Effect of Roscovitine Pretreatment on the Meiotic Maturation of Bovine Oocytes and their Subsequent Development after Somatic Cell Nuclear Transfer

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Abstract: Roscovitine, a specific inhibitor of M-phase promoting factor kinase activity was used to inhibit the completion of meiotic maturation of bovine oocytes. The objectives of this study were to evaluate the nuclear maturation of bovine oocytes pre-cultured with various concentrations (0, 50, 100 and 200 μM) of roscovitine before *in vitro* Maturation (IVM) and to examine the development of Somatic Cell Nuclear Transfer (SCNT) embryos derived from the oocytes pre-cultured with roscovitine. Before IVM, 72% of oocytes that were cultured without roscovitine (control) had reached the Metaphase II (MII) stage whereas culture with roscovitine decreased the rates of oocytes reaching MII (11-27%). After IVM, the maturation rate of oocytes pre-cultured with 200 μM roscovitine was significantly higher than that of control oocytes (79 vs. 58%). Moreover, significantly more oocytes extruded the first polar body in the 50 μM roscovitine group than in the control group (64 vs. 51%). The rate of blastocyst formation of reconstructed embryos derived from oocytes pre-cultured with 50 μM roscovitine was significantly higher than that from the control oocytes (14 vs. 6%). In this study, the addition of roscovitine to culture medium delays the completion of meiotic maturation of bovine oocytes and the cytoplasm derived from oocytes pre-cultured under meiotic inhibition can support the development of SCNT embryos.

Key words: Meiotic inhibition, prematuration, short storage, nuclear transfer, polar body, SCNT

INTRODUCTION

Since, the cloned sheep Dolly, various live births including mouse, bovine, goat, pig, rabbit, cat, horse and rat have been produced by Somatic Cell Nuclear Transfer (SCNT) techniques. SCNT techniques are valuable for wild animal conservation and production of transgenic animals. In general, bovine ovaries are collected at the slaughter house and brought to the laboratory. The oocytes are collected from the ovarian follicles and used for experiments. In the cloning technique, the matured oocytes with the first polar body are enucleated and used as a recipient oocyte for reconstructing embryos with donor cells.

If oocytes could be cultured without reducing their viability for 1-2 days before the onset of *in vitro* Maturation (IVM) culture, the temporal storage of oocytes at the Germinal Vesicle (GV) or early meiotic stage before IVM culture may allow a more convenient schedule for subsequent oocyte manipulations. However, bovine oocytes must be collected immediately after transportation of ovaries to the laboratory and subjected

to IVM culture for maintenance of the viability of oocytes. Roscovitine is a purine analog that blocks M-phase Promoting Factor (MPF) activation and can maintain the GV stage of bovine oocytes for 24 h (Mermillod *et al.*, 2000). It has been shown that early embryonic development is not compromised even when oocytes are pre-cultured with roscovitine for 24 h before IVM and *in vitro* Fertilization (IVF) (Mermillod *et al.*, 2000). Moreover, roscovitine treatment before IVM culture does not affect the establishment of pregnancy (Kasinathan *et al.*, 2001) or fetal development (Ponderato *et al.*, 2002) and subsequent birth of live animals (Coy *et al.*, 2005).

In other studies, similar approaches have already provided results of culture of bovine oocytes maintained at the GV stage for 24 h by inhibition of protein synthesis (cycloheximide) or phosphorylation (6-dimethylaminopurine) (Lonergan *et al.*, 1997; Avery *et al.*, 1998). However, roscovitine has been suggested to have less detrimental effects on the developmental competence of bovine oocytes after IVF compared with other inhibitors such as cycloheximide or

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6-dimethylaminopurine (Mermillod *et al.*, 2000). Although, the effect of roscovitine has been studied on IVF of bovine oocytes, less information is available concerning SCNT for bovine oocytes. The understanding of the inhibitory events involved in meiotic resumption of oocytes would offer meaningful information on the efficiency of SCNT procedures. In the present study, researchers examined the effects of various concentrations of roscovitine on the meiotic competence of oocytes pre-cultured for 24 h before IVM. Moreover, we investigated the development of SCNT embryos derived from oocytes pre-cultured with various concentrations of roscovitine.

MATERIALS AND METHODS

Oocyte recovery and *in vitro* maturation: Bovine ovaries were obtained from a slaughterhouse and transported to the laboratory in 0.9% (w/v) physiological saline at 35°C within 3 h of slaughter. Cumulus-Oocyte Complexes (COCs) were aspirated from follicles (2-5 mm in diameter) using a 5 mL syringe fitted with an 18 gauge needle. They were collected in Modified Phosphate-Buffered Saline (m-PBS; Nihonzenyaku, Fukushima, Japan) supplemented with 100 IU mL^{-1} penicillin G potassium (Meiji, Tokyo and Japan) and 0.1 mg mL^{-1} streptomycin sulfate (Meiji). Only COCs with uniform ooplasm and compact cumulus cells were used in this experiment. To examine the effects of roscovitine on the maintenance of meiotic competence of COCs, the collected oocytes were incubated in Tissue Culture Medium (TCM) 199 with Earle's salts (Invitrogen, Carlsbad, CA, USA) supplemented with 0 (control), 50, 100 and 200 µM of roscovitine (Sigma, St. Louis, MO, USA), 10% (v/v) Fetal Bovine Serum (FBS; Invitrogen), 2 mM L-glutamine (Sigma), 0.1 mM β-mercaptoethanol (Wako Pure Chemical Industries, Osaka, Japan) and 50 µg mL^{-1} gentamicin (Sigma) for 24 h under humidified 5% CO$_2$ in air at 38.5°C. After 24 h of incubation, the COCs were washed twice in maturation medium that consisted of TCM 199 supplemented with 0.02 AU mL^{-1} FSH (KawasakiMitaka K.K., Kawasaki, Japan), 5% FBS and 50 µg mL^{-1} gentamicin and then cultured for 21 h at 38.5°C in humidified 5% CO$_2$ in air.

Assessment of oocyte nuclear status: The meiotic stage of oocytes was evaluated before and after maturation culture in which the COCs had been incubated in medium with each concentration of roscovitine. The oocytes were completely denuded in TCM199 supplemented with 0.1% (w/v) hyaluronidase (Sigma). Irrespective of the morphology of the oocytes all oocytes were fixed and permeabilized for 15 min at room temperature in Dulbecco's Phosphate-Buffered Saline (PBS; Invitrogen)

supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton-X100 (Sigma) and then placed in PBS containing 0.3% (w/v) polyvinylpyrrolidone for 15 min at room temperature. The oocytes were transferred into the small drop comprising PBS supplemented with 90% (v/v) glycerol (Wako Pure Chemical Industries) and 1.9 µM bis-benzimide (Hoechst 33342; Sigma) on a glass slide. Subsequently, the oocytes were overlaid with a coverslip supported by 4 droplets of vaseline/paraffin and incubated for one night at 4°C. The oocytes were examined using a fluorescence microscope with a 355 nm wavelength excitation filter.

The meiotic stage of IVM oocytes was classified according to chromatin configuration as germinal vesicle, condensed chromatin, metaphase I or metaphase II. Those with diffusely stained cytoplasm characteristic of non-viable cells and those in which chromatin was unidentifiable or not visible were considered to be degenerated.

Nuclear transfer: Cumulus cells which were the source of donor nuclei were removed from the COCs by vortexing and were cultured in β-MEM (Invitrogen) supplemented with 10% FBS, 1% MEM non-essential amino acid solution (Invitrogen) and 50 µg mL^{-1} gentamicin for 7 days at 37.0°C under 5% CO$_2$ in air. Once the cumulus cells reached complete confluence, the cell monolayer was washed twice with α-MEM and then incubated in 0.25% (w/v) trypsin-EDTA (Sigma) for 3 min at 37.0°C. After trypsinization, 3 mL of washing medium (β-MEM supplemented with 10% FBS) was added to neutralize trypsin activity. The cells were pelleted by centrifugation at 500× g for 5 min, resuspended in the washing medium and then remained in this medium until SCNT manipulation. At 21 h after IVM onset, COCs were mechanically denuded in TCM 199 medium containing 0.1% (w/v) hyaluronidase (Sigma). Oocytes were separated according to the first polar body extrusion and then the number of oocytes with the first polar body was recorded. Prior to enucleation, oocytes were incubated for 10 min in 3 µg mL^{-1} Hoechst 33342 (Sigma) diluted in manipulation medium (PBS supplemented with 5% FBS and 5 µg mL^{-1} cytochalasin B (Sigma)). Oocytes were then washed, transferred to a drop of manipulation medium and enucleated at room temperature after minimal exposure to low-light filtered fluorescence. Oocytes were secured with a holding pipette and rotated as needed into a position suitable for enucleation. The zona pellucida above the first polar body was cut with a glass needle. A small volume (about 5-10%) of cytoplasm underneath the first polar body was squeezed out. Complete enucleation was confirmed by staining the squeezed out cytoplasm. After enucleation of each individual oocyte, the donor cell

(diameter: 14-16 μm) was then placed into the perivitelline space, adjacent to the plasma membrane of the oocyte. Karyoplast/oocyte couplets were transferred to modified Synthetic Oviduct Fluid (mSOF) medium (Kwun *et al.*, 2003) supplemented with 0.4% (w/v) Bovine Serum Albumin (BSA) and 50 μg mL^{-1} gentamicin (mSOF/BSA) and incubated for at least 30 min prior to fusion and activation.

Couplets were equilibrated for 3 min in the Zimmerman cell fusion medium (Wolfe and Kraemer, 1992), transferred into a drop of Zimmerman cell fusion medium and then manually aligned between the two electrode needles connected to the micromanipulator (MO-202D; Narishige, Tokyo and Japan). A single simultaneous fusion and activation by electrical pulse of 2.3 kV cm^{-1} for 30 μsec was applied to the couplets using an electro cell fusion (LF101, Nepagene, Chiba and Japan). After fusion, the couplets were incubated in mSOF/BSA for 20 min before chemical activation. The successfully fused couplets were activated in mSOF/BSA supplemented with 10 μg mL^{-1} cycloheximide (Sigma) and incubated for up to 5 h.

Following cycloheximide treatment, the couplets were washed, transferred into mSOF/BSA medium and then cultured for 72 h at 38.5°C in humidified 5% CO_2 and 5% O_2. After 72 h of culture only cleaved embryos were further co-cultured with bovine cumulus cells in mSOF supplemented with 5% FBS at 38.5°C in humidified 5% CO_2 for an additional 5 days to evaluate their ability to develop to the blastocyst stage.

Statistical analysis: About 5-8 replicate trials were carried out. Data are expressed as mean±SEM. The percentages of oocytes reaching each stage of meiosis before and after IVM, embryos cleaved and embryos developed to the blastocyst stage were subjected to arcsin transformation prior to the Analysis of Variance (ANOVA). The transformed data were tested by ANOVA followed by a post hoc Fisher's Protected Least Significant Difference (PLSD) test using the Statview program (Abacus Concepts, Inc., Berkeley, CA). Probability values (p) of 0.05 or less were considered to be significant.

RESULTS AND DISCUSSION

Meiotic status of oocytes before and after *in vitro* Maturation: Before IVM culture (Fig. 1a), significantly more oocytes that were pre-cultured without roscovitine had reached the MII stage (72.4%) as compared with other oocytes pre-cultured with roscovitine (10.7%-26.9%) (p<0.05). After IVM culture (Fig. 1b) when the oocytes were pre-cultured with 200 μM roscovitine, the maturation
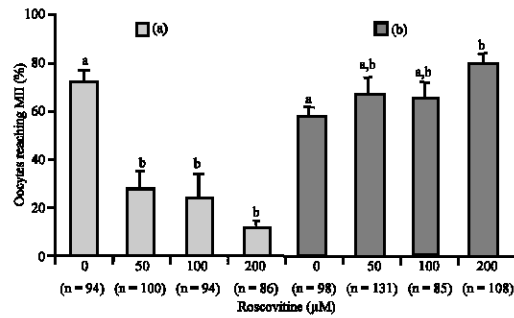

Fig. 1: The rates of oocytes reaching MII before (a) and after (b) *in vitro* maturation culture following pre-culture with various concentrations of roscovitine for 24 h. Numbers within parentheses indicate the total number of oocytes examined. Bars with different letters differ significantly (p<0.05)
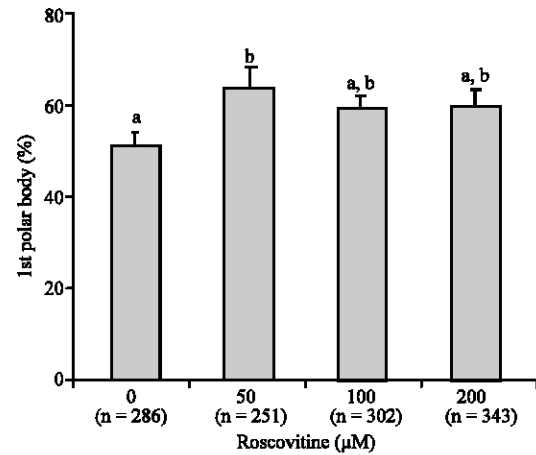

Fig. 2: The oocyte rates with extrusion of the first polar body after *in vitro* maturation culture. Numbers within parentheses indicate the total number of oocytes examined. Bars with different letters differ significantly (p<0.05)

rate of oocytes was significantly higher (p<0.05) than that of oocytes pre-cultured without roscovitine (79.4 vs. 57.9%). However, no significant differences in the rates of oocytes reaching MII were observed among the three groups with the addition of roscovitine.

Significantly, more oocytes with a visible polar body were observed in the 50 μM roscovitine group (63.8%) than in the control group (50.8%) (p<0.05). However, an increased concentration of roscovitine had no positive effects on the percentage of oocytes with a visible polar body (Fig. 2).

Development of SCNT embryos: There were no significant differences in the cleavage rates among the groups

Table 1: Development of somatic cell nuclear transfer embryos derived from oocytes pre-cultured for 24 h with various concentrations of roscovitine before *in vitro* maturation

| | | No. (Mean±SEM) of embryos | | |
Roscovitine concentrations (µM)	No. of fused couplets	Cleaved	Developed to blastocysts	Cell no. (Mean±SEM) of blastocysts
0	116	66 (56.6±6.1)	8 (5.8±1.7)[a]	74.6±7.10
50	74	35 (52.7±10.1)	10 (14.2±5.4)[b]	79.9±7.80
100	139	68 (46.1±6.0)	10 (6.8±3.0)[a,b]	106.9±22.5
200	145	77 (52.7±7.0)	13 (7.7±2.2)[a,b]	88.2±9.70

[a,b]Values with different superscript letters differ significantly (p<0.05)

(Table 1). The rate of development to blastocysts of reconstructed embryos derived from oocytes pre-cultured with 50 µM roscovitine was significantly higher (p<0.05) than that from control oocytes (14.2 vs. 5.8%). There were no significant differences in the cell numbers of blastocysts among the groups.

The results demonstrated that the addition of roscovitine to culture medium delays the completion of meiotic maturation of bovine oocytes and maintains the ability of cytoplasmic maturation of oocytes during/after pre-incubation for 24 h, resulting in a higher blastocyst formation of the reconstructed embryos derived from oocytes pre-cultured with 50 µM roscovitine before IVM.

Attempts to develop *in vitro* culture systems to maintain bovine or porcine oocytes at the GV stage using different meiotic inhibitors have been reported (Lonergan *et al.*, 1997; Avery *et al.*, 1998; Liu *et al.*, 1998; Dode and Adona, 2001; Faerge *et al.*, 2001).

If the oocytes could be maintained at the GV stage or early meiotic stage during the inhibitory period without negative effects on subsequent development to the blastocyst stage, the maturation schedules in the laboratory can be prolonged and more flexible (Coy *et al.*, 2004; Choi *et al.*, 2006).

Therefore, this method involving a two-step culture system (pre-culture in a medium with inhibitor and further culture in IVM medium) could be applied for *in vitro* embryo production of bovine embryos using not only IVF methods (Coy *et al.*, 2005) but also SCNT programs (Motlik *et al.*, 2000).

In the present study, researchers found that roscovitine delays the completion of meiotic maturation of bovine oocytes during incubation but 11-27% of oocytes had already reached MII at the end of the pre-culture period. It has been shown that the inhibitory effect of roscovitine varies according to the concentration but exposure to concentrations of roscovitine >50 µM can prevent meiotic resumption in ~60% of oocytes (Albarracin *et al.*, 2005).

It is well known that when the oocyte is removed from its follicular environment, it spontaneously resumes meiosis (Mermillod and Marchal, 1999). At oocyte collection, researchers collected and selected COCs which were placed in m-PBS without any inhibitors after follicle aspiration. It seemed that some oocytes had already resumed meiosis before pre-culture treatment with roscovitine.

Therefore, some MII stage oocytes observed in the present study might result from spontaneous meiotic resumption of oocytes before the treatment. It has been shown that after pre-incubation with roscovitine, the oocytes were released from the meiosis block by transferring them into the IVM medium (Adona *et al.*, 2008). In the present study, similarly we found that the maturation rates of oocytes pre-cultured with roscovitine increased from <27% at the onset of IVM culture to 66-79% at the end of IVM culture.

The maturation rates of oocytes observed in the present study were similar to other studies in which the rates of bovine oocyte s reached MII after incubation with roscovitine and subsequent maturation culture were 60-90% (Mermillod *et al.*, 2000; Donnay *et al.*, 2004; Albarracin *et al.*, 2005). Moreover, the incubation of oocytes with 200 µM roscovitine enhanced the meiotic competence of oocytes compared with control oocytes (79 vs. 58%). These results indicate that the meiotic inhibition of oocyte by roscovitine is fully reversible even when the oocytes did not developed further than the MI stage during inhibition culture.

At the end of maturation culture, more oocytes with a visible polar body were observed in the 50 µM roscovitine group (64%) than the control group (51%). The results are consistent with the result of Lagutina *et al.* (2002) who reported that about 70% of oocytes pre-incubated with roscovitine extruded their polar bodies after 11 h of maturation culture.

It has been suggested that some factors acting upstream of MPF activation may accumulate progressively during roscovitine inhibition and that their presence may allow a faster course of early stages of meiotic resumption, decreasing the whole maturation time (Vigneron *et al.*, 2004).

Therefore, incubation with roscovitine before IVM culture may increase the extrusion rates of oocytes. Roscovitine has been shown to be capable of reversibly

inhibiting meiotic resumption in bovine oocytes for 24 h without negative effects on subsequent development of IVF embryos to the blastocyst stage (Mermillod *et al.*, 2000). In contrast, Donnay *et al.* (2004) reported that pre-incubation treatment with roscovitine prevented the progression of meiosis in bovine oocytes but that this treatment led to a dramatic decrease in embryo development after IVF.

In the present study, similarly the development rate of reconstructed embryos derived from oocytes pre-cultured with roscovitine (7-14%) was significantly lower than that (blastocyst/fused couplets; 48/151, 33.5±7.7%) obtained from fresh oocytes without pre-culture treatment. Lonergan *et al.* (2003) reported that morphological changes occurred in immature and *in vitro* matured bovine oocytes following exposure to roscovitine. They demonstrated that pre-maturation treatment with roscovitine caused swelling of the mitochondrial cristae, degeneration of the cortical granules and convolution of the nuclear membrane in the oocytes.

Therefore, the decrease in the development rate of reconstructed embryos derived from pre-cultured oocytes might result in part from the cytoskeleton alterations caused by exposure to roscovitine. However, we found that the development of reconstructed blastocysts derived from oocytes pre-cultured with 50 µM roscovitine was significantly higher than that from control pre-cultured oocytes (14 vs. 6%).

It has been reported that enhancing the cytoplasmic maturation of recipient oocytes resulted in an improvement of SCNT outcome (Wongsrikeao *et al.*, 2007). Moreover, successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) improves the efficiency of preimplantation embryonic development as well as fetal development (Sagirkaya *et al.*, 2007). Therefore, pre-incubation of oocytes with roscovitine might maintain or improve cytoplasmic maturation, resulting in an increase in the development of reconstructed embryos. Moreover, the result indicates that the addition of 50 µM roscovitine as a meiotic inhibitor is effective for the development of SCNT embryos.

CONCLUSION

In this study, the addition of roscovitine to culture medium can delay the meiotic resumption of oocytes during inhibition culture and their cytoplasm can support the development of SCNT embryos even when the oocytes are pre-cultured for 1 day before the onset of IVM culture, allowing a more convenient schedule for subsequent oocyte manipulations.

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