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Research

Effects of roscovitine on maintenance of the germinal vesicle in horse oocytes, subsequent nuclear maturation, and cleavage rates after intracytoplasmic sperm injection

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This study was conducted to evaluate the effects of roscovitine on suppression of meiosis, subsequent meiotic maturation, and cleavage rates after intracytoplasmic sperm injection of horse oocytes. Oocytes were classified as having compact or expanded cumuli (Com or Exp oocytes) and were divided into three culture groups: 30 h culture in maturation medium (30 h Mat); 54 h culture in maturation medium (54 h Mat), or 24 h culture in medium containing 66 μmol roscovitine l^{-1} and then 30 h culture in maturation medium (Ros+M). After maturation, oocytes were subjected to intracytoplasmic sperm injection and cultured in G1.2 medium for 96 h. Among oocytes fixed immediately after roscovitine culture, 26 of 31 (84%) Com oocytes and 16 of 28 (57%) Exp oocytes were at the germinal vesicle stage ($P < 0.05$). After maturation culture, there were no differences in maturation rates or morphological cleavage rates among treatments. Among Com oocytes, significantly more embryos in the Ros+M treatment than in the 54 h Mat treatment had cleaved with ⩾ two normal nuclei (63 versus 36%; $P < 0.05$); whereas among Exp oocytes, significantly more embryos in the 30 h Mat treatment than in the Ros+M treatment (63 versus 42%; $P < 0.05$) had cleaved with ⩾ two normal nuclei. The average number of nuclei in embryos at 96 h was significantly higher ($P < 0.05$) in Ros+M Com oocytes (13.5) than in any other Com or Exp group. These results demonstrate that roscovitine can reversibly maintain equine oocytes in the germinal vesicle stage for up to 24 h, and that such suppression may increase the developmental potential of Com, but not Exp, oocytes.

Introduction

Little work has been carried out on the developmental competence of horse oocytes because methods for equine embryo production *in vitro* have not been repeatably successful. Effective techniques for conventional IVF have not been achieved in this species. Several assisted fertilization techniques have been used on equine oocytes, such as partial zona pellucida removal (Choi *et al*., 1994), zona pellucida drilling (Li *et al*., 1995) or intracytoplasmic sperm injection (ICSI, Squires *et al*., 1996; Dell'Aquila *et al*., 1997a; Grondahl *et al*., 1997; Cochran *et al*., 1998; Li *et al*., 2000, 2001; Choi *et al*., 2002a). Initial studies on equine ICSI yielded variable rates of pronucleus formation and cleavage; however, recent work using the Piezo drill for ICSI has resulted in good rates of activation, cleavage, and further embryo development (Choi *et al*., 2002a; Galli *et al*., 2002). These results indicate that ICSI may be a useful tool to study the developmental competence of equine oocytes after *in vitro* maturation.

The developmental competence of *in vitro* matured equine oocytes is low in comparison with that of oocytes matured *in vivo*, when evaluated after transfer to the oviducts of inseminated mares (Scott *et al*., 2001). Although a large proportion of oocytes will undergo nuclear maturation *in vitro* if removed from the follicular environment (Pincus and Enzmann, 1935), the developmental potential of these oocytes after fertilization is related to the extent of cytoplasmic maturation, which may not be synchronized with that of the nucleus. Oocyte cytoplasmic maturation appears to occur as the follicle grows toward preovulatory size. Oocytes recovered from less mature follicles may have the capacity to undergo nuclear maturation, but may not have adequate cytoplasmic maturation to develop normally after fertilization. In other species, cytoplasmic maturation has been induced *in vitro* by maintaining oocytes in meiotic arrest while allowing cytoplasmic changes to take place (Hyttel *et al*., 1997; Hashimoto *et al*., 2002). In horses, various biological methods such as addition of theca cells (Okolski *et al*., 1993), follicular

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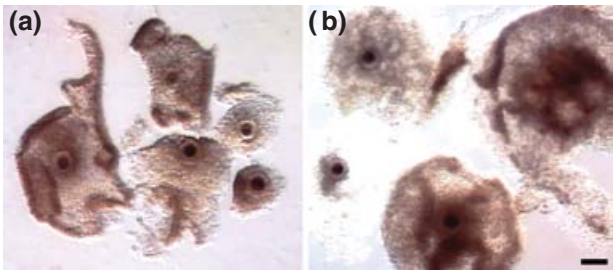

Fig. 1. Photomicrographs of horse cumulus–oocyte complexes immediately after recovery from the follicle: (a) compact (Com), (b) expanded (Exp). Scale bar represents 200 μm.

fluid (Hinrichs *et al*., 1995a; Schmid *et al*., 2000) and granulosa cells (Hinrichs *et al*., 1995a) have been used to maintain oocytes at the germinal vesicle stage, but due to variations in the cells, these procedures have low repeatability. In addition, pharmacological methods such as use of cycloheximide, a protein synthesis inhibitor (Alm and Hinrichs, 1996), and 6-DMAP, a phosphorylation inhibitor (Hinrichs *et al*., 2002), have been used to maintain meiotic arrest in equine oocytes. Although effective, these pharmacological agents may result in the loss of developmental potential at the end of the treatment (Lonergan *et al*., 2000).

During nuclear maturation, an increase in kinase activity initiates a cascade of specific phosphorylations and dephosphorylations of maturation-promoting factor (MPF) and microtubule-associated protein (MAP) kinase, which are ultimately responsible for the progression of meiosis (Nurse, 1990; Gotoh and Nishida, 1995). Both of these kinases have been identified in horse oocytes, and MPF activity is higher in mature horse oocytes than in immature horse oocytes (Goudet *et al*., 1998a,b). Roscovitine, a purine that specifically inhibits MPF kinase activity (Marchal *et al*., 2001), has been used successfully to maintain bovine oocytes temporarily at the germinal vesicle stage without compromising further development of the embryos (Mermillod *et al*., 2000; Ponderato *et al*., 2001). Roscovitine is also effective in suppressing meiosis in equine oocytes, but appears to have differential effects depending upon oocyte cumulus type (Hinrichs *et al*., 2002). The effect of roscovitine on the developmental competence of equine oocytes has not been evaluated.

Even though ICSI now provides a method for fertilization of horse oocytes, the study of developmental competence of these oocytes continues to be hindered by the lack of an *in vitro* culture system that supports proper embryo development. *In vitro* culture of equine ICSI embryos for 24 h reduces the pregnancy rate after transfer to the oviduct (McKinnon *et al*., 2000). Rates of blastocyst formation in ICSI embryos cultured *in vitro* have been low, ranging from 3 to 14% of injected oocytes (Dell'Aquila *et al*., 1997b; Guignot *et al*., 1998; Maclellan *et al*., 2001a; Galli *et al*., 2002; Choi *et al*., 2003) or 33% of cleaved embryos (Li *et al*., 2001). Unfortunately, embryos which have not formed blastocysts after culture for 7 days, degenerate; thus information about the maximum developmental stage is lost. For these reasons, authors have reported results after ICSI on the basis of pronucleus formation or early stages of cleavage (Dell'Aquila *et al*., 1997a; Li *et al*., 2000). Studies using the commercial embryo culture medium G1.2 have evaluated embryo development after 96 h culture (Choi *et al*., 2002a, 2003). Cleavage rates are best assessed by visualization after nuclear staining, which evaluates the presence of normal nuclei, indicating the potential for further development (Choi *et al*., 2002a).

The present study was conducted to evaluate the effectiveness of roscovitine in maintaining equine oocytes at the germinal vesicle stage for 24 h, and on subsequent nuclear maturation rates after release from inhibition. The developmental potential of oocytes that had undergone suppression was compared with that for oocytes matured immediately after collection, by assessing embryo development at 96 h after ICSI.

Materials and Methods

Collection of oocytes

Equine ovaries were collected at a local abattoir and rinsed in warm saline (35–37°C) upon collection. Ovaries were processed at the abattoir as they were removed post mortem, to minimize time to oocyte recovery. A maximum of eight follicles was used from each ovary; follicles ranged from 5 to 22 mm in diameter. Follicular contents were scraped with a bone curette and washed into individual Petri dishes with 20 μmol MOPS-buffered TCM-199 l^{-1}(Sigma, St Louis, MO) at 35–37°C. Dishes were searched with the aid of a dissection microscope at × 10–40, and oocytes were classified into expanded (Exp) and compact (Com) groups based on the appearance of the cumulus cells. Oocytes were considered to be in the Com group when most of the cumulus cells layers were tightly attached to the oocyte, and in the Exp group when the cumulus cells were loosely surrounding the oocyte in a mass (Fig. 1). Oocytes were placed in MOPS-buffered TCM-199 at 37°C and stored for up to 60 min. Every 60 min, oocytes collected during that period were transferred to 5 ml Falcon tubes (polystyrene) containing 1 ml of either maturation medium (Mat groups) or roscovitine-containing medium (roscovitine control and Ros+M groups; media are described below) covered with 2 ml light paraffin oil, both previously equilibrated in 5% CO_2 in air. The Falcon tubes were transported to the laboratory in a Minitub portable incubator (Minitube, Verona, WI) at 38.2°C. The total time from slaughter to

the placement of oocytes in culture medium was 30–90 min.

Upon arrival at the laboratory, oocytes were removed from the transport tubes and transferred to 500 μl of the same medium, in four-well multi-dishes (Nunc, Roskilde) and incubated at 38.2°C in 5% CO_2 in air. All components were purchased from Sigma unless stated otherwise. Base medium was a modified synthetic oviductal fluid (Maclellan *et al*., 2001b) containing 0.8% BSA, 11 mmol myoinositol l^{-1}, 10 μg streptomycin ml^{-1} and 1000 iu penicillin G ml^{-1}. Maturation medium was EMMI as described by Maclellan *et al*. (2001b), consisting of the base medium with 15 ng ovine FSH (NIDDK-oFSH-20) ml^{-1}, 1 μg bovine LH (AFP-11743B) ml^{-1}, 1 μg oestradiol ml^{-1}, 500 ng progesterone ml^{-1}, 100 ng epidermal growth factor ml^{-1} and 10 ng insulin-like growth factor-I ml^{-1}, with the addition of 10% fetal bovine serum (Gibco, Grand Island, NY). Medium containing roscovitine consisted of the base medium with 66 μmol roscovitine l^{-1}. Oocytes placed in roscovitine treatment were incubated for 24 h and then a third of the total number were fixed and evaluated to determine the proportion of oocytes maintained at the germinal vesicle stage (roscovitine controls). These oocytes were denuded of cumulus by pipetting with a fine bore glass pipette in a solution of 0.05% hyaluronidase in Hepes-buffered TCM-199 (Gibco) with 5% FBS, fixed in buffered formol saline, stained with 2.5 μg Hoechst 33258 ml^{-1} in 9:1 (v/v) glycerol:PBS, and observed under a E600 Nikon fluorescence microscope with a 365 nm excitation filter to evaluate chromatin configuration. Germinal vesicle chromatin was classified as condensed, intermediate, fibrillar, or diffuse, as described by Dell'Aquila *et al*. (2001). Other classifications included metaphase I (MI), MII and degenerating (abnormal chromatin or no chromatin visible). The remainder of the oocytes that had been incubated in roscovitine-containing medium was washed and transferred to 500 μl maturation medium for 30 h at 38.2°C in 5% CO_2 in air (Ros+M treatment).Oocytes in the Mat treatments were incubated for either 30 h (30 h Mat) or 54 h (54 h Mat) at 38.2°C in 5% CO_2 in air.

Oocyte preparation for ICSI

After *in vitro* maturation, oocytes were denuded of cumulus as described above. Oocytes with a polar body were selected for ICSI. Oocytes without a polar body were fixed and stained as described above.

Sperm preparation for ICSI

Fresh semen was collected from one stallion using a Missouri model artificial vagina, and then diluted to 80×10^6 spermatozoa per ml with a commercial skimmed-milk glucose extender (EZ-Mixing; Animal Reproduction Systems, Chino, CA) before further processing. Semen was then frozen using a skimmed-milk–egg-yolk based extender (Blanchard *et al*., 1998). Semen straws were thawed at 37°C for 30 s. Frozen–thawed semen (200 μl) was placed in the bottom of a 5 ml tube containing 1 ml modified TALP (Sp-TALP; Parrish *et al*., 1988) and incubated in an atmosphere of 5% CO_2 in air for swim-up. After 20 min, 0.6 ml medium was collected from the top of the tube, and centrifuged at 327 ***g*** for 3 min in a 1.7 ml polypropylene tube. The sperm pellet was resuspended and washed once with the same medium. The supernatant was removed, and the pellet was used for ICSI.

ICSI

Intracytoplasmic sperm injection was performed as described by Choi *et al*. (2002a). The outside diameter of the sperm injection pipette was 7–8 μm. A 120–140 μm (outside diameter) pipette was used to hold oocytes. Immediately before injection, 1 μl sperm suspension was placed in 3 μl Sp-TALP containing 10% PVP (molecular weight 360 000) under light white mineral oil. Sperm injection was carried out in a separate drop of Hepes-buffered TCM-199 containing 10% FBS. Each spermatozoon was immobilized by applying a few pulses with a Piezo drill (Prime Tech, Ibaraki) to the sperm tail immediately before injection (Kimura and Yanagimachi, 1995). All manipulations were performed at room temperature (22–25°C). Injected oocytes were held for 20 min at room temperature in the same medium to heal the broken membrane slowly, then transferred into G1.2 medium (IVF Science, Denver, CO) in microdroplets under mineral oil, at a ratio of 5 μl medium per oocyte, and incubated at 38.2°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 96 h.

Sham injection

In the last four replicates, 25% of the oocytes that had a polar body in the Ros+M and 30 h Mat treatments were subjected to sham injection. Sham injections were performed as described above, except that there were no spermatozoa in the injection pipette.

Evaluation of embryos

After 96 h of *in vitro* culture, sperm- or sham-injected oocytes were fixed and stained as described above to examine the number and status of nuclei. Only nuclei that appeared to be normal were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation or fragmentation) were disregarded. Two main parameters were used to evaluate embryo development: rate of normal cleavage (embryos which had cleaved with $\geqslant$ two normal nuclei), and the average number of nuclei achieved by normally cleaved

Table 1. Chromatin configuration of equine oocytes evaluated after 24 h of culture with roscovitine

		Germinal vesicle stage chromatin						
Oocyte type	*n*	Condensed (%)	Intermediate (%)	Fibrillar (%)	FN* (%)	MI (%)	MII (%)	Degenerating (%)
Compact	31	20 (65)[a]	5 (16)	0 (0)	1 (3)	0 (0)[a]	0 (0)	5 (16)
Expanded	28	11 (39)[b]	0 (0)	4 (14)	1 (4)	8 (29)[b]	3 (11)	1 (4)

*Fluorescent nucleus, homogeneous fluorescence throughout nucleus.
[a,b]Values with different superscripts within columns are significantly different ($P < 0.05$).

embryos. Embryos having only 2–7 nuclei at 96 h culture were considered to have arrested development.

Statistical analysis

Chi-squared analysis was used to evaluate differences in the following parameters: distribution of chromatin configurations after roscovitine treatment between Exp and Com oocytes; maturation rates among 30 h Mat, 54 h Mat and Ros+M treatments within cumulus type; maturation rate and proportion still at germinal vesicle stage over all treatments between Exp and Com oocytes; rates of morphological and normal cleavage after ICSI among treatments within cumulus type; and proportion of embryos arrested at 2–7 cells within cumulus type. A probability of $P < 0.05$ was considered significant. Comparisons with expected values of less than five were analysed using Fisher's exact test. Differences in the average number of embryonic nuclei among treatments were analysed using one-way ANOVA.

Results

Oocyte recovery and distribution

Out of a total of 211 ovaries, 1030 follicles were scraped (4.9 follicles per ovary), 611 oocytes were recovered (59% recovery rate) and 590 oocytes were used for this study. A total of 261 cumulus–oocytes complexes (COCs) was classified as Exp (44%), and 329 COCs were classified as Com (56%). The number of oocytes placed in each 1 ml tube at the abattoir ranged from seven to 21; the number of oocytes placed in each 500 μl well for culture after arrival at the laboratory ranged from seven to 32.

Effect of roscovitine in meiotic suppression

Com and Exp oocytes (33 and 28, respectively; five replicates) were fixed and evaluated as roscovitine controls, that is, after 24 h of incubation with roscovitine. A sixth replicate was disregarded due to a violation of protocol (failure to add roscovitine to the medium). Two Com oocytes fixed for evaluation were not suitable for analysis due to the presence of cumulus. The chromatin configurations seen in the analysed roscovitine control oocytes are presented (Table 1). Significantly more Com than Exp oocytes were at the germinal vesicle stage: 26 of 31 (84%) versus 16 of 28 (57%), respectively ($P < 0.05$). The five Com oocytes not at the germinal vesicle stage were degenerating. Of the 12 Exp oocytes not at germinal vesicle, eight were in MI, three were in MII and one was degenerating.

Table 2. Chromatin configurations of equine oocytes having compact or expanded cumuli after culture for *in vitro* maturation in three treatments

Maturation treatment	Oocyte type	*n*	MI (%)	MII (%)
30 h Mat	Com	117	5 (4)	78 (67)
54 h Mat	Com	46	0 (0)	37 (80)
Ros+M	Com	101	3 (3)	75 (74)
Total	Com	264	8 (3)	190 (72)[a]
30 h Mat	Exp	89	3 (3)	74 (83)
54 h Mat	Exp	40	0 (0)	30 (75)
Ros+M	Exp	74	1 (1)	63 (85)
Total	Exp	203	4 (2)	167 (82)[b]

Mat: culture in maturation medium.
Ros+M: oocytes were cultured in base medium with 66 μmol roscovitine l^{-1} for 24 h and then cultured in maturation medium without roscovitine for 30 h.
Com: oocytes with compact cumuli; Exp: oocytes with expanded cumuli.
[a,b]Values with different superscripts within columns are significantly different ($P < 0.05$).

In vitro *maturation*

The proportions of oocytes in MII (oocytes with a visible polar body plus those found to be in MII on fixation and staining, $n = 6$) in the 30 h Mat, 54 h Mat and Ros+M groups after *in vitro* maturation are provided (Table 2). There was no difference in maturation rates to MII among treatment groups. Over all treatments, a higher proportion of Exp than Com oocytes were in MII after maturation culture (167 of 203, 82%, versus 190 of 264, 72%, respectively; $P < 0.05$). Among oocytes

Table 3. *In vitro* embryo development of equine oocytes matured in different treatments and fertilized by intracytoplasmic sperm injection

Maturation treatment or oocyte type	*n*	Oocytes cleaved (%)	Embryos with ⩾ two normal nuclei (% of *n*)	Number of nuclei (% of embryos with normal nuclei)			Average number of nuclei (mean ± SEM)
				2–7	8–16	>16	
Com							
30 h Mat	55	45 (82)	30 (55)[a,b]	15 (50)[a]	10 (33)	5 (17)	9.3 ± 1.4[a]
54 h Mat	36	30 (83)	13 (36)[a]	12 (92)[b]	1 (8)	0 (0)	4.8 ± 0.7[a,b]
Ros+M	56	45 (80)	35 (63)[b]	13 (37)[a]	13 (37)	9 (26)	13.5 ± 2.1[c]
Exp							
30 h Mat	54	41 (76)	34 (63)[b]	19 (56)[a]	12 (35)	3 (9)	8.7 ± 0.9[a]
54 h Mat	29	18 (62)	13 (45)[a,b]	12 (92)[b]	1 (8)	0 (0)	3.6 ± 0.9[b]
Ros+M	48	34 (71)	20 (42)[a]	13 (65)[a,b]	6 (30)	1 (5)	7.2 ± 1.2[a,b]

Mat: culture in maturation medium.
Ros+M: oocytes were cultured in base medium with 66 μmol roscovitine l^{-1} for 24 h and then cultured in maturation medium without roscovitine for 30 h.
Com: oocytes with compact cumuli; Exp: oocytes with expanded cumuli.
Within columns, values with different superscripts are significantly different ($P < 0.05$).

that did not have a polar body, and were therefore fixed and stained, one Com and two Exp oocytes were lost during processing. In oocytes evaluated after fixation and staining, a significantly higher proportion of Com than Exp oocytes were still at the germinal vesicle stage after culture (23 of 73, 32%, versus three of 34, 9%; $P < 0.01$). The percentage of oocytes in MI ranged from 0 to 4%.

Sham injection

Sixty-six oocytes were sham-injected, of which 65 (98%) survived injection. After 96 h culture, no cleavage was observed in sham-injected oocytes in the Ros+M treatment. In the 30 h Mat treatment, two of 17 (12%) Exp oocytes and three of 18 (17%) Com oocytes showed morphological cleavage after sham injection. Upon evaluation after staining with Hoechst, one embryo in the Ros+M Com group that had been classified as uncleaved was seen to have two normal nuclei. Of the remaining 64 sham-injected oocytes, 45 contained one to multiple pronuclear-like structures, one was in MII, and the remainder were degenerating.

Cleavage rates after ICSI

ICSI was conducted on 285 oocytes. After injection, 279 oocytes (98%) survived. The *in vitro* development of oocytes cultured for 96 h after ICSI is shown (Table 3). There were no differences ($P > 0.05$) in morphological cleavage rates among treatments. The proportion of Com oocytes showing normal cleavage (⩾ two normal nuclei) was highest in the Ros+M group, and this was significantly higher than for the Com 54 h Mat treatment ($P < 0.05$). In contrast, the proportion of Exp oocytes showing normal cleavage was lowest in the Ros+M group, and this was significantly lower than in the 30 h treatment ($P < 0.05$).

Significantly fewer embryos arrested at 2–7 nuclei at 96 h in the 30 h Mat and Ros+M treatments than in the 54 h Mat for Com oocytes, and significantly fewer arrested in the 30 h Mat treatment than in the 54 h Mat treatment for Exp oocytes ($P < 0.05$). The average number of nuclei was significantly higher ($P < 0.05$) in the Ros+M Com treatment (13.5) than in any other Com or Exp treatment (3.6–9.3).

Discussion

The results of this study show that roscovitine is effective in maintaining equine oocytes at the germinal vesicle stage, but that there is a differential effect of roscovitine suppression on subsequent developmental competence, depending on the type of oocyte. When compared with 30 h maturation alone, roscovitine suppression before maturation significantly increased developmental competence of Com oocytes, as reflected by the average number of nuclei at 96 h, but significantly decreased developmental competence of Exp oocytes, as reflected by the proportion of oocytes showing normal cleavage after ICSI.

The method used in this study to classify COCs is based on the expansion of the cumulus only, without regard to morphology of mural granulosa cells. Other studies have used both mural and cumulus granulosa morphology for oocyte classification (Choi *et al*., 2002b; Hinrichs *et al*., 2002), resulting in a lower proportion of oocytes classified as having compact cumuli.

In preliminary studies with equine oocytes, roscovitine was effective in maintaining germinal vesicle stage for 24 h in 73% of Com and 38% of Exp at 66 μmol roscovitine l^{-1}; and in 65% of Com and 55% of Exp at 200 μmol roscovitine l^{-1}(Franz *et al*., 2002). In the present study, 66 μmol roscovitine l^{-1} was chosen to ensure that the treatment was reversible, and to reduce the chance of affecting subsequent developmental competence. The results show that this dose maintained 84% of Com and 57% of Exp oocytes at the germinal vesicle stage after 24 h incubation. The proportion of oocytes at the germinal vesicle stage at the time of recovery was not determined; however, previous studies have reported that this proportion is similar for oocytes with compact and expanded cumuli (78 and 73% respectively, Hinrichs *et al*., 1993; 68 and 69% respectively, Hinrichs *et al*., 1995a).

The results for meiotic suppression of Com oocytes in the present study were similar to those described for bovine oocytes (88% at 50 μmol l^{-1}; Mermillod *et al*., 2000) and pig oocytes (70–74%, 80 μmol l^{-1} and 120 μmol l^{-1}, respectively; Ju *et al*., 2001). In most species, oocytes surrounded by expanded cumulus cells are discarded during selection for culture; therefore, equine oocytes with expanded cumuli cannot be easily compared with those of any other species. In horses, oocytes with expanded cumuli originate from atretic follicles (Hinrichs and Williams, 1997), but may have higher rates of *in vitro* maturation (Hinrichs *et al*., 1995b; Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000) and mature more rapidly *in vitro* than oocytes with compact cumuli (Zhang *et al*., 1989; Hinrichs *et al*., 1995a). Oocytes with expanded cumuli also require a shorter period for protein synthesis (Alm and Hinrichs, 1996) before commitment to meiosis. A small proportion of these oocytes, those from the most atretic follicles, are already in MI or MII at the time they are recovered from the follicle (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000). Taken together, these results indicate that a proportion of oocytes with expanded cumuli at the time of collection are already maturing, committed to mature or committed to mature if removed from the follicle, and thus roscovitine is less effective in suppressing meiosis in this population than it is in oocytes with compact cumuli.

The suppressive action of roscovitine in Com oocytes in the current study is in contrast to the report of Hinrichs *et al*. (2002), in which 24 h culture with roscovitine maintained only 16–34% of compact oocytes at the germinal vesicle stage. In that study, suppression was related to the chromatin configuration within the germinal vesicle. Oocytes with condensed chromatin were capable of being suppressed, whereas oocytes with diffuse chromatin degenerated during culture. A major difference between these two studies is the delay from slaughter to oocyte recovery. In the present study, oocytes were placed into culture between 30 and 90 min after slaughter, whereas in the previous report, this period was 3–8 h, including time for ovary collection and transport. Oocytes with diffuse chromatin appear to undergo degeneration during ovary storage (K. Hinrichs and Y. H. Choi, unpublished); thus, the oocytes that failed to respond to roscovitine treatment in the previous study may have begun to degenerate before culture was initiated.

Roscovitine treatment in the present study did not affect the meiotic competence of the suppressed oocytes, as there was no difference in maturation rates between the Ros+M treatment and either 30 h or 54 h maturation controls. The overall maturation rates to MII in the present study (72–82%) were higher than typically reported for equine oocytes (68%, Fulka and Okolski, 1981; 67%, Zhang *et al*., 1989; 25–47%, Bruck *et al*., 1996; 55%, Goudet *et al*., 1998b; 35–64%, Hinrichs and Schmidt, 2000; 63% Carneiro *et al*., 2001; 42%, Tremoleda *et al*., 2001), which could possibly be related to rapid placement into culture (Dell'Aquila *et al*., 1997b, 2001). Results in nuclear maturation of equine oocytes are difficult to compare among laboratories due to the great variability of procedures used. Most workers in this field agree that equine oocytes require more than 24 h and less than 40 h of incubation *in vitro* to reach MII (Fulka and Okolski, 1981). Maturation times of 24 h for expanded COCs and 30–36 h for compact COCs (Zhang *et al*., 1989; Hinrichs *et al*., 1993) are generally accepted. A maturation time of 30 h was used for both groups of COCs in the present study to facilitate comparison and statistical analysis. However, as ICSI is now available as a method for fertilization of horse oocytes, work may be carried out to define the effect of duration of *in vitro* maturation on developmental competence of both types of oocyte.

Cleavage rates after ICSI in horses are difficult to compare among laboratories as many reports present only visual (morphological) cleavage, without assessing nuclear status. Morphological cleavage rates are also reported in the present study to allow comparison with these studies. However, it is evident both biologically and from the results of this study that normal cleavage (cleavage with $\geqslant$ two normal nuclei) is a much more valuable parameter for comparison of developmental competence than is morphological cleavage. On the basis of morphological cleavage alone, cleavage rates in this study varied between 62 and 83%, which is comparable with other reports in horses (Li *et al*., 2001; Choi *et al*., 2002a).

Studies on embryonic development *in vivo* in horses report that at 48 h, embryos have 4–6 cells; at 72 h, 7–11 cells, and at 96 h, 8–16 cells (Bezard *et al*., 1989; Betteridge, 2000). In the present study, it was considered that cleavage with seven or fewer normal nuclei after 96 h culture indicated retarded development. Almost all (92%) embryos from the 54 h Mat treatment were found in this category (2–7 nuclei). Thus, the developmental potential of both Com and Exp oocytes appeared to

be compromised by the extended maturation time. Exp oocytes in the Ros+M treatment also performed poorly, having a significantly lower cleavage rate and almost 10% more embryos arrested at the 2–7-cell stage than in the 30 h Mat treatment. This indicates that among Exp oocytes, the increased cytoplasmic maturation time allowed during roscovitine treatment was not helpful and may have resulted in over-maturity and loss of developmental potential.

The highest average number of nuclei per embryo was achieved in the Ros+M Com group; this was higher ($P < 0.05$) than for any other treatment. This indicates that culture with roscovitine allowed an increase in developmental competence (cytoplasmic maturation) within Com oocytes, while meiotic suppression was maintained. The average number of 14 nuclei in the Ros+M Com group compares favourably with embryo development *in vivo* as described above, and with the development of embryos cultured within the oviduct *in vivo* for 96 h after ICSI (average of 14 nuclei, Choi *et al*., 2002a).

In conclusion, culture with roscovitine efficiently maintained equine oocytes at the germinal vesicle stage for 24 h, and was more effective in oocytes originally having a compact cumulus than in those having an expanded cumulus. Suppression with roscovitine was reversible; it did not affect meiotic competence after subsequent maturation *in vitro* for either cumulus type. This is the first report on the effect of roscovitine on the developmental competence of horse oocytes. In oocytes originally having a compact cumulus, 24 h culture with roscovitine before maturation was associated with high rates of normal cleavage and a significantly increased average number of nuclei in resulting embryos. However, in oocytes originally having an expanded cumulus, culture with roscovitine before maturation significantly decreased rates of normal cleavage. Further studies to investigate the extent to which roscovitine may benefit research on equine oocytes, and the limitations of this treatment, should be pursued.

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