in ART facilities world-wide with little, if any, preliminary animal testing. Scientists are now questioning if the procedure is indeed safe. The ICSI procedure has been associated with an increased incidence of imprinting defects (Cox et al., 2002), Beckwith-Wiedeman Syndrome (DeBaun et al., 2003) and Angelman Syndrome (Orstavik et al., 2003).

For many years, slow-freezing with a mechanical freezer has been the industry standard for embryo cryopreservation. This time-tested technique is not without its drawbacks. These drawbacks include high expense, laboratory personnel time and

reduction of embryo development due to temperature differences within the freezing chamber. The initial cost of a mechanical freezer to slow-cool specimens is expensive, and does not include the yearly maintenance fees and unanticipated repairs. The time necessary to process embryos through a slow-freeze cycle is approximately 2 to 3 hours depending on the embryo stage. This time does not include the time spent moving embryos though freezing solutions or loading the embryos into straws. These additional steps also increase the risk of damage to the embryos. Vitrification allows the embryos to be moved through the vitrification media, into a freezing device and then plunged directly into liquid nitrogen without the aid of a mechanical freezer. This reduces the amount of time necessary for a vitrification cycle to 5 to 10 minutes; this includes the time to move the embryos through vitrification solutions.

Boone et al. (2004) reported that the location of embryos within a freezing chamber alters future developmental rates. These researchers also discovered that different positions within a freezing chamber demonstrated significantly different temperatures during cooling. Vitrification eliminates variations in temperature due to mechanical equipment as embryos are moved from the cryoprotectant medium directly into liquid nitrogen.

We compared the blastocyst rate for two-cell mouse embryos cryopreserved using a vitrification method and a slow-cooled method and compared DNA damage caused by both freezing methods using the Comet Assay. There were no significant differences observed in either the blastocyst rate or in the percentage of normal tail lengths (indicating DNA damage) between vitrified and slow-cooled two-cell mouse embryos.

We did not observe a significant difference between the controls and positive controls when the Comet Assay was performed; however, an increased level of hydrogen peroxide, for the positive controls, may be needed to demonstrate a significant difference. A significant difference observed between the controls and both freezing methods suggests that freezing causes DNA damage regardless of the cryopreservation protocol used.

A pitfall of the Comet Assay is the task of distinguishing damage to individual blastomeres, degeneration of blastomeres, or degeneration of polar bodies, all of which will be observed as a comet tail; therefore, the Comet Assay does not discriminate where the DNA damage is present. Although other researchers have used the Comet Assay to detect DNA damage in embryos (Takahashi et al., 1999; Takahashi et al., 2000; Fabian et al., 2003), we have not found other literature that describes the use of this assay for detection of DNA damage in cryopreserved mouse embryos. Stowinska et al. (2008) and Kalthur et al. (2008) have used the Comet Assay to analyze DNA damage in cryopreserved sperm.

In summary, we found no significant difference between the vitrification and slow-cooled methods when the blastocyst rates of frozen/thawed mouse embryos were compared. We also demonstrate that the vitrification and slow-cooled methods have a significantly lower percentage of normal embryos when compared to control embryos as assessed by the Comet Assay; however, neither freezing method is superior to the other in the production of normal embryos. With the reduction in cost, reduction in time for

laboratory personnel and absence of mechanical equipment, vitrification appears to be a better choice for freezing mammalian embryos.

CHAPTER SIX

COMPARISON OF DNA DAMAGE BETWEEN VITRIFIED AND SLOW-COOLED MOUSE EMBRYOS

INTRODUCTION

Vitrification has been in existence since 1985 (Rall and Fahy) and is currently being used to cryopreserve human embryos (Liebermann et al., 2002a; Vanderzwalmen et al., 2003; Kuwayama et al., 2005). A previous study performed by this laboratory determined that there was no significant difference ($P > 0.9$) between vitrification and slow-cooled methods when blastocyst rates were compared (66%; 64/97, 66%; 66/100, respectively), nor was either method superior in the production of normal embryos (56% [26/46], 40% [17/42], respectively; $P = 0.14$) when analyzed by the Comet Assay (Trevigen®; Graves-Herring and Boone, 2007). In contrast to this previous study, this study will culture thawed two-cell embryos to the eight-cell stage and analyze DNA damage with the use of the Comet Assay.

This laboratory currently uses mouse embryos that are cultured in 20% oxygen concentration. This oxygen tension reduces the number of embryos that develop to the blastocyst stage and the number of cells present in the blastocyst when compared to 5% oxygen concentration (Karagenc et al., 2004; Bavister 2004). Takahashi et al. (1999) observed a longer mean comet tail length (indicative of more DNA damage) in cattle embryos cultured to the blastocyst stage under 20% oxygen concentration (tail length - 149.9 µm) than 5% oxygen concentration (tail length - 43.3µm).

The objective of this study is to determine if there is more DNA damage caused by freezing with the vitrification method or the slow-cooled method. This is a randomized, prospective, pilot study with two-cell mouse embryos cultured to the eight-cell stage.

MATERIALS AND METHODS

Male and female mice (B6C3F1) were purchased from Jackson Laboratories (Bar Harbor, ME). This strain of mice was chosen because it is the strain that our laboratory uses for the mouse embryo-toxicity assay and we have had good survival results when the embryos were slow-cooled and thawed. All mice were handled according to an Institutional Animal Care and Use Committee protocol for this project. Once the female mice were acclimated, they were injected with pregnant mare serum gonadotrophin on Day 1, and then injected with human chorionic gonadotrophin and mated on Day 3. On Day 5, the female mice were euthanized, the oviducts removed, and embryos were retrieved at the two-cell stage.

Vitrification Procedure

Two-cell mouse embryos were exposed to a medium consisting of Dulbecco's Phosphate Buffered Saline without calcium and magnesium (DPBS; In Vitro Care, San Diego, CA), 7.5% ethylene glycol (Sigma, St. Louis, MO) and 7.5% dimethyl sulfoxide (DMSO; Sigma) for 3.5 minutes. The embryos then were transferred to a vitrification solution consisting of DPBS with 15% ethylene glycol and 15% DMSO (Boone and

Tucker 2003, personal communications). Both media were at 4°C immediately prior to use. Embryos were placed 10 at a time into a 150 μm Stripper Tip® (MidAtlantic Diagnostics Inc., Marlton, NJ) which then was sealed at both ends using a Cryo Bio Systems SYMS Sealing System (Cryo Bio Systems, L'Aigle, France). Within 1 minute of transferring the embryos into the 15% ethylene glycol and 15% DMSO, the sealed Stripper Tip® was placed vertically into a goblet on an aluminum cane which resided in 4 liters of liquid nitrogen in a Styrofoam container. Another goblet then was inverted and attached near the top of the cane to secure the Stripper Tip® within the goblets. The cane was covered with a cardboard sleeve and transferred to a storage tank containing liquid nitrogen.

To thaw vitrified embryos, Stripper Tips® were removed from liquid nitrogen and exposed to air. The area within the Stripper Tip® which contained the embryos was thawed quickly by rubbing this location between the thumb and index finger for 2 to 3 seconds. Both ends of the Stripper Tip® were cut off and the embryos were expelled from the device with the aid of a bolus of 0.1 mL of media using a 25 gauge needle attached to a 1 mL syringe. The embryos were collected and moved through a series of four thawing solutions at 37°C. The first solution consisted of DPBS with 1 M sucrose (Sigma) and 20% Synthetic Serum Substitute (SSS; Irvine Scientific, Santa Ana, CA). The second solution consisted of DPBS with 0.5 M sucrose and 10% SSS. The third solution consisted of DPBS with 0.25 M sucrose and 5% SSS and the fourth solution consisted of DPBS with 0.125 M sucrose and 2.5% SSS. The amount of time that the

embryos remained in each solution was 2 minutes, 3 minutes, 5 minutes and 5 minutes, respectively.

Slow-Cooled Procedure

Two-cell mouse embryos were exposed for 15 to 20 seconds to room temperature (23°C) Solution A which contained Dulbecco's Phosphate Buffer Solution (PBS, Sigma) with 20% human donor serum. Next, embryos were moved into a second rinse of Solution A for 15 to 20 seconds. Embryos then were moved into room temperature Solution B consisting of Solution A plus 1.5 M propanediol (PROH, Sigma). After 15 minutes, embryos were moved into room-temperature Solution C, which consisted of Solution B and 0.1 M sucrose (Testart et al., 1986). While in Solution C, ten embryos were placed into a Cryo Bio System (CBS) straw which was heat-sealed using the CBS SYMS Sealing System.

After a total of 15 minutes of exposure to Solution C, straws containing the embryos were placed into a Kryo 10 Series II Planer Freezer (Sunbury-on-Thames, Middlesex, United Kingdom) and the freezing protocol was initiated. Settings for the freezing protocol were as follows: Start temperature of 23°C with a decrease to -7°C at a rate of -2°C per minute; hold for 15 minutes; -7°C to -30°C at a rate of -0.3°C per minute; hold for 10 minutes; -30°C to -180°C at a rate of -50°C per minute; hold for 10 minutes; and plunge into liquid nitrogen. Manual seeding was performed 5 minutes after the -7.0°C hold had begun. At the conclusion of the freezing protocol, straws were removed from the programmable freezer and placed into a storage tank containing liquid nitrogen.

To thaw slow-cooled embryos, straws were removed from liquid nitrogen and placed on a counter at room temperature for 40 seconds. Thawing then was completed by rolling the straw between the fingers. Once thawing was complete, the exterior of the straw was dried and both ends were cut off. Embryos were expelled from the straw by attaching a 1 mL syringe to one end of the straw and pushing the medium out with a bolus of air. The embryos were moved through four solutions of decreasing osmolality (Thawing Solutions A, B, C and D) at 5-minute intervals. Solutions A, B and C were held at room temperature, while Solution D was held at 37°C. Thawing Solution A consisted of PBS, 1.0 M PROH, 0.2 M sucrose and 20% donor serum. Thawing Solution B consisted of PBS, 0.5 M PROH, 0.2 M sucrose and 20% donor serum. Thawing Solution C contained of PBS, 0.2 M sucrose and 20% donor serum. Thawing Solution D contained PBS and 20% donor serum.

Culturing Procedure

Once thawed, embryos cryopreserved using either method (vitrification or slow-cooled), were placed into 50 µL drops of Human Tubal Fluid (HTF; Irvine Scientific, Santa Ana, CA) overlaid with washed mineral oil and cultured in an incubator at 36.7°C with 5% CO_2 and air for 24 hours.

This study had three controls. The first control embryos were cultured in HTF (HTF control) for 24 hours, the second control embryos were cultured in HTF for 24 hours and then exposed to UV light (HTF/UV positive controls), and the third control embryos were embryos collected from females at the eight-cell stage (fresh controls).

Comet Assay

Part II of the study used the Comet Assay Kit (Trevigen®, Gaithersburg, MD) to evaluate the four groups of embryos (controls, positive controls, vitrified, slow-cooled) for comet tails. Manufacturer's recommendations to perform the assay have been optimized for our laboratory. Embryos were placed in 75 µL of melted agarose. The melted agarose containing the embryos then was placed on a Comet Assay slide. The slide was held at 4°C in the dark for 30 minutes and placed into a 4°C lysis solution for 1 hour. After this incubator, the slide was placed into an alkaline solution at 4°C for 30 minutes. This was followed by two 5 minute rinses of the slide with Tris-Borate-EDTA (Fisher Scientific, Pittsburg, PA).

Electrophoresis was performed for 20 minutes at 20 volts and 300 amps. The slide was rinsed in alcohol for 5 minutes, allowed to dry and placed into a desiccator. At the time of analysis, 50 µL of SYBR® Green I was added to the slide and observed using fluorescence microscopy with a fluorescein isothiocyanate filter.

All five groups (vitrification, slow-cooled, and three control groups) of embryos were exposed to the Comet Assay (Trevigen®) and evaluated for comet tails. Measurements were taken for the length of each embryo's comet tail using Slide Book Software (Intelligent Imaging Innovation, Inc., Denver, Colorado). In order to distinguish normal comet tails from abnormal comet tails, a confidence interval was created for the controls. The upper bound limit for the confidence interval was used as a boundary for undamaged embryos. The upper bound limit of the 95% CI for the controls was determined to be 119 µm; therefore, any tail length longer than 119 µm was

considered abnormal. Chi-square analyses were performed on percentage of normal tail lengths for all embryo groups.

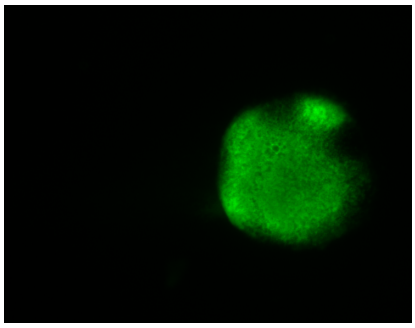


Figure 6.1. Fresh eight-cell mouse embryo with no comet tail (*in vivo* cultured).

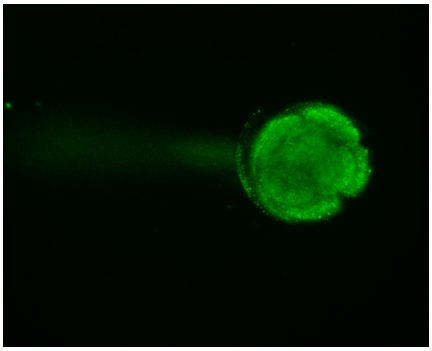


Figure 6.2. HTF control eight-cell mouse embryo with a large comet tail (*in vitro* cultured from two-cell stage).

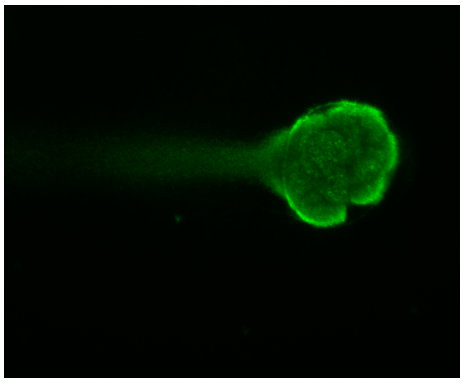


Figure 6.3. Eight-cell mouse embryo with large comet tail (vitrified at two-cell stage, thawed, and cultured *in vitro*).

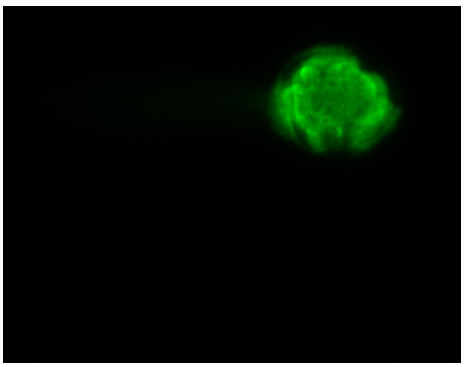


Figure 6.4. Eight-cell mouse embryo with no comet tail (slow-cooled at two-cell stage, thawed, and cultured *in vitro*).

RESULTS

When each group was compared to another, the only significant differences observed were when the fresh controls (90% [37/41]) were compared to HTF controls (56% [30/54]; $P < 0.001$), HTF/UV positive controls (59% [33/56]; $P < 0.001$), vitrified (53% [31/58]; $P < 0.001$) and slow-cooled (59% [19/32]; $P = 0.002$) groups.

DISCUSSION

This study determined that there is no significant difference in DNA damage between vitrified and slow-cooled two-cell mouse embryos when they are cryopreserved at the two-cell stage then thawed and cultured to the eight-cell stage. However, the significant difference observed in fresh embryos compared to all other groups is concerning because there appears to be DNA damage caused by culturing embryos.

This observed damage could be due to elevated oxygen tension as reported by Takahashi et al. (1999). These researchers observed more DNA damage in embryos cultured under 20% oxygen concentration than those under 5% oxygen concentration. A 20% oxygen tension has also been shown to reduce the number of embryos that develop to the blastocyst stage and the number of cells present in the blastocyst when compared to 5% oxygen concentration (Karagenc et al., 2004; Bavister 2004). Bavister (2004) indicated that the 20% oxygen concentration does not affect the trophectoderm, but does damage the inner cellular mass.

A pitfall of the Comet Assay is the lack of ability to distinguish among damage to individual blastomeres, degeneration of blastomeres, or degeneration of polar bodies, all

of which will be observed as a comet tail; therefore, the Comet Assay does not delineate location. Although other researchers have used the Comet Assay to detect DNA damage in embryos (Takahashi et al., 1999; Takahashi et al., 2000; Fabian et al., 2003), we have not found another researcher who has used this assay for detection of DNA damage in cryopreserved mouse embryos. Stowinska et al. (2008) and Kalthur et al. (2008) have used the Comet Assay to analyze DNA damage in cryopreserved sperm.

The outcome of this research warrants further investigation to determine if culturing mammalian embryos under a lower oxygen concentration would be beneficial. However, we have determined through this study and our previous study (Graves-Herring and Boone, 2007) that neither the vitrification nor slow-cooled method is superior to cryopreserve mouse embryos.

CHAPTER SEVEN

DISCUSSION

Vitrification has recently started to replace the commonly used slow-cooled method to cryopreserve mammalian embryos. The two methods to vitrify embryos are the open system and the closed system. The open-system method has the potential for contamination of embryos from liquid nitrogen whereas the closed-system method reduces this risk. Closed systems have become a commercial product within the past few years (Kuwayama 2005); however, when our research started in 2003, commercial vitrification devices were not readily available.

This research focused on concerns with vitrification. These concerns included using a closed system of vitrification, toxicity of the cryoprotectants, comparison of blastocyst rates and pup rates with vitrification and the slow-cooled method, and genetic damage with either freezing method. We tested the hypothesis that the two cryopreservation methods were not significantly different when comparing the above criteria, and we could replace the slow-cooled method of freezing with the vitrification method.

Development of a Closed System for Vitrification

The first study (Chapter 2) developed a closed system of vitrification from items purchased commercially. This study also vitrified two- and eight-cell mouse embryos to determine which stage to use for subsequent studies.

Overall, the eight-cell stage (84% [337/399]) provided a higher blastocyst rate ($P < 0.001$) than the two-cell stage (61% [243/395]). The open system (76% [291/384]) did not differ ($P > 0.09$) from the closed system (70% [289/410]). The Stripper Tip® did not differ from the CBS nozzle within systems (open or closed; $P > 0.05$) with the exception of the two-cell open-system Stripper Tip® vs. CBS nozzle (74% [73/98] vs. 53% [48/91]; $P = 0.002$).

Using these data, the device, system and cell stage were determined for subsequent studies. The Stripper Tip® produced a slightly higher blastocyst rate (although not significant) when cell stage and system were compared, and the Stripper Tip® was an easier device to manipulate; therefore, it was used in subsequent studies. Although the open systems produced a slightly higher blastocyst rate than the closed system, we elected to use the closed system to avoid potential embryo exposure to contaminates from liquid nitrogen. The eight-cell stage produced a greater blastocyst rate than the two-cell stage. However, the two-cell stage was used for subsequent studies because embryos at an early stage have more of a potential to demonstrate damage.

The developmental stage in which an embryo is frozen may be a determinant in the viability of the embryo after it has been thawed. Cseh et al. (1997) indicated that the morula to early blastocyst stage was the best stage to freeze in mice, and that the decrease in development rates in the early and late stage embryos was not due to toxicity or osmotic shock. In contrast to the report by Cseh et al. (1997), Emiliani et al. (2000) slow-cooled mouse zygotes, four-cell embryos and blastocysts, and indicated that four-cell embryos were the best stage to freeze mouse embryos.

The same year our research was presented (Graves et al., 2004), Walker et al. (2004) published a study demonstrating the Stripper Tip® as a device to vitrify mouse morula and early hatching blastocysts. These researchers first submerged the tip into liquid nitrogen and then secured the tip by sealing it in a CBS High Security Straw. This study yielded a 90.7% (97/107) survival rate after thawing.

Overall, our study determined that two-cell mouse embryos would be vitrified in the Stripper Tip® for the remaining studies. This study also determined that the closed system of vitrification is similar to the open system of vitrification.

Time in Vitrification Solution

The second study (Chapter 3) determined the length of time embryos needed to remain in vitrification solution to have a decrease in blastocyst rate. In addition, we used the determined time to study the effects on blastocyst rate, pup rate, and DNA damage.

The experimental time embryos needed to remain in vitrification solution to cause a decrease in blastocyst rate was 32 minutes. This time then was used in the second part of the study to observe blastocyst rate and pup rate, and used in the third part of the study to observe DNA damage in vitrified embryos.

When embryos remained in vitrification solution for 32 minutes and were compared to embryos that were not exposed to cryoprotectants, there was a significant difference ($P < 0.001$) in blastocyst rate (76% [193/255] vs. 95% [61/64], respectively). When embryos remained in vitrification solution for 32 minutes and were cultured to the blastocyst stage and then transferred to recipient mice, these embryos produced pups

(42% [20/48]) at the same rate as those embryos that were not exposed to vitrification solution, but were cultured to the blastocyst stage and transferred to recipient mice (31% [15/48]). Embryos exposed to vitrification solution for 32 minutes, but did not reach blastocyst stage when cultured, also were transferred to recipient mice. These embryos did not produce pups (2% [1/48]) at the same rate ($P < 0.05$) as the blastocyst stage group or the controls. This indicated that exposure to vitrification solution for 32 minutes caused a decrease in blastocyst rate compared to embryos that were not exposed to vitrification solution, demonstrating the high osmolarity vitrification solution compromised the embryos. However, if the embryos developed to the early or expanded blastocyst stage and were transferred, these embryos produced pups at the same rate as those that were not exposed to vitrification solution.

When blastocysts were exposed to a 20% ethylene glycol solution for 20 or 40 minutes, there was no significant difference in survival rate (Valdez et al., 1992), but our research observed a decrease in blastocyst rate when two-cell mouse embryos were exposed for 32 minutes. The difference between the two studies may be due to the different cell stages that were used.

Kasai et al. (1990) observed that when mouse morulae were exposed to vitrification solution composed of 40% ethylene glycol and 30% Ficoll for 5, 10, 15 or 20 minutes, embryos did not survive if they remained in vitrification solution for 15 minutes or longer. With the addition of sucrose, morulae were able to survive a 20 minute exposure but the survival rate was low (20% [10/50]). When these researchers used 40% ethylene glycol, 30% Ficoll and 0.5 M sucrose to vitrify embryos after 2, 5 or 10 minutes

exposure time to the solution, the 10 minute exposure (77% [43/56]) demonstrated a significant reduction ($P < 0.001$) in blastocyst development when compared to the 2 minute (98% [97/99]) and 5 minute (97% [103/106]) exposure time.

In contrast to Kasai et al. (1990) that observed a low survival rate at 20 minutes, Cseh et al. (1997) observed 92% (46/50) development of two-cell mouse embryos following exposure to 3 M ethylene glycol and 0.25 M sucrose for 20 minutes without freezing. However, embryos that were rapidly frozen after being exposed to the solution had fewer embryos to develop to the blastocyst stage (51% [43/84]).

The use of combinations of different cryoprotectants in vitrification solution may have caused different toxicity exposure times. For example, Kasai et al. (1990) observed toxicity at 15 minutes with ethylene glycol, while Valdez et al. (1992) did not observe toxicity even though embryos remained in vitrification solution for 40 minutes.

Not only did Kasai et al. (1990) alter the time in vitrification solution, these researchers also altered the concentration of ethylene glycol. These researchers used mouse embryos at the morula stage and exposed them to ethylene glycol in concentrations at 30, 40 and 50% without freezing, and determined the developmental rate at 98% (48/49), 84% (42/50) and 0% (0/50), respectively.

Although we were unable to find another researcher that used 7.5% ethylene glycol and 7.5% DMSO as a holding solution and 15% ethylene glycol and 15% DMSO as a vitrification solution, we were able to find researchers that used ethylene glycol and DMSO close to these concentrations. Kong et al. (2000) used 10% ethylene glycol and 10% DMSO as a holding medium and 16.5% ethylene glycol and 16.5% DMSO as a

vitrification medium. These researchers demonstrated that vitrified mouse blastocysts produced a 93.5% (58/62) reexpansion rate and 88.7% (55/62) hatched rate. Lane et al. (1999b) demonstrated that mouse blastocysts vitrified with 20% ethylene glycol and 20% DMSO produced 100% (160/160) reexpansion rate and a 95.5% hatched rate.

Overall, in each of the studies described above, different concentrations of ethylene glycol with a combination of other cryoprotectants were used and various results were observed; therefore, unless a researcher is using the exact combination and concentration of cryoprotectants, accurate comparisons cannot be made.

Blastocyst Rate and Pup Rate Comparison of Vitrification and the Slow-Cooled Method

The third study (Chapter 4) was performed to compare a vitrification method to a slow-cooled method using blastocyst rates and pup rates as determinants. Weights from pups produced via both methods were observed at 30, 60 and 90 days following birth.

When comparing the vitrification method to the slow-cooled method of cryopreservation, there were no significant differences ($P > 0.05$) in blastocyst rate (52% [69/132] vs. 58% [92/158]) nor in pup rate (31% [15/48] vs. 29% [14/48] respectively). No significant differences ($P > 0.3$) were observed for the three weights (30, 60 and 90 days) when comparing female pups produced from the various treatment groups. Similar findings were found for the weights of the male pups for the three weigh periods.

Our blastocyst rate results were similar to findings by Dinnyes et al. (1995) in which eight-cell mouse embryos were cryopreserved by a vitrification or slow-cooled method. They demonstrated that there was no significant differences in blastocyst

development after freeze/thawing using a vitrification or slow-cooled method. The findings by Dinnyes et al. (1995) and our research were different than Uechi et al. (1999) which indicated that the slow-cooled method was superior to the vitrification method and in contrast to Walker et al. (2004) which indicated blastocyst rate in the vitrification method was superior to the slow-cooled method. These differences between the researchers may be due to the combination of cryoprotectants used and the cell stages used at the time of cryopreservation.

Comparison of Vitrification and the Slow-Cooled Method Using the Comet Assay

The fourth study (Chapter 5) was performed to determine if there was DNA damage (as indicated by the presence of a comet tail) caused by freezing with the vitrification or slow-cooled method. The blastocyst rate was also determined for this study.

There were no significant differences ($P > 0.9$) in blastocyst rate between the vitrified (66% [64/97]) and slow-cooled (66% [66/100]) groups nor between the percentage of normal tail lengths ($P = 0.14$) in the vitrified (56% [26/46]) and slow-cooled (40% [17/42]) groups. Both cryopreservation methods produced blastocyst rates much lower ($P < 0.001$) than the control embryos (no exposure to cryoprotectants or freezing methods; 100% [97/97]), and both cryopreservation methods also had a lower percentage of normal embryos (those without DNA damage) than controls (78% [67/86]).

The fifth study (Chapter 6) was similar to the fourth study (Chapter 5) in which vitrified and slow-cooled mouse embryos were analyzed for DNA damage; however, the

embryos were only cultured to the eight-cell stage prior to analysis. Fresh controls (90% [37/41]) were significantly different ($P < 0.002$) than the HTF controls (56% [30/54]), HTF/UV positive controls (59% [33/56]), vitrified (53% [31/58]) and slow-cooled groups (59% [19/32]). These results warrant further investigation to determine if culturing using 20% oxygen concentration causes more damage to embryos than those cultured using 5% oxygen concentration.

These studies were similar to a study by Coutinho et al. (2007) that observed morula and blastocysts that were vitrified or slow cooled then thawed and assessed for viability and morphological changes due to necrosis or apoptosis injuries. These researchers demonstrated that both freezing methods resulted in injuries and that vitrified embryos (69.7%) had a higher percentage of dead cells when compared to the slow-cooled embryos (48.4%). They also determined that the slow-cooled method injuries were more apoptotic and the vitrification method injuries were more necrotic in morphological analyses indicating vitrification causes reduced viability.

Overall, the fourth and fifth studies indicate that both the vitrification method and the slow-cooled method cause DNA damage. However, there were no significant differences when comparing the percentage of normal blastocysts produced for either cryopreservation method.

Drawbacks in the Research

A drawback to using the Stripper Tip® as a device in which to vitrify embryos is the fact that it is a straw system. The straw system presents problems because the

operator must practice with the device for the procedure to be successful. Embryos can easily be destroyed if the operator does not pull a complete bolus of medium into the device or if the operator pulls the bolus of medium too quickly and the bolus splits, thus providing the potential for the embryos to stick to the sides of the straw.

The thawing method of the Stripper Tip® used in this research does not utilize a controlled thawing temperature. In order for this step to be controlled, it is recommended that the tip be removed from liquid nitrogen and remain at room temperature for 1 second to allow the liquid nitrogen that is trapped in the tip to evaporate to prevent possible explosion of the tip when it is submerged in a 37°C waterbath for 3 seconds. A waterbath will control the temperature of thawing and is recommended by others (Kuwayama et al., 2005).

The Comet Assay used in the fourth (Chapter 5) and fifth (Chapter 6) studies are indiscriminate on the type of DNA present or how large DNA fragments are; therefore, damage to individual blastomeres, degeneration of blastomeres, or degeneration of polar bodies, will be observed as a comet tail. Because the Comet Assay does detect DNA damage, it is a good starting point to determine if DNA damage is present. However, it should be used with another method to determine if the damage is apoptotic or necrotic.

Another drawback with the analysis of the Comet Assay performed in our laboratory is it's subjective. While measuring of the comet tail was performed by computer, the actual observation of the tail length was prone to human error. To avoid this subjective analysis, there are analyzers (Comet Assay IV, Perceptive Instruments, UK; AutoComet, TriTek Corporation, Sumerduck, VA) that observe each comet tail and

determine if that damage is normal or abnormal. This makes the analysis less subjective. Our laboratory did not have access to this program at the time of the analysis.

Length of Storage Time

Mozdarani and Moradi (2007) demonstrated that eight-cell mouse embryos that were not frozen, frozen for 24 hours, 1 week, 2 weeks, 1 month, 3 months or 6 months had decreased viability as storage length increased. The embryos that were not frozen had 98.3% viability (n=60), whereas those that were vitrified then stored for 24 hours had 90.1% viability (n=354). This is in contrast to those that were vitrified and then stored for 6 months which had 15.8% viability (n=355). These researchers demonstrated that as storage time increased, the number of normal embryos (those without chromosome abnormalities) also decreased, which may be due to ice crystal formation.

Although the length of storage time was not directly studied with our research, it did not seem to have an effect when embryos were frozen for approximately 1 year. The vitrification and slow-cooled studies used two-cell mouse embryos that were frozen over a period of one month. The study that observed blastocyst and pup rate used embryos that were thawed one week to one month later and produced a blastocyst rate from vitrified embryos of 52% (69/132). The study that observed blastocyst rate and utilized the Comet Assay used embryos that were thawed approximately 14 to 15 months later and produced a 66% (71/108) blastocyst rate from vitrified embryos.

Eum et al. (2008) reported that four-cell mouse embryos that were vitrified or slow-cooled then thawed after storage in liquid nitrogen or in liquid nitrogen vapor for 1

week, 1 month or 6 months produced a high survival rate (90% or greater, n=123 to 131 per group). This high survival rate was observed regardless of method, storage type or time interval.

Our research demonstrated that we are able to produce a closed system of vitrification that is similar to the standard slow-cooled method. We demonstrated that vitrification solutions are not as toxic as once believed and vitrification is equal to the slow-cooled method for cryopreserving embryos. Because we have demonstrated that both systems are comparable, we elected to use vitrification in our Assisted Reproductive Technology Laboratory at Greenville Hospital System. The vitrification method will reduce time needed for cryopreservation and eliminate the controlled-rate freezer.

FUTURE STUDIES

The TUNEL Assay

According to Chan et al. (2001), the Comet Assay still needs to be refined to produce "optimal sensitivity"; therefore, the analysis should be confirmed using another type of DNA fragmentation assay. One such assay is the terminal deoxynucleotidyl transferase mediated digoxigenin nick end labeling (TUNEL) Assay. The TUNEL Assay detects DNA damage by labeling double-stranded and single-stranded DNA breaks with fluorescein dUTP. The TUNEL Assay was used by Levy et al. (1998) to study chromosomal breakdown of arrested-development embryos and frozen-thawed embryos from humans.

Bovine embryos have been observed for DNA damage using the TUNEL Assay. Park et al. (2006) used the TUNEL Assay to compare the amount of DNA fragmentation in bovine blastocysts that were vitrified and thawed to those that were not frozen. Those that were vitrified indicated significantly more damage than those that were not frozen. Marquez-Alvarado et al. (2004) also observed a significantly higher number of TUNEL-positive cells in bovine embryos that were cryopreserved and thawed, when compared to those that were not frozen.

We hypothesize that the TUNEL Assay can be used to detect DNA damage in two-cell mouse embryos cryopreserved, thawed and cultured to the blastocyst stage by either the vitrification or slow-cooled method.

Calcein-AM and Ethidium Homodimer Assay

A concern with cryopreservation of embryos is their viability after thawing. Weil et al. (1996) used calcein-AM and ethidium homodimer to observe cell viability in 1-cell to 4-cell mouse embryos. Calcein-AM labels live cells whereas ethidium homodimer-1 labels dead cells. We hypothesize that calcein-AM and ethidium homodimer-1 can be used to detect the viability of individual blastomeres in two-cell mouse embryos cryopreserved using vitrification or slow-cooled methods.

Annexin V Labeling and Propidium Iodide Labeling

Vitrification causes more necrotic injuries to embryos than the slow-cooled method, while the slow-cooled method causes more apoptotic injuries than vitrification

(Coutinho et al. 2007). When a cell starts to undergo apoptosis, there are changes in the membrane. One of these changes is phosphatidylserine, which moves from the inner membrane to the outer membrane. Since the membrane should remain intact in cells undergoing apoptosis, Annexin V should be labeled on the outer membrane and propidium iodide should be excluded. If the cell is undergoing necrosis, phosphatidylserine will be labeled on the inner membrane and propidium iodide will label the nucleus (Hardy, 1999). We hypothesize that with the use of Annexin V labeling and propidium iodide labeling, investigators should be able to determine those embryos which undergo apoptosis and those which undergo necrosis.

Vitrification of Oocytes

The vitrification of oocytes may be a more favorable procedure than embryo vitrification due to legal and moral issues. Oocyte cryopreservation provides a chance for a fertile future for a female that may undergo chemotherapy, radiotherapy or surgery, all of which could results in the destruction of ovarian function (O'Neil et al., 1998; Chen et al., 2003). Since some patients may want to cryopreserve their oocytes instead of their embryos, future studies should include vitrification of oocytes to offer this service to patients.

Vitrification of oocytes in livestock and endangered animals would also be useful. This would allow the preservation of genetics for a female that has features that can be passed down to future generations.

Mouse oocytes have been successfully vitrified. Park et al. (2001) vitrified mouse oocytes of which 73.8% (59/80) survived after thawing and 47.5% (28/59) fertilized. Valojerdi et al. (2005) vitrified and thawed mouse oocytes and demonstrated that 80% (162/203) of the oocytes survived and 91.4% (148/162) were able to fertilize.

Future studies on vitrification of mouse oocytes should be similar to Park et al. (2001) and Valojerdi et al. (2005). Mouse oocytes should be vitrified and thawed then fertilized. These fertilized oocytes should be cultured to determine if they can produce blastocysts and the blastocyst transferred to fertile females to ensure that the process will result in live offspring. Futhermore, these offspring should be allowed to mate naturally to ensure that normal reproductive patterns have not been altered.

We hypothesize that the vitrification method we have used to successfully vitrify two-cell mouse embryos can be used to successfully vitrify mouse oocytes. The success of vitrifying mouse oocytes would then lead to vitrification of human oocytes.

In conclusion, we were able to use a device to vitrify mouse embryos in a closed system and determine that vitrification is similar to the slow-cool method; however, more research should occur that includes the TUNEL Assay, viability assay, Annexin V Assay and vitrification of oocytes.

APPENDICES

Appendix A

Vitrification Procedure Solutions

Table A.1. Holding and Vitrification Solution

Components	Holding Solution	Vitrification Solution
Synthetic Serum	6 mL	6 mL
Ethylene Glycol	2.25 mL	4.5 mL
Dimethyl Sulfoxide	2.25 mL	4.5 mL
Dulbecco's Phosphate Buffered Saline without Calcium or Magnesium	19.5 mL	15 mL

Holding Solution and Vitrification Solution are prepared at least one day prior to cryopreservation procedure. Osmolarity and pH are performed on each solution. Solutions are aliquoted into sterile tubes and stored in the refrigerator (4°C) until use. Solutions expire 4 weeks after preparation date.

Table A.2. Warming Solutions

Components	Warming Solution 1	Warming Solution 2	Warming Solution 3	Warming Solution 4
Synthetic Serum	10 mL	None	None	None
Sucrose	17.1 g	None	None	None
Dulbecco's Phosphate Buffered Saline without Calcium and Magnesium	40 mL	20 mL	20 mL	20 mL
Miscellaneous	None	20 mL Warming Solution 1	20 mL Warming Solution 2	20 mL Warming Solution 3

Warming Solutions are prepared at least one day prior to thawing procedure. Osmolarity and pH are performed on each solution. Solutions are aliquoted into sterile tubes and

stored in the refrigerator (4°C). A tube of each solution is placed in an incubator at 36.7°C and warmed overnight.

Table A.3. pH and Osmolarity Ranges

	pH	Osmolarity
Holding Solution	7.44-7.62	3184-3488
Vitrification Solution	7.56-7.72	6076-6934
Warming Solution 1	7.02-7.34	1474-1904
Warming Solution 2	7.06-7.34	737-788
Warming Solution 3	7.11-7.39	487-568
Warming Solution 4	7.20-7.47	382-422

Appendix B

Slow-Cooled Procedure Solutions

Table B.1. Freezing Solutions

Components	Solution A PBS + 20% DS	Solution B Solution A + 1.5 M PROH	Solution C Solution B + 0.1 M Sucrose
Sucrose	None	None	0.171 g
Phosphate Buffered Saline (PBS)	12 mL	None	None
Propanediol (PROH)	None	1.14 mL	None
Donor Serum (DS)	3 mL	None	None
Miscellaneous	None	8.86 mL Solution A	5 mL Solution B

Solutions are prepared at least one day prior to cryopreservation procedure and expire 4 weeks after preparation date. Solutions are sterile filtered and aliquoted into sterile tubes and stored in the refrigerator (4°C) until use. Solutions expire 4 weeks after preparation date.

Table B.2. Thawing Solutions

Components	Thawing Solution A 1.0 M PROH + 0.2 M Sucrose + 20% DS	Thawing Solution B 0.5 M PROH + 0.2 M Sucrose + 20% DS	Thawing Solution C 0.2 M Sucrose + 20% DS	Thawing Solution D PBS + 20% DS
Dulbecco's Phosphate Buffered Saline	3.7 mL	3.85 mL	4.0 mL	4.0 mL
Donor Serum	1.0 mL	1.0 mL	1.0 mL	1.0 mL
Propanediol	0.3 mL	0.15 mL	None	None
Sucrose	0.342 g	0.342 g	0.342 g	0.342 g

Solutions are prepared at least one day prior to thawing procedure and expire 4 weeks after preparation date. Solutions are aliquoted into sterile tubes and stored in the refrigerator (4°C) until use.

Table B.3. pH and Osmolarity Ranges

	pH	Osmolarity
Solution A	7.37-7.90	280-304
Solution B	7.37-7.90	2022-2541
Solution C	7.37-7.90	2101-2745
Thawing Solution A	7.34-7.89	1539-1756
Thawing Solution B	7.34-7.89	978-1165
Thawing Solution C	7.34-7.89	486-529
Thawing Solution D	7.34-7.89	278-314

Appendix C

Comet Assay Procedure

The Comet Assay is purchased as a kit by Trevigen®. The kit contains Lysis Solution, Comet LMAgarose, CometSlide™, 200mM EDTA and SYBR® Green 1 containing DMSO. Other reagents that must be purchased include Phosphate Buffered Saline without Calcium and Magnesium, Sodium Hydroxide Pellets, 10X Tris-Borate-EDTA (TBE) Buffer, ethanol, Tris-EDTA (TE) Buffer and deionized water.

Prior to performing the Comet Assay, some reagents must be prepared as follows. Lysis Solution should be refrigerated at 4°C or placed on ice. LMAgarose should be melted by placing the bottle in a 90-100°C water bath until liquid, then dividing into smaller containers such as Eppendorf 1.0 mL tubes. This will avoid continuous melting and solidifying of the bottle of LMAgarose. The smaller tubes of LMAgarose can then be used as needed. Once LMAgarose is melted, the tubes should be placed in a water bath at 37°C to allow LMAgarose to cool.

The Alkaline Solution is prepared with 49.75 mL deionized water, 0.6 g Sodium Hydroxide Pellets, and 250 µL of 200mM EDTA. The 10X TBE Buffer is diluted to 1X for a rinse solution and electrophoresis by diluting 50 mL 10X TBE Buffer with 450 mL of deionized water. Once all reagents are prepared, the Comet Assay is performed.

Steps to perform the Comet Assay.

1. Ensure Lysis Solution has been chilled for at least 20 minutes at 4°C or on ice.

2. Ensure LMAgarose has been melted and has remained in 37°C water bath for at least 20 minutes.
3. Place 1-2 mL of PBS into a Falcon 3001 dish at room temperature. Prepare enough dishes for each group of embryos to be observed.
4. Observe embryos for development and record information. Embryos are then placed into a designated Falcon 3001 dish with PBS.
5. Remove a tube of LMAgarose from the water bath. Pick up embryos using a Stripper Tip® with a minimum amount of PBS. Place into LMAgarose.
6. Using a pipettor with plastic tip, transfer LMAgarose containing embryos to a sample area on the Comet Slide.
7. Repeat steps 5 and 6 until all embryo groups have been placed in LMAgarose. Each Comet Slide has two sample areas.
8. Place slide(s) into refrigerator (4°C) for 30 minutes.
9. Place slide(s) into Lysis Solution for 1 hour. Remove slide(s) from Lysis Solution and blot edge of slide on cloth to remove excess solution.
10. Place slide(s) into Alkaline Solution for 30 minutes. Remove slide(s) from Alkaline Solution and blot edge of slide on cloth to remove excess solution.
11. Place slide(s) into TBE for 5 minutes. Remove slide(s) and place into a second TBE for 5 minutes.
12. Transfer slide(s) to electrophoresis unit. Fill unit with enough TBE until slide(s) are just covered. Assemble unit and perform electrophoresis at 20V, 300mA for 20 minutes.

13. Turn off unit and remove slide(s). Blot edge of slide(s) on cloth to remove excess TBE.
14. Place slide(s) into 70% ethanol for 5 minutes.
15. Remove slide(s) from ethanol and allow to air dry. Slides can be observed after air drying or can be stored at room temperature with desiccant.
16. Prior to analysis of comet tails, prepare SYBR® Green dilution by mixing 1 µL of SYBR® Green with 5 mL of TE. The dilution can be stored in the dark at 4°C for 4 weeks.
17. Place 50 µL of SYBR® Green dilution onto each area of dried LMAgarose.
18. Use a microscope with Fluorescein Isothiocyanate Filter to view comet tails.

Appendix D

Data Tables

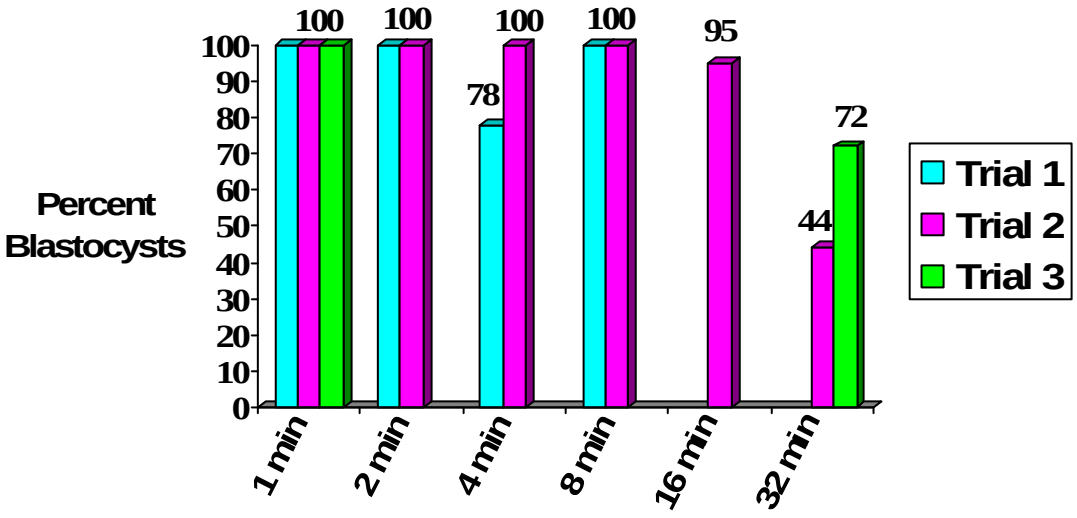


Table D.1. Time in Vitrification Solution. Determination of time in vitrification solution to cause a decrease in blastocyst rate. Embryos were collected at the two-cell stage and in trial 1 embryos remained in vitrification solution for 1, 2, 4 or 8 minutes; trial 2 embryos remained in vitrification solution for 1, 2, 4, 8, 16 or 32 minutes; trial 3 embryos remained in vitrification solution for 1 or 32 minutes. Embryos in all trials were cultured to the blastocyst stage.

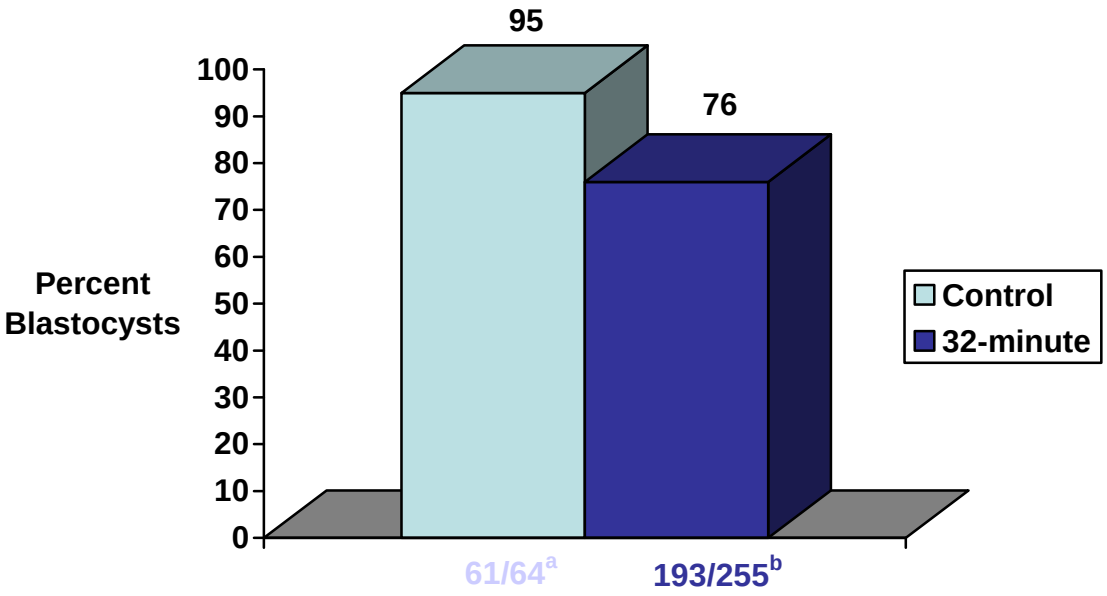


Table D.2. Time in Vitrification Solution Blastocyst Rate. Control embryos were not exposed to vitrification solutions, and 32-minute embryos remained in vitrification solution for 32 minutes prior to culturing to the blastocyst stage.
[a,b] Different superscripts indicate statistical difference ($P < 0.05$).

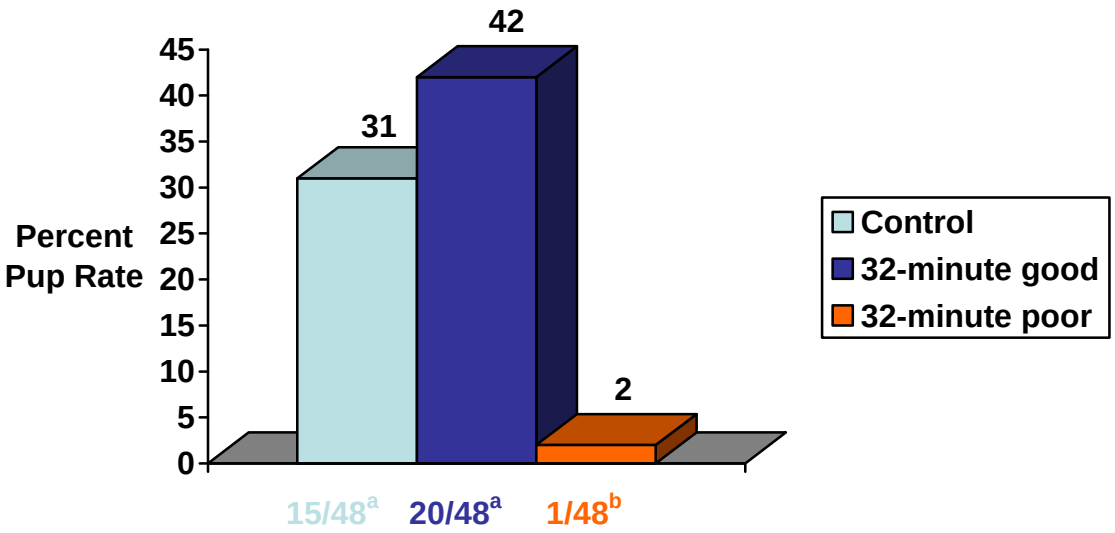


Table D.3. Time in Vitrification Solution Pup Rates. The control group was not exposed to vitrification solutions, cultured to the blastocyst stage and transferred to recipient female mice. The 32-minute good and 32-minute bad group remained in vitrification solution for 32 minutes prior to culture, divided into two groups; those at the blastocyst stage (32-minute good) and those that were at the multi-cell or morula stage (32-minute poor), were transferred into recipient female mice.
[a,b] Different superscripts indicate statistical difference ($P < 0.05$).

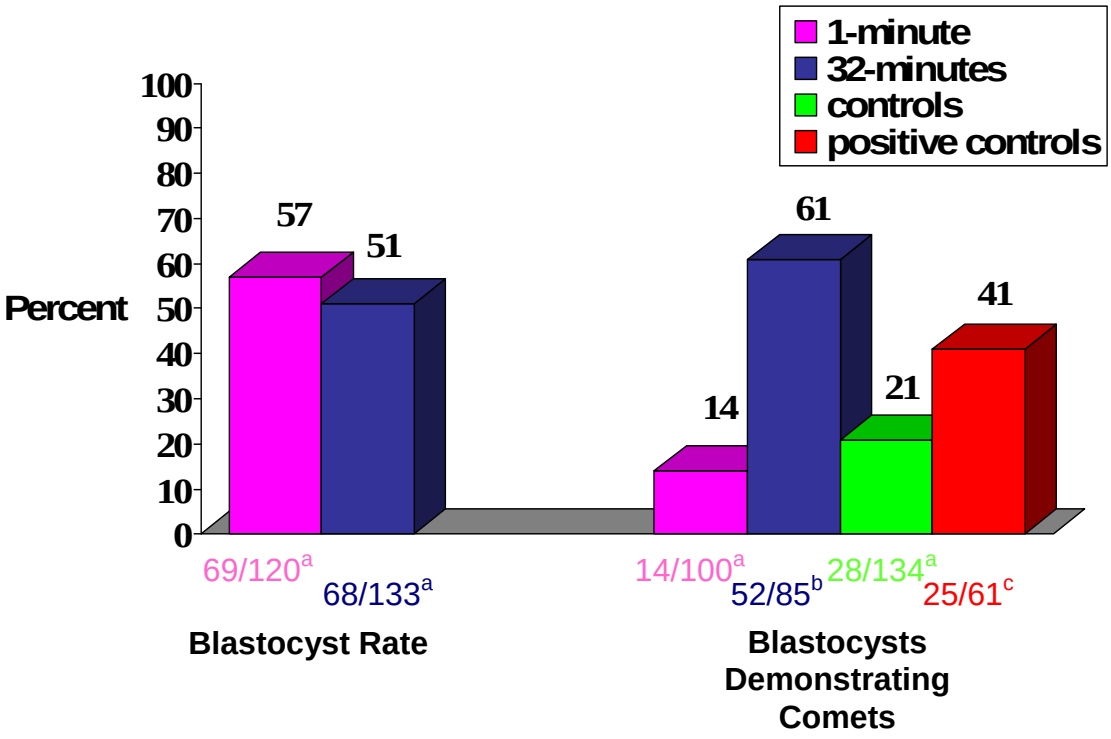
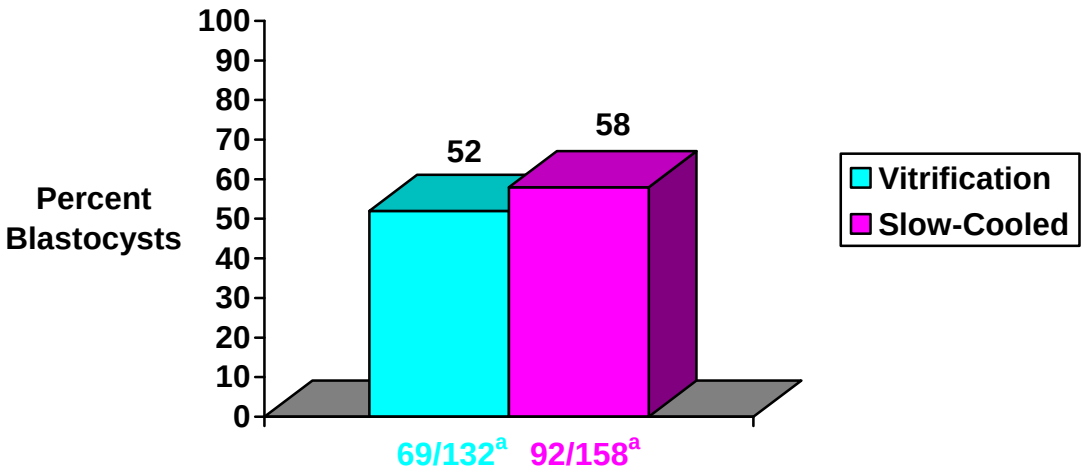
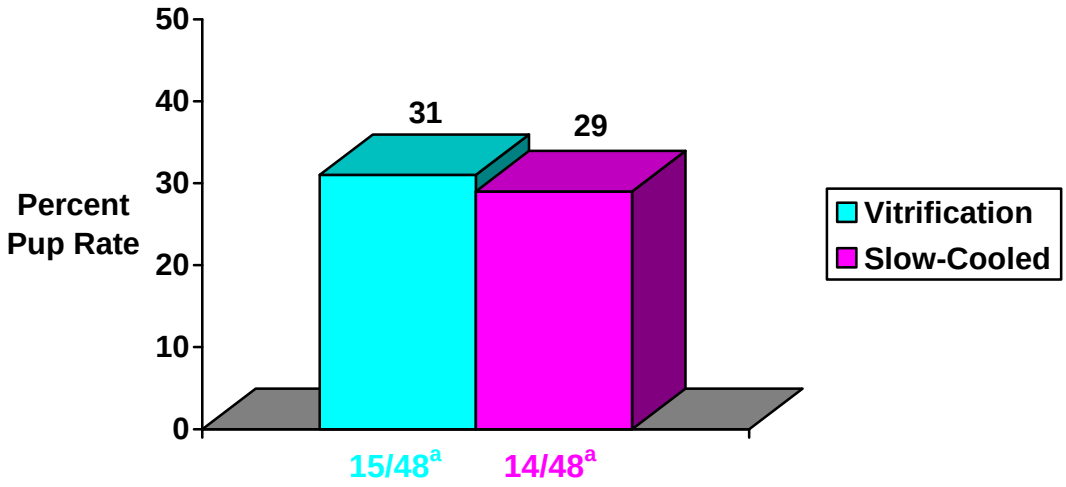


Table D.4. Time in Vitrification Solution Blastocyst Rate and Blastocysts Demonstrating Comets. Blastocyst rate was observed for two-cell embryos that remained in vitrification solution for 1 minute or 32 minutes. Controls were not exposed to vitrification solution, while positive controls were cultured with 1% hydrogen peroxide to induce DNA damage.

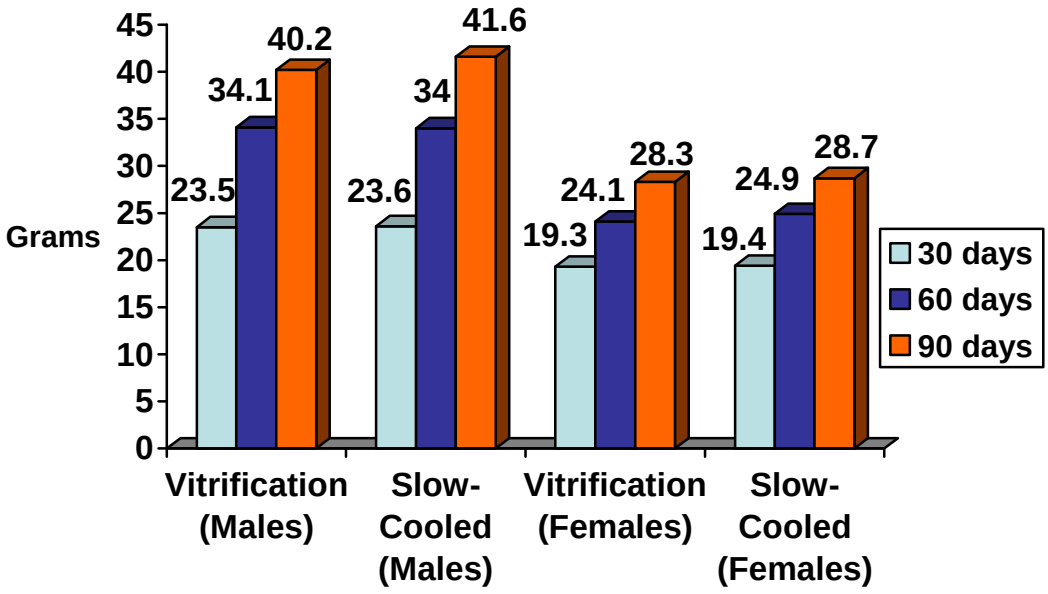
[a,b,c] Different superscripts in groupings indicate statistical difference ($P < 0.05$).



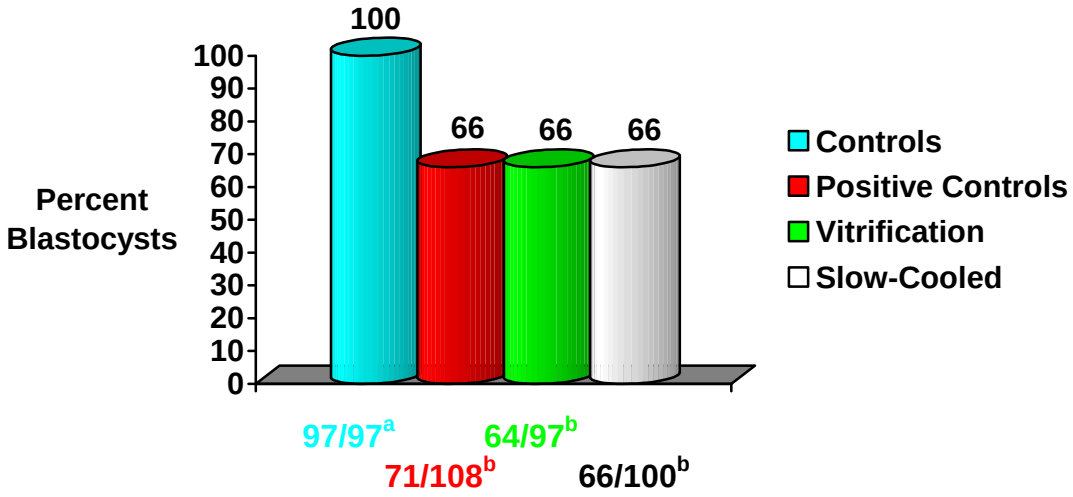
D.5. Blastocyst Rates from Vitrified and Slow-Cooled Mouse Embryos. Two-cell mouse embryos cryopreserved using either method and cultured to the blastocyst stage. [a] Similar superscripts indicate no statistical difference ($P > 0.05$).



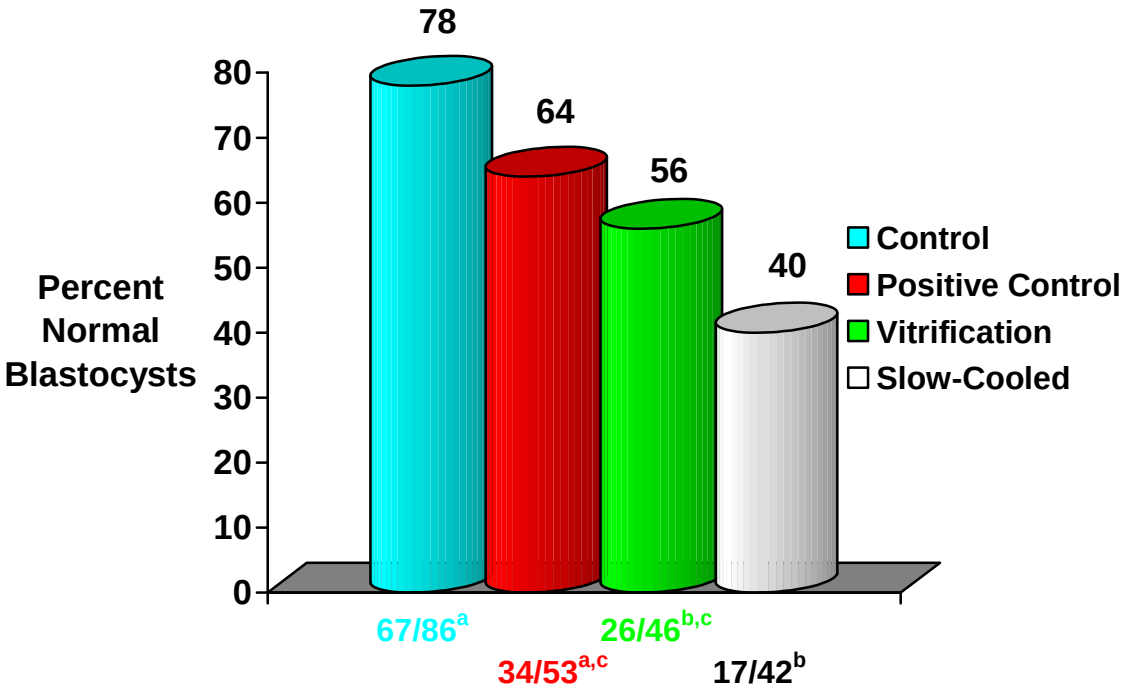
D.6. Pup Rate from Vitrified and Slow-Cooled Mouse Embryos. Two-cell mouse embryos cryopreserved using either method, cultured to the blastocyst stage and transferred into recipient mice.
[a] Similar superscripts indicate no statistical difference ($P > 0.05$).



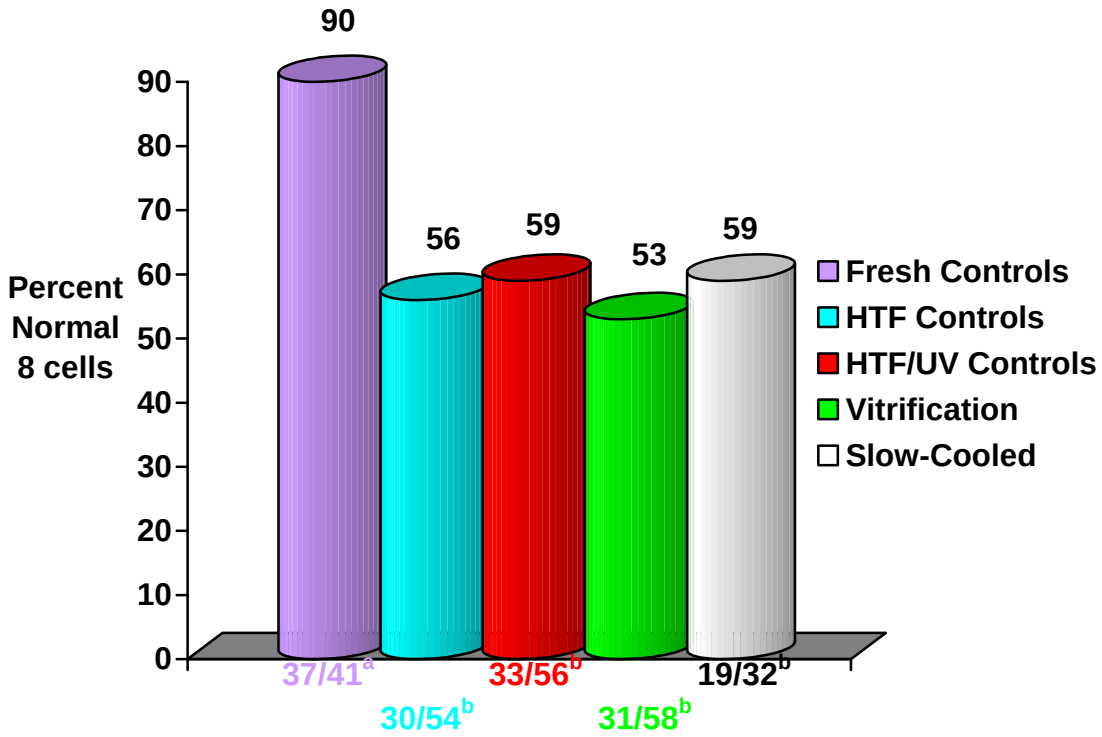
D.7. Pup Weights from Vitrified and Slow-Cool Mouse Embryos. Two-cell mouse embryos cryopreserved using either method, cultured to the blastocyst stage and transferred into recipient mice. Pups were weighed at 30, 60 and 90 days. Statistical comparisons between freezing methods for each gender, compared each day weighed, indicated no statistical differences ($P > 0.3$).



D.8. DNA Damage Study Blastocyst Rates from Vitrified, Slow-Cooled, Control and Positive Control Mouse Embryos.
[a,b] Different superscripts indicate statistical difference ($P < 0.05$).



D.9. DNA Damage Study Percentage of Normal Blastocysts Produced from Vitrified, Slow-Cooled, Control and Positive Control Mouse Embryos.
[a,b,c] Different superscripts indicate statistical difference ($P < 0.05$).



D.10. Comparison of DNA Damage Study Percentage of Normal Eight-Cell Mouse Embryos. Fresh control embryos were collected from mice at the eight-cell stage; Human Tubal Fluid (HTF) control embryos were collected at the two-cell stage and cultured in HTF overnight; HTF/UV control embryos were collected at the two-cell stage and cultured in HTF overnight then exposed to UV light; Vitrified and Slow-Cooled embryos were collected at the two-cell stage, cryopreserved then thawed and cultured overnight.

[a,b] Different superscripts indicate statistical difference ($P < 0.05$).

REFERENCES

Al-Hasani S, Ozmen B, Koutlaki N, Schoepper B, Diedrich K, Schultze-Mosgau A. Three years of routine vitrification of human zygotes: is it fair to advocate slow-rate freezing? RBM Online. 2007;14:288-93.

Ali J, Shelton N. Vitrification of preimplantation stages of mouse embryos. J Reprod Fertil 1993:98;459-65.

Ali J, Shelton J. Development of vitrification solutions. In: Vitrification in assisted reproduction: A user's manual and trouble-shooting guide. Editors Tucker MJ, Liebermann J. Informa Healthcare, London. 2007.

Bagis H, Sagirkaya H, Odaman Mercan H, Dinnyes A. Vitrification of pronuclear-stage mouse embryos on Solid Surface (SSV) versus in cryotube: Comparison of the effect of equilibration time and different sugars in the vitrification solution. Mol Reprod Devel. 2004;67:186-92.

Bagis H, Mercan HO, Cetin S, Sekmen S. The effect of equilibration time on survival and development rates of mouse pronuclear-stage embryos vitrified in solid surface (SSV) and conventional straws: in vitro and in vivo evaluations. Mol Reprod Devel. 2005;72:494-501.

Bavister B. Oxygen concentration and preimplantation development. RBM Online. 2004;9:484-6.

Begin I, Bhatia B, Baldassarre H, Dinnyes A, Keefer CL. Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. Theriogenology. 2003;59:1839-50.

Benifla JL, Letur-Konirsch H, Collin G, Devaux A, Kuttenn F, Madelenat P, Brun-Vezinet F, Feldmann G. Safety of cryopreservation straws for human gametes or embryos: a preliminary study with human immunodeficiency virus-1. Hum Reprod 2000;15:2186-9.

Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C. Viral contamination of embryos cryopreserved in liquid nitrogen. Crybiology. 2000;40:110-6.

Bielanski A, Bergeron H, Lau PCK, Devenish J. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology. 2003;46:146-52.

Boonkusol D, Baji Gal A, Bodo S, Gorhony B, Kitiyanant Y, Dinnyes A. Gene expression profiles and in vitro development following vitrification of pronuclear and 8-cell stage mouse embryos. Mol Reprod Dev. 2006;73:700-8.

Boone WR, Johnson JE, Blackhurst DW, Higdon HL. Cryopreservation Methodologies: freezing location and protocol alter development of mouse embryos. Contemp Topics. 2004;43:26-31.

Chan PJ, Calinisan JH, Corselli JU, Patton WC, King A. Updating quality controls assays in the assisted reproductive technologies laboratory with a cryopreserved hamster oocyte DNA cytogenotoxic assay. J Assist Reprod Gen. 2001;18:129-34.

Chen SU, Lien YR, Cheng YY, Chen HF, Ho HN, Yang YS. Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straw (OPS) and grids. Hum Reprod. 2001;16:2350-6.

Chen SU, Lien YR, Chao KH, Ho HN, Yang YS, Lee TY. Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing—a review article. Mol Cell Endocrinol. 2003;28:101-7.

Chung JT, Tosca L, Huang TH, Xu L, Niwa K, Chian RC. Effect of polyvinylpyrrolidone on bovine oocyte maturation *in vitro* and subsequent fertilization and embryonic development. RBM Online. 2007;15:198-207.

Coutinho ARS, Mendes CM, Caetano HVA, Nascimento AB, Oliveira VP, Hernadez-Blazquez FJ, Sinhorini IL, Visintin JA, Assumpcao MEO. Morphological changes in mouse embryos cryopreserved by different techniques. Microsc Res Tech. 2007;70:296-301.

Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B. Intracytoplasmic sperm injection may increase the risk of imprinting defects. Am J Hum Genet. 2002;71:162-4.

Cseh S, Corselli J, Nehlsen-Cannarella SL, Bailey LL, Szalay AA. The effect of quick-freezing in ethylene glycol on morphological survival and in vitro development of mouse embryos frozen at different implantation stages. Theriogenology. 1997;48:43-50.

DeBaun MR, Niemitz EL, Feinberg AP. Association of in vitro fertilization with Beckwith-Wiedemann Syndrome and epigenetic alterations of *LIT1* and *H19*. Am J Hum Genet. 2003;72:156-60.

Dinnyes A, Wallace GA, Rall WF. Effect of genotype on the efficiency of mouse embryos cryopreservation by vitrification or slow freezing methods. Mol Reprod Devel. 1995;40:429-35.

Edwards RG, Ludwig M. Are major defects in children conceived *in vitro* due to innate problems in patients or to induced genetic damage? RBM Online. 2003;7:131-8.

Emiliani S, Van den Bergh M, Vannin AS, Biramane J, Englert Y. Comparison of ethylene glycol, 1,2-propanediol and glycerol for crypreservation of slow-cooled mouse zygotes, 4-cell embryos and blastocysts. Hum Reprod. 2000;15:905-10.

Eum JH, Park JK, Lee WS, Cha KR, Yoon TK, Lee DR. Long-term liquid nitrogen vapor storage of mouse embryos cryopreserved using vitrification or slow cooling. Fertil Steril. 2008, Apr 26. (Epub ahead of print.)

Fabian D, Rehak P, Czikkova S, Il'kova G, Baran V, Koppel J. Induced cell death of preimplantation mouse embryos cultured in vitro evaluated by comet assay. Theriogenology. 2003;60:691-706.

Fountain D, Ralston M, Higgins N, Gorlin JB, Uhl L, Wheeler, C, Antin JH, Churchill WH, Benjamin RJ. Liquid nitrogen freezers: A potential source of microbial contamination of hematopoietic stem cell components. Transfusion. 1997;37:585-91.

Graves JE, Higdon III HL, Boone WR. A closed-system for vitrification that provides acceptable blastocyst rates. Fertil Steril. 2004;82:S272-3.

Graves-Herring JE, Higdon III HL, Boone WR. Extended time in vitrification solution still yields live births. Bull SC Acad Sci. 2005;67:80.

Graves-Herring J, Boone W. A comparison of DNA damage between vitrified and slow-cooled mouse embryos. Program for the Fortieth Annual Meeting of the Society for the Study of Reproduction. Biol Reprod. Special Edition 2007:200.

Han MS, Niwa K, Kasai M. Vitrification of rat embryos at various developmental stages. Theriogenology. 2003;59:1851-63.

Hardy K. Apoptosis in the human embryo. Rev Reprod. 1999;4:125-34.

Hotamisligil S, Toner M, Powers RD. Changes in membrane integrity, cryoskeletal structure and developmental potential of murine oocytes after vitrification in ethylene glycol. Biol Reprod. 1996;55:161-8.

Isachenko V, Montag M, Isachenko E, van der Ven H. Vitrification of mouse pronuclear embryos after polar body biopsy without direct contact with liquid nitrogen. Fertil Steril. 2005a;84:1011-6.

Isachenko V, Montag M, Isachenko E, Zaeva V, Krivokharchenko I, Shafei R, van der Ven H. Aseptic technology of vitrification of human pronuclear oocytes using open-pulled straws. Hum Reprod. 2005b;20:492-6.

Ishimori H, Saeki K, Inai M, Nagao Y, Itasaka J, Miki Y, Seike N, Kainuma H. Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. Theriogenology. 1993:40;427-33.

Kalthur G, Adiga SK, Upadhya D, Rao S, Kumar P. Effect of cryopreservation on sperm DNA integrity in patients with teratospermia. Fertil Steril. 2008;89:1723-7.

Karagenc L, Sertkaya Z, Ciray N, Ulug U, Bahceci M. Impact of oxygen concentration on embryonic development of mouse zygotes. RBM Online. 2004;9:409-17.

Kasai M, Komi H, Takakamo A, Tsudera H, Sakurai T, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fert. 1990;89:91-7.

Kola I, Kirby C, Shaw J, Davey A, Trounson A. Vitrification of mouse oocytes results in aneuploid zygotes and malformed fetuses. Teratology. 1988;38:467-74.

Kong IK, Lee SI, Cho SG, Cho SK, Park CS. Comparison of open pulled straw (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. Theriogenology. 2000;53:1817-26.

Kuleshova L, Gianoroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of small number of human oocytes. Hum Reprod. 1999;14:3077-9.

Kuleshova LL, Shaw JM, Trounson AO. Studies on replacing most of the penetrating cryoprotectant by polymers for embryo crypreservation. Cryobiology 2001;43:21-31.

Kumasako Y, Kumon M, Utsunomiya T, Araki Y. Successful pregnancy after the vitrification of zygotes using commercial vitrification solutions and conventional straws to protect against infections in liquid nitrogen. J Assist Reprod Genet. 2005;22:33-5.

Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. RBM Online. 2005;11:608-14.

Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: The Cryotop method. Theriogenology. 2007;67:73-80.

Lane M, Bavister BD, Lyons EA, Forest KT. Containerless vitrification of mammalian oocytes and embryos. Nat Biotechnol. 1999a;17:1234-6.

Lane M, Schoolcraft WB, Gardner DK, Phil D. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. Fertil Steril. 1999b;72:1073-8.

Levy R, Benchaib M, Cordonier H, Souchier C, Guerin JF. Annexin V labeling and terminal transferase-mediated DNA end labeling (TUNEL) assay in human arrested embryos. Mol Hum Reprod. 1998;4:775-83.

Liebermann J., Tucker MJ. Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. Reproduction. 2002a;124:483-9.

Liebermann J, Tucker MJ, Graham JR, Han T, Davis A, Levy MJ. Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipet. RBM Online. 2002b;4:146-50.

Liebermann J, Dietl J, Vanderzwalmen P, Tucker MJ. Recent developments in human oocyte, embryos and blastocyst vitrification: where are we now? RBM Online. 2003;7:623-33.

Lopez-Bejar M, Lopez-Gatius F. Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure. Theriogenology. 2002;58:1541-52.

Maertens A, Bourlet T, Plotton N, Pozzetto B, Levy R. Validation of safety procedures for the cryopreservation of semen contaminated with Hepatitis C virus in assisted reproductive technology. Hum Reprod. 2004;19:1554-7.

Marquez-Alvarado YC, Galina CS, Castilla B, Leon H, Moreno-Mendoza N. Evidence of damage in cryopreserved and fresh bovine embryos using the Tunel technique. Reprod Dom Anim. 2004;39:141-5.

Martinez AG, de Matos DG, Furnus CC, Brogliatti GM. In vitro evaluation and pregnancy rates after vitrification of in vitro produced bovine embryos. Theriogenology. 1998a;50:757-67.

Martinez AG, Matkovic M. Cryopreservation of ovine embryos: slow freezing and vitrification. Theriogenology. 1998b;49:1039-49.

Men HS, Chen JC, Shang EY, Yang SC, Zou RJ. Cryopreservation of Kunming mouse oocytes using slow cooling, ultrarapid cooling and vitrification protocols. Theriogenology. 1997;47:1423-31.

Misumi K, Suzuki M, Sato S, Saito N. Successful production of piglets derived from vitrified morulae and early blastocysts using a microdroplet method. Theriogenology. 2003;60:253-60.

Mochida K, Wakayama T, Takano K, Noguchi Y, Yamamotot Y, Suzuki O, Matsuda J, Ogura A. Birth of offspring after transfer of Mongolian Gerbil (*Meriones Unguiculatus*) embryos cryopreserved by vitrification. Mol Reprod Devel. 2005;70:464-70.

Moussa M, Bersinger I, Doligez P, Guingnot F, Duchamp G, Vidament M, Mermillod P, Bruyas J-F. In vitro comparisons of two cryopreservation techniques for equine embryos; Slow-cooling and open pulled straw (OPS) vitrification. Theriogenology. 2005;64:1619-32.

Mozdarani H, Moradi SZ. Effect of vitrification on viability and chromosome abnormalities in 8-cell mouse embryos at various storage durations. Biol Res. 2007;299-306.

Mukaida T, Wada S, Takahashi K, Pedro PB, An TZ, Kasai M. Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. Hum Reprod. 1998;13:2874-9.

Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, et al. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. Hum Reprod. 2003;18:384-91.

Naik BR, Rao BS, Vagdevi R, Gnanprakash M, Amarnath D, Rao VH. Conventional slow freezing, vitrification and open pulled straws (OPS) vitrification of rabbit embryos. Anim Reprod Sci. 2005;86:329-38.

Neglia G, Gasparrini B, CaracciolodiBrienza V, Di Palo R, Campanile G, Zicarelli L. First pregnancies established from vitrified blastocysts entirely produced in vitro in Mediterranean Italian buffalo cows (*Bubalus Bubalis*). Theriogenology. 2003;59:374.

Obserstein N, O'Donovan MK, Bruemmer JE, Seidel Jr GE, Carnevale EM, Squires EL. Cryopreservation of equine embryos by open pulled straw, cryoloop, or conventional slow cooling methods. Theriogenology. 2001;55:607-13.

O'Neil L, Paynter SJ, Fuller BJ, Shaw RW, DeVries AL. Vitrification of mature mouse oocytes in a 6 M Me_2SO solution supplemented with antifreeze glycoproteins: The effect of Temperature. Cryobiology 1998;37:59-66.

Orstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, Buiting K. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic sperm injection. Am J Hum Genet. 2003;72:218-9.

Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem and Biophys Res Commun. 1984;123:291-8.

Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340:17-8.

Park SE, Chung HM, Cha KY, Hwang WS, Lee ES, Lim JM. Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol™, a cytoskeleton stabilizer. Fertil Steril. 2001;75:1177-84.

Park SP, Kim EU, Kim DI, Park NH, Won YS, Yoon Sh, Chung KS, Lim JH. Simple, efficient and successful vitrification of bovine blastocysts using electron microscope grids. Hum Reprod. 1999;14:2838-43.

Park SY, Kim EY, Cui XS, Tae JC, Lee WD, Kim NH, Park SP, Lim JH. Increase in DNA fragmentation and apoptosis-related gene expression in frozen-thawed bovine blastocysts. Zygote. 2006;14:125-31.

Perez-Crespo M, Ramirez MA, Fernandez-Gonzalez R, Rizos D, Lonergan P, Pintado B, Gutierrez-Adan A. Differential sensitive of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Mol Reprod Devel. 2005;72:502-10.

Piltti K, Lindeberg H, Aalto J, Korhonen H. Live cubs born after transfer of OPS vitrified-warmed embryos in the farmed European polecat (Mustela putorius). Theriogenology. 2004;61:811-20.

Rall WF. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology. 1987;24:387-402.

Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at -196°C by vitrification. Nature. 1985;313:573-5.

Shaw JM, Ward C, Trounson AO. Evaluation of propanediol, ethylene glycol, sucrose and antifreeze proteins on the survivial of slow-cooled mouse pronuclear and 4-cell embryos. Hum Reprod. 1995:10;396-402.

Shaw JM, Oranratnachai A, Trounson AO. Cryopreservation of oocytes and embryos. In: Handbook of In Vitro Fertilization, Second Edition. Editors: Trounson AO, Gardner DK. CRC Press, Boca Raton, Florida. 2000.

Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175:184-91.

Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R, Kato O. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. RBM Online. 2005;11:53-7.

Stowinska M, Karol H, Ciereszko A. Comet assay of fresh and cryopreserved bull spermatozoa. Cryobiology. 2008;56:100-2.

Takahashi M, Saka N, Takahashi H, Kanai Y, Schultz RM, Okano A. Assessment of DNA damage in individual hamster embryos by comet assay. Mol Reprod Dev. 1999;54:1-7.

Takahashi M, Keicho K, Takahashi H, Ogawa H, Schultz RM, Okano A. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. Theriogenology. 2000;54:137-45.

Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. Fertil Steril. 2005;84:88-92.

Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, Irwin D, Blair S, Gorman AM, Patterson KG, Linch DC, Heptonstall J, Brink NS. Hepatitis B transmission from contaminated cryopreservation tank. Lancet. 1995;346:137-40.

Testart J, Lassalle B, Belaisch-Allart J, Hazout A, Forman R, Rainhorn JD, Frydman R. High pregnancy rate after early human embryo freezing. Fertil Steril. 1986;46:268-72.

Titterington JL, Robinson J, Killick SR, Hay DM. Synthetic and biological macromolecules: protection of mouse embryos during cryopreservation by vitrifcation. Hum Reprod. 1995;10:649-53.

Trounson A, Peura A, Freemann L, Kirby C. Ultrarapid freezing of early cleavage stage human embryos and eight-cell mouse embryos. Fertil Steril. 1988;49:822-6.

Tsujioka T, Otzdorff C, Braun J, Hochi S. Effect of post-IVF developmental kinetics on in vitro survival of vitrified-warmed domestic cat blastocysts. Reprod Domest Anim. 2008;43:323-7.

Uechi H, Tsutsumi O, Morita Y, Takai Y, Taketani Y. Comparison of the effects of controlled-rate cryopreservation and vitrification on 2-cell mouse embryos and their subsequent development. Hum Reprod. 1999;14:2827-32.

Vajta G, Booth PJ, Holm P, Greve T, Callesen H. Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. Cryo-Letters. 1997;18:191-5.

Vajta G, Holm P, Kuwayama, M, Booth PJ, Jacobsen H, Greve T, Callesen H. Open Pulled Straw (OPS) Vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Mol Reprod Dev. 1998;51:53-8.

Vajta G, Kuwayama M, Vanderzwalmen P. Disadvantages and benefits of vitrification. In:Vitrification in Assisted Reproduction: A user's manual and trouble-shooting guide. Editors Tucker MJ, Liebermann J. Informa Healthcare, London. 2007.

Valdez CA, Abas Mazni O, Takahashi Y, Fujikawa S, Kanagawa H. Successful cryopreservation of mouse blastocysts using a new vitrification solution. J Reprod Fert. 1992;96:793-802.

Valojerdi MR, Salehnia M. Developmental potential and ultrastructural injuries of Metaphase II (MII) mouse oocytes after slow freezing or vitrification. J Assist Reprod Gen. 2005;22:119-27.

Van Wagtendonk-de Leeuw AM, den Daas JHG, Kruip THAM, Rall WF. Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. Cryobiology. 1995;32:157-67.

Van Wagtendonk-de Leeuw AM, den Daas JHG, Rall WF. Field trial to compare pregnancy rates of bovine embryo cryopreservation methods: vitrification and one-step dilution versus slow freezing and three-step dilution. Theriogenology. 1997;48:1071-84.

Vanderzwalmen P, Bertin G, Debauche CH, Standaert V, Schoysman R. In vitro survival of Metaphase II oocytes and blastocysts after vitrification in Hemi Straw (HS) system. Fertil Steril. 2000;74(Suppl):215.

Vanderzwalmen P, Bertin G, Debauche CH, Standaert V, Bollen N, van Roosendaal E, Vandervorst M, Schoysman R, Zech N. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum Reprod. 2003;18:1504-11.

Walker DL, Tummon IS, Hammitt DG, Session DR, Dumesic DA, Thornhill AR. Vitrification versus programmable rate freezing of late stage murine embryos: a randomized comparison prior to application in clinical IVF. RBM Online. 2004;8:558-68.

Weil M, Jacobson MD, Coles HSR, Davies TJ, Gardner RL, Raff KD, Raff MC. Constitutive expression of the machinery for programmed cell death. J Cell Biol. 1996;133:1053-9.

Whittingham DG, Leibo SP, Mazur P. Survival of mouse embryos frozen to -196°C and -296°C. Science. 1972;178:411-4.

Yeoman RR, Gerami-Naini B, Mitalipov S, Nusser KD, Widmann-Browning AA, Wolf DP. Cryoloop vitrification yields superior survival of Rhesus monkey blastocysts. Hum Reprod. 2001;16:1965-9.

Yokota Y, Sato S, Yokota M, Yokota H, Araki Y. Birth of a healthy baby following vitrification of human blastocysts. Fertil Steril. 2001;75:1027-9.