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Current challenges in commercial equine embryo transfer

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Abstract

Embryo transfer (ET) is widespread in sport horse breeding, with established programs for both fresh and cooled-transported embryos offering high success rates (>80% pregnancy for transferred embryos). ET is also an integral step in advanced assisted reproductive technologies (ARTs) such as in vitro embryo production via ovum-pick up and intra-cytoplasmic sperm injection (OPU-ICSI), and cloning by somatic cell nuclear transfer. In fact, in some programs conventional embryo flushing has been replaced by OPU-ICSI because mean embryo recovery rates after insemination of donor mares with cooled-transported or frozen-thawed semen (~0.5 per cycle) cannot compete with the embryo yield after OPU-ICSI (~2 per session). The main reason for the limited number of embryos flushed per cycle is the continued absence of a commercially available product for inducing multiple ovulation. In addition, increasing donor mare age negatively impacts embryo recovery earlier for embryo flushing than for IVEP (~16 years compared to ~20 years). On the plus side, improved procedures for vitrifying expanded blastocysts simplifies the preparation of suitable, synchronized recipients and promotes embryo flushing outside the breeding season. Progress is also being made in techniques for collecting and testing embryonic DNA for genetic diseases, sex and selected phenotypic characteristics.

Keywords*: embryo transfer, multiple ovulation, synchronization, cryopreservation, genetic testing*

Introduction

Nowadays, ET is a routine procedure in horse breeding with roughly 30,000 equine embryos reported as being transferred annually by the International Embryo Transfer Society (IETS: e.g. Viana, 2023), of which more than 75% are performed in South America. Since reporting is incomplete, the IETS figures significantly underestimate the total number of horse embryos transferred worldwide; nevertheless, the fact that the numbers of transfers reported for the years 2000 and 2005 (Thibier, 2001 & 2006) were, respectively, 2750 and 13,600, emphasizes the enormous growth that equine ET has undergone since the start of the 21st century. A further relatively recent major advance in the equine ET industry is the development to commercial viability of *in vitro* embryo production (IVEP: Lazzari *et al*., 2020); as recently as 2013, all IETS reported horse embryos transferred were *in* vivo derived (i.e. flushed: Perry, 2014). By 2022, 25% of reported equine embryos transferred were blastocysts produced *in vitro* by OPU-ICSI (Viana, 2023). Indeed, in Europe and North America, the current numbers of IVEP embryos transferred are roughly equal to those of flushed embryos, with some breeders and programs switching entirely to IVEP. The reasons underlying this switch relate in large part to the continued unavailability of drugs to reliably stimulate development and synchronous ovulation of multiple preovulatory follicles in mares (Squires, 2019), meaning that embryo recovery rates per flush continue to average around 0.7 when mares are inseminated with fresh semen (Squires & McCue, 2007) and only around 0.5 when cooled-transported and frozen-thawed semen are used (Panzani *et al.*, 2014; Cuervo-Arango *et al.*, 2019a). Although these embryo yields are comparable to the 0.6 embryos per OPU session generated when IVEP was first reported as part of a commercial clinical program (Galli *et al*., 2007), they are a long way behind the 2 blastocysts per OPU-ICSI session that well-established IVEP programs now achieve (Lazzari *et al.*, 2020: Stout, 2020).

Surprisingly, the vast majority of flushed embryos are still transferred fresh or after cooled transport, with less than 5% cryopreserved for storage and delayed transfer (Stout, 2012). This contrasts markedly with the situation for IVP embryos, of which approximately 50% are cryopreserved and transported or stored for a period of days to years before transfer. Cryopreserving embryos has numerous advantages, related primarily to the fact that storage in liquid nitrogen allows embryo production and transfer to be separated in both place and time. Importantly, cryopreserving embryos means that synchronized recipients do not need to be prepared for every embryo flush or IVEP attempt, facilitates long distance transport of embryos and encourages embryo production outside the physiological breeding season

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(Stout, 2012). Cryopreserving embryos also enables genetic testing, since DNA can be recovered from an embryo and sent to the testing laboratory, with the embryo cryopreserved while waiting for the results of the test (Choi *et al*., 2010). This manuscript will review the current status of, and some of the major challenges facing, the equine ET industry.

Current status of equine ET

Equine embryo transfer was pioneered in the early 1970s (for reviews see Allen, 2005: Stout, 2006), but only really took off commercially in the 1990s following the demonstration that non-surgical transfer of embryos could yield similarly high pregnancy rates to surgical transfer (Pashen *et al*., 1993). Since the 1990s, little has changed in basic procedures for embryo flushing and transfer, except that it is now much easier to find commercially available materials for all steps of the process. Nowadays, embryo flushing is mostly performed using a closed system with an in-line filter and, although commercial embryo flushing media are available, many practitioners chose to use lactated Ringer's solution. While the technique for flushing embryos is relatively straightforward, there does appear to be an experience-related improvement in embryo recovery, a large part of which is having the skills to ensure that the whole of the uterine lumen is adequately filled and emptied more than once (Sala-Ayala *et al.,* 2024). Leaving the flushing medium in the uterus for several minutes and administering an ecbolic (e.g. oxytocin: McCue *et al*., 2003) can also help optimize embryo recovery. For the transfer process, while non-surgical transcervical transfer is now universally employed, learning the dexterity to perform manually guided transfer with high success rates can take some time (Cuervo-Arango *et al.,* 2018). To shorten the learning curve, Wilsher & Allen (2004) pioneered the use of a Polansky-type speculum and long grasping forceps, which enables beginners to achieve high pregnancy rates more quickly (Cuervo-Arango *et al*., 2018). Moreover, in a large commercial program, performing transfers with the Wilsher technique has the advantage of allowing multiple transfers to be performed in quick succession, although it does require an additional assistant. With access to sufficient recipients, experienced practitioners expect to achieve pregnancy rates after transfer of fresh or cooled-transported embryos of between 75% and 90% (Cuervo-Arango *et al*., 2018), such that there are few improvements to be made to this step of the process. Areas in which progress can be made include increasing the number of embryos recovered, cryopreserving embryos, genetic testing of embryos, improving the quality of embryos produced *in vitro* or flushed from aged mares, and selection of suitable recipient mares.

Multiple ovulation

One of the major limiting factors in embryo recovery is that mares generally only develop a single dominant follicle per estrous cycle and therefore ovulate only one oocyte. However, some mares will have spontaneous multiple (normally double) ovulations, with the frequency being breed and mare-dependent but reaching 30% of mares/cycles in some breeds or types, e.g. Warmbloods (Stout, 2006). Not surprisingly, spontaneous multiple ovulation improves the efficiency of commercial ET programs because it results in a higher total embryo yield and number of pregnancies (Losinno *et al.,* 2001), although the number of embryos recovered per ovulated follicle is lower if the follicles ovulate from the same ovary (Riera *at el.*, 2005). Many ovarian stimulation treatments have been tested in mares, but few yield more than an approximate doubling in the ovulation rate (Stout, 2006). Indeed, for a long time it was thought that pharmacologically induced multiple ovulation might be unachievable due to a combination of the mare's unusual ovarian anatomy and the relative insensitivity of horses to commercially available gonadotrophins. However, it was eventually established that twice daily treatment with Equine Pituitary Extract (EPE) starting from the time of dominant follicle emergence could stimulate a mean of 7.1 ovulations and 3.5 embryos per mare (Alvarenga *et al*., 2001). Subsequent studies have proven that multiple ovulation can be induced using a single daily administration of equine recombinant FSH, and it has been suggested that the optimal protocol should induce 2 pre-ovulatory follicles per ovary, with the hope of yielding a mean of 2 embryos per cycle (Squires & McCue, 2007). More is not better because overstimulation of the ovaries is associated with ovarian pain and poor embryo per ovulation recovery rates; both of which relate to the ovary of the adult mare being anatomically 'inside-out', with the follicle-containing cortex located centrally and the surface of the ovary being covered by a tough connective-tissue capsule that prevents ovulation anywhere except at the 'ovulation fossa'. The development of large numbers of pre-ovulatory follicles will cause tension inside the tunica albuginea, and it appears to be difficult for larger numbers of pre-ovulatory follicles to migrate and release their oocyte simultaneously into the oviductal fimbriae via the fossa. A novel recombinant equine FSH has been produced using single-chain expression techniques (Roser &

Meyers-Brown, 2012) but, while effective, this product has not yet made it on to the commercial market.

Competition from IVEP

One of the biggest changes in equine ET over the last 10 years has been the increase in IVEP (Stout, 2020). In 2014, IVEP was performed on a very limited scale by a handful of ICSI laboratories worldwide with only a couple of hundred embryos produced per year. In the subsequent decade, the IVEP market has grown rapidly such that the numbers of embryos produced per year are now in the tens of thousands. This is in part because IVEP has become much more accessible since it became clear that oocytes can be transported overnight to an established ICSI laboratory, and the embryos cryopreserved and shipped back in liquid nitrogen, with little or no detrimental effect on either embryo production or pregnancy rates (Galli *et al*., 2016). Moreover, IVEP now outcompetes conventional embryo flushing, since ICSI allows extremely efficient use of scarce or expensive frozen semen and delivers surprisingly good results from sub-fertile or old mares (Cuervo-Arango *et al*., 2019a); moreover, a number of laboratories now report embryo production rates of around 2 blastocysts per cycle (Lazzari *et al.*, 2020). Nevertheless, some mares are not suitable for IVEP, either because they do not tolerate the OPU procedure well, or because they develop very few follicles and yield fewer embryos per OPU-ICSI cycle than when inseminated and flushed (Perla Fleury, pers. comm.); therefore, conventional embryo flushing still has a place in commercial practice. Another changes to the ET industry that has been stimulated by IVEP, is embryo cryopreservation; because embryo development rates after ICSI vary (from 6, to 9 or more days to reach the blastocyst stage) and the number of blastocysts produced in one OPU-ICSI cycle can be high (>10), it is often necessary to freeze the embryos produced (Stout, 2020). Moreover, for ICSI embryos there is no apparent difference in pregnancy rates between embryos transferred fresh and after slow freezing or vitrification. In addition, the increased use of cryopreserved ICSI embryos has emphasized how much easier it is to prepare and manage recipients when using frozen embryos; because there is no need to manipulate the recipient mare's cycle, and the embryo only needs to be thawed or warmed when the recipient is on the desired day of the cycle and has been checked to make sure that everything looks good (e.g. no uterine fluid or edema, healthy corpus luteum).

Embryo cryopreservation

Whereas a high proportion of IVEP embryos are cryopreserved, the percentage of flushed embryos that are frozen is still quite small (<5%). This relates both to the fact that, without superovulation, there are usually not many flushed embryos available, and because until recently only small embryos (<300 mm) offered a reasonable likelihood of pregnancy after thawing or warming (Stout, 2012). Moreover, to collect small embryos required flushing the uterus of the donor mare on day 6.5-7 after ovulation when, particularly in older mares, the embryo may not yet have descended into the uterus and, as a result, embryo recovery rates were low (Allen, 2005). The biggest breakthrough in cryopreservation was the accidental discovery that puncturing expanded blastocysts (> 300 mm) and collapsing them by aspirating the blastocoele fluid, enabled them to be vitrified and warmed while yielding high pregnancy rates (>75%: Choi *et al.*, 2010). Since this discovery, numerous studies have been performed to modify the techniques and to try to remove the need for micromanipulation while still ensuring adequate cryosurvival. As a result, it now appears that expanded blastocysts up to 480 mm can be vitirified without puncture if they are incubated for extended periods in the first (cryoprotectant-containing) equilibration solution from commercial kits designed for vitrifying human (IVF) embryos (Kovasky *et al.*, 2024); for larger embryos, puncture and aspiration would still be recommended.

Recipient mare selection

The most important aspects of selecting recipient mares are age, inherent fertility, size, health and body condition together with adequate synchronization of the recipient mare's day of ovulation to that of the donor mare or to IVEP embryo development. While it is sensible to choose a healthy young recipient in moderate to good body condition, there is little difference in pregnancy rates for recipients up to the age of 16-17 years (Cuervo-Arango *et al.,* 2019b); in general, however, older mares will have a higher risk of subsequent pregnancy loss since they are more likely to suffer from chronic endometrial degeneration, but this could be checked for via a pre-season endometrial biopsy. For flushed embryos, there is a surprisingly wide range of acceptable synchrony, with no difference in pregnancy rates for day 8 embryos transferred to recipient mares that ovulated between 1 day before and up to 5 days after the donor (Cuervo-Arango *et*

al., 2019b). It also appears that the length and intensity of the estrus preceding the transfer is crucial, with pregnancy rates higher when the mare had at least 5-6 days of clear endometrial edema before ovulation (Cuervo-Arango *et al*., 2019b). This is one of the advantages of using frozen embryos or having access to a large herd of recipients, since the mares can be allowed to ovulate naturally with no need to induce ovulation and shorten the estrous period. For frozen-thawed ICSI embryos, the preferred days for transfer to the recipient are day 4, or day 3 if the mare has had more than one ovulation (Cuervo-Arango *et al*., 2019b); using day 5 or 6 recipients results in lower pregnancy rates even though the size of the conceptus vesicle a week later suggests that most ICSI blastocysts are at a developmental stage equivalent to a day 5-6 *in vivo* embryo at the time of transfer. This narrow window of acceptable synchrony presumably relates in part to the effects of freezing on the ICSI embryos, with better survival in a less advanced uterine environment which may allow them the opportunity to recover from any cryopreservation-induced damage. By the same logic, it is probably better to select recipients that are slightly behind embryo stage (e.g. day 6) for embryos vitrified after flushing on day 7 or 8.

Genetic testing

The discovery that expanded equine blastocysts survived vitrification better if they were first punctured and collapsed, was an inadvertent finding of a study in which biopsies of embryonic trophectoderm cells were recovered for genetic testing (Choi *et al*., 2010). There have been subsequent reports of the biopsy of both flushed and IVEP embryos to test for sex, phenotypic characteristics such as coat color, and for monogenetic diseases such as hyperkalemic periodic paralysis (HYPP) or hereditary equine regional derma asthenia (HERDA: Choi *et al*., 2010). While it is possible to hold the embryos at room temperature while waiting for the results of a PCR for the SRY gene (sex determination: Herrera *et al.,* 2014), it is more common for the embryos to be cryopreserved while the genetic tests are performed, and only warmed and transferred if the results are favorable. Biopsy of flushed embryos requires puncture with either a sharp needle or a Piezo drill, whereas for ICSI embryos cells can be allowed to herniate from the hole produced during ICSI and removed from the outside of the zona pelluicda (Lazzarri et al., 2020). At present, there is not enough data to definitively determine whether biopsy is detrimental to embryo quality; however, early results suggest that biopsied ICSI embryos are just as likely to yield a pregnancy as non-biopsied embryos (Lazzarri *et al*., 2020), although maintaining them in culture while waiting for trophectodermal cells to herniate may not be beneficial to their developmental competence. Moreover, because of concerns that biopsy may damage the embryos, studies are ongoing to see if it is possible to harvest sufficient DNA from blastocoele fluid (without removing any cells: Herrera *et al.*, 2015) or from the medium the embryo was cultured in. As yet, there are no reports of embryo biopsy for pre-transfer testing for chromosomal abnormalities (aneuploidy) or for genotyping.

Embryo Quality

A final area that is difficult to influence but can affect results is embryo quality. Fortunately, the majority (>90%) of flushed embryos are of high quality (Grade 1-2: Stout, 2006). On the other hand, embryos from old mares or that are smaller than expected on the day of flushing yield lower post-transfer pregnancy rates and higher early pregnancy loss rates (Cuervo-Arango *et al,* 2019c). This cannot be rectified by transferring the retarded embryos to a recipient mare that ovulated a day or 2 later and, therefore, seems to reflect an inherent problem in some of these smaller embryos. Similarly, ICSI embryos yield lower pregnancy rates than flushed embryos and, particularly for recently started laboratories, often result in higher pregnancy loss rates (15-25%: Cuervo-Arango *et al,* 2019a). For the IVEP embryos, improved culture media and protocols will presumably help to improve results in the future, but for both flushed and IVEP embryos and, in particular for older mares, it may be useful to develop feed supplements or other treatments that improve the follicular environment and oocyte health (Catandi *et al*., 2022), and/or more meaningful tests than morphological examination to determine whether an embryo has good or poor developmental competence.

Conclusions

ET is an established procedure in horse breeding. The development of IVEP has resulted in an increase in the total numbers of embryos transferred, but has encouraged parts of the industry to switch from conventional embryo flushing to IVEP because the latter offers significantly better mean embryo per procedure production rates. On the other hand, some mares are not good candidates for OPU and IVEP,

such that there is still a place for conventional ET in practice. Interest in conventional embryo flushing would be helped further by the commercial availability of a recombinant equine FSH preparation for reliably inducing multiple ovulation. The other major change to be expected in the near future is an increase in the number of flushed embryos being vitrified, to take advantage of the relative ease and lower costs of recipient mare management when using cryopreserved embryos; this will be facilitated by the recent development of protocols that offer competitive pregnancy rates after vitrifying expanded blastocysts. Finally, genetic tests for various embryo characteristics (e.g. sex, coat color, monogenetic diseases) have been developed, and various groups are working on genotyping and tests for more in-depth aspects of embryo quality (e.g. chromosomal normality), and it is possible that in the future increasing numbers of embryos will be tested for such characteristics prior to freezing and subsequent transfer.

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