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Nuclear maturation and mitochondrial distribution in equine oocytes matured *in vitro*

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Abstract

The improvement of efficiency of methods to mature equine oocytes *in vitro* is necessary for the application of biotechnologies in equine reproduction. Two maturation media, TCM-199 and follicular fluid (FF), were compared to mature equine cumulus-oocyte complexes (COCs) *in vitro*. Nuclear maturation and mitochondria distribution, and the ability to develop into embryos after transfer to inseminated mares were used as end points. In compact COCs, the percentage of oocytes that resumed meiosis (Metaphase I and II) was significantly higher in FF (77.3%) than in TCM-199 (46.8%). Compact COCs matured in FF reached a significantly ($P < 0.05$) higher percentage of Metaphase-II oocytes (31.8%) than those matured in TCM-199 (12.8%). Stages of nuclear maturation were associated with different patterns of mitochondrial distribution; 52.6% (40/76) of the oocytes found at germinal vesicle had dense clusters of mitochondria in the cortical area, whereas most oocytes at Metaphase I (63.6%, 42/66) showed either non-polarized or polarized patterns of mitochondria. Similarly, 76.7% (33/43) of Metaphase-II oocytes also had these patterns. After intrafollicular or intra-oviductal transfer to inseminated mares, few oocytes matured in FF (4.4%, 3/68) and developed to embryos *in vivo* compared to oocytes matured in TCM-199 (0/58). In conclusion, pure equine FF was suitable as an *in vitro* maturation medium, inducing higher rates of nuclear maturation than a semi-defined medium. In addition, distribution of mitochondria changed during maturation from dense clusters of mitochondria in the cortical region in germinal-vesicle oocytes to a more dispersed pattern in Metaphase-I and -II oocytes.

Keywords: embryo, equine, oocyte, mitochondria.

Introduction

Maturation of the female gamete *in vitro* is an essential procedure for a number of reproductive biotechnologies such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), and cloning. In the mare, results of *in vitro* oocyte maturation (IVM) are not consistent and reliable as in other species. This limits the development and application of reproductive biotechnologies in the mare. Highly variable nuclear maturation rates have been reported for mare oocytes (Dell'Aquila *et al.*, 1999; Gable and Woods, 2001) in contrast with other species, such as cows (Way and Killian, 2006), ewes (Grazul-Bilska *et al.*, 2006), and sows (Kobayashi *et al.*, 2006) where the procedure is repeatable and the results more satisfactory. Moreover, a few studies have been able to produce viable pregnancies and foals from *in vitro* matured equine oocytes using ICSI (Li *et al.*, 2001; Galli *et al.*, 2007), oocyte transfer (Carnevale *et al.*, 2003), and cloning (Galli *et al.*, 2003; Hinrichs *et al.*, 2006). In a similar approach, Woods *et al.* (2003) obtained oocytes from preovulatory follicles that were cultured *in vitro* for about 14 h before performing nuclear transfer to produce the first mule clones.

A reliable and consistent method for oocyte maturation *in vitro* would be of great benefit to the field of equine reproduction. A proportion of follicular fluid has been used to supplement synthetic IVM medium for equine (Dell'Aquila *et al.*, 1997, 1999; Gil *et al.*, 2005) and bovine (Ali *et al.*, 2004) oocytes with satisfactory results. In addition, pure equine follicular fluid (100%) used as undefined maturation medium has supported nuclear maturation of equine oocytes *in vitro* (Aguilar *et al.*, 1999, 2001; Bogh *et al.*, 2002; Conforti *et al.*, 2005) and to some extent also allowed subsequent IVF (Hinrichs *et al.*, 2002). It was shown that follicular fluid from cycling mares was more efficient to induce nuclear maturation than the conventional TCM-199 with hormones (Conforti *et al.*, 2005). Moreover, bovine follicular fluid supported *in vitro* nuclear maturation of bovine oocytes (Avery *et al.*, 2003) that subsequently were fertilized and developed to blastocysts *in vitro* (Choi *et al.*, 1998).

Progression during meiosis is normally utilized to evaluate the degree of oocyte nuclear maturation *in vitro*. Cytoplasmic maturation, which involves a complex reorganization of organelles within the ooplasm, is usually measured indirectly by the rate of male pronuclear formation or cleavage. One of the cytoplasmic changes that occur during oocyte maturation is the translocation and redistribution of mitochondria that seems to be essential to provide energy in specific cell regions where ATP is needed more (Van Blerkom and

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Runner, 1984) or where calcium release is required (Sousa et al., 1997). Mitochondria are the most abundant organelles and the main source of energy in oocytes. It has been shown that activity and redistribution of mitochondria in bovine oocytes influence the potential competence for reaching the blastocyst stage (Tarazona *et al.*, 2006) and that the number of mitochondria can directly affect the fertilization outcome of porcine oocytes *in vitro* (El Shourbagy et al., 2006). A recent study showed changes in mitochondrial distribution and mitochondrial activity during IVM related to the initial cumulus morphology in equine cumulus-oocyte complexes (COCs; Torner et al., 2007).

Regardless of the degree of cytoplasmic maturation, the capacity of an oocyte to be fertilized and to develop into a normal embryo is, eventually, the most relevant measure of its competence. Because no reliable IVF and culture systems for horse oocytes have been developed, in this study we evaluated the ability of *in vitro* matured oocytes to develop to blastocysts once transferred into recipient mares. Therefore, the objectives of this study were: a) to compare the efficiency of two maturation media, TCM-199 and 100% follicular fluid, to mature equine COCs *in vitro*; b) to study redistribution of mitochondria in equine COCs before and after IVM; and c) to test the capacity of *in vitro* matured COCs to develop to embryos after transfer to mares.

Materials and Methods

All animal procedures were performed with approval from the Science and Technology Secretary of the National University of Rio Cuarto, Cordoba, Argentina.

COC recovery

Ovaries from light, crossed type mares of unknown reproductive history were collected at a local slaughterhouse and transported to the laboratory at 25°C in saline solution within 2 h post mortem. Once in the laboratory, ovaries were dissected free of connective tissue, and the tunica albuginea was removed. Only individual follicles between 15 and 30 mm in diameter were isolated and opened on a sterile Petri dish as previously described (Okolski *et al*., 1987). Follicles smaller than 15 mm were discarded to reduce variability among oocytes. Cumulus-oocyte complexes were searched using a stereomicroscope and classified as compact (fully compacted cumulus and granulosa cells or with minor signs of expansion in the outer granulosa cells), expanded (signs of expansion in more than two thirds of the surrounded granulosa cells), or denuded (oocytes with only corona cells around the zona pellucida). After classification, COCs were placed in holding medium (TCM-199 with Hanks´ salts, Gibco Invitrogen, Buenos Aires, Argentina) at 37°C until they were randomly assigned to a maturation medium. Independent of cumulus state, oocytes with clear signs of degeneration such as cytoplasm fragmentation or condensation (increased perivitelin space) were discarded. A total of 168 ovaries were processed in 8 repetitions during this study.

Follicular fluid

Preovulatory follicular fluid (FF) was recovered from slaughterhouse ovaries as previously described (Aguilar *et al.*, 2001). Briefly, reproductive tracts were recovered from mares at a local slaughterhouse and transported to the laboratory on ice within 1 h post mortem. Preovulatory follicles were selected based on diameter (40 to 55 mm) and the absence of a mature corpus luteum per pair of ovaries. Follicular fluid from four selected follicles was aspirated, pooled, sterilized by filtration, and frozen at -70°C until use.

In vitro maturation

Groups of 10-16 COCs were matured in 125-µl droplets of medium under mineral oil (Sigma, Buenos Aires, Argentina) for 36 to 40 h at 38°C in an incubator with 5% CO_2 and humidified atmosphere. One hundred percent FF from preovulatory follicles or TCM-199 with Earl's salts containing oFSH 5 µg/ml, oLH 2 µg/ml, IGF-1 100 ng/ml, EGF 100 ng/ml, and 10% fetal calf serum (Sigma, Buenos Aires, Argentina) were used as maturation media.

Oocyte staining

At the end of the maturation period or immediately after recovery (control group), COCs were placed in a solution of 0.2% trypsin in calcium-free phosphate-buffered saline (PBS) for 5-10 min. During this time, the COCs were denuded of cumulus cells by repeated pipetting with a thin (250 to 300 µm in diameter) glass Pasteur pipette. Once denuded, the oocytes were rinsed twice with Dulbecco modified Eagle's minimal essential medium (DMEM, Gibco Invitrogen, Buenos Aires, Argentina) and then transferred to a 125-nM solution of MitoTracker Deep Red 633® (Molecular Probes, Invitrogen, Buenos Aires, Argentina) in DMEM at 37°C for 15 min in the dark. Oocytes were then rinsed again in DMEM and fixed in a freshly prepared buffered solution of 3.7% formaldehyde and kept in vials at 4°C until processing for confocal microscopy.

On the day confocal microscopy was performed, the oocytes were removed from the vials, rinsed twice in DMEM, and stained for DNA with a 1-µM solution of Sytox Green ® (Molecular Probes, Invitrogen, Buenos Aires, Argentina) in DMEM for 15 min in the dark. Then oocytes were individually mounted on a glass

slide in 50 µl of antifade solution (Molecular Probes) and covered with an imaging chamber (CoverWell imaging chambers, Sigma, Buenos Aires, Argentina).

Confocal microscopy

Stained oocytes were individually visualized at 20X magnification, and a series of 8 to12 optical slices were colleted with a confocal laser scanning microscope (LSM 5 Pascal, Zeiss, Jenna). For nuclear maturation, oocytes were classified as: a) Germinal Vesicle (GV) – condensed or diffuse chromatin; b) Metaphase I (MI) - clearly condensed and aligned chromosomes in the equatorial plane; c) Metaphase II (MII) aligned chromosomes in the equatorial plane plus a polar body (marginal area of dense chromatin); or d) Degenerated (Deg) - no detectable chromatin or undetermined patterns of chromatin that were usually dense irregular areas of fluorescence spread in the ooplasm. Three oocytes (1 from a compact COC and 2 from denuded COCs) found in anaphase II showing two distinct groups of chromosomes migrating in the ooplasm were categorized as Metaphase-II oocytes.

Distribution of mitochondria in the oocytes was classified in four patterns: a) Peripheral Clusters (PC) - peripheral clusters of dense mitochondria spread through the cortical region; b) Non-polarized (NP) - evenly distributed mitochondria throughout the cortical and subcortical cytoplasmic regions, usually almost no mitochondria in the center; c) Polarized (Pol) - uneven distribution of mitochondria throughout the cortical and subcortical regions, normally polarized occupying one half to two thirds of the oocyte; d) Other - any other undefined patterns including weak or no fluorescence, cytoplasmic fragments, or empty vesicles.

Intrafollicular transfer

After IVM, only COCs initially having a compact cumulus were used in this experiment. Six criollo-type mares were used as recipients. *In vitro* maturation was initiated when recipient mares showed signs of estrus and a growing follicle of 34 to 38 mm was detected by ultrasound. A 2500-IU dose of hCG (Ovusyn, Syntex SA, Argentina) was then administered i.v. to induce ovulation. The next day the recipient mares were artificially inseminated with fresh extended semen from a stallion of proven fertility, receiving at least 2000 million progressively motile spermatozoa. After 36 h of *in vitro* maturation, COCs were transferred to 300 µL of Hepes TCM-199 supplemented with 10% FCS and loaded in a flexible tip pipette connected to a 1-ml syringe with a 3-way stopcock. Recipient mares were administered 0.5 mg/kg, xylazine i.v. (Sedomin®, Xilacina 100mg/ml, König Labs, Buenos Aires, Argentina) and 0.02 mg/kg butorphanol i.v. (Torbugesic®, Butorfanol 10mg/ml, Fort Dodge Animal Health, México,D.F., Mexico) for sedation. In addition, 10 ml of lidocaine 2% (lidocaine HCl Elkins-Sinn, Inc., Cherry Hill, NJ) were administered subcutaneously and intramuscularly in the flank ipsilateral to the dominant follicle. An ultrasound-guided (6 Mhz) spinal needle (18 G) inserted through the flank was used to transfer 10 to 16 COCs into the preovulatory follicle while an operator was transrectally holding the ovary against the abdominal wall. Three to 5 ml of follicular fluid were aspirated to confirm that the tip of the needle was indeed within the follicular cavity. This volume of follicular fluid was kept in a 10-ml sterile syringe connected to the 3-way stopcock. Immediately after the COCs were injected into the follicle, 1 ml of the recently aspirated follicular fluid was pushed back into the follicle to flush the injecting circuit. Turbulence within the preovulatory follicle observed on the screen of the ultrasound machine was interpreted as a successful transfer. Ovulation of the “recipient follicle” was confirmed to occur within 6 h after the COC transfer.

Intraoviductal transfer

After detection of estrus and a preovulatory follicle in the recipient mares, hCG administration and artificial insemination were performed in four recipient mares as described above. Standing flank laparotomy (Squires and Seidel, 1995) was performed in the recipient mares to transfer only compact COCs matured *in vitro* into the ipsilateral oviduct of the dominant follicle. Surgeries were performed 24 h after hCG injection and 4 to 6 h after insemination. The animals were prepared and tranquilizing in the same way with the addition of paravertebral anesthesia (Muir and Hubbell, 1991) to achieve regional analgesia in the ipsilateral flank of the dominant follicle. Antibiotics (Estreptopendiben®, Estreptomicina-Penicilina 5.000.000 UI; Instituto San Jorge Bagó, Buenos Aires, Argentina) were administrated for 5 d beginning the day of the surgery. When the ovary was located and exposed through the incision, the *in vitro* matured COCs, loaded in 50 µl of Hepes TCM-199 supplemented with 10% FCS within a flexible tip pipette connected to a 1-ml syringe, were injected into the ampulla of the oviduct about 2 cm past the infundibular os (Carnevale *et al*., 2005). The ovary was immediately replaced and muscular planes and skin sutured at the surgical incision.

Embryo recovery

Embryo recovery in the recipient mares was performed by non-surgical uterine lavage (McKinnon and Squires, 1988) on Day 8 post ovulation. Three one-liter uterine flushes (PBS with 1% fetal calf serum) were repeated using a closed system with an inline filter. To calculate the number of embryos derived from *in vitro* matured COCs, the number of ovulations in

each recipient mare was subtracted from the number of embryos recovered during the uterine lavage.

Statistical analysis

Chi square analysis or Fisher exact test, where appropriate, were performed to compare nuclear maturation stages and mitochondrial patterns among the three groups (control, TCM-199 and FF). Differences were considered significant at $P < 0.05$.

Results

Nuclear maturation

In both maturation media, we observed progress in nuclear maturation but to different extents. The proportion of GV oocytes was significantly reduced in all oocyte groups in comparison to the non-cultured control oocytes. Nuclear maturation stages after IVM determined by confocal microscopy of oocytes before and after IVM are shown in detail in Table 1. In compact COCs matured in FF, the oocytes reached a significantly higher percent of MII (31.8%) than oocytes matured in TCM-199 (12.8%). The percentage of oocytes that resumed meiosis (MI and MII combined) was also significantly higher in FF (77.3%) than in TCM-199 (46.8%). The percentage of degenerated oocytes was significantly lower in FF than in TCM-199 (20.5% vs. 44.7%, respectively), but not different from the control (30.4%).

Table 1. Nuclear maturation patterns (percentages) of oocytes at Time 0 (Control) and after *in vitro* maturation in TCM-199 and follicular fluid for 36 to 40 h.

	Compact					Expanded					Denuded				
	n	GV	MI	MII	Deg	n	GV	MI	MII	Deg	n	GV	MI	MII	Deg
Control	56	67.9^{a}	0.0^{a}	1.8^{a}	30.4^{ab}	22	59.1^{a}	13.6	9.1^{a}	18.2	19	84.2^{a}	5.3^{a}	0.0^{a}	10.5
TCM-199	47	8.5^{b}	34.0^{b}	12.8^{b}	44.7^{a}	18	16.7^{b}	22.2	16.7^{ab}	44.4	17	5.9^{b}	47.1^{b}	17.6^{ab}	29.4
FF	44	2.3^{b}	45.5^{c}	31.8^{c}	20.5^{b}	16	0.0^{b}	25.0	43.8^{b}	31.3	26	0.0^{b}	38.5^{b}	26.9^{b}	34.6

^{a,b,c}Different superscripts indicate statistical differences within columns ($P < 0.05$).
Control: oocytes not matured *in vitro*.
FF: 100% preovulatory follicular fluid.
GV: germinal vesicle; MI: Metaphase I; MII: Metaphase II; Deg: degenerated.

For expanded COCs, the proportion of oocytes that reached MII after IVM in FF (43.8%) was not higher than those matured in TCM-199 (16.7%), but in comparison to the control, the difference was significant (43.8 vs. 9.1%). In denuded COCs, the proportion of oocytes that reached MI in FF (38.5%) was not statistically different from those matured in TCM-199 (47.1%), but these rates were higher than that in the control oocytes (5.3%). The rate of MII oocytes did not differ either cultured in FF (26.9%) or in TCM-199 (17.6%), but it was significantly increased in comparison to the non-cultured control oocytes (0.0%). Images of nuclear stages and mitochondria patterns are shown in Fig. 1.

Mitochondrial distribution

Patterns of mitochondrial distribution in oocytes before and after IVM are shown in detail in Table 2. In compact COCs, control oocytes showed a higher rate of peripheral clusters (PC) of mitochondria (51.8%) compared with oocytes matured in TCM-199 (4.3%) or FF (2.3%). Oocytes matured in FF showed a significantly higher rate of non-polarized (NP) pattern (27.3%) compared with TCM-199 (10.6%) or the control group (3.6%). Polarized distribution of mitochondria did not differ in oocytes matured in FF (43.2%) or in TCM-199 (29.8%), but these rates were statistically higher than that found in the control group (7.1%). In expanded COCs, oocytes with PC of mitochondria were observed only in the control, but not in matured oocytes, in TCM-199 or in FF. However, in denuded COCs, the proportion of PC was significantly higher in control oocytes (52.6%) than in matured oocytes (TCM-199 11.8%; FF, 3.8%).

To detect any possible association between nuclear maturation stages and mitochondria patterns, all COC types and treatments were combined (Table 3). Forty of 76 (52.6%) oocytes at the germinal vesicle stage of nuclear maturation had PC of mitochondrial distribution. Most of the oocytes at MI showed either NP or Pol patterns of mitochondria (63.6%, 42/66); a similar picture was observed in oocytes at MII (76.7%, 33/43). Fifty-three of 80 (66.3%) oocytes classified as degenerated for nuclear stage showed indefinable patterns of mitochondrial distribution and were classified as “other”. An area of high density of mitochondria was observed in 56.1% (37/66) of oocytes at MI and in 65.1% (28/43) of oocytes at MII. The polar body also contained dense mitochondria in 48.8% (21/43) of the MII oocytes.

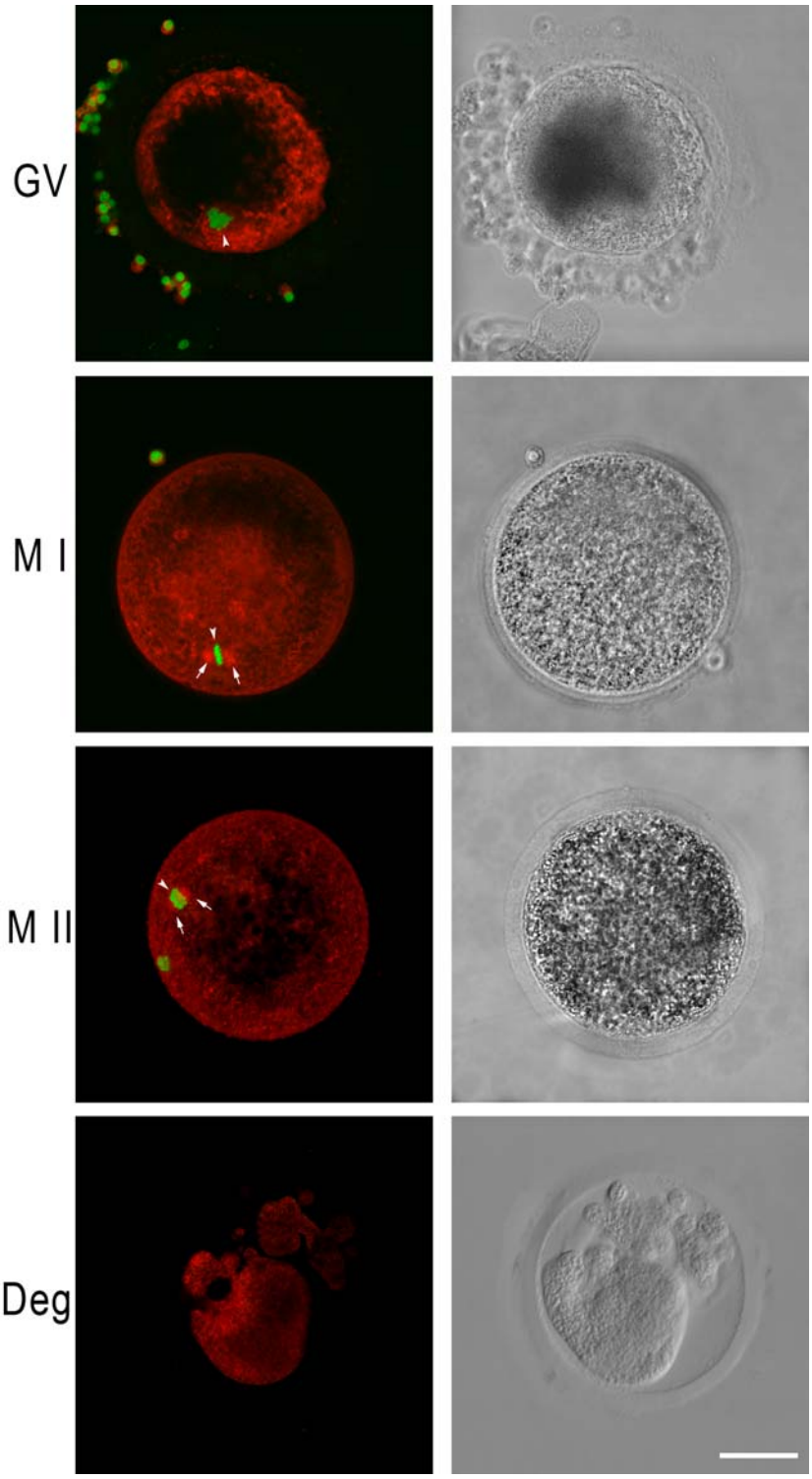

Figure 1. Photomicrographs with transmitted light (right) and laser scanning confocal images (left) at the middle focus plane showing mitochondria distribution within equine oocytes. Scale bar represents 50 μm. GV: a non-matured oocyte from a compact COC at germinal vesicle stage with peripheral dense clusters of mitochondria in the cortex region. MI: a Metaphase I oocyte from a compact COC matured *in vitro* in follicular fluid (FF) showing polarized mitochondria. MII: at Metaphase II oocyte from a compact COC matured *in vitro* in FF showing non-polarized mitochondria. Deg: a degenerated oocyte from a denuded COC matured *in vitro* in FF showing no detectable chromatin and several cytoplasmic fragments and vesicles. Arrow heads indicate condensed chromatin or chromosomes, and arrows indicate areas of mitochondria in high density next to the chromosomes.

Table 2. Mitochondrial patterns (percentages) of oocytes at Time 0 (Control) and after *in vitro* maturation in TCM-199 and follicular fluid for 36 to 40 h.

	Compact					Expanded					Denuded				
	n	PC	NP	Pol	Other	n	PC	NP	Pol	Other	n	PC	NP	Pol	Other
Control	56	51.8[a]	3.6[a]	7.1[a]	37.5[ab]	22	31.8[a]	13.6	27.3	27.3[a]	19	52.6[a]	5.3	26.3	15.8
TCM-199	47	4.3[b]	10.6[a]	29.8[b]	55.3[a]	18	0.0[b]	11.1	27.8	61.1[b]	17	11.8[b]	11.8	41.2	35.3
FF	44	2.3[b]	27.3[b]	43.2[b]	27.3[b]	16	0.0[b]	12.5	37.5	50.0[ab]	26	3.8[b]	19.2	46.2	30.8

[a,b]Different superscripts indicate statistical differences within columns ($P < 0.05$).
Control: oocytes non-matured *in vitro.*
FF: 100% preovulatory follicular fluid.
PC: peripheral clusters of dense mitochondria spread in the cortical region.
NP: non-polarized, evenly distributed mitochondria throughout the cortical and subcortical regions, with almost no mitochondria in the center.
Pol: polarized, uneven distribution of mitochondria throughout the cortical and subcortical regions, normally polarized to one half to two thirds of the oocyte.
Other: any other undefined patterns including fragments, vesicles or weak fluorescence.

Table 3. Number and proportion of mitochondria patterns associated with nuclear maturation stages for compact, expanded and denuded oocytes combined.

	Peripheral clusters		Non-Polarized		Polarized		Other		
	n	%	n	%	n	%	n	%	Total (n)
GV	40	52.6	5	6.6	12	15.8	19	25.0	76
MI	4	6.1	9	13.6	33	50.0	20	30.3	66
MII	1	2.3	14	32.6	19	44.2	9	20.9	43
Deg	7	8.8	6	7.5	14	17.5	53	66.3	80

PC: peripheral clusters of dense mitochondria spread in the cortical region.
NP: Non-polarized, evenly distributed mitochondria throughout the cortical and subcortical regions, with almost no mitochondria in the center.
Pol: polarized, uneven distribution of mitochondria throughout the cortical and subcortical.
regions, normally polarized to one half to two thirds of the oocyte.
Other: any other undefined patterns including fragments.
GV: germinal vesicle; MI: Metaphase I; MII: Metaphase II; Deg: degenerated.

Embryo recovery after intrafollicular transfer

Table 4 presents detailed results of *in vitro* matured COCs transferred into the preovulatory follicle of inseminated recipient mares and the subsequent embryo recovery in vivo. Only two normal, healthy-looking blastocysts classified as Grade 1 (McKinnon and Squires, 1988) were obtained from 39 transferred oocytes that were matured *in vitro* in FF, which represents an embryo development rate of 5.1%. No embryos were recovered from the oocytes matured in TCM-199.

Embryo recovery after intraoviductal transfer

Table 5 shows detailed results of *in vitro* matured COCs transferred into the oviduct of inseminated recipient mares and subsequent embryo recovery. Out of the 29 oocytes matured *in vitro* in FF, only 1 expanded blastocyst (Grade 1; 3.4%) was recovered from the uterine lavage. No oocytes matured *in vitro* in TCM-199 developed *in vivo* into embryos.

Table 4. Number of *in vitro* matured COCs transferred into the preovulatory follicle of recipient mares, embryos recovered by uterine lavage, number of ovulations and embryos developed from *in vitro* matured COCs.

IVM Medium	COCs transferred	Embryos recovered	Ovulations in recipient mares	Embryos from IVM COCs	Development rate
TCM-199	12	1	1	0	0/32 (0%)
	10	0	2	0	
	10	0	1	0	
FF	16	3*	1	2	2/39 (5.1%)
	13	0	1	0	
	10	0	1	0	

FF: 100% preovulatory follicular fluid.
*One large expanded blastocyst and 2 early blastocysts, all Grade 1 embryos.

Table 5. Number of *in vitro* matured COCs transferred into the oviduct of recipient mares, embryos recovered by uterine lavage, number of ovulations and embryos developed from *in vitro* matured COCs.

IVM Medium	COCs transferred	Embryos recovered	Ovulations in recipient mares	Embryos from IVM COCs	Development rate
TCM-199	12	1	1	0	0/26 (0%)
	14	1	1	0	
FF	13	2*	1	1	1/29 (3.4%)
	16	0	1	0	

FF: 100% preovulatory follicular fluid.
*Two expanded blastocysts.

Discussion

Nuclear maturation rates in this study were lower than those reported by other researches (Carneiro *et al.*, 2001; Choi *et al.*, 2004). However, differences in maturation rates between the two maturation media were detected. Pure equine preovulatory FF used as maturation medium was apparently more successful than TCM-199 to induce nuclear maturation of equine COCs *in vitro*. This effect was mainly observed in compact COCs, and with less intensity, in expanded and denuded COCs. Follicular fluid was able to induce a marked progression in meiosis of compact COCs, reducing the rate of oocytes at germinal vesicle and increasing the proportion of oocytes in metaphase I and II. In addition, the rate of degenerated oocytes was not increased during maturation in FF, but did occur when compact COCs were matured in TCM-199.

In expanded COCs, the difference between FF and TCM-199 was not so pronounced. Both media reduced the proportion of oocytes graded as GV at a similar level; however, only FF increased the number of MII oocytes significantly in comparison to the control group. In denuded COCs, the rate of GV oocytes in both media was decreased by significantly increasing the proportion of MI oocytes; however, only FF was able to increase the rate of MII oocytes over the control group. These results complement previous studies (Aguilar *et al.*, 1999, 2001; Conforti *et al.*, 2005) that demonstrated beneficial effects of FF on cumulus expansion and nuclear maturation of equine COCs *in vitro*.

In bovine IVF, the importance of classifying the COCs based on compactness and amount of cumulus cells, as well as the quality of the ooplasm, has been shown in several studies (Hazeleger and Stubbings, 1992; Blondin and Sirard, 1995). Recently it has been shown that COCs with minor signs of atresia led to maturation and embryo developmental rates as high as those obtained with fully compact bovine COCs (de Wit *et al*., 2000; Bilodeau-Goeseels and Panich, 2002). Structural changes in the oocyte associated with early degeneration in subordinate follicles are similar to those occurring in the oocyte of the dominant follicle prior to the LH surge (Assey *et al.*, 1994; Hyttel *et al.*, 1997). Nevertheless, it is also important to consider that highly expanded equine COCs come from very atretic follicles (Hinrichs and Williams, 1997) and may produce lower embryo development rates.

The significance of morphological features of cumulus cells and ooplasm are not well understood for equine COCs. It was shown that expanded COCs come from atretic follicles (Hinrichs and Williams, 1997) and that they can reach higher rates of IVM than compact COCs (Hinrichs *et al.*, 1995; Alm and Hinrichs, 1996). However, other studies did not find differences in MII oocytes after IVM between compact and expanded COCs (Del Campo *et al.*, 1995; Guignot *et al.*, 1999). Moreover, other researchers have selected only compact COCs for IVM (Carneiro *et al.*, 2001; Brinsko *et al.*, 1995) or ICSI studies (Dell'Aquila *et al.*, 1997). In an ICSI study, oocytes derived from compact COCs showed better developmental competence than those from expanded COCs, indicated by the proportion of embryos having more than 4 normal nuclei after 48 h of culture (Choi *et al.*, 2004).

Mitochondrial distribution undergoes stage-specific changes during oocyte maturation, fertilization, and early embryo development in mouse (Van Blerkom and Runner, 1984) and hamster (Bavister and Squirrell, 2000) oocytes. Moreover, it appears that distinct mitochondrial distribution patterns indicate developmental capacity in bovine and porcine oocytes (Schatten *et al.*, 2005). In the mouse, mitochondria are found polarized within the unfertilized oocyte (Calarco, 1995), but later during fertilization become homogeneously distributed in the ooplasm (Muggleton-Harris and Brown, 1988). Immature porcine oocytes at GV seem to have the mitochondria aggregated in the cortical region, whereas in fully-grown oocytes, mitochondria accumulate in the peripheral cytoplasm and migrate around the GV. As meiosis progresses, mitochondria accumulate in the perinuclear area from germinal vesicle breakdown (GVBD) to Anaphase I (Sun *et al.*, 2001). Additionally, in comparison with *in vivo* matured oocytes, it was concluded that IVM may cause incomplete movement of mitochondria to the inner cytoplasm and thus affect cytoplasmic maturation (Sun *et al.*, 2001).

In this study, associations between nuclear stages and mitochondrial patterns are similar to those observed in porcine oocytes (Sun *et al.*, 2001) but differ substantially from goat oocytes (Velilla *et al.*, 2006).

Most of the oocytes (52.6%) found at the germinal vesicle stage showed dense clusters of mitochondria in the cortical region. When the oocytes advanced in meiosis (MI and MII), the mitochondria were found spread more towards the subcortical region in a more dispersed manner (dense clusters dispersed), which resulted in the polarized and non-polarized patterns. Almost 64% of oocytes at MI and 77% of oocytes at MII showed either polarized or non-polarized patterns of mitochondrial distribution. The meaning and importance of polarized mitochondria during maturation of equine oocytes remains unknown. However, it has been demonstrated that polarization of mitochondria in human oocytes may cause disproportional mitochondrial inheritance in 2-4 cell embryos resulting in lysis of blastomeres with deficient numbers of mitochondria (Van Blerkom *et al.*, 2000). Mitochondrial patterns in prepuberal goat oocytes seem to differ dramatically from our findings in mare oocytes. Most immature goat oocytes at GV had mitochondria homogeneously distributed through the cytoplasm before IVM. After maturation, most MII oocytes had mitochondria aggregated in the peripheral region and polarized opposite to the metaphase spindle (Velilla *et al.*, 2006). Homogeneous and heterogeneous patterns of mitochondrial distribution in recently ovulated mare oocytes were described (Aguilar *et al.*, 2002). Based on this description, the polarized (heterogeneous) pattern found in the present study can probably be considered as normal. Supporting this point, a very detailed equine electron microscopy study by Grondahl *et al.* (1995) describes a series of cytoplasmic changes during IVM that involved the rearrangement of mitochondria from a predominantly peripheral distribution (in immature oocytes from small follicles) to a more central or semilunar domain (in MII oocytes from preovulatory follicles). Dense areas of mitochondria were also commonly observed in close association with chromosomes and within the polar body. Whether these patterns of mitochondrial distribution observed in equine oocytes are related to oocyte competence should be a matter of further investigation.

Results of embryo development after intrafollicular and intraoviductal transfer of *in vitro* matured COCs were disappointing. Due to the low numbers of embryos recovered, statistical tests were not performed. However, although only IVM in FF led to the development of embryos *in vivo*, this result should be taken with precaution. Embryo recovery rates after intrafollicular transfer are not consistent with only one third of the transferred mares producing multiple embryos (Hinrichs and DiGiorgio, 1991). Inflammation or hemorrhage may occur in some mares after follicular puncture, which may alter the follicular environment and therefore affect oocyte competence. In addition, the mechanism of ovum pick up by the oviductal fimbria might be altered when inflammation occurs in this area. Intra-oviductal oocyte transfer was very successful in supporting embryo development (11/12 91.7%) in young mares that received only 1 to 4 oocytes per oviduct (Carnevale and Ginther, 1995). In the present study, the high number of oocytes transferred per oviduct (12-16) may have interfered with the oviductal physiology, preventing the development of multiple embryos. Conversely, in spite of the level of nuclear maturation observed after IVM, deficient oocyte competence cannot be ruled out as a cause of low embryo recovery.

In conclusion, pure equine follicular fluid was shown to be suitable as an IVM medium, inducing higher rates of nuclear maturation than a semi-defined medium (TCM-199 with serum and hormones). In addition, mitochondrial reorganization occurred during maturation, changing from dense clusters of mitochondria in the cortical region of germinal vesicle oocytes to a more central and dispersed pattern in Metaphase-I and -II oocytes. Finally, FF as maturation medium led to the development of few embryos *in vivo* after transfer of COCs to inseminated recipient mares.

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References

Aguilar JJ, Woods GL, Miragaya MH, Olsen LM. 1999. Follicular fluid and follicular shells followed by DMEM increased nuclear maturation of equine denuded cumulus oocyte complexes *in vitro*. *Theriogenology*, 51**:**366. (abstract).

Aguilar JJ, Woods G, Miragaya M, Olsen L, Vanderwall D. 2001. Effect of homologous follicular fluid on *in vitro* maturation of equine cumulus-oocyte complexes. *Theriogenology*, 56:745-758.

Aguilar JJ, Losinno L, Koncurat M, Miragaya MH. 2002. Nuclear, cytoplasmic and mitochondrial patterns of ovulated oocytes in young and aged mares. *Theriogenology*, 58:689-692.

Ali A, Coenen K, Bousquet D, Sirard MA. 2004. Origin of bovine follicular fluid and its effect during *in vitro* maturation on the developmental competence of bovine oocytes. *Theriogenology*, 62:1596-1606.

Alm H, Hinrichs K. 1996. Effect of cycloheximide on nuclear maturation of horse oocytes and its relation to initial cumulus morphology. *J Reprod Fertil*, 107:215-220.

Assey RJ, Hyttel P, Greve T, Purwantara B. 1994. Oocyte morphology in dominant and subordinate

follicles. *Mol Reprod Dev*, 37:335-344.

Avery B, Strobech L, Jacobsen T, Bogh IB, Greve T. 2003. *In vitro* maturation of bovine cumulus-oocyte complexes in undiluted follicular fluid: effect on nuclear maturation, pronucleus formation and embryo development. *Theriogenology*, 59:987-999.

Bavister B, Squirrell J. 2000. Mitochondrial distribution and function in oocytes and early embryos. *Hum Reprod Suppl*, 15:189-198.

Bilodeau-Goeseels S, Panich P. 2002. Effects of oocyte quality on development and transcriptional activity in early bovine embryos. *Anim Reprod Sci*, 71:143-155.

Blondin P, Sirard MA. 1995. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol Reprod Dev*, 41:54-62.

Brinsko SP, Ball BA, Ellington JE. 1995. *In vitro* maturation of equine oocytes obtained from different age groups of sexually mature mares. *Theriogenology*, 44:461-469.

Bogh IB, Bezard J, Duchamp G, Baltsen M, Gerard N, Daels P, Greve T. 2002. Pure preovulatory follicular fluid promotes *in vitro* maturation of in vivo aspirated equine oocytes. *Theriogenology*, 57:1765-1779.

Calarco PG. 1995. Polarization of mitochondria in the unfertilized mouse oocyte. *Dev Genet*, 16:36-43.

Carneiro G, Lorenzo P, Pimentel C, Pegoraro L, Bertolini M, Ball B, Anderson G, Liu I. 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. *Biol Reprod*, 65:899-905.

Carnevale EM, Ginther OJ. 1995. Defective oocytes as a cause of subfertility in old mares. *Biol Reprod Mono*, 1**:**209-214.

Carnevale EM, Maclellan LJ, Coutinho da Silva MA, Squires EL. 2003. Pregnancies attained after collection and transfer of oocytes from ovaries of five euthanatized mares. *J Am Vet Med Assoc*, 222:60-62.

Carnevale E, Coutinho da Silva M, Panzani D, Stokes J, Squires E. 2005. Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. *Theriogenology*, 3:519-527.

Choi YH, Takagi M, Kamishita H, Wijayagunawardane MP, Acosta TJ, Miyazawa K, Sato K. 1998. Developmental capacity of bovine oocytes matured in two kinds of follicular fluid and fertilized *in vitro*. *Anim Reprod Sci*, 50:27-33.

Choi YH, Love LB, Varner DD, Hinrichs K. 2004. Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction*, 127:187-194.

Conforti VA, Vanderwall DK, Woods GL. 2005. Effect of homologous follicular fluid from medium-sized and large follicles on *in vitro* maturation of equine cumulus-oocyte complexes. *Reprod Fertil Dev*, 17:651-658.

Del Campo MR, Donoso MX, Parrish JJ, Ginther OJ. 1995. Selection of follicles, preculture oocyte evaluation and duration of culture for *in vitro* maturation of equine oocytes. *Theriogenology*, 43:1141-1153.

Dell'Aquila ME, Cho YS, Minoia P, Traina V, Lacalandra GM, Maritato F. 1997. Effects of follicular fluid supplementation of in-vitro maturation medium on the fertilization and development of equine oocytes after *in-vitro* fertilization or intracytoplasmic sperm injection. *Hum Reprod*, 12:2766-2772.

Dell'Aquila ME, De Felici M, Massari S, Maritato F, Minoia P. 1999. Effects of fetuin on zona pellucida hardening and fertilizability of equine oocytes matured *in vitro*. *Biol Reprod*, 61:533-540.

de Wit AAC, Wurth YA, Kruip TA. 2000. Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *J Anim Sci*, 78:1277-1283.

El Shourbagy SH, Spikings EC, Freitas M, St John JC. 2006. Mitochondria directly influence fertilisation outcome in the pig. *Reproduction*, 131:233-245.

Gable TL, Woods GL. 2001. Increasing culture time from 48 to 96 or 144 hours increases the proportions of equine cumulus oocyte complexes with negative or fragmented nucleus morphology. *Theriogenology*, 55:1549-1560.

Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G. 2003. Pregnancy: a cloned horse born to its dam twin. *Nature*, 424:635.

Galli C, Colleoni S, Duchi R, Lagutina I, Lazzari G. 2007. Developmental competence of equine oocytes and embryos obtained by *in vitro* procedures ranging from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim Reprod Sci*, 98:39-55.

Gil L, Saura S, Echegaray A, Martinez F, de Blas I, Akourki A, Gonzalez N, Espinosa E, Josa A. 2005. Effect of the *in vitro* maturation medium on equine oocytes: comparison of follicular fluid and oestrous mare serum. *Acta Vet Hung*, 53:241-248.

Grazul-Bilska AT, Pant D, Luther JS, Borowicz PP, Navanukraw C, Caton JS, Ward MA, Redmer DA, Reynolds LP. 2006. Pregnancy rates and gravid uterine parameters in single, twin and triplet pregnancies in naturally bred ewes and ewes after transfer of *in vitro* produced embryos. *Anim Reprod Sci*, 92:268-283.

Grondahl C, Hyttel P, Grondahl ML, Eriksen T, Gotfredsen P, Greve T. 1995. Structural and endocrine aspects of equine oocyte maturation in vivo. *Mol Reprod Dev*, 42:94-105.

Guignot F, Bezard J, Palmer E. 1999. Effect of time during transport of excised mare ovaries on oocyte recovery rate and quality after *in vitro* maturation. *Theriogenology*, 52:757-766.

Hazeleger NL, Stubbings RB. 1992. Developmental potential of selected bovine oocyte cumulus complexes. *Theriogenology*, 37:219. (abstract).

Hinrichs K, DiGiorgio LM. 1991. Embryonic development after intra-follicular transfer of horse oocytes. *J Reprod Fertil Suppl*, 44:369-374.

Hinrichs K, Martin MG, Schmidt AL, Friedman PP. 1995. Effect of follicular components on meiotic arrest and resumption in horse oocytes. *J Reprod Fertil*, 104:149-156.

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

Hinrichs K, Love CC, Brinsko SP, Choi YH, Varner DD. 2002. *In vitro* fertilization of *in vitro*-matured equine oocytes: effect of maturation medium, duration of maturation, and sperm calcium ionosphore treatment, and comparison with rates of fertilization in vivo after oviductal transfer. *Biol Reprod*, 67:256-262.

Hinrichs K, Choi YH, Love CC, Chung YG, Varner DD. 2006. Production of horse foals via direct injection of roscovitine-treated donor cells and activation by injection of sperm extract. *Reproduction*, 131:1063-1072.

Hyttel P, Fair T, Callesen H, Greve T. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, 47:23-32.

Kobayashi M, Lee ES, Fukui Y. 2006. Cysteamine or beta-mercaptoethanol added to a defined maturation medium improves blastocyst formation of porcine oocytes after intracytoplasmic sperm injection. *Theriogenology*, 65:1191-1199.

Li X, Morris LH, Allen WR. 2001. Influence of co-culture during maturation on the developmental potential of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI). *Reproduction*. 121:925-932.

McKinnon AO, Squires EL. 1988. Equine embryo transfer. *Vet Clin North Am Equine Pract*, 4:305-333.

Muggleton-Harris AL, Brown JJ. 1988. Cytoplasmic factors influence mitochondrial reorganization and resumption of cleavage during culture of early mouse embryos. *Hum Reprod*, 3:1020-1028.

Muir WW, Hubbell JA. 1991. Local anaesthetics and local anaesthetics techniques in horses. *In*: Equine anaesthesia: monitoring and emergency therapy. St. Louis, MI: Mosby. pp.231.

Okolski A, Babusik P, Tischner M, Lietz W. 1987. Evaluation of mare oocyte collection methods and stallion sperm penetration of zona-free hamster ova. *J Reprod Fertil Suppl*, 35:191-196.

Schatten H, Prather RS, Sun QY. 2005. The significance of mitochondria for embryo development in cloned farm animals. *Mitochondrion*, 5:303-321.

Sousa M, Barros A, Silva J, Tesarik J. 1997. Developmental changes in calcium content of ultrastructurally distinct subcellular compartments of preimplantation human embryos. *Mol Hum Reprod*, 3:83-90.

Squires EL, Seidel GE. 1995. Collection and transfer of equine embryos. Fort Collins, CO: Colorado State University, Animal Reproduction and Biotechnology Laboratory. pp.24-26. (Bulletin, 8).

Sun QY, Wu GM, Lai L, Park KW, Cabot R, Cheong HT, Day BN, Prather RS, Schatten H. 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development *in vitro*. *Reproduction*, 122:155-163.

Tarazona AM, Rodriguez JI, Restrepo LF, Olivera-Angel M. 2006. Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced *in vitro*. *Reprod Domest Anim*, 41:5-11.

Torner H, Alm H, Kanitz W, Goellnitz K, Becker F, Poehland R, Bruessow KP, Tuchscherer A. 2007. Effect of initial cumulus morphology on meiotic dynamic and status of mitochondria in horse oocytes during IVM. *Reprod Domest Anim*. 42:176-183.

Van Blerkom J, Runner M. 1984. Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. *Am J Anat*, 171**:**335-355.

Van Blerkom J, Davis P, Alexander S. 2000. Differential mitochondrial inheritance between blastomeres in cleavage stage human embryos: determination at the pronuclear stage and relationship to microtubular organization, ATP content and developmental competence. *Hum Reprod*, 15:2621-2633.

Velilla E, Rodriguez-Gonzalez E, Vidal F, Izquierdo D, Paramio MT. 2006. Mitochondrial organization in prepubertal goat oocytes during *in vitro* maturation and fertilization. *Mol Reprod Dev*, 73:617-626.

Way AL, Killian GJ. 2006. Sperm binding, *in vitro* fertilization, and *in vitro* embryonic development of bovine oocytes fertilized with spermatozoa incubated with norepinephrine. *Anim Reprod Sci*, 96:1-9.

Woods GL, White KL, Vanderwall DK, Li G-P, Aston KI, Bunch TD, Meerdo LN, Pate BJ. 2003. A mule cloned from fetal cells by nuclear transfer. *Science*, 22:1063.