Abstract
Collection and transfer of embryos is common in equine veterinary practice. Keys to success are attention to detail, optimal reproductive management of donor mares, careful selection and management of recipient mares, adherence to guidelines for embryo recovery, evaluation and handling, plus a gentle transcervical transfer technique.

Key Words: Equine, embryo transfer, pregnancy

Introduction
Embryo transfer is a common technique in broodmare practice. The goal of this review is to provide a practical clinical perspective to various aspects of equine embryo transfer.

Embryo collection

Collection days
Equine embryos enter the uterus through the utero-tubular junction (UTJ) between 5.5 and 6 days after ovulation, at which time most equine embryos will be at morula or early blastocyst stage of development. Embryo recovery attempts in clinical practice are usually performed 6.5 to 9 days after ovulation. A collection attempt may be performed on day 6.5 or early on day 7 to procure a small (i.e. < 300 μm) embryo for cryopreservation.

A majority of mares in the United States are flushed on day 7.5 or 8 after ovulation because embryo recovery rates are high and a majority of embryos are blastocysts or expanded blastocysts and are easily observed under the microscope. The collection procedure is often delayed by one-half day for mares bred with frozen semen because of a slight delay in embryonic development (Table 1). In some cases collection is also delayed for older mares and mares bred after ovulation, although the data do not support either situation as routinely resulting in recovery of smaller embryos.

Equine embryos approximately double in size from day 7 to day 8 (Table 2) and double again between day 8 and day 9. Consequently, embryo collection on day 9 after ovulation usually results in the recovery of large embryos (i.e. > 1 to 2 mm in diameter).

Flush media
Options for flush media include a variety of commercially available ‘complete’ flush media that contain a Zwitterion-based buffer system, antibiotics and purified albumen or polyvinyl alcohol (PVA) as a surfactant or lactated Ringer’s solution (LRS) or Hartmann’s solution without additives. A recent study at CSU showed no difference in either embryo recovery rate or pregnancy rate after transfer using a complete flush medium or Hartmann’s solution (Figure).

Embryos adhering to search dish
One potential advantage of a complete flush solution containing a surfactant versus a crystalloid solution devoid of surfactant is the perception that embryos may stick to the catheter, tubing, cup or Petri dish if flush fluids without a surfactant are used. Of the first 21 embryos recovered in 2017 using Hartmann’s solution without a surfactant, 11 embryos adhered to the search Petri dish. All 11 embryos were gently ‘unstuck’ from the original search dish, washed in a commercial embryo holding medium containing polyvinyl alcohol (PVA) and transferred into a recipient mare. Nine of the 11 embryos (81.8 %) transferred resulted in a pregnancy. By comparison, 7 of the 10 embryos (70 %) that did not adhere to the original Petri dish resulted in a pregnancy after transfer.
Due to the high proportion of embryos adhering to the Petri dish when flushed with Hartmann’s solution, a trial was performed evaluating embryo adherence to a variety of commercial plastic search dishes. It was noted that equine embryos tended to stick to dishes from some manufacturers and not to others. Consequently, our clinical embryo transfer program now utilizes a plastic search dish that is not associated with embryo adherence.

Extra flush procedure

If an embryo is not recovered following an initial series of three uterine lavages, one to two additional liters of medium are immediately infused into the uterus and 20 units of oxytocin is administered intravenously. The medium is allowed to remain in the mare for three minutes before being recovered by gravity flow aided by uterine massage per rectum. In a recent retrospective study of 208 embryo flush attempts, an embryo was collected during the first round of three lavages on 89 occasions (42.8 %). An embryo was collected on 30 ‘re-flush’ attempts, yielding a total of 119 positive flushes in 208 attempts, for a 57.2 % overall embryo collection rate.

Next-day re-flush

In rare circumstances a mare may be re-flushed the day after an initial negative embryo collection attempt. Usually this would be a mare that had a great cycle, semen was of high quality, the mare ovulated on schedule, no post-mating fluid accumulation was noted, and yet no embryo was recovered. In addition, a ‘next-day re-flush’ may be performed if that was the only estrous cycle in which the mare was bred that year. In a retrospective study at CSU, three embryos were recovered during a total of 31 ‘next-day reflushes’ (9.7 %). In general, the recovered fluid is slightly cloudy and mild to moderate debris is present in the search dish on ‘next-day re-flushes’.

Unfertilized oocytes

An unfertilized oocyte (UFO) is occasionally recovered during an embryo collection attempt. Since prostaglandin E2 is required for embryo transport through the isthmus and utero-tubular junction, a UFO is usually only recovered if it passively follows a viable embryo. Two UFOs were recovered in a retrospective study of 208 flush attempts; in both instances an embryo was also recovered.

Embryo evaluation

Assessment of embryo grade, determination of developmental stage and measurement of embryo size are key components of an embryo evaluation. Accurate evaluation of embryos is important in a clinical embryo transfer program. For example, recovery of a small morula stage embryo from a Day-8 donor may dictate transfer into a Day-5 recipient mare that is synchronized more on embryo stage than on ovulation date. Another example is good cryopreservation success with embryos ≤ 300 µm in diameter and progressively less success with embryos significantly larger than 300 µm. Photographing embryos has become routine in many practices and the photograph is part of the donor mare’s medical record.

Recipient management

Acquisition of quality recipient mares remains one of the most important components of a successful equine embryo transfer program. The best advice is to be very careful and selective when acquiring recipients, only maintain good quality recipients and find alternative careers for recipient mares that do not fit into an embryo transfer program.

Culture and cytology

Collection of samples for uterine culture and cytology is an important part of the evaluation process for recipient mares. Samples for culture and cytology may also be collected at other points throughout the breeding season for recipient mares that do not become pregnant after transfer or mares that have echogenic fluid in their uterus on ultrasound examination. It is estimated that 5 to 10 % of potential recipient mares have light to moderate growth of a potentially pathogenic bacterium or a
positive cytology or both. Once identified, affected mares should be treated and recultured before ever receiving an embryo. Mares that continue to have a positive culture should be culled.

**Hormone treatment**

Recipient mares are ‘put under lights’ beginning on December 1 to advance the first ovulation of the year. Unfortunately, it is not always possible to have a sufficient number of recipients with natural ovulations in the first month or two of the breeding season for all of the embryos that need to be transferred. Consequently, hormonal management of recipient mares is a necessity. In 2016, a total of 40 embryos were transferred into deep anestrus or transitional mares that had been treated with 6.6 mg estradiol 17β for two consecutive days followed by 5 to 7 days of a short-acting progesterone preparation (200 mg, IM, q 24h). Over the same time period 65 embryos were transferred into recipient mares with natural ovulations. Pregnancy rates at day 14 were 85 % and 75 %, respectively (p>0.05).

Long-acting progesterone preparations (i.e. 1,500 mg, IM, once per week) may be considered after a recipient mare is determined to be pregnant. Administration of a long-acting progesterone preparation at the time of transfer is discouraged as mares that do not become pregnant may take a long time to return to estrus.

**Donor-recipient synchrony**

Historically, the range of synchrony between a donor and a recipient was considered to be +1 (recipient ovulated one day ahead of the donor) to -2 (recipient ovulated 2 days behind the donor). Recent work at several ET facilities around the world has expanded the range to -3 and occasionally -4. For example, in 2016, a total of 14 embryos were transferred into recipient mares that had ovulated 3 days behind the donor and 12 of those recipient mares became pregnant (85.7 %).

**Recipient size**

Although research has shown that a recipient mare can carry a pregnancy from a much larger donor mare to term and give birth to a healthy foal, clinical experience has indicated that owners expect that a recipient mare should be approximately the same size as the donor.

**Progesterone concentrations in recipient mares**

It is usually assumed that after an ovulation is detected, a normal corpus luteum will form and that adequate progesterone will subsequently be produced. An early study at CSU showed that 3.7% (9 of 242) of estrous cycles in recipient mares were associated with progesterone values of < 4.0 ng/ml at 5 days after ovulation. In 2016, progesterone concentrations were determined 5 days after ovulation during 218 estrous cycles in recipient mares. The mean progesterone value 5 days after ovulation was 9.4±3.7 ng/ml. A progesterone concentration of < 4.0 ng/ml was noted in 5.5 % of mares and a progesterone concentration of < 1.0 ng/ml was detected in 0.9% of mares (2 of 218). In the latter two cases, a corpus luteum apparently never formed after ovulation.

‘Two-strike rule’

In the CSU embryo transfer program, a recipient mare is usually not utilized again if she does not become pregnant after having received two Grade 1 or Grade 2 embryos within a single breeding season. Historical data have indicated that pregnancy rates are low following transfer of a third embryo within the same season.

**The 5-day check**

Recipient mares should be examined 5 days after ovulation to determine if they qualify to receive an embryo on that cycle. Mares that are graded as ‘acceptable’ on this examination are available for use as recipients for the next 3 to 4 days. Criteria for evaluation of a mare on the 5-day check include:

- Quality of estrous cycle (follicle growth pattern, edema pattern, ovulation detection)
- Presence and ultrasonographic quality of the corpus luteum
- Progesterone level (if available)
- Tone of the uterus
- Tone of the cervix
- Absence of uterine edema
- Size of the recipient relative to size of the donor mare
- General physical health
- Behavioral characteristics
- Absence of reproductive abnormalities, medical issues or behavioral concerns

Factors that disqualify or decrease the likelihood of using an individual recipient mare include:
- Poor quality cycle
- Ovulation within 2 days after receiving prostaglandin
- Ovulation of an abnormally small follicle
- Failure of ovulation or development of a hemorrhagic anovulatory follicle
- Absence of uterine edema during the cycle
- Presence of echogenic fluid within the uterine lumen during estrus
- Presence of fluid within the uterine lumen during diestrus
- Positive uterine culture or positive cytology
- Presence of a significant medical condition or behavioral issue

Transfer of embryos
Transfer of an equine embryo into a recipient is where the science and art of embryo transfer merge together. There are numerous minor variations on the general theme of how to transfer an embryo. A few clinical suggestions include:
- Always ultrasound the recipient mare immediately prior to transfer
- Lightly sedate the recipient mare (i.e. acepromazine, 20 mg, i.v.)
- Administer a non-steroidal anti-inflammatory drug prior to transfer (i.e. 500 mg flunixin meglumine, i.v.)
- Utilize a chemise to protect the transfer instrument from contamination
- Gently pass the transfer gun or pipette through the cervix without inserting a finger into or through the cervix
- Check the position of the instrument tip by palpation per rectum
- Gently deposit the embryo while slowly withdrawing the transfer instrument
- Consider using a Cassou gun and 0.25 ml straw for embryos ≤ 1,000 µm; use an insemination pipette for embryos > 1,000 µm
- Always rinse out the tip of a Cassou gun into a Petri dish after transfer to make sure that the embryo was not retained in the instrument

Management of the recipient mare after transfer
Minimize stress
Minimizing stress in the recipient mare after transfer is important to optimize pregnancy rates. It may be beneficial to keep the recipient mare in her original herd after transfer as opposed to moving her immediately to a different herd of mares. Anecdotal evidence suggests that social stress may adversely affect pregnancy rates in recipient mares.

Progesterone supplementation
It is probable that most embryo transfer recipient mares do not need supplemental progesterone. However, administration of exogenous progesterone or progestins to recipient mares following embryo transfer is routinely performed at some embryo transfer facilities and used sparingly or not at all at other facilities. The decision whether or not to supplement with progesterone is based on clinical experience,
value of the embryo and the perceived risk that luteal insufficiency may adversely affect embryonic survival in a given mare. Supplementation may include altrenogest (0.044 mg/kg; orally, once daily), short-acting progesterone (200 mg, IM, once daily), or long-acting progesterone (1,500 mg, intramuscularly, once every 7 days).

Progesterone supplementation may be discontinued at any time in early pregnancy provided that endogenous levels are measured and determined to be sufficient to maintain pregnancy (i.e. ≥ 4.0 ng/ml). Progesterone therapy may be discontinued between 45 to 70 days of pregnancy if an ultrasound examination confirms the presence of secondary corpora lutea or may be discontinued at 100 to 120 days of gestation without testing since the equine placenta produces sufficient progestins by day 90 to maintain the pregnancy.

An advantage of progesterone supplementation is that failure of maternal recognition of pregnancy (MRP) occasionally occurs in recipient mares, especially if the mare received a small, morula or early blastocyst stage embryo. Exogenous progesterone/progestin support can maintain a pregnancy in the absence of MRP.

Pregnancy examinations on recipient mares

Ultrasound pregnancy examinations are performed at very specific intervals based on embryo age (days 11, 12, 14, 16, 25 and 35). A majority of ET pregnancies can be detected by day 11 or 12. Small morula or early blastocyst embryos 150 to 250 µm in diameter may not become a visible embryonic vesicle in the recipient mare until day 14. The final check for early pregnancy status is on day 16 (embryo age). Early identification of pregnancy status allows for an early notification to an owner and facilitates subsequent breeding management decisions regarding the donor mare (i.e. rebreeding to the same stallion, switching to a new stallion or being finished for the season). Equine embryos grow at an average rate of 3 to 5 mm per day from day 11 to day 16 of pregnancy (Table 3). Failure to grow, reduced growth rate, or failure to advance in developmental stage may be associated with eventual embryo loss.

Empty trophoblastic vesicles

Empty trophoblastic vesicles (ETV) are occasionally noted after transfer of an equine embryo. An empty trophoblastic vesicle is defined as an embryonic (trophoblastic) vesicle without an embryo proper. An embryo destined to form an ETV often exhibits normal early growth (i.e. day 11 to 16). Ultrasonographically, an ETV is recognized as a static oval or irregular embryonic structure without an embryo proper after day 25 of gestation. Empty trophoblastic vesicles do not form endometrial cups, even if the vesicle is still present in the uterus after day 35. Once an ETV has been confirmed, the recipient mare should be administered prostaglandin to cause luteolysis and allow for a return to estrus. In a retrospective study of 820 embryo transfers at Colorado State University, 20 ETVs were detected on day 25 (embryo age), representing 2.4 % of all transfers and 3.3 % of day 16 pregnancies.

Pregnancy rate after transfer

Initial pregnancy rate

Pregnancy rate after transfer, pregnancy loss rate, and live foal rate are key statistics used to evaluate success of an embryo transfer program. Pregnancy data are difficult to compare among different publications, embryo transfer centers and years because of differing conditions and management systems. Table 4 is presented as a general guideline for initial pregnancy rates (i.e. day 16) after transfer of equine embryos in clinical practice.

Live foal rate

The ultimate outcome of a breeding program is birth of a live healthy foal. A retrospective study showed that there was no significant difference in live foal rate for embryo transfer recipient mares.
pregnant at day 16 (144 of 171; 84.2 %) as compared to mares pregnant at day 16 carrying their own foal (116 of 134; 86.6 %).

**Suggested reading**

Table 1. Embryo size on day 7 or day 8 after ovulation for mares bred with cooled or frozen semen.

<table>
<thead>
<tr>
<th>Semen Type</th>
<th>Day 7 (µm)</th>
<th>Day 8 (µm)</th>
</tr>
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<tbody>
<tr>
<td>Cooled</td>
<td>401.9 ± 19.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>716.9 ± 104.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frozen</td>
<td>258.2 ± 33.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>383.5 ± 54.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,bp<0.05; c,dp=0.0553</sup>

![Embryo Recovery Rates](image)

Figure. Embryo recovery and pregnancy rates following uterine lavage with complete flush medium or Hartmann's solution. There were no significant differences in either embryo recovery or pregnancy rates (p >0.05).

Table 2. Embryo diameter (µm) relative to day of the collection procedure.

<table>
<thead>
<tr>
<th>Collection Day</th>
<th># Embryos</th>
<th>Mean ± S.D. (µm)</th>
<th>Range (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>20</td>
<td>191.8 ± 13.2</td>
<td>150 to 325</td>
</tr>
<tr>
<td>7</td>
<td>183</td>
<td>354.0 ± 13.9</td>
<td>150 to 900</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>623.9 ± 72.9</td>
<td>150 to 2,500</td>
</tr>
</tbody>
</table>

Table 3. Diameter of the embryonic vesicle in pregnant recipient mares on days 11 to 16 (embryo age).

<table>
<thead>
<tr>
<th>Day of Pregnancy</th>
<th>Embryonic Vesicle Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>5.5 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>8.5 ± 0.1</td>
</tr>
<tr>
<td>14</td>
<td>14.9 ± 0.2</td>
</tr>
<tr>
<td>16</td>
<td>23.5 ± 0.2</td>
</tr>
</tbody>
</table>
Table 4. Guideline for evaluation of success of an equine embryo transfer program.

<table>
<thead>
<tr>
<th>Pregnancy Rate</th>
<th>Evaluation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 90 %</td>
<td>Outstanding</td>
<td>Difficult to consistently achieve with large numbers of transfers</td>
</tr>
<tr>
<td>80 – 90 %</td>
<td>Excellent</td>
<td>Achievable with significant effort</td>
</tr>
<tr>
<td>75-80 %</td>
<td>Very Good</td>
<td>A solid goal</td>
</tr>
<tr>
<td>70-75 %</td>
<td>Good</td>
<td>Work on details</td>
</tr>
<tr>
<td>60-70 %</td>
<td>Fair</td>
<td>Need to improve</td>
</tr>
<tr>
<td>&lt; 60 %</td>
<td>Marginal</td>
<td>Need significant improvement</td>
</tr>
</tbody>
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