v. 2, n. 14, 2022

Journal of Agricultural Sciences Research

ICSI – INTRACYTOPLASMIC SPERM INJECTION IN EQUINES – A LITERATURE REVIEW

Taís Cristina De Oliveira Atanazio
Student of the Veterinary Medicine Course at Centro Universitário Nossa Senhora do Patrocínio - CEUNSP – Salto SP
https://orcid.org/0000-0003-3725-7136

Victoria Nunes dos Santos
Student of the Veterinary Medicine Course at Centro Universitário Nossa Senhora do Patrocínio - CEUNSP – Salto SP
http://lattes.cnpq.br/2940184002314150

Enore Augusto Massoni
Veterinarian at the Veterinary Complex at Centro Universitário Nossa Senhora do Patrocínio CEUNSP – Salto SP
http://lattes.cnpq.br/8241147224183513

Fernando Dal Sasso
Lecturer in the discipline of animal reproduction at the Veterinary Medicine Course at Centro Universitário Nossa Senhora do Patrocínio CEUNSP – Salto SP
http://lattes.cnpq.br/0059632837327140

Abstract: Intracytoplasmic sperm injection (ICSI) is a biotechnology applied in assisted reproduction in horses, in which it overcomes the barrier of inefficiency of *in vitro fertilization* showing better pregnancy results in the equine species. Biotechnology allows the use of high value-added semen that has low motility, it also allows the use of oocytes from old mares with high genetic value, or mares that have already died. The objective of this work was to carry out a review of the technique, and to show how efficient it is in the equine species, thus showing its advantages and disadvantages.
Keywords: ICSI, horses, breeding biotechniques.

INTRODUCTION

Equine farming has great economic and social importance and grows significantly every year. Data from the PPM (PESQUISA PECUÁRIA MUNICIPAL) show that the equestrian herd in Brazil increased by 1.9% in 2020 compared to 2019, in which a total of 5,962,126 animals were recorded in the country, with the Midwest being the region with greater number of horses. The equine industry proves to be important in the economy, and they see it as an innovative means of business in the market (MAPA, 2016). Due to these circumstances, the search for high genetic standards and a high added value to these animals, increased interest in the advancement of reproductive biotechnologies in horses (RUA et al., 2016).

With advances in science, man managed to bring about assisted reproduction techniques, instead of using *in vivo methods,* which would be fertilization as normally happens, the use of *in vitro fertilization was sought,* thus bringing genetic improvements, contributing to the search and optimization of better products (foals), and with desirable characteristics.

For *in vitro fertilization* of oocytes in horses, it is necessary to use intracytoplasmic sperm injection (ICSI), being an alternative to traditional IVF, using co-culture (environment that remove toxic metabolites and protect cells against oxidative stress) of oocyte and spermatozoa (PALHARES, 2019), therefore, ICSI is a more assertive technique in Brazilian equinoculture, in which a male gamete is required for each oocyte.

In this article, we seek to carry out a literature review on intracytoplasmic sperm injection (ICSI) in horses, describing the technique, the main advantages and disadvantages and its applicability in equine culture.

DEVELOPMENT

The bibliographic survey used the Scielo, Web of Science, PubMed, Science Direct, Google Scholar databases, data from a private laboratory and reference textbooks on Reproductive Biotechnology. The following terms were used as descriptors for the research: ICSI, biotechnologies in assisted reproduction, embryo transfer, in vitro production, equine culture. The searches were not limited by languages, and most of the articles chosen were in Portuguese, English and Spanish. In total, 24 scientific articles published in the last 6 years were used.

LITERATURE REVIEW

ICSI

Intracytoplasmic sperm injection was described in 1962 in sea urchins. In 1992, it was described in humans, being the technique elective in severe sperm alterations (SILVA; VERZELETTI, 2017). ICSI came to circumvent male infertility adversities in different species (KOCH, 2017), in horses it occurred for the first time in 2002 by Galli in Italy, 10 years later (2012) Brazil produced the first filly using the ICSI technique, through of an ovary of a mare that died. This tool has been used in horses, as it overcomes the inefficiency of

other biotechnologies, especially because this species has a lower reproductive capacity than the others (CARNEIRO, 2016), proving to be an effective technique and providing better pregnancy results using matured oocytes. *in vitro*

The application of the ICSI technique, after ET (embryo transfer), offers a high pregnancy rate, ranging from 50% to 80% (SALAMONE et al., 2017), in horses results in 60% accuracy. The use of biotechnologies, such as ICSI, has as its main objective to promote better reproductive efficiency (CARNEIRO, 2016), enabling the use of animals with better zootechnical and genetic indices, which have some reproductive disorder (RUA et al., 2016)

SELECTION OF DONORS

When we think of infertile mares they are less likely to produce an embryo in the ICSI technique and when compared to subfertile or fertile mares they produce 4 to 6 times less embryos. If infertility is the result of serious uterine abnormalities, such as chronic infections, embryo production is even less likely (CLAES et al., 2016). According to Cuervo -Arango et al. (2019), the donor's productivity history is extremely important, because mares with a high rate of embryo production tend to produce better results. It has already been described about mares that did not produce an embryo in a first attempt at OPU and ICSI, these were considered unfit to produce in the second (10/26; 38%). While mares that produced an embryo on the first attempt were able to repeat production on the next attempt (66/86; 77%) (CUERVO-ARANGO et al, 2019).

SELECTION OF MALE STALLIONS AND GAMET

As for the selection of stallions, those that stand out for their excellence and that have a good genetic background are chosen. For older stallions, it is recommended to use frozen semen that was collected when the respective stallion was younger (HINRICHS, 2016). Another important point is that the fertilization rates of oocytes are different according to the breeds, animals of the Arabian breed have lower fertilization rates, whereas the Quarter Horses and the Hot-Blooded have high fertilization rates within the technique. of ICSI (GALLI et al., 2016). The variation in actual fertility between breeds and the use of subfertile animals in the biotechnology result in low rates of development of zygotes to the blastocyst stage, which becomes the main factor limiting the technique. To increase the probability of success in the second OPU-ICSI session, it is very interesting to use semen from a different stallion, which can increase the chance of producing at least one embryo by up to 3 times (HINRICHS, 2012; CUERVO-ARANGO et al. al., 2019)

ICSI STEPS

TYPES OF FOLLICLE ASPIRATION

The technique relies on two types of follicular aspiration to obtain oocytes.

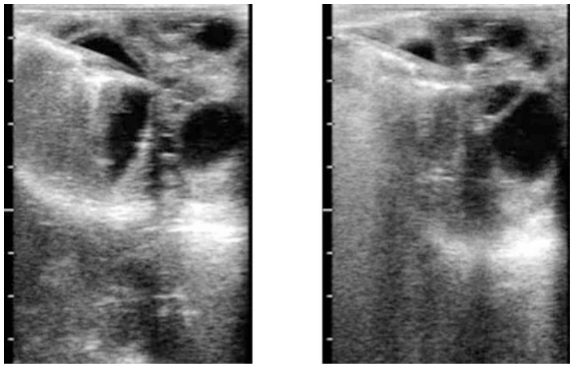

Figure 1 - Ultrasonography at the time of follicular aspiration (Source: Personal Archive).

ASPIRATION OF FOLLICLES *ANTE MORTEM*

Pre-ovulatory follicles are aspirated closer

to the time of ovulation, where they will naturally be ready to be fertilized. Because of this, this mare must be monitored and examined regularly to monitor the estrous cycle (MAK et al., 2018). The advantage of aspirating the pre-ovulatory follicle is that it is already ready to be fertilized, it is a mature follicle, in metaphase II. The disadvantage is the continuous monitoring and aspiration of this type of follicle, which is low compared to the other type of follicle.

The aspiration of immature follicles (rest phase of meiosis, which would be the prophase) can be performed at any stage of the mare's estrous cycle, it is not necessary to monitor and control follicular growth (HINRICHS, 2018). Aspiration at this stage is more complicated, with a greater degree of difficulty, as they are smaller follicles and form a cumulus- oophorus (agglomeration of granulosa cells that allow the oocyte to remain on the follicle wall).

When aspiration of immature follicles, which still need to undergo the *in vitro maturation process, is performed,* the follicles are subjected to ICSI 36-42 hours after collection, using gonadotropins and growth factors to help the oocytes reach metaphase II, in this way they are ready to be fertilized (SANSINENA, 2020). According to Carnavale, 2016, only about 50% of the oocytes are able to mature, reaching metaphase II, due to the high variation in the developmental state of these oocytes.

The OPU (ovum pick up) consists of keeping in mind the anatomy of the mare's reproductive tract, with the mare's ovaries having variable positions, and also thinking that ovulation occurs on the ventral surface of the ovary, in the ovulation fossa.

Before starting, it is necessary to administer sedative and analgesic (α 2-adrenergic: detomidine hydrochloride 0.01mg/kg IV and opioid: butorphanol 0.01mg/kg IV), (CARNEVALE, 2016). Good sedation must be performed to cause good rectal relaxation, facilitate manipulation of the ovaries, improve the process and prevent rectal lacerations.

After sedation, the mare's tail is hung up, the rectum must be emptied and the vulva and perineum are cleaned aseptically, and the bladder is emptied with a urinary catheter, enabling good handling (ORELLANA-GUERRERO et al., 2022)..

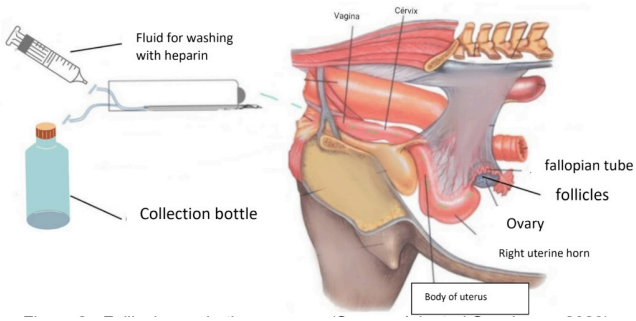

Figure 2 - Follicular aspiration process. (Source: Adapted Sansinena, 2020)

Aspiration is performed by a veterinarian, who must use a microconvex probe, inserted into a needle, so as to increase the rate of oocytes collected as shown in figure 2. 3 professionals are needed, the first manipulates the ovary per rectum and brings it caudally against the probe, facilitating the visualization of the ovarian follicles on ultrasound, keeping the ovarian ligament between the fingers, preventing the needle from reaching any important vessel or intestinal segment. Another assistant manipulates the needle, which advances through the wall of the vagina, entering the ovary and reaching the follicles. Another professional is responsible for washing and recording the aspirated follicles, using a needle connected to a suction pump, in which they are stored in a heated collection bottle (LOSINNO et al., 2019).

When the follicle is emptied, it is washed 8 to 10 times with heparin to avoid clots and clogging of the needle or aspiration system

(RODRIGUEZ et al., 2021). It is also necessary to rotate the needle to release the cumulus-oocyte complex from the follicle wall. After washing, a blood-stained liquid is expected, due to follicular scraping and proper washing.

After the procedure, it is ideal that you administer an anti-inflammatory due to the discomfort of the procedure, and use of antibiotics, avoiding inoculation of bacteria from the vagina to the intraperitoneal cavity (HERRERA, 2018). It is important to monitor this mare every 2 hours on the OPU day and twice a day on the following days, at least for 3 days (RODRIGUEZ et al., 2021). It is ideal for this mare to rest for at least 2 days after aspiration of follicles, and for sport mares for at least 7 to 10 days of rest (LOSINNO et al., 2019).

POST-MORTEM ASPIRATION

Figure 3 - OPU technique (ovum pick up), in ovary of a mare that died (Source: Personal Archive)

In females that died, the ovaries are removed and sent to the laboratory at controlled temperature. According to Hinrichs, the best results are when the ovary arrives at the laboratory in less than 6 hours, if the trip is longer than 6 hours, the ideal is to aspirate the oocytes immediately and send them. Oocytes are transferred to a Petri dish and searched for under a stereoscopic microscope. Finally, they are sent to the laboratory to be matured and fertilized (HINRICHS, 2018).

MATURATION OF OOCYTES

When we talk about follicular aspiration, we mention that we can aspirate two types of follicles, the pre ovulatory and immature follicles. Immature follicles, as they are smaller follicles, are at rest during meiosis, so their *in vitro maturation must be carried* out before performing ICSI (LOSINNO et al., 2019).

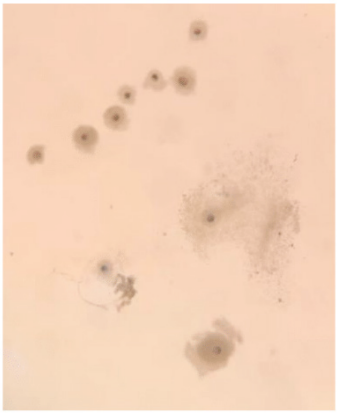

Figure 4 - Immature Oocytes (Source: Personal Archive)

The immature oocytes remain in the culture medium for their maturation in a time of 24-30 hours, in which they will develop to metaphase II, which is the ideal stage for performing ICSI (HINRICHS, 2018). This maturation takes place inside incubators, with temperature (38ºC) and controlled atmosphere (5-6% CO2 and 5% O2 to reduce the formation of superoxide radicals) (SANSINENA, 2020). At the end of the oocyte culture, the cumulus- oocyte complex is denudated by means of repeated pipetting with enzymes that help dissolve the thick intracellular matrix, allowing the polar body to be visualized and ICSI being able to perform (LOSINNO et al., 2019).

SEMEN PREPARATION

The semen can be fresh or cryopreserved (thawed in a water bath, with water at

a temperature of 37ºC for 20 seconds) and must be washed, placed in PVP (polyvinylpyrrolidone) medium, in order to reduce motility to facilitate the handling of the sperm where it will be aspirated by the pipette conducted into the cytoplasm of the oocyte. Finally, it will be transferred to a Petri dish, considering it as a micro injection chamber.

SPERM INJECTION

In this step, both gametes are manipulated by micromanipulators coupled to an inverted microscope and injected with a pipette associated with Piezo (device used to promote the insertion of a needle into the oocyte by piezoelectric movements) or the conventional method can be used. (SALAMONE et al., 2017).

The immobilization of the sperm is done by breaking the plasma membrane of the sperm tail, making it immobile, thus allowing communication between the cytoplasm of the sperm and the oocyte. (LOSINNO et al., 2019). In the conventional method, a micropipette with a sharp and beveled tip is used. The sperm is immobilized against the Petri dish and collected with the micropipette, due to the sharp tip of the micropipette, it enters the zona pellucida and plasma membrane of the oocyte until it reaches the cytoplasm, where the sperm will be injected. (HINRICHS, 2018). The method that uses piezoelectric units, the micropipette penetrates the plasma membrane of the oocyte more easily, because the piezoelectric units used cause electrical vibrations, and the micropipette works as a "drill". The sperm amid PVP, and suffering piezo pulses, undergoes a permeabilization in its plasma membrane, cutting its tail, mobilizing it, being good for its handling and for better communication with the cytoplasm of the sperm and oocyte (BRISKI & SALAMONE, 2022; RADER et al., 2016).

EMBRYO CULTURE

After ICSI, oocytes fertilized are placed in an incubator in a culture medium, so that a good embryonic development occurs, having control of the environment (CARNEVALE et al., 2019; LOSINNO et al., 2019).

Many laboratories that do not contain so much technology, evaluate the oocyte injected on the fifth day after ICSI, in which they must be in a morula stage, which is the moment when the embryos need to change culture medium, at this stage, embryos that have or have not undergone cell division are already visualized. Later, on days 7, 8 and 9 after ICSI, the stage to be reached is blastocyst, and in horses this stage is different from other species, being a structure with hundreds of cells, without a clearly differentiated blastocoel, which is recognized by the apparent organization in an outer layer.

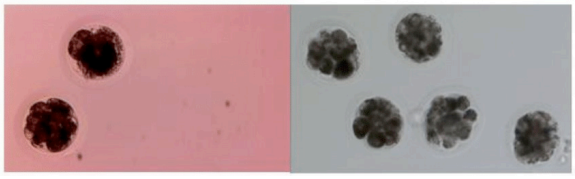

Figure 5 - Embryos in mitotic divisions (cleavage) (Source: Personal Archive)

Figure 6 - Embryos already packaged for commercialization (Source: Personal Archive).

ICSI ADVANTAGES AND DISADVANTAGES

As a great advantage when thinking about mares, we have the optimization of the reproductive management of

competing mares, in addition to allowing the reproduction of mares incapable of producing embryos, either due to reproductive problems associated with the uterus, cervix or oviduct, the technique also has the advantage of eliminating the risk of endometritis in the donor mares as they do not have contact with the ejaculate in them. In stallions ICSI allows the use of males with low quality and seminal production, with testicular degeneration due to age or those who died and have limited samples of frozen semen.

On the disadvantages, the first is that the number of oocytes aspirated from the follicles is low, as most mares only have one pre-ovulatory follicle, thus resulting in a lower efficiency in the rate of blastocyst formation (HINRICHS, 2018). The equipment and specializations that the technique requires limit its availability, and the time and cost of the technique also add to the list of disadvantages (HERRERA, 2018). We cannot fail to mention that the OPU procedure is invasive and has a considerable risk of laceration of the rectum and contamination of the abdominal cavity, causing peritonitis (STOUT, 2020).

FINAL CONSIDERATIONS

We conclude through this work that intracytoplasmic sperm injection has overcome the reproductive difficulties of the equine species. Showing that the probability of success of this biotechnology is influenced by the steps of each process. Finally, it is extremely important for the veterinarian to understand the factors that influence the effectiveness of the technique, thus ensuring the progress of equine reproduction.

REFERENCES

AIRES, A. R.; ROCHA, R. X.; MORESCO, R. N.; MENEGAR, C.; BERTO, T.; LEAL, M. L. R. **Efeito da suplementação de colina protegida no perfil metabólico e intervalo entre parto e concepção de vacas leiteiras**. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, v. 72, n. 2, p.553-559,2020. https://www.scielo.br/j/abmvz/a/hJWVpc5dCvLHqZZNxd764cb/abstract/?lang=pt

AIRES, A. R.; ROCHA, X. R.; TORBITZ, V. D.; MORESCO, R.; SOUSA, R. S.; SEVERO, S. L. S.; NAIBO, W.; SOSSANOVICZ, R. A.; PRETTO, A.; ORTOLANI, E. L.; LEAL, M. L. R. **Efeito da suplementação com colina protegida sobre parâmetros bioquímicos, produção e reprodução de vacas leiteiras no periparto.** Arquivo Brasileiro de Medicina Veterinária e Zootecnia, v. 68, n. 6, p. 1573-1589, 2016. https://www.scielo.br/j/abmvz/a/mT6ZsNGG4Dv5rnCYpMYqFcR/?lang=pt#

ARÉVALO, R. I.; RUIZ, G. L.; CHAGRAY, A. N.; SANDOVAL, M. R. **Asociación entre la presentación de endometritis y los niveles de calcio sérico en vacas lecheras de crianza intensiva** (Lima, Perú). Revista de Investigaciones Veterinarias del Perú, v. 32, n. 1, p. 1-9, 2021. http://www.scielo.org.pe/scielo.php?pid=S1609-91172021000100018&script=sci_abstract

BLOCH, V.; LEVIT, H.; HALACHMI, I. Assessing the potential of photogrammetry to monitor feed intake of dairy cows. Journal of Dairy Research, v. 86, n. 1, p. 34-38, 2019. https://www.cambridge.org/core/journals/journal-of-dairy-research/article/abs/assessing-the-potential-of-photogrammetry-to-monitor-feed-intake-of-dairy-cows/F0F896DF471A5F1D7171CA78D11E3A37

CARNEIRO, G. F. **Produção in vivo e in vitro de embriões em equinos.** Revista Brasileira de Reprodução Animal [online], Minas Gerais, MG, v.40, n.4, p.158-166, 2016. Disponível em: <http:// cbra.org.br/portal/downloads/publicacoes/rbra/v40.pdf>. Acesso em 08 abril. 2020.

CARNEVALE, Elaine M., Maclellan, L. J., & Stokes, J. A. E. (2019). **In Vitro Culture of Embryos from Horses.** Methods in Molecular Biology, *2006*, 219–227. https://doi.org/10.1007/978-1-4939- 9566-0_16

CLAES, A.; CUERVO-ARANGO, J.; COLLEONI, S.; LAZZARI, G.; GALLI, C.; STOUT, T. A. **Speed of in vitro embryo development affects the likelihood of foaling and the foal sex ratio**. Reproduction, Fertility and Development, [S.l.], v. 32, n. 5, p. 468-473, fev. 2020.

CUERVO-ARANGO, J.; CLAES, A. N.; STOUT, T. A. E. **Mare and stallion effects on blastocyst production in a commercial equine ovum pick-up–intracytoplasmic sperm injection program**. Reproduction, Fertility and Development**,** [S.l.], v. 31, n. 12, p. 1894-1903, oct. 2019a.

DIEGO, R., DOUET, C., REIGNER, F., BLARD, T., COGNIÉ, J., DELEUZE, S., & GOUDET, G. (2016). **Influence of transvaginal ultrasound-guided follicular punctures in the mare on heart rate, respiratory rate, facial expression changes, and salivary cortisol as pain scoring.** Theriogenology, *86*(7), 1757–1763. https://doi.org/10.1016/j.theriogenology.2016.05.040

FOSS, ROB. (n.d.). Retrieved May 17, 2022, from https://equmed.com/reproductive-services/icsi/

HiNRICHS, K. (2016). **Equine oocyte recovery and ICSI in clinical practice: what can I expect?** Proceedings of the Society for Theriogenology Annual Conference 2016.

HINRICHS, KATRIN. (2018). Assisted reproductive techniques in mares. Reproduction in Domestic Animals, 53(May), 4–13. https://doi.org/10.1111/rda.13259

KOCH, J. **Produção in vitro de embriões bovinos através de técnicas de micromanipulação**. 2017. 58 f. Monografia (Medicina Veterinária) – Universidade Federal de Santa Catarina. Curitibanos, 2017.

LAZZARI, G., COLLEONI, S., CROTTI, G., TURINI, P., FIORINI, G., BARANDALLA, M., LANDRISCINA, L., DOLCI, G., BENEDETTI, M., DUCHI, R., & GALLI, C. (2020). Laboratory **Production of Equine Embryos.** Journal of Equine Veterinary Science, 89, 103097. https://doi.org/10.1016/j.jevs.2020.103097

LOSINNO, L; SANCHEZ,; STOUT, T. (2019). **Equine Assisted Reproduction**

ORSOLINI, M. F. MEYERS, S. A. DINI, P. An Update on Semen Physiology, Technologies, and Selection Techniques for the Advancement of In Vitro Equine Embryo Production: Section II. Journal Animals, v11, n3319, 2021

PALHARES, R. C. F. T.; GONZAGA, D. R. N. **Injeção intracitoplasmática de espermatozoides aplicada à reprodução equina**. Revista V&Z Em Minas, Belo Horizonte, n. 142, jul./ago./set. 2019.

RODRIGUEZ, J., MASERATI, M., ROBILOTTA, T., AUGUSTO, G., ALONSO, M. A., REDOAN, M., TIBARY, A., & FlEURY, P. (2021). **Recovery of Equine Oocytes in Ambulatory Practice and Potential Complications**. Journal of Equine Veterinary Science, 98, 103324. https://doi.org/10.1016/j.jevs.2020.103324

RUA, M. A. et al. **Aspiração folicular, maturação in vitro e injeção intracitoplasmática em éguas**. Pubvet. Campos dos Goytacazes, RJ, v.10, n.3, p.248-256, Mar., 2016.

RUA, M. A. S.; QUIRINO, C. R.; PACHECO, A.; JÚNIOR, A. B.; VEGA, H. O.; RIBEIRO, M. S. **Aspiração folicular, maturação in vitro e injeção intracitoplasmática em** éguas**.** Pubvet, Londrina, v.10, n.3, p.248-256, mar. 2016.

SALAMONE, D. F.; RODRÍGUEZ, N. G. C. a. M. B. **Intracytoplasmic sperm injection in domestic and wild mammals.** Society for Reproduction and Fertility. Buenos Aires, Argentina, v.154, p.111-124, 2017.

SILVA, C. P. D.; VERZELETTI, F. B. **Avaliação do desenvolvimento embrionário através da técnica de ICSI (injeção intracitoplasmática de espermatozoide).** Cadernos da Escola de Saúde. Curitiba, PR. 11: 168-178. 2017.

STOUT, T. A. E. (2020). **Clinical Application of in Vitro Embryo Production in the Horse.** Journal of Equine Veterinary Science, 89, 103011. https://doi.org/10.1016/j.jevs.2020.103011

STOUT, T. A., CLAES, A., COLLEONI, S., LAZZARI, G., GALLI, C., & CUERVO-ARANGO, J. (2018). **Mare and Stallion Effects on Blastocyst Production in a Commercial OPU-ICSI Program.** Journal of Equine Veterinary Science, 66, 166. https://doi.org/10.1016/j.jevs.2018.05.060