Equine oocyte recovery and ICSI in clinical practice: what can I expect?
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Abstract
Embryo production by ICSI is becoming a common clinical procedure in the horse, but little information is available on the expected outcomes of this procedure. Our clinical ICSI program at Texas A&M University sees about 450 cases per year (case = all the oocytes recovered from the ovaries of a mare on one aspiration session), both of oocytes recovered from immature follicles and those recovered from the dominant stimulated follicle. Over half of these cases are of oocytes shipped to the laboratory by referring veterinarians. The average number of oocytes recovered per aspiration of immature follicles at Texas A&M University is seven; after in vitro maturation this yields 4.5 mature oocytes per aspiration. The blastocyst rate achieved is 23% per injected mature oocyte, or about one blastocyst per aspiration session. Approximately half of these blastocysts produce ongoing pregnancies after transfer, for about one foal produced for every two aspiration sessions performed. These averages reflect only the findings of our laboratory; other laboratories may have different results.

Keywords: Embryo, equine, in vitro embryo production, intracytoplasmic sperm injection

Introduction
The first report on commercial equine use of intracytoplasmic sperm injection (ICSI) for foal production was presented by Galli and coworkers in 2007. They reported a 58% oocyte recovery rate on transvaginal ultrasound-guided aspiration (TVA) of immature follicles, providing an average of 10 immature oocytes per aspiration, of which 6.6 matured after in vitro culture. The blastocyst rate was 12% per injected oocyte, providing 0.85 blastocysts per aspiration, with a 55% ongoing pregnancy/foaling rate per blastocyst transferred. We presented a brief report on the findings from our clinical ICSI service for in vitro embryo production after aspiration of immature follicles for February through May of 2013. During this period, we performed 103 aspiration sessions and produced 119 blastocysts, a 20% blastocyst production per injected oocyte. Fresh transfer of 101 embryos yielded 82 pregnancies (82%). The final number of foals produced from these 82 pregnancies was 60, for a foaling rate per embryo transferred of 59%. Galli and coworkers summarized more recent results from their laboratory in a review paper in 2014, in which they reported a 66 to 70% oocyte recovery rate from immature follicles, providing nine to 12 oocytes per aspiration, depending upon mare breed. The blastocyst rate was 2% to 8% per injected oocyte, again depending upon breed, providing 0.3 to 0.8 blastocysts per aspiration. To the best of our knowledge, these are the only reports available on the expected efficiency of ICSI as a clinical method for foal production.

Currently, there is increasing interest in recovery of oocytes by referring practitioners for shipment to ICSI laboratories, and increasing awareness of ICSI on the part of horse breeders. Our laboratory at Texas A&M University started research on in vitro embryo production via ICSI in 2000, and has been offering ICSI clinically since 2009. We saw < 60 cases per year from 2009 to 2012. In 2013, the owner of a popular American Quarter Horse sire which had aged to the point of subfertility announced that the stallion was available for breeding only via ICSI with frozen semen. This had a notable effect on our ICSI caseload; it went from 63 cases in 2012 to 176 cases in 2013. In 2014, two more owners of popular aged stallions followed suit; our program increased in participation to over 450 cases. We have limited the caseload to this level subsequently. We define “case” as all the oocytes recovered from a given mare at one aspiration; for the purpose of this summary, if both immature and mature oocytes were recovered at the same time, i.e., subordinate follicles were aspirated at the time of aspiration of a dominant stimulated follicle (DSF), then this is counted as two cases, an immature oocyte case and a DSF case.
Here we discuss, based on our laboratory findings over the last three years, the basic parameters that can be expected by the referring practitioner in offering embryo production via ICSI to their client. These parameters are applicable only to our laboratory, as other laboratories may have different results.

**Oocyte recovery**

Recovery from the dominant stimulated follicle

Aspiration of the oocyte from the dominant stimulated follicle (DSF) immediately before ovulation is fairly easy to learn, so is a good place to start for the practitioner wanting to apply oocyte aspiration in their practice. Due to the large size of the follicle and the fact that it is being aspirated after a gonadotropin stimulus (thus the oocyte-cumulus complex is expanding and loosening from the follicle wall), the procedure is relatively simple and the recovery rate is high. Oocyte recovery from the DSF was first described by Vogelsang et al., in 1983; they used a needle placed through the flank. Aspiration of the DSF via transvaginal ultrasound-guided aspiration (TVA) was first described by Brück et al., in 1992. Subsequently, aspiration of the maturing oocyte from the DSF has been performed, both via the flank puncture and via TVA, for many purposes including study of oocyte maturation, study of follicle-oocyte interactions, recovery of oocytes for oocyte transfer, and recovery of oocytes for ICSI.

Detailed methods for oocyte recovery from the DSF, by both flank approach and TVA, have been presented previously. Whether done by flank approach or by TVA, the oocyte recovery rate from DSF should be 70% or better. Because the oocyte is maturing (is in the process of meiosis) at the time of recovery, care should be taken to keep the oocyte at body temperature (37 to 38.2°C) during handling and transport to the ICSI laboratory. Detailed methods for handling and packaging of DSF oocytes for shipment have been presented previously.

Briefly, to recover oocytes from the DSF, mares are monitored for follicle development during estrus, and an ovulatory stimulus (human chorionic gonadotropin [hCG] or a gonadotropin releasing hormone [GnRH] analog such as deslorelin) is administered when the operator thinks that the follicle will respond to it. The stimulus must be given before the mare’s endogenous luteinizing hormone (LH) signal has been received by the follicle, so that the stage of oocyte development at the time of aspiration can be timed definitively from the time of administration of the stimulus. Typically the DSF is aspirated 24 to 35 h after the ovulatory stimulus was administered. The oocyte will be cultured in vitro until the anticipated time that the donor would have ovulated, i.e. until about 40 h after administration of the ovulatory stimulus, and then fertilized.

However, because of the need for a) monitoring of estrus and follicle activity in the mare; b) timing of gonadotropin stimulation in relation to follicle maturity; c) timing of aspiration at a set point after administration of gonadotropin stimulation; d) special handling of the maturing oocyte; e) inability to schedule these procedures beforehand, due to variations in follicle growth, and f) the fact that aspiration of the DSF recovers at most only one oocyte, our laboratory rarely performs aspiration of the DSF. Aspiration of immature oocytes from non-ovulatory (immature) follicles allows exact scheduling of aspiration times, increases the number of oocytes available and thus the blastocysts produced per aspiration session, and simplifies oocyte handling.

Recovery from immature follicles

Aspiration of oocytes from immature follicles in the mare, via TVA, was first described by Cook et al. in 1992. Use of TVA is essentially required when immature follicles are aspirated; aspiration through the flank, which is done based on transrectal palpation, can be used to attempt to aspirate larger immature follicles (>20 mm) but visualization via ultrasound is necessary to locate and puncture smaller follicles. Typically, on TVA of immature follicles, all follicles ≥ 5 mm in diameter are aspirated.

Detailed methods for performing TVA of immature follicles has been presented previously. Because TVA involves placing a needle through the vaginal wall into the ovary numerous times per aspiration session, we evaluated the effect of this procedure on mare health in mares in our research herd over a three year period. Over almost 400 aspiration procedures and > 3,000 follicle punctures,
performed on all immature follicles on the ovary once every 14 days throughout the breeding season and without administration of antibiotics, only one complication, an ovarian abscess, was noted. Evaluation of ovaries on laparoscopy and after removal via ovariectomy showed minimal gross or histological changes. It should be noted, however, that ovarian abscess, rectal tear, peritonitis and even death due to hemorrhage remain potential complications of performing TVA.

The most important facet of TVA of immature follicles that the practitioner should take note of is that there is a very long learning curve, during which the procedure may be both frustrating and unproductive. At first, simply holding the ovary and probe in such a way as to puncture a follicle can be difficult, even for an experienced equine reproduction practitioner. We find that practitioners may attempt to perform TVA in their practice several times, and after finding it not possible to do effectively, abandon it. However, in our laboratory, we have found that essentially every veterinarian, given enough experience, can learn this technique. Since the closure of all equine slaughterhouses in the US in 2007, the source of oocytes for our research has been TVA in our herd of research mares. Thus we perform TVA of immature follicles, typically on three to five mares per day, one or two days per week, to obtain oocytes for our studies. All veterinarians in the laboratory participate in TVA, including those employed by the laboratory, graduate students, and visiting scholars. Given this schedule, and that for laboratory members there is no option not to perform TVA, we have found that after a variable period (typically 10 to 20 TVA attempts) all operators learn to perform the technique effectively.

In performing TVA of immature follicles, each follicle punctured should be recorded so that the operator can evaluate the rate of oocyte recovery per aspirated follicle. The operator should attempt to aspirate all follicles ≥ 5 mm diameter. This should lead to an average about 12-14 follicles aspirated per mare for Quarter-type mares; other breeds may have a higher average follicle number. Young mares may have more than this number of follicles, and older mares fewer. The operator should strive for an average recovery rate over a series of mares of 50% or greater. The recovery rate depends greatly on the technique of both the operator manipulating the ovary and the operator manipulating the needle. For each follicle we puncture, we aspirate the fluid, then rotate the needle to knock granulosa cells (and hopefully the cumulus-oocyte complex) free of the follicle wall, while moving the ovary so that the needle contacts different aspects of the follicle. We then fill the follicle with flush medium to suspend these cells, aspirate the fluid out, and repeat, for a total of six flushes. We also record the size of each follicle punctured. The recovery rate from smaller follicles is higher than that from larger follicles, probably because in the larger follicles the surface area is greater, decreasing the likelihood of knocking the cumulus-oocyte complex free with the needle.

The recovered immature oocytes are sent to the ICSI laboratory, where they will be subjected to in vitro maturation. Oocytes that mature (progress to metaphase II) will undergo ICSI. Detailed methods for handling, packaging and shipping immature oocytes have been presented previously.12 The most important thing for the practitioner to note is that oocytes are exquisitely sensitive to toxins; these can include volatile organic compounds in the air, fly spray, lingering antiseptic from washing the perineum, or deodorant or perfume from handling dishes or medium in a non-sterile manner. All media and supplies used for oocyte collection and shipment should be embryo-quality (tested to be non-toxic to embryos). Before sending client oocytes to ICSI laboratories for fertilization and embryo development, it is strongly recommended that the referring veterinarian send “practice” oocytes, recovered from non-valuable mares such as embryo recipient mares, to determine if the oocyte developmental competence has been maintained during handling, as evidenced by successful blastocyst production after ICSI with a control stallion.

**Blastocyst production by ICSI**

Immature oocytes

When immature oocytes are recovered, they are matured in vitro in the ICSI laboratory. About 65% of oocytes, on average, will mature to metaphase II and go on to ICSI. In about 9% of cases with
which we deal, no oocytes mature to undergo ICSI; this can be due to a low number of oocytes recovered, or to a low maturation rate for that lot of oocytes.

The results from ICSI with oocytes recovered from aspiration of immature follicles are variable. Overall, 20-25% of mature oocytes that undergo ICSI will produce blastocysts. However, the variability in blastocyst production is reflected in our finding that no blastocysts are produced in about 40% of cases. The proportion of cases resulting in one, two, three to five, and six or more blastocysts per case are approximately 30%, 15%, 13%, and 2%, respectively. The most blastocysts we have produced from one aspirate was 10 (from 15 recovered oocytes). Thus, the AVERAGE blastocyst production is 1.1 blastocysts per case, but the most likely outcome for any given mare is to produce no blastocyst. Currently there is little information about why this variation exists, for example it is not known whether aspiration of follicles at a certain time of the cycle, or a certain time in relationship to follicle wave growth, provides oocytes with greater developmental competence. We have found no difference in blastocyst development between oocytes recovered at the same time as is a DSF (thus, the immature follicles are at the decline of their follicular wave, and oocytes are recovered from them after hCG or deslorelin administration) and those aspirated during the luteal phase.

Oocytes from the dominant stimulated follicle

Since only one oocyte is available for recovery from the DSF, blastocyst production is limited to “yes” or “no.” The DSF oocyte has a higher intrinsic developmental competence, because it is the oocyte naturally selected to ovulate, and has undergone the majority of its meiotic progression in vivo. This is in contrast to immature oocytes, which may be from growing or atretic follicles at any stage of their development.

Theoretically, the DSF oocyte should give us the same blastocyst rate in vitro as we see with it after oocyte transfer, that is, surgical transfer of the mature oocyte to the oviduct of an inseminated recipient mare. This is not the case, however: Pregnancy rates after OT when research mares and stallions are used are typically ≥ 75%,9, 10 whereas the blastocyst rate after ICSI and in vitro culture of DSF oocytes in our laboratory, even for research animals, is only ~40%.18

In our clinical program, the blastocyst rate for DSF oocytes is higher than that for in vitro-matured oocytes (38% vs. 23%); however since only one DSF oocyte may be recovered per aspiration, and is recovered only about 80% of the time, the blastocyst rate per aspiration is much lower than is the blastocyst rate after recovery of immature oocytes (estimated 0.8 x 0.38 blastocysts/oocyte = 0.3 blastocysts per DSF aspiration, vs. 1.1 blastocysts per aspiration of all immature follicles).

Mare and stallion effects

Blastocyst production varies by both mare and stallion used. Colleoni et al. reported no difference in blastocyst rate after ICSI for eight mares with reproductive disorders compared to 22 normal mares.1 Unfortunately, we do not have good histories to determine which mares are presented for subfertility and which are presented because of stallion semen availability, especially in oocytes shipped to us by practitioners.

While both practitioners and ICSI operators are quick to point to mare age as a possible reason for low efficiency, the only significant finding we have had in relation to advancing mare age is a decrease in the number of follicles present on the ovaries at the time of aspiration.2 However, this can significantly affect results, because when few follicles are present, few oocytes are recovered, few mature oocytes are available and thus few blastocysts are produced.

We sometimes find low cleavage and blastocyst rates for sperm from a given stallion.19 This may not be related to the field fertility of that stallion or of that frozen semen; low cleavage rates could be due to either inadequate sperm quality, or, theoretically, to sperm having robust membranes that resist dissolution after ICSI. We have found that the method used for sperm preparation can significantly affect embryo development after ICSI in stallions with low embryo production.19

In stallions that have “aged out” and for whom only frozen semen is available, we recommend using semen that was frozen as early as possible in the stallion’s reproductive life. In humans, it has been
shown that paternal age can affect both the ability of sperm to produce embryos in vitro, and the health of the offspring produced, both in vitro and by natural conception.20

Results from shipped oocytes

Currently, over half of the cases in our clinical program originate from oocytes shipped to us from referring veterinarians. These include both oocytes recovered from the DSF and oocytes recovered from immature follicles. Recovery and shipment of oocytes from referring veterinarians has produced results equal to those for in-house aspiration. The maturation rate of shipped and in-house-recovered immature oocytes is similar at 60-65%, and the blastocyst rate achieved in our program with shipped immature oocytes is similar to that for immature oocytes recovered in-house (20 to 25% per injected mature oocyte). Results with recovery and shipment of oocytes from DSF by referring veterinarians (36 to 48% blastocysts per injected oocyte) have also been equivalent to what we obtain with in-house DSF aspirations (41%).

Pregnancy and pregnancy loss

In our program, all blastocysts produced are shipped for transfer, or are vitrified for later transfer, as we do not have a recipient mare herd. We recommend that in vitro-produced blastocysts be treated as for Day-6 in vivo embryos; that is, transferred to recipients that have ovulated 4 to 6 days previously. This is true no matter what day of culture the in vitro-produced blastocyst is seen to develop.

In our program, we find that in vitro-produced blastocysts have a good initial pregnancy rate (over 70%) but that about 25% of these pregnancies are lost, most before a heartbeat is recognized. This loss rate appears to be intrinsic to the in vitro system as the rates are almost exactly the same between oocytes collected from immature follicles and those collected from DSF, which should have optimal viability. A major current focus of our laboratory is to modify the in vitro system to minimize this pregnancy loss.

Conclusion

Overall, given a blastocyst rate of about 1 blastocyst per immature follicle aspiration procedure and an ongoing pregnancy/foaling rate of about 50% per transferred blastocyst, the clinical ICSI program at Texas A&M produces on average one foal for every two immature-follicle aspiration procedures. This is approximately the same efficiency as the per-cycle pregnancy rate for embryo transfer in normally-fertile mares; however, the potential for injury to the donor mare, the labor involved, and the cost are much greater for aspiration and ICSI than for direct ET. For this reason, ICSI should not be considered as a method to produce more foals from a normal mare in a given year. Intracytoplasmic sperm injection is an effective method to produce foals from mares that cannot produce embryos for transfer, or from stallions with limited semen stores.

The averages presented here are applicable to our laboratory only; other laboratories may have very different findings. Equine oocyte maturation, ICSI and embryo culture are complex systems that take both equipment and expertise to address effectively. Laboratories seeking to develop equine ICSI programs may find it difficult to establish an effective system, even when utilizing embryologists experienced with in vitro embryo production in other species.21 When using a laboratory for clinical ICSI, the referring veterinarian should first gain information on the number of cases the laboratory has done, the blastocyst rate per injected oocyte, and the ongoing pregnancy rate per transferred embryo, to determine if the laboratory is efficient enough to justify the expense to the client.

References