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Equine Reproduction

Mini Series

Session Three: Embryo Transfer and Associated Advanced Reproductive Techniques

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CPD Solutions: **Embryo transfer and associated advanced reproductive techniques:**

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Learning objectives

- How to set up an embryo transfer programme at your practice
- Assessment of potential recipient mares
- Management of donor mares
- Synchronisation of donor and recipient mares
- Embryo flushing
- Embryo packaging for chilled shipment
- Embryo transfer

ESTABLISHMENT OF AN EMBRYO TRANSFER PROGRAMME IN PRACTICE

Applications of embryo transfer

The major application of embryo transfer is in enabling top class sports horse mares to carry on competing whilst producing foals, rather than having to defer their stud careers until their competitive careers have finished, by which time their fertility may have declined. Embryo transfer can also increase the number of foals produced per mare per year, and enhance the impact of individual mares on the breed. ET may also be appropriate for valuable mares which are unable to carry a foal safely to full term themselves e.g. due to previous colic surgery / a ventral rupture. Some subfertile mares with specific problems eg cervical injuries may be good candidates for embryo transfer. However, recent research shows that transferring embryos from old mares with a history of early embryonic death into young recipients did not increase foaling rates i.e. embryo transfer is not a useful technique in old mares with embryonic failure (because the fundamental problem is at the level of the aged oocyte)

Generally subfertile mares have lesser embryo recovery rates than normal mares, and their embryos have a higher failure rate post-transfer than embryos of normal mares, and this needs to be explained to clients if the ET process is not to be disappointing for everyone.

Equine embryo transfer has become fashionably popular over the last 10 years in the UK, and there is a demand for it which probably outstrips the number of mares who are genuinely successful enough to warrant using as embryo donors. ET is not a cheap technique (despite the facts that many vets are charging inappropriately little for performing it), and many of foals/youngsters being produced by ET in the UK at the moment may never sell for a price which is greater than the cost of producing them to saleable age. The increase in demand for ET has led to an increased desire of Practitioners to provide the service to their own clients. Embryo transfer work is time-consuming and labour-intensive, and Practices will need to charge appropriately to provide an economically viable service.

Minimum requirements for setting up an embryo transfer programme in Practice

Personnel
- Experienced AI Vet
- Enough lay staff to handle mares and foals at all times of night and day
- ? dedicated Lab technician

Facilities
- Stocks for mare examination / flushing / transfer
- Laboratory for embryo handling / assessment
- ? stabling / fields for donor mares
- ? stabling / fields for recipient mares
- ? recipient herd
- Frozen semen storage facilities

Equipment
- Ultrasound equipment
- A.I. equipment (fresh/chilled/frozen)
- Disposable ET equipment (see equipment list)
- E.T. guns (if not using disposable guns) and method of re-sterilisation
- Dissecting microscope

Sourcing of recipients
- Maintenance of a recipient herd (management: land; deworming; farriery; vaccination; disease control; dentistry)
- Owner provides own recipients
- ? ship cooled embryos to recipient herd

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SYNCHRONISATION OF DONOR AND RECIPIENT MARES

Initial Breeding Soundness Exams

In an ideal world, the Veterinarian would have input into which mares are suitable for use as ET donors and recipients. The reality is that the decision to use a mare as an ET donor is often made by the owner alone, who may be impervious to veterinary advice! Clinical experience in the twenty or more years since equine ET first started has proved conclusively that old mares which have failed to become pregnant by any others means are poor candidates for embryo transfer donation. Donor mares would ideally be not old (<12 years), in good general health and of normal fertility. Recipient mares should ideally be either young (<12 years) maiden mares in good general health or proven broodmares who have historically conceived and foaled easily. All of the normal considerations about a mare's ability to carry a foal to term (no COPD; not laminitic; no previous colic surgery etc) obviously apply to recipient mares. Ovariectomised, hormonally-treated mares have been used as recipients, but this is not common clinical practice. Because of the problems that can be experienced in synchronising mares, it is best to have at least 2 recipient mares available per donor mare for any given cycle.

Ideally, both donor and recipient should undergo a breeding soundness examination before the embryo transfer programme begins. In practice, this is often limited to the recipient mare. Proper selection of recipient mares is the most crucial part of an ET programme.

Breeding soundness examinations should include:

- Rectal exam
- Vaginal exam (with particular attention to the cervix in dioestrus)
- Ultrasonography of the reproductive tract
- Clitoral and uterine swabs for venereal diseases and uterine swab for aerobic microbiology.
- Uterine cytology
- ? uterine biopsy
- Screening for diseases such as EVA, EIA and Strangles

Synchonisation

Aim: recipient to ovulate one day ahead or up to three day behind the donor mare.

Ideal is for recipient to ovulate one day behind donor as the embryo transfer process seems to retard the embryo slightly.

Methods of synchronisation

Synchrony can be achieved naturally if a large enough band of mares is available as recipients. Otherwise, hormonal administration is required.

(i) If both mares are in mid- dioestrus with similar sized ovarian follicles when the programme begins:

- inject the donor with prostaglandin on day one and the recipient with prostaglandin on day two (ie 24 hours later). Three days after the donor was injected with prostaglandin, start examining the mares on a daily basis (ultrasound and palpation) for follicular development and signs of oestrus.
- When both mares are in oestrus and have at least one dominant follicle of at least 35mm diameter, inject the donor mare with an ovulation-induction agent such as hCG (Chorulon) or Deslorelin (ovuplant).
- Inject the recipient mare with an ovulation-induction agent such as hCG (Chorulon) or Deslorelin (ovuplant) 24 hours later than the donor mare.
- Inseminate the donor mare 24 hours after ovulation induction (fresh/chilled semen) or within 6 hours of ovulation (frozen semen). Monitor the donor mare and the recipient mare for ovulation – if the programme has worked well the donor will ovulate within 24 hours of insemination and the recipient will ovulate 24 hours after the donor.
- Monitor / treat the donor mare for post-breeding endometritis.

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(ii) If the donor and recipient are at disparate stages of the oestrus cycle when the programme begins:

- Treat both mares for 10-14 days with altrenogest (Regumate®) to lengthen the luteal phase of the cycle
- Scan and administer a prostaglandin injection to the donor on the penultimate day of progestin treatment and on the last day day of progestin treatment to the recipient, to regress any retained corpora lutea.(NB donor and recipient should be examined by ultrasonography on day 13 of progestin treatment, and if the size of their largest ovarian follicles is very disparate the timing of the prostaglandin injection should be adjusted accordingly e.g. if the donor has a very large dominant follicle and the recipient only small follicles, administer prostaglandin to both on the same day rather than a day apart as in this scenario the donor will reach the stage of ovulation more quickly than the recipient following prostaglandin).
- Proceed with monitoring, ovulation induction and insemination as for (i) above.

(The following regime has also been described but is not used by this author:

- Administer 150 mg progesterone + 10 mg oestradiol-17-beta in oil daily for 10 days.
- Follow by prostaglandin on the 10th day.
- Administer hCG when a 35 mm follicle is detected →70-75% of mares ovulating on days 10-12 after treatment termination.)

BREEDING THE DONOR MARE

Breeding an ET donor mare is no different from breeding any other mare except that particular attention must be paid to the timing of ovulation. Assess donors for post-breeding endometritis, and treat appropriately.
See notes for the first webinar in this mini-series.

SUPEROVULATION IN DONOR MARES

One of the major limitations to ET in the mare is the fact that mares physiologically ovulate once or twice on a given cycle, and that the number of embryos available for recovery is therefore limited. This in in contrast to other species, e.g. cattle. It is ironic that equine chorionic gonadotrophin (eCG) can be used to stimulate superovulation in other species such as cattle, but not in horses. Considerable research has been devoted to finding a superovulatory agent which works in mares. Pituitary extract is effective, but is not available to concerns about transmissible encephalopathies. A purified, commercially available equine follicle-stimulating hormone (eFSH) product was available in the USA, and is used as follows: 12.5 mg of eFSH is administered intramuscularly, twice daily beginning 5 to 7 days after ovulation when the diameter of the largest follicle is 20 to 25 mm. Prostaglandins are administered on the second day of eFSH therapy. Treatment with eFSH is continued for 3 to 5 days until follicle(s) are ≥35 mm in diameter. The mare is subsequently allowed to 'coast' for 36 h, after which human chorionic gonadotropin is administered to induce ovulation. Though this standard dose regime is reported to increase both the number of ovulations and the number of embryos recovered, regimes involving fewer days of administration of eFSH seem to increase the number of ovulations but not the number of embryos recovered.

EMBRYO FLUSHING IN THE MARE

Embryo flushing in the mare is a non-surgical trans-cervical technique which is minimally invasive and generally well tolerated. The flush is usually performed at day 7 or 8 post-ovulation for immediate transfer or chilling of embryos, and day 6 if the embryo is to be vitrified. Embryos are recovered more efficiently later but are more robust earlier. Recovery rates from aged mares may be improved by flushing on day 8 rather than day 7.

The technique will be covered in detail in the webinar. Briefly:

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Preparation

- Restrain donor in stocks, wrap and elevate tail, aseptically prepare hindquarters. All residues must be rinsed off.
- Using a sterile plastic sleeve-covered hand and arm, delicately introduce a uterine flushing catheter (80 or 150 cm long, balloon-tipped, silicone catheter) through the cervical opening and into the posterior uterine body.
- Fill the balloon cuff with 60-80 ml of air or water and pull the catheter caudally to establish a seal at the internal cervical os.

Technique

- Use 1000-3000 mls of commercially prepared equine embryo flushing media for each flush.
- Run the flush into the uterine lumen by gravity using Y-tubing attached to the foley catheter
- Depending on the parity of the mare and the size of the mare's uterus it may help to massage the filled uterus *per-rectum* before retrieval, to ensure that the fluid has reached all of the uterus.
- Run the fluid from the uterus back through the y-tubing and into an in-line embryo filter/cup.The filter must always be bathed in fluid to prevent embryo dessication and mus not be allowed to overfill.
- Depending on the parity of the mare and the size of the mare's uterus an i.v. injection of oxytocin may be given prior to the final flush to aid uterine evacuation.
- After the final flush, rinse the tubing before disconnecting it, in case the embryo is trapped between the cuff and the cup.
- At least 90% of the infused fluid should be retrieved.

EMBRYO PREPARATION AND TRANSFER

Where embryos are either to be transferred directly or chilled for shipment to a recipient mare, the initial processing and assessment is the same.

- Mark the underside of sterile Petri dishes with a concentric pattern to aid orientation during searching
- The fluid retained in the embryo cup/filter is placed in sterile Petri dishes by gently swirling the fluid in the cup and tipping in quickly into the Petri dish
- Rinse the cup using some of the flush media which has been retained for the purpose
- Examine the contents of the Petri dishes using a dissecting microscope (x 5- 10) to locate the embryo(s).
  - Gentle swirling of the Petri dish brings the embryo and any endometrial cells to the centre
  - Embryos are heavy and so sink to the bottom of the Petri dish
  - Push any cellular debris out the way during searching with a sterile semen straw NB Anytime an embryo is drawn into a handling instrument, the medium containing the embryo should be surrounded on each side by an air bubble and blank medium. This prevents the embryo from accidentally being lost from the instrument should the tip touch something absorbent. The process of picking up and depositing an embryo should be observed under the microscope to avoid "losing" the embryo.

- Transfer embryo(s) into a small Petri dish containing commercial embryo holding medium.

- Wash up to 6 times by transfer between small Petri dishes containing commercial embryo holding medium.

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- Assess embryo stage and morphology and grade 1 (good to 4 (poor).
- .The embryos can be stored up to 2-4 hours at room temperature (20-25°C) in holding media in the covered Petri dish before transfer.

Preparation of an embryo for direct transfer

- Under microscopic control, load the embryo into a 0.5 or 0.25 ml semen straw from the holding media as follows: medium / air/ medium+embryo/ air/medium.
- Place the loaded straw into an ET gun (sterilised or disposable).

Preparation of an embryo for chilling and shipment

- Place commercial embryo holding medi at room temperature in a 5 ml "snap-cap" tube, leaving a small air gap at the top of the tube.
- Carefully transfer the embryo into the medium and secure the cap by snapping shut and wrapping with parafilm ®.
- Fill a 50-ml centrifuge tube with commercial embryo holding media at room temperature
- Place the 5-ml tube containing the embryo is placed into the 50-ml centrifuge tube. Seal the cap of the 50-ml centrifuge tube, eliminating as much air as possible, and wrap with parafilm®.
- Place the packaged embryo into an Equitainer® ,which passively cools the embryo to 5ºC.
- Embryos can remain viable for at least 24 hours, during which time they can be transported to the embryo transfer facility.

For the process of embryo vitrifying see later in these notes.

Transfer of embryo into recipient mare

- Under microscopic control, load the embryo into a 0.5 or 0.25 ml semen straw from the holding media as follows: medium / air/ medium+embryo/ air/medium.
- Place the loaded straw into an ET gun (sterilised or disposable).
- Cover the ET gun with a sterile sheath
- Having prepared the recipient mare by emptying the rectum and washing, rinsing and drying the perineum, introduce the ET gun through the vulval lips and identify the cervix
- Without penetrating the cervix with a finger, introduce the ET gun through the cervix into the uterine body
- An assistant should pull back the sterile sheath
- Depress the ET gun to release the embryo whilst slowly moving the gun caudually so that the embryo does not get rammed against the uterine wall as it is released.

NB:

- Some clinicians sedate recipients with ACP
- Some clinicians pre-treat recipients with an “anti-prostaglandin” does of flunixin meglumine
- Some clinicians treat recipients with Altrenogest before and for some time after transfer.

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EQUINE EMBRYO FREEZING / VITRIFICATION

Long-term storage of embryos can be achieved by conventional cryopreservation or vitrification. Such techniques can be used to preserve valuable genetics, or to facilate prolonged transportation e.g. during importation or exportation or when recipients are not available. With conventional cryopreservation techniques require specialized equipment and ~1.5 h to complete. On thawing, the embryo is usually placed through a series of solutions to dilute cryoprotectants before transfer into a recipient's uterus, which makes the technique very difficult to perform in the field. In contrast, vitrification is a rapid procedure requiring <15 min. During vitrification, embryos are exposed to high concentrations of cryoprotectants before being plunged into liquid nitrogen. Thawing occurs within the straw without additional solutions or use of a microscope, and the embryo can be transferred directly into a recipient from the straw in which it was vitrified. Thus the vitrification procedure is more practical in the field than conventional cryopreservation of embryos.

Cryopreservation of embryos >300 µm has not been as successful as that of embryos ≤300 µm, and the success of the vitrification technique is dependent upon embryos ≤300 µm being collected. For this reason, and because they are more robust than older embryos, embryos for vitrification are usually recovered form the donor mare at 6 days post-ovulation.

A commercial vitrification kit is available, although there have been supply problems recently. Briefly, the vitrification process as described by Carnevale in the Proceedings of the 50th AAEP Annual Convention 2004 is as follows:

"*The embryo is first removed from the holding medium and transferred into the first vitrification solution (VS1) containing 1.4 M glycerol. The embryo can be gently moved in the drop of solution to dilute any transferred holding medium. After 5 min, the embryo is moved from VS1 to a second solution (VS2) that contains 1.4 M glycerol and 3.6 M ethylene glycol. The embryo will remain in VS2 for 5 min. The embryo is then placed in a 30-µl drop of the final vitrification solution (VS3) that contains 3.4 M glycerol and 4.6 M ethylene glycol. The embryo should remain in VS3 for <1 min, including time for loading the straw, until exposure to liquid nitrogen vapor. The final vitrification solution (30 µl) containing the embryo is loaded into a 0.25-µl, non-irradiated, polyvinyl chloride straw First, a 90-µl drop of dilution solution (0.5 M galactose [Gal] in PBS) is pulled into the straw followed by an air bubble (5 - 10 µl), the embryo in 30 µl of VS3, another air bubble, and a second 90-µl drop of Gal (Fig. 1B). Second, the open end of the straw is closed with two heat seals. Third, the straw is placed into a cooled plastic goblet (10 x 120 mm)] held vertically in liquid nitrogen within an insulated container (Fig. 2). Two large forceps can be used to suspend the goblet, with one forceps attached to the goblet and the second forceps holding the first forceps in position (Fig. 2). Liquid nitrogen will surround the goblet, but only vapor will surround the straw. Finally, after 1 min, the goblet and straw are plunged into the liquid nitrogen. The straw can then be transferred into a holding tank.*"

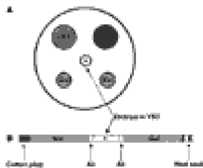

Figure 1. (A) A petri dish containing droplets of VS1 (200 µl), VS2 (200 µl), Gal (90 µl), and VS3 (30 µl) and the embryo. (B) The straw is loaded. VS3 (30 µl) containing the embryo is in the center. Dilution solution, Gal (90 µl), is loaded at both ends. The straw is closed by a cotton plug and two heat seals.

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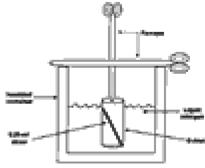

Figure 2. The straw is placed inside the goblet. The goblet is submerged in liquid nitrogen and held by two forceps.

Text and figures from Carnevale, E (2004) “How to collect and vitirify equine embryos for direct transfer” Procs 50th AAEP Convention 2004.

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