Zygote **14** (February), pp. 1–8. © 2006 Cambridge University Press
doi:10.1017/S0967199406003534 Printed in the United Kingdom

Blastocyst development in equine oocytes with low meiotic competence after suppression of meiosis with roscovitine prior to *in vitro* maturation

Y.H. Choi[1], *L.B. Love*[1], *D.D. Varner*[2] *and K. Hinrichs*[1,2]
College of Veterinary Medicine, Texas A&M University, College Station, Texas, USA

Date submitted: 11.08.05. Date accepted 06.10.05

Summary

This study was conducted to evaluate the *in vitro* development of equine oocytes with compact cumuli that had been subjected to a period of meiotic suppression with roscovitine before *in vitro* maturation. In experiment 1, oocytes were recovered from slaughterhouse-derived ovaries and held in M199 + 10% fetal bovine serum containing 66 μM roscovitine with or without an overlay of mineral oil in 5% CO_2 in air at 38.2 °C for 16–18 or 24 h. No oocytes treated with roscovitine in the absence of an oil overlay for 16–18 h were maturing, compared with 2–4% of oocytes in other treatments. In experiment 2, oocytes were either fixed immediately after recovery, or were cultured for 18 h in the presence or absence of roscovitine. Oocytes cultured in the absence of roscovitine had a significantly higher rate of meiotic resumption (18%) than was found in the other two treatments (0). In experiment 3, oocytes were matured immediately or after 16–18 h culture with roscovitine. Maturation rates were similar between oocytes previously treated with roscovitine (22%) and control oocytes (19%). Mature oocytes were fertilized by intracytoplasmic sperm injection and then cultured, with or without oviductal epithelial cells, for 7.5 days. There was no significant effect of roscovitine treatment on blastocyst development. Development to blastocyst of roscovitine-treated oocytes in DMEM/F-12 + co-culture (37%) was significantly higher than that of control oocytes in DMEM/F-12 without co-culture (14%). These data indicate that equine oocytes with compact cumuli can be held in roscovitine before maturation without any harmful effect on blastocyst formation.

Keywords: Horse, Intracytoplasmic sperm injection, Meiosis, Oocyte, Roscovitine

Introduction

The success of assisted reproduction techniques is dependent upon the developmental competence of the oocytes utilized for these procedures. Although assisted reproduction is becoming an important clinical and research tool in the horse, factors affecting the developmental competence of horse oocytes are poorly understood. This is because, until recently, there was no efficient method to fertilize horse oocytes *in vitro*. In the past few years, however, techniques for intracytoplasmic sperm injection (ICSI) using the piezo drill have been developed, which result in high fertilization and cleavage rates (69–89%) after injection of equine oocytes (Galli *et al.*, 2002; Choi *et al.*, 2002*a*). In addition, techniques to support efficient development of horse embryos to the blastocyst stage in culture have very recently been reported (Hinrichs *et al.*, 2005). These now provide us with a method to study oocyte developmental competence in the horse.

At the time of collection from the ovary, horse oocytes may be classified as having a compact cumulus (Cp) or expanded cumulus (Ex). Typically, 25–40% of recovered oocytes are Cp, 50–60% Ex, and approximately 10% are degenerating (Choi *et al.*, 2003*a*, 2004*a*). Oocytes classified as Cp originate in viable follicles, but

All correspondence to: K. Hinrichs, Department of Veterinary Physiology & Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4466, USA. Tel: +1 (979) 8621338. Fax: +1 (979) 8456544. e-mail: khinrichs@cvm.tamu.edu
[1]Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA.
[2]Department of Large Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843, USA.

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

have lower maturation rates (Alm & Hinrichs, 1996; Hinrichs & Williams, 1997; Hinrichs & Schmidt, 2000), mature more slowly *in vitro* (Zhang *et al.*, 1989; Hinrichs *et al.*, 1993), and take more time to produce proteins necessary for maturation (Alm & Hinrichs, 1996) than do Ex oocytes. Maturation rates of Cp oocytes increase with increasing follicle size; however, it is not until the follicle reaches approximately the diameter seen at divergence of the dominant preovulatory follicle (≥20 mm; Gastal *et al.*, 1997) that the *in vitro* maturation rate of Cp oocytes is equal to that for Ex oocytes (Hinrichs & Schmidt, 2000). We have termed Cp oocytes from smaller follicles 'juvenile'; they may be compared to bovine oocytes originating from follicles less than 3 mm in diameter. Unfortunately, over 95% of visible follicles present on equine ovaries are less than 20 mm diameter (Hinrichs & Schmidt, 2000); thus understanding of factors affecting meiotic and developmental competence of juvenile Cp oocytes is important in the efficient use of equine oocytes for assisted reproduction.

The better performance of equine Ex oocytes may be attributed to their recovery from atretic follicles; oocyte changes associated with follicle atresia mimic those of oocytes in viable follicles approaching ovulatory size (Hinrichs & Schmidt, 2000). One of the most marked of these changes is the gradual condensation of chromatin within the germinal vesicle (GV) into one dense mass (the condensed chromatin configuration; CC). When collected from the ovary immediately post-mortem, the most juvenile Cp oocytes have chromatin distributed in strands within the GV (fibrillar configuration) and have low meiotic competence (20%), while those with some chromatin condensation ('intermediate' configuration) have normal maturation rates (80%; Hinrichs *et al.*, 2005). A proportion of oocytes in both these juvenile configurations undergo degeneration to non-viable chromatin configurations over time post-mortem, as occurs when ovaries are transported from the abattoir before oocyte recovery. This results in a lower observed maturation rate for Cp oocytes recovered from transported ovaries compared with those recovered immediately post-mortem. However, the blastocyst development per recovered Cp oocyte is not affected by ovary transport (Hinrichs *et al.*, 2005).

Because the number of horse abattoirs is limited, horse ovaries are often transported for long distances to the laboratory. Oocyte recovery is difficult to perform at the abattoir, as equine oocytes are harvested by opening and scraping of follicles (because follicle aspiration damages the cumulus and yields low recovery rates; Hinrichs, 1991; Choi *et al.*, 1993) and identification of the oocyte, via dissection microscope, among the recovered granulosa cells. When horse ovaries are transported to the laboratory for oocyte collection, the time involved in oocyte collection typically renders oocytes available for culture in the late afternoon or evening. Manipulations such as ICSI or nuclear transfer, performed after 24–30 h maturation, must then be carried out the following evening and night. Thus, a reversible method for suppression of meiosis of horse oocytes would be extremely useful as a scheduling tool in the laboratory. Adjustment of the time of onset of oocyte maturation, via a transient period of meiotic suppression, would allow manipulations to be scheduled when personnel are more readily available. In addition, it has been suggested that allowing oocytes a period of culture under meiotic suppression before the onset of *in vitro* maturation may increase embryo development from juvenile oocytes (review; Sirard, 2001).

Treatment with roscovitine, a purine analogue that specifically inhibits M-phase promoting factor activity, effectively suppresses meiosis in bovine oocytes, with no detrimental effect on subsequent blastocyst development (Mermillod *et al.*, 2000). The effectiveness of roscovitine in suppressing meiosis in horse oocytes is not clear. There have been two studies published on culture of horse oocytes with roscovitine. In the first study, roscovitine was found to have only low efficiency in maintaining equine oocytes at the GV stage (Hinrichs *et al.*, 2002); however, in a second study (Franz *et al.*, 2003), it was found to be effective. One major difference between the two studies was that in the study of Franz *et al.* (2003) oocytes were collected at the abattoir and placed into culture immediately, whereas in the former report, oocytes were collected from ovaries transported to the laboratory. As noted above, changes undergone by the oocytes while being held within the ovary affect the observed rate of maturation *in vitro*; in addition, ovary storage affects the kinetics of subsequent maturation (stored oocytes mature more quickly) and the developmental competence of Ex oocytes after ICSI (Hinrichs *et al.*, 2005). It is possible that changes occurring in Cp oocytes during ovary transport affect their ability to respond to roscovitine treatment. Because horse ovaries are typically transported before oocyte recovery, it is essential to determine whether roscovitine treatment is effective in suppressing meiosis in horse oocytes collected from ovaries after a delay. Another factor that may have influenced the differences in observed effectiveness of roscovitine between the two above-referenced studies is that oocytes were cultured in microdroplets of medium with an oil overlay in the first study (Hinrichs *et al.*, 2002), whereas Franz *et al.* (2003) cultured oocytes in wells without oil. Roscovitine activity may diminish when it is used under oil, presumably because it is lipid-soluble and is lost into the oil layer (Phillips *et al.*, 2002). It is important to establish whether these culture conditions affect the ability of roscovitine to suppress meiosis in horse oocytes.

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

The effect of roscovitine suppression of horse oocytes on embryo development has been evaluated in only one previous study, that of Franz *et al.* (2003). At the time of that study there was no effective method available for culture of equine embryos to the blastocyst stage, therefore embryo development was assessed by determining cleavage rates and nucleus number at 96 h after ICSI. While roscovitine-treated Ex oocytes showed impaired development, embryos from roscovitine-treated Cp oocytes had a significantly higher nucleus number than did controls. This suggested that cytoplasmic maturation in Cp oocytes was improved by roscovitine treatment. However, we have subsequently found that embryo status at 96 h is not an accurate measure of further developmental competence in the horse; for example, equine embryos cultured in a modified CZB medium showed normal development at 96 h (86% cleavage and 7 nuclei per embryo) but 0/112 developed to blastocyst (Choi *et al.*, 2003*b*, 2004*a*). Similarly, embryos showed normal development in G1.2/G2.2 media at 96 h but had only 2–9% development to the blastocyst stage when cultured further (Choi *et al.*, 2003*a*). Before roscovitine treatment can be recommended as a method for meiotic suppression of horse oocytes for research or clinical applications, it is necessary to ascertain its effect on the development of horse embryos to the blastocyst stage. As noted above, we have recently identified a culture system, using DMEM/F-12 medium (Choi *et al.*, 2004*a*), that supports over 30% blastocyst development in horse embryos (Hinrichs *et al.*, 2005). This system may now be used to evaluate factors affecting blastocyst development after ICSI of horse oocytes.

This study was performed to investigate the ability of roscovitine treatment to suppress meiosis in Cp horse oocytes recovered from transported ovaries. Rates of suppression after culture in microdroplets under oil, or in medium without an oil overlay, were compared. The effect of transient meiotic suppression with roscovitine on subsequent blastocyst development after ICSI was then evaluated under two different embryo culture conditions (with and without co-culture with oviductal epithelial explants).

Materials and methods

Oocyte collection

Oocytes were collected from slaughterhouse-derived ovaries (3–4 h transportation time) by scraping the granulosa layer from opened follicles. Classification of oocytes was conducted as described previously (Hinrichs & Williams, 1997). Briefly, oocyte–cumulus complexes were classified as compact (Cp), expanded, or degenerating depending on the expansion of both mural granulosa and cumulus cells. Oocytes with any sign of expansion of either the cumulus or the mural granulosa (from having individual cells visible protruding from the surface to having full expansion with copious matrix visible between cells) led to the classification of expanded. Oocytes with both compact cumulus and compact mural granulosa were classified as Cp. Only Cp oocytes were used for this study.

Experiment 1: Effect of holding method and duration on suppression of maturation

Oocytes were placed in M199 buffered with Hanks' salts and 25 mM Hepes (Gibco Life Technologies, Grand Island, NY) until all oocytes had been collected. Oocytes were then washed twice in basic M199 culture medium (M199 buffered with Earle's salts (26.2 mM $NaHCO_3$; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 25 μg/ml gentamicin) and twice with M199-Ros (basic culture medium with the addition of 66 μM roscovitine (Sigma Chemical, St Louis, MO)). Two roscovitine treatments were performed: culture in 500 μl M199-Ros in a 4-well multi-dish (Nunc, Roskilde, Denmark) without mineral oil (Well treatment); or in microdroplets of M199-Ros under light white mineral oil (Sigma) in a 35 mm Petri dish (Oil treatment). Microdroplets were used to closely mimic the conditions used in the report of Hinrichs *et al.* (2002). Oocytes were assigned randomly to Well or Oil treatments. All oocytes assigned to the Well treatment on a given day were placed in the same well; this number varied among replicates. The size of the microdroplet in the Oil treatment was manipulated to achieve the same ratio of medium to oocyte as in the Well treatment on that day. Oocytes were incubated at 38.2 °C in 5% CO_2 in air for 16–18 h, then were fixed in buffered formol saline, mounted on a slide with 6.5 μl of 9:1 glycerol:PBS containing 2.5 μg/ml Hoechst 33258, and examined using fluorescence microscopy to determine the chromatin configuration. Chromatin configurations were classified as previously described (Dell'Aquila *et al.*, 2001; Love *et al.*, 2002); briefly as (1) Fibrillar: distinct strands of intertwined chromatin visible throughout the GV; (2) Intermediate: with chromatin strands spread over approximately half the area of the GV; (3) Condensed: with chromatin condensed into one mass within the GV; (4) Metaphase I (MI): including stages up to telophase I; (5) Metaphase II (MII): showing extrusion of the polar body; (6) FN: homogeneous fluorescence throughout the GV; (7) Fibrous GV: with a distinct but irregular outline of the GV with heterogeneous or net-like staining within; and (8) Degenerating: with abnormal chromatin, chromatin distributed throughout the oocyte, or no visible chromatin. For analysis, classifications 1 through 3 were grouped together as representing viable GV-stage oocytes, and classifications 6 and 7 were

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

considered to represent non-viable GV-stage oocytes, as reported previously (Hinrichs *et al.*, 2005).

Because the data from the Oil treatment for 16–18 h conflicted with that of our previous study (Hinrichs *et al.*, 2002), in which oocytes had been held for 24 h before evaluation, the experiment was repeated but with a 24 h culture period before fixation and staining, to determine whether the duration of incubation was responsible for the difference in results.

Experiment 2: Comparison of selected roscovitine treatment with immediately fixed oocytes and non-roscovitine-treated controls

Oocytes were recovered as described for experiment 1. After all oocytes were recovered, they were divided randomly into three groups. Immediate control oocytes were denuded of cumulus and immediately fixed in buffered formol saline. Based on the results of experiment 1, oocytes in the roscovitine treatment were washed in M199-Ros and cultured in 500 μl M199-Ros in a 4-well multi-dish without mineral oil for 18 h. Oocytes in the culture control group were washed in basic M199 culture medium without roscovitine and were cultured in 500 μl of this medium in a 4-well multi-dish without mineral oil for 18 h. At the end of the culture period, oocytes were denuded of cumulus and fixed in buffered formol saline. Oocytes in all three groups were stained and evaluated for chromatin configuration as described above.

Experiment 3: Effect of roscovitine meiotic inhibition on subsequent blastocyst development

In vitro *maturation*

Oocytes were recovered as described for experiment 1. After all oocytes were recovered, oocytes in the control group were washed in maturation medium (basic medium with 5 mU/ml FSH (Sioux Biochemicals, Sioux Center, IA)) and cultured in microdroplets of the same medium at a ratio of 10 μl medium per oocyte, under mineral oil for 30 h. Oocytes in the roscovitine treatment were washed and cultured in M199-Ros as for experiment 2, for 16–18 h. Roscovitine-treated oocytes were then washed in maturation medium and cultured in this medium for an additional 30 h. After 30 h of maturation culture, oocytes were denuded by pipetting in 0.05% hyaluronidase and those with a polar body were used for ICSI. Oocytes not having a polar body were fixed and stained for evaluation of chromatin status as described above.

Sperm preparation

Frozen semen from one stallion was used for the entire experiment. Semen straws were thawed at 37 °C for 30 s and 200 μl of semen was put in the bottom of a 5 ml tube containing 1 ml of Sp-CZB (Choi *et al.*, 2003*b*) for swim-up. After 20 min incubation at 38.2 °C in an atmosphere of 5% CO_2 in air, 0.6 ml of 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 then resuspended and washed once with the same medium. The percentage of motile spermatozoa after washing was 60–75%.

Intracytoplasmic sperm injection

The ICSI procedure and media used were as described previously (Choi *et al.*, 2003*b*). Briefly, 1 μl of sperm suspension was placed in a 3 μl droplet of Sp-CZB containing 10% polyvinylpyrrolidone (Sigma) under oil. Sperm injection was carried out in a separate 50 μl drop of CZB-M containing 10% FBS. Spermatozoa were immobilized by applying a few pulses with a piezo drill (Prime Tech, Ibaraki, Japan) to the tail, and were injected into the oocytes. All manipulations were performed at room temperature. Injected oocytes were held in CZB-H containing 10% FBS in 5% CO_2 in air at 38.2 °C until all manipulations were done.

Preparation of oviductal epithelial explants

Equine oviducts were transported from the slaughterhouse to the laboratory on the same day that the ovaries were obtained for oocyte recovery. The connective tissues surrounding oviducts were trimmed with scissors and the oviducts were straightened, then opened longitudinally using the scissors. The oviductal tissue was recovered by scraping the luminal surface of the ampulla using 0.5 cm bone curette as described previously (Choi *et al.*, 2004*a*). The recovered tissue was washed twice in Hepes-buffered M199, and placed in 500 μl DMEM/F-12 (Sigma) with 10% FBS in a 4-well dish in 5% CO_2 in air at 38.2 °C. After 24 h, the dish was examined for presence of vesicle-like structures. Thirty to 50 of these structures were recovered from the culture dish, washed twice in the same medium, and placed in 500 μl wells of the same medium, under oil, for embryo culture.

In vitro *culture*

After sperm injection, oocytes were placed into culture wells in DMEM/F-12 + 10% FBS, either alone or in co-culture with oviductal epithelial vesicles, under mineral oil in 5% CO_2 in air at 38.2 °C. Half of the medium in each well was replaced every 48 h. The oocyte density was 1–10 oocytes in the total volume of 500 μl. Uncleaved or retarded embryos were removed at the time of each medium change. Embryos were cultured for total of 7.5 days. At the end of the culture period, embryos were evaluated morphologically under a dissection microscope at ×63. If there was a question on morphological classification, the embryos were stained as described for oocytes, above, for evaluation of nucleus number. Embryos

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

Table 1 Chromatin configuration of equine Cp oocytes held in roscovitine in microdroplets under mineral oil or in wells without oil overlay for 16–18 or 24 h

Duration of culture (h)	Presence of oil overlay	n	Viable GV[a] (%)	MI (%)	Non-viable GV[b] (%)	Degenerated (%)
16–18	+	68	19 (28)	2 (3)	12 (18)	35 (51)
16–18	–	67	16 (24)	0	14 (21)	37 (55)
24	+	46	8 (17)	2 (4)	10 (22)	26 (57)
24	–	48	9 (19)	1 (2)	11 (23)	27 (56)

GV, germinal vesicle; MI, metaphase I.
[a]Includes fibrillar, intermediate and condensed configurations.
[b]Includes fluorescent nucleus and fibrous GV configurations.

Table 2 Chromatin configurations of equine Cp oocytes fixed immediately upon recovery from the follicle, or cultured for 18 h in the presence or absence of roscovitine

Treatment	n	Viable GV[a] (%)	MI (%)	MII (%)	Non-viable GV[b] (%)	Degnerated (%)
Immediate fix	52	15 (29)	0[c]	0	21 (40)[c]	16 (31)[c]
18 h control	51	9 (18)	9 (18)[d]	0	4 (8)[d]	29 (57)[d]
18 h roscovitine	53	17 (32)	0[c]	0	8 (15)[d]	28 (53)[d]

GV, germinal vesicle; MI, metaphase I; MII, metaphase II.
[a]Includes fibrillar, intermediate and condensed configurations.
[b]Includes fluorescent nucleus and fibrous GV configurations.
[c,d]Values with different subscripts are significantly different ($p < 0.05$).

with ≥64 nuclei and with an outer layer of apparent differentiating trophoblast cells were considered to be blastocysts; those with ≥32 nuclei but lacking apparent organization were classified as morulae.

Statistical analysis

Three replicates were performed for each trial (16–18 h and 24 h cultures) in experiment 1. Five replicates were performed in experiment 2. Due to low maturation rates for Cp oocytes, 18 and 19 replicates were conducted for roscovitine and control maturation treatments, respectively, in experiment 3 to supply an adequate number of oocytes for ICSI (Table 3). The proportions of oocytes with each chromatin configuration after suppression, proportions of oocytes at metaphase I and II after culture for *in vitro* maturation, proportions of oocytes with normal cleavage, and proportions of oocytes developing to the blastocyst stage were compared among groups using chi-square analysis, with Fisher's exact test used when the value of any parameter was 5 or less.

Results

One thousand two hundred and forty-one ovaries were processed, and 7170 follicles were scraped, for an average of 5.8 follicles per ovary. Of the 3953 oocytes recovered, 1184 (30%) were classified as Cp, 2400 (61%) were classified as Ex, and 369 (9%) were degenerating. For this study, 1157 Cp oocytes were used; 27 Cp oocytes were used for a different project.

Experiment 1

A total of 229 Cp oocytes were evaluated after culture in M199-Ros. In the Well roscovitine treatments, the number of oocytes cultured together in each replicate varied from 12 to 30. In the Oil treatments, 6–14 oocytes were cultured per microdroplet; drop size varied from 190 to 266 μl. The ratio of medium to oocyte varied from 17 to 42 μl medium per oocyte among replicates; however, within each replicate the ratio of medium to oocyte was the same for Well and Oil treatments. There were no significant differences in the percentages of oocytes with total viable, metaphase I, non-viable or degenerating chromatin configurations among treatments or culture times (Table 1). No oocytes (0/67) cultured in roscovitine for 16–18 h without oil cover had progressed to metaphase I. Thus, this treatment was chosen for use in experiment 2.

Experiment 2

A total of 156 Cp oocytes were evaluated (Table 2). Culture, in either the presence or absence of roscovitine, was associated with a significant increase in the proportion of degenerating oocytes ($p < 0.05$) and

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

Table 3 Chromatin configurations of equine Cp oocytes held in roscovitine for 16–18 h and then cultured for *in vitro* maturation for 30 h

Presence (+) or absence (−) of roscovitine	n	MI (%)	MII (%)[a]	MI + MII (%)
+	376	9 (2)	82 (22)	91 (24)
−	395	14 (4)	74 (19)	88 (22)

MI, metaphase I; MII, metaphase II.
[a]The number of oocytes with MII was based on the sum of the number of oocytes with a polar body and the number of oocytes with MII after fixation and staining.

a significant decrease in the proportion of oocytes showing non-viable GV configurations ($p < 0.01$). There were no significant differences in the proportions of viable GV-stage oocytes, or in oocytes resuming meiosis, between oocytes that were fixed immediately and those that were cultured in the presence of roscovitine. The proportion of oocytes resuming meiosis (in MI; no oocytes progressed to MII during the culture period) was significantly higher in the control culture group (9/51, 18%) than in either immediately fixed oocytes (0) or in oocytes cultured in the presence of roscovitine (0; $p < 0.01$).

Experiment 3

A total of 772 oocytes were examined after 30 h culture in maturation medium. Of these, 1 was broken during denudation and 771 were evaluated for the presence of a polar body. The maturation rate of oocytes held in roscovitine before maturation was not different from that for control oocytes (22% and 19% at MII, respectively; Table 3). Of 153 oocytes with a polar body that were subjected to ICSI, 150 (98%) survived intact as determined by the presence of an intact plasma membrane. Of 618 oocytes classified as not having a polar body, 3 (0.5% of total oocytes) were found to be in MII and 23 (4% of total oocytes) in MI on fixation and staining, and the remainder were at a GV stage or were degenerating.

Eight injected oocytes were used for another trial, thus 142 injected oocytes were cultured. The extent of embryo development after 7.5 days *in vitro* culture is presented in Table 4. There was no significant difference in cleavage rates (72–78%) among treatments. Overall development to blastocyst of roscovitine-treated oocytes (19/69, 28%) was not significantly different from that of control oocytes (15/73, 21%; $p > 0.1$). Co-culture with oviductal epithelial vesicles tended to increase blastocyst development over culture with medium alone (13/75, 17% vs 21/67, 31%, respectively; $p = 0.08$). Blastocyst development rate in roscovitine-treated oocytes in co-culture was significantly higher than that in control oocytes without co-culture (37 vs 14%, $p < 0.05$).

Discussion

The results of this study demonstrate that roscovitine treatment can suppress meiosis in Cp horse oocytes recovered from transported ovaries, while maintaining the meiotic and developmental competence of the oocytes. Roscovitine treatment may thus be a useful tool for scheduling the timing of onset of maturation in Cp horse oocytes to be used for further manipulations.

The chromatin of Cp oocytes in the most juvenile GV configurations degenerates to non-viable configurations (FN or fibrous GV) during ovary holding or transport (Hinrichs *et al.*, 2005). Culture (with or without roscovitine) was associated with a decrease in the proportion of oocytes in the non-viable GV chromatin configurations in comparison with immediately-fixed control oocytes (experiment 2). The concomitant increase seen in the proportion of degenerating oocytes after culture suggests that oocytes in the non-viable GV configurations continue to deteriorate during culture. Roscovitine treatment was not effective in preventing this deterioration.

Table 4 *In vitro* development of equine Cp oocytes held in roscovitine before *in vitro* maturation and then matured, fertilized and cultured in DMEM/F-12 with or without equine oviductal epithelial explants for 7.5 days

Presence (+) or absence (−) of roscovitine	Groups	Oocytes cultured n	Oocytes cleaved n (%)	Oocytes developed to: Morula n (%)	Oocytes developed to: Blastocyst n (%)
+	DMEM/F-12	39	28 (72)	4 (10)	8 (21)[a,b]
+	Co-culture	30	23 (77)	1 (3)	11 (37)[a]
−	DMEM/F-12	36	27 (75)	0	5 (14)[b]
−	Co-culture	37	29 (78)	3 (8)	10 (27)[a,b]

[a,b]Values with different subscripts are significantly different ($p < 0.05$).

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

Roscovitine treatment was found to suppress meiosis in Cp oocytes in the current study even when used in microdroplets under oil, in contrast to our previous results (Hinrichs *et al.*, 2002). In the current study, we used a roscovitine concentration of 66 μM, the concentration used in the study of Franz *et al.* (2003), whereas Hinrichs *et al.* (2002) used concentrations of 25 and 100 μM. Other conditions were similar between studies. The reason for the discrepancy between the results of the current study and previous results (Hinrichs *et al.*, 2002) is unclear; one possible explanation is that 25 μM under oil leaves insufficient roscovitine in the medium for meiotic suppression, yet 100 μM is potentially toxic to the oocytes.

Roscovitine treatment did not affect the meiotic competence of viable GV-stage oocytes, as the proportion of oocytes reaching metaphase I and II after maturation *in vitro* was similar between control and roscovitine-treated oocytes (Table 3). The low rate of maturation seen for Cp oocytes in this study, in both control oocytes and those previously suppressed with roscovitine, is typical of Cp oocytes in our laboratory, which are recovered from transported ovaries and classified using stringent morphological criteria (Love *et al.*, 2002; Choi *et al.*, 2002*b*, 2004*b*). This reflects the proportion of viable, meiotically competent GV-stage oocytes present in this population after ovary transport (Hinrichs *et al.*, 2005).

Roscovitine inhibition did not negatively affect the ability of Cp oocytes to form blastocysts after maturation and ICSI; in fact, roscovitine-treated oocytes tended to yield a higher proportion of advanced embryos (morulae + blastocysts) than did control oocytes ($p = 0.13$). In cattle, inhibition of meiosis by roscovitine, or roscovitine plus butyrolactone, has also been found not to compromise embryo development (Mermillod *et al.*, 2000; Ponderato *et al.*, 2001). The current report is the first to examine the blastocyst development rate in equine oocytes subjected to meiotic suppression with roscovitine before maturation. Franz *et al.* (2003) reported that, when assessed at 4 days post-ICSI, roscovitine-treated equine Cp oocytes had a significantly increased nucleus number compared with non-treated Cp oocytes; however, further development was not evaluated. It is possible that roscovitine treatment may be most effective in increasing developmental competence when oocytes are collected immediately after slaughter, as was done by Franz *et al.* (2003). If collected and cultured immediately after slaughter, some Cp oocytes that would deteriorate if ovaries were transported may be rescued, and a proportion of these will mature in culture. The ability of roscovitine treatment to increase blastocyst development in this population of oocytes, which were not present in the current study, warrants further investigation.

Embryos resulting from roscovitine-treated oocytes, and that were co-cultured with oviductal epithelial cells, achieved a 37% blastocyst development rate; this was significantly higher than the rate for oocytes having neither treatment (14%). This represents only the second published report of treatments yielding *in vitro* equine blastocyst development of over 30%. We found previously that co-culture did not affect blastocyst development in Ex oocytes (16% and 15% with and without co-culture, respectively; Choi *et al.*, 2004*a*). Expanded-cumulus and Cp oocytes differed as well in their response to roscovitine treatment when embryos were evaluated at 4 days of culture (Franz *et al.*, 2003); this treatment improved embryo development in Cp oocytes and reduced it in Ex oocytes. It is possible that because Cp oocytes are less cytoplasmically mature than are Ex oocytes, they respond positively to treatments aimed at increasing their developmental competence whereas Ex oocytes do not. Future studies should be directed at determining culture factors which differentially affect these two oocyte types.

In conclusion, roscovitine treatment reversibly suppressed meiosis in equine Cp oocytes recovered from transported ovaries. Oil overlay during culture did not significantly affect the degree of suppression. Roscovitine-treated Cp oocytes matured and fertilized by ICSI developed to the blastocyst stage in proportions similar to those of control oocytes. Co-culture with oviductal epithelial explants tended to increase blastocyst development; however blastocyst development of up to 27% was achieved when embryos from roscovitine-treated oocytes were cultured in medium alone. Roscovitine treatment of Cp oocytes may be of great use in synchronizing the onset of maturation of oocytes to aid scheduling of assisted reproduction techniques such as oocyte transfer, ICSI and nuclear transfer in the horse.

Acknowledgements

This research was supported by the Link Equine Research Endowment Fund (Texas A&M University).

References

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**, 215–20.

Choi, Y.H., Hochi, S., Braun, J., Sato, K. & Oguri, N. (1993). *In vitro* maturation of equine oocytes collected by follicle aspiration and by the slicing of ovaries. *Theriogenology* **40**, 959–66.

Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S. & Hinrichs, K. (2002*a*). Developmental competence

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press

in vivo and *in vitro* of *in vitro*-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. *Reproduction* **123**, 455–65.

Choi, Y.H., Shin, T., Love, C.C., Johnson, C., Varner, D.D., Westhusin, M.E. & Hinrichs, K. (2002*b*). Effect of co-culture with theca interna on nuclear maturation of horse oocytes with low meiotic competence, and subsequent fusion and activation rates after nuclear transfer. *Theriogenology* **57**, 1005–11.

Choi, Y.H., Love, C.C., Varner, D.D., Love, L.B. & Hinrichs, K. (2003*a*). Effects of gas conditions, time of medium change, and ratio of medium to embryo on *in vitro* development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59**, 1219–29.

Choi, Y.H., Chung, Y.G., Walker, S.C., Westhusin, M.E. & Hinrichs, K. (2003*b*). *In vitro* development of equine nuclear transfer embryos: effects of oocyte maturation media and amino acid composition during embryo culture. *Zygote* **11**, 77–86.

Choi, Y.H., Roasa, L.M., Love, C.C., Varner, D.D., Brinsko, S.P. & Hinrichs, K. (2004*a*). Blastocyst formation rates *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilized by intracytoplasmic sperm injection. *Biol. Reprod.* **70**, 1231–8.

Choi, Y.H., Love, L.B., Varner, D.D. & Hinrichs, K. (2004*b*). Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127**, 187–94.

Dell'Aquila, M.E., Masterson, M., Maritato, F. & Hinrichs, K. (2001). Influence of oocyte collection technique on initial chromatin configuration, meiotic competence and male pronucleus formation after intracytoplasmic sperm injection (ICSI) of equine oocytes. *Mol. Reprod. Dev.* **60**, 79–88.

Franz, L.C., Choi, Y.H., Squires, E.L., Seidel, G.E. Jr & Hinrichs, K. (2003). Effects of roscovitine on maintenance of the germinal vesicle in horse oocytes, subsequent nuclear maturation, and cleavage rates after intracytoplasmic sperm injection. *Reproduction* **125**, 693–700.

Galli, C., Crotti, G., Turini, P., Duchi, R., Mari, G., Zavaglia, G., Duchamp, G., Daels, P. & Lazzari, G. (2002). Frozen-thawed embryos produced by ovum pickup of immature oocytes and ICSI are capable to establish pregnancies in the horse (abstract). *Theriogenology* **58**, 705–8.

Gastal, E.L., Gastal, M.O., Bergfelt, D.R. & Ginther, O.J. (1997). Role of diameter differences among follicles in selection of a future dominant follicles in mares. *Biol. Reprod.* **57**, 1320–7.

Hinrichs, K. (1991). The relationship of follicle atresia to follicle size, oocyte recovery rate on aspiration, and oocyte morphology in the mare. *Theriogenology* **36**, 157–68.

Hinrichs, K. & Schmidt, A.L. (2000). Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. *Biol. Reprod.* **62**, 1402–8.

Hinrichs, K., Schmidt, A.L., Friedman, P.P., Selgrath, J.P. & Martin, M.G. (1993). *In vitro* maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. *Biol. Reprod.* **48**, 363–70.

Hinrichs, K. & Williams, K.A. (1997). Relationships among oocyte–cumulus morphology, follicular atresia, initial chromatin configuration, and oocyte meiotic competence in the horse. *Biol. Reprod.* **57**, 377–84.

Hinrichs, K., Love, C.C., Choi, Y.H., Varner, D.D., Wiggins, C.N. & Reinoehl, C. (2002). Suppression of meiosis by inhibitors of m-phase proteins in horse oocytes with low meiotic competence. *Zygote* **10**, 37–45.

Hinrichs, K., Choi, Y.H., Love, L.B., Varner, D.D., Love, C.C. & Walckenaer, B.E. (2005). Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. *Biol. Reprod.* **72**, 1142–50.

Love, C.C., Love, L.B., Varner, D.D. & Hinrichs, K. (2002). Effect of holding at room temperature after recovery on initial chromatin configuration and *in vitro* maturation rate of equine oocytes. *Theriogenology* **57**, 1973–9.

Mermillod, P., Tomanek, M., Marchal, R. & Meijer, L. (2000). High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 h in culture by specific inhibition of MPF kinase activity. *Mol. Reprod. Dev.* **55**, 89–95.

Phillips, K.P., Petrunewich, M.A.F., Collins, J.L., Booth, R.A., Liu, X.J. & Baltz, J.M. (2002). Inhibition of MEK or cdc2 kinase parthenogenetically activates mouse eggs and yields the same phenotypes as $Mos^{-/-}$ parthenogenotes. *Dev. Biol.* **247**, 210–23.

Ponderato, N., Lagutina, I., Crotti, G., Turini, P., Galli, C. & Lazzari, G. (2001). Bovine oocytes treated prior to *in vitro* maturation with a combination of butyrolactone I and roscovitine at low doses maintain a normal developmental capacity. *Mol. Reprod. Dev.* **60**, 579–85.

Sirard, M.A. (2001). Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology* **55**, 1241–54.

Zhang, J.J., Boyle, M.S., Allen, W.R. & Galli, C. (1989). Recent studies on *in vivo* fertilisation of *in vitro* matured horse oocytes. *Equine Vet. J. Suppl.* **8**, 101–4.

https://doi.org/10.1017/S0967199406003534 Published online by Cambridge University Press