Animal (2013), **7:9**, pp 1493–1499 © The Animal Consortium 2013
doi:10.1017/S175173111300116X

animal

Influence of equine growth hormone, insulin-like growth factor-I and its interaction with gonadotropins on *in vitro* maturation and cytoskeleton morphology in equine oocytes

G. R. Pereira[1,2†], P. L. Lorenzo[3], G. F. Carneiro[1], B. A. Ball[1], L. M. C. Pegoraro[4], C. A. Pimentel[2] and I. K. M. Liu[1]

[1]*Department of Population Health and Reproduction, School of Veterinary Medicine, University of California, Davis, CA 95616, USA;* [2]*Department of Animal Pathology, Animal Reproduction Laboratory, School of Veterinary Medicine, Federal University of Pelotas, Capão do Leão s/n – Mailbox 354, Pelotas, RS 96010-900, Brazil;* [3]*Animal Physiology Department, Veterinary School, Universidad Complutense de Madrid, Madrid 28040, Spain;* [4]*Animal Reproduction Laboratory, Temperate Climate Research Corporation-EMBRAPA, Pelotas, RS 96001-970, Brazil*

(Received 22 October 2012; Accepted 22 April 2013; First published online 21 June 2013)

In horses, successful in vitro *fertilization procedures are limited by our inability to consistently mature equine oocytes by* in vitro *methods. Growth hormone (GH) is an important regulator of female reproduction in mammals, playing an important role in ovarian function, follicular growth and steroidogenesis. The objectives of this research were to investigate: the effects of equine growth hormone (eGH) and insulin-like growth factor-I (IGF-I) on the* in vitro *maturation (IVM) of equine oocytes, and the effects of eGH in addition to estradiol (E_2), gonadotropins (FSH and LH) and fetal calf serum (FCS) on IVM. We also evaluated the cytoskeleton organization of equine oocytes after IVM with eGH. Equine oocytes were aspirated from follicles <30 mm in diameter and matured for 30 h at 38.5°C in air with 5% CO_2. In experiment 1, selected cumulus–oocyte complexes (COCs) were randomly allocated as follows: (a) control (no additives); (b) 400 ng/ml eGH; (c) 200 ng/ml IGF-I; (d) eGH + IGF-I; and (e) eGH + IGF-I + 200 ng/ml anti-IGF-I. In addition to these treatment groups, we also added 1 μg/ml E_2, 5 IU/ml FSH, 10 IU/ml LH and 10% FCS* in vitro *(experiment 2). Oocytes were stained with markers for microtubules (anti-α-tubulin antibody), microfilaments (AlexaFluor 488 Phalloidin) and chromatin (TO-PRO_3-iodide) and assessed via confocal microscopy. No difference was observed when eGH and IGF-I was added into our IVM system. However, following incubation with eGH alone (40%) and eGH, E_2, gonadotropins and FCS (36.6%) oocytes were classified as mature* v. *17.6% of oocytes in the control group ($P < 0.05$). Matured equine oocytes showed that a thin network of filaments concentrated within the oocyte cortex and microtubules at the metaphase spindle showed a symmetrical barrel-shaped structure, with chromosomes aligned along its midline. We conclude that the use of E_2, gonadotropins and FCS in the presence of eGH increases the number of oocytes reaching oocyte competence.*

Keywords: equine, oocyte maturation, equine growth hormone, cytoskeleton distribution

Implications

As successful *in vitro* fertilization has not yet been well established in horses, efficient techniques for *in vitro* oocyte maturation are crucial to define culture conditions that promote oocyte maturation competence and to provide new strategies to improve the efficiency of assisted reproductive technologies (ART) in horses. This study emphasizes the importance of using equine growth hormone to mature equine oocytes *in vitro* and its important role on the cytoskeleton distribution that may accelerate advances in ART. It also provides strong evidences of a potential role of eGH in reproductive function for oocyte development procedures *in vitro* in horses.

† E-mail: gabriel.pereira@ufpel.edu.br, gpereira@ucdavis.edu

Introduction

In horses, *in vitro* maturation (IVM) rates of equine oocytes differ between laboratories; this may be partially owing to inappropriate methods for oocyte selection, poor *in vitro* culture conditions or both (Hinrichs and Williams, 1997; Hinrichs, 2010). In addition, there is limited information regarding developmental competence and specific requirements during IVM of equine oocytes recovered from abattoir-derived ovaries (Dell'Aquila *et al.*, 1997).

Growth hormone (GH), an important regulator of female reproduction in mammals, plays an important role in ovarian function, follicular growth and steroidogenesis (Hull and Harvey, 2001). Recently, Shirazi *et al.* (2010) reported a positive effect of GH added *in vitro* to mature ovine oocyte that resulted in 73% of hatched blastocyst development. Insulin-like growth factor-I (IGF-I) has also been known for stimulating oocyte maturation and promoting blastocyst development in several species, such as rabbits (Yoshimura *et al.*, 1994), bovine (Matsui *et al.*, 1995) and sheep (Guler *et al.*, 2000); therefore, those findings demonstrated that IGF-I is critical for embryo culture. In horses, IGF-I is known to promote nuclear and cytoplasm maturation of *in vitro* matured equine oocytes (Carneiro *et al.*, 2002). The interaction between GH and IGF-I is important, as IGF-I has been shown to play an important role in ovarian function in humans and animal models (Erickson *et al.*, 1989). Studies in our laboratory demonstrated that more equine oocytes resumed meiosis in the presence of eGH and IGF-I in the IVM system after 30 h of culture in serum-free culture medium (Pereira *et al.*, 2006 and 2012).

Reports of horse oocyte development have focused on evaluating changes in chromatin configuration (Hinrichs *et al.*, 1993; Siddiqui *et al.*, 2009), mitochondrial organization (Torner *et al.*, 2007), as well as nuclear and cytoplasmic changes during oocyte maturation (Carneiro *et al.*, 2002). However, there is limited evidence on whether cytoskeleton distribution remains intact after IVM of equine oocytes cultured with additional growth factors. In fact, the number of *in vitro* matured equine oocytes used to evaluate oocyte development and organelle distribution is so minimal that it is difficult to determine oocyte integrity and viability using various treatment groups as an experimental design.

In most studies, maturation rates have been based on the number of oocytes that developed to metaphase II (M-II; Choi *et al.*, 2002; Hinrichs *et al.*, 2005). Assessments of the migration of cortical granules, formation of meiotic spindle and integrity of the cytoplasmic membrane have been used as indicators of oocyte maturity and integrity (Tremoleda *et al.*, 2001; Carneiro *et al.*, 2002; Siddiqui *et al.*, 2009). However, for an oocyte to achieve maturity, none of these parameters alone is a true indicator of oocyte competence for fertilization. To the best of our knowledge, there are no reports on the cytoskeleton structure of equine oocytes after the addition *in vitro* of eGH and its interaction with IGF-I, gonadotropins (FSH and LH), estradiol (E_2) and fetal calf serum (FCS). We hypothesize that the limited success achieved with IVM of horse oocytes is partly because of abnormalities associated with cytoskeleton structures, which influence cell viability and fertilization potential. The main objective of this research was to investigate the effects of eGH and its interaction with IGF-I, gonadotropins, E_2 and FCS on the integrity of cytoskeletal organization following IVM.

Materials and methods

This experiment was conducted in the Southern Brazil (latitude 27°28′S, longitude 51°55′W), from November to December, during the physiological breeding season. All chemicals used were purchased from Sigma Chemicals Company, St. Louis, MO, USA, unless otherwise indicated in the text.

Collection of cumulus–oocyte complexes (COCs)

Fresh equine ovaries were obtained from a slaughterhouse located 20 min from EMBRAPA Research Center, RS, Brazil, and immediately transported in warmed saline solution to the laboratory in an insulated container at 25°C to 30°C. A total of 144 ovaries were used to conduct this study, and the interval between ovary collection from the slaughterhouse and retrieval of COCs ranged from 2 to 4 h. Tunica albuginea from the ovaries selected for oocyte aspiration were stripped and follicular fluid from each follicle $<$30 mm in diameter was aspirated with an 18 G needle connected to a 35-ml syringe. After 20 min of sedimentation, the pellet was collected using a Pasteur pipette and placed into 100-mm Petri dishes.

All recovered COCs were evaluated for their morphology and structural integrity using a dissecting stereomicroscope and classified as being compact (having a tight, complete compact multilayer of cumulus cells), expanded (having granular or expanded layers of cumulus cells) and partially or completely denuded (having a partial or no cumulus cells, or only corona radiata, surrounding the oocytes; Hinrichs and Williams, 1997). We decided to use only compact COCs with homogeneous cytoplasm having a lower mitotic competence in comparison with expanded oocytes, we used only compact COCs in this experiment to test whether different supplementation of maturation media would be effective in improving the maturation rate of equine oocytes *in vitro*.

Culture of COCs

The COCs intended for incubation with maturation media were randomly allocated to each treatment group ($\sim$8 to 12 COCs per group). eGH (NHPP; Harbor-UCLA, CA, USA) was diluted in tissue culture media-199 (TCM-199) and used at a concentration of 400 ng/ml (Pereira *et al.*, 2012) for *in vitro* procedures. IGF-I was also diluted in TCM-199 and used at 200 ng/ml, in accordance with a study by Carneiro *et al.* (2001). Base medium was composed of TCM-199, 1 mg/ml bovine serum albumin (BSA), 100 IU/ml penicillin G and 50 μg/ml streptomycin sulfate. The medium was filtered through a 0.22 μm pore diameter filter into 50 ml centrifuge tubes and allowed to equilibrate for at least 1 h under 5% CO_2 in air before being used as the base culture medium. The COCs were washed four times in maturation medium before culture. This study comprised two experiments as described below. Both experiments undergoing IVM were cultured for 30 h at 38.5°C in air with 5% CO_2, as described below.

Experiment 1: Effect of eGH and IGF-I on equine COCs

COCs were cultured for 30 h and randomly assigned to one of five experimental groups as follows: (1) base medium (control); (2) 400 ng/ml eGH; (3) 200 ng/ml IGF-I; (4) 400 ng/ml eGH + 200 ng/ml IGF-I; and (5) eGH + 200 ng/ml IGF-I and 200 ng/ml anti-IGF-I (GR11T; Calbiochem, San Diego, CA, USA) antibody. We decided to investigate the effect of eGH using

neutralizing anti-IGF-1 antibodies in the presence of IGF-I to rule out any toxic effect on their interactions. This first experiment was replicated six times.

Experiment 2: Effect of eGH, E_2, FSH, LH and FCS on equine COCs

Equine COCs were randomly assigned to one of four experimental groups: (1) base medium (control); (2) eGH; (3) eGH + 1 mg/ml E_2, 5 IU/ml porcine FSH (Catalogue #915; Sioux Biochemical Inc., Sioux Center, IA, USA), 10 IU/ml of equine LH, and 100 mg/ml heat-inactivated FCS; and (4) eGH + FSH + LH + FCS at the same concentration cited above. The second experiment was replicated five times.

Oocyte processing and staining

After IVM, cumulus cells were removed with 1% hyaluronidase in DPBS-w/o solution for 1 min and mechanically stripped with a microcapillary glass pipette. Oocytes were permeabilized by treatment with medium M (0.5 M PIPES, 25 mM magnesium chloride, 0.01% aprotinin, 1 mM dithiothreitol, 50% deuterium oxide, 1 mM taxol and 0.1% Triton-X-100 in DPBS-w/o), a glycerol-based microtubule-stabilizing solution for 1 h at 39°C and fixed in 2% solution of paraformaldehyde in PBS-w/o. After fixation, they were maintained in paraformaldehyde at 4°C for 2 days and stored in blocking solution (0.2% of sodium azide, 1 mg/ml of polyvinyl alcohol and 100 mM of glycine in PBS-w/o) until immunofluorescent staining.

Microfilaments, microtubules and chromatin assessment were evaluated as described (Tremoleda *et al.*, 2001), with some modifications as given below. Microtubules were labeled first by incubating the oocytes with a primary antibody against microtubules (dilution 1 : 250, monoclonal anti-α-tubulin, Sigma; T-5168) for 90 min at 37°C. Then, oocytes were washed three times in a PBS containing 1% Triton-X-100 in DPBS-w/o and 5 mg/ml BSA before being incubated for 1 h in block solution. Next, oocytes were exposed to a secondary antibody conjugated with AlexaFluor 555 (dilution 1 : 250, goat anti-mouse IgG (#A21422; Molecular Probes, Eugene, OR, USA)) for 1 h at 37°C. Microfilaments were stained by oocyte incubation with AlexaFluor 488 Phalloidin (15 IU/ml, goat anti-mouse IgG_1 (#A21121; Molecular Probes)) for 1 h at 37°C. Finally, to enable chromatin visualization, oocytes were evaluated by counterstaining with TO-PRO_3-iodide (1 μg/ml (#T3605; Molecular Probes)) for 15 min at 37°C. Each step of immunolabeling of equine oocytes was performed in a dark chamber and followed by washing for 1 h in block solution.

After immunostaining, oocytes were placed on glass microscope slides with a space between the coverslip, filled with 25 μl drop of antifade mounting medium (#H1300; Vector Laboratories, Burlingame, CA, USA) and sealed with clear fingernail polish. Laser-scanning confocal microscopy was performed using a Bio-Rad MRC 1024 ES equipped with a Krypton–argon ion laser. Laser-scanning microscope equipped with three excitation filter sets of 488/555/650 nm were engaged simultaneously to excite AlexaFluor 488 Phalloidin, AlexaFluor 555 and TO-PRO-3 iodide (650 nm) for visualization of microfilaments, microtubules and chromatin detection, respectively. Each oocyte was fully observed with optical sections of 0.1 to 2.0 μm intervals.

Interpretation of results

Descriptive analyses of cytoskeleton integrity were used to determine the oocyte maturation rate upon culture condition as described earlier in the manuscript. All the parameters described below remained constant for all oocytes observed in this study. Assessment of oocyte integrity was evaluated by the configuration of the spindle, alignment of the chromosomes in the equatorial plane, and the amount and location of fluorescent microtubules and microfilaments in the oocyte. Oocytes in germinal vesicle (GV) stage were classified by having a diffuse appearance of their chromatin and weakly labeled microtubules and microfilaments distributed uniformly throughout the ooplasm. Metaphase-I (M-I) oocytes were characterized by the presence of highly condensed chromosomes, microtubules were radially elongated around the chromosomes and microfilaments were distributed throughout the oocyte. Metaphase-II (M-II) oocytes were characterized by the presence of well-organized chromosomes aligned along a second meiotic spindle, microtubules observed at the spindle and microfilament distribution concentrated in the cortex of the oocyte were classified as oocyte reaching M-II stage of development. Abnormal oocytes were characterized by the presence of a disorganized or disperse spindle elements or accumulation of microtubules mass near the condensed chromatin arrangements.

Statistical analyses

The differences in oocyte maturation were carried out by the χ^2 and Fisher's exact tests. Cytoskeleton distribution was merely descriptive and used to evaluate maturation status of equine oocytes after IVM. Statistical analysis was realized using Minitab 14 software (Minitab Inc., State College, PA, USA) and differences were considered significant when $P \leqslant 0.05$.

Results

From recovered oocytes, 360 (80.5%) were classified as compact, 52 (11.6%) were classified as degenerated and 35 (7.9%) were classified as expanded. After selection, 301 oocytes with compact cumulus cells were used in the experiment. We observed a lower number of evaluated oocytes (55%) used for final interpretation of the results owing to the fact that equine oocytes were preserved in block solution, transported to University of California, Davis, USA, and recovered from the eppendorf microtubes for staining and further confocal evaluation.

Chromatin configuration at the GV stage showed a diffused appearance without any visible organization into distinct chromosomes. Oocytes in the GV stage displayed a complex network of microtubules distributed uniformly throughout the ooplasm. Oocytes that had progressed to metaphase of the first meiotic division showed individual chromosomes as aggregated dots of condensed chromatin. A complete

Table 1 *Effects of eGH and IGF-I on maturation status of equine oocytes* in vitro *matured after 30 h of culture*

Culture treatment	Oocytes cultured (n)	Oocytes evaluated (n (%))	Abnormal (n (%))	GV (n (%))	M-I (n (%))	M-II (n (%))
Control	35	14 (40.0)	4 (28.6)[a]	6 (42.9)[a]	1 (7.1)[a]	3 (21.4)[a]
eGH	37	22 (59.4)	2 (9.1)[a]	7 (31.8)[a]	5 (22.7)[a]	8 (36.4)[a]
IGF-I	32	14 (43.7)	2 (14.3)[a]	5 (35.7)[a]	2 (14.3)[a]	5 (35.7)[a]
eGH + IGF-I	31	17 (54.8)	0[a]	9 (52.9)[a]	2 (11.8)[a]	6 (35.3)[a]
eGH + IGF-I + anti-IGF-I	34	18 (52.9)	1 (5.5)[a]	7 (38.9)[a]	5 (27.8)[a]	5 (27.8)[a]

GV = germinal vesicle; M-I = metaphase I; M-II = metaphase II; eGH = equine growth hormone (400 ng/ml); IGF-I = insulin growth factor-I (200 ng/ml); anti-IGF-I = anti-insulin growth factor-I antibody (200 ng/ml).
Data from six replicates were pooled.
[a]No statistical significant difference was observed between treated groups ($P > 0.05$).

Table 2 *Maturation status of equine oocytes matured* in vitro *with estradiol, gonadotropins and FCS in the presence and absence of eGH after 30 h of culture*

Culture treatment	Oocytes cultured (n)	Oocytes evaluated (n (%))	Abnormal (n (%))	GV (n (%))	M-I (n (%))	M-II (n (%))
Control	27	17 (62.9)	3 (17.6)[a]	8 (47.1)[a]	3 (17.6)[a]	3 (17.6)[a]
eGH	33	20 (60.6)	1 (5.0)[a]	6 (30.0)[a]	5 (25.0)[a]	8 (40.0)[b]
E_2 + FSH + LH + FCS	30	15 (50.0)	2 (13.3)[a]	6 (40.0)[a]	4 (26.7)[a]	3 (20.0)[a,b]
eGH + E_2 + FSH + LH + FCS	42	30 (71.4)	3 (10.0)[a]	6 (20.0)[a]	10 (33.3)[a]	11 (36.7)[b]

GV = germinal vesicle; M-I = metaphase I; M-II = metaphase II; eGH = equine growth hormone (400 ng/ml); E_2 = estradiol (1 μg/ml); FSH = follicle-stimulating hormone (5 IU/ml); LH = luteinizing hormone (10 IU/ml), FCS = fetal calf serum (10 mg/ml).
[a,b]Different superscripts within a column indicate significant differences between groups ($P < 0.05$).
Data from five replicates were pooled.

rearrangement of the microtubules was observed after GV breakdown (GVBD) that displayed radial-like microtubules in close association with the recent condensed chromosomes (Supplementary Figure S1a, b). Oocytes that reached metaphase of the second meiotic division showed compact chromosomes aligned along the equatorial plane of the spindle structure. Metaphase-II oocytes had a classic symmetrical barrel-shaped structure, with chromosomes aligned along the equatorial plane and microtubules forming a clear spindle (Supplementary Figure S1c, d). Abnormal spindle structures were associated with lack of chromosomes and an accumulation of a microtubular mass near the condensed chromatin. Microtubules appeared to be detached from the spindle poles and spread into the ooplasm (Supplementary Figure S1e, f). When microfilaments were analyzed, oocytes at GV stage showed a distribution throughout the subcortical region of the ooplasm (Supplementary Figure S2a). Microfilaments appeared as a layer immediately beneath the plasma membrane and as discrete foci on the cortical region as oocytes progress to maturity (Supplementary Figure S2b, c). Abnormal actin filaments distribution was observed as an irregular mass close to the condensed chromatin (Supplementary Figure S2d). Cytoskeleton configuration was used to provide the basic description of cytoskeleton modification during IVM, and to determine the maturation status of each oocyte for its final interpretation.

Experiment 1: Effects of eGH and IGF-I on equine COCs

A total of 169 COCs were cultured *in vitro*, but only 85 oocytes were stained. Overall, the percentage of 10.6%, 40.0%, 17.6% and 31.8% oocytes were classified as abnormal, GV, M-I and M-II stages of development, respectively. The addition of eGH, IGF-I and eGH + IGF-I in the base medium had no significant effect on the percentage of equine oocytes reaching M-II compared with the control group, whereas the highest maturation rates (36.3%) were observed when eGH alone was added into maturation medium. The highest percentage of GV oocytes was observed when the eGH and IGF-I (9/17; 52.9%) were added in maturation medium. In this experiment, only 10.6% (9/85) COCs evaluated showed the presence of abnormal chromatin configuration with dispersed chromosomes. Data are presented in Table 1.

Experiment 2: Effect of eGH, E_2, FSH, LH and FCS on equine COCs

A total of 132 COCs were matured *in vitro*, but only 82 oocytes were stained. Overall, the percentage of 11.0%, 31.7%, 26.8% and 30.5% oocytes were classified as abnormal, GV, M-I and M-II stages of development, respectively. More ($P < 0.05$) oocytes reached M-II stage after maturation in the presence of eGH, E_2, FSH, LH and FCS than the control group (11/30; 36.7% *v.* 3/17; 17.6%, respectively). However, no difference ($P > 0.05$) was observed when COCs were treated with eGH, E2, FSH, LH and FCS compared with eGH alone. Incubation with eGH alone had the highest maturation rate (40.0%, 8/20) when compared with control (3/17; 17.6%) ($P < 0.05$; Table 2).

Discussion

The effects of eGH with IGF-I, gonadotropins, E_2 and FCS on IVM and cytoskeleton organization of equine oocytes were

investigated in this study. The results showed that eGH added in the presence of E_2, gonadotropins and FCS had a positive effect on nuclear maturation compared with equine oocytes cultured *in vitro* without additional growth factors. The addition of eGH to an IVM media containing hormones promotes nuclear maturation and cytoplasmic segregation during the maturation of equine oocytes *in vitro*.

In the current study, we found that eGH in the presence of IGF-I added to IVM media caused no change in the percentage of equine oocytes reaching M-II (35.29%) compared with the control group (21.43%); however, the highest maturation rates (36.36%) were observed when eGH alone was added into culture. Hinrichs and Williams (1997) reported that equine oocytes with compact cumulus cells are not competent to mature *in vitro*; however, expanded oocytes have a high meiotic competence. In contrast, Carneiro *et al*. (2002) observed that compact equine oocytes were able to mature in the presence of IGF-I and gonadotropins *in vitro*. In previous studies, we have demonstrated that the addition of eGH to media containing IGF-I significantly increased the maturation of equine oocytes to M-I and M-II stages of development when compared with control (Pereira *et al*., 2006, 2012). Carneiro *et al*. (2001, 2002) used an IVM system in the presence of IGF-I and observed an improved cytoplasmic maturation on the basis of the migration of cortical granules to the periphery of horse oocytes and an increased cleavage rate after parthenogenetic activation. IGF-I has been reported to stimulate bovine oocyte maturation and fertilization *in vitro* (Lorenzo *et al*., 1994), and to promote rabbit blastocyst development (Herrler *et al*., 1998). Moreover, the effects of eGH could be possibly mediated by IGF-I in equine oocytes matured *in vitro*.

We also observed in our study that compact COCs were effective for maturation under culture with eGH associated with E_2, FSH, LH and FCS. The percentage of oocytes cultured with eGH in the presence of E_2, FSH, LH and FCS significantly increased the number of oocytes reaching M-II stage (36.67%) compared with oocytes in the control group (17.65%). The low maturation rate observed in the control group may be partly explained by the absence of serum in the media containing COCs. Thus, the addition of hormones and factors into IVM might suggest an important role to trigger oocyte maturation *in vitro*. However, when eGH was added alone into IVM, maturation rates were similar compared with the addition of eGH to an IVM media containing hormones (40.0% *v.* 36.7%, respectively). Marchal *et al*. (2003) reported a significant increase in the percentage of oocytes reaching M-II (43%) when eGH and equine-luteinizing hormone (eLH) were used *in vitro* to promote equine oocyte development. Gonadotropins are the main regulators of nuclear maturation of oocytes *in vivo*; however, recent observations suggest that gonadotropins are only part of a complex system of autocrine and paracrine factors that may influence oocyte competence. In agreement with Marchal *et al*. (2003), we also observed a maturation rate of 40.0% in oocytes reaching M-II stage when eGH was added into culture.

The present study showed that microtubules and microfilaments play an important role on the distribution of components of the cytoskeleton that can facilitate nuclear maturation and cytoplasmatic segregation of horse oocytes cultured *in vitro*. We observed that cytoskeleton changes were more evident in the majority of oocytes matured in the presence of eGH, E_2, gonadotropins and FCS group. Siddiqui *et al*. (2009) reported rates of 89% of oocytes reaching M-I and M-II stages of development on the basis of nuclear configuration, spindle morphology and cytoskeleton organization using fluorescent probes to evaluate horse oocytes collected *in vivo* from human chorionic gonadotropin-stimulated mares. The results from the present study are in agreement with a report (Tremoleda *et al*., 2003) that described cytoskeletal changes of equine oocytes before fertilization by intracytoplasmic sperm injection and also in equine embryos.

In our study, oocytes in GV stage displayed a complex of microtubule networks distributed uniformly throughout the ooplasm, and the chromatin configuration had a diffused appearance without any visible organization into distinct chromosomes. Similar observations have also been described in GV mouse oocytes where cytoplasmic microtubules began to extend into the subcortical regions of the ooplasm when meiotic progression toward GVBD occurred (Messinger and Albertini, 1991). In contrast, goat and pig oocytes showed no microtubule distribution in early GV stage, although they become apparent in the meiotic spindle during the later meiotic stages (Kim *et al*., 1997; Sui *et al*., 2005).

A complete rearrangement of the cytoplasmic microtubules was observed after GVBD that displayed radial-like microtubules in close association with condensed chromosomes. Oocytes that had progressed to the metaphase of the first meiotic division showed individual chromosomes visible as aggregated dots of condensed chromatin. This finding is associated with the development of meiotically competent oocytes and the phosphorylation of centrosomes in other mammalian species (Sui *et al*., 2005). Viable oocytes in the current study showed microtubules at the meiotic spindle as a symmetrical barrel-shaped structure with chromosomes aligned along its center. Our description of the meiotic spindle of horse oocytes at M-II stage was very similar to that reported for other species such as cows (Kim *et al*., 2000) and pigs (Ma *et al*., 2003). In horses, Goudet *et al*. (1997) and Tremoleda *et al*. (2001) also reported similar microtubule organization that was also concentrated in the meiotic spindle surrounding the aligned chromosomes.

The presence of disorganized microtubules at the meiotic spindle seen in the present study has also been described in women oocytes (Li *et al*., 2006). Damage to microtubule organization results in depolymerization of the meiotic spindles in mammalian oocytes, leading to an irreversible process that results in chromosomal abnormalities after fertilization, such as aneuploidy and polyploidy (Wang and Sun, 2006). Mammalian oocytes that are atypical in the progression of meiotic maturation displayed aberrant configurations of microtubules and abnormal chromosome

morphology (De La Fuente, 2006). Recently, Siddiqui *et al*. (2009) reported a rate of 11% of atypical equine oocytes recovered *in vivo* showing apparent abnormalities of the microtubules organization. In agreement with reports on mouse oocytes, we also observed that a few oocytes (around 10%) had a scattered and disorganized aggregation of chromatin and microtubules.

Oocytes reaching M-II stage showed the distribution of microfilaments with a stained layer of actins observed immediately beneath the plasma membrane. In the present study, we observed the presence of strands of microfilaments within the zona pellucida; a similar pattern has been described as part of transzonal processes extending from the cumulus cells, which is thought to be essential to the production of a developmentally competent female gamete (Allworth and Albertini, 1993).

Conclusions

In summary, the present study provides a description of microtubules, microfilaments and chromatin configuration on compact horse oocytes after culture with additional growth factors *in vitro*. On the basis of the results described in this study, we conclude that the use of eGH in the presence of E_2, gonadotropins and FCS has a positive effect on the proportion of oocytes reaching M-II stage of compact equine oocytes during 30 h of culture *in vitro*. These observations suggest a synergistic interaction between eGH, gonadotropins, E_2 and FCS in nuclear maturation and cytoskeleton segregation of equine oocytes. These findings emphasize that further studies at the molecular level are needed to understand different oocyte handling procedures *in vitro* in horses.

Acknowledgments

This research was supported by The Del Amo Program Grant (University of California, Davis and Universidad Complutense de Madrid, Spain), The Temperate Climate Research Corporation (EMBRAPA) and The Coordination for the Improvement of Higher Education Personnel (CAPES), Brazil. The authors thank Dr Parlow from the National Hormone and Peptide Program (NHPP) at Harbor UCLA Medical Center to provide the eGH hormone for this study. The authors thank Frank Ventimiglia (UCDavis) for assistance with confocal microscopy and imaging.

Competing Interests

The author(s) declare that they have no competing interests.

Supplementary materials

For supplementary material referred to in this article, please visit http://dx.doi.org/10.1017/S175173111300116X

References

Allworth AE and Albertini DF 1993. Meiotic maturation in cultured bovine oocytes is accompanied by remodeling of the cumulus cell cytoskeleton. Developmental Biology 158, 101–112.

Carneiro GF, Liu IK, Hyde D, Anderson GB, Lorenzo PL and Ball BA 2002. Quantification and distribution of equine oocyte cortical granules during meiotic maturation and after activation. Molecular Reproduction and Development 63, 451–458.

Carneiro GF, Lorenzo PL, Pimentel CA, Pegoraro LC, Bertolini M, Ball BA, Anderson GB and Liu IK 2001. Influence of insulin-like growth factor-I and its interaction with gonadotropins, estradiol, and fetal calf serum on *in vitro* maturation and parthenogenic development in equine oocytes. Biology of Reproduction 65, 899–905.

Choi YH, Lee BC, Lim JM, Kang SK and Hwang WS 2002. Optimization of culture medium for cloned bovine embryos and its influence on pregnancy and delivery outcome. Theriogenology 58, 1187–1197.

De La Fuente R 2006. Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. Developmental Biology 292, 1–12.

Dell'Aquila ME, Fusco S, Lacandra GM and Mariato F 1997. Intracytoplasmic sperm injection (ICSI) versus conventional IVF on abattoir-derived and *in vitro*-matured oocytes. Theriogenology 47, 1139–1156.

Erickson GF, Garzo VG and Magoffin DA 1989. Insulin-like growth factor-I regulates aromatase activity in human granulosa and granulosa luteal cells. The Journal of Clinical Endocrinology and Metabolism 69, 716–724.

Goudet G, Bezard J, Duchamp G and Palmer E 1997. Transfer of immature oocytes to a preovulatory follicle: an alternative to *in vitro* maturation in the mare? Equine Veterinary Journal Supplement 25, 54–59.

Guler A, Poulin N, Mermillod P, Terqui M and Cognie Y 2000. Effect of growth factors, EGF and IGF-I, and estradiol on *in vitro* maturation of sheep oocytes. Theriogenology 54, 209–218.

Herrler A, Krusche CA and Beier HM 1998. Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis. Biology of Reproduction 59, 1302–1310.

Hinrichs K 2010. The equine oocyte: factors affecting meiotic and developmental competence. Molecular Reproduction and Development 77, 651–661.

Hinrichs K and Williams KA 1997. Relationships among oocyte-cumulus morphology, follicular atresia, initial chromatin configuration, and oocyte meiotic competence in the horse. Biology of Reproduction 57, 377–384.

Hinrichs K, Schmidt AL, Friedman PP, Selgrath JP and Martin MG 1993. *In vitro* maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. Biology of Reproduction 48, 363–370.

Hinrichs K, Choi YH, Love LB, Varner DD, Love CC and Walckenaer BE 2005. Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. Biology of Reproduction 72, 1142–1150.

Hull KL and Harvey S 2001. Growth hormone: roles in female reproduction. Journal of Endocrinology 168, 1–23.

Kim NH, Chung KS and Day BN 1997. The distribution and requirements of microtubules and microfilaments during fertilization and parthenogenesis in pig oocytes. Journal of Reproduction and Fertility 111, 143–149.

Kim NH, Cho SK, Choi SH, Kim EY, Park SP and Lim JH 2000. The distribution and requirements of microtubules and microfilaments in bovine oocytes during *in vitro* maturation. Zygote 8, 25–32.

Li Y, Feng HL, Cao YJ, Zheng GJ, Yang Y, Mullen S, Critser JK and Chen ZJ 2006. Confocal microscopic analysis of the spindle and chromosome configurations of human oocytes matured *in vitro*. Fertility and Sterility 85, 827–832.

Lorenzo PL, Illera MJ, Illera JC and Illera M 1994. Enhancement of cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I. Journal of Reproduction and Fertility 101, 697–701.

Ma W, Hou Y, Sun QY, Sun XF and Wang WH 2003. Localization of centromere proteins and their association with chromosomes and microtubules during meiotic maturation in pig oocytes. Reproduction 126, 731–738.

Marchal R, Caillaud M, Martoriati A, Gerard N, Mermillod P and Goudet G 2003. Effect of growth hormone (GH) on *in vitro* nuclear and cytoplasmic oocyte maturation, cumulus expansion, hyaluronan synthases, and connexins 32 and 43 expression, and GH receptor messenger RNA expression in equine and porcine species. Biology of Reproduction 69, 1013–1022.

Matsui M, Takahashi Y, Hishinuma M and Kanagawa H 1995. Insulin and insulin-like growth factor-I (IGF-I) stimulate the development of bovine embryos fertilized *in vitro*. Journal of Veterinary Medical Science 57, 1109–1111.

Messinger SM and Albertini DF 1991. Centrosome and microtubule dynamics during meiotic progression in the mouse oocyte. Journal of Cell Science 100, 289–298.

Pereira GR, Lorenzo PL, Carneiro GF, Ball BA, Goncalves PB, Pegoraro LM, Bilodeau-Goeseels S, Kastelic JP, Casey PJ and Liu IK 2012. The effect of growth hormone (GH) and insulin-like growth factor-I (IGF-I) on *in vitro* maturation of equine oocytes. Zygote 20, 353–360.

Pereira GR, Lorenzo PL, Carneiro GF, Bilodeau-Goeseels S, Kastelic JP, Pegoraro LM, Pimentel CA, Esteller-Vico A, Illera JC, Silvan G, Casey P and Liu IK 2006. Effect of equine growth hormone (eGH) on *in vitro* maturation of equine oocytes and on steroidogenesis by their cumulus-oocyte complexes. Animal Reproduction Science 94, s364–s365.

Shirazi A, Shams-Esfandabadi N, Ahmadi E and Heidari B 2010. Effects of growth hormone on nuclear maturation of ovine oocytes and subsequent embryo development. Reproduction in Domestic Animal 45, 530–536.

Siddiqui MA, Gastal EL, Ju JC, Gastal MO, Beg MA and Ginther OJ 2009. Nuclear configuration, spindle morphology and cytoskeletal organization of *in vivo* maturing horse oocytes. Reproduction in Domestic Animal 44, 435–440.

Sui HS, Liu Y, Miao DQ, Yuan JH, Qiao TW, Luo MJ and Tan JH 2005. Configurations of germinal vesicle (GV) chromatin in the goat differ from those of other species. Molecular Reproduction and Development 71, 227–236.

Torner H, Alm H, Kanitz W, Goellnitz K, Becker F, Poehland R, Bruessow KP and Tuchscherer A 2007. Effect of initial cumulus morphology on meiotic dynamic and status of mitochondria in horse oocytes during IVM. Reproduction in Domestic Animal 42, 176–183.

Tremoleda JL, Schoevers EJ, Stout TA, Colenbrander B and Bevers MM 2001. Organisation of the cytoskeleton during *in vitro* maturation of horse oocytes. Molecular Reproduction and Development 60, 260–269.

Tremoleda JL, Van Haeften T, Stout TA, Colenbrander B and Bevers MM 2003. Cytoskeleton and chromatin reorganization in horse oocytes following intracytoplasmic sperm injection: patterns associated with normal and defective fertilization. Biology of Reproduction 69, 186–194.

Wang WH and Sun QY 2006. Meiotic spindle, spindle checkpoint and embryonic aneuploidy. Frontiers in Bioscience 11, 620–636.

Yoshimura Y, Iwashita M, Karube M, Oda T, Akiba M, Shiokawa S, Ando M, Yoshinaga A and Nakamura Y 1994. Growth hormone stimulates follicular development by stimulating ovarian production of insulin-like growth factor-I. Endocrinology 135, 887–894.