Review Report

Intracytoplasmic sperm injection zone: insights and applications from a university-based assisted reproduction laboratory

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Abstract

Intracytoplasmic sperm injection (ICSI) has become one of the most important tools used for in vitro production of embryos (IVP) in equine reproduction management programs around the world. This procedure is often performed in the US for several breeds and is used primarily to optimize foal production for broodmares and performance mares but also for stallions with limited semen availability or poor semen quality. As such, there are a limited number of US laboratories capable of using this method, which requires advanced training of personnel and specialized equipment. Many veterinarians and breeding farms currently aspirate ovarian follicles in cycling mares and ship oocytes to ICSI-capable laboratories, where embryos can be produced and typically vitrified for ultralow temperature storage and transport for transfer to recipient mares and establishment of surrogate pregnancies. The Veterinary Assisted Reproduction Laboratory in the School of Veterinary Medicine at the University of California, Davis, maintains a 3-dimensional approach to equine assisted reproductive technology. We offer commercial solutions to breeders, educational advice and training to other laboratories, veterinarians, and visiting scholars. Moreover, as a research laboratory, our objective is to analyze and apply observations from IVP to elucidation of complex developmental problems such as embryonic and fetal loss. In 2018, we reported the birth of the first foal produced at UC Davis using ICSI, and we have since developed a commercial ICSI program for practitioners and horse breeders in the US. Today, our laboratory receives thousands of immature oocytes for ICSI sessions every year, with an average of 2 embryos per mare-session. Our research is focused on molecular, cellular and genetic aspects of gamete biology, perifertilization events, and early equine development using an array of tools including advanced microscopy, sequencing, and time-lapse imaging of developing embryos. In this review, we have highlighted our laboratory's current methods for commercial equine IVP and research and clinical studies conducted to optimize IVP in horses.

Keywords: Equine embryo, intracytoplasmic sperm injection, noninvasive time-lapse microscopy

Introduction

In the late nineteenth century, experiments in artificial insemination in animals were conducted in multiple research centers across Europe. The pioneering work of Ilya Ivanov and Walter Heape inspired an emerging generation of scientists to further develop this and other gamete techniques, with the first American Artificial Insemination (AI) cooperative in cattle established in 1938.[1] It took almost a century to commercialize embryo transfer and embryo transportation; embryo vitrification was introduced only in 1996.[2,3] Today, advanced technological solutions such as IVP, biopsy for preimplantation genetic diagnosis, embryo vitrification, and time-lapse microscopy have become routine procedures for equine embryologists in their endeavors to optimize pregnancy outcomes and further understand equine development, with the potential for involvement of artificial intelligence in the near future.[4]

Intracytoplasmic sperm injection (ICSI) was initially developed in 1992[5] as a natural development of subzonal

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insemination.[6] The resulting manuscript was published in the medical journal Lancet, which highlighted the worldwide impact of advanced reproductive technologies. As of 2024, ICSI remains the only commercially reliable method for IVP in horses.[7-10] In this species, several laboratories in the US and Europe routinely report ~ 20-25% success rate in blastocyst production with an average of ~ 2 blastocysts per successful ICSI session for most mares.[11-13] However, there has been at least one recent report regarding successful conventional in vitro fertilization (IVF) in horses with viable offspring obtained in a research setting.[14]

The Veterinary Assisted Reproduction Laboratory in the School of Veterinary Medicine at the University of California, Davis maintains a 3-dimensional approach to equine assisted reproductive technology. First, as a clinical service, we offer reproductive solutions to horse breeders and veterinarians (transvaginal follicular aspiration, oocyte in vitro maturation, postmortem gamete retrieval, in vitro embryo production, embryo biopsy and vitrification, intracytoplasmic sperm injection, cell isolation and storage for posterior cloning, and embryo transfer services). Secondly, as an academic institution, we provide educational support and training to veterinarians and visiting scholars. Finally, as a research facility, we aim to apply conclusions and findings from in vitro production of embryos to the elucidation of complex developmental problems such as embryonic and fetal loss. In the last ~ 5 years, our laboratory has placed special emphasis on the study of embryo morphokinetics using noninvasive imaging incubation technology, which allowed us to monitor embryo development in near real-time without removing embryos from the incubator to visualize them.[13] This innovation provides insight into not only fundamental equine biology, which is applicable to other species, but also allows us to optimize equine embryo production, serving the horse industry directly. In each ICSI session, time-lapse imaging provides an insight into the development from zygote to blastocyst stage, when the embryo is transferable into a recipient mare, and predict which embryos are most likely to develop to a healthy foal. We have also been focusing on advanced microscopy techniques, such as conventional and laser-scanning confocal immunofluorescence as well as genomic and transcriptomic approaches to gametes, embryos, and single-cell dynamics.

In 2018, we reported the birth of the first foal produced at UC Davis using ICSI, and we have since focused on developing a commercial ICSI program for horse breeders in the US. Today, our laboratory performs hundreds of ICSI sessions every year, with an average of 2 embryos per mare-session. An essential value of ICSI is that the shipment of oocytes and sperm does not require transportation of the animal, and valuable mares and stallions are not subjected to the risks and costs associated with transportation. Moreover, multiple embryos can be obtained in a single ICSI session and transferred into recipients located in different parts of the country. The demand for this service in the US and abroad is continuing to increase.

In this review, we discuss our practical approach to ICSI, lessons learned, and potential applications of this reproductive technology.

Laboratory protocols, general commentary

Embryologists in our laboratory are responsible for all media preparation, which is performed in a laminar-flow biosafety cabinet using reported preparations for maturation medium (discussed below).[11,15] Since our main interest is optimizing the use of gametes and ultimately producing more embryos, it is necessary to maintain the highest standard of hygiene as to not pose a threat to gamete, zygote, or embryo health. Positive pressure ventilation in the ICSI laboratory is useful to prevent the ingress of airborne particles when the entry door is opened. We refrain from the use of ethanol-containing solutions and instead, we use Oosafe® disinfectant (Oosafe, Inc, Hingham, MA 02043) for cleaning surfaces. Access is restricted to essential personnel, who use dedicated sets of shoes, scrubs, and laboratory coats when working in the ICSI room. Commonly, we avoid using gloves; thus, rigorous hand-scrubbing is required before entering the room. Use of scented soap, perfume, or lotions is forbidden, even when not working directly with embryos/gametes. We maintain a sticky floor mat by the door to control foot-carried lint and debris, and end-of-day daily sweeping for dust and debris using a Swiffer floor mop with dry pads sprayed with Oosafe® disinfectant after all incubators are closed for the night. When gametes or embryos are outside the incubator, dim light is used, and the door remains closed.

Science of intracytoplasmic sperm injection

In broader terms, there is a universal scheme for ICSI, regardless of the protocol or species specificity: gamete acquisition, in vitro maturation (IVM), sperm selection, fertilization using microinjection of a single sperm, in vitro culture (IVC) to the blastocyst stage, vitrification (optional), and embryo transfer into recipient mares. Although commercial media and their strictly prescribed protocols are readily available, embryo production methods in our laboratory are based on previously reported equine culture systems.[11,15] For purposes of this discussion, we refer to 'holding' medium as an oocyte transportation culture medium that is commercially available and used for oocyte shipping to our laboratory, and in which metabolic state is supported but does not allow oocytes to develop or otherwise mature. After a brief period, typically 24 hours in a commercial holding medium, the oocytes are transferred to a medium that supports IVM from the MI stage (no visible polar bodies) to the MII stage of gametogenesis, revealing a single polar body that indicates the resumption of meiosis. At this stage of development, the oocyte is prepared to undergo fertilization by a single sperm.

Although, many aspirations are performed in-house at UC Davis, our labortory, like most ICSI facilities, receives numerous shipped oocytes from referral practitioners performing aspirations in distant locations. We also obtain oocytes from postmortem ovarian retrieval. After IVM, both oocytes and sperm are evaluated. Oocytes that extruded their first polar body and motile sperm of normal morphology are then moved into the ICSI dish and the microinjection procedure is performed by an embryologist. In a typical session, the embryo is vitrified when it reaches the blastocyst stage of development at 7 to 10 days. Vitrified embryos are then express-shipped to veterinarians managing recipient mares for thawing and embryo transfer.

In special circumstances, alternative options are available depending on the animal's status. Although rare, we retrieve ovaries at the time of or shortly after euthanasia. Inevitably, postmortem oocyte recovery has a negative impact on the quality of oocytes that can be reflected downstream on the success of the ICSI procedure. The most critical factors for ICSI success in such cases were the number of collected oocytes

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and euthanasia being due to a nonacute medical condition.[16] Furthermore, pentobarbital penetrates follicles (detectable soon after injection), and our research suggested that this may impede both bovine and equine in vitro embryo production.[17]

Acquiring gametes for intracytoplasmic sperm injection

Transvaginal aspiration of oocytes

Transvaginal aspiration (TVA) of oocytes or ovum pick-up (OPU) is necessary to obtain oocytes from ovarian follicles. It is performed on standing, sedated mares, using an ultrasound-guided transvaginal probe with needle channel attached to an ExaPad ultrasound scanner (IMV Technologies, Inc.). Our group uses a Rocket Craft™ DUO-VAC vacuum suction pump (Rocket Medical plc, UK) connected to a 12G x 25″ double-lumen needle that is manually directed into each follicle (5-20 mm diameter) which is punctured and followed by manual needle agitation (necessary to scrape the follicular wall) accompanying follicular lavage. Each follicle is lavaged by injecting a small volume adequate to visibly expand the follicle and subsequently draining the fluid while observing the ultrasound flat-screen display. Although numerous factors contribute to variations in pump settings (length and diameter of tubing, needle length and inner diameter, and mare's movement/sedation status), we typically set the pump to aspirate at a flow rate of 20 ml/40 seconds, but the pressure measurement can vary under those circumstances. Follicles > 20 mm diameter are aspirated after the small to medium follicles (5-20 mm), and as the collapse of larger follicles distorts the ovarian size and shape, they are best aspirated last to optimize the smaller follicle aspiration. Although mares with very active ovaries with small to medium antral follicles are recommended as oocyte donors (10-15 follicles of 5-20 mm diameter), this is not always possible. When less than an optimal number of follicles are visualized (typically < 5-10 visible follicles), we recommend postponing transvaginal aspiration of oocytes by 2 weeks to allow for the mare's natural cycle to recover and generate a new cohort of aspiratable follicles, and then rechecking the mare's ovarian activity.[18-20] However, with owner consent, we occasionally schedule mares with ~ 3 immature follicles of 5-20 mm diameter; we make sure the owner understands that these mares typically yield lower numbers of transferrable embryos. For optimal results, which include at least 1 transferrable blastocyst, our recommendations are that between both ovaries, at least 12 follicles with 10-20 mm diameter be visualized at the day of aspiration. Regardless of the outcome, at least 14 days between sequential aspirations are required for health and safety concerns for donor mares. During that period, a new wave of follicles can develop as well. We can obtain oocytes from antral follicles throughout the year, including fall and spring transition periods, but this is not possible from most winter anestrous mares in Northern California.

For a TVA procedure at our facilities, mares are placed into a well-padded stock for standing restraint. Perineum, vulva, and tail base are aseptically scrubbed using a mild soap such as Ivory soap. Detomidine (0.01-0.02 mg/kg) and butorphanol (0.01-0.02 mg/kg) are used for standing sedation. Intravenous buscopan (0.32 mg/kg) is given to further relax the rectum. The procedure takes 30-45 minutes, but mares are usually monitored overnight prior to release from the hospital. Although safe, our research revealed that there was a significant transitory increase in serum lactate, total protein, and peritoneal neutrophil count after TVA. Furthermore, mares can develop low-grade fever, mild colic, and transient anorexia.[21] From a safe practice perspective, we recommend 24 hours postTVA monitoring of all mares undergoing the procedure for appetite, feed intake, output of feces and urine, and signs of discomfort. As a prophylactic method, we give oral Equisul (sulfadiazine and trimethoprim, 24 mg/kg) prior to the procedure and 2 extra doses every 12 hours after the procedure (total of 3 doses). We also use flunixin meglumine (Banamine®, 1.1 mg/kg) before the procedure and an extra dose 12 hours prior to a mare's discharge from the Veterinary Medical Teaching Hospital.

Transvaginal aspiration of oocytes can be performed by a single highly experienced person; but as a teaching hospital, we prefer a team approach to provide training in all aspects of the process for residents and other veterinarians. Our clinicians follow an established protocol, with 2 veterinarians operating the rectal and vaginal part of TVA, respectively. First person maneuvers the ovaries transrectally and then manipulates 1 ovary to fix it against the transvaginal probe for optimal visualization. Second person operates the needle and aspiration pump, being responsible for the puncture and scraping of each follicle (Figure 1). The double-lumen needle allows a separate channel for inflow and outflow from follicles. The latter person typically operates the vacuum pump for follicular lavage using a foot pedal. A third person manually manages the fluid injection into the punctured follicle using EquiPlus OPU Recovery Medium (Minitube). All procedures are under strict aseptic conditions with care to maintain them at all times. Typically, 5-20 oocytes are retrieved per TVA session (12 ± 7 [average ± SD]; n = 136). In the case of a single mature preovulatory follicle (> 35 mm) present on 1 or both ovaries, we will aspirate that follicle and search for an oocyte. Since such an oocyte has likely already started maturation in vivo, they generally have a higher developmental potential

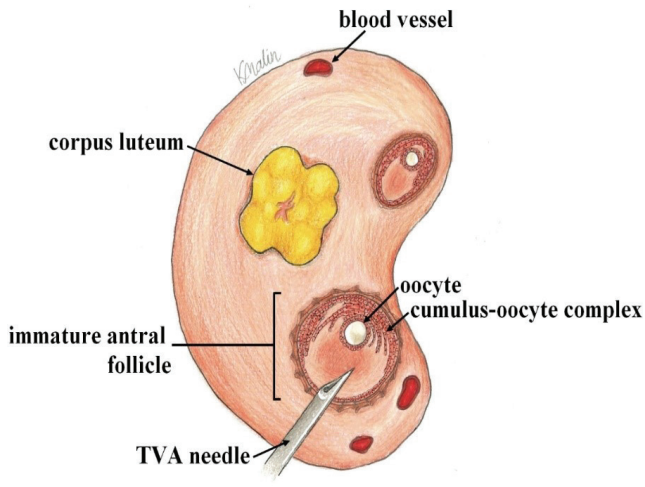

Figure 1. Illustration of a mare's ovary having 2 immature antral follicles in addition to a corpus luteum. An aspiration needle tip is depicted within the larger follicle during aspiration and scraping (nonscale representation).

with ICSI injection of sperm, but the blastocyst yield per mare is slightly lower.

After TVA, the aspirated fluid is transported to laboratory at ambient temperature, filtered using OPU recovery medium and an EmCon™ embryo filter that is carefully rinsed and flushed into large Petri dishes which are then searched using a bright-field stereomicroscope. Identified oocytes are then placed into holding medium (see below) overnight (12-16 hours) in sterile glass vials at ambient temperature (22°C) until they are placed into maturation medium in a trigas environment for 27-30 hours in droplets of maturation medium at a ratio of 10 µl per oocyte under light paraffin oil at 38.2°C in a humidified atmosphere of 5.8% CO_2, 5%O_2, and 89.2% N_2. Oocytes are then denuded from cumulus cells and evaluated for extrusion of the first polar body into the perivitelline space, indicating that prophase II of meiosis has reached and oocytes are ready for fertilization by sperm microinjection.

Oocyte transport

Typically, > 80% of oocytes used for ICSI are shipped to our facility from veterinarians in the US. Our laboratory receives oocytes in sterile glass vials in 1 or 3 ml volume of EmCare™ (ICPbio Reproduction, Spring Valley, WI) or EquiHold (Minitube, Verona, WI) commercial media, as they are available to most clinics shipping oocytes, and oocytes are stable in these media at ambient temperature for at least 24 hours.[8,11] Oocytes are express-shipped overnight in holding medium with minimal air in either a battery-powered shipping incubator (MicroQ Technologies, Mesa, AZ 85204) or in the Hamilton Biovet Equitainer or Equocyte shippers (Ipswich, MA) using 22°C insert cans (Figure 2). Generally speaking, isothermal devices maintain stable temperature that is critical to oocyte's survival and further developmental competence. We recently observed that MicroQ and Equitainer supported oocyte stability during shipping and their subsequent maturation, cleavage, and blastocyst rates, comparing them to those held overnight in the laboratory in a battery-powered incubator. Interestingly, the overall blastocyst rates were consistent across all devices, but oocytes transported in EquOcyte device had a lower maturation rate.[22]

Cryopreservation of oocytes remains an active research area for several laboratories; however, vitrification of equine oocytes[23–25] has not yet yielded commercially acceptable results. Regardless, viable offspring from such a pregnancy was reported in 2018.[26]

Sperm handling

Nearly all ICSI sessions at our laboratory are performed with frozen/thawed sperm. Although full motility cannot be restored from cryopreserved sperm, this technique requires a very small amount of thawed sperm for successful fertilization of all oocytes. This is especially important in cases where issues with collection or consistent low-quality sperm occur, and when limited numbers of straws are available (i.e. from deceased or castrated stallions). In our observations, some stallions perform better than others, having higher fertilization, cleavage, and blastocyst rates after ICSI sessions. Furthermore, certain mares paired with specific stallions achieve highly variable blastocyst rates, depending on the specific breeding cross. Similarly, different batches of cryopreserved semen from the same stallion can be of dramatically different quality. Typically, when possible, we request batches of frozen semen from stallion management that have known ICSI success rates. Our laboratory maintains a commercial sperm bank from highly desired sires for the mare clients' convenience, but every ICSI cycle requires legally binding contracts.

Our optimized technique allows us to perform straw cuts such that only 1/12 to 1/8 of a straw is used in each session, conserving semen samples that oftentimes are limited and highly valuable. For the same reason, we try to schedule oocyte collections on the same day so they can be fertilized with sperm from a single straw cut. The cutting is performed under liquid nitrogen to prevent thawing of the remaining straw segment. In our laboratory, we perform swim-up as the selection method for high-quality sperm. Swim-up is performed by layering the thawed sample underneath 2.0 ml G-MOPS (Vitrolife, Inc. Sweden) containing 10% Fetal Bovine Serum (FBS) and incubated for 30 minutes at 37°C. The sperm are aspirated from the upper swim-up layer after incubation, and their quality is evaluated by the embryologist. If needed due to poor quality, another sample can be readily processed. Selected sperm are suspended in the ICSI dish in a droplet of polyvinylpyrrolidone (PVP, 7%), an inert macromolecule that helps prevent sperm agglutination and facilitates sperm aspiration into the injection needle. It is important to note that the semen is thawed and processed after oocytes are denuded and maturation is assessed.

Intracytoplasmic sperm injection procedures

Oocyte in vitro maturation

Immature oocytes are placed in droplets of maturation medium consisting of 54.0% Dulbecco's modified Eagle's

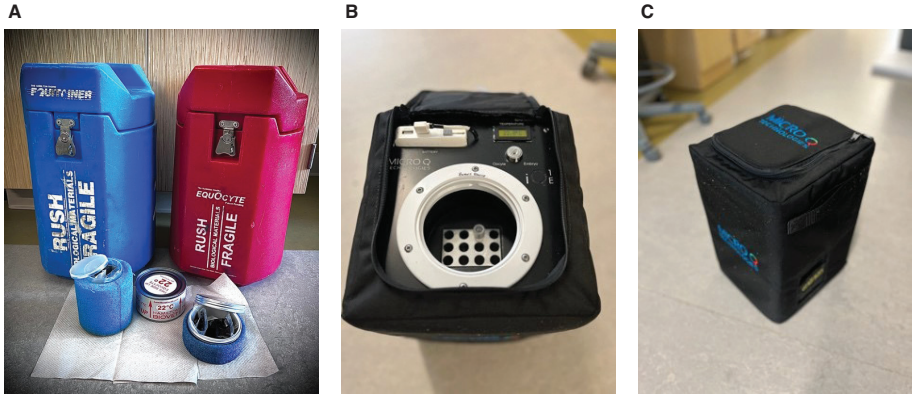

Figure 2. Isothermic shipping containers for equine oocyte transport to the ICSI laboratory. A. Hamilton Research Equitainer (blue) and EquOcyte (red) transporters. B and C. MicroQ battery-powered warmed transporter system.

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medium (DMEM)/F-12 with 15 mM HEPES, 25 mg ml^{-1} gentamicin, 36.0% Global medium (LifeGlobal), 0.1 mM sodium pyruvate, 6.0% fetal bovine serum (FBS; F2442; Sigma), 10 ml ml^{-1} insulin–transferrin– selenium (ITS) solution, 10% dominant stimulated follicle follicular fluid, 8.8 mU ovine FSH (National Hormone and Peptide Program) and 1.1 mU ml^{-1} porcine somatotropin (Harbor- UCLA Research and Education Institute), as described.[11,13]

The morning after aspiration and overnight holding, protected from light, oocytes are moved from holding medium in 22°C to 38.2°C in a triple gas incubator using N_2 gas to lower oxygen tension (5.8% CO_2, 5%O_2, and 89.2% N_2) for maturation. Oocytes remain in maturation medium for 27-30 hours, then cumulus oocyte complexes are denuded using hyaluronidase (hyaluronidase 0.2%/ml [Sigma Aldrich H3506-1G]; 2-minute incubation) and mechanical pipetting in a 4-well dish. The first polar body, if present, can then be visualized between the oolemma and zona pellucida, known as the perivitelline space, using a magnification of 20 x with brightfield optics on an Olympus IX-70 microscope. Denuding the surrounding cumulus cells is necessary to visualize the oolemma and perform ICSI. This is in contrast to traditional IVF, in which the cumulus supports, and is necessary for fertilization, as it provides natural sperm selection and prevents polyspermy. After the maturation period of a batch of oocytes, they cannot be assumed to be in the same stage of meiosis. Occasionally, we observe that even 10 minutes of additional IVM culture may be sufficient for the first polar body extrusion to occur if not observed during the initial assessment. On the other hand, some oocytes might already be atretic, and thus fail to extrude a polar body regardless of any additional time. The appearance of the first polar body defines the meiotic stage of the oocyte and consequently its readiness to be fertilized by injection of a single sperm.

All oocytes presenting this morphology are injected with a single sperm (Figure 3). Our laboratory uses a time-lapse imaging system to observe oocyte maturation inside the incubator which has allowed us to observe cumulus expansion (Figure 4) and polar body extrusion in near-real time. We have measured rates of cumulus-oocyte complex (COC) expansion. Similarly, we elucidated the dynamics of genetic expression in the cumulus cells and oocytes during maturation in vitro and in vivo after induction with GnRH analogs. This unraveled important biological pathways critical for the proper development of the oocyte and provided some clues about the main difference between the in vitro and in vivo environments.[27]

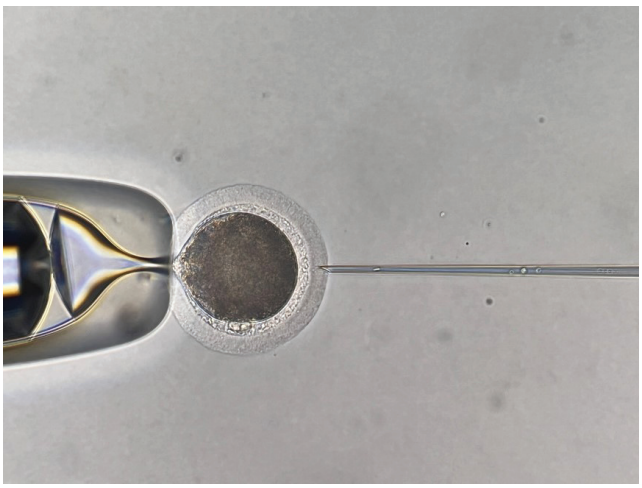

Figure 3. Mature oocyte held by holding pipette at the 9 o'clock position. First polar body is observed in the bottom part of the oocyte (6 o'clock), injection needle holding a single sperm about to be injected in the ooplasm. This image was obtained in an Olympus microscope (IX-70) 20 x objective.

Intracytoplasmic sperm injection

For every ICSI procedure, we prepare a dish with microdroplets of G-MOPS-10% FBS for injection and PVP 7% for semen pickup; both of these media are buffered to maintain the pH while they are outside the incubator environment. Although the micromanipulator-microscope system (Figure 5) is equipped with a heating stage, each minute spent outside the incubator decreases the chances of obtaining blastocysts, thus this step needs to be performed efficiently and quickly.

When the swim-up process ends and a good quality sample of sperm is obtained, mature oocytes are individually placed in microdroplets (4 µl) of G-MOPS 10% FBS that are laid out in the ICSI dish, while 3 µl of semen is placed in the droplet of PVP (sperm droplet). The first step is the immobilization of the targeted sperm by pressing or trapping the midpiece/tail with the injection needle against the bottom of the dish. This immobilizes the sperm and creates a visible bend in the tail. Then, the sperm is picked up with the injection needle by aspirating the tail first. Next, the dish is repositioned to center the image in a droplet containing one of the MII oocytes, and with the holding pipette, the oocyte is held from one side (9 o'clock). Oocyte is rotated in a manner that the polar body is situated at either 6 or 12 o'clock position, leaving the other side (3 o'clock) free for inserting the injection needle and depositing the sperm. In our laboratory, we use conventional ICSI that requires an injection needle sharp enough to penetrate the zona pellucida and oocyte plasma membrane (oolemma). Some laboratories report improved embryo cleavage and subsequent development using a piezo drive that gently 'drills' the injection pipette into and through the zona pellucida while maintaining the membrane integrity of the oolemma and inducing activation of the oocyte to some extent.[7,11] In our hands, the piezo drive has not resulted in improved blastocyst rates for equine zygotes and appears to be highly dependent on the skills of the embryologist performing ICSI.

After the sperm is injected, all steps are repeated for all oocytes in the dish; then, they can be moved into the embryo culture media dish in embryo culture medium (EC) and placed back in the incubator. It is critical that the injection itself is performed with a sense of urgency while still being careful to not cause mechanical damage to the oocyte, and the fertilized oocytes be placed in EC (54% DMEM/F-12, 40% Global, 6% FBS, 10 µl/ml1 ITS solution, and 0.1 mM sodium pyruvate) immediately after the microinjection.

In vitro culture of equine embryos

Our laboratory uses time-lapse embryo imaging system (Miri®TL, Esco Technologies) for observation of cell division timing and cellular integrity during embryo culture immediately after the ICSI procedure. This noninvasive system captures bright-field images at 5-minutes intervals in 5 various focal planes using a single red light-emitting diode (635 nm), with total light exposure of 0.064 second per captured image.

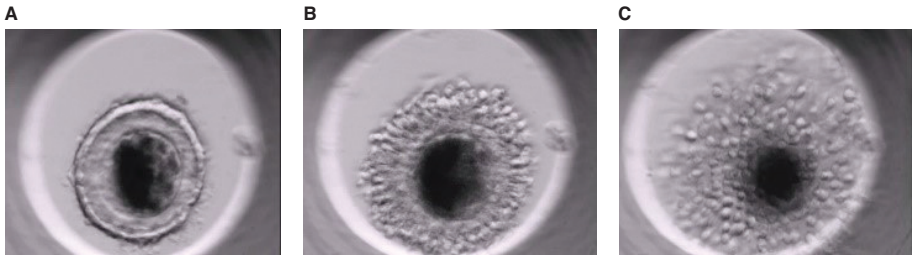

Figure 4. Cumulus expansion during the in vitro maturation culture for 27-30 hours. A. initial state of the cumulus surrounding the oocyte as a compacted group of cells known as the corona radiata (0 hours). B. Cumulus expansion at mid-culture, or 11.5 hours expansion of cumulus layer during oocyte maturation. C. End of culture, at ~ 27 hours, demonstrates the maximum expansion of the cumulus cell layer. Images were obtained in the MiriTL® time-lapse incubator.

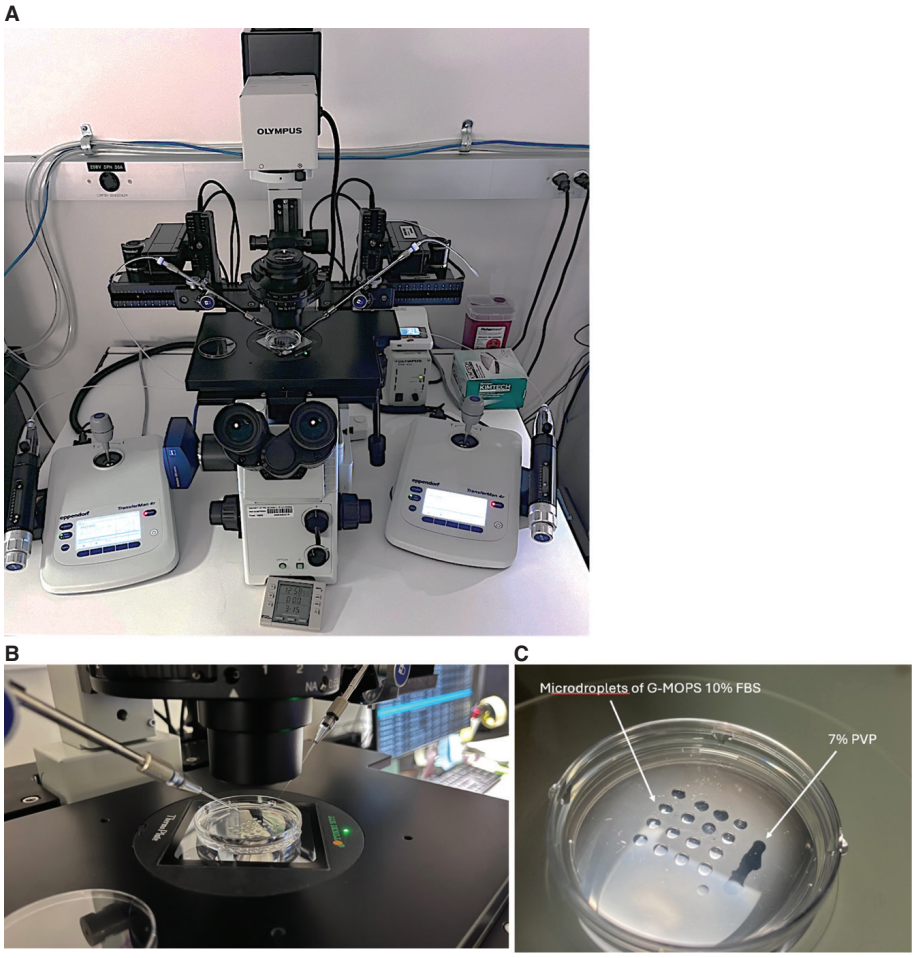

Figure 5. A. Olympus IX-70 with digital Eppendorf micromanipulators and microinjectors and brightfield optics and heated stage. B. Microscope stage during setup for ICSI with injection dish under sterile embryo grade mineral oil. C. Injection dish ready for injection with oocytes in G-MOPS medium, swim-up-selected sperm in 7% PVP medium.

Images are then assembled into videos that can be used for retrospective morphokinetic analysis in near real-time. There are multiple benefits to time-lapse embryo culture, but the most important is that continuous embryo evaluation is possible without an interruption to remove embryos from the incubator environment, except for day 4, when the culture medium is replaced. Embryos are vitrified when blastocysts are visualized to expand (zona thinning) along with the presence of pulsation (see below description and Video). From our general observations, a normally developing embryo is expected to reach the blastocyst stage on day 7, and embryos that take longer have lower chances for pregnancy.[28] The smaller the blastocyst, the higher the potential viability, thus throughout their monitoring, we make sure to vitrify as soon as possible once a blastocoel is observed in conjunction with pulsing.

Our laboratory has been researching time-lapse embryo evaluation since 2014, and in addition to documenting key cell division and mitotic events at specific hours after ICSI

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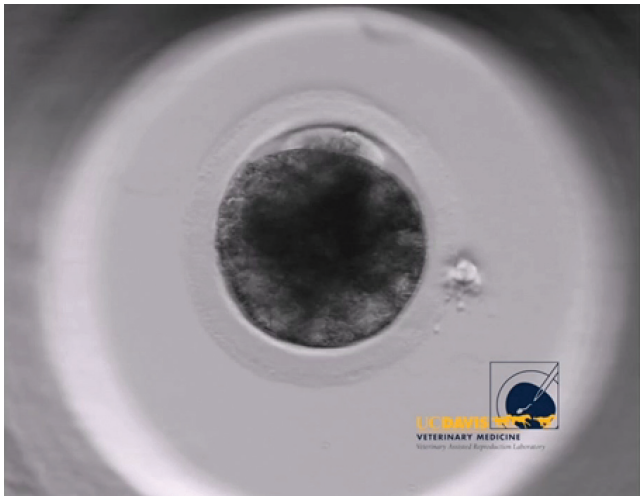

Video. Time-lapse development in an equine early embryo. To view the video, read the HTML version of the article where the video is embedded.

(Figure 6A-F), we have identified several morphokinetic parameters of successful blastocysts that are consistent with blastocyst formation and pregnancy success rates.[13,29] We have described 2 cleavage-related phenomena that are closely associated with the completion of development to a blastocyst. First, a precleavage event known as cytoplasmic extrusion occurs in equine zygotes, whereby extraneous cytoplasmic zygotic material is released into the perivitelline space from the single-celled zygote just before cleavage division (Figure 6B). Embryos that fail to become blastocysts have a lower rate of this precleavage extrusion.[13] Second, as the embryo reaches the late morula and early blastocyst stage, we routinely observe the successful embryo begin a pulsation sequence[30] at regular intervals (0.22 hour), in which an overall growth in embryo diameter occurs with expansion and subsequent contraction. The pulsation also results in thinning of the zona pellucida and can only be observed using the time-lapse videos rather than single observations. We have reported this dynamic phenomenon to occur in both in vitro and in vivo derived embryos (flushed from the uterus of mares at 6.5 days postovulation and cultured in the TL system).[30] While irregularities in mitosis can

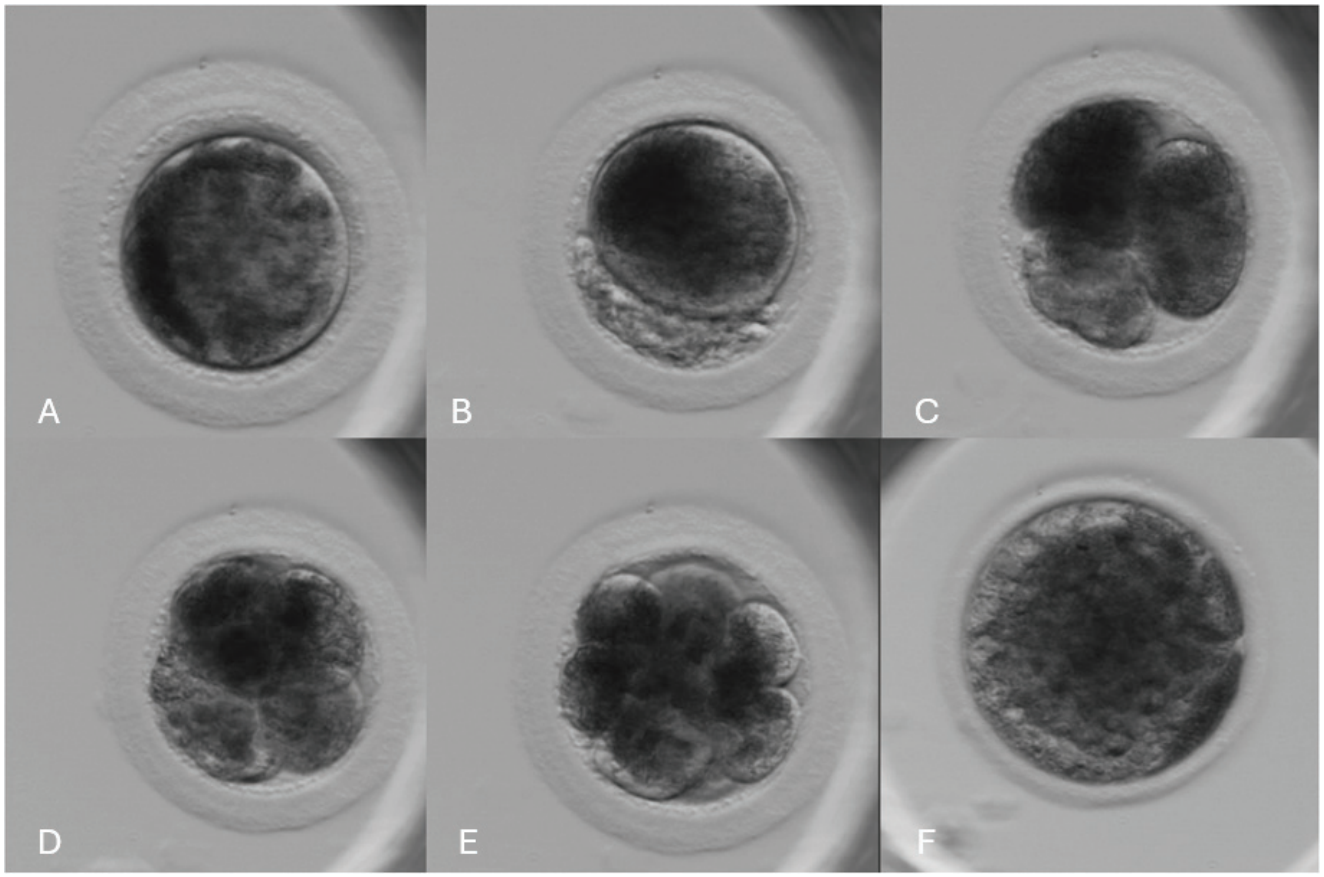

Figure 6. Time-lapse development in an equine zygote after ICSI (A) and progressing to blastocyst stage (B-F) as visualized in the MiriTL® incubator. A. Equine oocyte immediately after sperm injection and placement into time-lapse incubator. We observe cellular extrusion (B) to occur soon before the first cleavage where the extruded material occupies the perivitelline space. In (C), 2 blastomeres are visualized alongside the extruded material, giving an impression of a third blastomere. D. Formation of a 4-cell embryo. However, in contrast to human embryos, it quickly becomes impossible to judge the number of blastomeres beyond the 8-cell stage due to the equine blastomeres' dark color (E, F). F. ICSI-derived blastocyst.

often indicate aneuploidy or polyploidy in developing embryos, embryo pulsing is linked to successful implantation in human embryos.[31,32] Our observations are in agreement with these reports.[29,30]

The TL system has allowed researchers to document the embryo development timeline for each cell division of growing human, bovine, and equine embryos.[13,31,33-35] As mentioned above, studies from our laboratory and others have determined the precise timing of embryo cleavage from a single cell fertilized zygote from the moment of sperm injection to 2-cell, 4-cell, 8-cell, morula and blastocyst.[13] Recent work from our laboratory has extended the observations of the first few equine cell divisions and their influence on blastocyst development to that of equine pregnancy outcomes. With ~ 70 pregnancies from transferred vitrified blastocysts, we observed that those stemming from embryos with low rates of abnormal early-stage cleavages (at the 2-cell stage) were sustained at a higher percentage. These pregnancies demonstrated a lower rate of pregnancy loss further on during pregnancy demonstrating that early cleavage abnormalities likely lead to subsequent pregnancy loss.[28] We cannot, at this point, determine the cost-effectiveness of the time-lapse system as this new technology for equine development has not been fully evaluated for adequate numbers of clinical pregnancy outcomes.

Blastocyst vitrification and thawing for embryo transfer

A great majority of the blastocysts produced in our laboratory are vitrified and stored in liquid nitrogen or shipped to other clinics. Embryos are vitrified as soon as they reach the blastocyst stage (i.e. a clear trophectoderm and blastocoele structures, and pulsation can be observed), particularly if no recipient mares are available or the client desires storage of the embryos for a later transfer. We use a 2-step vitrification protocol with 1.5 M ethylene glycol (Solution A) and 7.0 M ethylene glycol, and 0.6 M galactose (Solution B). Cryolock® vitrification devices (Biotech, Inc, Alpharetta, GA) are used to store the frozen blastocysts which are then stored in individual canes in liquid nitrogen dewars.

Vitrified embryos, shipped overnight in dry shipping liquid nitrogen containers, are thawed and warmed after a simple 1-step, but effective, field protocol, which only requires the use of a commercial holding media by veterinarians in the field. A sterile, 30-mm culture dish is filled with room temperature holding media and the Cryolock® is quickly moved from liquid nitrogen to the dish, where the embryo will thaw and remain protected from the light for ~ 5 minutes to allow reexpansion before loading into a 0.25-ml embryo transfer straw. Then, the embryo can be loaded into an embryo transfer gun.

Summary and future plans

The modern equine ICSI laboratory has become essential for complete reproductive management of horses in the US and around the world. Oocytes can be readily express-shipped to any ICSI laboratory using a variety of shipping containers and media. The success of ICSI depends on a multitude of factors discussed, including mare and stallion effects, media, culture conditions, environmental conditions, technical experience, knowledge, and hygiene.

The time-lapse embryo imaging system used in our laboratory allows us to optimize our commercial and research programs, and it also delivers an abundance of embryo development data for individual embryo analysis. This allows us to track each embryo's cell division timing that provides detailed data for each embryo's success from fertilization to establishment of pregnancy. Although there is no universally-agreed upon grading system for equine oocytes or in vitro produced embryos, recent research from our laboratory indicated that video morphokinetics may provide a novel method to dynamically grade equine embryos that correlates well with pregnancy rates after embryo transfer.[13,28] Our main field of interest for future research is the series of complex events taking place around the first cleavage division,[13] such as cytoplasmic extrusion and pronuclei formation, which seem to be good predictors of the time of cleavage, aneuploidy, and overall developmental potential. In addition to time-lapse characterization of early embryonic events, we and others utilize immunofluorescence and transcriptomics[13,25,27,36,37] to broaden our insight into this period of equine development.

Conflict of interest

None to report

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