Flushing and transfer of equine embryos in practice

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Introduction

The purpose of this article is to give the reader an overview of an embryo transfer program and the factors that determine its success. The process involves five separate phases: (1) Monitoring and insemination of the donor mare, (2) Monitoring of a minimum of two recipient (surrogate) mares (in season with the donor) to determine when they ovulate, (3) Flushing of the donor mare to retrieve the embryo, (4) Transfer of the embryo into a recipient mare, and (5) Pregnancy determination of the recipient mare.

The freezing of stallion sperm to preserve valuable genetics is commonplace and the freezing of sperm from the testicles of castrated, seriously ill or dead stallions is also available. The opportunities for preserving mare genetics are however much more limited. Embryo transfer opens up the possibilities of preserving genetic material from mares through the process of embryo freezing. The freezing of mare eggs (oocytes) although not yet commercial may be available in the future.

Embryo transfer in practice

The applications of embryo transfer include:

- Obtaining foals from performance mares that continue to compete
- Obtaining multiple foals from individual mares each year
- Obtaining foals from young (two-year-old) mares
- Obtaining foals from reproductively unsound mares
- Obtaining foals from mares with non-reproductive health problems
- Obtaining embryos for cryopreservation

It is important to have a good grasp of what the expectations for embryo transfer are. Likely success determined by donor conception rate, embryo transfer rate (successfully transferred embryos) and pregnancy failure rate in the recipient mare; likely success can be calculated using the following formula:

Donor conception rate x transfer rate x pregnancy success rate (100 - pregnancy failure rate)

Internationally recognised conception rates are 60–77% for fresh, 44% for chilled and 46% for frozen semen inseminations. If we assume a 50% embryo recovery rate, 80% transfer rate and 10% pregnancy failure rate the per-cycle success rate of an embryo transfer program would be:

Per-cycle success = 50 x 0.8 x 0.9 = 36%

Therefore, to ensure that one is successful the client may have to budget and plan for three breeding phases to give them the best chance of achieving a foal; of course, some will be successful on the first attempt but some will require three or more breeding phases. This formula also highlights that if a practitioner can maximise embryo recovery and embryo transfer rates and minimise pregnancy failure rate one can anticipate greater success within any given embryo transfer program. It is worth acknowledging that some ‘competition mares’, especially

the older ones do suffer from poor embryo recovery rates (Campbell 2014) and temporarily suspending training and competition, for some mares at least, may be necessary to optimise embryo recovery rates. The average recovery rate in a commercial embryo transfer program is approximately 50% but when one selects young, reproductively healthy donor mares and uses fresh or chilled semen the rate can be over 70%.

Other than the inherent fertility of the donor mare and stallion semen used a number of factors will influence the embryo recovery rate, including, the status and age of the donor, day of embryo recovery/flushing, number of ovulations and clinical expertise (McCue *et al.* 2010).

Donor mare age

Older mares with poor reproductive histories produce fewer embryos and age related degeneration of the oocyte is a potential explanation as is chronic degenerative endometrial disease which is more likely as mare age increases. One can also expect an increased likelihood of oviductal pathology and/or blockage. A couple of useful stats to consider are that young mares less than 12 years are 10% more likely to produce embryos than mares over 18 years and a higher percentage of embryos are recovered from mares aged <10 years than from mares aged >15 years (Uliani *et al.* 2010, McCue and Squires 2015). The rate of pregnancy loss (early embryonic death) following transfer from older donor mares may also higher than that obtained from younger mares. The combined low embryo recovery rate from the older mare coupled with a potentially higher rate of loss of transferred embryos from these mares greatly limit the number of foals that can be produced from this category of mare in any given breeding season.

Day of flushing and number of ovulations

The equine embryo enters the uterus between 132 and 168 hours post ovulation (5.5 to 7 days) and the time (day) of embryo flushing and collection will determine the size of the embryo collected (Squires *et al.* 1985). A number of studies, reviewed by Vanderwall and Woods (2007), have studied the effect of days post-ovulation on equine embryo recovery rate and determined that on day 6 the embryo recovery rate was on average 58%; day 7, 61%; day 8, 65%; day 9, 71%. Although recovery rates are lower on day 6 than day 9 one must take into account the variation in the size of embryos that are recovered. Smaller (younger) embryos may be more technically demanding to handle and come with a lower recovery rate however, these are necessary for cryopreservation. Larger (older) embryos may come with a higher recovery rate and be easier to detect and handle however very large embryos can be too big, being more technically demanding and may be too large for the transfer equipment you have. As a routine in our practice we flush mares bred with chilled semen on the 7th day post ovulation and mares inseminated with frozen semen on the 8th day post ovulation. Individual and some older mares may have delayed oviductal transport and release and these may be flushed a day later than the norm. For colleagues new to the process day 8 may offer the best balance between recovery and ease of transfer. Mares that double ovulate have higher embryo recovery rates than single ovulating mares however multiple ovulations from one ovary paradoxically tend to reduce recovery rates compared to mares ovulating from both ovaries (Riera *et al.* 2006).

Embryo flushing procedure

The mare is generally restrained in stocks, a well behaved can be flushed without sedation however the handler generally plays an active role in keeping younger mares' attention during a process which can take between 20 and 45 minutes. Unlike the bovine patient epidural anaesthesia not necessary. The process starts with general cleaning of the perineum and buttocks to minimise contamination of dust and hairs, one must be careful not to leave chemical or soap residues on the perineum of the mare. In our practice a vestibular tampon is placed prior to evacuation of faeces from the mare's rectum after which the mares is palpated and scanned. The

tail is then ‘bagged’ in a glove and a bandage applied and the tail clipped to one side. Gross contamination is removed from the perineum prior to focussed cleaning of the vulva with particular attention to the vulval lips using clean gauze swabs. Once the mare is clean, the vulval lips are parted and the tampon removed.

The mare is flushed using an in-out procedure using flushing media instilled via one branch of a commercial Y-tubing set and drained from the other via a 70-90 cm 32 french gauge foley catheter with a balloon cuff inserted through the mare’s cervix. The efflux is passed through one of a number of commercially available embryo filters which prevent the passage of the embryo, trapping it in the filter. Media for flushing is generally a balanced salt solution and can vary from Hartman’s or lactated Ringer’s solutions with or without additives (surfactant) through to ‘complete’ commercial flushing media containing pH stabilisers (+/- indicators), heavy metal chelators bovine serum albumin (BSA) or polyvinyl alcohol (PVA) as surfactant and antibiotics.

The foley catheter may be open ended or bullet tipped, the author generally uses an open ended foley with one or two additional side holes; cuffs vary from 50 to 100ml volumes, generally the author uses a 75 ml cuff distended with 50mls of flushing fluid and a lager 150ml cuff distended with 100ml of flushing fluid for post foaling or mares with a semi-relaxed cervix (usually known from prior experience). Checking the cuff prior to insertion is wise before the tubing lines and filter are primed with pre-warmed flushing fluid. Insertion is performed in a sterile (clean) fashion using a sterile (clean) rectal sleeve or, over-glove technique. A minimal amount of sterile ‘embryo-safe’ lubricant is used to aid insertion and an assistant may part the vulval lips if necessary. The external cervical ostia is located and the catheter placed into and advanced through the cervix with minimal manipulation. Once passed through the internal cervical ostia the cuff is inflated and withdrawn back to the internal ostia. Whilst one’s hand remains in the vagina the fluids are allowed to flow by gravity; once no leakage is detected one can remove the hand from vagina.

Whilst the fluids are infused into the uterus per-rectum palpation and manipulation of uterus can be performed to ensure even filling of the uterus. It is important to provide enough volume to comfortably fill the uterus (range between 1-6L). Generally speaking, the author fills the uterus to full distension with between 1-3L of flushing fluids before draining. When full one closes the inflow line and opens the outflow line to allow retrieval of fluids; retrieval is aided by manipulation of uterus and/or catheter during the process, occasionally ultrasound guidance can aid in this process. This process is repeated a minimum of three times prior to drainage of the system through the filter concluding the process. Flushing can be an active or a passive process. It is the author’s perception is higher recovery rates can be achieved with active involvement from the operator; the uterus is agitated and balloted gently to vigorously to aid in ‘release’ of embryo, this also ensures even and complete filling and emptying of both horns and the body of the uterus. Occasionally the catheter tip can become occluded by close apposition to the endometrium and the uterine body can collapse leaving the fluid isolated in the horns. Aids to fluid recovery include: manual deflection of the balloon and catheter tip, advancement of the catheter by digital pressure, retraction of the catheter by applying tension straightening the uterine body, rotation of the catheter by twisting the external portion and partial deflation of the balloon and advancement into the horn. The goal is to achieve maintaining a rapid and even outward flow. On the final fill of the process it is common for some practitioners to fill the uterus and allow the fluid to sit for a few minutes prior to drainage, oxytocin can be administered 20 IU, IV or IM simultaneously to aid in fluid drainage.

A number of methods of filtration are available operating in a closed, semi closed or open fashion. Generally the author prefers a closed fashion using an EmCon style filter or a semi-open method using an EZWay filter. The efflux which passes through the filter is collected in a

receptacle (bucket) with measuring lines to allow visual assessment of the volume of fluids that have passed.

Once the flush is finished one should administer a luteolytic dose of prostaglandin to lyse the CL(s) and induce oestrous. A follow up check of the mare to ensure, in follicular phase, free from fluid/infection and not pregnant should be mandatory (Martinez-Bovi *et al.* 2024).

Embryo searching and handling

Embryo searching requires a lab or workspace to set up in, this can be a dedicated room or area which should be a warm, ideally 20-25ºC, draught free clean space with no embryo-toxic disinfectants or residues. Ideally there will be no through traffic and it should be quiet! Purpose built laboratories may have a laminar-flow hood (filtering air) but this is not absolutely necessary for domestic embryos. The workflow from body temperature to room temperature should be gradual and a heated stage/plate and/or incubator can be helpful for warming equipment, media and dishes. A good quality stereomicroscope with 10x to 15x magnification is necessary to search for the embryo(s).

At the end of the flushing process there will be a volume of flushing media in the filter. Some filters double-up as a searching dish, whilst others need to be emptied into a gridded searching dish. One should adopt a methodical, slow and steady approach. The filter is drained via the outflow so that approximately 20ml remains in filter, Using pre-warmed flushing media the lid and sides of the filter are rinsed with 20ml of media using a 20g needle prior to and post pouring into a gridded searching (petri) dish. The filter in and EZWay filter is flushed into the bottom of the dish and the outflow 'plugged'. Do not let any of the filters run dry.

The embryo is searched for using the stereo-microscope in a methodical grid approach, fluid can be 'swirled' to differentiate rolling from floating objects and collects debris and embryo(s) in centre of dish. The author would start in the centre of the dish and work outwards ensuring that the dish is checked to the edges. If no embryo is located the search is repeated perhaps at a higher magnification and ideally by a second operator.

Once an embryo is located it should be retrieved from the flushing dish, be washed to remove any particulate matter and debris and remove excess flushing media before placing into a clean holding solution prior to loading for transfer. washing and holding solutions/media are often the same media which are enriched formulations of flush medium containing amino acids, nutrients, surfactant, energy sources such as glucose and pyruvate, growth factors, antibiotics +/- hyaluronan. These media are capable of maintaining embryos for a number of hours in an air atmosphere at temperatures from 18-25ºC and up to 20 hours at 4ºC (product specific). Embryo(s) are handled using a variety of devices from syringes and pipettors to specific handling devices. The author routinely uses a 1ml syringe and a Steripipette (Minitüb). It is ideal for one to check tip and rinse pipette/handling straws prior to use. Ideally one needs to work in a methodical manner, being slow and steady and take care to avoid catching the tip on edges of the dish/filter. Firstly one should separate and isolate the embryo from large debris in searching dish/filter dish and then move to a separate dish for washing.

Embryo washing involves dispensing 8-10 drops of pre-warmed embryo holding media. The embryo is then serially transferred between the drops to 'wash 'embryo before transfer to a holding dish. This is normally done under the stereomicroscope but can be done with the naked eye with larger embryos. One aims for minimal transfer of media between drops. The procedure is as follows:

1. Pick up a small volume of drop aiming to advance embryo into
2. Target embryo and not debris

3. Expel small volume of media; 'blow' around embryo and then aspirate embryo
4. Progressively separate from any debris
5. Prior to advancing draw up small volume of air to avoid embryo sinking to meniscus
6. Advance embryo to next drop
7. Repeat through each drop
8. Finally transfer into holding dish

Embryo grading

The quality of the recovered embryo has a major effect on pregnancy rates. Embryos with poor quality scores result in poor pregnancy rates, in addition, embryos that are smaller than normal for their age, or those that have morphological abnormalities, also result in lower pregnancy rates (McCue and Squires 2015)

Embryo grading system (adapted from McCue *et al.* 2009)

1. Excellent; ideal embryo - spherical, with cells of uniform size, colour and texture
2. Good; minor imperfections - few extruded blastomeres, irregular shape
3. Fair; definite but not severe problems - degenerate cells, extruded blastomeres, collapsed blastocoele
4. Poor; severe problems - collapsed blastocoele, numerous extruded blastomeres, degenerated cells
5. A degenerating embryo or Unfertilised oocyte (UFO)

Transfer of the embryo

The embryo is loaded into a 0.25 or 0.5ml embryo (semen) straw; the straw should be rinsed and the tip checked for sharp edges prior to loading. The straw is filled with three columns of media with embryo in middle column with air column either side, a moistened PVA straw plug stops fluid escaping by capillary action. The embryo is then ready to load into a transfer device and the author prefers to use a traditional Cassou gun with a bullet nosed transfer sheath with side holes (IMV) for non-surgical transfer.

Embryos may be transferred either manually or using a vaginal speculum and cervical forceps. Manual transfer is just as it sounds and involves passage of the transfer device through the recipient's dioestrous cervix and deposition of the embryo in the body of the uterus. Manual transfer starts with a clean preparation as previously described, using a sterile rectal glove or over-glove and a minimal volume of sterile embryo-safe lubricant one passes the transfer device, minimising vaginal contact prior to puncturing the chemise at the external cervical ostia. The device is advanced through cervix and the embryo deposited in body with the transfer device being drawn backwards slightly at point of transfer. The skill required to manually transfer embryos, with minimal cervical manipulation and achieve good transfer rates should not be underestimated however with experience this can achieve very good transfer rates.

The speculum and forceps technique is often referred to as the Wilsher technique (Wilsher and Allen 2004). This can be referred to as a minimal contamination technique and requires the recipient to be sedated to place a vaginal duck billed or Polansky speculum. The cervix is then grasped under visual guidance with a pair of cervical grasping Wilsher forceps; traction is then applied to pull the cervix caudally straightening the cervical canal. The chemise cut rather than punctured prior to the transfer device being advanced through cervix and the embryo being deposited in body of the uterus. It has been demonstrated in a commercial embryo transfer program that this technique can achieve higher recipient pregnancy rate (transfer rate) *versus* conventional manual transfer with 92 *vs.* 71% respectively (Cuervo-Arango *et al.* 2018). Cuervo-Arango and co-workers demonstrated a significant operator effect with the conventional

manual technique ranging from 50.9 to 79.7%, however no operator effect was apparent for the Wilsher technique with transfer rates of 90.9 to 93.4% suggesting that once trained in the technique it is suitable for beginners. This technique does however come with an increased equipment cost, requires a minimum of two people and requires sedation of the recipient mare.

Post transfer of an embryo, using either method of transfer, the tip of the device should be checked to determine that the embryo has been deposited in the recipient and not retained in the tip of the transfer device as embryos can ‘stick’ to, or within, the tip of the transfer device. Risk factors for this have not really been defined however the author perceives an increase when media without surfactant is used. To do this, one needs to rinse the tip of the transfer device with warm holding media into a dish. If an embryo is found it should be re-washed, re-loaded and re-transferred; if undamaged, embryos can and will survive a second transfer attempt.

Recipient mare management and selection

The ideal recipient will be in good health and body condition and free from disease and of equal health status to the donor, ideally they will also be easy to handle and halter broken! Their body size should be equal to or greater than that of the donor and they will ideally be between 4 and 10 years of age. They need to be in sound breeding condition, have good endometrial health a history of regular cyclic activity. Preferably they will have had a foal already avoiding any size issues surrounding maiden mares and also demonstrating that they are a good mother that lactates normally.

Recipient synchronisation and selection

An appropriately synchronised recipient should have ovulated 1 day before (-) to 0 to 3 days after (+) the donor mare (+5 day recipients possible; Foss *et al.* 1999; Jacob *et al.* 2010). The author ideally aims for 0 to +2. Ideally two recipient mares will be selected per donor ovulation and on the day of transfer the ideal recipient selected. The recipient should have a well defined *corpus luteum* with good to excellent uterine and cervical tone.

Medication of the recipient mare

Sedation is administered if required (acetylpromazine may aid cervical handling). Historically flunixin meglumine has been administered as an anti-prostaglandin therapy on the basis that cervical manipulation may cause prostaglandin release and premature luteolysis. In a study of 230 flunixin treated *versus* 179 untreated recipients with manually transferred embryos, Okada and co-workers (2018), demonstrated no significant difference in pregnancy rates at days 15 and 60 with and without flunixin treatment at transfer. In fact, they noted a tendency for increased pregnancy failure in treated mares (p=0.085). As far as the author is aware there are no studies looking at the use of flunixin when the Wilsher method is used.

Altrenogest therapy may help when recipients of lower quality (low endogenous P4) and/or be used to advance the post-ovulatory progesterone rise to aid in synchronising asynchronous recipients, however one has to acknowledge that exogenous altrenogest may suppress endogenous progesterone and therefore care needs to be taken when withdrawing therapy (Crabtree and Serrano 2020).

The overall success of the transfer phase of the process is dependent on the quality of the embryo recovered and the quality of recipient chosen. Proper selection and management of recipient mares is the most important factor affecting the success of a commercial equine embryo transfer program.

Transportation of embryos for transfer at another facility

When adequate recipients are not available it is possible to ship embryos to another facility where recipients can be made available; this does require close liaison to synchronise recipient and donor cycles. In controlled studies there are no significant differences in pregnancy rates between embryos transferred fresh and those chilled and transported before transfer (McCue *et al.* 2011, Moussa *et al.* 2002). Embryos can be shipped in commercial holding media at ambient temperature or 5°C in an Equitainer (for the best cooling curve), depending on the time/distance in transport. Embryos sealed in a transport vial in a falcon tube with media as a physical and thermal buffer.

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