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Use of confocal microscopy to evaluate equine zygote development after sperm injection of oocytes matured *in vivo* or *in vitro*

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Abstract

Confocal microscopy was used to image stages of equine zygote development, at timed intervals, after intracytoplasmic sperm injection (ICSI) of oocytes that were matured *in vivo* or *in vitro*. After fixation for 4, 6, 8, 12 or 16h after ICSI, zygotes were incubated with α/β tubulin antibodies and human anticentromere antibody (CREST/ACA), washed, incubated in secondary antibodies, conjugated to either Alexa 488 or Alexa 647 and incubated with 561-Phalloidin and Hoechst 33258. An Olympus I \times 81 spinning disk confocal microscope was used for imaging. Data were analyzed using χ^2 and Fisher's exact tests. Minor differences in developmental phases were observed for oocytes matured *in vivo* or *in vitro*. Oocytes formed pronuclei earlier when matured *in vivo* (67% at 6h and 80% at 8h) than *in vitro* (13% at 6 and 8h); 80% of oocytes matured *in vitro* formed pronuclei by 12h. More ($p=0.04$) zygotes had atypical phenotypes, indicative of a failure of normal zygote development, when oocyte maturation occurred *in vitro* versus *in vivo* (30% and 11%, respectively). Some potential zygotes from oocytes matured *in vivo* had normal phenotypes, although development appeared to be delayed or arrested. Confocal microscopy provided a feasible method to assess equine zygote development using limited samples.

Keywords

zygote; confocal microscopy; equine; ICSI; cytoskeleton

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Introduction

Conventional *in vitro* fertilization has had poor success for the horse (Leemans, et al., 2016; Squires, et al., 2003). However, assisted fertilization using intracytoplasmic sperm injection (ICSI) resulted in experimental pregnancies and limited offspring by the mid-1990s (Galli, et al., 2007; Squires, et al., 2003). Subsequently, ICSI was developed for clinical use in horses, with the production of pregnancies and offspring from subfertile mares and/or stallions with limited or poor sperm quality (Carnevale, et al., 2007; Colleoni, et al., 2007; Hinrichs, 2013). As the demand for ICSI increased in the equine industry, methods for maturing oocytes and culturing embryos *in vitro* were explored (Carnevale & Sessions, 2012; Galli, et al., 2014; Hinrichs, 2013). However, our understanding of equine fertilization and early embryo development is still limited.

Oocytes can be matured *in vivo* or *in vitro* for equine assisted reproduction. Oocyte maturation is induced *in vivo* by administration of compound(s) to the donor mare that initiate follicle and oocyte maturation within the dominant follicle during the follicular phase, and the oocyte can be collected from the follicle before ovulation (Carnevale, 2016). Theoretically, the resulting oocytes should be of optimal quality, and collection, oviductal transfer, and fertilization *in vivo* of similar oocytes result in high pregnancy rates (Carnevale & Ginther, 1995). *In vitro* maturation of oocytes has been widely used in domestic animals and is of interest in human reproduction (Arlotto, et al., 1996; Edwards, 1965; Galli, et al., 2007; Hinrichs, et al., 1993; Lonergan & Fair, 2016). In the horse, immature oocytes are collected from live mares or excised ovaries by collecting oocytes from numerous follicles of various sizes for *in vitro* maturation, fertilization, and foal production (Carnevale, 2016; Galli, et al., 2013; Hinrichs, 2013). The extent that the artificial environment, associated with *in vitro* maturation, affects the oocyte has not been fully determined. In addition, immature oocytes from small follicles are removed from their natural environment prior to conditions associated with follicle growth and hormonal stimulation. Consequently, oocytes from immature follicles are more variable in quality and developmental competency (Hinrichs, 1991; Hyttel, et al., 1997).

An understanding of differences in zygotes developing from oocytes matured *in vivo* (IVO) and *in vitro* (IVM) would further our knowledge of the normal progression of postfertilization events and of potential alterations in cytoskeletal and nuclear maturation prior to the first mitotic division. In our study, we used confocal microscopy to examine equine zygote development at timed intervals after ICSI of IVO and IVM.

Materials and Methods

Oocyte Collections

IVO were collected between April and August in Fort Collins, CO, USA (40° latitude) from light-horse mares between 4 and 16 years (mean \pm SEM of 10.8 ± 0.7 year). Reproductive tracts were imaged using ultrasonography to evaluate follicular growth. Oocytes were collected from dominant follicle(s) during the follicular phase and between 18 and 25 h (21 ± 0.3 h) after administration of human chorionic gonadotropin, (1,500 IU, iv; Intervet Inc, Millsboro, DE, USA) and deslorelin acetate (SucroMate™, 0.75 mg, im; Bioniche Life

Sciences Inc., Belleville, Ontario, Canada). Oocytes were collected by ultrasound-guided, transvaginal follicle aspirations as previously described (Carnevale, et al., 2000), but using a commercial embryo flush solution (ViGRO™ Complete Flush, Bioniche Animal Health USA, Inc., Pullman, WA, USA) to lavage the follicle. Upon collection, the oocytes were cultured for 19.5 – 27.0h (22.0 ± 0.3 h) in Tissue Culture Medium 199 with Earle's salts (Gibco, Life Technologies, Grand Island, NY, USA) with additions of 10% fetal calf serum (FCS, Cell Generation LLC, Fort Collins, CO, USA), 0.2 mM sodium pyruvate, and 25 µg/mL gentamicin sulfate (Sigma Aldrich, St. Louis, MO, USA) at 38 or 38.5 °C in a humidified atmosphere of 6% CO₂ and air.

IVM were collected from excised ovaries in Cremona, Italy (45° latitude) during the natural breeding season. Ovaries were obtained from mares of diverse breeds and unknown ages from a local abattoir and transported at 24 °C for < 4h before collection of oocytes at the laboratory. Retrieved oocytes were placed in culture medium [Dulbecco's modified Eagle's medium (DMEM)/F12 (D8900; Sigma Aldrich, Milan, Italy) with 10% serum replacement (Life Technologies, Monza, Italy) and 0.1 IU/ml of human menopausal gonadotropin (Menopur 75, Ferring, Milan, Italy)] for 28 h at 38.5°C in humidified atmosphere of 5% CO₂ and air.

ICSI and Zygote Culture

Prior to ICSI of IVO or IVM, cumulus cells were removed and extrusion of the first polar body was confirmed. For both labs, ICSI was performed using a piezo drill. Frozen106 thawed semen from one stallion in each laboratory was used for all sperm injections, and a motile sperm with normal morphology was selected for each injection. After ICSI, potential zygotes were cultured under standard conditions for each laboratory. For IVO, potential zygotes were cultured individually in 30-µL drops of medium [DMEM/F12 (Sigma Aldrich, St. Louis) with 10% FCS] under mineral oil at 38.5 °C and in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Potential zygotes from IVM were placed as a group into 300 µL of a modified synthetic oviductal fluid medium with bovine serum albumin (BSA; Sigma-Aldrich, Milan) and modified Eagle Medium amino acids (Sigma-Aldrich, Milan) in four-well dishes at 38.5 °C and in 5% CO₂, 5% O₂ and 90% N₂ (Colleoni, et al., 2011; Lazzari, et al., 2002).

Samples Fixation and Immunostaining

Presumptive zygotes were fixed at room temperature in a microtubule stabilizing buffer (MTSB-XF, 0.1 M Pipes, 5 mM MgCl₂·6H₂O, 2.5 mM EGTA at pH 6.9) containing 2% formaldehyde, 1 µM taxol, 10 units/mL aprotinin, 50% deuterium oxide and modified with 0.1% Triton X-100, and 1 mM 1,4-Dithiothreitol (DTT) (Messinger & Albertini, 1991). Zygotes were fixed at 4 ($n = 5$), 6 ($n = 6$), 8 ($n = 5$), 12 ($n = 5$) and 16h ($n = 7$) (ICSI = 0h) for IVO and at the same time points, 4 ($n = 8$), 6 ($n = 8$), 8 ($n = 8$), 12 ($n = 10$) and 16h ($n = 10$) for IVM. After fixation, oocytes were rinsed in wash solution [(phosphate buffered saline containing 0.2% powdered milk, 2% normal goat serum, 0.1 M glycine, and modified to contain 1% BSA and 0.1% Triton X-100 (Messinger & Albertini, 1991)] for IVO or a modified wash solution (phosphate buffered saline containing 1% BSA and 0.1% Triton

X-100) for IVM, and stored at 4°C until immunostaining. The same procedure for immunostaining was used for IVO and IVM.

Oocytes were incubated with diluted primary antibodies in wash solution and positioned in four-well plates on rotating platform shaker for 4h at 37°C at the following concentrations: $\alpha\beta$ tubulin cocktail (1:100, mouse; Sigma Aldrich, St. Louis) and human-anti centromere antibody-CREST/ACA (1:100; Life Technologies, Grand Island). After primary incubation, oocytes were rinsed in wash solution for a minimum of 12h at 4°C, then incubated with secondary antibodies conjugated to either Alexa 488 or Alexa 647 (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in wash solution for 4h. When secondary incubation was complete, the oocytes were held in wash solution for 5h and then incubated with phalloidin (Alexa 561; Life Technologies) and Hoechst 33258 (1 μ g/mL; Life Technologies, Grand Island, NY, USA) for another 5h at 37°C. For confocal imaging, samples were mounted onto coverslips in 50% glycerol in phosphate buffered saline with 25 mg/mL sodium azide and 1 μ g/mL of Hoechst 33258 (Barrett & Albertini, 2007).

Confocal images were acquired on an Olympus Ix81 microscope (Waltham, MA, USA) fitted with a Yokogawa spinning disk (CSU22 head) using either a 60X/1.42 NA, DIC Planapochromatic or a 40X/1.35NA planapochromatic oil lens. Entire oocytes were imaged in 1- μ m intervals at 40 \times and 0.2- μ m intervals at 60 \times . Images were captured with a Photometrics Cascade II EM CCD camera (Tucson, AZ, USA) and analyzed using SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA).

Determination of Zygote Development Stages and Abnormalities After ICSI

Five sequential developmental events were categorized for presumptive equine zygotes: (1) condensed sperm chromatin with maternal chromosomes and extruded first polar body (PB1); (2) anaphase to telophase transition including anaphase/telophase shift of maternal chromosomes, condensed sperm chromatin, PB1, and second polar body (PB2) in the process of extrusion or fully extruded; (3) formation of the male pronuclei with maternal chromosomes, PB1 and PB2; (4) male and female pronuclei at distant positions; (5) male and female pronuclei in close apposition. Pronuclei (presumed male and female) were identified by localization of DNA and a human anticentromere antibody (CREST polyclonal antibody).

Development of presumptive zygotes were considered abnormal if the following images were observed: (1) premature chromosome condensation of the sperm with male chromosomes flanked by a bipolar spindle; (2) multiple pronuclei; (3) sperm chromatin induced ectopic-polar body; (4) multipolar spindles with incorrect separation of chromosomes and presence of multiple centrosomes; (5) scattered maternal chromosomes and intact sperm head.

Statistical Analysis

χ^2 analysis was used to determine overall effects across all time points, and Fisher's exact test was used within each category for comparisons among time points if the overall difference was significant at $p < 0.05$.

Results

Presumptive Zygote Developmental Events and Pronuclei Assessment

A similar series of developmental events were observed during zygote development after ICSI of IVO or IVM (Fig. 1, Table 1). Because of the relatively low number of samples, differences in the number of potential zygotes at specific developmental stages were observed over time only for IVO with condensed sperm chromatin, maternal chromosomes and PB1 ($p = 0.02$, Figs. 1a–c), IVO for presence of distant pronuclei ($p = 0.06$, Fig. 2b), and IVM for apposed pronuclei ($p = 0.008$, Fig. 2a, Table 1). The number of zygotes with pronuclei (distant or apposed) was significantly elevated at 12 and 16h when compared to earlier time points for IVM, with the numerically highest percentage of pronuclei observed at 12h (80%). Although not different over time, the highest numerical percentages of pronuclei were observed at 6 (67%) and 8h (80%) for IVO (Table 1).

At the initial observation after ICSI, many zygotes had a female spindle arrested at metaphase II with aligned and compact chromosomes, an extruded first polar body, and paternal DNA as identified by the presence of the sperm head and, in some samples, the tail (Figs 1a, 1b). Kinetochores were associated with maternal chromosomes at the metaphase plate and in the extruded first polar body (Fig. 1c). This early phase of development was observed predominantly at 4h after ICSI, including 80 and 38% of presumptive zygotes derived from IVO and IVM, respectively. One zygote from each of the two groups had not progressed past this developmental stage at 6h after ICSI, and two zygotes from each group appeared delayed or arrested in development and had not progressed from this stage by 8h after ICSI.

The anaphase to telophase transition was imaged as maternal chromosomes approaching anaphase or extruding the second polar body during telophase, with the presence of a sperm head (Figs 1d–f). This stage was observed as early as 4h after ICSI for IVO and IVM, with some presumptive zygotes arrested at this point in later hours.

Decondensation of male chromatin with a microtubule array around the sperm head nuclei, female chromosomes aligned at the metaphase plate, and PB1 with or without PB2 (Figs. 1g–i) was only imaged in three oocytes, including IVO oocytes that appeared delayed in development at 12h after ICSI.

The presence of two pronuclei, presumed male and female, were imaged as early as 6h after ICSI (Fig. 2b). At 8h for IVO and 12h for IVM, 80% of zygotes had pronuclei. When the pronuclei were in different areas of the ooplasm (distant), they were surrounded by a complex tubulin net, which diffused throughout the developing zygote. When the two pronuclei were close, the tubulin net was concentrated and contracting at the site of apposition (Fig. 2a). The number of nucleolus precursor bodies (NPB) in both parental pronuclei varied from three to seven; NPB were imaged in all pronuclei when PB2 was not extruded and three pronuclei were present (Fig. 3b).

Abnormalities Observed in Presumptive Equine Zygotes After ICSI

Failure of zygote development was observed for IVO and IVM and was associated with abnormal cytoskeletal and chromatin configurations (Figs. 3, 4). IVO had an incidence of abnormalities during zygote development of 3/28 (11%), with no significant differences for the various phenotypes over time (Table 2). Of the 44 potential zygotes from IVM, 13 (30%) had abnormal phenotypes, with the most common abnormalities being premature chromosome condensation and sperm chromatin induced ectopic polar body. Overall, potential zygotes from IVM had a higher number of abnormal phenotypes per total injected oocytes than IVO ($p = 0.04$, Table 2).

Discussion

Equine zygotes are difficult to obtain, and confocal microscopy provides a potentially efficient method to analyze the limited numbers of samples. The equine oocyte is a large cell with a high lipid content (Ambruosi, et al., 2009). The ooplasm measures $\sim 85 \mu\text{m}$ in diameter and is surrounded by the zona pellucida, a glycoprotein layer about $12\text{-}\mu\text{m}$ in thickness; the outer zona pellucida is $\sim 117 \mu\text{m}$ in diameter (Altermatt, et al., 2009). The size and properties of the equine oocyte affect immunostaining and confocal imaging, adding difficulty to the procedures. In addition, relatively few antibodies have been tested with the equine oocyte and zygote. However, we were able to successfully incorporate antibodies that had previously been used in other species.

Historically, development of the equine zygote and early embryo has been challenging to study. The equine conceptus will remain in the oviduct for 5 – 6 days before entering the uterus as a morula or early blastocyst (Freeman, et al., 1991). Initial observations of equine zygotes were done using transmission electron microscopy, with limited numbers of samples collected from oviducts based on timing of breeding or administration of ovulation inducing compounds (Enders, et al., 1987; Grondahl & Hyttel, 1996). Later, ICSI was used as a method to fertilize equine oocytes matured *in vitro*, prior to analysis by electron microscopy (Grondahl, et al., 1997) or confocal microscopy (Tremoleda, et al., 2003). However, at the time of these observations, procedures for equine oocyte maturation, ICSI and embryo culture were not yet easily repeatable or proven to produce viable embryos and foals, and timing of embryo development after ICSI was not always optimal. In the present study, proven methods were used in laboratories with records of producing viable foals after ICSI of IVO and IVM (Carnevale, et al., 2007; Galli, et al., 2007).

In our study, we observed zygote development at timed intervals after ICSI and noted similar events after IVO or IVM. In previous studies using ICSI and microscopic evaluations of zygotes, oocytes comparable to our IVM group were used, with oocytes collected from ovaries that were obtained from abattoirs and matured *in vitro* (Grondahl, et al., 1997; Tremoleda, et al., 2003). Our first observations were done 4h after ICSI, and in most presumptive zygotes, the maternal metaphase II spindle with aligned chromosomes, first polar body, and sperm head were imaged. In the study by Tremoleda et al. (2003), oocytes remained at metaphase II directly after sperm injection, with signs of oocyte activation noted in most oocytes at 6h and leading to the second meiotic division with extrusion of the second

polar body. We observed the anaphase to telophase transition, prior to extrusion of a second polar body, by 4h in approximately one quarter of injected oocytes.

In our study, IVO seemed to develop to pronuclei sooner after ICSI than IVM, with the highest percentages of pronuclei noted after ICSI at 6 and 8h for IVO in comparison to 12 and 16h for IVM. Within pronuclei were distinct areas with no CREST anticentromere staining, signifying NPB (Fig. 2a). The timing of pronuclei formation agreed with previous reports using IVM oocytes, with half of sperm-injected oocytes reaching the pronuclear stage by 12h, while the remaining presumptive zygotes were still in earlier stages of development (Tremoleda, et al., 2003). In another study, half of the sperm-injected IVM oocytes had two pronuclei at 20h after ICSI, but none had two pronuclei at 10h after ICSI (Tremoleda, et al., 2003). A significant percentage (36%) of these sperm-injected, IVM oocytes did not cleave into two cells until 48h after ICSI (Tremoleda, et al., 2003). In our laboratory, we would expect cleavage or indicators or impending cleavage for IVO and IVM oocytes by 24h after ICSI (Carnevale, personal communication, 2017).

The timing of pronuclei formation for IVO and IVM could be impacted by many factors other than maturation method, including differences in sperm, procedures, inherent oocyte quality, and total time of maturation. However, oocyte quality and cytoplasmic maturation could also differ between IVO and IVM oocytes (Gilchrist & Thompson, 2007; Sutton, et al., 2003). Oocytes collected from all follicles from excised ovaries represent a heterogeneous group of oocytes in different stages of competence, maturation, and atresia (Hinrichs, 1991). In contrast, oocytes collected from dominant, maturing follicles are in a relatively consistent stage of maturation, with ovulation of a metaphase II oocyte anticipated ~38h after administration of induction drugs (Figueiredo, et al., 2011). In this study, IVO oocytes were collected during the process of maturation, probably at metaphase I, and cultured in medium for the completion of maturation, in a system that has been proven to result in developmentally competent oocytes (Carnevale, et al., 2004). In contrast IVM oocytes were collected from follicles of various sizes and from ovaries of mares of unknown ages or stages of their reproductive cycle or pregnancy. Oocytes collected from small- or medium-sized, immature follicles can be less developmentally competent, as they complete maturation in artificial conditions over a limited time in contrast to natural conditions within the dominant follicle (Hyttel, et al., 1997). In addition, synchronization of nuclear and cytoplasmic maturation can be impacted when oocytes are matured *in vitro*. Epigenetic modifications occur in gametes and in early embryos (Bromfield, et al., 2007; Cantone & Fisher, 2013; Reik, et al., 2001), and ART procedures and *in vitro* culture could cause epigenetic disturbances that affect oocyte competence, pregnancy, and offspring health (Bromfield, et al., 2007; El Hajj & Haaf, 2013). In our study, no definitive differences in zygote development were noted with the limited numbers of IVO and IVM oocytes, although zygotes from IVM subjectively appeared to develop slower. However, oocyte maturation groups differed in that significantly more zygotes from IVM, than IVO, had abnormalities suggestive of developmental failure. One potential risk of maturation *in vitro* is that nuclear and cytoplasmic maturation are not synchronized and can result in collateral effects on embryonic development (Sanfins, et al., 2015; Smitz, et al., 2011). Transcriptional profiling after *in vitro* maturation of oocytes from cattle, women and mice has confirmed changes in genes and pathways, which could affect post fertilization events (El Hajj & Haaf,

2013; Mamo, et al., 2011; Rinaudo & Schultz, 2004). The extent that maturation *in vitro* affected oocyte quality in this study could not be directly determined, primarily because of the inherent variability within the population of immature oocytes.

After sperm injection, factors associated with the sperm and oocyte are critical for oocyte activation, decondensation of the sperm chromatin, and initiation of embryo development (Choi, et al., 2004; Galli, et al., 2007). Alterations in these processes can result in abnormal development, and abnormal morphologies were observed in some potential zygotes. Sperm-transmitted DNA damage leads to diverse abnormal reproductive outcomes and paternal genome loss, and it can be caused by different sperm chromatin defects, including premature sperm condensation and ectopic polar body extrusion after ICSI (Deng & Li, 2009; Marchetti, et al., 2015; Marchetti & Wyrobek, 2005; Schmiady, et al., 1996). After aneuploidy, the most common cause of fertilization failure in human *in vitro* fertilization and ICSI is premature sperm condensation (Edirisinghe, et al., 1997; Moghbelinejad, et al., 2013). Proper meiotic resumption of the oocyte is required to avoid premature condensation of the sperm head, which leads to DNA damage and aneuploidy in human oocytes (Manandhar & Toshimori, 2003). In our study, premature sperm condensation was observed only in IVM, as previously reported (Tremoleda, et al., 2003). Another sperm-related abnormality, specific to IVM in our study, was sperm chromatin induced ectopic polar body extrusion, with a spindle forming around sperm chromatin and leading to failure of normal zygote development because of the loss of paternal chromatin (Deng & Li, 2009). Premature sperm chromatin condensation and sperm chromatin induced ectopic polar bodies were only observed in IVM, suggesting that progression to the pronuclei stage was disrupted during oocyte activation in IVM oocytes or alterations of sperm DNA specific packaging and protamine deficiency (Deng & Li, 2009; Schmiady, et al., 1996). However, we cannot exclude the potential that intrinsic oocyte quality or the stallion affected these results, as these variables were different for the two ICSI systems. Additionally, the two post insemination culture conditions differed between laboratories where the experiments were performed. Both DMEM/F12 and SOF media are used clinically with high success rates, but we acknowledge that diverse culture media, and therefore the environment of the embryo development, could have affected the results observed in this study.

Additional zygote abnormalities included multiple pronuclei and multipolar spindle or presence of scattered maternal chromosomes and intact sperm head (chromosome fragmentation). Multipolar spindles suggest failure in spindle assembly checkpoints, and they were observed in a single oocyte from IVO and IVM (Courtois, et al., 2012; Sluder, et al., 1997). Scattered chromosomes and a sperm head were also observed in two potential zygotes from IVO and two from IVM. Multiple pronuclei suggest the failure of extrusion of the second polar body, possibly due to poor oocyte quality or sperm chromatin defects (Rosenbusch, 2001). Overall, we observed more morphological abnormalities in zygotes from IVM than IVO. This could have been caused by alterations in normal oocyte maturation *in vitro*; however, the increased diversity of oocytes collected from equine ovaries is also likely to affect developmental potential.

Although the clinical use and success of ICSI has dramatically increased in the equine industry over the last decade, this is one of few studies that has focused on factors that

impact zygote development. The lack of ongoing research is primarily due to our lack of knowledge regarding normal equine zygote formation and the expense and difficulty in obtaining equine oocytes, making projects that require large numbers of oocytes impractical and overly expensive. Often, the end points of projects studying equine ICSI are cleavage and blastocyst rates. Although these end points are important, they do not assess if fertilization was normal or why fertilization failure might have occurred. Therefore, more research is needed in this area to increase our understanding of equine fertilization and embryo development and to optimize success of ICSI. In this study, we demonstrated that confocal microscopy could provide meaningful data when using relatively low numbers of sperm-injected oocytes. Although multiple factors were different in regard to IVO or IVM, the results demonstrated some of the benefits and drawbacks for oocyte collection and maturation methods that are currently being used for clinical and research ICSI programs. The timelines provided by this research will allow future investigators to pick optimal time point(s) for observations of equine zygotes after ICSI of IVO or IVM. This information can be used for future investigations, especially as related to sperm and oocyte quality. The research demonstrates that confocal microscopy of individual zygotes can be a powerful tool in assessing factors impacting fertilization and zygote development in the horse, and it provides methodology that can be used by researchers to practically investigate treatment effects.

Conclusions

In conclusion, we used confocal microscopy to observe equine zygotes at timed intervals after ICSI of IVO or IVM. A similar progression of events during zygote development occurred in both groups. IVO when compared with IVM appeared to have a more rapid progression to pronuclei, although some of the potential zygotes were delayed or arrested in development. Abnormal zygote morphologies occurred more frequently in IVM than IVO. Confocal microscopy provided a feasible method to assess zygote development after *in vivo* or *in vitro* oocyte maturation using a limited number of samples.

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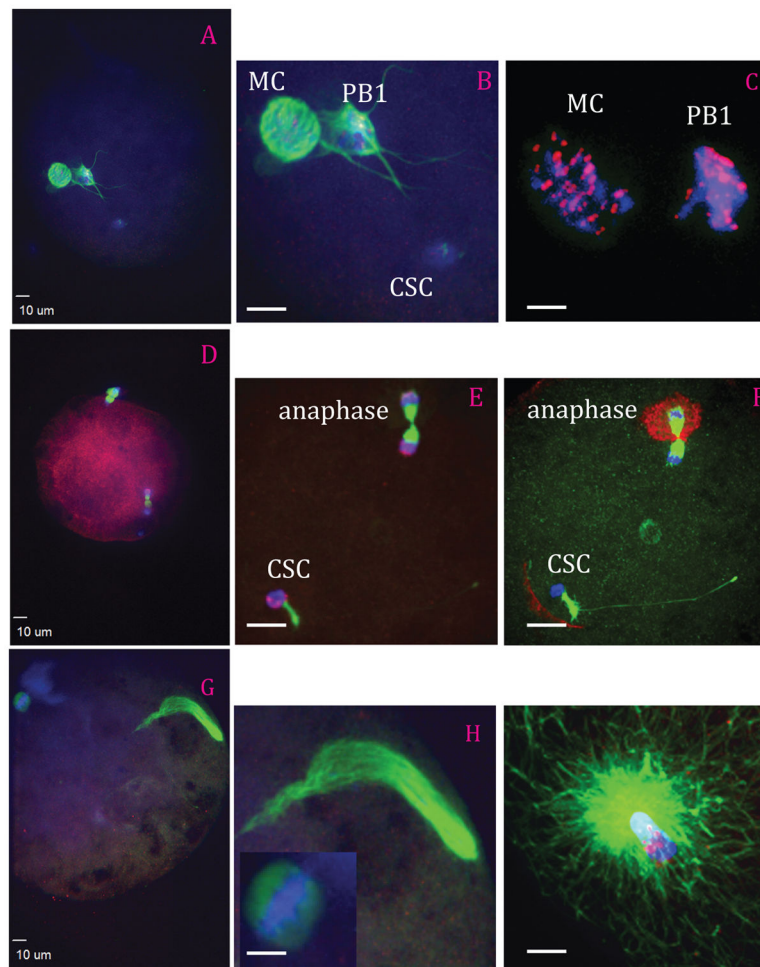


Figure 1.

Events during zygote development observed between 4 and 16h after ICSI. **a,b**: Condensed sperm chromatin (CSC, lower right), maternal chromosomes (MC, left) and first polar body (PB1, just to right of MC); **(c)** representation of the kinetochores localized on MC and PB1; **(d,e)** anaphase to telophase transition with CSC with acrosome portion of the sperm (lower left), anaphase/telophase of MC, extruding the second polar body; **(f)** actin cap surrounding the set of chromosomes being extruded in the second polar body, sperm tail visible next to CSC; **(g,h)** formation of the male pronucleus (indicated by elongated tubulin structure), MC **(h, left insert)**; and **(i)** tubulin aster formation around the sperm head at the time of sperm DNA decondensation (blue, DNA; red, centromeres and kinetochores, except in panel **f** red indicates actin; green, tubulin).

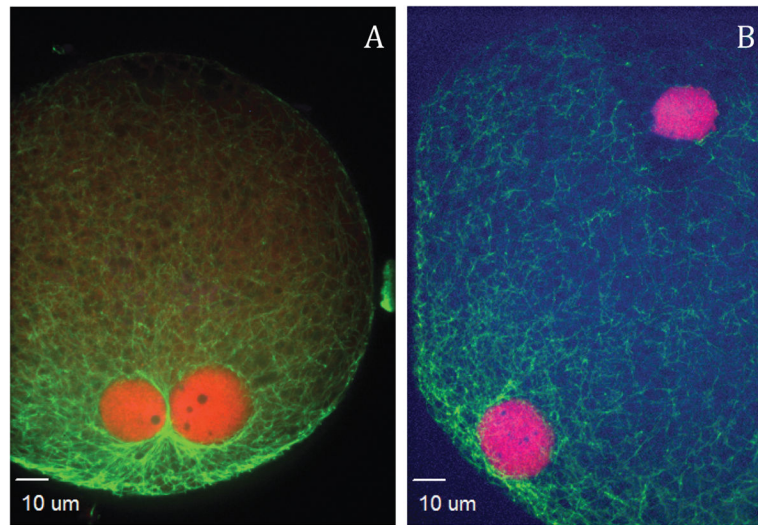


Figure 2. Nucleolus precursor bodies were observed within pronuclei at apposed (**a**) or distant (**b**) locations. (red, centromere; green, tubulin).

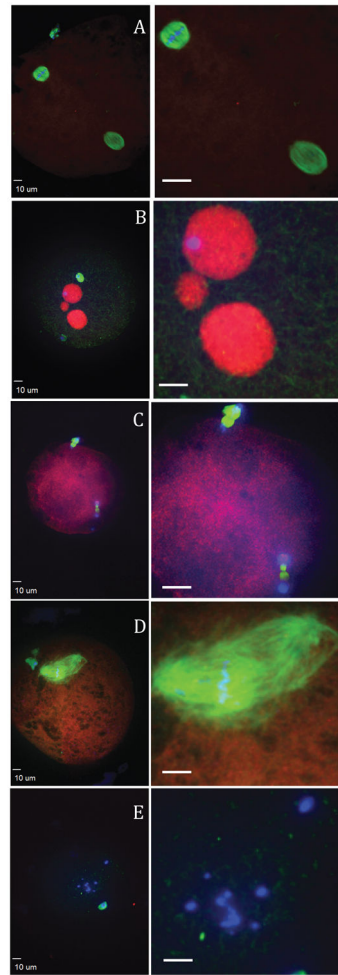


Figure 3.

Abnormal morphologies of potential equine zygotes after intracytoplasmic sperm injection include (a) premature chromosome condensation, with female spindle (top left) and male chromosomes flanked by a spindle structure (lower right); (b) multiple pronuclei; (c) sperm chromatin induced ectopic polar body, represented by the two sets of chromosomes undergoing anaphase; (d) multipolar spindle; (e) scattered maternal chromosomes (blue, DNA; red, centromeres and kinetochores; green, tubulin).

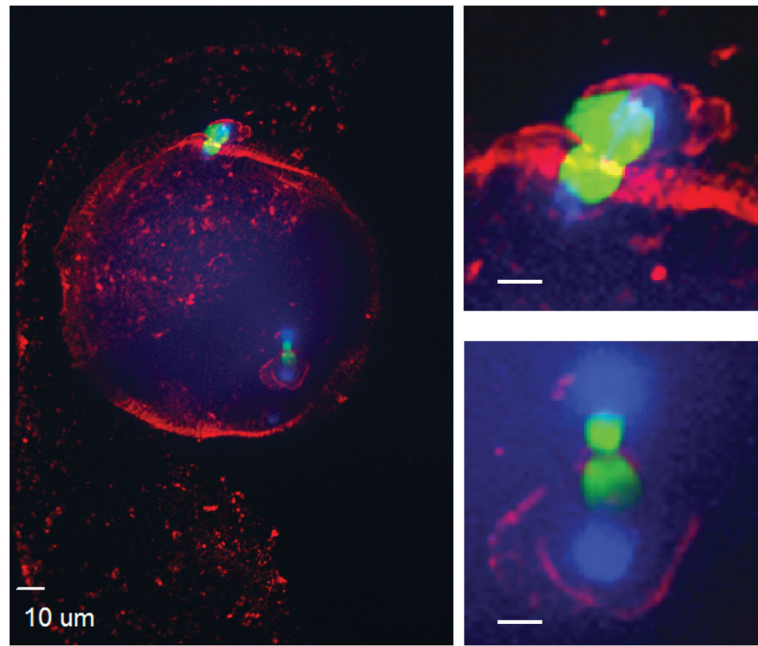


Figure 4. Actin cytoskeleton localization during ectopic polar body extrusion. In the left panel, cortical actin is concentrated around the two points of DNA extrusion. Top right and bottom right panels are magnified images of the two points of extrusion (blue, DNA; red, actin; green, tubulin).

Numbers of Potential Equine Zygotes at Different Points of Development at 4, 6, 8, 12 and 16h from All Oocytes Matured *In Vivo* (IVO) or *In Vitro* (IVM) And Fertilized by Intracytoplasmic Sperm Injection (0h).

Table 1

| Stages of development | 4 h | 6 h | 8 h | 12 h | 16 h | p Value |
|--|--------------------------|------------------------|------------------------|------------------------|------------------------|---------|
| Condensed sperm chromatin, maternal chromosomes and first polar body | | | | | | |
| IVO | 4/5 (80) ^a | 1/6 (17) ^{ab} | 0/5 (0) ^b | 0/5 (0) ^b | 2/7 (29) ^{ab} | 0.02 |
| IVM | 3/8 (38) | 1/8 (13) | 1/8 (13) | 1/10 (10) | 0/10 (0) | 0.23 |
| Anaphase to telophase transition, condensed sperm chromatin, first polar body | | | | | | |
| IVO | 1/5 (20) | 1/6 (17) | 1/5 (20) | 1/5 (20) | 0/7 (0) | 0.81 |
| IVM | 2/8 (25) | 2/8 (25) | 1/8 (13) | 0/10 (0) | 0/10 (0) | 0.24 |
| Male pronuclei chromatin decondensation, maternal chromosomes, first and second polar bodies | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 1/5 (20) | 0/7 (0) | 0.31 |
| IVM | 1/8 (13) | 2/8 (25) | 0/8 (0) | 0/10 (0) | 0/10 (0) | 0.16 |
| Distant male and female pronuclei | | | | | | |
| IVO | 0/5 (0) | 3/6 (50) | 1/5 (20) | 0/5 (0) | 0/7 (0) | 0.06 |
| IVM | 0/8 (0) | 0/8 (0) | 0/8 (0) | 2/10 (20) | 1/10 (10) | 0.32 |
| Apposed male and female pronuclei | | | | | | |
| IVO | 0/5 (0) | 1/6 (17) | 3/5 (60) | 2/5 (40) | 3/7 (43) | 0.26 |
| IVM | 0/8 (0) ^a , * | 1/8 (13) ^{ab} | 1/8 (13) ^{ab} | 6/10 (60) ^b | 6/10 (60) ^b | 0.008 |
| Pronuclei stages (combined distant and apposed) | | | | | | |
| IVO | 0/5 (0) | 4/6 (67) | 4/5 (80) | 2/5 (40) | 3/7 (43) | 0.103 |
| IVM | 0/8 (0) ^a | 1/8 (13) ^a | 1/8 (13) ^a | 8/10 (80) ^b | 7/10 (70) ^b | 0.001 |

Some potential zygotes had abnormal morphologies (see Table 2) and did not fit within the developmental stages.

* One zygote from IVM had three pronuclei imaged at 4h (see Table 2).

^{ab} Values within rows with different superscript letters differ with time at $p < 0.05$.

Table 2
 Number of Potential Equine Zygotes with Abnormal Morphologies at 4, 6, 8, 12 and 16h After Intracytoplasmic Sperm Injection (0h) Per Total Injected Oocytes Matured *In Vivo* (IVO) or *In Vitro* (IVM).

| Abnormality | 4 h | 6 h | 8 h | 12 h | 16 h | Total Zygotes |
|---|----------|----------|----------|-----------|-----------|-------------------------------|
| Premature chromosome condensation | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 0/5 (0) | 0/7 (0) | 0/28 (0) |
| IVM | 1/8 (13) | 1/8 (13) | 2/8 (25) | 0/10 (0) | 1/10 (10) | 5/44 (11) |
| Multiple (>2) pronuclei | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 0/5 (0) | 0/7 (0) | 0/28 (0) |
| IVM | 1/8 (13) | 0/8 (0) | 0/8 (0) | 0/10 (0) | 0/10 (0) | 1/44 (3) |
| Sperm chromatin induced ectopic polar body | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 0/5 (0) | 0/7 (0) | 0/28 (0) |
| IVM | 0/8 (0) | 1/8 (13) | 3/8 (30) | 0/10 (0) | 0/10 (0) | 4/44 (9) |
| Multipolar spindle | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 1/5 (20) | 0/7 (0) | 1/28 (4) |
| IVM | 0/8 (0) | 0/8 (0) | 0/8 (0) | 0/10 (0) | 1/10 (10) | 1/44 (2) |
| Scattered chromosomes and sperm head intact | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 0/5 (0) | 2/7 (29) | 2/28 (7) |
| IVM | 0/8 (0) | 0/8 (0) | 0/8 (0) | 1/10 (10) | 1/10 (10) | 2/44 (5) |
| Total abnormalities | | | | | | |
| IVO | 0/5 (0) | 0/6 (0) | 0/5 (0) | 1/5 (20) | 2/7 (29) | 3/28^a (11) |
| IVM | 2/8 (25) | 2/8 (25) | 5/8 (63) | 1/10 (10) | 3/10 (30) | 13/44^b (30) |

The total number of zygotes with morphologic abnormalities was higher ($p = 0.04$) for IVM when compared to IVO (as denoted by bold numbers and different superscripts).