Integration of advanced assisted reproduction in the equine practice
Elaine M. Carnevale
Equine Reproduction Laboratory, Colorado State University, Fort Collins, CO

Abstract
Assisted reproductive technologies (ART) have been developed for the horse and can aid in the production of offspring from mares or stallions that are subfertile or infertile using standard breeding procedures or embryo transfer. Many ART procedures require special equipment or expertise, limiting their distribution in veterinary practices. However, as equine ART continue to develop, the equine practitioner has opportunities for new clinical endeavors and referral relationships. Established procedures for equine ART will be reviewed, with emphasis on clinical relevance, and the potential for ART in equine practices will be discussed.

Keywords: Equine, ART, oocyte, oocyte transfer, ICSI

Oocyte collection
The basis of many ART procedures is the collection and manipulation of equine oocytes. Oocytes can be collected from the maturing or immature follicles of live mares or from the harvested ovaries of deceased mares.

Oocytes collected from maturing follicles are typically collected from the dominant follicle of the estrous mare between two and 18 h before the expected time of ovulation. Maturation of the follicle and oocyte is often induced by the administration of a gonadotropin releasing hormone (GnRH) analog and/or human chorionic gonadotropin (hCG) after the follicle reaches > 33 mm in diameter. Most oocytes are at Metaphase I, when collected at approximately 12 h prior to the anticipated ovulation time, and at Metaphase II when collected within a few hours of the anticipated time of ovulation. Oocytes collected 12 h before the anticipated time of ovulation are cultured in vitro for 12 to 16 h before transfer or sperm injection to allow the completion of maturation. Because maturation is induced in vivo, the addition of hormones to the culture medium is not required.

Oocytes are easier to collect as the follicle approaches maturation, and the attachments of the granulosa and cumulus cells loosen. When oocyte collections were performed in our laboratory by personnel experienced in palpation techniques, but with no previous experience in transvaginal oocyte recoveries, oocyte collection rates were significantly higher when collection attempts were done at approximately 36 versus 24 h after hCG and deslorelin (65% versus 38%, respectively; unpublished data). However, with experience at the technique, oocyte collection rates between 70 to 80% are expected.

Procedures to collect oocytes are not overly difficult, but they do require practice for proficiency and training of support personnel. Minimal equipment is needed if oocytes are collected from large, preovulatory follicles through the mare’s flank. A trocar is used to place a cannula through the flank. The cannula will be used as a needle guide, as the ovary is manipulated per rectum. An advantage to the flank approach is that the needle does not puncture the vaginal wall of mares with chronic or severe uterine infections and potential vaginal contamination. The most common procedure for oocyte collections is transvaginal, ultrasound-guided follicular aspirations. This method requires an ultrasound console and probe; linear, curvilinear and sector probes have been used. The probe is placed in a casing containing a needle guide and positioned within the anterior vagina. The ovary is manipulated per rectum to position the follicle, while the procedure is imaged on the ultrasound monitor.

In most mares, the preovulatory follicle is relatively easy to position, and the needle can be used to puncture the follicular antrum while avoiding adjacent structures, such as the oviduct or broad ligament. When oocytes are collected from the dominant follicles of estrous mares, only one or two oocytes are available per cycle. The mare can be short cycled through the use of prostaglandin at five or six d after the oocyte collection, resulting in oocyte collections at approximately two-week intervals. Oocyte collections have to be scheduled to assure the follicle will not ovulate before collection of the oocyte. Scheduling of oocyte collections is dictated by follicular development and availability of semen.
Often, a window of only a few days is available to reliably induce follicular maturation and collect the oocyte prior to initiation of oocyte maturation in vivo and subsequent ovulation. Oocytes can also be collected without exogenous initiation of maturation and from the entire population of follicles on the ovary. This provides more follicles and, potentially, more oocytes per collection. The immature oocyte is closely adhered to the follicular wall within a hillock of cumulus cells. Oocyte collections are done using the ultrasound-guided technique to image and position the small follicles for needle punctures. Because the oocyte is closely adhered to the follicular wall, the follicle is usually repeatedly flushed and scraped. Oocyte collection rates (per follicle) vary with techniques and individual mares, but oocyte collection rate from immature follicles is often approximately half of the expected rate from preovulatory follicles. Oocytes can be collected at 10- to 15-d intervals. Hormones and growth factors are usually added to the maturation medium, with oocytes matured between 24 and 30 h. The viability of oocyte can vary with the different follicles and stages of development. Although we anticipate an almost 100% maturation rate (extrusion of a polar body with maturation to Metaphase II) for oocytes collected from preovulatory follicles, the maturation rate of oocytes collected from immature follicles is approximately half. Collection of oocytes from small follicles requires numerous punctures over the ovarian surface. Consequently, it is more difficult to avoid structures adherent to the ovary, such as the oviduct or broad ligament.

**Identification and handling of oocytes**

The identification and handling of oocytes is important for the success of subsequent procedures. In general, oocytes are more sensitive to temperature changes than embryos. The temperature of media and equipment should be carefully maintained at approximately body temperature, and fluctuations in temperature should be avoided. Collections from maturing follicles are not filtered in our laboratory. The flush medium contains granulosa cells that have become mucoid during maturation and will adhere to the filter and prevent media flow. The follicular flush from preovulatory follicles usually contains blood, as the complex vasculature network surrounding the follicle is disrupted. Identification of the maturing oocyte is facilitated by the clear mass of cumulus cells surrounding the oocyte. The oocyte is often polarized in appearance with a surrounding ring of cells, the corona radiata, in a very clear mass of cumulus cells that are translucent in appearance. Large numbers of granulosa cells can be obtained; they are usually in sheets, with a slightly dark and grainy appearance when compared to cumulus cells.

Follicular fluid and flush medium collected from small follicles can be filtered to help in finding the associated oocytes. Embryo collection filters can be used, but the mesh has to be carefully rinsed to remove the follicular cells and oocytes. Immature oocytes are often surrounded by a compact mass of cumulus cells which are difficult to differentiate from granulosa cells; therefore, care must be taken to properly identify the oocyte. After identification, oocytes should be quickly rinsed in appropriate medium and placed into culture.

**Oocyte collections in a clinical practice**

It is feasible to do oocyte collections in a clinical setting. However, conditions should be carefully controlled to assure cleanliness and attention to detail. Although infrequent, complications reported after oocyte collections include fatal hemorrhage, peritonitis and a thickening of the ovarian serosa. Careful sedation and handling of the mare and ovarian manipulations are important to avoid problems during oocyte collections. Equipment and procedures to handle oocytes need to be prepared in advance, as even short intervals of altered temperature or less than ideal media conditions can be deleterious to the oocyte.

**Oocyte transfer**

Oocyte transfer involves the collection and transfer of an oocyte from a donor mare into the oviduct of an inseminated recipient. Fertilization, embryo and fetal development occur within the recipient. Oocyte transfer is primarily used to obtain pregnancies from mares that have severely reduced fertility because of various problems within the reproductive tract, such as cervical, uterine or oviductal
pathology. However, the donor mare must still cycle and produce viable oocytes. The oocyte is transferred into the infundibular os of the oviduct during a standing flank laparotomy to expose the ovary and oviduct. In our laboratory, the recipient is inseminated 12 to 18 h prior to transfer and again at two or three h after transfer if a second dose of semen is available. When mares were inseminated before and after oocyte transfer with semen from two fertile stallions, 92% of the oocytes were fertilized from the insemination prior to transfer. We prefer to inseminate at least one billion progressively motile sperm to optimize the success of oocyte transfer. The transfer of preovulatory oocytes from the donor to recipient is the least manipulative ART procedure to produce pregnancies from otherwise infertile mares, as oocyte maturation is initiated in vivo, and fertilization and embryo development occurs within a mare’s reproductive tract. However the procedure is not effective for subfertile stallions.

For oocyte transfer, ovulation of a donor mare’s follicle has to be avoided to prevent fertilization of the incorrect oocyte. Different types of recipients have been used. Noncyclic or early estrous mares or mares with hormonally suppressed ovarian activity can be induced into estrus with the administration of exogenous estrogen while only small follicles are present on the ovaries; after transfer, progesterone or a synthetic progestin is administered to maintain the pregnancy. Cyclic mares can also be used after the collection of their own oocyte from their preovulatory follicle. In our laboratory, pregnancy rates after oocyte transfer are not different for cyclic or noncyclic mares.

Oocyte transfer is feasible under controlled clinical conditions. Although laparoscopic methods have been attempted, most transfers are done by standing flank laparotomy. The surgeon exposes and holds the ovary, while a technician manipulates the transfer pipette into the oviduct and expels the oocyte. Pregnancy rates are not different for oocytes cultured for the completion of maturation or collected just prior to ovulation and directly transferred into recipients’ oviducts. Collection of oocytes from preovulatory follicles and immediate transfers negate the necessity of equipment for oocyte culture. However, the oocyte must be maintained in proper medium and temperature conditions to maintain developmental potential even if it is only held outside of the mare for a short interval of time.

Gamete intrafallopian transfer and intracytoplasmic sperm injection

Although oocyte transfer is quite successful when good quality semen is available, other procedures must be used if sperm numbers are limited or sperm quality is poor. Classic methods for in vitro fertilization, in which the sperm and oocyte are co-incubated, have had limited success in the horse. Oviductal insemination with placement of a limited number of sperm and the oocyte into the oviduct have been tried in a process called gamete intrafallopian transfer (GIFT). Under experimental conditions, GIFT was successfully used to produced pregnancies, using $2 \times 10^5$ fresh sperm that had not been exposed to a semen extender and when transferred with oocytes into recipients’ oviducts. Although 82% of oocytes were fertilized when fresh sperm were used, fertilization rates were significantly lower for cooled and frozen sperm (25 and 8%, respectively). Therefore, although procedures for GIFT are feasible in a clinical settings, the semen requirements can make the procedure difficult.

Currently, the most reliable method of assisted fertilization in vitro is through the technique of intracytoplasmic sperm injection (ICSI). The procedure involves the selection and injection of a sperm into the ooplasm of a mature oocyte. Use of ICSI provides a sperm efficient method of fertilization, allowing offspring to be produced with limited quantities of sperm or poor quality semen. Typically, sperm used for ICSI are frozen and thawed, but the procedure can be done with cooled or fresh semen. Immature and maturing oocytes have been collected from donors and used to produce pregnancies for clinical cases with subfertile mares or limited or poor-quality semen.

Fertilized oocytes usually cleave into two cells within a day of the sperm injections. The early embryos can be transferred into the oviduct of a synchronized, cyclic recipient by standing flank laparotomy. Alternately, embryos can also be cultured in vitro until the morula or blastocyst stage of development; at this time, they can be transferred into recipients’ uteri. Embryos remaining in culture too long often try to unsuccessfully “hatch” from the zona pellucida. In vivo, the zona pellucida will thin as the blastocyst expands and an acellular layer, called the capsule, is formed between the trophoblast and zona pellucida. Under current culture conditions, the capsule is not formed, and the zona pellucida does
not fully expand. Therefore, embryo should be transferred into a recipients’ uterus before the trophoblast tries to extrude from the zona pellucida. The embryos can be transferred using standard procedures for transcervical embryo transfers, with the recipient synchronized to be four to six d after ovulation. Cells in the ICSI-produced embryos usually cleave at 12- to 24-h intervals, with formation of a blastocyst at approximately six d. However, some ICSI-produced embryos in our laboratory have been delayed by days in development and still resulted into viable pregnancies.

The ICSI procedure is difficult in most clinical situations. The micromanipulation and incubation equipment is expensive and requires clean conditions, a controlled environment, diligent monitoring, and regular upkeep. Specific expertise is required to conduct the procedure and maintain quality control. However, some options exist for the equine practitioner to incorporate the procedure into their practice without investing in the equipment and expertise for ICSI. Oocytes can be collected at one facility and shipped to a central facility for ICSI and embryo culture. When shipping maturing oocytes, we instruct referring veterinarians to ship them in a portable incubator maintained at approximately body temperature. The oocytes can be collected in the afternoon at approximately 20 to 24 h after hCG or deslorelin and shipped by ground parcel overnight. Upon arriving at their destination, ICSI can be performed. More flexibility may be possible for immature oocytes.

Once an embryo is produced, it can be transferred at the central facility, cryopreserved or shipped back to the facility of origin. We have shipped ICSI-produced embryos in cooled containers at approximately 5ºC or in portable incubators set at 37ºC.

**Harvesting of oocytes**

The equine practitioner can be tasked with having to euthanize a mare because of a medical condition. In an attempt to produce additional offspring, the ovaries can be harvested, and the remaining gametes collected. Equine practices intending to offer these services to clients should be prepared for ovarian removal and shipment and with contact information of referral facilities. The practitioner can make a general assessment of potential success based on ovarian activity and other factors associated with the mare. The ovaries should have some follicular activity. Immature fillies or old mares, mares under protracted stress, pain or medical treatments, and anestrous mares often do not provide good quality oocytes. When possible, the referral facility should be contacted prior to euthanasia for recipient preparation, timing of procedures, and shipping instructions. Ovaries are usually removed directly after euthanasia; however, if the potential occurs to obtain the gonads prior to euthanasia, for example during colic surgery, it could be beneficial to remove the ovaries prior to the administration of the euthanasia solution. After euthanasia, the easiest method to remove the ovaries is often to make an incision in the flank area, locate the ovary, and cut the surrounding ligaments and connections. The second ovary can usually be identified and removed through the same incision. Alternately, a second incision can be made in the opposite flank or a midline incision can be used. The ovaries should be kept as clean as possible to avoid contamination of the tissue and gametes. Upon collection, the ovaries should be rinsed in a physiological salt solution, such as an embryo collection medium. The ovaries are then placed in clean, plastic bags with a small amount of medium and secured.

If the interval to the laboratory is less than one h, a temperature of 37 to 38ºC is preferred; if a longer shipment is required, the shipment temperature should be lowered to cool room temperature or approximately 20ºC. The ovaries should not be held at 5ºC or colder for any length of time. In our laboratory, offspring have been produced from ovaries reaching the facility after almost one d after removal. However, the shortest possible interval of transport is preferred. In some cases, shipment by overnight ground courier is more economical and reliable than connecting air flights.

Alternately, oocytes can be collected at the facility of origin and shipped in culture medium in a portable incubator or at room temperature. For oocytes to be collected at the facility of origin, proper techniques for oocyte collection and handling are needed. The firm attachment of the cumulus oocyte complex to the follicular wall prevents the oocyte from being easily aspirated from follicles, and the follicles are often incised and scraped using a bone curette. Oocytes must be placed in an appropriate
medium for shipment, which can usually be obtained from the referral facility if sufficient time is available. Harvested oocytes are usually cultured for 24 to 30 h for in vitro maturation prior to oocyte transfer or ICSI. Equine oocytes have been successfully cryopreserved, warmed and resulted in viable foals; the efficiency is not high. Therefore, harvested oocytes are usually transferred, or ICSI-produced embryos are shipped to another facility or cryopreserved if appropriate recipients are not available. The first clinical offspring from harvested, shipped ovaries was born in 2002, and more offspring have been successfully produced from some referral facilities with oocytes or ICSI-produced embryos transferred at the facility or after shipment to another location.

Summary

As advances are made in ART, the equine practitioner has new opportunities to promote new clinical procedures or provide referral consultations. Establishing a facility for some ART procedures, such as ICSI, is not feasible for many clinics. However, establishing methods to collect and ship oocytes or transfer ICSI-produced embryos can be incorporated. Services, such as harvesting and shipping ovaries or oocytes, can be relatively easily incorporated into most equine practices.

References