Effects of Roscovitine on Nuclear Maturation, Spindle Configuration, and Chromosome Alignment in Porcine Oocytes

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ABSTRACT

In the present study, effects of concentration and time of culture in presence of roscovitine on nuclear maturation and meiotic spindle configuration, chromosomal alignment were examined in porcine oocytes. In experiment 1, porcine cumulus oocyte complexes (COCs) were cultured at 39℃ in a 5% CO_2 atmosphere in North Carolina State University 23 (NCSU-23) supplemented with 25, 50, 75 or 100 μM roscovitine for 22 h and then were cultured for additional 22 h after removal of roscovitine. Nuclear maturation and morphology of the meiotic spindle and chromosomal alignment were examined to determine the optimal concentration of roscovitine in oocyte maturation. In experiment 2, COCs were cultured in NCSU-23 supplemented with 50 μM roscovitine for 17, 20, 27 or 42 h and then an additional 22 h without roscovitine was followed to determine the optimal time of culture. The optimal concentration of roscovitine to arrest and resume meiosis of porcine oocyte was 50 μM by examining nuclear status ($p<0.05$) and normal spindle and chromosome configuration. The optimal time of culture in presence of roscovitine to arrest meiosis of porcine oocyte was 17 h ($p<0.05$), although MII rates and normal morphology of the meiotic spindle and chromosomal alignment were not significantly different among various times of culture. In conclusion, the optimal concentration and time of culture in presence of roscovitine to arrest porcine oocytes are 50 μM and 17 h, respectively.

(Key words : roscovitine, nuclear maturation, meiotic spindle configuration, chromosome alignment)

INTRODUCTION

Oocyte maturation is one of the most important stages for *in vitro* production of embryos. Various methods to improve developmental competence of porcine oocytes *in vitro* have been reported (Yoshiba *et al.*, 1992; Abeydeera *et al.*, 2000; Tatemoto *et al.*, 2004).

Synchronization of meiotic progress in porcine oocytes has been attempted to overcome a lesser developmental competence of *in vitro* matured oocyte. Various meiotic inhibitors, cycloheximide (CHX), 6-dimethylaminopurine (6-DMAP), roscovitine, dibutryl cyclic adenosine phosphate (dbcAMP) have been used (Motlik *et al.*, 1984; Le beux *et al.*, 2003; Bagg *et al.*, 2006; Romar and Funahashi, 2006).

Roscovitine is a purine known as a specific inhibitor of cyclin-dependent protein kinase that prevents $p34^{cdc2}$ dephosphorylation and inhibits MPF kinase activity (Meijer and Kim, 1997). Roscovitine has been successfully used to arrest meiotic activation without compromising further embryo development in cattle and pigs (Motlik *et al.*, 1984; Mermillod *et al.*, 2000). On the contrary, Marchel *et al.* (2001) concluded that roscovitine may not protect oocytes against loss of fertilisability. Schoevers *et al.* (2005) reported that roscovitine treatment reduced embryonic development.

On the other hand, most of studies on roscovitine have been focused on nuclear maturation and *in vitro* fertilization in various animals, pig as well as cattle, goat, and horse by blocking meiosis activation (Krischek and Meinecke, 2001; Marchal *et al.*, 2001; Ju *et al.*, 2003; Le Beux *et al.*, 2003; Donnay *et al.*, 2004; Schoevers *et al.*, 2005). The process of spindle formation and chromosomal segregation are believed to be particularly sensitive to chemical environment, highlighting the importance of the maturation environment in the genesis of aneuploidy (Roberts *et al.*, 2005). Spindle and chromatin alterations of oocytes affected by roscovitine have not completely been understood, although some studies determined effect on cytoskeleton of oocytes. Schoevers *et al.* (2005) assessed morphology of microtubule and microfilaments of porcine oocytes exposed to 50 μM roscovitine.

Therefore, the major objective of the present study was to

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determine the optimal concentration of roscovitine and time of culture in presence of roscovitine by observing spindle configuration and chromosomal alignment of oocytes. In addition, nuclear maturation to develop fertilization capacity was assessed.

MATERIALS AND METHODS

1. Culture Media

Unless otherwise noted, all chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA). The medium used for collection and washing of porcine oocyte-cumulus complexes (COCs) was modified Tyrode's lactate-HEPES-polyvinyl alcohol (TL-HEPES-PVA) composed of 114 mM NaCl, 3.2 mM KCl, 0.4 mM $Na_2H_2PO_4$, 2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 5 mM $NaHCO_3$, 20 mM HEPES, 16.6 mM sodium lactate (60% syrup), 0.5% PVA, 10 IU/ml penicillin, and 10 μg/ml streptomycin. Oocyte maturation was accomplished in North Carolina State University 23 medium (NCSU-23) supplemented with 10% porcine follicular fluid (PFF), 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml pregnant mare serum gonadotropin (PMSG), and 10 IU/ml human chorionic gonadotropin (HCG). PFF was collected from ovarian follicles that ranged from 3~6 mm in diameter by centrifugation at 1,600 × g for 30 min at room temperature and filtration through a 1.2 μm syringe filter. The final product was stored at −20℃ until use.

2. *In Vitro* Maturation

Porcine ovaries were collected from a local abattoir and were transported to the laboratory at 34~36℃ in 0.9% saline supplemented with 100 IU/ml penicillin G and 100 mg/ml streptomycin. COCs were aspirated through an 18 gauge needle. Oocytes with a compact cumulus mass and a dark, evenly graduated cytoplasm were washed three times in TL-HEPES-PVA medium and maturation medium, respectively. Oocytes (n=20~25) were cultured in a 100 μl droplet of maturation medium supplemented with 10 IU/ml PMSG and 10 IU/ml HCG for 22 h, and then were cultured for further 22 h in hormone-free maturation medium. Oocyte maturation was carried out under sterile mineral oil at 39℃ in a 5% CO_2 atmosphere in NCSU-23.

3. Assessment of Meiotic Stage

Oocytes denuded by vigorous shaking in 3% sodium citrate solution were fixed in a 4-well culture dish (Nunc, Rochester, NY, USA) containing 500 μl of ethanol:acetic acid (3:1 v/v) for 48 h. They were then dispensed onto a slide to which a cover slip was added. Each sample was stained with aceto-orcein [1% (w/v) orcein in 45% (v/v) acetic acid] and destained with glycerol:acetic acid:distilled water (1:1:3, v/v). Oocytes were evaluated under a light microscope at 400 × magnification. Meiotic stages were classified as previously described (Romar and Funahashi, 2006) as being at germinal vesicle, germinal vesicle breakdown, metaphase I, anaphase I, telophase I, or metaphase II.

4. Meiotic Spindle and Chromosome Immunostaining

Immunoflurorescence staining of oocytes was performed by the method described by Shi *et al.* (2007). Cumulus cells of oocytes were removed with 3% sodium citrate and were fixed in 4% formaldehyde containing 0.3% Triton x-100 in PBS for 40 min. They were then washed in PPB (PBS containing 0.1% PVA, 1% BSA and 1% sodium azide) three times at room temperature. The fixed oocytes were exposed to PPB containing 10% goat serum for 10 min at room temperature, and then were stored in PPB containing mouse anti-α-tubulin monoclonal antibody (1:100) at 4℃ overnight. After three washes in washing buffer, the oocytes were incubated in PPB containing fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:40) for 1 h in the dark at room temperature. The oocytes were washed with PPB for three times and were incubated with in PPB containing 10 μg/ml propidium iodide for 15~20 min. the oocytes were washed with PPB for three times. Finally, the oocytes were mounted on glass slide and spindle morphology and chromosome organization were examined under florescence inverted microscope at 400 × magnification. Spindle morphology and chromosome alignment were classified by the analysis described by Shi *et al.* (2007). Percentage of metaphase II was evaluated from existence of first polar body with chromosome.

5. Statistical Analysis

Six replicates were conducted for experiment. All data and data sets are presented as the mean ± SE, and were analyzed by Duncan's multiple range test using the Statistical Analysis System ver. 8 X (SAS, Cary, NC, USA). Percentage data were subjected to an arcsin transformation before analysis. p<0.05 was considered to be statistically significant.

6. Experimental Design

Experiment I determined the effect of roscovitine concentration on nuclear maturation, spindle configuration, and chro-

mosome alignment. Oocytes were cultured in maturation medium supplemented with 25, 50, 75 or 100 μM roscovitine and hormones for 22 h, and then were cultured in maturation medium free of roscovitine and hormones for additional 22 h. Oocytes cultured in roscovitine-free maturation medium for 44 h constituted the control. A portion of the oocytes from each group were removed, denuded, and fixed at 22 h; the remaining was fixed at 44 h after denuding cumulus cells. The meiotic stages were assessed in the populations and configuration of spindle and chromosomes were examined. Six replicates of each group were assessed.

Experiment II determined the effect of time of culture in presence of roscovitine on nuclear maturation, spindle configuration, and chromosome alignment. The oocytes were cultured in maturation medium supplemented with 50 μM roscovitine and hormones for 17, 22, 27 or 42 h, and then were cultured in maturation medium free of roscovitine and hormones for additional 22 h. Oocytes cultured in roscovitine-free maturation medium for 44 h represented the control. A proportion of oocytes from each group were removed, denuded, and fixed at 17, 22, 27 or 42 h; the remaining was fixed at 39, 44, 49 or 64 h after denuding cumulus cells. The meiotic stages were assessed in the populations and spindle configuration and chromosomes alignment were examined. Six replicates of each group were assessed.

RESULTS

1. Effect of Roscovitine Concentration on Nuclear Maturation, Meiotic Spindle Configuration, and Chromosome Alignment

Nuclear status of oocyte was examined after 22 h of culture to assess the meiotic arrest of oocytes exposed to various concentrations of roscovitine. After 22 h of exposure to 50, 75 or 100 μM roscovitine, the proportion of oocytes that remained at the GV stage was significantly higher ($p<0.05$) than that of oocytes cultured in absence of roscovitine (Table 1). Oocytes cultured without roscovitine for 22 h showed significantly higher proportions of GVBD stage oocytes ($p<0.05$).

Oocytes were cultured for additional 22 h after removal of roscovitine to assess the meiotic resumption and configuration of spindle and chromosomes. M II rate of oocytes that had previously not been treated (control), or treated with 25 or 50 μM roscovitine was significantly higher ($p<0.05$) than that of oocytes treated with 100 μM roscovitine (Table 2). Table 2 also showed the effect of roscovitine concentration on spindle morphology and chromosome alignment. Oocytes in stage of metaphase II showed higher percentage of normal spindle configuration. However, there were not significantly different among groups with various roscovitine concentrations. When chromosome alignment was compared, there was no significant difference among groups.

2. Effect of Various Times of Culture in Presence of 50 μM Roscovitine on Nuclear Maturation, Meiotic Spindle Configuration, and Chromosome Alignment

Oocytes were cultured for various times of incubation to determine the optimal time of culture for roscovitine-mediated meiotic arrest and resumption. The proportions of oocytes in the GV stages were significantly higher at 17 h ($p<0.05$, Table 3). However, the proportion of oocytes in the GVBD stage was significantly higher at 27 h.

Table 1. Effects of various concentrations of roscovitine on nuclear maturation of porcine oocytes cultured for 22 h

Conc.(μM)	No. of oocytes	Nuclear maturation (%)			
		GV	GVBD	MI	AI-TI
C	95	52.0 ± 9.8^{c}	39.0 ± 9.0^{a}	8.8 ± 3.8^{a}	0.0 ± 0.0
25	101	74.4 ± 5.5^{bc}	21.5 ± 6.6^{ab}	3.4 ± 2.3^{ab}	0.8 ± 0.8
50	92	86.0 ± 4.1^{ab}	13.9 ± 4.1^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0
75	94	88.4 ± 3.3^{ab}	10.5 ± 2.9^{b}	1.1 ± 1.1^{ab}	0.0 ± 0.0
100	87	89.2 ± 5.3^{a}	10.8 ± 5.3^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0

Six replicates were assessed.
Values with different superscript are significantly different ($p<0.05$).
The data represent mean ± SE.
C: Oocytes cultured for 22 h without roscovitine in maturation medium.
GV: geminal vesicle, GBVD: geminal vesicle break down, MI: metaphase I, AI-TI: anaphase I-telophase I.

Table 2. Effects of various concentrations of roscovitine on morphology of meiotic spindle and chromosome alignment in porcine oocytes cultured for 44 h

Conc. (μM)	No. of oocytes	MII, n (%)	Spindle configuration, n (%)			Chromosome alignment, n (%)		
			Normal	Abnormal	Absent	Dispersed	Decondensed	Absent
C	96	82(86.7)[a]	75(92.6)	7(7.4)	(0)	1(1.0)	6(6.3)	(0)
25	96	87(90.7)[a]	83(95.5)	4(4.5)	(0)	1(1.1)	3(3.4)	(0)
50	96	85(88.4)[a]	82(96.5)	3(3.5)	(0)	(0)	3(3.5)	(0)
75	102	84(83.1)[ab]	80(95.0)	3(3.7)	1(1.3)	(0)	3(3.7)	1(1.3)
100	91	59(63.6)[b]	55(95.2)	4(4.8)	(0)	(0)	4(4.8)	(0)

Six replicates were assessed.
Values with different superscript are significantly different ($p<0.05$).
C: Oocytes cultured for 44 h in roscovitine-free maturation medium. MII: metaphase II.

Meiotic resumption of oocytes incubated additionally for 22 h in roscovitine-free maturation medium was assessed (Table 4). The proportions of MII stage-oocytes at control, 39 or 44 h were higher than those of MII stage-oocytes at 49 or 64 h without significant difference. Table 4 also showed the effect of time of culture in presence of roscovitine on spindle morphology and chromosome alignment. Oocytes at stage of metaphase II showed higher percentage of normal spindle configuration. However, there were not significantly different among various times of incubation in presence of 50 μM roscovitine. When chromosomes alignment was compared, there was no significant difference among groups.

In the interaction between roscovitine and cumulus cells, cumulus cells expansion of oocytes treated with roscovitine for 22 h were inhibited regardless of concentration (Fig. 1). Limited expansion of cumulus cells with some clump of cumulus cells was shown from 50 to 100 μM, although expansion of cumulus cells were resumed after removal of roscovitine. Morphology of COCs cultured in 50 μM roscovitine for various times of culture was observed. Cumulus cells of oocytes treated with 50 μM roscovitine showed limited expansion regardless of time of culture. Clump of cumulus cells was observed from 22 to 42 h of culture.

DISCUSSION

In this study, we compared various concentrations of roscovitine to determine optimal concentration for porcine oocyte

Table 3. Effect of various times of culture in presence of 50 μM roscovitine on nuclear maturation of porcine oocytes

Time of incubation (h)	No. of oocytes	Nuclear maturation (%)					
		GV	GVBD	MI	AI	TI	MII
C	109	62.2 ± 5.5[b]	31.1 ± 4.6[b]	5.6 ± 1.4	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 1.1
17	94	96.1 ± 3.9[a]	3.9 ± 3.9[b]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22	100	70.4 ± 6.2[b]	27.6 ± 5.8[b]	1.1 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.9
27	105	29.9 ± 5.5[c]	59.1 ± 5.9[a]	11.2 ± 5.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
42	111	8.2 ± 2.3[c]	3.9 ± 1.9[b]	11.8 ± 2.2	0.8 ± 0.8	0.9 ± 0.9	74.4 ± 2.2

Six replicates were assessed.
Values with different superscript are significantly different ($p<0.05$).
The data represent mean ± SE.
C: Oocytes cultured for 22 h in roscovitine-free maturation medium.
GV: germinal vesicle, GBVD: germinal vesicle break down, MI: metaphase I, AI: anaphase I, TI: telophase I, MII: metaphase II.

Table 4. Effect of various times of culture in presence of 50 μM roscovitine on morphology of meiotic spindles and chromosome alignment

Time* of incubation (h)	No. of oocytes	MII, n (%)	Spindle configuration, n (%)			Chromosome alignment, n (%)		
			Normal	Abnormal	Absent	Dispersed	Decondensed	Absent
C	105	89(84.1)	87(97.9)	2(2.1)	(0)	1(1.0)	2(2.1)	(0)
39	98	83(84.0)	82(98.7)	1(1.3)	(0)	1(1.3)	(0)	(0)
44	98	81(82.9)	81(100)	(0)	(0)	(0)	(0)	(0)
49	105	80(76.2)	80(100)	(0)	(0)	(0)	(0)	(0)
64	103	76(74.5)	76(100)	(0)	(0)	(0)	(0)	(0)

Six replicates were assessed.
C: Oocytes cultured for 44 h in roscovitine-free maturation medium.
*Time of incubation in presence of roscovitine and additional incubation in absence of roscovitine.

maturation. Nuclear maturation rate was significantly lower (63.6%) after removal of roscovitine, although 100 μM roscovitine showed the highest meiotic arrest at 22 h of culture. Meiotic arrest has been increased as dose-dependent manner. This result showed the similar tendency to the result by using roscovitine in goat oocytes (Han *et al.*, 2008). Meiotic arrest by roscovitine is fully reversible after removal of roscovitine in maturation medium (Ponderato *et al.*, 2002; Le Beux *et al.*, 2003; Albarracin *et al.*, 2005). In the present study, some oocytes which had been treated with higher concentration of roscovitine could not be recovered from meiotic arrest. This result indicated appropriate concentration of roscovitine should be used in maturation medium to resume meiosis after removal. Ju *et al.* (2003) found that levels of 80~120 μmol/l roscovitine were necessary to inhibit GV in 83~91% of oocytes. However, in our study, doses of 50 μmol/l roscovitine were enough to block GV (88.4%) after 22 h of culture. Fifty to 75 μM might be the appropriate concentration in considering resumption of meiosis.

Spindle microtubules are known to involve pronuclear migration in fertilized oocytes in pigs (Kim *et al.*, 1996a; 1996b). In the effect on meiotic spindle of oocytes, 4~7% oocytes showed abnormal spindle configuration. Roscovitine did not fully affect meiotic spindle configuration and chromosome alignment regardless of concentrations. In referring to Ju *et al.* (2003)'s report, 15~21% oocytes showed abnormal MII morphology with aberrant meiotic spindles after culture in 80~120 μmol/l roscovitine. There was difference in time of culture compared to our result. Ju *et al.* (2003) incubated oocytes in maturation medium with roscovitine for 44 h and then incubated for additional 44 h after removal of roscovitine. Oocytes were exposed to higher concentration of roscovitine for additional 22 h compared to time of incubation we used. We suggest that oocytes should not be incubated for longer than 22 h in higher concentration of roscovitine in porcine. On the other hand, there was difference among species. Albarracin *et al.* (2005) examined the effect of roscovitine on morphology of the meiotic spindle in calf oocytes. They reported that meiotic spindles showed the typical MII morphology in oocytes prematured with 50 μM roscovitine, differing significantly from 20 or 100 μM treatment. In effect on chromosomal alignment, roscovitine did not affect chromosome regardless of concentration in our study. Albarracin *et al.* (2005) reported that chromosome disorganization was observed in a higher proportion of oocytes treated with roscovitine in calf oocytes.

We determined the effect of time of culture on nuclear maturation in experiment 2. Time-dependent decreases in the GV rate have been reported in porcine oocytes treated with roscovitine (Schoevers *et al.*, 2005). These results are consistent with our findings that the rate of meiotic arrest decreased as time of culture increased. Seventeen hour showed the highest meiotic arrest rate ($p<0.05$). However, MII rate (>75%) was not significantly different among various times of incubation. Romar and Funahashi (2006) assessed nuclear progression of porcine oocytes cultured in 50 μM roscovitine for 48 h and subsequently cultured in roscovitine-free medium for 22, 30, 38 or 44 h. Nuclear progression was inhibited after 22 or 30 h of culture (M II rate: 0~18%). Nuclear maturation rate was rather decreased as time of culture increased in our study (Table 4). We speculate that too longer time of culture in presence of roscovitine might inhibit reversibility after removal, although appropriate concentration is used for meiotic arrest of oocytes.

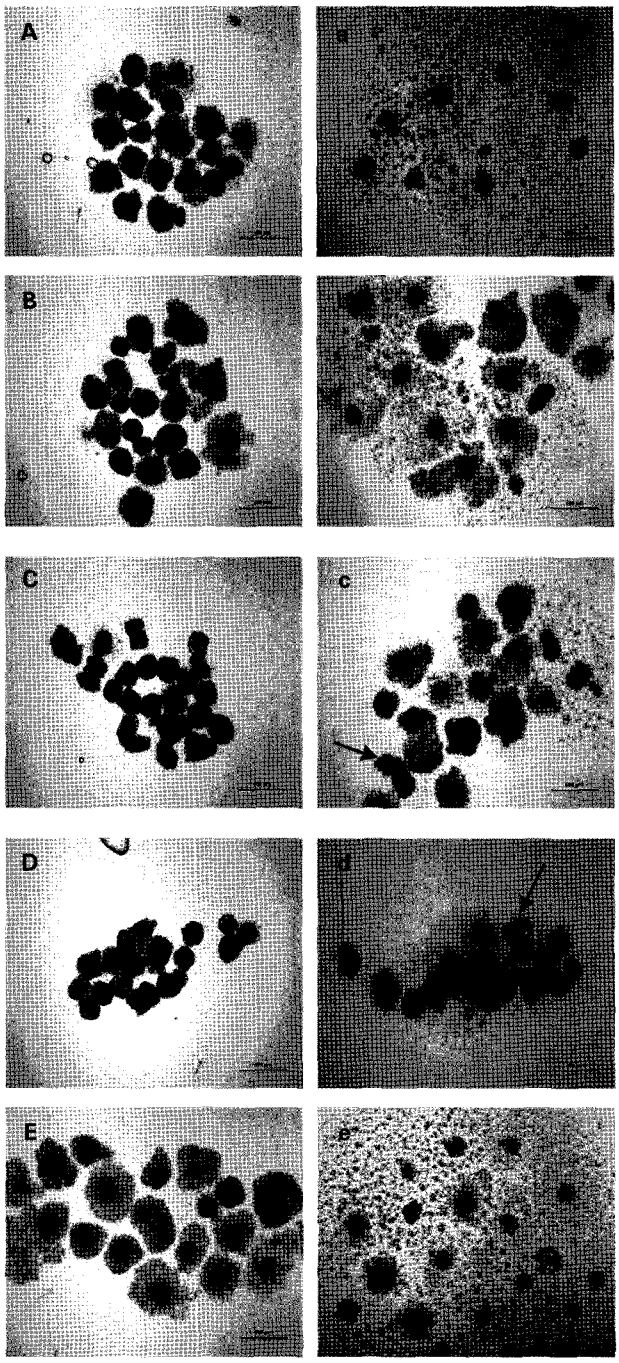

Fig. 1. Morphology of porcine COCs cultured in various concentrations of roscovitine, 25, 50, 75 or 100 μM (A, B, C, D) for 22 h and then additionally cultured (a, b, c, d) for 22 h after removal of roscovitine. (A, B, C, D): showing less expanded cumulus compared to those of control, (a): showing fully expanded cumulus cells, (b): showing partially expanded cumulus cells, (c, d): showing clotted cumulus cells (arrows), (E) control (22 h of culture): showing expanded cumulus cell, (e): control (44 h of culture): showing fully expanded cumulus cell. Magnification: 40 X.

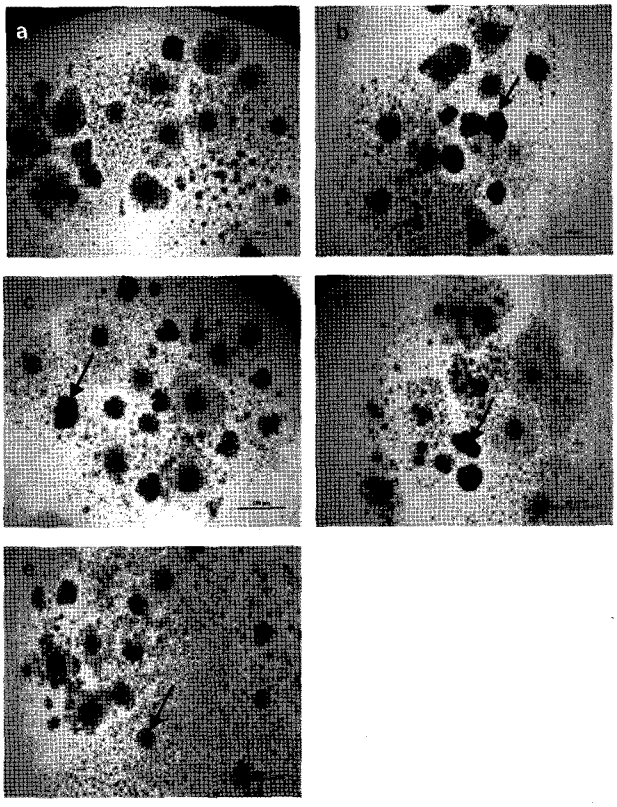

Fig. 2 Morphology of porcine COCs cultured in presence of 50 μM roscovitine for 17, 22, 27 or 42 h and then additionally cultured for 22 h after removal of roscovitine. (a): 39 h, COCs with less expanded cumulus, (b, c, d): 44 h (b), 49 h (c), and 64 h (d), respectively, showing clotted cumulus cells (arrows), (e): control showing fully expanded cumulus cell (arrow). Magnification: 40 X.

On the other hand, several authors have observed that nuclear maturation of porcine and bovine oocytes were faster after meiotic inhibitors, such as, butyrolactone-I (Hashimoto *et al.*, 2002; Wu *et al.*, 2002), roscovitine (Marchal *et al.*, 2001), BLD-MAP (Kalous *et al.*, 1993; Avery *et al.*, 1998), cycloheximide (Kubelka *et al.*, 1988), dbcAMP treatment (Funahashi *et al.*, 1997). In the present study, 17 h of incubation in presence of 50 μM roscovitine and additional 22 h of incubation in roscovitine-free medium showed the highest maturation rate. Alfonso *et al.* (2008) reported GV rate was 67% of oocytes treated with 50 μM roscovitine for 5 h. However, GV rate reported by Alfonso *et al.* (2008) is similar to that of control (62% GV rate) not treated with roscovitine in our study. We infer that too short time of culture in presence of roscovitine might not fully affect meiotic arrest of oocytes.

Marchal *et al.* (2001) reported that cumulus expansion was

inhibited by roscovitine and this effect was reversed after additional 44 h of culture in basal maturation medium. Schoevers *et al.* (2005) also indicated that cumulus cells expansion was delayed in presence of 50 μM roscovitine for 22 h and expansion of cumulus cells occurred after removal of roscovitine for additional 22 h of culture. The similar tendency was observed in the present study; expansion of cumulus cells was inhibited after treatment of roscovitine regardless of concentration. Expansion of cumulus cells at 44 h was rather decreased as concentration of roscovitine used for 22 h of culture increased. This result was related to maturation rate (Table 2). In other words, expansion of cumulus cells affect nuclear maturation of oocytes. In addition, cumulus cells were expanded after removal of roscovitine. However, some clumps of cumulus cells were observed as time of culture increased (Fig. 2). The presence of viable cumulus cells during maturation is required for complete cytoplasmic maturation of oocytes (Marchal *et al.*, 2001). However, we suggest that prolonged contact with cumulus cells does not improve oocyte development.

Roscovitine has been known as a specific inhibitor of MPF that efficiently and reversibly prevents meiosis resumption in porcine oocytes *in vitro*, and protects oocytes from aging. However, we conclude that reversibility of the treatment with roscovitine in arresting meiotic maturation is dependent of concentration of roscovitine and time of culture in presence of roscovitine. In the present study, the appropriate concentration and the time of culture are 50 μM and 17 h, respectively. Further studies are necessary to improve the developmental competence of oocytes maintained at the GV stage by roscovitine for *in vitro* fertilization, parthenogenic activation.

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