THESIS

PREIMPLANTATION GENETIC DIAGNOSIS OF EQUINE EMBRYOS

Submitted by

Erika L. Cullingford

Department of Biomedical Sciences

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY ERIKA L CULLINGFORD ENTITLED PREIMPLANTATION GENETIC DIAGNOSIS OF EQUINE EMBRYOS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate work

[redacted]

Jason Ahola

[redacted]

Gerrit Bouma

[redacted]

Advisor: George Seidel, Jr.

[redacted]

Co-Advisor: Patrick McCue

[redacted]

Department Head: Barbara Sanborn

ABSTRACT OF THESIS

PREIMPLANTATION GENETIC DIAGNOSIS OF EQUINE EMBRYOS

In horses, determination of certain genetic traits/alleles in embryos before embryo transfer would be advantageous due to the costs of resulting pregnancies. An attractive option is preimplantation genetic diagnosis (PGD), but to date few biopsied equine embryos have resulted in pregnancies. In the current experiment, 37 embryos ranging from 160 - 575 μm in diameter were biopsied. To obtain embryos, donor mares were monitored using transrectal ultrasonography. When a follicle > 35 mm in diameter was observed, 2,500 IU hCG or 1.5 mg deslorelin acetate was administered, and mares were inseminated daily until ovulation was detected. Embryos were recovered nonsurgically on days 6.5 – 7 (day 0 = ovulation). Trophoblast biopsies were collected in a 30 μl droplet of Syngro Holding Medium (Bioniche, Belleville, ON) using a piezo drill and beveled injection pipette. After removal of the embryo, the droplet containing the biopsied cells was moved into an Eppendorf tube and centrifuged. Supernatant was removed leaving ~5 μl sample, which was snap frozen for later genetic testing. Fifteen biopsied embryos were immediately transferred nonsurgically into uteri of synchronized recipients. Day 16 pregnancy rate for embryos ≤ 300 μm was 75.0% (6 of 8; 175 – 240 μm), which was not significantly different from control embryos of the same size (77.3%; 17 of 22). For embryos > 300 μm, day 16 pregnancy rate was 28.6% (2 of 7; 320 and 400 μm), which was not significantly different from control embryos of the same size (62.5%; 10 of 16).

Additionally, 22 embryos (150 - 440 μm) were vitrified by standard procedures after biopsying and later warmed and transferred directly. No embryos > 300 μm (n = 3) became

pregnancies after vitrification. Day 16 pregnancy rate for ≤ 300 μm was 47.4% (9 of 19; 150 – 225 μm), which was significantly different ($p < 0.05$) from direct transfer and control embryos of the same size (75.0% and 77.3%, respectively). Three of these pregnancies (150 - 200 μm) resulted in the formation of empty trophoblastic vesicles by 25 d. All pregnancies were terminated on or after 25 d to collect embryos for further genetic testing.

For preimplantation genetic testing, a duplex nested polymerase chain reaction (PCR) was developed for amplification of the DNA from the biopsied cells using primers for sex chromosome-linked zinc finger protein genes (*ZFx*/*ZFy*; 445 bp), and 2 pairs of primers for equine-specific sex-determining region on the Y-chromosome (*SRY*; 217 bp, 121 bp). Experiments on XX and XY genomic DNA from white blood cells revealed accurate genetic testing on as little as ~9 pg DNA, which equals ~1 cell. Sex determination on biopsied material occurred for 30% of samples, one of which was confirmed from a placental sample. Low PGD results indicate either lack of sensitivity of the test, or more likely the loss of cells during the steps of transfering the biopsied cells to Eppendorf tubes. We concluded that biopsy collection, preimplantation genetic diagnosis, and direct transfer can be performed on equine embryos without compromising pregnancy rates when performed on embryos ≤ 300 μm. Vitrification lowered pregnancy rates of biopsied embryos ($p < 0.05$). Continued effort in improving genetic tests and in vitrifying equine embryos, especially those > 300 μm, is warranted.

Erika L Cullingford
Department of Biomedical Sciences
Colorado State University
Fort Collins, CO 80523
Spring 2010

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CHAPTER ONE

REVIEW OF LITERATURE

Introduction

Preimplantation genetic diagnosis (PGD), the ability to determine an embryo's genotype before implantation, has been performed on many species, including mice and humans. However, successful PGD of equine embryos has been limited, as the numbers available for testing and costs of obtaining embryos and procedures are problematic compared to many other species. Nevertheless, by applying biotechnological techniques already performed routinely on equine embryos, PGD can be developed for this species, as well. Effective PGD would save owners and breeders not only the costs associated with unwanted pregnancies, but also could increase the percentage of desirable offspring. Genetic disorders such as Hyperkalemic Periodic Paralysis (HYPP) or Severe Combined Immunodeficiency Disease (SCID) could be eradicated without removing heterozygous carriers from the breeding stock. Desirable traits could also be determined, such as sex or color, thus increasing the value of the offspring before birth.

Biopsy Techniques

With the primary goal of retrieving a DNA sample to test, the biopsy technique must not compromise the health of the embryo. Several techniques are outlined below that were developed primarily in human, bovine, and murine models.

Zona Pellucida Drilling

The oocyte and early embryo are protected by a glycoprotein structure known as the zona pellucida. All biopsy techniques require opening the zona pellucida before genetic material can be removed. Zona drilling can be performed by three methods: chemical, mechanical, and laser-assisted.

Chemical Drilling

Chemical zona pellucida drilling is performed using acidic Tyrode's solution (Inzunza et al., 1998). Application to the zona pellucida is performed by loading a micropipette with the solution and gently expelling the solution onto a single location of the zona pellucida until a hole is created. The resulting acidification of the culture medium requires that the embryo be washed

after the procedure or that the medium be replaced (Inzunza et al., 1998; De Vos and Van Steirteghem, 2001). This technique is rarely used because there is increased blastomere lysis (Inzunza et al., 1998) and embryo viability is compromised (Kim et al., 2008).

Mechanical Drilling

Mechanical zona drilling is performed using either a microblade to cut through the zona or a micropipette to pierce the zona. Direct puncture can be used to either directly aspirate cells (Krzyminska et al., 1990) or to displace precompaction cells by a second puncture used to gently inject medium into the embryo until cells are extruded (Tarin and Handyside, 1993). Partial zona dissection can be performed using a microblade or fine needle to make longitudinal slices in the zona pellucida. Based on methods developed for zona removal and to assist sperm penetration (Malter and Cohen, 1989), dissection is not as elegant as piercing the zona, which allows penetration and cellular removal to be performed with the same micropipette.

Laser-Assisted Drilling

Laser-assisted drilling requires additional equipment but can make more predictable holes than those obtained by acidic Tyrode's solution (Joris et al., 2003). Additionally, using a laser rather than Tyrode's solution allows the biopsy to be done without changing the culture dish or micropipettes. The laser beam is emitted at the edge of the zona, away from the embryonic cells, to create a hole using various intensities and durations.

Tadir et al. (1989 & 1990) were the first to use a solid-state laser on sperm in IVF, while the first report of oocyte/embryo micromanipulation with a laser was by Palanker et al. (1991). These lasers, however, are considered risky because of the potential mutagenic effects of the ultraviolet (UV) radiation (Kochevar, 1989; Rasmussen et al., 1989). Infrared lasers are now routinely used for a variety of IVF procedures including assisted hatching, spermatozoa immobilization, assisted fertilization, and biopsy collection (Ebner et al., 2005). In addition to zona drilling, a laser can be used for blastocyst biopsy by using the laser to dissociate

trophectoderm cells that have herniated through a hole in the zona (Veiga et al., 1997; Kokkali et al., 2007).

Genetic Material Retrieval

After zona penetration, the biopsy sample can be taken, generally using a micropipette to aspirate the genetic material. Distinctions between various procedures are dependent on developmental stage, and are outlined below.

Polar Body Biopsy

During the process of oogenesis, two polar bodies are extruded by the maturing fertilized oocyte before the male and female pronuclei form. The first polar body is the result of meiotic resumption of an oocyte formerly arrested in prophase I (Schatten and Constantinescu, 2007). Meiotic resumption occurs due to the preovulatory luteinizing hormone (LH) surge, which also induces ovulation. The oocyte is in a duplicated diploid (4N) state prior to this surge. The first polar body contains one duplicated homologous chromosome from each pair and is extruded following the LH surge. Both the polar body and the oocyte contain half of the genetic material, in a duplicated haploid state. The oocyte remains arrested at metaphase II until fertilization or degeneration.

Activation occurs at fertilization, where meiosis II is completed by the introduction of phospholipase C-zeta from the sperm causing an intracellular rise of Ca^{2+}. After the second round of meiosis induced by fertilization, where the sister chromatids are separated, the embryo extrudes the second haploid polar body. The diploid embryo is formed by the haploid female pronucleus joining with the haploid male pronucleus.

After fertilization, two polar bodies are available for biopsying by aspirating with a fine glass micropipette. The major advantage of this technique is that both polar bodies consist of genetic material that will have no biological role in the development of the future fetus. Therefore their removal does not alter the viability of the embryo, as was demonstrated in a mouse model by Kaplan et al. (1995) and Montag et al. (1998). The polar bodies, however, are

only comprised of the maternal genome (Verlinksy et al., 1990; De Vos and Van Steirteghem, 2001). In order to evaluate the paternal contribution or the combination of maternal and paternal alleles, it is necessary to biopsy the embryo at later developmental stages.

Blastomere Biopsy

Cells of the early dividing embryo can be collected to evaluate the entire genome. Human (Tarin et al., 1992) and mouse (Krzyminska et al, 1990; Somers et al., 1990) studies have shown that removing blastomeres at the 2- and 4-cell stage reduces the proportion of cells in the inner cell mass (ICM), and thus are not good stages for biopsying. Biopsying at the 8-cell stage, however, does not compromise embryo viability, as all the cells are still totipotent. Also, retrieval of genetic material at that stage is still relatively easy, as compaction has not yet occurred in most species. To perform the biopsy at the 8-cell stage, a holding pipette is used to fix the embryo in place while a fine glass micropipette is used to aspirate 1 or 2 blastomeres through the hole in the zona pellucida for genetic testing. Cleavage stage biopsies can be performed in addition to polar body biopsy without affecting pregnancy rates (Magli et al., 2004). The most important disadvantage of biopsying a single cell is that allele drop-out – the random non-amplification of one of the alleles of a heterozygous sample during genetic testing– can occur, giving an incorrect diagnosis (Findlay et al., 1995). Although infrequent, this is particularly relevant for autosomal dominant disorders, where allele drop-out of the affected allele can result in misdiagnosis and therefore transfer of an affected embryo.

Blastocyst Biopsy

Embryos further along in development have undergone compaction and cell adhesion, and thus biopsying runs the risk of removal of genetic material destined to become the fetus. The advantage of these stages, however, is that relatively more genetic material can be collected.

The morula stage is theoretically inappropriate for biopsying, as the cells that will form the ICM, the cell line destined to become the fetus, are not easy to differentiate because of extensive compaction. Biopsying at this stage was detrimental in mouse embryos, reducing

implantation rate, fetal viability, and the mean fetal weight (Krzyminska et al., 1990). However, this may not apply to all species (Williams et al., 1984; Skidmore et al., 1989).

The blastocyst stage allows trophoblast cells to be removed without damaging the ICM. Human IVF clinics primarily perform blastocyst stage transfers as higher pregnancy rates are achieved compared to the transfer of earlier stages (de Boer et al., 2004; Papanikolaou et al., 2005). The success of later stage transfers likely is due to embryo self-selection at each developmental stage as some degenerate while the viable ones progress. Although cleavage-stage embryo biopsies are the most prevalent in human clinics (Goossens et al., 2009), there are an increasing percentage of blastocyst stage embryo biopsies. The most common method in recent years is laser-assisted biopsy, where cells are allowed to herniate through a hole in the zona produced by a laser before the cells are removed (Kokkali et al., 2007). Murine (Monk et al., 1988) and bovine (Hasler et al., 2002) embryos also have been biopsied at the blastocyst stage using different techniques, such as the aforementioned aspiration technique or by microblade, which involves slicing a portion of cells off the embryo using a sharp micropipette or microblade.

A major problem with biopsying blastocysts is the limited time for genetic analysis, as embryos typically must be transferred shortly after the biopsy is taken. However, increased accuracy of genetic predictions due to the increase in cell numbers outweighs this downfall. Also, the proportion of cells removed is far smaller in blastocyst (about 5 cells removed from 100) versus blastomere (1 cell removed from 8) biopsies.

Equine Embryo Transfer Techniques

The techniques described for early cleavage-stage embryos are not applicable to the majority of equine embryos as *in vitro* fertilization has had little success (Bezard, 1989; Dell'Aquila, et al., 1999; McPartlin et al., 2009). Although intracytoplasmic sperm injection (ICSI) can be performed on equine oocytes (Squires et al., 1996; Grondahl et al., 1997; Choi et al., 2002), thereby allowing both polar body and blastomere removal, the most common assisted reproduction technology in the horse, other than artificial insemination (AI), is collection and

transfer of morula or blastocyst stage embryos. Currently, most horse breeds accept the registration of foals produced by embryo transfer (ET). While ET remains an expensive procedure, it has been estimated that over 26,000 equine embryos were transferred in 2008, mostly in Argentina, Brazil, and the United States (Squires et al., 1999; Thibier, 2009).

Management of donor mares for ET begins with breeding, either by live cover or AI, generally 1 or 2 days (d) prior to ovulation. Ovaries are monitored daily via palpation or ultrasonography per rectum until ovulation is detected. The day of ovulation designates the start of the cycle (d = 0). Embryo recovery is performed nonsurgically 6 to 8 d post-ovulation. A catheter is placed through the mare's cervix, into the caudal uterine body, and a balloon cuff is inflated. Flush medium is infused throughout the entire uterus, and then recovered through a cup-filter. The cup contents are examined microscopically for presence of an embryo. Embryo recovery rates are dependent on many factors including ovulation rate, donor mare age and reproductive status, skills of the practitioner, and semen quality (Squires et al., 1999).

The day of recovery generally dictates the size and stage of the embryo. The equine embryo reaches the uterus 144 to 156 h after ovulation in the late morula or early blastocyst stage of development (Battut et al., 1998; Stout et al., 2005). Therefore, attempts at embryo recovery from the uterus should not normally occur until 6.5 d or later. Early embryo (6.5 - 7 d) recovery procedures are pivotal for cryopreservation, as larger embryos (> 300 μm) have a poor prognosis for survival after vitrification (Carnevale, 2006) or conventional cryopreservation (Takeda et al., 1984; Seidel et al., 1989; Hochi et al., 1994).

Recovered embryos are measured and evaluated for morphology (McKinnon and Squires, 1988), as these parameters can affect pregnancy rates (Carnevale et al., 2000). The most influential factor for sustained pregnancy is embryo quality. Embryos are graded with a quality score of 1 to 4: 1 = excellent, no morphological abnormalities; 2 = good, minor imperfections; 3 = fair, distinct abnormalities; and 4 = degenerate, dead, or unfertilized ova. Morphologic features that affect this score include: compactness of blastomeres, color of embryo, size of

perivitelline space, embryo shape, and damage to the zona pellucida. Stage of development relative to embryo age can also affect this score. A quality score of 1 or 2 is considered normal, as 12 d pregnancy rates were higher (68.5% and 65.1%, respectively) than those for embryos graded 3 or 4 (40.4% combined; Carnevale et al., 2000).

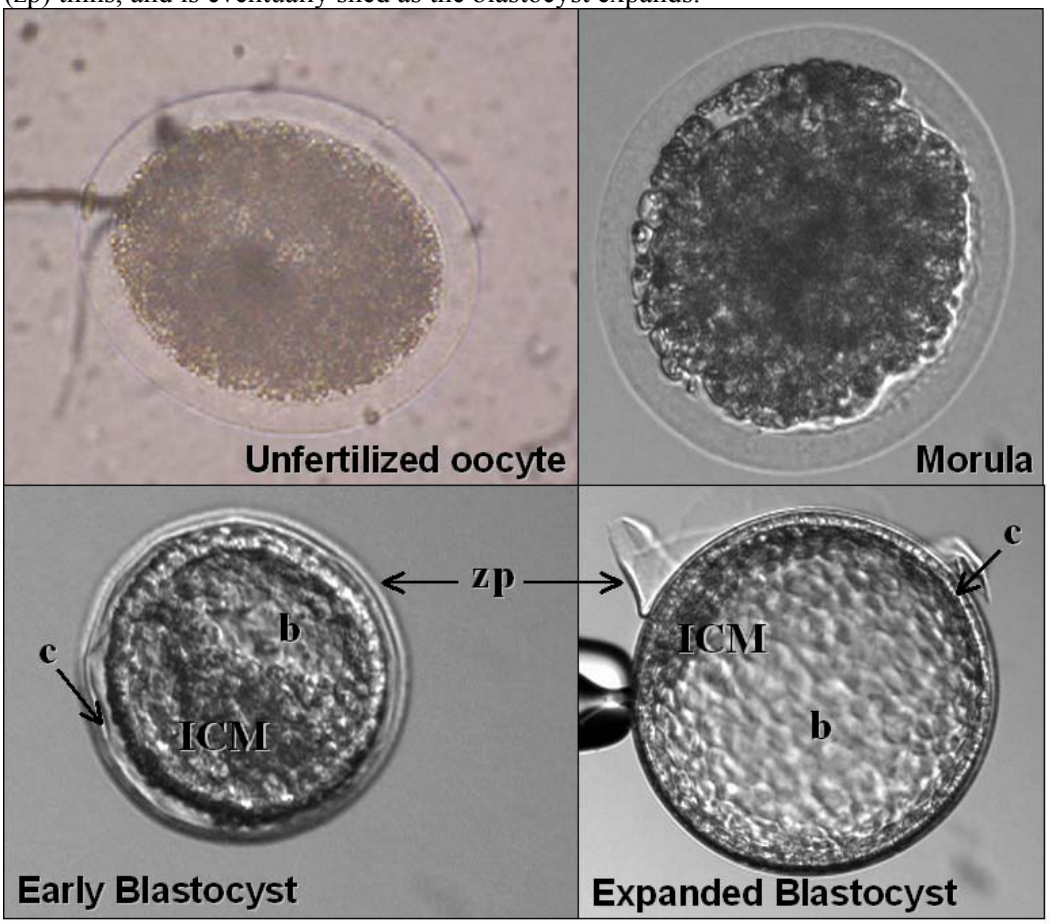
At the inception of equine ET procedures, embryos were deposited into the tip of the uterine horn by surgical flank incision (Imel et al., 1981). Since then, nonsurgical embryo transfer techniques have been developed, in which the embryo is deposited into the uterus transcervically by use of an embryo transfer gun or insemination pipette.

Recipient quality is one of the most important determinants of success of an ET program. Quality is determined by several factors, including reproductive history and status, but also, perhaps most importantly, correct management of the recipient's estrous cycle. Recipients should ovulate 1 d prior to 2 d after the donor's ovulation date (Squires et al., 1995). Pregnancy rates are highest when embryos are transferred into recipient mares that ovulate 1 to 2 d after the donor (Carnevale et al., 2000).

Early Equine Embryo Development

As in most species, fertilization in the horse occurs in the ampullary portion of the oviduct. Following fertilization, the embryo goes through regular cell divisions within the oviduct approximately every 24 h to reach the 2-cell stage within 24 h, 4 to 6-cells by 48 h, and 8 to 10-cells after 72 h (Bezard et al., 1989). Between 4 to 5 d a morula is formed as the yet undifferentiated cells compact. By late day 5 or early day 6, a central cavity, the blastocoele, begins to form as fluid is produced by blastomeres and the cells begin to differentiate into trophectoderm and ICM cells. At or around the same time, the embryo finally reaches the uterus (Battut et al., 1998). (Fig 1)

Fig 1. Characteristics of equine embryos. The single-cell, unfertilized oocyte (picture courtesy of Elaine Carnevale DVM, PhD), which rarely reaches the uterus, looks similar to the multi-cell morula. An embryo progresses to the blastocyst stage when a blastocoele develops (b). At this stage, the inner cell mass (ICM) can be differentiated. Around the same time the capsule (c) develops. As the embryo grows, the zona pellucida (zp) thins, and is eventually shed as the blastocyst expands.



The embryo does not increase in diameter from the oocyte to early blastocyst stage (7 d), remaining about 150 μm in diameter. The mare is unique in that unfertilized oocytes remain in the oviduct (Betteridge and Mitchell, 1974). Their appearance in embryo flushes is normally associated with co-transport to the uterus with a fertilized embryo (Wilson et al., 1985). An unfertilized oocyte can be similar in initial appearance to a morula stage embryo. The zona pellucida is thick and both are round or slightly oval in shape. However, the degenerated unfertilized oocyte consists of only one cell and is usually flat. A morula is spherical in shape and consists of many blastomeres resulting in the classic "scalloped" edge appearance.

With blastocoele development (6.5 d), the embryo begins to rapidly grow in diameter. During this time the zona pellucida thins and eventually is shed (Carnevale, 2006). The spherical embryo expands in diameter from 150 μm at 6 d to 2.5 - 2.8 cm by 21 d (Betteridge et al., 1982).

Just after blastocoele development, the capsule, an acellular glycoprotein matrix, begins to form between the trophectoderm and the zona pellucida. The capsule remains until 23 d (Oriol et al., 1993). Equine embryos produced *in vitro* do not form a complete capsule (Tremoleda et al., 2003). However, embryos produced *in vitro* and then transferred into the uteri of recipient mares can develop a capsule *in vivo* (McKinnon et al., 1989). Although the function of the capsule is still unknown, it may be involved in maternal recognition of pregnancy and the migration of the embryo throughout the uterus (Oriol et al., 1993; Stout et al., 2005). The formation of a functional capsule appears to be necessary for a successful pregnancy (Tremoleda et al., 2003; Stout et al., 2005).

The equine embryo is unlike ruminant or swine embryos, which exhibit rapid elongation of the blastocyst (starting at 11 or 12 d) whereas the equine embryo remains spherical. It has been proposed that the capsule maintains the spherical shape of the conceptus after zona loss (Stout et al, 2005). Another important characteristic of the equine embryo is that it migrates throughout the uterus from when it enters at 5 or 6 d until 17 d when the embryo's position becomes fixed (Ginther, 1983). Embryo migration is essential to facilitate maternal recognition of pregnancy (McDowell et al., 1985).

Transrectal ultrasonography can be used to identify the embryonic vesicle as early as 10 – 12 d after ovulation and the embryo proper at approximately 20 d (Ginther, 1986). The embryonic heartbeat is generally detectable by 24 or 25 d (Ginther, 1986). Although fixation occurs around 17 d, when the embryo ceases its migration and plants itself at the base of one of the uterine horns, implantation does not occur until 35 d. At this stage, a few trophoblast cells begin to invade the maternal endometrium to stimulate formation of the endometrial cups, which secrete chorionic gonadotrophin (eCG; Allen and Stewart, 2001). Endometrial cups are usually

present from 35 d to 100-120 d when they slough off. They reach their maximum productivity at 70 d of pregnancy (Allen and Stewart, 2001). Pregnancy loss after 35 d is associated with retention of the cups and continued eCG production. Consequently, affected mares will not exhibit normal estrous cycles, preventing the mares from being re-bred or used as a recipient mare for the remainder of the season (Daels and Hughes, 2005).

Embryo Cryopreservation

Embryo cryopreservation is a significant roadblock to success in assisted reproductive technologies in the horse. Pregnancy rates after the transfer of cryopreserved embryos are just over 50% when using small embryos (< 300 μm) and are significantly lower after the transfer of larger embryos (Hochi et al., 1995 & 1996; Eldridge-Panuska et al., 2006; Hudson et al., 2006; Carnevale, 2006). Vitrification, a shorter procedure that does not require special equipment, is the most practical cryopreservation method for practitioners, and is starting to replace conventional cryopreservation. Ice crystal formation is prevented by using a high concentration of permeating cryoprotectants and rapid cooling. The success of vitrification is dependent on embryo size, as there is still no repeatable method of cryopreservation of large, expanded equine embryos (> 300 μm). It has been proposed that the chief barrier to success is the capsule, which likely impedes transfer of cryoprotectant into the blastocoele. The larger volume of the blastocoele at this stage is another possible issue. A recent report by Choi et al. (2010) suggests higher pregnancy rates can be achieved by collapsing the blastocoele cavity before vitrification. They reported 5 of 7 pregnancies (71%) at 14 d after warming and transferring large (> 300 μm) equine embryos.

Because of the relative ease and established pregnancy rates from vitrification, vitrification solutions (VS) are available commercially or can be produced using a base medium of modified phosphate-buffered saline (mPBS) without calcium and magnesium and supplemented with 20% fetal calf serum (Table 1).

Table 1. Vitrification protocol and solutions (Eldridge-Panuska et al., 2006). Vitrification solutions (VS) are presented in order of use. The base medium is modified phosphate-buffered saline (mPBS) without calcium and magnesium and supplemented with 20% fetal calf serum.

Solution	Glycerol	Ethylene Glycol	Galactose	Duration of exposure	Load in straw
VS1	1.36 M			5 min	
VS2	1.36 M	3.58 M		5 min	
VS3	3.26 M	4.65 M		45-60 sec	30 µl column in center
Diluent (D)			0.5 M		2 columns of 90 µl at ends

Vitrified embryos can be transferred directly from the straw following warming (thawing). Warming involves holding the straw in air for 10 sec and then submerging it in a water bath at 20º to 22º C for an additional 10 sec (Hudson et al., 2006). Contents of the straw should be well mixed to dilute the concentrated cryoprotectants and draw them out of the embryo before transfer. The embryo can then be transferred transcervically by the same method as fresh embryos.

Superovulation

Superovulation in horses is relatively inefficient (Squires et al., 1999; Cullingford et al., 2010) compared to other species. As a monovular species (Ginther, 1992), superovulation in horses might be considered successful with 3 – 4 ovulations per cycle and 2 or more embryos recovered per flush (Squires and McCue, 2006). Gonadotropin releasing hormone (GnRH), porcine follicle stimulating hormone (pFSH), purified equine FSH (eFSH), equine pituitary extract (EPE), inhibin immunoneutralization, and recombinant equine FSH (reFSH) all have been used to attempt to superstimulate mares with varied results.

Partially-purified EPE and purified pituitary eFSH have been the most successful agents for superovulation of the mare (Squires and McCue, 2006; Scoggin et al., 2002), but are impractical to obtain since the closing of equine slaughterhouses in the United States. The use of reFSH (produced *in vitro* by Chinese hamster ovary (CHO) cells) for superovulation was also

promising (Deluca et al., 2008; Jennings et al., 2009), but the product is no longer commercially available.

The standard method to superovulate mares involves treatment twice daily for 4 or 5 d to stimulate multiple follicles to grow (McCue et al., 2007). The treatment phase sometimes is followed by a coasting period, without treatment for 42 h (Welsh et al., 2006), at which point an ovulation induction agent is administered, and insemination follows. Niswender et al. (2003) reported more success using human chorionic gonadotropin (hCG) than a gonadotropin-releasing hormone (GnRH) agonist (Deslorelin) for inducing ovulation in mares superstimulated with eFSH.

Research is ongoing to strategize new improvements to superovulation protocols. These have included using a follicular suppression model (Jennings et al., 2009), or techniques used to superovulate cattle such as a decreasing dosage, step-down approach (Scoggin et al., 2002; McCue et al., 2007) or low dose protocol (Araujo et al., 2009). Recently, an increase in the number of daily injections has been proposed to mimic the natural hormonal profile of FSH. Verver et al. (2009) found that four times a day injections of 0.5 mg reFSH resulted in significantly higher ovulation rate (4.0 ± 3.3 ovulations per cycle) and embryo recovery rate (1.8 ± 2.5 embryos per flush) (mean ± S.D.) in comparison to controls (1.11 ± 06 ovulations per cycle and .66 ± .11 embryos per flush).

The incidence of ovulation failure increases in superovulated mares (Cullingford et al., 2010). This phenomenon has been noted in several studies using different superstimulating agents (Roser et al., 2008; Deluca et al., 2008, Verver et al., 2009; Cullingford et al., 2010). Another proposed inhibitor to superovulation success is the anatomical configuration of the mare's ovary (Squires and McCue, 2006). It is believed that because only a small portion of the ovary able to accommodate ovulation (the ovulation fossa), superovulation is anatomically constrained. Also attributed to the anatomical configuration is the ovulation failure and decreased

embryo recovery per ovulation that occurs in mares with greater than 5 preovulatory follicles (Squires and McCue, 2006).

Without an excess of embryos from superovulation, few equine embryos are cryopreserved yearly (379 in 2008; Thibier, 2009). Lacking the ability to increase embryo yield and with limited ability to cryopreserve equine embryos, PGD could be important for genetic advancement. Its incorporation into ET practices could decrease generation intervals while increasing the number of genetically superior animals.

Evaluation of Biopsied Material

A draft of the equine genome sequence was first deposited into public databases in 2007 (Perrocheau et al., 2006; Wade et al., 2009). Sequencing began in 2006 as a combined effort of the Equine Genome Project on DNA of a mare known as Twilight, from Cornell University. The Y chromosome, the male genetic sex-determining chromosome, was mapped by Raudsepp et al. (2004a) and is the only domesticated animal species for which a detailed map is available.

With the knowledge these sources have provided and various PGD techniques, new genetic tests for the horse can be developed. Below is an outline of the most common methods of PDG, as performed in other species, and how they can be adapted for horses.

Karyotyping

Chromosome analysis by karyotyping has been used for genetic testing of individuals for decades. This involves staining the chromatin of cells arrested in metaphase (the stage of mitosis when the chromosomes are condensed and highly coiled, and therefore visible under a high-resolution microscope). The chromosomes can be stained by several techniques, but the most common is C-, G-, R-banding, to identify the segmentation of chromosomes. The standard equine karyotype has 31 pairs of autosomal chromosomes and a pair of sex chromosomes (Richer at al., 1990). Chromosomes are identified by their banding pattern and size. The equine sex chromosomes are very similar in size to other autosomal chromosomes, making genetic sex diagnosis difficult by this means.

The history and reasons for cytogenetic analysis in horses has been covered in depth by Lear and Bailey (2008). Cytogenetic analysis is generally unreasonable for single cells because of the inefficiency of achieving a metaphase spread. For example, a study with bovine embryos only achieved a metaphase spread two-thirds of the time (Hare et al, 1976). Biopsies were taken on day 14 or 15 from the ends of embryos, which had elongated up to 80 mm. While biopsying the elongated bovine embryo can provide significantly more cells than from a spherical embryo, sex-determination rates were inadequate.

A new method for karyotyping developed by Verlinsky et al. (2002) involves nuclear transfer of a single blastomere into an enucleated mouse zygote. By following the division of the resulting heterokaryons, the cells could be fixed upon the entry of mitosis. A higher percentage of metaphase spreads can be achieved (88% in that study). However, this technique is impractical for most situations.

Fluorescence In-Situ Hybridization

Fluorescence in-situ hybridization (FISH) relies on identifying a unique DNA sequence that takes up a large section of a chromosome. By identifying these areas, unique fluorescent DNA probes can be developed to bind to the sequences of interest. The procedure for FISH is similar to that of karyotyping: the biopsied cells are fixed to a slide, and the nucleus is isolated for analysis (Baart et al., 2004). This technique is commonly used to detect aneuploidy, structural chromosomal abnormalities, or for sex-selection (Harper and Delhanty, 1996). It is inadequate, however, for detection of single-nucleotide polymorphisms (SNPs) or mutations that involve smaller portions of DNA.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) mimics the natural DNA replication machinery to amplify DNA of interest to detectable levels. This technique involves development of primers, short (19 - 27 nucleotide) single-stranded oligonucleotides, that can hybridize to a targeted strand of DNA and act as a “docking site” for the DNA polymerase. The DNA polymerase or

replication enzyme attaches to the end of this primed sequence and begins adding the appropriate deoxyribonucleosides triphosphates (dNTPs) that pair to the original DNA sequence going in a 3' to 5' direction. Replication ends by reaching the 5' terminus, dictated by either the polymerase detaching (run-off) or reaching a second primer. (Mullis and Faloona, 1987)

The PCR reaction usually involves three-steps repeated up to 30 or 40 times, cycling between different temperatures. Each step of replication is temperature dependent, starting with a denaturation temperature (94 - 96º C), where the double-stranded DNA (dsDNA) separates, allowing for primer binding. Next is the primer hybridization temperature (~55 - 65º C) that results in a "primed" DNA sequence. The final step is extension, when the temperature (~74º C) is held to allow the polymerase to replicate the entire strand of interest. The primer hybridization and extension steps can be combined for a two-step reaction. The series of temperatures is repeated with each round being referred to as a cycle. A thermal cycler is used to heat and cool the reaction tubes to the designated temperature of each step. Amplification of the DNA of interest relies on the dsDNA product of a previous cycle becoming the reagent for the next cycle, resulting in an exponential increase in the DNA sequence of interest. For example, if starting with 2 copies of DNA, by the second cycle there are 4 copies, for the third there are 8, and by the fourth there are 16, so that after many cycles there is a detectable number of copies. (Mullis and Faloona, 1987)

A PCR reaction involves several reagents: a DNA template of interest; primers to identify the target DNA; DNA polymerase or taq polymerase to drive the reaction; dNTPs (A, T, C, G) for DNA extension; and a buffer solution that will provide a stable and optimum environment for the polymerase, which includes Mg^{2+} and K^{+}. The DNA sequence of interest is generally 100-1000 bp in length. (Mullis and Faloona, 1987)

Identification of the resulting product can be performed by several approaches. Typical identification relies on the charged backbone of DNA allowing the product to be separated by electrophoresis, typically through agarose or polyacrylamide (higher resolution, up to 1 bp

difference). By running a charge through the gel in buffer, DNA products migrate through the agarose matrix at a speed dependent on the size of the fragment. Long strands move more slowly than short strands. The strength of the charge will also dictate how fast the DNA products will pass through the matrix. A standard ladder of different DNA lengths is run through the gel at the same time to identify lengths of amplified products by comparison with known sizes from the ladder. Multiple regions of template DNA can be amplified in the same PCR reaction, so long as the primers and products do not anneal to each other. By having products of different sizes, electrophoresis separates them into different bands. (Barlett, 2003)

DNA of the same length will move at the same rate through the matrix; thus, if enough DNA of interest is present, the fragments will be visible as bands on the gel. To see the bands, dyes are used to stain the DNA by intercalating into the DNA sequence. The most common dye used is ethidium bromide, which can be visualized under ultraviolet light. The intensity of the bands can be used to estimate DNA concentration. (Barlett, 2003)

The use of PCR relies on a knowledge of the sequence of interest. Primers must be developed that will only anneal to that sequence and not on other regions of the genome. With most of the equine genome sequenced, researchers usually can identify regions of interest and design primers to anneal to these segments for PCR analysis. Much work still needs to be done to sequence genetic abnormalities. Currently 9 monogenic disorders have been sequenced and can be tested for in the horse (American Association of Equine Practitioners, May 2009):

Autosomal Dominant

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

Autosomal Recessive

4. Overo Lethal White Syndrome in Paint Horses
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 Asthenia (HERDA) in Quarter Horse-related breeds

Genetic tests are also available for many different coat colors. While coat color does not affect quality of life (except perhaps grey, which has a predisposition for melanomas), coat color tests help owners guarantee the color of offspring by having a homozygous parent. Currently there are genetic tests for the mutations for: Extension (Red Factor), Agouti, Cream Dilution, Pearl Dilution, Champagne Dilution, Silver Dilution, Gray, Sabino 1, Tobiano and Lethal White Overo. A complete reference for coat color patterns, genes, and testing can be found at http://www.vgl.ucdavis.edu/services/coatcolorhorse.php.

Whole genome amplification (WGA) can be used for scarce amounts of DNA to generate a new sample with a higher concentration of DNA. Various methods to WGA are covered in depth by Spits and Sermon (2009), but the ideal WGA technique would amplify cellular DNA up to the microgram level while maintaining fidelity and homogenous replication of the original sequence. While unnecessary for singular genetic tests, incorporating WGA into a PGD program enables the practitioner to do several genetic tests over long periods of time, rather than just a single round. The amplified products may also be useful in the future for use with microarray or SNP chips which require higher quantities of DNA than PCR alone.

Because of its specificity and ease of use, PCR usually is the most appropriate diagnostic tool for PGD. It certainly is used extensively in scientific research and diagnosis. A historical perspective on PCR by Bartlett and Stirling (2003) can be found in PCR Protocols.

Additional Notes

Illumina, Inc (San Diego, CA) is the first company to offer a SNP array in the form of EquineSNP50 Genotyping BeadChip. Using the publicly available genome map, Illumina developed a chip with 54,602 somewhat evenly distributed SNPs that can provide a genome-wide

genotyping array. This powerful tool can be used to assay for a specific trait in affected and unaffected animals, allowing genome-wide association analysis to map that trait. Many more equine diseases and traits will soon be understood at the molecular level so that genetic tests can become available.

The primary obstacle for genetic testing in the horse is the lack of governing bodies to approve tests and offer quality control for testing laboratories (Bannasch, 2008). Although some mutations and their appropriate tests are published in peer-reviewed journals, some tests are offered without supporting scientific publication. Frequently this is due to patent-protection, where only licensed laboratories may perform the tests. The implications of patent-infringement on the future of genetic testing are not completely known, but is recognized as detrimental to innovation and increasing clinical knowledge (Klein, 2007).

Sex Specific Genes

Although testing for hereditary diseases is important, the primary trait of interest to owners and practitioners is genetic sex. In horses, evaluation of fetal gonads for sex determination by ultrasonography is best performed between 59 to 68 d of pregnancy (Curran, 1992). At this point, sex selection is not practical because the presence of endometrial cups will prevent the mare from cycling even if the pregnancy is terminated (Daels and Hughes, 2005). Preimplantation diagnosis for sex selection can be achieved by looking for the presence or absence of genetic sex- specific genes, specifically those on the Y chromosome.

Compared to the X chromosome, which is highly conserved across species (Charlesworth, 1991; Raudsepp et al., 2004b), Y chromosomes have undergone extensive degeneration, duplication, translocation, inversion and loss of gene content (Graves, 1998; Skaletsky et al., 2003). Consequently, comparative mapping between species is rarely useful.

One exception is the zinc finger genes from the X (ZFx) and pseudoautosomal region of the Y (ZFy) chromosome (region capable of recombining with the X chromosome). Poloumeinko (2004) showed by cloning and comparative analysis that the bovine, porcine, and

equine ZFx and ZFy genes show a high degree of conservation. Zinc fingers are small protein structural motifs that coordinate one or more zinc ions to help stabilize their folds. They are one of the most prevalent protein motifs in mammalian cells, repeated throughout the genome (Krishna et al., 2003). Though the functions of ZFx and ZFy remain unknown, they have been proposed as probable transcriptional activators (Schneider-Gaedicke, et al., 1989). The first equine embryo preimplantation sex diagnosis study looked at restriction enzyme sites of the sex chromosome ZF genes to identify an enzyme that could distinguish between the sexes. After looking at 38 different restriction enzymes, only *Bsm I* yielded sex-specific banding patterns by gel electrophoresis (Pieppo et al., 1995).

Another possible PGD marker is the sex-determining region of the Y chromosome (SRY), the primary determinant of phenotypic sex. It was first located in humans in 1990 by Sinclair et al. The equine SRY transcript was sequenced by Hasegawa et al. (1999) and localized on the Y chromosome using FISH by Hirota et al. (2001). Many cases of sex reversal have examined this gene as a possible culprit, by translocation or mutation (Kent et al., 1996).

Both ZFy and SRY are present on the Y chromosome in one copy, and therefore single cell PCR identification is difficult. Repeated sequences, such as mammalian testis specific protein, Y-encoded (TSPY), or RNA binding motif Y (RBMY) could greatly enhance the sensitivity of the test. Copy numbers of TSPY range from 20 to 60 in men and up to 200 in bulls, and have been suggested for equine (Manz et al., 1998) and bovine (Lemos et al., 2005) preimplantation sex diagnosis.

Biopsying Equine Embryos

The groundwork has been laid for equine PGD as equine embryo micromanipulation is well researched. In the 1980's, the splitting of equine embryos garnered much attention, as it could provide genetically identical twins for research. In 1984 monozyotic twins were born after the separation of blastomeres at the 4-cell stage (Allen and Pashen, 1984). In 1989, day 6 embryos were spit using a microblade or micromanipulator and then both halves were transferred

into the same recipient. These "demi-embryos" resulted in lower ($p < 0.05$) 11 d pregnancy rates (5 of 22) than the controls (16 of 22; McKinnon et al., 1989). That same year Skidmore et al. (1989) announced that a satisfactory pregnancy rate (67%) could be achieved by splitting morulae, but not from splitting blastocysts. They speculated that inadequate capsule development prevented the blastocyst demi-embryos from becoming pregnancies.

Interest in equine embryo biopsying began with Huhtinen et al. (1997), who published the first report of biopsied embryo pregnancies. This group started with micropipette penetration and aspiration, but found the technique too cumbersome due to the difficulty in penetrating the capsule. They went on to use a microblade, followed by 2 h of incubation before transferring embryos nonsurgically into recipient uteri. Pregnancy rates were 6 of 8 for the control group and 3 of 14 for the microblade group. Sample sizes ranged from 10 - 30 blastomeres. Pregnancy rates following biopsy were not considered high enough to allow the procedure to be used in a commercial program.

The most recent studies returned to the micropipette aspiration technique (Choi et al., 2009 & 2010). Choi et al. (2009) made two or three biopsy manipulations per embryo. This group did not achieve pregnancies with biopsied-vitrified embryos. However, 3 of 3 embryos that were biopsied and transferred directly without vitrification resulted in pregnancies (25 d). In their 2010 abstract, the same group used the biopsy process to collapse the blastocoele of larger equine embryos. Using this technique followed by vitrification, they achieved a 71% pregnancy rate at 14 d after warming and transferring of large equine embryos.

While there is much interest in equine embryo biopsy for PGD, development trails behind that for other species. Embryo biopsy is yet to be integrated into commercial practice, although its incorporation with currently available tests for monogenetic traits or future molecular techniques could be extremely valuable.

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CHAPTER TWO

PREIMPLANTATION GENETIC DIAGNOSIS OF EQUINE EMBRYOS

Introduction

Preimplantation genetic diagnosis (PGD) of embryos could be a highly advantageous technique to incorporate into embryo transfer programs. The most common procedure for recovery of equine embryos is a transcervical uterine lavage performed 6.5 to 8 d after ovulation of the donor mare (Squires et al., 1999). Previous reports have indicated that only early embryos (6.5 – 7 d, which result in morulae or early blastocysts) have a good prognosis to survive the biopsy procedure (Huhtinen et al., 1997; Choi et al., 2009) and vitrification (Hochi et al., 1995 & 1996; Eldridge-Panuska et al., 2005; Hudson et al., 2006; Carnevale, 2006). Thus, PGD techniques must be fine-tuned for these stages.

Polymerase chain reaction (PCR) provides a rapid means of genetic testing, and is already in use for an array of monogenetic disorders and coat color tests for the horse. Vitrification of biopsied embryos would allow sufficient time to perform genetic testing and to decide which embryos to transfer.

The objective of this study was to develop a procedure for biopsying equine embryos for PGD that would not compromise pregnancy rates. Additionally, vitrification was incorporated to provide logistical flexibility. Genetic sex diagnosis was performed by PCR as proof of principle that genetic testing of the biopsied material was possible. We hypothesized that direct transfer of biopsied embryos would result in similar pregnancy rates to contemporary commercial controls. We also hypothesized that pregnancy rates would be lower for biopsied then vitrified embryos compared to biopsied-nonvitrified and untreated control embryos.

Materials & Methods

Embryo Collection

A total of 37 embryos were collected for this study; 25 embryos were collected from non-stimulated cycles while 12 embryos were recovered from mares superovulated with recombinant equine follicle stimulating homormone (0.25 mg 4x/d or 0.5 mg 2x/d of reFSH, AspenBio

Pharma, Inc., Castle Rock, CO; Verver et al., 2009). Estrous cycles were monitored using transrectal ultrasonography from May to August, 2009. Mares were administered 2,500 IU hCG (Chorulon®, Intervet/Schering-Plough, Millsboro, DE) i.v. or 1.5 mg deslorelin acetate (Franck's Pharmacy, Ocala, FL) i.m. to induce ovulation once a follicle > 35 mm in diameter was observed. Mares were inseminated once daily with 500 x 10^6 progressively motile fresh spermatozoa beginning on the day of hCG or deslorelin administration until ovulation was detected (day 0 = ovulation). Embryos were recovered by transcervical uterine lavage on 6.5 or 7 d using a commercial flush medium (ViGRO Complete Flush Solution, Bioniche, Belleville, ON or Emcare® Complete Flushing Solution, ICPbio Ltd., Auckland, New Zealand). Embryos were identified, washed through 4 - 6 droplets of commercial holding medium, and evaluated for size, grade, and developmental stage (McKinnon and Squires, 1988; Carnevale et al., 2000) prior to biopsy collection.

Embryo Biopsy Technique

All 37 embryos were biopsied by the following technique. Trophoblast biopsies were collected in a 30 µl droplet of Syngro® Holding Medium (Bioniche) using a piezo drill and beveled injection pipette (7 - 10 µm outer diameter) attached to a Narishige micromanipulator. Biopsy technique was similar to that described by Choi et al. (2009). The micropipette was used to puncture the zona pellucida and capsule, opposite from the ICM in blastocyst-stage embryos, and to aspirate trophoblast cells adjacent to the site of entry through the zona. The piezo device was used occasionally to aid in penetrating the zona and capsule, as well as to separate cells. Biopsy samples ranged from 4 to 10 cells by visual assessment and were expelled from the micropipette back into the droplet for visualization. The biopsied embryo was then transferred to a new holding dish containing Syngro® Holding Medium, leaving the biopsied cells in the original 30 µl droplet of media. The droplet was aspirated with a pipette and moved into an Eppendorf tube which was centrifuged at 11,000 x g for 10 min. The supernatant was removed leaving approximately 5 to 10 µl, which was stored at -80º C for later genetic testing.

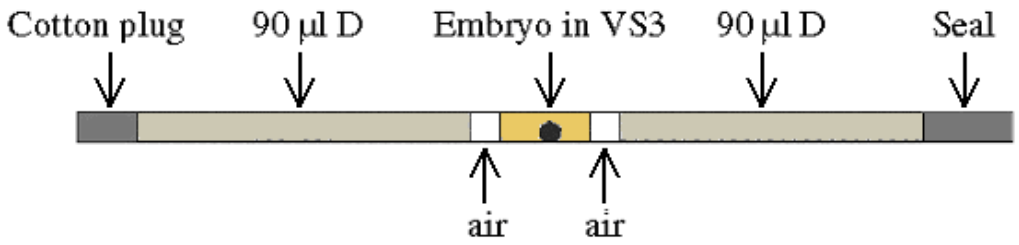
Embryo Vitrification

Twenty-two biopsied embryos were vitrified according to the protocol of Eldridge-Panuska et al., 2005 (Table 1 and Fig 2.) with one variation. The final step of the vitrification procedure, the cooling process, was either performed by suspension of the 0.25 ml straw in liquid nitrogen-cooledair or by a novel technique using an aluminum block immersed in liquid nitrogen (Kruse and Seidel, 2010). The block, containing a hole precisely the diameter of the straw, was nearly submerged in liquid nitrogen at least 10 min prior to use to achieve temperature equilibrium. After confirming the hole in the block was free of liquid nitrogen and condensation, the straw containing the embryo was inserted directly into the block. The straw remained in the block for at least 1 to 2 min before transfer into liquid nitrogen for storage. Ten embryos were vitrified using the aluminum block while 13 were vitrified by the traditional technique.

Table 1. Vitrification protocol and solutions. Vitrification solutions (VS) are presented in order of use. The base medium is modified phosphate-buffered saline (mPBS) without calcium and magnesium and supplemented with 20% fetal calf serum.

Solution	Glycerol	Ethylene Glycol	Galactose	Duration of exposure	Load in straw
VS1	1.36 M			5 min	
VS2	1.36 M	3.58 M		5 min	
VS3	3.26 M	4.65 M		45-60 sec	30 µl column in center
Diluent (D)			0.5 M		2 columns of 90 µl at ends

Fig 2. Diagram of vitrification straw. Straw is loaded with 90 µl D, then an air bubble, followed by 30 µl of VS3 containing the embryo, another air bubble, and finally 90 µl D before being plugged or sealed. The straw is cooled by liquid nitrogen cooled-air or in the aluminum block beginning 45 – 60 sec after placing the embryo in VS3.



Embryo Transfer & Pregnancy Examination

Embryos were transferred from June to September 2009 by standard nonsurgical technique (Carnevale et al., 2000) into recipient mares. Recipient reproductive tracts were evaluated on day 5 for uterine and cervical tone, corpus luteum (CL) morphology and the presence of uterine edema, cysts, fluid or air. All recipients were classified as having a well-defined CL and good to excellent uterine and cervical tone. For the embryo transfer procedure, recipients were administered 200 mg xylazine (Tranquived, Vedco Inc., Saint Joseph, MO) or 20 mg acepromazine (Vedco Inc., Saint Joseph, MO) and 500 mg flunixin meglumine (Banamine®-S, Intervet/Schering-Plough, Millsboro, DE) i.v. Recipients were administered 12 ml (2.2 mg/ml) altrenogest (Regu-mate®, Intervet/Schering-Plough Animal Health, Millsboro, DE) P.O. daily for the duration of pregnancy. Mares were housed in dry lots and provided free-choice alfalfa hay.

Vitrified embryos were warmed by holding the straw in room temperature air for 10 sec followed by 10 sec in a 20 - 22° C water bath. The warmed straw was then flicked vigorously several times to combine the fluid columns. After 5 min, contents of each straw were observed under a microscope to evaluate survival of the embryo after vitrification. Warmed embryos were transferred directly from the vitrification straw within 7 to 15 min of warming. Fresh embryos were drawn into a straw between columns of air and medium to assure expulsion of the embryo during transfer.

Embryos were transferred as singletons into a recipient mare synchronized to ovulate 0 to 2 days after the donor. Each transfer was performed by loading the straw containing the embryo onto a Cassou gun (IMV Technologies, Maple Grove, MN) and depositing the embryo transcervically into the uterus of the recipient mare.

Pregnancy examinations were performed via ultrasonography on days 11, 12, 14, 16, 20, and 25 (embryo age). Mares were considered to be not pregnant if no embryonic vesicle was observed by 16 d. All pregnancies were terminated on or after 25 d to collect embryos for further genetic testing.

Polymerase Chain Reaction

For preimplantation genetic diagnosis of sex, a duplex nested PCR was developed for amplification of the DNA from the biopsied cells using primers for sex chromosome-linked zinc finger protein genes (*ZFx*/*ZFy*; 445 bp), and two pairs of primers for the equine-specific sex-determining region on the Y-chromosome (*SRY*; 217 bp, 121 bp). (Table 2) Specificity of the primers was confirmed during a blinded study of 18 blood samples collected equally from mares and stallions. The sex of all samples was correctly identified. Experiments on diluted samples of XX and XY genomic DNA from white blood cells revealed accurate genetic testing on as little as 9 pg DNA, which is the equivalent of ~1 cell (~3N, in a rapidly dividing embryo). PCR cycle conditions are presented in Table 3. Biopsy samples were put through 3 freeze-thaw cycles (- 80º to 60º C) to lyse cells. No further DNA extraction technique was used.

Table 2. Primer sequences used for preimplantation sex determination PCR.

Name	Sequence
ZFx/ZFy-F	ATAATCACATGGAGAGCCACAAGCT
ZFx/ZFy-R	GCACTTCTTTGGTATCTGAGAAAGT
SRY-F1	ACAAACGGGAGGAGCGGTTA
SRY-R1	CAGGGACTCTGAAGCCACCA
SRY-F2	CCATTCGGGTAACGTTGGCTA
SRY-R2	CAGGGACTCTGAAGCCACCA

Table 3. Cycle conditions for PCR. Highlighted sections are repeated each cycle. For first set of primers, 10 cycles were performed. With the nested primers, 40 cycles were performed.

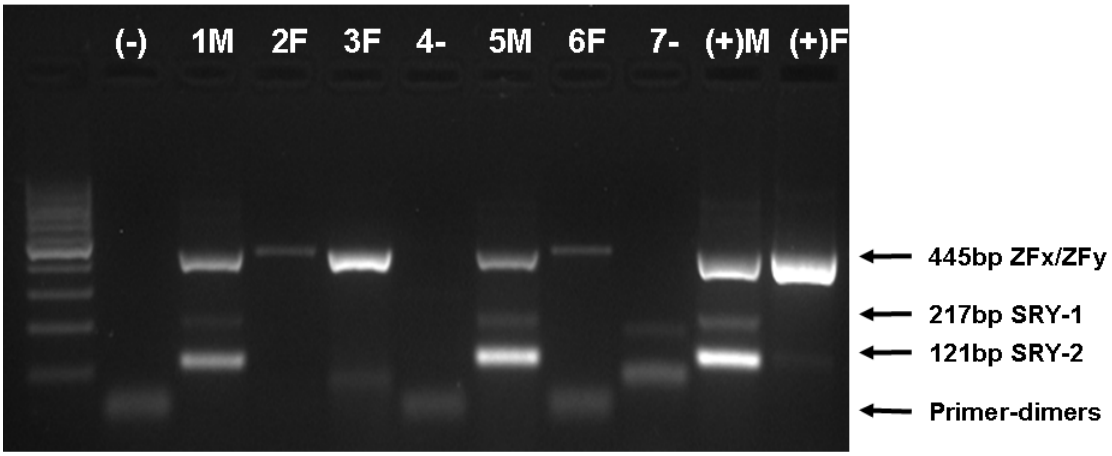
Step	Temp	Time (min:sec)
Initial Dissociation	95º	2:00
Dissociation	95º	00:45
Primer Annealling	62º	00:45
Extension	72º	1:00
Final Extension	72º	9:00
Hold	4º	Hold

The PCR reaction was carried out in a final volume of 25 µl using GoTaq® DNA Polymerase (Promega, Madison, WI) with a halved version of the GoTaq® standard procedure. The composition of the PCR mixture was as follows: 5 µl Green GoTaq® reaction buffer (5X), 2

µl of ZFx/ZFy primers (forward and reverse) at 10 mM, 2 µl of appropriate SRY pair of primers at 10 mM , 0.5 µl of dNTP mix at 10 mM each, 0.12 µl GoTaq® polymerase (5 µ/µl), 13 µl water, and 2 µl template (biopsy sample). Water was adjusted if template volume changed. PCR reactions were run on 5 or more samples at a time from a master mix. The 10 cycles was performed with the SRY-1 set of primers. This was used as the template for a second reaction (2 µl from original reaction), using the embedded pair of SRY-2 (2 µl at 10 mM) primers, for 40 cycles.

Following thermocycling, PCR products were loaded on a 2% agarose gel containing ethidium bromide and electrophoresed for 2 h at 60 V or 1 h at 110 V. Results were visualized and photographed using UV light transillumination. Presence of a ZFx/ZFy band confirmed DNA in the sample. Presence or absence of a SRY band determined genetic sex (Fig 3).

Fig 3. Representative gel electrophoresis results with controls in parentheses. The ZFx/ZFy is present in both male (M) and female (F) samples while the SRY bands indicates presence of a Y chromosome. No results were present for lanes 4 and 7. Notice the SRY-1 band is lighter than SRY-2, as it was only amplified for ~10 cycles. A DNA ladder of 100 - 1000 bp is in the left lane.



Cell Line Establishment and Maintenance

The media used in these experiments were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Of the 16 pregnancies, twelve 25+ d embryos were recovered from pregnant recipient mares and used to generate cells lines for further genetic testing.

The embryos were collected in sterile saline, separated from the placental tissue, placed in 1.5 ml of ATV trypsin (1X in PBS) for 30 min and gently vortexed. Cells were then rinsed with 30 ml PBS and centrifuged at 100 x g for 5 min twice. Pelleted cells were resuspended in 6 ml Dulbecco's Modified Eagle Meadium (DMEM) + 10% FBS (Gemini Bio-Products, West Sacramento, CA) + penicillin G, streptomycin sulfate, gentamicin sulfate and amphotericin B (to a final concentration of 100 units/ml, 50 μg/ml, 50 μg/ml, 2.5 μg/ml respectively) in two wells of a six-well plate, and incubated in 5% CO_2 for up to 2 d. To replate the cells, wells were washed with PBS before adding 0.2 ml of ATV for 10 min. Each well was then resuspended in 16 ml of the DMEM solution and plated in a 10 cm tissue culture dish. Incubation took place for up to 4 d, until the cells became confluent.

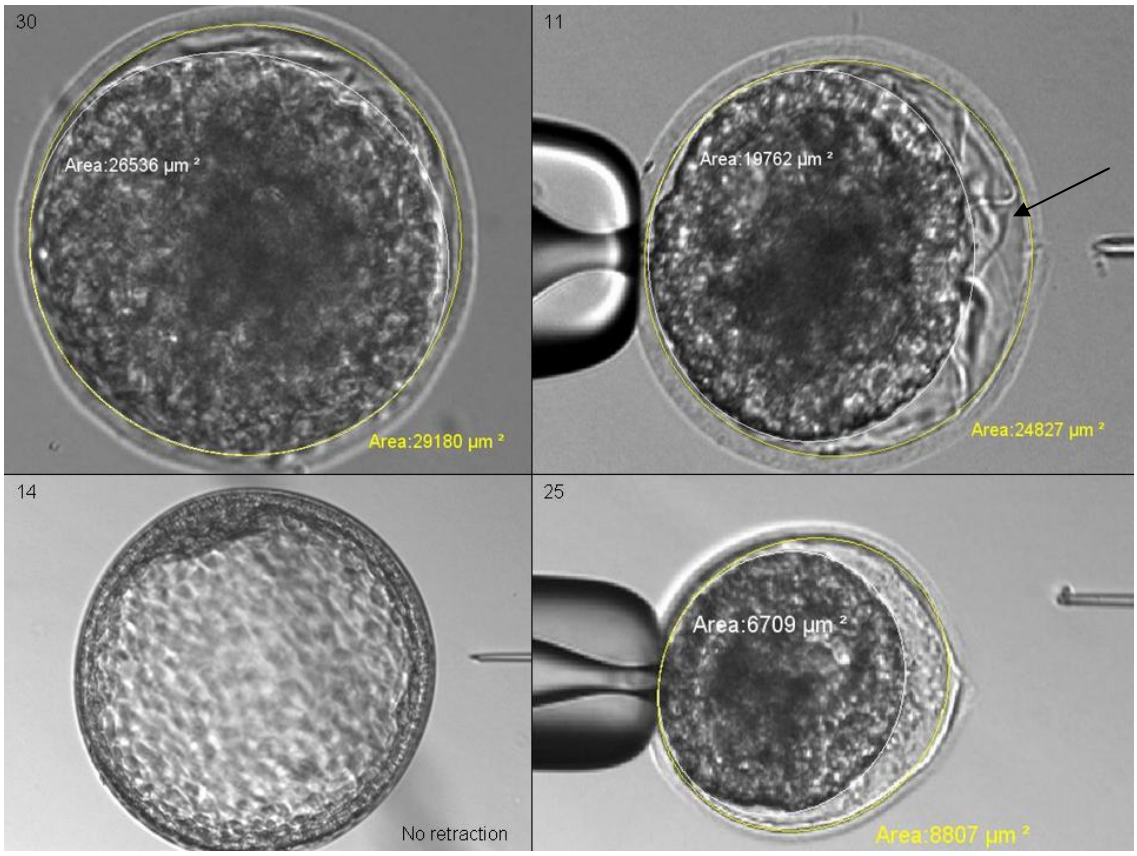
To slow cool the cell lines for cryopreservation, plates were rinsed with PBS before adding 0.4 ml ATV for 10 min. Cells were resuspended in 4 ml DMEM + 10% FBS and the plates were placed on ice for 20 min. Chilled dimethyl sulfoxide (DMSO) was then added as 1 ml DMEM + 400 μl DMSO to bring the final DMSO concentration in the plates up to 8%. Cells were slow cooled at -80°C in eight 1.2 ml cryogenic vials (Corning, Corning, NY) overnight, and then transferred into liquid nitrogen for storage. A portion of each sample was submitted for PCR evaluation of sex. The remainder was stored for later genetic testing. Empty trophoblastic vesicles were not created into cell lines, as they were mostly dead by the time of collection. Rather, these were immediately submitted for genetic evaluation by PCR.

Statistical Analysis

Control data for non-biopsied, direct transfer embryos were from embryos of similar size (150 – 600 μm) collected from donor mares in the clinical embryo transfer program during the same season. Embryo collection methods were the same as described above, without superovulation. Pregnancy data were compared using Fisher's Exact Test. One-way analysis of variance was used to compare embryo sizes and morphologies. Retraction percentages were generated using Spot Imaging Software (Diagnostic Instruments, Inc, Sterling Heights, MI) to

measure embryo area versus inner zona pellucida diameter from photographs taken within 2 min of biopsy (Fig 4). Retraction percentages were calculated as (inner zona area – embryo area)/inner zona area x 100%. Regression equations were generated using Minitab (Minitab Inc, State College, PA).

Fig 4. Examples of retraction. Notice that in many cases the capsule is disturbed (arrow). Photos were taken within 2 min of biopsy. Ellipse option was used in Spot Imaging Software with calibration set at 1 pixel = 0.5 µm.



Results

No significant difference ($p > 0.1$) was found between the aluminum block (5 of 10 16 d pregnancies) and cooled air (4 of 13) methods to vitrify embryos; therefore, results were pooled into one biopsied-vitrified group. A detailed breakdown of embryo sizes can be found in Table 4. Group sizes were too small to compare; therefore, embryos were compared as ≤ 300 µm or > 300 µm, the sizes normally compared during embryo vitrification.

Table 4. Summary embryo diameters by group.

Embryo diameter	Biopsy only	Biopsy-Vitrify (LN_2 cooled-air)	Biopsy-Vitrify (Aluminum)
< 200 µm	3	5	3
200 - 250 µm	5	4	4
251 - 300 µm	1	1	1
≥ 300 µm	6	2	2
Total	15	12	10

Biopsy of embryos ≤ 300 µm transferred directly did not affect ($p > 0.05$) the 16 d pregnancy rate compared to contemporary controls (75.0 vs 77.3%; Table 5). Transfer of biopsied-vitrified embryos ≤ 300 µm resulted in a decreased ($p < 0.05$) pregnancy rate (47.4%) compared to contemporary controls and the direct transfer group. Biopsied embryos > 300 µm did not have satisfactory pregnancy rates whether directly transferred (28.6%) or vitrified and then transferred (0%). (Table 5)

Table 5. Summary of day 16 pregnancy rates. Embryos are broken down by size (small vs large) and treatment.

	Small Embryos (≤ 300 µm)				Large Embryos (> 300 µm)		
Treatment	**Pregnant**	**Total**	**Preg Rate**		**Pregnant**	**Total**	**Preg Rate**
Unbiopsied Controls	**17**	**22**	**77.3%a**		**10**	**16**	**62.5%a**
Biopsied-Direct Transfer	**6**	**8**	**75.0%a**		**2***	**7**	**29.6%ab**
Biopsied-Vitrified	**9º**	**19**	**47.4%b**		**0**	**3**	**0.0%b**

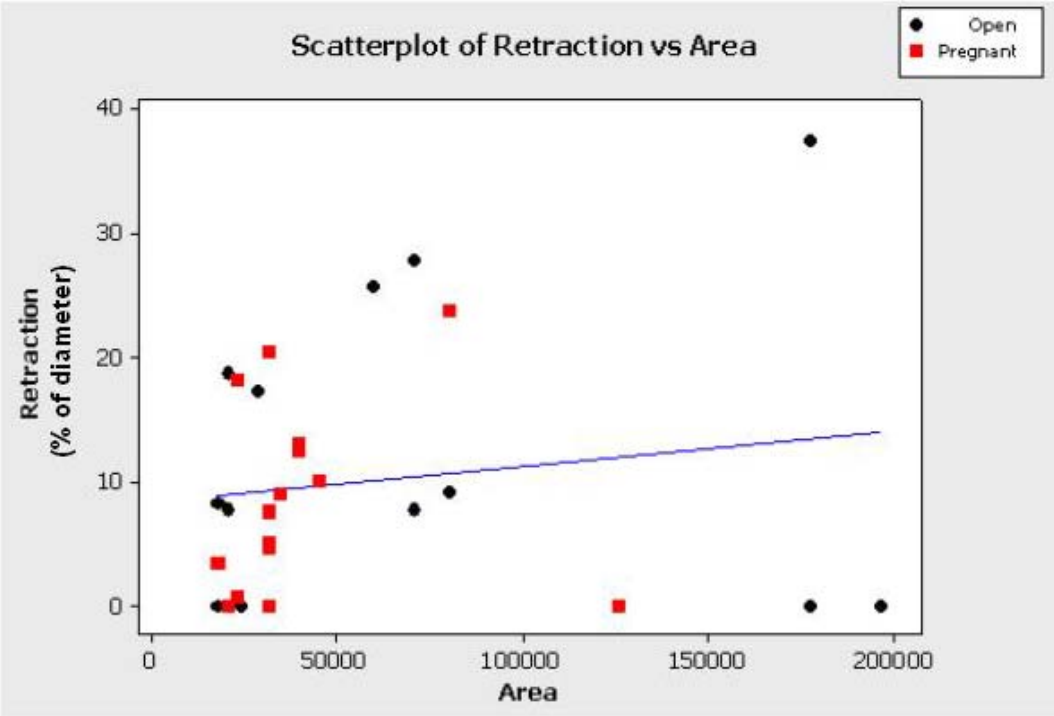
ab ($p < 0.05$), 1 tail Fisher's Exact Test.
* Pregnancies were 320 and 400 µm in diameter.
º Three pregnancies became empty trophoblastic vesicles by 25 d.

One direct transfer pregnancy (160 µm) was lost between 16 – 20 d. Three embryos (150 – 200 µm) vitrified in the aluminum block appeared as normal pregnancies until 25 d when no heartbeat was detected. By 27 d they were reported as empty trophoblastic vesicles (ETV).

The percent collapse of an embryo after biopsying (retraction percentage) ranged from 0 to 37.4%, for the 30 embryos for which clear, measurable post-biopsying photographs were available. Percent retraction was not correlated with embryo area (Fig 5). One-way analysis of variance showed that percent retraction was similar ($p > 0.1$) for embryos that did or did not result in a pregnancy in both the direct transfer and vitrified groups. Furthermore, retraction was

unrelated ($p > 0.1$) to pregnancy outcome whether grouped as >10% vs. <10% or >15% vs. <15% retraction.

Fig 5. This figure illustrates the lack of correlation between retraction percentage and embryo area. The regression equation generated was Percent Retraction = 8.42 + 0.000028 * Area, but was not different ($p > 0.1$) from zero.



Stage of development (morula vs. blastocysts) did not affect ($p > 0.1$) the embryo's chances of becoming a viable pregnancy. Additional embryo and pregnancy data are in Tables 6 and 7.

Genetic sex detection by PCR occurred in 30% (11/37) of biopsy samples. No DNA was detected for the other 26 samples. Of the samples with a detectable ZFx/ZFy band, 55% (6/11) were male and 45% (5/11) were female (Table 6). However, only one of these samples coincided wit available cell lines from aborted embryos. Analysis of the aborted embryos collected from 25+ d pregnancies resulted in presumptive sex diagnosis of all samples, with 64% (9/14) male and 35% (5/14) female.

Table 6. Summary embryo data and PGD results from biopsy samples and placental tissue from aborted pregnancies. If no result is reported, genetic test can back negative.

#	Flush Date	Morph *	Grade	† Size	Group	Flush Day	Transfer Date	Recip Synchr	25d ‡ vesicle	day of § HB	day of ‖ abort	Retraction Percentage	Biopsy result	Placental result
1	6/7/09	Blast	1	390	Direct	7	6/7/09	-1	-					
2	6/25/09	Blast	2	160	Direct	6	6/25/09	-1	-¶			18.7	Male	
3	6/25/09	Blast	1	240	Direct	6	6/25/09	-1	+	25	28	10		Male
4	7/8/09	Morula	2	210	Direct	7	7/8/09	-1	-					
5	7/8/09	Blast	1	210	Direct	6	7/8/09	0	+	25	27	9		
6	7/10/09	Blast	1	575	Direct	7	7/10/09	-1	-				Female	
7	7/13/09	Blast	1	200	Direct	7	7/13/09	-2	+	25	29	0		Male
8	7/22/09	Blast	1	190	Direct	7	7/22/09	-1	-			17.4	Male	
9	7/25/09	Blast	1	175	Direct	6	7/25/09	-1	+	25	29			Male
10	7/28/09	Blast	2	225	Direct	6	7/28/09	-1	+	25	26	12.5	Female	Female
11	8/10/09	Blast	1	320	Direct	7	8/10/09	-2	+	27	28	23.8		Male
12	8/16/09	Blast	1	500	Direct	7	8/16/09	-2				0		
13	8/16/09	Blast	1	475	Direct	7	8/16/09	-1				0	Female	
14	8/25/09	Blast	1	400	Direct	7	8/25/09	-2	+	25	28	0		Male
15	8/26/09	Blast	1	475	Direct	7	8/26/09	-2	-			37.4	Male	
16	7/10/09	Blast	1	200	Aluminum	7	8/1/09	-2	+	ETV	30	7.6		
17	7/28/09	Blast	1	200	Aluminum	6	8/4/09	-1	+	25	26			Female
18	7/28/09	Blast	1	200	Aluminum	6	8/3/09	-1	-			4.8	Male	
19	8/16/09	Blast	1	300	Aluminum	6	8/30/09	-1	-					
20	8/16/09	Blast	1	375	Aluminum	7	8/30/09	-2	-			27.9	Male	
21	8/22/09	Morula	1	150	Aluminum	6	9/1/09	-1	-			0		
22	8/25/09	Blast	1	225	Aluminum	7	9/1/09	-2	+	25	28	13.1		Female
23	8/25/09	Morula	1	150	Aluminum	7	9/1/09	-2	+	ETV	28	3.5		Male
24	8/28/09	Blast	1	160	Aluminum	6	9/2/09	-1	+	ETV	29	0		Male
25	8/30/09	Blast	2	275	Aluminum	7	9/4/09	-2	-			25.7		
26	7/23/09	Blast	1	200	Vitrified	6	7/31/09	-1	+	25	26	20.4		Female
27	7/24/09	Morula	1	150	Vitrified	6	8/2/09	-1	-			8.3		
28	7/28/09	Blast	2	160	Vitrified	6	8/5/09	-1	-			7.8	Female	
29	7/29/09	Blast	1	300	Vitrified	6	8/7/09	-1	-			7.8		
30	7/29/09	Blast	1	200	Vitrified	6	8/21/09	-1	-			0		
31	8/9/09	Blast	1	440	Vitrified	7	8/21/09	-2	-					
32	8/10/09	Blast	1	270	Vitrified	6	8/22/09	-1				9.1	Male	
33	8/12/09	Blast	1	240	Vitrified	7	8/23/09	-2	-					
34	8/13/09	Blast	1	200	Vitrified	6	8/25/09	-1	+	26	27	5.2		Female
35	8/26/09	Morula	1	170	Vitrified	7	9/1/09	-2	+	25	28	18.3		Male
36	8/27/09	Blast	1	170	Vitrified	6	9/3/09	-1	+	25	27	0.8		Male
37	8/30/09	Morula	1	175	Vitrified	6	9/4/09	-1	-			0		

* Morphologies are given as either blastocyst (blast) or morula.
† Size of embryo is measured as diameter in μm.
‡ Presences or absence of a 25 d vesicle is indicated with + (pregnant) or – (not pregnant).
§ Indicates day that heartbeat was detected in pregnancies. Empty trophoblastic vesicles (ETV) were noted at 27 d once no heartbeat had been detected.
‖ Day pregnancies were aborted for further genetic testing.
¶ Pregnancy was present at 16 d, but not at 25 d.

Table 7. Summary of pregnancy data as detected by ultrasonography. Sizes are given as diameter of embryonic vesicle in mm and +/- indicates presence or absence of embryonic vesicle. Mares were considered not pregnant if no embryonic vesicle was seen by 16 d.

#	Flush Date	Group	Transfer Date	size of 11d (mm)	size of 12d (mm)	size of 14d (mm)	size of 16d (mm)	day of HB
1	6/7/09	Direct	6/7/09	-	-	-	-	-
2	6/25/09	Direct	6/25/09	-	-	-	11	-
3	6/25/09	Direct	6/25/09	8	11	20	30	25
4	7/8/09	Direct	7/8/09	-	-	-	-	-
5	7/8/09	Direct	7/8/09	6	8	19	22	25
6	7/10/09	Direct	7/10/09	-	-	-	-	-
7	7/13/09	Direct	7/13/09	-	6	10	20	25
8	7/22/09	Direct	7/22/09	-	-	-	-	-
9	7/25/09	Direct	7/25/09	5	10	18	22	25
10	7/28/09	Direct	7/28/09	4	10	18	21	25
11	8/10/09	Direct	8/10/09	3	8	11	34	27
12	8/16/09	Direct	8/16/09	-	-	-	-	-
13	8/16/09	Direct	8/16/09	-	-	-	-	-
14	8/25/09	Direct	8/25/09	5	9.5	18	26	25
15	8/26/09	Direct	8/26/09	-	-	-	-	-
16	7/10/09	Aluminum	8/1/09	4	6	13	20	ETV
17	7/28/09	Aluminum	8/4/09	-	3	6	22	25
18	7/28/09	Aluminum	8/3/09	-	-	-	-	-
19	8/16/09	Aluminum	8/30/09	-	-	-	-	-
20	8/16/09	Aluminum	8/30/09	-	-	-	-	-
21	8/22/09	Aluminum	9/1/09	-	-	-	-	-
22	8/25/09	Aluminum	9/1/09	2	7	15	24	25
23	8/25/09	Aluminum	9/1/09	-	-	10	21	ETV
24	8/28/09	Aluminum	9/2/09	-	4	-	8	ETV
25	8/30/09	Aluminum	9/4/09	-	-	-	-	-
26	7/23/09	Vitrified	7/31/09	5	6	8	22	25
27	7/24/09	Vitrified	8/2/09	-	-	-	-	-
28	7/28/09	Vitrified	8/5/09	-	-	-	-	-
29	7/29/09	Vitrified	8/7/09	-	-	-	-	-
30	7/29/09	Vitrified	8/21/09	-	-	-	-	-
31	8/9/09	Vitrified	8/21/09	-	-	-	-	-
32	8/10/09	Vitrified	8/22/09	-	-	-	-	-
33	8/12/09	Vitrified	8/23/09	-	-	-	-	-
34	8/13/09	Vitrified	8/25/09	-	8	18	28	26
35	8/26/09	Vitrified	9/1/09	-	5	10	21	25
36	8/27/09	Vitrified	9/3/09	6	9	19	24	25
37	8/30/09	Vitrified	9/4/09	-	-	-	-	-

Discussion

Based on the successful pregnancy rates of the small biopsied direct transfer group (75.0%), embryo biopsy could be incorporated into commercial practice. This would involve consideration of two important factors. First, embryos ≤ 300 µm would need to be consistently recovered. This can be achieved by closely monitoring time of ovulation and flushing 6.5 d later (Squires et al., 1995). Timed flushing can also be performed with the use of an ovulation induction agent to estimate time of ovulation (Carnevale, 2006). The second important factor is the timing of biopsy and the transfer of the embryo in relation to receiving PGD results. Genetic testing may take several hours or days to perform. Unless the PGD equipment and expertise are available on-site, the biopsied embryo either needs to be transferred before the results arrive or

the embryo could be cryopreserved and results obtained prior to transfer. The former option is currently the most viable, as biopsied-vitrified embryos did not have an acceptable pregnancy rate (47.4% for small embryos). However, an owner must decide if pregnancy termination is an option if undesirable results are received.

Capsule damage is the most likely cause of pregnancy loss with the large biopsied equine embryos. The formation of a functional capsule appears to be necessary for a successful pregnancy (McKinnon et al., 1989; Tremoleda et al., 2003; Stout et al., 2005). McKinnon et al. (1989) first noted that the only embryos that developed into pregnancies after capsule disruption were those that had an intact capsule when recovered later. Similarly, Stout et al. (2005) reported no pregnancies after complete removal of the capsule by micromanipulation.

Capsule development begins with the onset of blastulation (6.5 d), and ceases around 18 d (Oriol et al., 1993). The micromanipulations made in this study were performed around the same time as the onset of capsule development. The larger embryos (> 300 μm), having been flushed on day 7 or later, already had significant capsule development. We could clearly visualize the capsule in many of the expanded blastocysts, and noted that it was harder to penetrate and frequently stretched (Fig. 4) by the biopsy procedure. It is possible that biopsying at later stages damages the capsule beyond its ability to repair and reform. The results from Skidmore et al. (1989) agreed with this assessment, because bisected morulae were capable of becoming pregnancies while bisected blastocysts were not.

Huhtinen et al. (1997) performed the first successful equine embryo biopsies. They first attempted an aspiration technique similar to that used in the current study, but found it difficult to penetrate the capsule (we bypassed this problem with the piezo drill and beveled micropipettes). By using a microblade instead, 6 of 8 pregnancies were achieved in the control group and 3 of 14 in the microblade biopsy group. Successful PCR amplification was achieved for all samples, likely because 10 to 30 blastomeres were reported to be removed during each biopsy.

The first biopsied embryo pregnancies achieved using a piezo drill were performed by Choi et al. (2009). They reported 3 of 3 pregnancies for 6 d biopsied embryos transferred into recipient mares. All embryos had heartbeats at 25 d. Biopsy micromanipulations were performed 2 or 3 times to retrieve an adequate number of cells. However, Choi et al. reported no pregnancies following transfer of 5 *in vivo*- and 5 *in vitro*-produced blastocysts that were biopsied and then vitrified. No PGD results were reported.

To our knowledge, our study is the first to report pregnancies resulting from biopsied-vitrified equine embryos. The pregnancy rate for small biopsied-vitrified embryos (47.4%) is lower than published pregnancy rates for vitrified embryos. Eldridge-Panuska et al. (2005) used the same vitrification procedure as the current study and reported a 16 d pregnancy rate of 62%. Hudson et al. (2006) reported a slightly higher pregnancy rate of 70%. Conventional cryopreservation also achieves similar pregnancy rates. For example Hochi et al. (1996) reported a 16 d pregnancy rate of 63.6% using a cryopreservation solution of ethylene glycol + sucrose and direct transfer.

Pregnancies in the current study were carried out until 25 d in order to find a heartbeat while the previous studies reported 16 d pregnancies. The highest rate of embryonic loss detectable by ultrasonography occurs between days 17 and 25. At this stage 6% of embryos are lost, which represents 38.5% of all embryonic loss (Carnevale et al., 2000). Indeed we lost 4 embryos during this time period, one embryonic loss in the direct transfer group and three ETV developed in the biopsied-vitrified group.

Similar empty vesicles appear in 4.4% of conventional equine pregnancies (Vanderwall et al., 2000), and though their incidence was not statistically significant in the current study, it could be clinically relevant. It is unknown whether vitrification in the aluminum block contributed to the ETV formation; further research is necessary to determine if there is excess ETV development with the biopsy-vitrification process.

Retraction of the embryo from the zona pellucida post-biopsying was measured because Choi et al. (2010) suggested that collapsing the blastocoele before vitrification increases the embryo's chance of survival. They used blastocysts > 300 μm and collapsed the blastocoele using a biopsy procedure before vitrifying by a novel method developed by Sun et al. (2008) for ferret embryos. Results of the current study showed no relationship between retraction and the embryo's survival after vitrification. However, the retraction was an artifact of the biopsy procedure rather than the aim, and further work is necessary to determine if increased retraction percentages performed intentionally during the biopsy procedure would increase the embryo's chance of survival after vitrification.

The novel vitrification method used by Sun et al. (2008) involved the use of the tip of an Eppendorf microloader (Eppendorf, catalog no. 5242-956.003) as the embryo chamber after cutting it to a diameter of 0.25 mm, which provides a constant and uniform wall thickness of 0.03 mm. Vitrification was performed using two steps of ethylene glycol + dimethyl sulfoxide (DMSO) in increasing concentrations, and warming using 3 step-down concentrations of sucrose. Two studies on equine embryos have been performed using similar solution concentrations in open pulled straws (OPS) (Oberstein et al., 2001; Moussa et al., 2005). Embryos were cultured after warming, rather than transferred, and viability was assessed by live-dead stains and percentage of cells entering S-phase. Both studies indicated that OPS may be an efficient vitrification method for equine embryos. Choi et al. (2010) used the same cryopreservation solutions in the Eppendorf tip and indicated that this procedure may be useful for cryopreservation of both biopsied and large equine embryos.

It is interesting that our results to do not show a correlation between stage of development and an embryo viability, as the cells of a morula destined to become the ICM cannot be easily visualized while biopsying and could be damaged by the process. Krzyminska et al. (1990) found that biopsying murine morulae resulted in reducing implantation rate, fetal viability, and the mean fetal weight significantly. This might not be for horses, since Skidmore et al.

(1989) produced pregnancies from bisected equine morulae. In the current study, with an n = 6 and the majority of morulae vitrified, it is difficult to conclude if morula is an appropriate stage for biopsy.

The percentage of genetic sex detection from the biopsy samples was disappointing. Two possible causes were identified: lack of sensitivity of the test or loss of biopsied material. To examine the first cause, serial dilutions were performed using a known concentration of extracted DNA to reach the concentration of a single cell (~9 pg of genetic material in a dividing cell). On the extracted DNA, genetic sex detection occurred nearly 100% of the time.

Therefore, lack of detection was likely due to loss of biopsied material, especially since the ZFx/ZFy control band, used for confirmation of presence of DNA, was missing in all unsuccessful samples. We identified three opportunities for loss. First, cells were not visualized as the biopsy droplet was moved "blindly" to the Eppendorf tube. Second, by removing the supernatant, genetic material may have been lost. Lastly, the material needed to be transferred to a PCR Eppendorf tube, risking another opportunity to leave the material behind. To confirm that the PCR reaction would work on unextracted DNA, and to test a more direct method of transferring the biopsied material, an additional experiment was performed. Previously vitrified embryos were warmed and biopsied twice, and the remainder of the embryo was divided in half by microblade. The cells were then picked up with a pipette while visualizing them under a microscope, and moved directly to a PCR Eppendorf. Great care was taken to keep the volume moved under 2 μl, and to deposit the material at the bottom of the tube. Genetic sex analysis on these samples yielded 74% (23/31) detection rate. Based on these results, it appears that both of the proposed problems are likely. Future research needs to be done to increase the sensitivity of the PCR reaction, perhaps by incorporation of whole-genome amplification. Guignot et al. (2009) reached a 96.6% sex detection rate in bovine embryos by use of primer extension pre-amplification PCR (PEP-PCR).

Guignot et al. (2009) also used Proteinase K in an extraction step prior to PCR. We also investigated incorporation of a Proteinase K step, but found that for whole blood, this did not result in better detection rates than the use of freeze-thaw cycles. Further research is warranted in this area using other tissue samples.

Another alternative for optimizing the genetic test is to develop primers for repeated sequences, which would drastically increase the amount of amplified product. Examples of these repeated sequences include mammalian testis specific protein, Y-encoded (TSPY) or RNA binding motif Y (RBMY) and using them as the gene of interest could greatly enhance the sensitivity of the test. Copy numbers of TSPY range from 20 to 60 in men and up to 200 in bulls, and have already been suggested for equine preimplantation sex diagnosis (Manz et al., 1998).

More genetic testing methods are being developed that would be easier for genetic evaluation. These include the use of SYBR-green in a real-time PCR reaction (Pfaffl, 2001), which takes out the electrophoresis step to visualize the product, or loop-mediated isothermal amplification (LAMP), which replaces traditional PCR with a single-step reaction (Notomi et al., 2000). Zoheir and Allam (2010) performed preimplantation sex diagnosis 100% of the time (with 3+ cells) on bovine embryo biopsies by adding ethidium bromide or $CuSO_4$ to their LAMP reaction. This allowed detection of the results within a single tube by visualizing a color change and a precipitate. With the entire process taking 45 min, LAMP reactions may be the most practical method to PGD currently available.

There are many potential benefits from incorporation of PGD into a commercial embryo transfer program. Currently available diagnostic tests include tests for monogenetic disorders (HYPP & SCID), coat color alleles, and parentage testing. Research is also ongoing to develop tests for performance (Gu et al., 2009) and fertility (Giesecke et al., 2009) alleles, as well as an association analysis map from SNP arrays. Very soon, performing these tests on cells from an embryo will provide detailed genetic information.

Perhaps the biggest hindrance to progress is the licensing of many of the currently available monogenetic tests. A major obstacle for genetic testing in the horse is the lack of governing bodies to approve tests and offer quality control for testing laboratories (Bannasch, 2008). Although some mutations and their appropriate tests are published in peer-reviewed journals, some tests are offered without supporting scientific publication. Frequently this is due to patent protection, where only licensed laboratories may perform the tests. The implications of patent infringement and its applications to genetic testing is not completely known, but is recognized as detrimental to innovation and increasing clinical knowledge (Klein, 2007).

Conclusions

Biopsy collection, preimplantation genetic diagnosis, and direct transfer can be performed on equine embryos without compromising pregnancy rates using embryos ≤ 300 μm in diameter. Biopsying larger embryos and incorporating vitrification lowered pregnancy rates.

To maintain small embryo size, embryos should be recovered between 6.5 – early 7 d. Trophoblast biopsies can be taken using a beveled micropipette attached to a micromanipulator with piezo drill used to separate a few cells from the opposite side of the ICM. Biopsy samples must be directly transferred into PCR Eppendorf tubes, but can flash frozen for later genetic testing. A duplex PCR reaction testing for genetic sex by evaluating ZFx/ZFy and SRY was created for the current study, but may not be the most practical for future testing. Improvements to this genetic test increased sensitivity from 30 to 74% detection rate. Further optimization may include whole-genome amplification or loop-mediated isothermal amplification. Continued effort to develop more sophisticated genetic tests and to improve the vitrification process (especially for embryos > 300 μm) is warranted.

By incorporating PGD into commercial practice, significant progress could be made in equine genomics as higher quality embryos could be selected. Heterozygous carriers of diseases such as Hyperkalemic Periodic Paralysis (HYPP) or Severe Combined Immunodeficiency (SCID)

could remain in the breeding stock without the risk of passing on the defective allele, thus increasing the size of the gene pool. Also desirable traits, such as color, sex, and perhaps someday performance traits, could be selected to improve the value of offspring. The potential applications are endless and would greatly benefit the equine reproductive industry.

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