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Cytoskeletal alterations associated with donor age and culture interval for equine oocytes and potential zygotes that failed to cleave after ICSI

Elena Ruggeri^A, Keith F DeLuca^B, Cesare Galli^{C,D}, Giovanna Lazzari^D, Jennifer G DeLuca^B, and Elaine M Carnevale^{A,E}

^ADepartment of Biomedical Sciences, College of Veterinary Medicine & Biomedical Sciences, Colorado State University, 1601 Campus Delivery, Fort Collins, CO 80523 USA

^BDepartment of Biochemistry and Molecular Biology, College of Natural Sciences, Colorado State University, 1870 Campus Delivery, Fort Collins, CO 80523, USA

^CDepartment of Veterinary Medical Sciences, University of Bologna, Via Tolara di sopra, 50, 40064, Ozzano Emilia (Bologna), Italy

^DAvantea srl, Laboratory of Reproductive Technologies, Via Porcellasco 7f, 26100 Cremona, Italy

Abstract

Intracytoplasmic sperm injection (ICSI) is an established method to fertilize equine oocytes, but not all oocytes cleave after ICSI. The aims of the present study were to examine cytoskeleton patterns in oocytes after aging in vitro for 0, 24 or 48 h (Experiment 1) and in potential zygotes that failed to cleave after ICSI of oocytes from donors of different ages (Experiment 2). Cytoplasmic multiasters were observed after oocyte aging for 48 h ($P < 0.01$). A similar increase in multiasters was observed with an increased interval after ICSI for young mares (9–13 years) but not old (20–25 years) mares. Actin vesicles were observed more frequently in sperm-injected oocytes from old than young mares. In the present study, multiasters appeared to be associated with cell aging, whereas actin vesicles were associated with aging of the oocyte donor.

Keywords

actin; maternal aging; oocyte senescence; tubulin

Introduction

Intracytoplasmic sperm injection (ICSI) is an established assisted reproductive technology in human fertility clinics, and in the past decade, ICSI has been modified for equine clinical use, to produce offspring from stallions with limited or poor quality sperm and older, subfertile mares (Grondahl *et al.* 1997; Choi *et al.* 2002; Galli *et al.* 2002; Hinrichs *et al.* 2002; Tremoleda *et al.* 2003; Altermatt *et al.* 2009; Palermo *et al.* 2014). However, not all

^ECorrespondence at: Elaine.Carnevale@colostate.edu.

sperm-injected oocytes undergo the first cleavage division. The cause of this developmental failure is probably multifactorial and has not been adequately studied in either species.

Maternal aging is associated with a decline in fertility in both the mare and woman. Fertility is reduced in mares as they enter their teen years, but the decline in fertility is marked in old (> 20 years) mares and associated with a high incidence of early embryo loss (Carnevale and Ginther 1992; Ginther 1992; Carnevale 2008). The primary factor associated with reduced fertility in the old mare is oocyte developmental quality (Carnevale and Ginther 1995). Recent findings suggest chromosomal misalignment frequently occurs in MII oocytes of old mares (Carnevale *et al.* 2012). A similar decline in fertility is noted in women and is associated with reduced oocyte quality and, specifically, increased aneuploidy (Battaglia *et al.* 1996; Kuliev *et al.* 2003). Although the age-associated decline in oocyte quality is generally accepted in both species, relatively little is known regarding the inherent capabilities of oocytes from donors of different ages for fertilization and zygote development.

Nuclear and cytoskeletal rearrangements are involved in oocyte maturation and competence acquisition (Combelles *et al.* 2002; Sun and Schatten 2006; Barrett and Albertini 2010; Yi and Li 2012). Actin and microtubules have been proposed as main contributors to spindle organization and the oocyte's ability to activate after fertilization (Schatten 1994; Dell'Aquila *et al.* 2001; Tremoleda *et al.* 2001; Yi *et al.* 2013; Yu *et al.* 2014). The union of paternal and maternal genomes in equine zygotes is characterized by a series of cytoskeleton-mediated events, mostly involving microtubule and chromatin remodeling (Tremoleda *et al.* 2003). The effects of aging of the oocyte donor and cell senescence on changes in actin and tubulin patterns have not been studied in the equine oocyte before or after fertilization, and understanding modifications of the actin and microtubule cytoskeleton that are associated with donor aging in relationship to oocyte maturation and fertilization has been untouched (Coticchio *et al.* 2014).

The aim of the present studies was to elucidate changes in the oocyte associated with failure of zygote development after equine ICSI, with a focus on cytoskeletal changes associated with donor aging and cellular senescence. We hypothesized that cytoskeletal alterations are associated with oocyte aging *in vitro* and affected by age of the oocyte donor. The specific objectives of the present study were to: (1) determine cellular alterations, especially with regard to actin and microtubules, that occur with oocyte aging *in vitro*; (2) examine oocytes that failed to cleave after sperm injection to determine potential causes of failure of zygote development; (3) identify characteristics of oocytes that fail to cleave after ICSI that are associated with cellular aging or aging of the oocyte donor; and (4) determine whether cytoskeleton remodeling and chromosome organization are different in oocytes that failed to cleave after sperm injection that were acquired from young (9–13 years) versus old (20–25 years) mares.

Materials and Methods

Samples used to examine cytoskeleton patterns in oocytes after aging *in vitro* for 0, 24 or 48 h (Experiment 1) were collected at Avantea (Cremona, Italy), whereas those used to examine

potential zygotes that failed to cleave after ICSI of oocytes from donors of different ages (Experiment 2) were collected at Colorado State University, Equine Reproduction Laboratory (Fort Collins, CO, USA)

Oocyte collection and manipulation

Experiment 1—For Experiment 1, samples were collected in Cremona (Italy; 45° latitude) during the natural breeding season (March and April 2014). Ovaries from mares of different breeds were collected from a local abattoir and transported within approximately 2 h at 24°C to the laboratory, where all cumulus oocyte complexes (COCs) were collected within an approximate 2-h interval. After retrieval, the COCs were placed in culture media (Dulbecco's modified Eagle's medium (DMEM)/F12 (D8900; Sigma Aldrich, Milan, Italy) containing 10% serum replacement (Life Technologies, Monza, Italy) and 0.1 IU ml⁻¹ human menopausal gonadotropin (HMG) (Menopur 75; Ferring, Milan, Italy) at 38.5°C in 5% CO₂ and air. After culture for 28 h, oocytes were denuded of cumulus cells. Only oocytes with a well-defined polar body were used for the study, with some oocytes fixed at 28 h and others returned to culture for an additional 24 or 48 h prior to fixation. Oocytes were fixed as described previously in a solution containing, in part, 2% formaldehyde and 0.1% Triton X-100 (Microtubule Stabilization Buffer Extraction Fix (MTSB-XF); Messinger and Albertini 1991) at room temperature at three different time points: (1) Time 0, equivalent to the expected time of MII (28 h in culture; $n = 11$); (2) Time 24, after an additional 24 h of culture, equivalent to 52 h in culture ($n = 14$); and (3) Time 48, after an additional 48 h culture after Time 0, equivalent to 76 h in culture ($n = 13$). After fixation in MTSB-XF, oocytes were rinsed and stored at 4°C in wash solution (phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100).

Experiment 2—The mares used in Experiment 2 were between 9 and 25 years of age, were housed in Fort Collins (CO, USA; 40° latitude) and were part of a clinical ICSI program. Reproductive cycles were monitored by ultrasonography to determine follicular growth and stage oocyte collections. Oocytes were collected during the follicular phase and from the dominant follicle(s) approximately 35 mm in diameter. Oocytes were collected by ultrasound-guided transvaginal follicular aspirations approximately 24 h after administration of human chorionic gonadotropin (hCG; 1500 IU, i.v.; Intervet, Millsboro, DE, USA) and deslorelin acetate (SucroMate; 0.75 mg, i.m.; Bioniche Life Sciences, Belleville, Canada). Collected oocytes were cultured in medium (TCM-199; Gibco BRL Life Technologies, Grand Island, NY, USA) containing 10% fetal calf serum (FCS; Cell Generation, Fort Collins, CO, USA), 0.2 mM pyruvate, and 25 µg mL⁻¹ gentamicin (Sigma Aldrich, St. Louis, MO, USA) at 38°C or 38.5°C in an atmosphere of 6% CO₂ and air.

Approximately 44 h after the time of deslorelin and hCG administration to donor mares, oocytes were denuded of cumulus cells and injected with frozen spermatozoa from various stallions. Potential zygotes were cultured in medium DMEM/F12 (Sigma-Aldrich) with 10% FCS under oil at 38.5°C and in 5% CO₂, 5% O₂ and 90% N₂. Injected oocytes were assessed after approximately 24 h for initial cleavage. Between 24 and 51 h after ICSI, injected oocytes that had not cleaved and did not show signs of cleavage (fragmentation or indentation of oolemma) were fixed in MTSB-XF at room temperature before being washed

with a solution containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 and stored at 4°C until being moved into wash solution before immunostaining.

Immunostaining and confocal analysis

Primary antibodies were diluted in 2% normal goat serum and incubated with the oocyte on a rotating platform shaker for 4 h at 37°C. Oocytes were stained with the following primary antibodies: α/β -tubulin cocktail (1:100, mouse; Sigma-Aldrich) and human-anti centromere antibody CREST/ACA (1:100; Life Technologies). Following incubation with the primary antibody, oocytes were washed in 2% normal goat serum for at least 12 h at 4°C, then incubated for 4 h with secondary antibodies conjugated to either Alexa 488 or Alexa 647 (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in 2% normal goat serum. Oocytes were rinsed for 5 h in 2% normal goat serum and incubated for another 5 h with phalloidin (Alexa 561; Life Technologies) and Hoechst 33258 ($1\mu\text{g mL}^{-1}$; Life Technologies) at 37°C. Oocytes were then mounted onto coverslips (50% glycerol in PBS with 25 mg mL^{-1} azide and $1\mu\text{g mL}^{-1}$ Hoechst 33258) for imaging (Barrett and Albertini 2007).

Images were acquired on an Olympus (Waltham, MA, USA) IX81 spinning disk confocal (CSU22 head) microscope using either a $\times 60/1.42$ Numerical Apperture (NA) Differential Interference Contrast (DIC) planapochromatic or a $\times 40/1.35\text{NA}$ planapochromatic oil lens. Z-stacks were acquired at a magnification of $\times 60$ and at $0.2\text{-}\mu\text{m}$ intervals. Magnification at $\times 40$ was used to image the entire oocyte at $1\text{-}\mu\text{m}$ intervals. Images were acquired with a Photometrics (Tucson, AZ, USA) Cascade II CCD camera and analyzed using SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA).

Actin and α/β -tubulin morphometric analysis oocyte measurements

Oocytes were imaged for: the presence or absence of sperm head or tail; tubulin multiasters and their respective shapes (gamma, twisted elliptical or star); actin vesicles and their position within the oocyte (cortex, cortex and internal, or internal); the presence of the meiotic spindle and the orientation for the first and, if present, the second spindle observed in the oocyte with polarity (bipolar or multipolar); and transzonal projections (TZPs; dense or thin filaments of actin at the projections).

The total integrated fluorescence intensity of actin was determined from compressed images collected at $1\text{-}\mu\text{m}$ intervals at $\times 40$, where actin vesicles were selected using the “draw function” in Slidebook. For spindle perimeter and area measurements, the entire oocyte was imaged at $\times 40$ magnification at $1\text{-}\mu\text{m}$ intervals. SlideBook software was used to determine the perimeter and area measurements.

Widths of the zona pellucida (ZP) and perivitelline space (PVS) were determined by taking the average of two measurements at the widest and the narrowest widths of the oocyte. The inner zona pellucida (IZP) was determined by subtracting the measurement of the ZP from the full oocyte diameter, which was the average of four separate measurements.

Statistical analysis

For Experiment 1, data were analyzed using Chi-square analysis. In Experiment 2, continual data (using mares of all ages and potential zygotes fixed at all hours after ICSI) were compared using Student's *t*-test, and correlations were analyzed using Spearman correlation. Additional analyses were performed for Experiment 2 using subsets of the data for mare age (Young (9–13 years) and Old (20–25 years) mares) and time from ICSI to fixation (24–28 h and 44–51 h); analyses were performed using Fisher's exact test (proportions) or Student's *t*-test (continual data). For analyses of oocytes having multiple spindles, analyses were performed on each spindle.

Results

Experiment I—No significant differences were observed in the number of oocytes at Time 0 ($n = 11$), Time 24 ($n = 14$) or Time 48 ($n = 13$) in terms of the presence of actin vesicles or kinetochores, position and polarity of spindles, or configuration of DNA. However, more ($P < 0.05$) oocytes had microtubule multiasters at Time 48 than at Times 0 and 24 (Table 1; Fig. 1).

Experiment II—All potential zygotes ($n = 52$) were used to compare mares of all ages (9–25 years) and number of hours from ICSI (ICSI = 0 h) to fixation (24–51 h) for various morphology parameters. Similar morphological parameters were also compared for subgroups of mares classified as Young (9–13 years; $n = 11$) and Old (20–25 years; $n = 32$) and potential zygotes fixed at 24–28 h ($n = 29$) or 44–51 h ($n = 17$) after ICSI. The time to fixation was not different for mares classified as Young or Old (mean ($\pm$ SEM) of 38.1 ± 3.7 and 33.6 ± 1.8 h, respectively; $P = 0.24$).

Sperm head or tail

An intact sperm head or tail was observed in 9 of 52 (17%) presumptive zygotes after ICSI. Donor age did not affect the number of observations; however, the mean hour of fixation was lower for oocytes with than without an intact sperm head or tail (Table 2), and the frequency of an observed sperm was higher ($P = 0.04$) for oocytes fixed at 24–28 h than 44–51 h (Table 3).

Spindle measurements, number and polarity

Of the 52 presumptive zygotes, 44 (85%) had one spindle, nine (17%) had two spindles, and three (6%) had no observed spindle (Fig. 2). The total number of spindles observed was 58; of these, 28 spindles (48%) were bipolar and 30 (52%) were multipolar (Fig. 3). The number of spindles, spindle orientation, and size were not affected by donor age (Tables 2–4), but the mean area of the spindle was greater ($P = 0.01$) and the mean length of the perimeter was longer ($P = 0.03$) if fixation occurred at 44–51 than 24–28 h after ICSI (Table 4).

Chromosome configurations and presence of kinetochore proteins

Chromosome configuration was determined and categorized based on the state and localization of the chromatin within the spindle (Table 5). Of the 58 spindles observed, 19 (33%) had chromosomes not aligned on the spindle plate, with chromatin dispersed on the spindle microtubules. Another 19 spindles (33%) had dispersed chromatin on the spindle microtubules, but no chromosomes. Although most of the chromosome configurations were in these two categories, no significant differences were found among all categories. Misaligned chromosomes were observed in 43% of the sperm-injected oocytes, but chromosome misalignment did not differ with donor age or hours after ICSI. Kinetochore proteins were observed in only 8 of 58 (14%) spindles.

Multiasters

Within the 52 oocytes analyzed, 32 (62%) contained microtubule asters with differing shapes, including gamma, twisted elliptical, and star shapes. Of the 32 oocytes with multiasters, 25 (78%) had gamma-shaped asters, 17 (53%) had twisted elliptical shaped asters, and 23 (72%) had star-shaped asters. There were no significant differences found between the different categories observed. The mean number of multiasters per potential zygote was higher ($P = 0.03$) in Young versus Old mares (Table 6), and the mean age of the oocyte donor was younger ($P = 0.05$) when multiasters were observed compared with when they were not observed (Table 2; Fig. 4). A negative correlation between mare age and number of multiasters was observed: as mare age increased, the number of multiasters decreased ($P = 0.04$, $r = -0.28$). Multiasters were more prevalent at 44–51 h than 24–28 h after ICSI ($P = 0.04$; Table 3). The presence of multiasters was not correlated to the number of spindles, the presence of multipolar spindles or the presence of actin vesicles.

Actin vesicles: presence, position, dimensions, number and intensity

Actin vesicles were present in 37 of 52 oocytes (71%; Fig. 5). The position of actin vesicles within the ooplasm was affected by time after ICSI, because the mean hour of fixation was significantly sooner when actin vesicles were observed within the ooplasm and later when vesicles were observed at the cortex (Table 2). Within the presumptive zygotes from Old versus Young mares, actin vesicles occupied a larger ($P = 0.05$) area, and more ($P = 0.05$) actin vesicles were observed (Table 4). The mean perimeter around the actin vesicles was greater ($P = 0.05$) in presumptive zygotes from Old than Young mares (Table 4). The sum intensity of actin vesicles was greater in oocytes fixed at 44–51 than 24–28 h ($P = 0.01$; Table 4). The mean number of actin vesicles and the area occupied by actin vesicles were greater when fixation occurred at 44–51 than 24–28 h after ICSI ($P = 0.05$ and $P = 0.04$, respectively; Table 4).

Discussion

ICSI has been successfully applied to equine and human oocytes when fertilization needs to be improved and for cases of infertility (Squires *et al.* 2003; Butts *et al.* 2014). At the Colorado State University's Equine Reproduction Laboratory, cleavage rates after experimental equine ICSI have ranged from 61% to 85%, but a percentage of sperm-injected equine oocytes will fail to complete fertilization and cleave (Altermatt *et al.* 2009; Sessions-

Bresnahan *et al.* 2014). Zygote arrest and failure of embryo development was previously reported after equine ICSI, and the researchers determined that in the 24% of sperm-injected oocytes that did not complete fertilization, most defects were associated with failures of sperm integration or completion of meiosis, and oocyte activation did not occur (Tremoleda *et al.* 2003). In the present study, we examined equine potential zygotes from our clinical program that failed to complete the first cleavage division or show signs of cleavage (fragmentation or indentation of the oolemma) starting at approximately 24 h after ICSI. Samples were collected during one breeding season in which an overall cleavage rate of 60.5% was obtained. In our experience, after the first check, sperm-injected oocytes displaying no signs of cleavage will fail to cleave if they are kept in culture, and if delayed cleavage does occur, the embryos will arrest at an early stage. However, we did maintain some potential zygotes in culture for up to 51 h to confirm the failure of cleavage. In our clinical program, mares were used as oocyte donors because of: (1) mare subfertility, which is often associated with aging; (2) availability of limited or poor quality spermatozoa; or (3) a combination of the two. Sperm factors, which we were not able to analyze, would have had an effect on fertilization failure, as many sperm injections were done with spermatozoa with unknown or poor viability. Consequently, we focused on two aspects of aging related to the oocyte (i.e. cellular senescence associated with time in culture and maternal aging of the oocyte donor) and their influence on chromosome integrity and cytoskeletal structure.

The cytoskeleton has an essential role in fertilization and zygote development, and cytoskeletal remodeling should be in synchrony with nuclear events, which include fusion of the female and male genomes (Tremoleda *et al.* 2001). In the present study, we evaluated two major cytoskeletal components, microtubules and actin. Microtubules are present in the oocyte during fertilization, and assemble in the cytoplasm soon after sperm incorporation (Schatten *et al.* 1985; Tremoleda *et al.* 2003). The role of the microtubules at fertilization and during zygote development has been studied in mammalian murine, porcine, bovine, equine and primate oocytes (Schatten *et al.* 1985; Schatten 1994; Kim *et al.* 1996; Tremoleda *et al.* 2003; Delimitreva *et al.* 2011; Hara *et al.* 2012). In the oocyte, microtubule multiasters are required for zygote and embryo development, and they are involved in multiple rearrangements and configuration changes in the cytoskeleton microtubule matrix (Schatten *et al.* 1985; Schatten *et al.* 1986; Schatten 1994). Depending on species, centrosomes are located at the center of microtubule multiasters and are of both maternal and paternal origin, as in the mouse and cow, or are of paternal origin, as in humans (Schatten 1994). During fertilization of equine oocytes, microtubule organization is predominantly triggered by the sperm forming the sperm aster; therefore, the zygote's centrosome, as in most other mammalian species, is predominantly paternally inherited (Tremoleda *et al.* 2003). However, both sham-injected equine oocytes and parthenogenetic zygotes assemble a microtubule network in the form of multiasters, leading to the conclusion that the equine oocyte itself has the ability to form functional centrosomes, supporting the origin of the horse zygote centrosomes from both parents (Tremoleda *et al.* 2003). This finding was also supported in the present study, because we observed multiasters in aged oocytes, which could be interpreted as a sign of parthenogenetic activation of the oocyte.

In the horse, as in the mouse, microtubules can be generated independently by the oocyte, and they play a role in proper congression of maternal chromosomes, activation and

positioning of the male and female pronuclei (Schatten *et al.* 1985; Tremoleda *et al.* 2003). The presence of multiple microtubule foci, as we also observed, can originate from fragmentation of defective meiotic spindles, resulting in meiotic arrest of oocytes (Tremoleda *et al.* 2003). Observations from the studies on equine zygotes that fail to cleave after ICSI demonstrate defective organization of microtubules into multiple asters and incorrect reconstruction of centrosomes, with zygotic arrest and failure of further development.

Actin is a key component in the migration of the meiotic spindle and asymmetrical positioning in murine and porcine oocytes (Yi *et al.* 2013; Yu *et al.* 2014). In particular, the roles of actin vesicles as pushing forces and actomyosin contractile machinery have been suggested as regulators of the oocyte maturation process (Yi and Li 2012; Holubcova *et al.* 2013; Coticchio *et al.* 2014). Cytoplasmic actin morphodynamics have been studied and linked to quality of the oocyte and ability to progress through maturation. Although the role of actin is poorly defined during and after fertilization in mammalian species, it has been evaluated in sea urchins. A few minutes after fertilization in the sea urchin, actin bundles move centrally with the incorporation of the spermatozoa (Vasilev *et al.* 2012; Santella *et al.* 2014). Once the spermatozoon enters the egg, the fertilization cone forms and contains actin filaments. If the actin cytoskeleton is perturbed at this point, multiple fertilization cones form, and they fail to incorporate the spermatozoa (Santella *et al.* 2014). Actin cytoskeleton remodeling and localization is involved in human oocyte maturation, and IVM affects the actin network and amount of actin present before fertilization (Coticchio *et al.* 2014). The role of actin in equine oocyte maturation and fertilization has not been studied.

After attainment of MII, the oocyte has a limited lifespan. Because injected oocytes were cultured for various intervals in the present study, we anticipated alterations associated with cellular aging. In Experiment 1, equine oocytes were cultured up to 48 h after MII to determine the features associated with aging *in vitro*. With the limited number of samples, the only significant change was an increase in microtubule multiasters by 48 h of culture. However, other cellular changes were beginning to occur in association with cell aging, with non-significant increases in the percentages of multipolar spindles, failure to observe kinetochores, movement of the spindle from a cortical to central position, and more diffuse chromatin.

In Experiment 2, microtubule multiasters were also observed more frequently as the time from ICSI to fixation increased. The time groups in Experiment 2 (fixation at 24–48 and 44–51h after ICSI) approximately correspond to the two time groups of aged oocytes in Experiment 1 (24 h and 48 h after polar body extrusion). Tubulin multiasters appeared to be associated with oocyte activation after sperm injection or parthenogenetic activation if the oocyte was aged *in vitro*. Polymerization and depolymerization of the microtubule filaments is essential for stable chromosome-tubulin connections, and energy from GTP hydrolysis is needed to correctly organize the microtubule stability and instability (Delimitreva *et al.* 2012). The stabilization of microtubule filaments is mediated by various factors such as kinesins, dyneins and pericentrin that determine polarity of the spindle and correct stabilization or destabilization of microtubule filaments attached to the chromosomes during different maturation stages of the oocyte (Brunet and Maro 2005; Sun and Schatten 2006;

Delimitreva *et al.* 2012). Perturbation of these components cause altered microtubule organization and a disorganized microtubule network, leading to oocyte spindle abnormality and incapability to succeed in fertilization (Delimitreva *et al.* 2012).

In the present study, microtubule multiasters were more often present in oocytes retrieved from young donors compared to old donors. The multiasters observed primarily in oocytes from young donors at 48–51 h after ICSI in Experiment 2 were similar to what was observed for oocytes aged *in vitro* for 48 h in Experiment 1, suggesting this was a consistent indicator of cellular aging and potentially an attempt of activation by the oocyte. Energy (GTP and ATP) storage supply and active presence of microtubule stabilizing factors and motor proteins, as kinesins and dyneins, could be sufficient in oocytes from a young healthy donor to trigger microtubule aster formation, and therefore, activation of the equine oocyte. It has been proposed that mitochondrial functionality and quantity is perturbed and decreased with maternal aging of the mare, which results in reduced fertility (Tilly 2001; Heffner 2004; Rambags *et al.* 2014). Perturbations of any of the components involved in microtubules organization and stabilization could lead to fragmentation of existent spindle structure and incorrect reconstruction of the zygote centrosome, as reported in both human IVF oocytes and equine ICSI zygotes (Asch *et al.* 1995; Tremoleda *et al.* 2003).

Because of the predominance of older, subfertile mares in our ICSI program, maternal age of oocyte donors could have a substantial impact on results. The association of maternal aging with reduced fertility has been well documented in mares and women (Carnevale *et al.* 1993; Carnevale 2008; Butts *et al.* 2014; Stoop *et al.* 2014). Aging-related changes in chromosome and cytoskeletal organization in the oocyte can contribute to failure of fertilization and fusion of the two gametes in the woman (Mau-Holzmann 2005). The effect of mare aging on cytoskeletal aspects of the oocyte has not been studied; however, the incidence of chromosomal misalignment is significantly increased for old mares (20 years) versus young mares (4 to 11 years) (Carnevale *et al.* 2012). In the present study, various chromosome patterns were observed with no significant effect of donor age or hours from ICSI to fixation. However, the ‘normal’ chromosome arrangement, with chromosomes compact and aligned at the metaphase plate, was observed in <10% of potential zygotes. In addition, the chromosomes differed in their condensation and/or decondensation status, possibly due to nuclear remodeling after sperm injection and failure to develop into the two parental pronuclei. We acknowledge that some of the oocytes that failed to cleave after ICSI were injected with a sperm of poor or compromised quality, which could have led to sperm-specific defective decondensation patterns and delayed formation of the male pronuclei. Kinetochores were observed in only 14% of the spindles, probably due to the failure of zygote development and resulting degradation of these proteins, which are attached on the sister chromatids. In contrast, the kinetochores were observed in all oocytes fixed at MII (Time 0) in Experiment 1, and even after 48 h of aging *in vitro*, 85% of the oocytes had discernible kinetochores. Our findings suggest that cellular changes were occurring after ICSI, which were not exclusively consistent with cell or donor aging. Our findings support the hypothesis that microtubule cytoskeletal organization does play a role in oocyte quality and ability to succeed in fertilization.

We observed actin vesicles, spherical filamentous actin structures, in 37 of 52 samples (71%) analyzed. In eukaryotic cells, actin filaments determine the movement of vesicles at specific cell locations and contribute to transport of intracellular proteins and the secretory pathway (Khaitlina 2014). Actin is a mediator and sensor of apoptosis (membrane blebbing and cell rounding) and it regulates apoptosis signaling (Desouza *et al.* 2012). In the oocyte, actin filaments modulate asymmetric spindle positioning in the mouse. In the starfish egg, actin filaments help chromosome collection, creating an actin network at the germinal vesicle breakdown stage (Lamash and Eliseikina 2006; Yi and Li 2012); networks of different density can be generated by adjusting vesicle volume, modulation that is essential for spindle positioning (Holubcova *et al.* 2013). In the present study, the presence of actin vesicles was associated with advanced donor age and did not appear to be as relevant during the senescence process of the oocyte. The results suggest that actin vesicle structures are a cytoskeletal component in zygote development, but their function at this stage of development is not well known. The increased presence of actin structures in oocytes collected from older mares could be interpreted as a marked remodeling of the oocyte cytoskeleton to compensate for the inability of the oocyte to organize chromosomes and cytoskeleton before and after the introduction of spermatozoa.

The actin filament structures in Experiment 2 were observed centrally within the ooplasm, although the location of the actin vesicles was more peripheral with increased time after ICSI. The trafficking of actin vesicles from the inner cytoplasm to the cortical area could be an attempt from the cytoskeleton to organize the actin filaments and facilitate the oocyte's degenerative progression. Directionality of this process seems to follow the asymmetrical cortical polarization and positioning of the spindle by actin filaments, as presented in the literature, which leads us to believe that there is a possible one-directional movement and build-up of these vesicles as the aging process continues (Yi and Li 2012; Coticchio *et al.* 2013; Holubcova *et al.* 2013).

Maternal aging causes a decline in reproductive success in various species and age-associated alterations, including mitochondrial changes and oxidative stress (Tilly 2001; Heffner 2004; Rambags *et al.* 2014). In yeast, a decrease in actin turnover, and consequent production of aggregates of F-actin, causes an increase in reactive oxygen species in the cytosol, which leads to both senescence of the cell and apoptosis (Gourlay and Ayscough 2005). We observed components of cell aging and programmed cell death, because actin vesicle number, area and sum intensity were significantly higher in injected oocytes from older mares and at a later time after ICSI. The increase in actin filaments in oocytes from older mares could indicate a suboptimal environment, causing a cascade of events to develop and trigger failure of zygote and embryo development. Quantity and diffusion of actin spherical filaments within the oocyte could be altered and incorrectly timed during zygote early development in oocytes from older donors due to altered energy generation and lack of correct maturation progression and, therefore, cytoskeleton remodeling. The present study is the first to investigate the role of actin spherical filaments in oocyte senescence and alterations due to maternal aging. Although aspects of oocyte aging *in vivo* (donor aging) and *in vitro* (culture) have been postulated to be similar, our findings demonstrate that different mechanisms are occurring, at least in some aspects of the cytoskeleton. Understanding the modifications of both microtubules and actin due to natural cell aging or

failure of the oocyte to be fertilized will allow us to determine what role the cytoskeleton plays in fertilization and early zygote development and the effects of maternal aging on zygote cytoskeletal modifications.

In conclusion, we found that microtubule multiasters were associated with oocyte aging *in vitro* and oocyte maternal centrosome activation. Multiasters were more prevalent in sperm-injected oocytes collected from younger versus older donors, and the tubulin multiasters appeared to be involved in cell aging and oocyte activation in oocytes from younger mares. Actin vesicles were associated with sperm-injected oocytes from older donor mares; these structures could be indicative of a deleterious process within the fertilized oocytes of older donors. Further determination of the normal diffusion and quantity of microtubule and actin structures during the first 24 h of normal equine zygote development is necessary to establish how these cytoskeleton alterations differ from normal embryo development and potentially why cytoskeletal remodeling appears to be different in oocytes from old and young donors.

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Fig. 1. Equine oocytes cultured *in vitro* for 28 h (Time 0; predicted time of MII) with no tubulin multiasters and for a further 48 h (Time 48) with diffuse foci of tubulin asters. Blue, chromatin; Green, tubulin; Red, kinetochores. Scale bar = 20 μ m.

Fig. 2.

Sperm-injected oocytes had different numbers of spindles after failing to cleave. Two spindles represent the female and male sets of chromosomes; one spindle represents the female set of chromosomes; no spindles were present after degeneration and decondensation of the spindle structures. Blue, chromatin; green, tubulin; red, actin. Scale bar= 20 μ m.

Fig. 3. Multipolar and bipolar spindles in equine failed zygotes, with four poles imaged in the multipolar spindle. Blue, chromatin; green, tubulin. Scale bar= 20 μ m.

Fig. 4. Tubulin multiasters in failed equine oocytes that failed to cleave after intracytoplasmic sperm injection, with representative high and low numbers of tubulin multiaster foci. Blue, chromatin; green, tubulin. Scale bar= 20 μ m.

Fig. 5. Equine oocytes that failed to cleave after intracytoplasmic sperm injection, with (a) low and high numbers of actin vesicles and (b) small and large areas of actin vesicles. Green, tubulin; red, actin. Scale bar= 20 μ m

Table 1

Number of oocytes with specific morphologies after maturation and culture *in vitro* to Time 0 (expected time of MII), Time 24 (culture for 24 h after MII) and Time 48 (culture for 48 h after MII)

Morphological end-point	0 h	24 h	48 h	Overall <i>P</i> -value
Actin vesicles	1/11 (9)	2/14 (14)	2/13 (15)	0.89
Chromatin				
Condensed	11/11 (100)	12/14 (86)	10/13 (77)	0.25
Diffuse	0/11 (0)	2/14 (14)	3/13 (23)	0.25
Kinetochores	11/11 (100)	13/14 (93)	11/13 (85)	0.38
Spindle				
Bipolar	11/11 (100)	13/14 (93)	10/13 (77)	0.16
Multipolar	0/11 (0)	1/14 (7)	3/13 (23)	0.16
Spindle position				
Centered	1/11 (9)	4/14 (29)	5/13 (39)	0.26
Cortical	10/11 (91)	10/14 (71)	8/13 (62)	0.26
Tubulin multiasters	1/11 (9) ^a	2/14 (14) ^a	11/13 (85) ^b	<0.001

With rows, values with different superscript letter differ significantly ($P < 0.05$)

Comparison of mean donor age (years) and hours from intracytoplasmic sperm injection to fixation on the presence or absence of observed morphological end-points ($n = 52$)

Morphological end-point	Donor age			Hours after ICSI		
	Yes	No	P-value	Yes	No	P-value
Actin vesicles	18.73±0.85 (37)	19.67±1.34 (15)	0.56	35.19±1.83 (37)	32.23±2.40 (15)	0.37
Actin vesicle position						
Cortex	16.20±3.61 (5)	19.30±0.82 (33)	0.22	47.20±1.24 (5)	33.12±1.85 (33)	0.01
Cortex and inside	19.00±1.90 (8)	18.71±0.94 (31)	0.89	39.50±4.71 (8)	33.58±1.83 (31)	0.18
Inside	19.17±0.92 (24)	18.13±1.62 (15)	0.55	31.25±1.87 (24)	40.47±2.96 (15)	0.01
Aster shape						
Gamma	17.80±1.02 (25)	18.14±2.20 (7)	0.88	35.16±2.20 (25)	39.57±3.71 (7)	0.35
Twisted elliptical	18.18±1.24 (17)	17.53±1.38 (15)	0.73	35.29±2.63 (17)	37.07±2.83 (15)	0.65
Star	18.87±1.06 (23)	15.33±1.55 (9)	0.08	36.30±2.29 (23)	35.67±3.57 (9)	0.88
Multiasters	17.88±0.91 (32)	20.80±1.06 (20)	0.05	36.13±1.90 (32)	31.50±2.24 (20)	0.13
No. of spindles						
One	19.23±0.78 (40)	-	0.45	34.13±1.61 (40)	-	0.80
Two	17.78±1.95 (9)	-	-	35.11±4.16 (9)	-	-
Sperm head or tail	19.78±1.77 (9)	18.84±0.79 (43)	0.62	26.33±0.41 (9)	36.02±1.67 (43)	0.01
Spindle orientation						
Bipolar spindle 1	18.50±1.09 (22)	19.33±0.98 (27)	0.57	32.59±2.27 (22)	35.70±2.00 (27)	0.31
Bipolar spindle 2	19.50±1.88 (6)	14.33±4.37 (3)	0.23	30.83±4.27 (6)	43.67±7.84 (3)	0.16
Multipolar spindle 1	19.33±0.98 (27)	18.78±1.08 (23)	0.70	35.70±2.00 (27)	32.35±2.19 (23)	0.26
Multipolar spindle 2	14.33±4.37 (3)	17.00±3.00 (2)	0.69	43.67±7.84 (3)	26.00±2.00 (2)	0.18
TZPs						
Dense	18.24±0.79 (37)	-	0.10	32.65±1.60 (37)	-	0.07
Thin	20.87±1.45 (15)	-	-	38.53±3.05 (15)	-	-

Data show the mean ± s.e.m., with the number of observations in parentheses. Spindle 1, first spindle; spindle 2, second spindle; TZPs, transzonal projections

Table 3

Number of injected oocytes in which different morphological endpoints were observed according to donor age group and time of fixation after intracytoplasmic sperm injection

Morphological end-point	Donor age (years)			Hours (h)		
	Young (n = 11)	Old (n = 32)	P-value	24–28 h (n = 29)	44–51 h (n = 13)	P-value
Actin vesicles	9/11 (82%)	22/32 (69%)	0.70	19/29 (66%)	10/13 (77%)	0.72
Actin vesicle position						
Cortex	3/9 (33%)	2/23 (9%)	0.12	0/20 (0%)	5/10 (50%)	0.002
Cortex and inside	1/9 (11%)	5/23 (22%)	0.65	3/21 (14%)	1/10 (10%)	1.00
Inside	5/9 (56%)	15/23 (65%)	0.70	16/21 (76%)	4/10 (40%)	0.10
Aster shape						
Gamma	6/8 (75%)	11/15 (73%)	1.00	12/14 (86%)	7/11 (64%)	0.35
Twisted elliptical	3/8 (38%)	8/15 (53%)	0.67	10/14 (71%)	5/11 (45%)	0.24
Star	5/8 (63%)	13/15 (87%)	0.30	9/14 (64%)	8/11 (73%)	1.00
Multiasters	8/11 (73%)	15/32 (47%)	0.18	14/29 (48%)	11/13 (85%)	0.04
Sperm head or tail	2/11 (18%)	7/32 (22%)	1.00	9/29 (31%)	0/13 (0%)	0.04
Spindle orientation						
Bipolar spindle 1	5/10 (50%)	13/30 (43%)	0.73	15/27 (56%)	4/13 (31%)	0.18
Bipolar spindle 2	0/2 (0%)	4/5 (80%)	0.14	5/6 (83%)	0/1 (0%)	0.28
Multipolar spindle 1	5/10 (50%)	17/31 (55%)	1.00	12/28 (43%)	9/13 (69%)	0.18
Multipolar spindle 2	2/2 (100%)	1/2 (50%)	1.00	1/3 (33%)	1/1 (100%)	1.00
TZPs						
Dense	8/11 (73%)	20/32 (63%)	0.72	22/29 (76%)	8/13 (62%)	0.46
Thin	3/11 (27%)	12/32 (38%)		7/29 (24%)	5/13 (38%)	

Young, mares 9–13 years of age; Old, mares 20–25 years of age; ICSI, intracytoplasmic sperm injection; Spindle 1, first spindle; spindle 2, second spindle; TZPs, transzonal projections

Table 4

Spindle and actin vesicle parameters according to donor age group and time of fixation after intracytoplasmic sperm injection

Morphological end-point	Donor age			Time after ICSI (h)		
	Young (n = 11)	Old (n = 32)	P-value	24–28 (n = 29)	44–51 (n = 13)	P-value
Actin vesicles						
Area (μm^2)	573±226	1570±377	0.05	744±200	1720±491	0.04
Perimeter (μm)	109±20	215±35	0.05	145±30	225±42	0.06
No. of actin vesicles	2.00±0.24	8.11±1.75	0.05	3.71±1.03	8.50±2.48	0.05
Sum intensity of actin vesicles ($\times 10^7$ ADU)	4.63±1.35	29.8±15.0	0.12	6.43±1.99	81.6±40.8	0.01
Sum intensity per actin vesicle ($\times 10^7$ ADU)	2.56±0.70	6.17±2.29	0.91	4.46±1.98	7.49±2.51	0.10
Time after ICSI (h)	34.7±4.0	32.1±2.7	0.78	-	-	-
Spindle						
Area (μm^2)	124±16	123±14	0.46	119±14	270±96	0.01
Perimeter (μm)	285±37	258±22	0.42	227±14	318±38	0.03
Time after ICSI (h)	35.7±4.5	32.5±4.5	0.90	-	-	-

Data show the mean $\pm$ s.e.m. Young, mares 9–13 years of age; Old, mares 20–25 years of age; ICSI, intracytoplasmic sperm injection; Spindle 1, first spindle; spindle 2, second spindle; ADU, Analog-Digital-Unit

Table 5

Number of injected oocytes in each category of chromatin localization according to donor age group and time of fixation after intracytoplasmic sperm injection

Endpoints	Donor age		Time ICSI to Fixation		
	Young (%)	Old (%)	P-value	24–28 h (%)	44–51 h (%)
Spindle 1					
1	1/10 (10)	2/30 (7)		2/27 (7)	1/13 (8)
2	1/10 (10)	5/30 (17)		5/27 (19)	0/13 (0)
3	1/10 (10)	5/30 (17)	0.76	1/27 (4)	4/13 (31)
4	2/10 (20)	8/30 (27)		7/27 (26)	4/13 (31)
5	5/10 (50)	7/30 (23)		10/27 (37)	3/13 (23)
6	0/10 (0)	3/30 (10)		2/27 (7)	1/13 (8)
Spindle 2					
1	0/2 (0)	0/5 (0)		0/6 (0)	0/1 (0)
2	0/2 (0)	0/5 (0)		1/6 (17)	0/1 (0)
3	0/2 (0)	0/5 (0)	1.00	0/6 (0)	0/1 (0)
4	2/2 (100)	3/5 (60)		3/6 (50)	1/1 (100)
5	0/2 (0)	2/5 (40)		2/6 (33)	0/1 (0)
6	0/2 (0)	0/5 (0)		0/6 (0)	0/1 (0)

Chromatin localization was categorized as follows: 1, chromosomes aligned compact at the metaphase plate; 2, chromosomes aligned and compact at the metaphase plate and chromatin dispersed throughout the spindle; 3, compact chromosomes scattered and not aligned at the metaphase plate; 4, compact chromosomes scattered, not aligned at the metaphase plate and chromatin dispersed throughout the spindle; 5, chromatin only dispersed throughout the spindle; 6, compact chromosomes aligned at the metaphase plate and elongated chromosomes positioned out of the plate. Young, mares 9–13 years of age; Old, mares 20–25 years of age; ICSI, intracytoplasmic sperm injection; Spindle 1, first spindle; spindle 2, second spindle

Number of actin vesicles and tubulin multiasters and oocyte measurements according to donor age group and time of fixation after intracytoplasmic sperm injection

Table 6

	Donor age		Time after ICSI (h)			P-value
	Young (n = 11)	Old (n = 32)	24-28 (n = 29)	44-51 (n = 13)	P-value	
No. of actin vesicles	1.73 ± 0.30	6.37 ± 1.60	3.45 ± 1.35	6.54 ± 2.15	0.22	
No. of multiasters	9.45 ± 2.47	4.47 ± 1.01	5.14 ± 1.24	8.85 ± 1.77	0.10	
Width (×10 ³ μm)						
IZP	87.0 ± 1.4	88.8 ± 1.5	90.2 ± 1.4	86.2 ± 2.9	0.17	
PVS	8.03 ± 1.62	8.37 ± 0.52	8.43 ± 0.66	9.53 ± 1.43	0.43	
ZP	19.9 ± 0.7	19.3 ± 0.6	18.9 ± 0.5	19.9 ± 1.0	0.15	

Data show the mean ± s.e.m. Young, mares 9–13 years of age; Old, mares 20–25 years of age; ICSI, intracytoplasmic sperm injection; IZP, inner zona pellucida; PVS, perivitelline space; ZP, zona pellucida.