The Effect of Embryo Biopsy and Vitrification on the Development Potential of Equine Embryos

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ABSTRACT

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This study investigated the development potential of equine embryos in vitro after biopsy and vitrification. Twenty embryos were obtained from Quarter Horse, Thoroughbred, and mix-breed light mares between three and ten years old. The twenty embryos were divided into a biopsy (n=10) and control group (n=10). The biopsy group underwent microaspiration biopsy using a micromanipulator to obtain a small tissue sample from the embryo. Both groups were then vitrified using a commercially available technique originally described by Carnevale (2006) at Colorado State University.

All 20 embryos were cultured in DMEM/Hams F-12 medium under oil at 37°C in 5% CO_2 in air (Hinrichs et al., 1990). Embryos were monitored for expansion and hatching. Embryo development was statistically different between the two groups ($p<0.05$). The biopsy procedure did result in a much lower development potential in the biopsy group as compared to the control group (20% vs. 80%). However, embryos in the biopsy group did show expansion and hatching therefore the combined procedure did not preclude development potential in vitro. Based on these

findings, more research needs to be done to increase the success of the combined procedure and the ultimate viability of the embryos needs to be confirmed with the establishment of successful pregnancies.

Key Words: Vitrification, embryo biopsy, microaspiration, micromanipulation, and embryo culture.

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Introduction

Rationale

The development and use of embryo transfer in the horse has progressed steadily and foals resulting from embryo transfer are now permitted in most breed registries (Squires et al., 2003). However embryo technologies in the horse have lagged behind that of other livestock species. Several unique biological features as well as technical problems have limited embryo technology advancement in the horse as compared to that of the cattle industry (Squires, 1999). The lack of an effective superovulation technique in the horse combined with a reluctance of some breed registries to embrace newer embryo technologies has limited the development and commercialization of techniques now considered to be commonplace in the cattle industry. In vitro fertilization, intracytoplasmic sperm injection, oocyte freezing, embryo biopsy, and embryo cryopreservation can still be considered developing technologies in the horse (Squires et al., 2003).

It is likely that advancements will continue to be made in the future as breed registry rules change and newer techniques are developed to allow superovulation in the mare. It also seems sensible that the most economical way of moving genetic material throughout the world is in a cryopreserved state (Squires et al., 2003). The use of pre-implantation genetic diagnosis

could be used to enhance the value of embryo cryopreservation and lead to more successful commercialization of embryo technologies. Preimplantation genetic testing would allow for the culling of embryos with genetic mutations and the marketing of embryos known to result in foals of a specific color or gender.

Purpose

The unique characteristics of equine embryo development make equine embryo cryopreservation a labor intensive and expensive process. The equine embryo forms a thin glycoprotein capsule as it expands into a blastocyst. The capsule is resistant to cryoprotectants, therefore successful equine embryo cryopreservation must be carried out prior to capsule formation. This presents a problem unique to the horse as the equine embryo normally passes from the oviduct into the uterus 5.5 days post ovulation and the glycoprotein capsule begins to form 6.5 days post ovulation (Freeman et al., 1991). The short duration between the arrival of the embryo in the uterus and capsule formation presents a very narrow window of opportunity during which the embryo can be collected and frozen. As embryos are collected using non-surgical uterine lavage, mares must be monitored closely by ultrasound to determine the time of ovulation. Even with close monitoring, the expected number of embryos recovered is fewer than 1 per lavage.

Because of the expense and low number of embryos recovered, equine embryo cryopreservation is not economically viable and research for better techniques has not resulted in success similar to other livestock species, although an effective and commercially available vitrification process that allows for quick cryopreservation of equine embryos without specialized equipment has been developed (Carnevale, 2006).

Pre-implantation genetic diagnosis could add value to equine embryo cryopreservation. If equine embryos could be genetically tested prior to cryopreservation, it could confirm the resulting foal would be genetically normal and even determine the potential foal's gender and color. Genetic tests are currently available to test for several inherited diseases commonly found in various horse breeds including Hyperkalemic Periodic Paralysis (HYPP) Disease, Glycogen Branching Enzyme Deficiency (GBED), Junctional Epidermolysis Bullosa (JEB), and HERDA - Hereditary equine regional dermal asthenia.

The purpose of this study is to determine if embryo biopsy performed prior to cryopreservation will affect embryo viability in vitro.

Research Goals

The main goal of this study is to investigate the effect of embryo biopsy and subsequent cryopreservation (vitrification) on the development

potential of equine embryos. The working hypothesis tested in the study is that combining vitrification and biopsy of equine embryos does not alter the development potential of equine embryos in vitro.

The specific objectives are:

(1) To determine if embryo biopsy affects in vitro development of the equine embryo.

(2) To determine if embryo biopsy and vitrification treatment combined inhibits in vitro development of equine embryos more than embryos treated by vitrification alone (no biopsy).

Demonstrating the potential for development in vitro of equine embryos after combining the two techniques would help establish the potential for further research in vivo.

Embryo Technologies in the Horse

Although artificial insemination was probably used on the horse before any other large animal species, it was the last to undergo embryo recovery and transfer (Allen, 2005). The techniques of embryo collection and transfer in horses were developed in the 1970's (Squires et al., 2003). The lag in further development of embryo technologies in the horse compared to other livestock species is often blamed on the initial unwillingness of breed registries to accept foals produced by embryo transfer and the lack of an effective superovulation protocol for mares. In addition, the first attempts at non-surgical embryo transfer had relatively low success (Allen & Rowson, 1972; Squires et al., 1985). It appears as horse producers discovered the potential commercial benefits of embryo technologies, research increased. Great progress in the non-surgical technique was made in the 1990s especially in Argentina where high value polo ponies are produced (Pashen et al., 1993; Riera & McDonough, 1993). Progress began to increase in America when the restrictions limiting the number of offspring that could be registered as a result of embryo transfer was lifted from the American Quarter Horse Registry (Hudson & McCue, 2004). Preimplantation genetic testing has the potential to increase the

commercial appeal of embryo technologies even more and thus encourage research.

Embryo Collection

The recovery of embryos from a donor mare is a straightforward process. The relatively straight and easily distensible cervix of a mare make it a simple process to pass a two-way catheter into the body of the uterus (Allen, 2005). An inflatable cuff on the end of the catheter allows the internal opening of the cervix to be sealed. An embryo-flushing medium is then used to distend the uterus and dislodge the embryo. The medium is recovered by gravity flow through an in-line embryo filter cup. The contents of the filter cup is carefully searched under a microscope and the embryo recovered. The direct transfer to a recipient mare is also a straightforward procedure and with proper embryo handling can result in acceptable pregnancy rates of 75%-85% (Pashen et al. 1993; Meadows et al. 2000; Jasko 2002). However, the transfer of frozen equine embryos presents some additional challenges.

Equine embryos take an unusually long time to pass from the oviduct into the uterus (Allen, 2005). Because equine embryos remain in the oviduct for 144 to 168 hours, most are collected 7 to 8 days post ovulation (Battut et al., 2000). Multiple studies have shown that embryos greater than 300μm

are unsuitable for cryopreservation (Squires et al., 2003). Blastocyst formation and expansion begins 6-7 days post ovulation (Grondahl & Hytell, 1996). This creates a problem for collecting morula or early blastocyst (<300μm) stages needed for successful cryopreservation. There is a very narrow window of opportunity when the embryo is in the uterus, but is still small enough for cryopreservation. Carnevale (2006) described a method to increase the number of morulae and early blastocysts recovered smaller than 300μm by timing the flushes from ovulation induction. Ovulation occurs approximately 36 hours after the administration of human chorionic gonadotropin (hCG) and approximately 40 hours after administration of a gonadotropin-releasing hormone (GnRH) analogue (deslorelin). Carnevale (2006) demonstrated that embryo flushes can be carried out exactly eight days from administration of an ovulation-inducing hormone to collect embryos small enough for vitrification (<300μm). But even with the improved methods of embryo collection, the mean embryo recovery per cycle is approximately 50% (Squires et al., 2003).

Superovulation

The failure of the mare and other equids to undergo genuine superovulation in response to treatment with exogenous gonadotrophic hormones has been a major limiting factor to the commercial development

of equine embryo transfer from the outset (Allen, 2005). There are numerous accounts of as many as 20-30 viable embryos from cattle, sheep, and pigs from a single uterine flush when superovulation was induced. Despite a commercial, partially purified extract of equine FSH being available, the effectiveness remains disappointingly low. Alvarenga et al. (2000) reported an improved embryo recovery of 3.5 embryos per cycle in mares treated twice daily for 6-8 days with eFSH. However commercial embryo recovery remains at a mean of just 1.8 per flush for treated mares compared to a recovery of .85 embryos per cycle from non-treated donor mares (Allen, 2005). While research is still being conducted on mare ovarian stimulation, there appears to be a basic physiologic challenge to superovulation. Allen (2005) notes that equine follicles can only ovulate and release their oocytes through the ovulation fossa because of the very tough and fibrous tunica albuginea covering the external surface of the ovary. Even with increased follicle development, the number of embryos recovered is low. According to Allen this implies that the follicles compete with one another in their attempt to track through the ovary toward the ovulation fossa. While it is possible to slightly increase the number of ovulations per cycle, it still doesn't approach the 20 embryos per cycle demonstrated in other livestock species.

With the low response to treatment with expensive hormones, superovulation still is not commercially viable in the equine. Most embryo recoveries in equids are performed on spontaneously ovulating mares (Squires et al., 2003). The recovered embryos are then directly transferred to recipient mares. In other livestock species, the number of recovered embryos often exceeds the number of available recipients resulting in excess embryos. With a lack of extra embryos in the equine, research on cryopreservation and other embryo technologies has continued to be slow to develop. Without the ability to produce large numbers of embryos through superovulation, opportunities for research have also been limited.

Embryo Cryopreservation

Techniques for the transfer of frozen equine embryos have lagged well behind that of bovine embyos mainly because of biological reasons (Squires et al., 2003). Because of the lack of an effective superovulation protocol in horses, typically only a single embryo is recovered and immediately transferred to a recipient mare. However in bovine embryo transfer, donor cattle often produce in excess of 20 embryos (Allen, 2005). The number of embryos collected often exceeds the number of recipients available. The lack of recipients created a need for embryo cryopreservation research (Galli et al., 2003). According to Galli et al. (2003), large numbers

of bovine embryos can also be produced using ovaries of slaughtered donors. The ovaries are often pooled and processed as a batch. This simplifies the process and consequently embryos are produced at low cost. This gives researchers an abundant source of embryos.

Equine embryos also form a unique, thin glycoprotein capsule which resists the penetration of cryoprotectants and makes the freezing of blastocysts and expanded blastocysts very difficult (Legrand et al. 2000). Squires et al., (2003) reported that several investigators have demonstrated embryos <300μm in size (before the capsule forms) survive freezing and thawing, whereas larger embryos have much lower survival rates.

A significant advance occurred in embryo freezing with the application of vitrification (Allen, 2005). Vitrification is a process that uses high concentrations of permeating cryoprotectants to flash freeze the embryo. Damaging ice crystals do not form and instead a glass-like state is achieved (Carnevale, 2006). Carnevale (2006) reported pregnancy rates of 60-75% when vitrifying embryos <300 μm diameter. Unlike conventional embryo freezing, vitrification is a quick procedure that requires no special equipment and can even be carried out in field conditions. Vitrified embryos can be rapidly warmed within a straw and directly transferred into the uterus of a donor mare (Carnevale, 2006).

Embryo Culture

Hinrichs et al. (1990) were the first to describe equine embryo development in long-term culture (10-20 days). The investigators recovered morulae stage embryos via non-surgical lavage and placed them in culture media. The embryos were maintained at 37°C in a 5% CO_2 atmosphere. The embryos expanded by the third day of culture and later hatched from their zona, rather than the zona thinning and flaking off as occurs in vivo (Hinrichs et al., 1990). According to Hinrichs et al. (1990), the results of their study indicate that horse embryos can consistently maintain growth and expansion for 10 days in vitro.

Embryo Biopsy

The use of embryo biopsy for pre-implantation genetic testing has been around for quite some time in horses and other livestock species. Huhtinen et al. (1997) were able to establish successful pregnancies that resulted in live foals in 1994. The embryos were biopsied using a microblade procedure removing 10-30 blastomeres from day 6 embryos (Huhtinen et al., 1997). DNA from the biopsied cells was amplified using polymerase chain reaction (PCR) to determine the resulting foals gender. The pregnancy rate as reported by Huhtinen et al. (1997) was significantly lower in the biopsy group (3/14) vs. the control group (6/8), but the authors

pointed out that better biopsy techniques and earlier embryo recovery (before the glycoprotein capsule formed) could potentially improve pregnancy rates. The authors also noted that improved pregnancy results along with more identified genes associated with particular diseases or inherited characteristics could be used for preimplantation genetic diagnostics (Huhtinen et al., 1997). A biopsy technique that utilized a micro-needle to remove a smaller portion of the embryo was described by Agca et al. in 1995. Embryos were held in place by suction applied to a glass holding pipette. The pipette was controlled by a joystick-type micromanipulator. An aspiration pipette was then inserted through the zona pellucida and 2-3 blastomeres were removed by suction. The cells were left in the media drop and later recovered for genetic testing (Agca et al., 1995).

PCR

Polymerase chain reaction (PCR) is a tool used to amplify specific DNA sequences from minute quantities of cellular material. Using PCR, genes have been identified from embryonic material of many species (Huhtninen, et al. 1997). Successful DNA amplification has been carried out on bovine and murine blastocysts (King & Wall, 1988), murine demi-morulae (Ninomiya et al., 1990), bovine demi-blastocysts (Peura et al., 1991), ovine 3-cell biopsies (Bredbacka & Peippo, 1992), equine demi-

blastocysts (Huhtinen et al., 1997), and single blastomeres from human (Verlinsky et al., 1991) and bovine (Machaty et al., 1993) embryos.

Genetic Testing

The American Association of Equine Practitioners (AAEP) supports the use of genetic testing by veterinarians or breed associations to identify genetic mutations in animals so that owners can make informed decisions about breeding, purchase and specific treatments (American Association of Equine Practitioners, 2009). According to the association, there are genetic tests for 9 diseases.

Autosomal Dominant Diseases

1. Hyperkalemic Periodic Paralysis (HYPP) in the Quarter Horse
2. Type 1 Polysaccharide Storage Myopathy (PSSM) in numerous breeds
3. Malignant Hyperthermia in Quarter Horse related breeds

Autosomal Recessive Diseases

4. Overo Lethal White Syndrome in the Paint Horse
5. Combined immunodeficiency in Arabian Horses
6. Glycogen Branching Enzyme Deficiency (GBED) in Quarter Horse related breeds
7. Junctional Epidermolysis Bullosa (JEB) in Belgians

8. JEB in Saddlebred horses
9. Hereditary Equine Regional dermal Asthemia (HERDA) in Quarter Horse related breeds

AAEP also notes that there are numerous other conditions strongly suspected to be due to mutations in genes of major effect, but genetic tests are not yet available. Genetic testing for all of these diseases could also be presumably carried out on single blastomeres using the PCR amplification technique. In addition, there are tests for coat color genetics and gender.

Chapter 2

Materials and Methods

Embryo Collection

Embryos were obtained between February and September during the regular breeding season from Quarter Horse, Thoroughbred, and mix-breed light mares between three and ten years old. Mares and stallions underwent reproductive evaluation to ensure fertility before being included in the study. Follicular growth was monitored daily via transrectal palpation and ultrasonography. Mares with follicles >35 mm were bred via artificial insemination with fresh semen collected from Quarter Horse and Welsh stallions. After insemination, mares were injected with hCG (2500 IU administered IV) or deslorelin (1.5mg IM). Mares were monitored via transrectal ultrasonography at 24 and 48 hours post insemination to confirm ovulation.

Mares were re-checked via ultrasound 5 days post ovulation. If the mares appeared free of fluid and uterine edema, they were flushed via non-surgical, trans-cervical uterine lavage 6.0-6.5 days post-ovulation. Sedation was provided prior to embryo recovery using 0.5mg/kg zylazine administered intravenously. An embryo catheter with a 60-75cc balloon was attached to Y tubing and inserted transcervically into the uterine body of the mare. The balloon was inflated to capacity with flush media to seal the cervix and mild pressure was applied in a caudal direction to seat the

catheter against the internal cervical os. Four 1L increments of flush media (BioLife Advantage Complete Flush Media, Agtech, Inc., Manhattan, KS) were used to flush the uterus. Fluid was collected as it exited the uterus in a cup with a 75-micron filter. After the flush was complete, 20 ml of flush media was allowed to remain in the cup. The cup was then rinsed with additional flush media into a search dish.

Embryo Identification

The dish was examined under a microscope at 10x power. Embryos were identified by developmental stage and grade (Table 1). Once identified, embryos were washed four times through embryo holding media (Vigro Holding and Transfer Medium, Bioniche Animal Health USA Inc., Pullman, WA).

The embryos were temporarily placed in holding media in a 2ml cryotube and held at room temperature for transport to the laboratory. Total time from identification to treatment was less than 1 hour.

Biopsy and Vitrification

Embryos underwent micromanipulation and biopsy prior to being cryopreserved. Embryos were placed in biopsy media (Embryo Biopsy Medium, Irvine Scientific, Santa Ana, CA) under oil and viewed under a microscope at 20x power. A small amount of tissue was removed from each

embryo using a microaspiration technique. Embryos were held in position by gentle suction from a holding pipette using a micromanipulator. A small hole was mechanically made in the zona pellucida using a second micromanipulator and a microneedle. A small amount of tissue was gently aspirated and placed in the media near the embryo for later removal (Fig. 1). Once the biopsy procedure was complete, the tissue samples were frozen in 1ml cryotrubes and stored at -20°C.

The biopsied embryos and control embryos (embryos not undergoing biopsy) were then vitrified according to the protocol described by Carnevale and co-workers (2006) at Colorado State University. During the vitrification procedure, embryos are sequentially moved through three vitrification solutions containing various combinations of glycerol and ethylene glycol as cryoprotectants. To facilitate movement of the embryos from one vitrification solution to the next and the rapid loading of the freezing straw, the vitrification and diluent solutions can be set up on the lid of a Petri dish (Fig. 2). A timer is necessary to ensure the proper exposure time to each of the three vitrification solutions. Embryos are removed from the biopsy media and placed into vitrification solution 1 (glycerol [1.4 M]). Movement of the embryo is made with a small volume of media (<1μL) to ensure the embryo is exposed to the proper concentration of cryoprotectants. The

embryo remains in vitrification solution 1 for 5 minutes and then promptly moved to vitrification solution 2 (glycerol [1.4 M] plus ethylene glycol [3.6 M]) for 5 minutes. The embryo is then placed in vitrification solution 3 (glycerol [3.4 M] plus ethylene glycol [4.6 M]). The embryo is exposed to vitrification solution 3 for less than 1 minute before being placed in liquid nitrogen vapor. To facilitate rapid loading of the freezing straw, the embryo is placed in a 30μL drop of vitrification solution 3, the exact amount to be drawn up into the straw (Carnevale, 2006).

A 0.25-ml semen straw (nonirradiated, polyvinyl chloride) is loaded with the embryo in the final vitrification solution between two sections of 90 -μL of diluent solution (galactose; 0.5M) to allow dilution of cryoprotectants in the straw after warming (Fig. 2). The straw is loaded for vitrification with 90 μL galactose, air (5 μL), the embryo in vitrification solution 3 (30 μL), air (5 μL), and 90 μL galactose. The straw is then sealed with a heat sealer and placed in liquid nitrogen vapor (Carnevale, 2006). No special equipment is required for vitrification; the straw containing the embryo is exposed to vapor for 1 minute before plunging it into liquid nitrogen. Embryos were stored in liquid nitrogen until thawing and culture.

Embryo Thawing

Warming of the straw requires no special equipment. Biopsied and control embryos were warmed within the straw. The straw was held in the air for 10 seconds and then plunged in water (37° C) for 10 seconds. The straw was then shaken to mix the contents (Carnevale, 2006). The contents of the straw including the embryo were expelled into holding media (BioLife Holding and Transfer Medium, Agtech, Inc., Manhattan, KS).

Embryo Culture

The embryos were identified and then transferred to a 200-µl droplet of culture media (HyClone HyQ DMEM/Ham's F-12 medium 1:1, Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal calf serum and 100 mcg penicillin and 100 mcg streptomycin per ml, in a 35 mm petri dish under a film of filtered paraffin oil. The culture technique used in this study was described by Hinrichs et. al (1990). Embryos were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was not changed during the culture period. Using a dissection microscope at 10-50x power, embryos were evaluated for normal development daily for 5 – 10 days of culture (until the embryos stopped expanding or degenerated). Embryo morphology was recorded. Embryo viability could not be assessed as no pregnancies were established, however development potential was defined as blastocele expansion with thinning of the zona pellucida (Fig. 3).

According to Hinrichs (personal communication 2006) evidence of expansion (increase in diameter) especially with hatching from the zona should be sufficient to prove that the embryos survive vitrification and thus have development potential.

Statistical Analysis

The development potential of the study group (biopsy combined with vitrification) was compared to the development potential of the control group (vitrification only) using a chi-square goodness of fit test.

The statistical model used was:

$$X^2 = \Sigma [(O_i - E_i)^2 / E_i]$$

where O_i is the observed frequency count for the *i*th level of the categorical variable, and E_i is the expected frequency count for the *i*th level of the categorical variable. For this study, the significance level is 0.05.

Carnevale et al. (2004) reported that 75% of vitrified embryos <300μm resulted in established pregnancies. Therefore, more than 75% of the vitrified embryos in this study should show development potential.

Hypothesis:

H_0: The development potential of the vitrification only group and the development potential of the vitrification and biopsy group is at least 80%.

H_a: The development potential of the vitrification and biopsy group is less than the development potential of the vitrification only group.

Chapter 3

Results

Embryo Collection

Embryos (<300μm) were recovered from 20 of 43 flushes (47%) including one set of multiple embryos. All embryos were classed morphologically as excellent (15/20) or good (5/20) (grade 1 or 2) (Table 1; Fig. 3). The embryos were sequentially assigned to either the biopsy or control group.

Embryo Development

Embryos were monitored daily for development potential; defined as expansion of the blastocele, thinning of the zona pellucide and in some cases hatching from the zona pellucida (personal communication Hinrichs, 2006). Although equine embryos do not hatch from the zona like other species in vivo, the embryos do hatch when cultured in vitro (Hinrichs et al., 1990).

The embryos that did show development potential had developed a well defined blastocele by day 3 in culture and those that hatched had either partially or completely broken free of the zona pellucida by day 5 in culture. None of the embryos showed any further development after 5 days in culture. Figure 4 shows an example of the incomplete hatching of an embryo after vitrification and culture in vitro. Figure 5 shows the complete hatching of the embryo. Once free of the zona pellucida, the blastocyst expanded quite rapidly until it degenerated on day 5 in culture. While

hatching was common, there were examples of embryos that showed noticeable expansion and thinning of the zona pellucida, but without hatching (Fig. 6).

The embryos with no development potential showed no signs of expansion or zona pellucida thinning during culture. Figure 7 is an example of an embryo that did not increase in diameter and showed more degeneration of the blastomeres while in culture.

The embryos that underwent the combined vitrification and biopsy treatment were much less likely to show expansion and hatching, but those that did typically showed hatching through the zona tear created during the biopsy process. Figure 8 is a biopsy embryo that hatched through the zona tear. Two of the embryos in the combined treatment were not intact after thawing. Neither embryo showed any kind of subsequent development while in culture. Figure 9 is an example of an embryo without a defined zona pellucida after thawing.

None of the embryos in this study was transferred to a recipient and thus no pregnancies were established.

Statistical Findings

There was a statistically significant difference ($P<0.05$) between the development potential of the biopsy group (2/10 embryos) compared to the

control group (8/10 embryos) (Table 2). While the development of the embryos was significantly different than the control group, it was not zero.

Chapter 4

Discussion

Equine embryo transfer was developed in the 1970s (Squires et al., 2003). Since then, researchers have been able to establish successful pregnancies after biopsy (Huhtinen et al., 1997), culture equine embryos before transfer (Weimer et al., 1989), and develop a commercially available vitrification method (Carnevale, 2006). What has not been accomplished is combining biopsy with vitrification. The combined procedure is important because any genetic testing of the embryo should be carried out before transfer to a recipient.

This study looked at embryo development in vitro after biopsy and vitrification as a combined procedure. Because the embryos were not transferred to a recipient mare and no pregnancies were established, it did not determine the ultimate viability of the embryos after the combined procedures. Instead this study determined development potential in vitro. Culturing the embryos in vitro eliminates variables with the embryo transfer process itself. It is important to establish whether embryos can survive the combined procedure before attempting to establish pregnancies. Failure to establish successful pregnancies could be the result of the transfer process itself, rather than the biopsy/vitrification procedures. Because Hinrichs and co-workers (1990) showed that equine embryos do develop in vitro, this

study was able to determine whether the combined procedure affected embryo survivability in vitro.

Although growth and hatching rates were much lower in comparison to the control group, the results do show that equine embryos can grow in vitro after undergoing biopsy and vitrification together thus it does not preclude the potential to establish successful pregnancies after the combined procedures. It does however point to the need for further research to refine the combined procedure.

Because both vitrification and biopsy are established procedures separately, the lower success rates achieved in the treatment group can be attributed to the combination of the two. The biopsy procedure requires creating a small tear in the zona pellucida. The resulting tear creates an opening that may affect how cryoprotectants interact with the embryo. Changes to the vitrification procedure may be needed. Different vitrification solution concentrations or a change in the exposure times may lead to greater success in the future.

The biopsy procedure may be damaging the embryo. The two embryos that showed expansion and hatching after the combined procedures were the last two to undergo micromanipulation. Technician experience has been shown to affect pregnancy rates after non-surgical embryo transfer in

the horse (Iuliano et al., 1985). Technician experience with the micromanipulation procedure could have affected success in this study as well.

Embryos had to be transported from the collection location to the lab. Carnevale (2006) recommends vitrification procedures be carried out as quickly as possible. The transport time combined with the time required for the biopsy procedure may have affected embryo survivability. Reducing the time between collection and vitrification enhance development potential.

Research in equine intracytoplasmic sperm injection (ICSI) has shown that the use of a piezo drill improves cleavage rates (Hinrichs, 2005). The device causes minute vibrations in the injection pipette facilitating passage through the zona pellucida (Hinrichs, 2005). The same technology could improve success rates with the microaspiration technique. The technique used in this study requires pressure on the embryo to tear a small opening in the zona pellucida. The piezo drill makes smaller opening while exposing the embryo to less pressure (Hinrichs, 2005).

Due to budget limitations and limited facilities, this study included a small number of equine embryos. A larger sample size may have provided a more accurate assessment of the development potential of embryos after the combined procedure.

Implications

Because this study did not preclude in vitro development of equine embryos after biopsy and vitrification, it shows promise for the eventual development of a preimplantation genetic diagnosis technique for horses. The ability to ensure that embryos are free from known genetic mutations would increase the value of embryo transfer and embryo cryopreservation to breeders. Predetermining the gender and color of potential offspring could also make the embryos more valuable.

However, to take advantage of the genetic testing available, it would need to be carried out prior to pregnancies being established. The ability to biopsy and genetically test the embryo is not practical unless the embryo can be stored while the testing is conducted. This study lays the groundwork for the ability to store embryos that have undergone biopsy. While Hinrichs and co-workers are carrying out a similar study at Texas A & M University, no known published research exists on combining vitrification and biopsy in the equine embryo.

Embryo technologies in the horse lag behind that of other livestock (Allen, 2005). Much of this can be blamed on the lack of available equine embryos for research. The lack of an effective superovulation protocol

means that there are very few extra embryos available. Typically a single embryo is collected from a donor mare and immediately transferred to a recipient. Adding value to the embryo transfer procedure could lead to additional research. The ability to eliminate known genetic mutations by culling embryos before transfer would add value for breeders. Embryos with genetic mutations could be eliminated from storage before being transferred to a recipient. Additionally, the ability to predetermine the genetic health, gender and coat color of embryos could add value for the potential import/export of equine embryos. Increasing the value of embryo cryopreservation would open the door to further research in superovulation and other embryo technologies in the horse.

Chapter 5

Tables

Table 1. System used to grade the quality of equine embryos*

Grade 1	Excellent or good: Symmetrical and spherical embryo mass with cells of uniform size, color, and density. Embryo mass has clear edges without indentation. No evidence of cellular fragmentation or loose cytoplasmic granules in the perivitelline space.
Grade 2	Fair: Moderate irregularities in the overall shape of the embryo, for example, a minor degree of cytoplasmic fragmentation evident as slight convolution of the edges of the embryo and small cytoplasmic granules in the perivitelline space.
Grade 3	Poor: Major irregularities in the shape of the embryonic mass or in the size, color, or density of the constituent cells. High degree of cytoplasmic fragmentation with extrusion or degeneration of cells of variable size reflected by highly lobulated, irregular edges of the embryonic mass and a large perivitelline space.
Grade 4	Degenerate or dead: High degree of cytoplasmic fragmentation. Embryonic cells of irregular size and color. Absence of cellular compaction and failure to form a clear embryonic mass. This group includes embryos with irregularly sized cells of a low number inconsistent with the expected stage of development.

* Tremoleda et al., 2003

Table 2. Chi-Square Goodness-of-Fit Test for Observed Counts

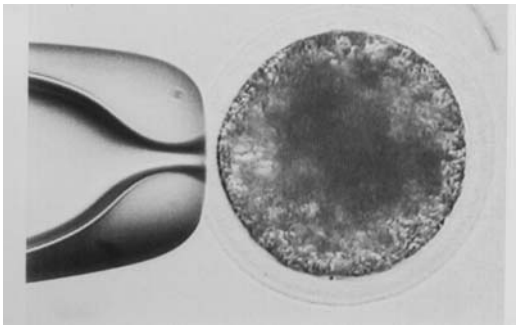
Category	Observed	Historical Counts	Test Proportion	Expected	Contribution to Chi-Sq
1	8	8	0.4	8	0.0
2	2	2	0.1	2	0.0
3	2	8	0.4	8	4.5
4	8	2	0.1	2	18.0

N	DF	Chi-Sq	P-Value
20	3	22.5	P<0.05

Chapter 6

Figures

A.



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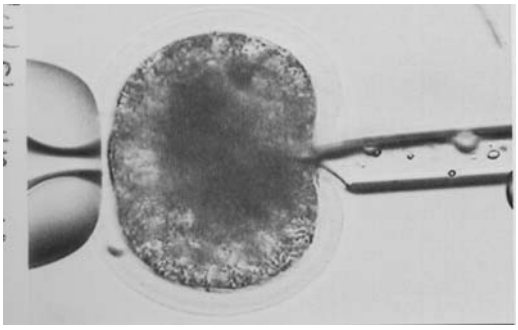

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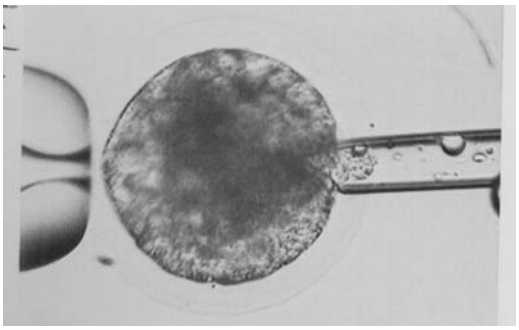

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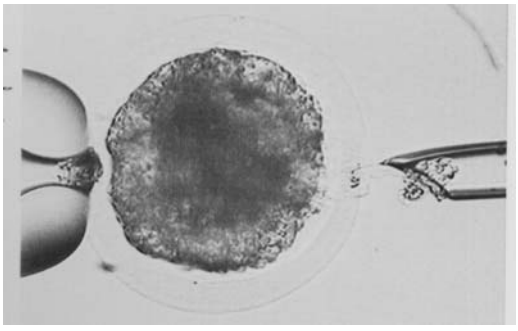


Figure 1. Microaspiration biopsy technique. The embryo is placed on a holding pipette (A). A small hole is created in the zona pellucida with the biopsy needle (B). Gentle suction is applied to aspirate a small amount of tissue (C). The tissue sample is released next to the embryo for later retrieval (D).

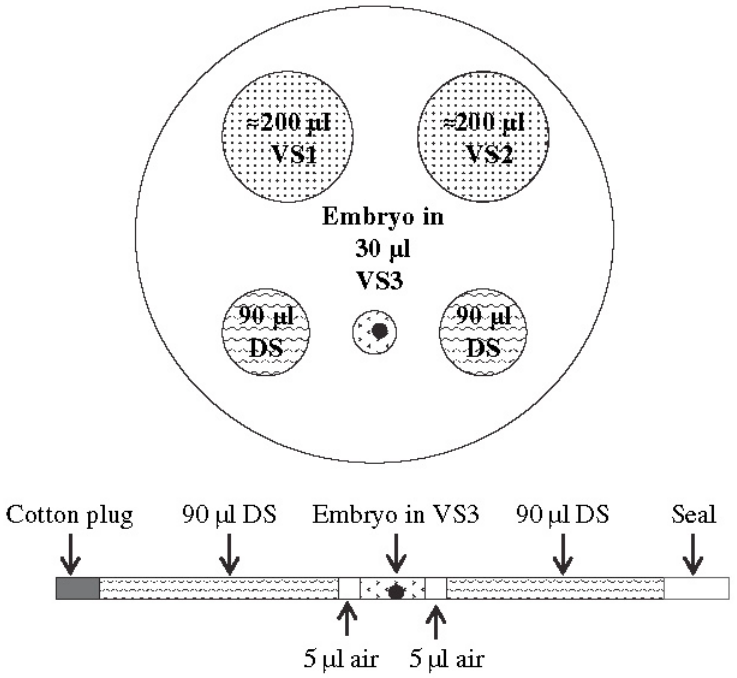
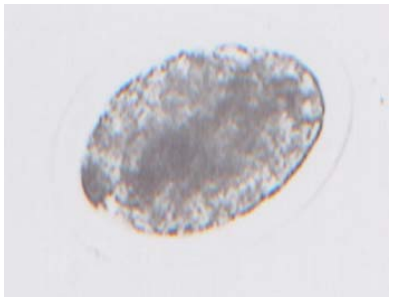
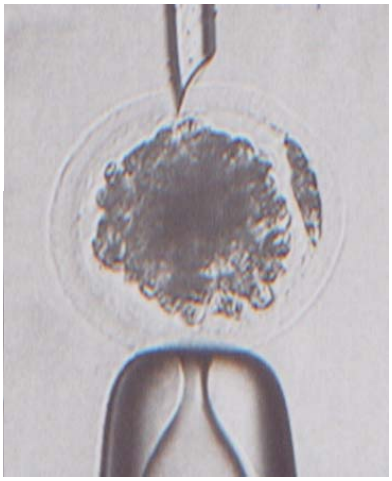


Figure 2. Organization of the vitrification solutions and diluent solution in a petri dish. Volumes of vitrification solutions 1 & 2 (VS 1; VS 2) can be approximate; however, precise amounts are needed of vitrification solution 3 (VS 3) and the diluent solution (DS) when loading the straw (Carnevale, 2006).

A.  B. 

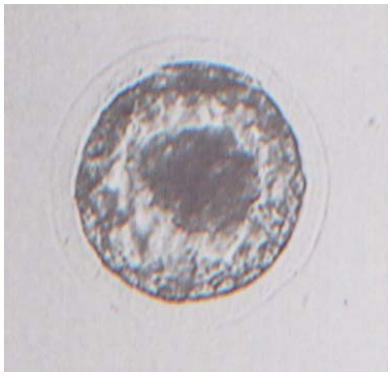
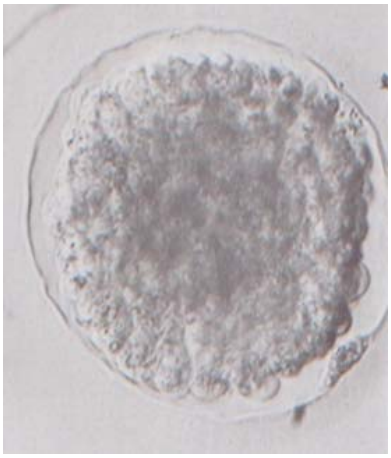
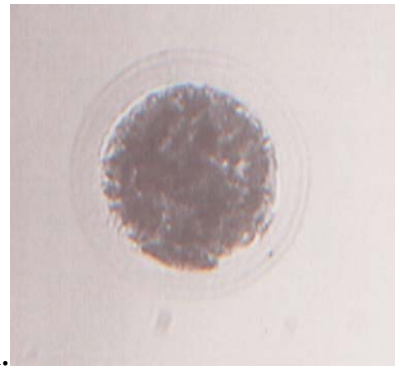
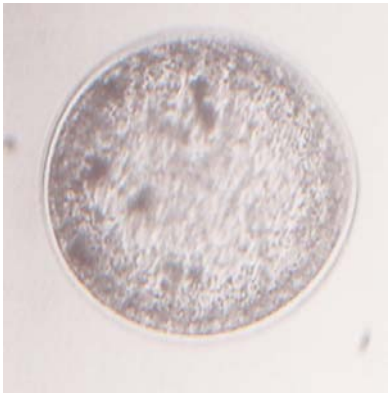
C.  D. 

Figure 3. Examples of embryos before treatment and their morphological assessment. Morula grade 1(A). Morula grade 2 (B). Early blastocyst grade 1 (C). Expanding blastocyst grade 2 (D).

A.  B. 

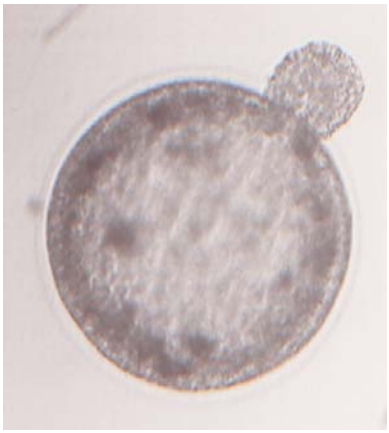
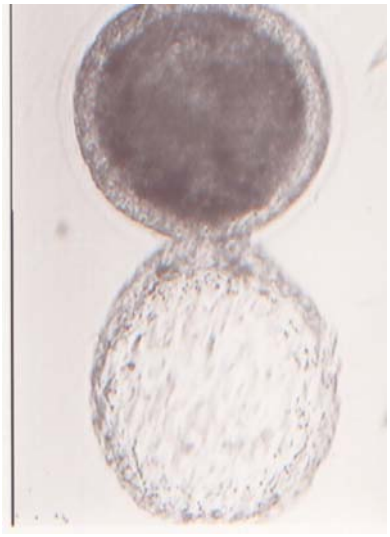
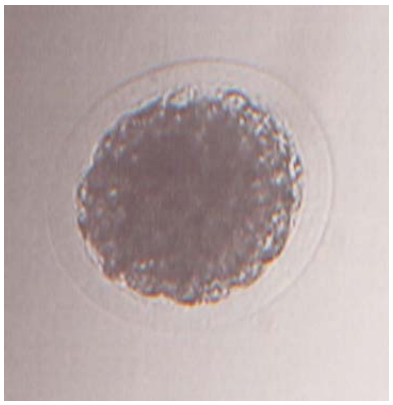
C.  D. 

Figure 4. An example of the expansion and hatching seen during in vitro culture. This embryo was vitrified without biopsy. The embryo is a very early blastocyst with only a small blastocele visible after thawing (A). By day 2, the embryo shows significant re-expansion (B). A small hole has formed in the zona pellucida by day 3 (C). Almost complete hatching by day 5 (D).

A.  B. 

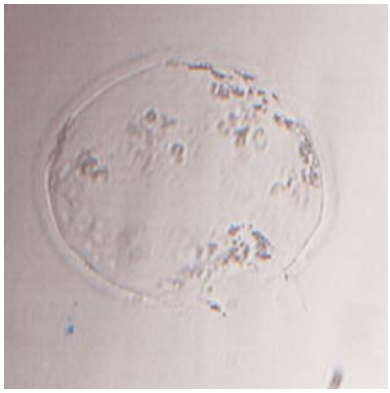
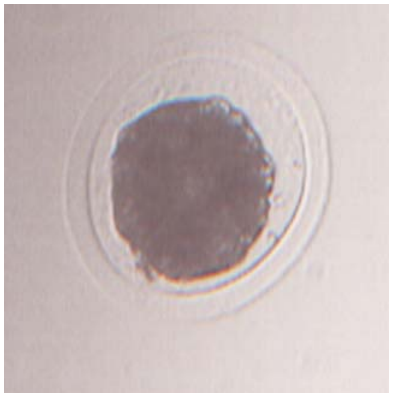
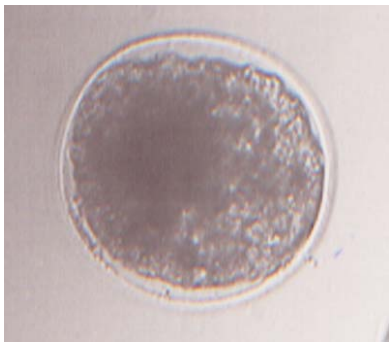
C.  D. 

Figure 5. Embryos that hatched earlier expanded to a much greater size before degenerating. This morula (vitrification without biopsy) showed some blastomere contraction upon thawing (A), but quickly re-expanded and completely hatched by day 3 in culture (B). The empty zona pellucida was visible right next to the expanded blastocyst (C). Without the glycoprotein capsule, the blastocyst rapidly expanded (D).

A.  B. 

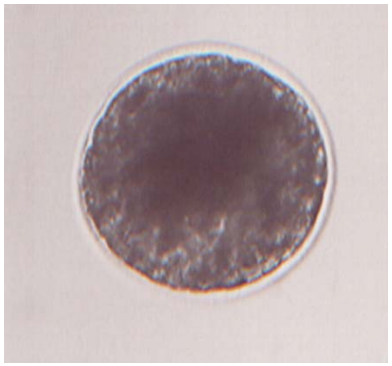
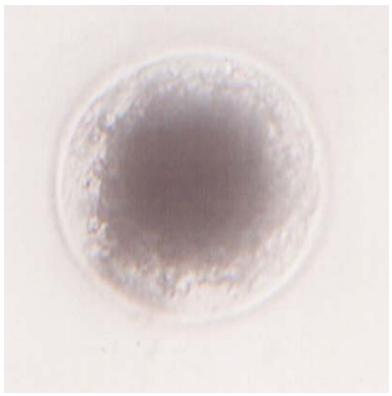
C.  D. 

Figure 6. Some of the embryos showed blastocele expansion and thinning of the zona pellucida without hatching. This morula (vitrification without biopsy) show a great deal of contraction of the blastomeres upon thawing (A). By day 3 a blastocele had developed with thinning of the zona pellucida (B). On day 4, even greater expansion and zona thinning occurred (C), but complete degeneration was seen on day 5 (D).

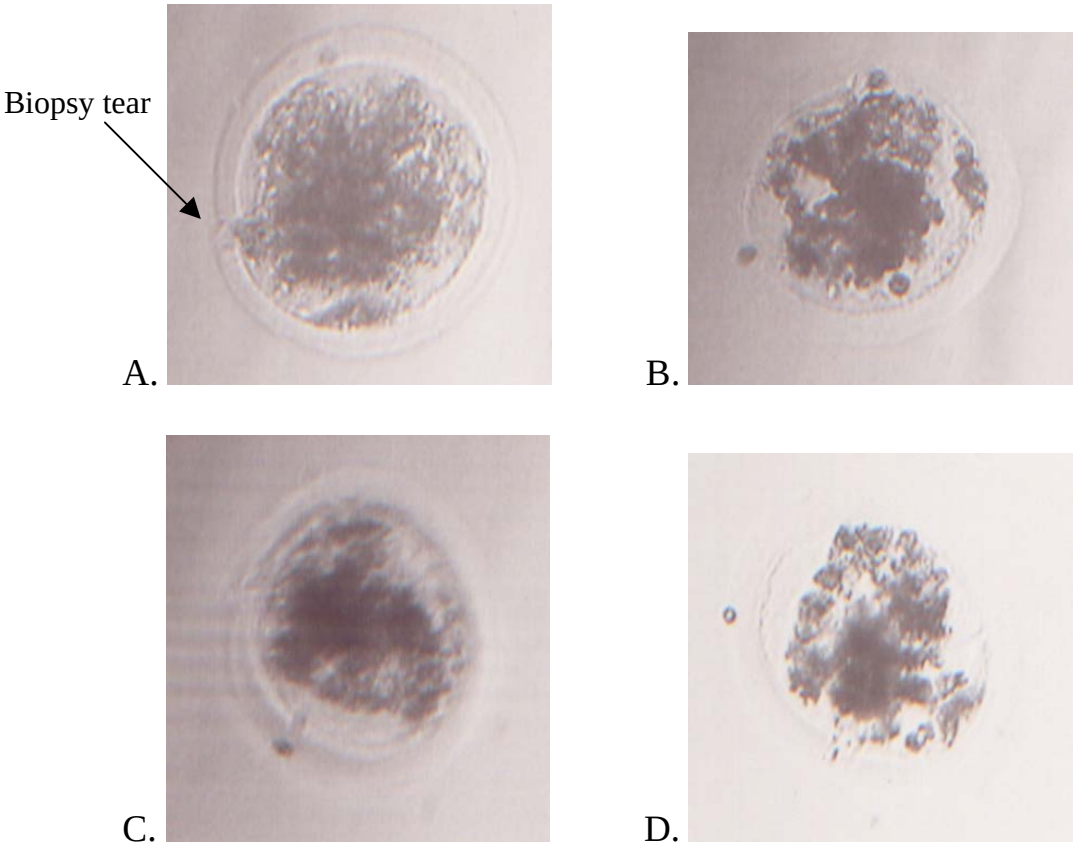
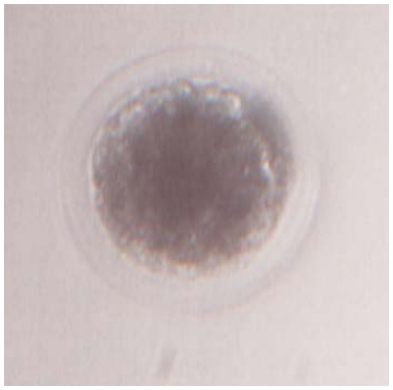
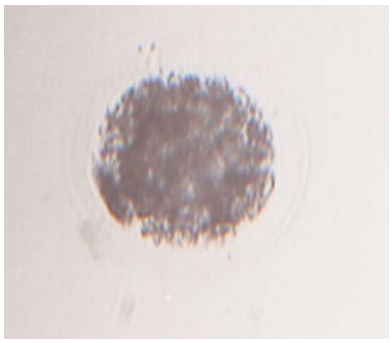
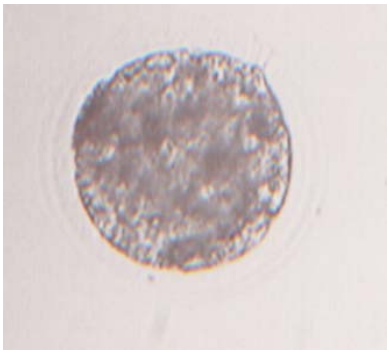


Figure 7. Embryos without developmental potential showed no expansion during in vitro culture. This embryo (biopsy with vitrification) was a grade 1 morula but showed considerable degeneration upon thawing and the tear in the zona pellucida as a result of the biopsy procedure can be clearly seen(A). Even greater degeneration can be seen by day 3 (B), day 4 (C), and day 5 (D) with no zona thinning or embryo expansion.

A. 

B. 

C. 

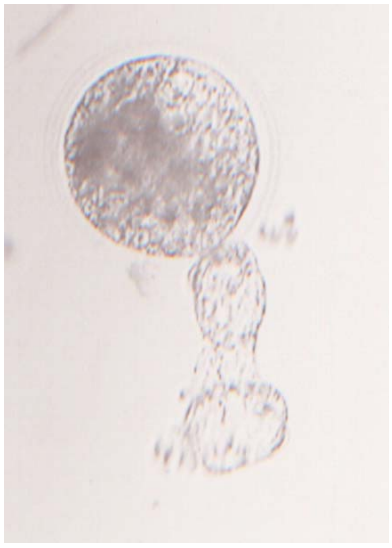
D. 

Figure 8. The embryos that underwent both biopsy and vitrification hatched through the opening created in the zona pellucida by the biopsy procedure. This embryo (biopsy with vitrification) was a grade 1 morula that showed some degeneration upon thawing (A). By day 2, it had expanded to a small blastocyst (B). By day 3, greater expansion and zona pellucida thinning was evident (C). Incomplete hatching could be seen by day 5 (D).

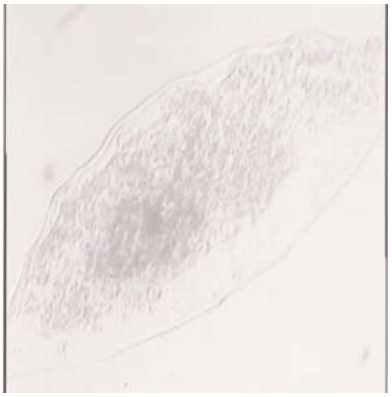
A.  B. 

Figure 9. This embryo did not survive the vitrification with biopsy procedure. At thaw, the embryo was degenerated with no evidence of an intact zona pellucida (A). It continued to degenerate through day 5 in culture (B).

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