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Introduction

The commercial availability of equine embryo transfer has recently led to tremendous growth in breeder’s requests for desired genotypic and phenotypic specificity of offspring as well as embryo cryopreservation. These scientific advances come at a most opportune time for the horse industry because producing desired offspring not only reduces the “unwanted horse” population, but it favors a decrease of inherited disease, as well as permits the preservation of desired embryos until an owner can provide an optimal environment for their development.

Trophectoderm biopsy and preimplantation genetic diagnosis

Accurate gender identification of the equine fetus can be readily accomplished by transrectal ultrasonographic examination after 60 days of gestation. If the gender is not acceptable to an owner, the mare is often given an abortifacient agent and the unwanted fetus is aborted. While some consider abortion at this stage of gestation based on gender to be unethical, it should also be recognized that the practice is reproductively inefficient as well. Not only are pregnant recipient mares in use for at least 60 days carrying a fetus of unknown gender, but if aborted, they may fail to cycle again during the breeding season due to endometrial cup formation and the continued secretion of equine chorionic gonadotropin (eCG). Furthermore, the semen used to produce these pregnancies is wasted—it may be in limited supply, whereby the breeding dose for one mare precludes the use in another, or very valuable, as is often the case with frozen semen. Furthermore, stallion owners may not be aware of or in agreement with the abortion of their stallion’s offspring due to gender preference.

Although sex-sorted semen has the potential to produce the desired gender, its use has not yet become commercially available in most parts of the world, nor does the processed sperm appear to tolerate cryopreservation. Therefore, trophectoderm (TE) biopsy of an embryo followed by preimplantation genetic diagnosis (PGD) represents an excellent alternative for early gender selection and heritable disease surveillance. The TE biopsy procedure requires specialized equipment that is capable of micromanipulation of the embryo as well as experienced technicians, for puncture and biopsy of the expanded blastocyst can be challenging.

The unique equine capsule, formed by in vivo, but not in vitro-produced embryos, poses a significant barrier to obtaining trophectoderm cells and must be effectively penetrated so as not to compromise embryo viability during the biopsy procedure. The laser-assisted biopsy procedure used in other mammalian embryos (Figures 1, 2), humans included, has been largely ineffective in penetrating the equine capsule and maintaining viability of the embryo. The earliest report of an equine embryo biopsy and successful pregnancy following transfer was published by Huhtinen et al. These researchers used a microblade to obtain cells from Day 6 embryos. The pregnancy rates following transfer were low in this study, with only 3 out of 16 biopsied embryos resulting in a pregnancy. Several more recent studies have followed, reporting an improvement over these initial pregnancy rates following embryo biopsy. Seidel et al² compared pregnancy rates following the microblade embryo biopsy technique versus aspiration of TE cells using a glass 6-10um glass pipette. They reported a pregnancy rate of 40% following the microblade biopsy technique versus 28% for embryos >300um diameter and 75% for embryos < 300 um diameter following aspiration. These authors concluded that both the biopsy technique and the size of the embryo contributed to pregnancy rate, and that embryos <300um diameter had a more favorable outcome following needle aspiration. However, Choi et al³ reported normal pregnancy rates following biopsy and transfer using a piezo drill and aspiration needle biopsy technique in embryos that ranged in size from the morula stage to the expanded blastocyst stage (up to 1300um in diameter). Herrera et al⁴ recently reported similar results; they found no significant difference in 25 day pregnancy rates between transferred biopsied embryos versus nonbiopsied embryos (59% versus 62% respectively), nor any
difference based on embryo size in either group. Furthermore, in this latter study, the capsule was breached without the use of a piezo drill.

The piezo drill is an expensive piece of equipment and is not necessary for successful equine trophectoderm biopsy and resultant pregnancy, even when used with in vivo produced large blastocysts. Using a micromanipulator with a large holding pipette to secure the embryo, a 25 micron inner diameter glass micropipette or polar body biopsy needle, one can penetrate the capsule and retrieve sufficient numbers of cells from the trophectoderm by scraping and aspiration (Figure 3).

From a commercial viewpoint, it is important to note that embryos can be held overnight in warmed holding medium prior to biopsy as well as held for as much as 7-10 hours after biopsy without affecting viability. Therefore, embryos can be recovered at one farm, shipped overnight for biopsy at a laboratory, and then returned or shipped elsewhere for transfer into a surrogate mare. Herrera et al reported that PCR gene amplification of biopsied cells required 6 hours for gender determination, so ideally only embryos of desired gender would be transferred. In this study, however, the researchers were only able to determine gender in 50% of their samples (52 of 104 samples) and the specificity of their assay for females was 84.3%.

Presently, the Veterinary Genetic Laboratory (VGL) at the University of California-Davis (https://www.vgl.ucdavis.edu/services/horse.php) provides a service to detect not only gender, but also several lethal or crippling inherited diseases, from biopsied TE cells with >97% accuracy. Through a process of whole gene amplification, specific gene loci for HERDA, HYPP, lethal white, cerebellar abiotrophy, etc are reliably identified. Furthermore, prediction of coat color of the resultant offspring is also possible. Unfortunately, at the time of printing, the detection of combined immunodeficiency (CID) loci is under patent protection and the holders will not grant a license to UC Davis to test embryos for this disease.

Embryo vitrification

An unforeseen, but ultimately beneficial, consequence of trophectoderm biopsy is blastocoel fluid aspiration and rapid collapse of the blastocyst (Figure 4). This discovery has revolutionized equine embryo vitrification techniques and the successful cryopreservation of expanded equine blastocysts.

Vitrification provides an attractive option for many in the breeding industry under the following circumstances:
1. A synchronized recipient mare is unavailable.
2. To hold an embryo while awaiting PGD results.
3. Allows multiple embryos to be collected (via embryo flush or oocyte collection) without the expense of a synchronized recipient mare.
4. Sale of the vitrified embryo.
5. International distribution of genetics.
6. Enhance genetic variability and hybrid vigor.
7. Assess offspring phenotype--“temporary genetic banking”
8. Late or offseason cycling mares can produce embryos for more desirable transfer date
9. Unexpected double or triple ovulation
11. Production of embryonic stem cells

Until several years ago, successful pregnancy following vitrification was limited to embryos that were <300 um in diameter. These embryos were most often recovered from donor mares 6-6.5 days following ovulation and were at the late morula or early blastocyst stage of development. This size limitation affects reproductive efficiency for two reasons. First, embryos at this stage, even before vitrification, have been shown to have a lower pregnancy rate and higher rate of early embryonic loss when compared to later stage blastocysts. Furthermore, the recovery rate of day 6 embryos has been shown to be significantly lower than that of day 8 embryos, presumably because these early embryos
have not yet entered the uterus. Therefore, delaying an embryo flush until day 7 or day 8 offers an
improved rate of embryo recovery success.

An early study\(^7,8\) reported 16-day pregnancy rates of 67% after day 6 embryos were vitrified in
0.25 ml straws, warmed and then transferred into the uterus of recipient mares. These researchers
exposed embryos to increasing concentrations of ethylene glycol and glycerol (EG/Gly) vitrification
solutions before loading the embryos into straws. Based on this study, vitrification kits and a standard
vitrification technique became available commercially. However, using commercial protocols, several
studies did not report pregnancy rates as high as reported in the initial study. Troedsson et al\(^9\) reported
that only a single pre-capsule embryo (1/8) that had undergone biopsy and vitrification survived to day 45
pregnancy in a recipient mare. Choi et al\(^10\) reported that, using the standard EG/Gly protocol for
vitrification following biopsy, no day 6 embryos (0/5) resulted in pregnancy at 16 days.

In this latter study, the researchers also explored alternative methods for vitrification for embryos
different sizes and developmental stage. Their work with successful TE biopsy in expanded
blastocysts had resulted in the serendipitous discovery of blastocoel collapse. Scherzer et al\(^11\) attempted
tovitrify embryos by replacing blastocoel fluid with cryopreservation solution using a laser system to
penetrate embryonic capsule; however, only 1/9 recipient mares was pregnant at 23 days after transfer .
Campos-Chilton\(^12\) et al also studied an alternative method using ethylene glycol for vitrifying and
warming very young equine embryos (two-eight cell stage) before transferring them into the oviduct of
recipient mares. This group vitrified the embryos in open pulled straws, which limited the final volume to
1-2ul of vitrification media that bathed the embryo. The pregnancy rate was 62% (6/8) at day 20
following transfer. Using the same protocol and medium for vitrification, a sucrose gradient described by
Sun et al\(^13\) for warming, and a minute volume of media to surround the embryo, Choi et al\(^10\) reported an
86% pregnancy rate (6/7) from expanded blastocysts 407 to 565 um in diameter that were collapsed,
vitrified and warmed before transfer. Instead of loading the embryos into an open pulled straw, these
researchers loaded the collapsed embryo into a fine-diameter microloader tip\(^b\) as described by Sun et al\(^13\)
before plunging the pipette into liquid nitrogen. When warming the embryo, the tip of the microloader
was immersed in a warmed drop of thawing medium, releasing the embryo.

This author has found similar pregnancy rates using the protocol described by Choi et al for the
vitrification and warming of collapsed blastocysts as well as earlier stage embryos. However, the
equipment is different. The embryos are loaded onto a beveled trough of a device called the Cryolock\(^ac\).
The trough of the Cryolock\(^a\) tip holds the embryo in place, surrounded by less than 0.2 ul final
vitrification medium at the time of plunging into liquid nitrogen. When warming, the tip of the trough is
immersed into a 10 ul warmed drop of the initial sucrose warming solution and agitated gently, releasing
the embryo. The author has found that a healthy embryo gradually reexpands over the next 30 minutes
during transport in a warmed Equitainer\(^ad\).

Summary
The research cited in this paper has transformed the equine embryo transfer industry, especially
with respect to preservation of valuable and healthy equine genetic lines. The ability to successfully
biopsy and cryopreserve early equine embryos up to the expanded blastocyst stage of development allows
not only selection of offspring by breeders but enhances their commercial enterprise and avails for a
healthier population of horses.

\(^a\)Origio Humagen Pipets, Charlottesville, VA
\(^b\)Eppendorf NA Hapauge NY
\(^c\)Biotech Inc, Alpharetta, GA
\(^d\)Hamilton Research Inc Ipswitch, MA
References

Figure 1: Mouse trophectoderm biopsy following laser assisted penetration. Courtesy of Keith Masterson, MS.
Figure 2: Mouse trophectoderm biopsy. Cells are gently aspirated into the biopsy pipette. Courtesy of Keith Masterson, MS.

Figure 3: Trophoderm biopsy of an equine day 8 expanded blastocyst.
Figure 4: A collapsed day 8 expanded equine blastocyst following blastocoel aspiration, preparing for vitrification.

(Editor’s note: Photographs in this manuscript are available in color in the online version of Clinical Theriogenology.)