Biopsy and vitrification of equine expanded blastocysts
Katrin Hinrichs
Departments of Veterinary Physiology and Pharmacology and Large Animal Clinical Sciences
College of Veterinary Medicine, Texas A&M University, College Station, TX

Abstract
Preimplantation genetic diagnosis and embryo cryopreservation have extensive clinical application in the horse. Biopsy of equine embryos is complicated by the presence of the equine embryonic capsule. Recently, we developed a technique to puncture the capsule and obtain cells from the trophoblast of expanded equine blastocysts, using micromanipulation with the Piezo drill. Biopsies were obtained without affecting the pregnancy rate of the blastocysts after transfer. Genetic analysis after whole genome amplification of the biopsied cells demonstrated some failure to detect signal, and allele dropout, and work is currently underway to improve the accuracy of genetic analysis. Observation that the blastocysts collapsed after biopsy, yet maintained viability, led to the hypothesis that the collapsed blastocyst might be successfully vitrified. Further work showed that vitrification of collapsed blastocysts using an ethylene glycol-containing medium was associated with high pregnancy rates after transfer (71% of embryos produced pregnancies that developed normally to the heartbeat stage), thus providing the first successful procedure for cryopreservation of expanded equine blastocysts. These advances in embryo manipulation techniques should find wide application in clinical equine practice.

Keywords: Embryo, cryopreservation, horse, preimplantation genetic diagnosis

Biopsy of embryos for preimplantation genetic diagnosis
Devastating genetic diseases such as hereditary equine regional dermal asthenia (HERDA), glycogen branching enzyme deficiency, hyperkalemic periodic paralysis (HYPP), polysaccharide storage myopathy, severe combined immunodeficiency disorder, and cerebellar abiotrophy have been identified in the horse.1-3 With the exception of polysaccharide storage myopathy, all of these diseases are inherited in a recessive manner. This, and the fact that some of the top-performing horses in their breeds carry these recessive mutations, make it difficult to eliminate the causative alleles from the population.

Preimplantation genetic diagnosis (PGD) via embryo biopsy is commonly utilized in humans to determine the genetic status of at-risk embryos.4 In humans, to perform PGD, embryos must be produced in vitro. In the horse, while in vitro embryo production is possible, it is performed at only a few centers, is expensive, and is inefficient relative to in vivo embryo collection. Thus, for equine PGD to be a viable clinical procedure, it must be performed on embryos recovered from mares after uterine flush. However, the presence of the equine embryonic capsule, which starts to develop as soon as the embryo enters the uterus, on late Day 5 after ovulation,5,6 may complicate both the performance of the biopsy and the survival of the embryo after biopsy.7,8

Using unexpanded (e.g., Day-6) embryos for PGD avoids the presence of the capsule, and several laboratories have reported successful biopsy of early (Day-6 to 7.0) embryos.7-9 Transfer of the biopsied embryos resulted in pregnancy rates of 21 to 75%. The cells recovered on biopsy were successfully analyzed for genetic sex in these studies. However, biopsy of larger embryos was associated with lower pregnancy rates (29%).7 One hypothesis that might be made from these data is that younger (e.g. Day-6) equine embryos can repair damage to the capsule, whereas older, fully encapsulated embryos, cannot. This hypothesis is supported by the finding that pregnancy rates after embryo bisection, in which the capsular material and zona pellucida are lost, were 23 to 67% for embryos bisected at the morula or early blastocyst stages, but 0/12 for embryos bisected at the expanding blastocyst stage.10,11

Unfortunately, most clinicians do not perform equine embryo recovery before Day 7, as the small size of the earlier embryo makes the embryo harder to locate, thus increasing search time, and recovery rates have been reported to be lower when uterine flush is performed on Day 6 than on later days.12-14 For clinical application of PGD, it is important to determine whether it is possible to obtain biopsy samples from Day-7 embryos, having fully formed capsules, and if so, whether these embryos can maintain
viability after biopsy. In addition, to apply PGD clinically, embryos must be shipped to the laboratory from the field. Equine embryos are commonly shipped overnight to embryo transfer facilities in passive heat exchange devices (Equitainer®, Hamilton Research, Inc., South Hamilton, MA), and thus it is important to examine the effect of overnight shipping on the survival of embryos after subsequent biopsy.

We performed studies to determine if embryo biopsy using micromanipulation could be performed on Day-6 and Day-7 equine embryos, and if this method was effective in recovering sufficient cells for genetic analysis. Biopsy was performed by micromanipulation, using a Piezo drill to penetrate the zona and/or capsule, then cells were aspirated from the trophoblast layer through the manipulation pipette. We began by performing biopsies on Day-6 embryos. Pregnancy rates for these embryos, which had intact zonae pellucidae at the time of recovery, were 3/3 for those biopsied immediately after recovery, and 0/3, 1/3 and 2/3 for embryos shipped overnight at cold, room temperature, and warm temperatures, respectively. We utilized shipping warm for subsequent studies. We then evaluated the effect of biopsy on expanded, encapsulated blastocysts. Pregnancy rates for Day-7 expanded blastocysts were 5/6 for those biopsied immediately after recovery, and 5/6 for those biopsied after being shipped overnight warm. All pregnancies developed normally to the heartbeat stage. We also biopsied four Day-8 blastocysts; two, 790 and 1350 µm in diameter, established normal pregnancies (to the heartbeat stage) after biopsy. Nine mares carrying Day-6 and Day-7 biopsied embryos were allowed to maintain pregnancy, resulting in birth of nine normal foals. From these findings, we concluded that biopsy of expanded equine blastocysts with the Piezo drill is possible and does not compromise embryo viability.

Genetic analysis of the biopsied cells met with some problems. We saved the biopsies from the nine embryos that produced foals, and then compared the genetic analysis of these cells, after whole-genome amplification, with that of the resulting foals. One embryo biopsy was lost when the vial cracked on thawing. Sex was successfully determined from amplified DNA in 8/8 embryos.

The eight embryo biopsy/foal pairs were also evaluated for HYPP status (SCN4A gene) and for HERDA status (PPIB gene). In evaluating the accuracy of detection of allele status, the major problem associated with analysis of small samples, such as embryo biopsies, is “allele drop-out,” that is, the amplification of only one allele in an animal which is in fact heterozygous. When only one allele is detected, the sample is interpreted as being homozygous for that allele, rather than being accurately detected as heterozygous. In our embryos, the SCN4A gene failed to amplify altogether in two embryos, and the PPIB gene failed to amplify in one embryo. Analysis of the remaining embryo/foal pairs was confounded by the fact that all foals were homozygous normal for the normal SCN4A gene, and six of eight foals were homozygous normal for the PPIB gene; thus there were only two foals that were heterozygous for a disease-causing gene and could be used to test for allele drop-out. In these two samples, one was accurately detected as being heterozygous, but the other was interpreted as being homozygous for the affected allele, thus it had suffered from allele drop-out.

At the time of writing, we are exploring alternative methods of whole-genome amplification with Dr. Cecilia Peneda of the University of California at Davis Genetics Laboratory, and the results are very promising. In a recent trial of 16 biopsy samples from four embryos, one sample was lost on thawing, and one failed to amplify, but the remaining 14 samples had excellent fidelity in detection of heterozygous coat color and parentage identification loci when compared to the results obtained by analyzing the whole embryo. We will continue these studies with additional embryos that are heterozygous for important disease-causing mutations, to ensure that these loci are also detected with good fidelity.

From this study, we can conclude that the capsule of the equine embryo can be breached without impairing viability. Embryos may be collected from mares on Day 6 or 7 after ovulation, and shipped to the laboratory overnight in standard commercial embryo holding medium, biopsied, and shipped back for transfer with no detrimental effect on pregnancy rates or live foal rates. We anticipate that PGD may soon become a viable clinical procedure.
**Embryo vitrification**

Embryo vitrification is a relatively simple technique that may be performed in practice without extensive equipment, and has been well described previously. Freezing and vitrification of early equine embryos (less than 300 µm in diameter) is effective; however, freezing or vitrification of embryos >300 µm diameter has resulted in low pregnancy rates after transfer. Unfortunately, this has necessitated recovery of embryos on Day 6 after ovulation if cryopreservation is to be performed, which, as noted above, may be problematic.

While conducting the above studies on embryo biopsy, we realized that to apply the biopsy procedure clinically, the embryo would have to be held before transfer, while the genetic analysis of the biopsied cells was conducted. Cryopreservation seemed like the most logical method to hold the embryo. We hypothesized that while cryopreservation of expanded Day-7 blastocysts has low success, perhaps the breach in the capsule induced by the biopsy procedure would aid in cryopreservation of blastocysts after biopsy. We evaluated viability of three blastocysts that had been biopsied, allowed to reform in culture (reformation of the blastocoele occurred with 3 h), then vitrified, and compared this to the viability of a blastocyst that was vitrified immediately after biopsy, while still collapsed. Only the blastocyst that was vitrified while collapsed grew normally in culture after warming. This was surprising to us, as we thought of biopsy, collapse and vitrification as a series of insults that might be additive; however, on further consideration, it made biological sense that collapse eliminated the large volume of blastocoele fluid, which might aid the speed of vitrification, and perhaps allowed penetration of the cryoprotectant through the biopsy-induced defect in the trophoblast.

To further investigate, we conducted a study on the vitrification of embryos after blastocoele collapse. We started by evaluating different methods of vitrification using non-biopsied, small embryos (<300 µm). A recent paper had reported success in vitrification of ferret embryos, which contain high amounts of lipid similar to horse embryos, using a fine-diameter microlader pipette tips. We vitrified small, non-biopsied embryos using dimethylsulfoxide-containing medium (DM), as described in the paper of Sun et al. on ferrets, or using ethylene glycol-containing medium (EG), which had been used successfully in early equine embryos. Both media were used in conjunction with the micropipette tips. After we realized that embryos in the EG group were not developing well after warming, we introduced a third group, EG-vitrified embryos warmed by the procedure described by Sun et al., using sucrose (EG/s). Embryos in the DM and EG/s treatments grew in culture after vitrification, and so we investigated the pregnancy rates after transcervical transfer. Both groups of embryos established pregnancies after transfer (3/12 and 3/6 for DM and EG/s respectively).

These vitrification methods were then applied to expanded blastocysts collected on Day 7 after ovulation (300-730 µm in diameter). The blastocysts were biopsied, then vitrified immediately while in the collapsed state. The blastocysts were subsequently warmed and transferred to recipient mares. Rates of normal pregnancy (detection of embryonic heartbeat) were 2/16 (13%) and 6/13 (46%) for DM and EG/s treatments, respectively. We conducted further studies using only the EG/s treatment.

The estimated percentage of blastocoele fluid lost after biopsy was recorded for the 13 embryos in the EG/s group, and this was evaluated in relationship to the pregnancy status after transfer for these embryos. The pregnancy rates were 0/3, 2/5 and 4/5 for embryos losing <10%, 20 to 30%, and 70 to 100% of their blastocoele fluid after biopsy, indicating that greater loss of blastocoele fluid after biopsy was associated with higher survival. Therefore, in the next study, we evaluated an altered (“Central”) biopsy technique, in which the biopsy pipette was introduced into the center of the blastocoele and the fluid aspirated, then cells biopsied from the central area. This was followed by vitrification in EG/s. However, after thawing and transfer, pregnancy rates were only 1/8 (13%) for embryos cultured after warming and 4/7 (57%) for embryos transferred immediately after warming. This suggested that the Central technique may have been damaging the embryo, perhaps from the vigorous aspiration and suction to eliminate fluid or obtain cells from the inner surface of the blastocoele.

In our final study, therefore, we returned to biopsy of cells from the periphery of the blastocyst, but performed gentle suction to assure the removal of blastocyst fluid. Expanded blastocysts 407 to 565 µm in diameter were biopsied in this manner, then vitrified with EG/s. Transfer of these embryos after
warming resulted in a pregnancy rate of 6/7 (86%), with a rate of normal pregnancy (embryos with heartbeats) of 5/7 (71%).

These findings are exciting, demonstrating for the first time that expanded equine blastocysts can be successfully vitrified. Much further work is needed to refine the method for blastocoele collapse, hopefully to eliminate the need for micromanipulation, and to determine whether such collapsed blastocysts may be vitrified using standard techniques.

Acknowledgements

Supported by a grant from the American Quarter Horse Foundation, and by Ms. Kit Knotts

References