How to add oocyte collection to your equine reproductive practice
M. R. Schnobrich
Rood and Riddle Equine Hospital, Lexington KY

Introduction
There is an increased demand for intracytoplasmic sperm injection (ICSI) derived embryos in recent years in the equine industry. The increased popularity of this procedure is likely due to several factors including: increased familiarity of equine breeders with the procedure, an increase in personnel able to perform oocyte collection, and an improvement in the technique leading to increased success with the procedure. The following paper will outline the rationale for introducing oocyte aspiration for ICSI in clinical practice, and provide one facility’s equipment, methods, results and clinical impressions regarding development and implementation of this procedure (transvaginal aspiration of oocytes from immature follicles) in a private practice setting.

Background
In vitro fertilization (IVF) in the horse is most commonly performed using ICSI. Traditional methods of gamete co-incubation for fertilization, as performed in bovine or human assisted reproduction is not reliably successful in the horse, with this difference due to low rate of sperm penetration through the equine zona pellucida. Intracytoplasmic sperm injection was developed in the human field of reproduction to address male subfertility by allowing injection of sperm into the oocyte, with the first child born from ICSI in 1992. The first report for commercial equine production of ICSI derived embryos was presented in 2007, this study reported a 58% oocyte recovery rate on transvaginal ultrasound-guided aspiration (TVA) of immature follicles, an average of 10 immature oocytes per aspiration session, a 12% blastocyst development rate per injected oocyte, and a 55% pregnant following transfer into recipients. Several studies have followed which have reported an improvement in oocyte recovery from immature and dominant stimulated follicles (aspirated after administration of an ovulation inducing agent), blastocyst development rates and pregnancy rates following transfer. The increased success of ICSI from TVA oocytes has prompted several practices to become proficient in the procedure of oocyte collection, with shipped oocytes making up more than half of the oocytes used for ICSI in some centers.

In general the procedure for the creation of ICSI derived embryos includes the following steps: 1) recovery of oocytes from immature or dominant stimulated follicles, 2) maturation (in-vitro for immature, in-vivo for mature) of the oocyte to a point when metaphase II (M II) of meiosis (intact oolema and visible polar body) has occurred and 3) fertilization (ICSI) performed by injection of an immobilized spermatozoan into the cytoplasm of the oocyte 4) culture of embryo for approximately seven days until the blastocyst stage is reached, and finally 5) transfer of the embryo in the blastocyst stage into a recipient mare. Several complete reviews of this process have been previously described, and the reader is referred there, as well as a discussion on the pros and cons of collecting immature versus mature oocytes for ICSI.

The results of several commercial ICSI centers have been made available and have demonstrated reasonable efficiency of the procedure. Communication of reasonable expectations are based on these studies, and the client should be informed on the current expectations of success for each stage of the procedure to prevent unnecessary disappointment or expectations. In general research has shown that for oocyte aspiration in clinical practice a 50-70% recovery rate of each immature follicle aspirated should be expected, and an 80% recovery rate for dominant stimulated follicles (DSF). For immature oocytes collected from normal mares, in-vitro maturation rate to the MII stage has been reported to be approximately 65%. Following ICSI, the percentage of embryos that proceed to blastocyst range has been reported from 12-23%, and is highly dependent on the laboratory used. Once the embryo has reached the blastocyst stage and has been transferred into a recipient, there is a reported increase in pregnancy loss in ICSI derived embryos with an expected 50-65% live foal rate. When explaining to the client what to expect, we usually inform them that it will likely take two-three aspirations sessions,
recovering 8-12 oocytes to result in a live offspring. Obviously mare age, health, semen quality, shipping conditions and laboratory processing, recipient mare health and quality can all deleteriously effect these numbers.

The clients’ mares that benefit from the option of obtaining of oocyte aspiration/ICSI derived embryos compared to traditional embryo transfer include the ability to bypass the uterus, oviducts, and abnormalities in ovulation. Mares with chronic uterine infections, suspected oviductal pathology, or mares that repeatedly have hemorrhagic anovulatory follicles and have had reproductive success prior to these pathologies make good candidates. In addition, for clients with limited access to semen (deceased stallion, subfertile stallion, limited semen available), ICSI derived embryos may be a reasonable alternative, as only one sperm is required for fertilization. In the case of untimely death of a mare, or impending euthanasia, oocytes can be harvested from the ovaries and used for ICSI as well.

The costs associated with the procedure are generally considered more expensive than traditional embryo transfer and for most practitioners the cost of oocyte aspiration will be separate from the cost of ICSI and recipient mare fees. In general we advise clients that the foal should be worth approximately $10,000.00 to justify the cost of the procedure. Comparison of several oocyte collection and ICSI centers have given the following range of costs:

1) Transvaginal ultrasound guided aspiration of immature oocytes (1 session: $500-$1,000)
2) Packaging and shipment overnight of immature oocytes (US domestic $300-$600)
3) Maturation, ICSI and embryo culture ($2,500-$4,000)
4) Transfer into recipient ($300-$700),
5) Cost of pregnant recipient mare ($1,200-$3,600)

Deciding if oocyte aspiration is right for your practice

The decision to proceed with the addition of oocyte aspiration in your practice will obviously vary with the individual practice. Addition of oocyte aspiration services allows a practice to provide clients the option of maintaining mares at home/nearby, and still having performing ICSI for reproductive management. The expense of the equipment required and the time needed to master TVA and achieve acceptable oocyte recovery rates, as well as access to mares to practice the procedure on, should not be overlooked. It is also vital to the success of setting up an oocyte aspiration service to establish a working relationship with a reputable ICSI center, one that is consistently achieving expected maturation, and blastocyst rates and is willing to assist in training or troubleshooting when needed. Many of the materials noted below were suggested in a previous review of how to start TVA in practice in 2013, but have been repeated here with some additional notes. In addition, what follows is a very simplistic explanation of the materials and protocols involved in transvaginal ultrasound-guided aspiration of oocytes from immature follicles, much discussion and prior research has been conducted and is debated as to the optimal conditions, methods and materials to use, and the reader is referred to the resources listed as the end of the review for a more in-depth discussion.

Materials needed for TVA of immature oocytes

1) Ultrasound machine and transducer with the ability to achieve a reasonable image for transvaginal ultrasound guided aspiration. Often machines with poor resolution make it difficult to determine if follicles have been successfully entered with the needle, and air and artifact on some machines will make an already difficult procedure, fruitless and frustrating. In our practice we use an Sonosite M turbo with a micro-convex transducer (5-8 Mhz frequency range, 10 cm depth). Others have successfully used a linear rectal transducer with fitted adapter for needle guide, that can be found supplied by several companies. The cost of an ultrasound machine ($6,500-$100,000), and microconvex transducer ($2,000-$12,000) ranges greatly and depends most on the quality of image you are willing to work with.
2) Transducer/needle guide. There are many variations of cases/guards that provide a rigid case to enclose the transducer and needle, maintaining a static relative position and the ability to manipulate the case easily to facilitate ovarian/transducer/needle positioning. It is important that the case/guard can be opened and the components properly cleaned between each aspiration, with gluteraldehyde being the disinfectant of choice. These cases are often supplied by ultrasound companies, companies that supply TVA equipment, and some practitioners have even molded their own from plastic with success. The guide we use is a plastic pre-manufactured needle guide for a microconvex probe. The cost of the needle guide ranges approximately from $200-$3,000.

3) Needle for aspiration. In our practice we use a 60cm, double-lumen,12 gauge stainless-steel oocyte aspiration needle. One will also need to purchase a needle sharpener, that is recommended with the needle, to prevent dull or burred edges which make precise follicle puncture difficult. Cost is approximately $300-$500 per needle.

4) Aspiration source with foot controlled on-off and adjustable control of negative pressure. The aspiration device should have a pressure relief valve and the ability to maintain 150-300 mmHg for follicular aspiration. The pressure should be monitored and should ideally not exceed 300mmHg. An aspiration source with foot pedal allows the aspirator and assistant to control when negative pressure is applied and prevents continuous aspiration when the needle is not in a follicle. Cost ($300-$2,000).

5) Plastic or glass collections bottles (250ml-500ml), maintained at 37⁰C, with stopper. Cost is $6-$60/container, depending on if you use disposable or glass and re-sterilize.

6) Tubing for connection of needle to collection vial and collection vial to vacuum. Cost ranges from $40-$100, and disposable tubing is recommended.

7) Embryo transfer low-volume filter (75 micron pore diameter) is used to filter the collected follicular fluid following aspiration of all follicles. The filter is rinsed into a Petri dish and evaluated with a dissecting microscope for oocyte isolation. All rinsing of flush dish and filter is performed using flush media and non-latex syringes. Cost of the filter dish can range from $15-$35, and some are resterilized and used again.

8) Dissecting microscope with 40x magnification ability. A heated stage is recommended if one will be aspirating mature oocytes to ensure minimal fluctuations in temperature. Cost ranges from $2,000-$16,000, and is highly dependent on image quality desired.

9) Follicle flush medium is a standard embryo flush medium with heparin 5 IU/mL added. It is recommended to use a standard embryo flush medium with human medical grade heparin added. We use approximately 2-4 liters per aspiration session. Cost of flush fluid ($15-$35).

10) 20mL all plastic, non-latex syringes for flushing follicles and rinsing filter following oocyte recovery.

11) Oocyte holding medium: This medium will be used for oocyte holding and overnight shipment for immatute oocytes. We use a standard embryo holding medium. The cost of a single 6mL vial is approximately $3.

12) Oocyte shipping container: Oocytes must be maintained (room temperature immature, approximately 37⁰C for dominant stimulated/mature) at the same temperature during shipment to protect from temperature fluctuations. These incubators/shippers must be reliable and be able to
maintain a set temperature for a minimum of 24 hours. The cost of different incubators/shipping containers ranges from $2,000-$6,000 and though some use standard equine semen shipping containers, it is thought these are not adequate to hold temperature stable.

Method of immature oocyte collection

Mare preparation

Prior to the day of aspiration, the mare is evaluated by transrectal palpation and ultrasound to determine the number of follicles present. In general aspiration is attempted when the largest number of follicles (5-20mm) are present due to the higher oocyte recovery from follicles of this size, and that very large follicles can increase the difficulty of the procedure, and too few follicles will lower the number of oocytes potentially recovered. The mare is administered sedation (detomidine HCl 0.01-0.02 mg/kg and butorphanol tartrate (0.01-0.02 mg/kg IV) and systemic antibiotics (ceftiofur crystalline free acid (Excede) 6.6 mg/kg IM), immediately prior to entering stocks. Fecal material is evacuated from the rectum and transrectal ultrasound and palpation are performed, with all follicles >5mm measured and recorded. The mare is then administered N-butylscopalammonium bromide (0.9 mg/kg IV) to facilitate rectal relaxation, and the perineum, vulva and vestibule are aseptically prepared with Ivory™ soap.

Equipment set-up

The aspiration device/vacuum, collection vials, and the ultrasound with transducer are placed on a cart to the opposite side of the dominant palpation arm. The general set-up for transvaginal ultrasound guided aspiration of immature oocytes is depicted below, and letters denote sources of products listed in the footnotes above.

![Figure. Drawing of general set-up, and equipment for trans-vaginal aspiration of immature oocytes.](image-url)
Once transrectal ultrasound is completed with the linear transducer, the microconvex transducer is placed, in its case is changed into the ultrasound and the guard protected with a sterile sleeve. The person who will be performing the aspiration places a sterile sleeve and glove on and using copious sterile lubricant at the level of the transducer footplate, covers the probe with a sterile sleeve.

The needle is placed through the guard and flush medium with heparin is flushed through the needle and all tubing and containers flushed and primed so minimal air will enter the follicle upon aspiration. The needle is retracted into the guard to avoid trauma while positioning the case with transducer and needle in the anterior vagina.

**Follicle aspiration**

Sterile lubricant is applied to the hand and the guard with transducer is advanced through the vulva and vestibule to the anterior vagina. The footplate of the transducer is positioned to the 10 or 2 o’clock position of the anterior vaginal wall. Mild pressure is applied with the non-dominant hand to ensure contact with the transducer and vaginal wall and the image is monitored as the dominant hand is withdrawn and placed rectally for transrectal positioning of the ovary against the anterior vagina and transducer.

The follicle that will be aspirated is manipulated so that the needle, when extruded, will enter at the widest diameter of the follicle. Rotation of the probe and ovary together can facilitate positioning, and once ideal positioning occurs the hand holding the ovary can be used to brace the ovary against the probe to minimize movement. The needle is extruded into the follicle carefully by the same operator with the non-dominant hand and aspiration is initiated. Once the follicle has completely collapsed, fluid is pulsed by an assistant who is also controlling the foot pedal for aspiration pressure and the screen is monitored to ensure positioning. Complete aspiration and refilling of the follicle is performed ideally 5-10 times and then repeated another 5-10 times with the ovary manipulated and needle rotated to ensure scraping of the follicular wall at the time of follicular collapse. Once the follicle has been aspirated additional follicles in the same path of the needle are attempted, or the ovary is manipulated with needle still in place until another follicle aligns appropriately and the needle is then advanced into the next follicle. Negative pressure/aspiration is applied only when the needle is in the ovary or a follicle or when the lines are flushed to facilitate clot removal.

**Filtration**

Following aspiration all fluid is filtered through the embryo filtration cup and rinsed with additional flush fluid if hemorrhagic until fluid is clear. As much fluid as possible is removed and the contents of the dish are rinsed into a search dish, and oocytes are identified using the dissecting microscope.

**Identification and packaging of immature oocytes**

Oocytes are identified as characteristic, approximately 150 micron round oocytes with or without the cumulus cells at 40x magnification with a dissecting microscope. It is crucial to thoroughly tease out clots and debris as often oocytes become adhered and can be easily missed. Once identified, the oocytes are aspirated into a a glass micropipette or 0.25mL frozen semen straw, and transferred to a dish containing embryo holding medium (1-3mL). Ideally the holding medium is temperature matched to the flush fluid (by the end of the search this is room temperature), and all oocytes are placed in the dish until one is confident no more can be found in the initial search dish. The oocytes are transferred to a 5mL plastic Eppendorf tube containing fresh embryo holding medium, filled to within a few mm of the top, sealed in parafilm and packaged into the temperature controlled shipment container which is maintained and set at ambient temperature. The entire procedure from start to finish takes an average of 1-3 hours. Shipment is made for overnight delivery.
Results

Three boarded theriogenologists participate in oocyte aspiration in our practice. Two visited an ICSI center for training for 2-5 days within the first month of practice to receive guidance on the procedure, and one had prior experience. To train, each practitioner performed 5-15 aspirations on recipient mares prior to performing client aspirations. The first year 2 mares (average age 23 years) were aspirated with a total of 5 sessions with an average of 2.4 oocytes recovered. Two ICSI centers were sent oocytes and neither had a live foal produced.

The second year 12 mares (average age 16.6 years) were presented for oocyte aspiration with a total of 28 sessions with an average of 3.8 oocytes collected/session. Oocytes were sent to three ICSI centers and the number of live foals produced could not be verified. The third year 31 mares (average age of 18.4 years) were presented for oocyte aspiration with a total of 80 aspiration sessions with an average of 7.1 oocytes recovered/session.

Only one mare had complications associated with the procedure, as the mare was startled and jumped from the stocks with the vaginal probe in place and was euthanized to complications associated with the probe rupturing her aorta. Approximately 2-5% of the aspiration sessions will result in mild rectal irritation (evidence of blood on the sleeve), but no complications have been associated with this. No abscesses or systemic illness have been caused to our knowledge.

Discussion

The greatest advantage to adding this service to our practice is the flexibility it gives clients who want to pursue ICSI but do not want to move their mares at a remote site for oocyte aspiration. The greatest difficulty of providing this service was becoming proficient with the aspiration procedure, which was aided by the availability of recipient mares in the off season. One considering the addition of this service should expect a training period to master immature follicle aspiration, and close communication with an experienced and reputable ICSI center to ensure optimal results. The addition of this slightly time consuming procedure should be weighed in light of current case load, and client need, as often the time required to perform an aspiration can be very disruptive during the busiest time of the breeding season. The income provided by this additional service has paid for the equipment cost and is now a profitable service for our practice. In addition we have felt that the service has been beneficial to our clientele and ourselves as practitioners.

References


Footnotes
aUJIFILM Sonosite, Inc. Bothell, WA
bCidexPlus, Johnson and Johnson, Irvine, CA
cNeedle guide for micro-convex transducer, Minitube of America, Inc, Verona, WI
dAspiration needle: 60-cm, double-lumen, 12-gauge oocyte aspiration needle, Minitube of America, Inc, Verona, WI
eEquine Follicular Aspiration pump, Minitube of America, Inc, Verona, WI
fOocyte 500mL collection bottles (19009/4107), Minitube of America, Inc, Verona, WI
gNon-latex stopper for collection bottle (19009/4102), Minitube of America, Inc, Verona, WI
hTubing for Equine Oocyte Aspiration Set (19009/4101), and Equine Follicular Aspiration and Injection Device Tubing Set (19884/0614), Minitube of America, Inc, Verona, WI
iControlled Flushing Set CFS36, vented spike, 90cm tubing, and bi-directional valve, Mila International, Florence, KY
jEmbryo Low Volume Filter (04137), ICPbio Reproduction, Spring Valley, WI
kEmbryo holding solution, 6mL vial , ICPbio, Reproduction , Spring Valley, WI
lHeparin sodium injection, Sagent Pharmaceuticals, Schaumburg, IL
mMicro Q oocyte shipping incubator, Micro Q Technologies, Scottsdale, AZ