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Application of equine oocyte recovery and assisted reproductive techniques to clinical practice
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Assisted reproductive techniques (ART) are increasingly used to produce foals from mares with reproductive abnormalities, from stallions with limited semen availability or quality, or both.1-5 While currently available in limited locations, it is possible to provide access to these services in private clinical practice. Most sophisticated reproductive practices can add a little equipment and, with proper preparation and practice, be able to recover oocytes useful for ART. These can then either be used for oocyte transfer or shipped to an ICSI (intracytoplasmic sperm injection) laboratory. Blastocysts resulting from ICSI can be transferred to recipient mares, frozen for future use, or shipped back to the referring veterinarian for transfer.

Oocyte development and follicular dynamics
A diverse population of follicles can exist on a single ovary at any given time during the estrous cycle, during transition, or at any time other than deep anestrus. Other than oocytes in pre-ovulatory follicles, oocytes in antral follicles are in the germinal vesicle (GV) stage. Germinal vesicle oocytes will differ in cytoplasmic maturity or atresia and thus vary in competency to resume meiosis. Oocytes are held in meiotic arrest by the follicle until luteinizing hormone (LH) activation, but the cytoplasm will mature over time. When removed from follicles oocytes will attempt to resume meiosis as soon as they are released from follicular inhibition.

Oocytes and associated cumulus masses in dominant follicles undergo significant changes as ovulation approaches. The granulosa cells surrounding the oocyte become the cumulus cells as part of the cumulus oocyte complex (COC). The cumulus mass expands in size as the cells secrete extra-cellular matrix, decreasing the firmness of the attachment of the oocyte to the follicular wall until the COC is released at ovulation. As a result of the LH surge the oocyte resumes meiosis characterized by germinal vesicle breakdown and formation of the meiotic spindle. The oocyte reaches metaphase II, characterized by extrusion of the first polar body, shortly before ovulation, and remaining in metaphase II until fertilization.

The right oocyte for the job
Oocyte requirements for ART vary between the individual techniques and the laboratories performing them. Oocyte transfer is most frequently performed with oocytes from dominant follicles stimulated (DSF) with an ovulation-inducing agent such as deslorelin or human chorionic gonadotropin (hCG). Oocyte recovery, transfer, and insemination of the recipient is based on the timing of the ovulation inducing agent maturation of the oocyte.6 Based on laboratory preference, ICSI can either be performed with DSF oocytes with similar timing or with immature oocytes (IMM) in the GV stage recovered from small follicles and matured in the laboratory. The DSF oocyte has the advantage of having undergone natural selection and maturation, generally being the highest quality oocyte available from a given mare. It has the disadvantage of being temperature sensitive (the meiotic spindle can depolymerize from only mild and brief temperature fluctuations) as well as a limitation in numbers with only one or two dominant follicles per cycle. Immature oocytes can frequently be recovered in greater numbers, have decreased temperature sensitivity until meiosis resumes, and recovery is not dependent on the stage of estrus, simplifying scheduling. However, IMM oocytes have greater variation in competence.7

Oocyte culture basics
Basic oocyte culture should provide for temperature, pH maintenance, osmolality, electrolyte balance, and nutrition. Most oocyte culture systems are based on Medium 199 (M199), a classic tissue culture medium. There are two general formulations, Hanks’ salts and Earle’s salts. The Hanks’ formulation, generally with 25mM HEPES, uses a phosphate buffer system to maintain pH in room
atmosphere, while Earle’s includes a bicarbonate buffer system that maintains physiological pH in a 5% CO₂ atmosphere. Serum is added at 10% for pH stabilization, cellular nutrition, and growth factors. Pyruvate is added, being an important energy source for the oocyte and cumulus cells. Since phosphates and HEPES at a concentration above 10mM have been shown to be detrimental to oocyte viability in culture, Hanks’ salts M199 is generally reserved for short-term culture or handling in room atmosphere, while Earle’s M199 is more commonly used for longer culture and maturation in a 5% CO₂ atmosphere.

Immature oocytes will attempt to resume meiosis once removed from the follicle, but require additional hormones for most effective maturation. Formulations for in vitro maturation (IVM) media vary but invariably include follicle stimulating hormone (FSH). Conversely maturation of IMM oocytes can be delayed by holding them at room temperature overnight for one to two nights. This can be particularly useful for appropriate timing of ICSI in the laboratory, since most IVM protocols call for 30 hours of maturation, helping to limit laboratory procedures to normal business hours. The ability to delay maturation also greatly facilitates transport of these oocytes to an ICSI laboratory. Holding at room temperature without the benefit of a CO₂ atmosphere is usually accomplished with either EH medium (a combination of 40% Earle’s salts M199, 40% Hanks’ salts M199, and 20% serum) or embryo holding medium.

Temperature maintenance is critical for the preservation of the meiotic spindle in oocytes following GV breakdown. While some repair may be possible over time, a decrease in temperature for 1.5 minutes to 32°C can depolymerize the meiotic spindle. Reduced culture temperature in a DSF oocyte shipping protocol has been shown to dramatically reduce ICSI blastocyst production, even when oocytes were allowed time for spindle repair.

What techniques are applicable to clinical practice?

Oocyte transfer (OT) is the procedure most applicable to clinical practice if the entire procedure is to be carried out in an individual practice. Oocyte transfer requires the ability to recover DSF oocytes, maintain them in appropriate conditions prior to transfer, the availability of a synchronized recipient whose own oocyte has been removed or a hormone-treated non-cyclic recipient mare, good quality semen, and the ability to perform surgical transfer of the oocyte into the recipient oviduct. With practice, many practitioners can successfully perform this procedure. It is however, logistically demanding, time consuming, and can be recipient mare intensive. Like all ART procedures results can be significantly affected by mare age and oocyte quality.

Oocyte recovery and transport to a laboratory for ICSI is probably applicable to many more practice situations. This allows the veterinarian to recover oocytes from client mares with reproductive abnormalities as well as for the production of embryos from semen of limited availability or in vivo fertility. Practitioners equipped for embryo transfer need only add a few pieces of equipment in addition to practice time to recover useful oocytes. Recovery of IMM oocytes can be scheduled in advance on a regular basis, simplifying addition to a busy practice schedule. Protocols for transport of both DSF and IMM oocytes for ICSI have been shown to provide similar results to on site collection and ICSI, and are currently being successfully commercially used.

The establishment of ICSI program itself can be a quite daunting project for any practice and should not be entered into lightly. The equipment expense and technical skills are significant, but even without these considerations, the creation of a complete successful laboratory program is an elusive goal that has escaped many. The ability to create and maintain an effective ICSI program that is a service to clients is probably not practically attainable in most situations.

Oocyte recovery

Oocyte recovery is a limiting factor in any ART program, the methods will vary according to practitioner preference and the type of oocyte required. Flank aspiration of DSF oocytes for either OT or ICSI using a trocar, cannula, and needle has been shown to be highly effective, and requires very little equipment. It is of limited usefulness however for aspiration of IMM oocytes from small follicles. Collection of oocytes immediately following the death or euthanasia of mares by scraping follicles with a
bone curette can provide a useful service to mare owners, since the GV oocytes recovered can be held and transported at room temperature in a less harsh environment than in a disembodied ovary. The most versatile technique for oocyte recovery is ultrasound guided transvaginal follicular aspiration (TVA) since this technique allows for collection of oocytes from both dominant and small follicles under ultrasound visualization. It does however require additional equipment and some time to develop the skills for proficient oocyte collection. The complete TVA procedure has been described.\textsuperscript{18}

Transvaginal aspiration TVA utilizes an ultrasound probe holder and needle guide to allow the operator to place the ultrasound probe and ovary in close proximity, separated only by the vaginal wall. This provides the visualization necessary to accurately direct a needle into follicles 6mm and larger. Practitioner preference and equipment availability will influence the selection of micro-convex or linear ultrasound probe. The orientation of the micro-convex probe allows easier access to ovaries of mares with short suspensory ligaments and vaginal vaults while the linear probe may allow the operator additional surface area to stabilize the ovary for needle penetration. A double lumen 12 gauge needle is used to allow simultaneous aspiration and flushing of the follicle. Suction is maintained with a vacuum pump regulated to -150mm mercury. Transvaginal aspiration will vary somewhat depending on the follicle being aspirated. Since DSF oocytes are very temperature sensitive, warmed flushing solution (usually heparinized embryo flush medium) and warmed collection bottle, search dishes, and microscope stage are warranted. Since DSF are generally aspirated 24-30 hours following gonadotropin stimulation, the attachment of the DSF COC to the follicle wall is decreased relative to those in IMM follicles. Turbulence caused by infusion of flushing medium and by manual manipulation of the follicle and aspiration needle facilitates dislodging and collection of the COC. Conversely, aspiration of IMM follicles requires turbulence and scraping of the follicle wall with the aspiration needle for good recovery, as the COC is more compact.

Transvaginal aspiration with successful and efficient oocyte recovery takes not only an experienced operator and proper equipment, but also assistants that are well versed in the procedure. The operator may handle not only the ultrasound probe and the rectal manipulation of the ovary, but the needle advancement and placement itself. Other practitioners prefer to have a separate individual handling the needle. While a foot pedal may operate the aspiration pump, the infusion of flushing medium requires another person with the ability to read the ultrasound image to provide adequate filling and refilling of each follicle.

Oocyte transport

Oocyte transport can provide clients access to ART programs through their reproductive veterinarians. Mares that otherwise would be retired or shipped to an ICSI facility can stay in the care of their veterinarian and have oocytes recovered and shipped to an ICSI facility. One study showed no reduction in blastocyst production rates utilizing transport protocols compared to the standard on site protocols.\textsuperscript{11} Timing as well as temperature and pH management are critical in the collection and transport of the DSF oocyte since meiosis has resumed following gonadotropin stimulation. The timing of stimulation will dictate the timing of collection and transport so that the oocyte arrives at the ICSI laboratory prior by 40-42 hours following stimulation at a time when the laboratory is prepared to perform ICSI. Oocytes from DSF have the highest rates of blastocyst formation, but the timing and transport requirements can be difficult to fit into some practice environments. Collection, shipping temperature and timing requirements of IMM oocytes are somewhat more forgiving, while aspiration of small follicles may be somewhat more difficult for the beginning aspirator.

What to expect

Initiation of an oocyte recovery program for ART can be both a stimulating and frustrating proposition for the practitioner. The ability to deliver a greater level of service to the client by utilizing new techniques can be quite rewarding. Conversely the learning curve and time required to develop these techniques is somewhat surprising to many individuals. These are the first things to expect.
Transvaginal aspiration oocyte recovery rates for an experienced operator should be around 70% or more per DSF follicle. This is dependent on appropriate timing of gonadotropin injection, the response by the mare, and aspiration skill. Oocyte recovery from small follicles should be generally around 60% overall, but this is influenced dramatically by follicle size, with the highest recovery rates from follicles < 20mm. The number of IMM oocytes recovered is dependent on both the number and size of the small follicle cohort present on the ovaries of an individual mare. This in turn is influenced by the season of year, the breed, the age, and prior aspirations of the mare. Older mares will tend to have a smaller follicular reserve and subsequently fewer follicles present on the ovaries. Follicles tend to be more numerous during transition, although this is variable between individuals.

Blastocyst production from oocytes submitted to ICSI will vary from mare to mare and also between stallions. Research situations can produce blastocysts from 70% of DSF oocytes and 35% of IMM oocytes, but clinical results are lower than this in most situations, relative to mare age, oocyte quality, and semen quality. Based on this author’s experience, overall production of 35% from DSF oocytes and 20% from IMM oocytes may be anticipated with wide variations for individual stallions and mares.

References