

# Maintenance of meiotic arrest in bovine oocytes using the S-enantiomer of roscovitine: effects on maturation, fertilization and subsequent embryo development *in vitro*

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## Abstract

The overall objective was to evaluate the effectiveness of the S-enantiomer of roscovitine (inhibitor of p34<sup>cdc2</sup>/cyclin B kinase) to maintain bovine cumulus–oocyte complexes at the germinal vesicle (GV) stage for extended times after removal from antral follicles without compromising subsequent maturation, fertilization and embryo development. Oocytes were cultured in 0, 12.5, 25 or 50 µmol/l S-roscovitine for 24 h. Hoechst staining showed that 50 µmol/l S-roscovitine maintained >90% of oocytes at the GV stage and inhibited gonadotropin-induced cumulus expansion. Fewer oocytes underwent nuclear maturation after *in vitro* maturation (Hoechst staining) when cultured in 50 µmol/l S-roscovitine for 66 versus 21 or 42 h. Zona pellucida (ZP) hardening (pronase resistance), cortical granule types (lens culinaris agglutinin–fluorescein isothiocyanate), nuclear maturation and fertilization with frozen-thawed spermatozoa (Hoechst staining) were assessed after culture of oocytes in 50 µmol/l S-roscovitine for 0, 24 or 48 h. Neither ZP hardening, nor nuclear maturation nor fertilization were altered by roscovitine culture for 48 h. A higher proportion of oocytes had a type III cortical granule pattern (premature translocation to the oolemma) after roscovitine culture for 48 h. However, embryo development was not compromised as cleavage, development to 8–16 cell and blastocyst stages were at least comparable in control and roscovitine-treated oocytes. In conclusion, the studies have shown that S-roscovitine reversibly maintained bovine oocytes at the GV stage for 48 h. However, maintenance of oocytes in static culture for 48 h was not sufficient to improve development above non-treated controls.

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## Introduction

Abattoir-derived ovaries provide an abundant source of oocytes to identify the factors necessary for oocyte maturation, fertilization and early embryo development. Premature removal from antral follicles (i.e. 3–8 mm) induces the oocyte to undergo spontaneous resumption of meiosis (Pincus & Enzmann 1935) which may not be without consequence as 60–70% fail to develop to the blastocyst stage after *in vitro* maturation, fertilization and embryo culture. The higher development of oocytes when obtained from later stage follicles (Hagemann *et al.* 1999) suggests that removal from earlier stages deprives the cumulus–oocyte complex (COC) of important differentiative events necessary for acquisition of full developmental competence. As such, intensive efforts have focused on the development of two-step culture systems to allow for

increased culture time to ensure appropriate cumulus–oocyte interactions (Motlik *et al.* 2000).

Use of the majority of meiotic inhibitors tested to date compromises embryo development (Lonergan *et al.* 1997, Saeki *et al.* 1997, Avery *et al.* 1998, Dode & Adona 2001). However, results obtained with selective inhibitors of cyclin-dependent kinases (CDKs) have shown promise (Motlik *et al.* 2000). In 2000, Mermillod *et al.* reported that 25 µmol/l of the R-enantiomer of roscovitine (inhibitor of p34<sup>cdc2</sup>/cyclin B) maintained >80% of bovine oocytes at the germinal vesicle (GV) stage for 24 h after removal from antral follicles without compromising nuclear maturation, embryo cleavage and blastocyst development. Additional efforts have shown that roscovitine culture does not compromise the establishment of pregnancy (Kasinathan *et al.* 2001) or fetal development during organogenesis (Ponderato *et al.* 2002).

Most recently, Coy *et al.* (2004) reported the birth of live piglets derived from roscovitine-treated oocytes.

Because COC originate from antral follicles at various stages (i.e. 3–8 mm), an *in vitro* inhibitory period of 24 h may not allow enough time to acquire full developmental competence. Meiotic inhibition for several days may be necessary (Motlik *et al.* 2000). Efforts to characterize the reversibility of roscovitine longer than 32 h in a static culture (Lagutina *et al.* 2002) have not been reported using bovine oocytes. Until this information is known, inferences about the usefulness of roscovitine to improve the development of otherwise developmentally 'incompetent' oocytes may be limited.

The S-enantiomer of roscovitine was obtained by Edwards' laboratory for use as an *in vitro* model to maintain bovine oocytes at the GV stage to study the effects of environmental stressors over time (Payton *et al.* 2004). After S-roscovitine had been obtained, it was noted that differences in the R- versus the S-enantiomer of roscovitine to inhibit p34<sup>cdc2</sup> kinase activity existed ( $IC_{50} = 650$  and 800 nM respectively; Havlicek *et al.* 1997). De Azevedo *et al.* (1997) reported that R-roscovitine was almost twice as potent as the S-enantiomer. A racemic mixture of the R- and S-enantiomers showed intermediate activity. Moreover, unpublished results (L Meijer, personal communication) suggest that the S-enantiomer, although slightly less potent at inhibiting CDK1, CDK2 and CDK5, may be more selective towards other protein kinases (ERKs). Enantiomer-dependent differences, along with the absence of studies describing the use of roscovitine to meiotically inhibit oocytes for prolonged time-periods prompted us to conduct a series of experiments to (1) establish the effective dose of the S-enantiomer of roscovitine to maintain bovine oocytes at the GV stage and inhibit gonadotropin-induced cumulus expansion, (2) determine the length of time that oocytes from antral follicles can be held at the GV stage in a static culture with S-roscovitine, (3) examine the effects of prolonged culture with S-roscovitine on zona pellucida (ZP) hardening, nuclear and cytoplasmic maturation of the oocyte, fertilization (penetration, pronuclear formation and monospermic and putative embryos) and (4) assess the effects of prolonged static culture with S-roscovitine on embryo cleavage and blastocyst development.

## Materials and Methods

### Materials

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tissue culture medium-199 (M199), nucleosides and penicillin–streptomycin were purchased from Specialty Media (Phillipsburg, NJ, USA). Bovine ovaries were purchased from a commercial abattoir (Gaffney, SC, USA). Harrogate Genetics (Harrogate, TN, USA) donated frozen bovine semen. Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD, USA). The USDA (Beltsville, MD, USA) provided luteinizing hormone (LH).

Follicle-stimulating hormone (FSH) was obtained from VetrepHarm Inc. (London, Ontario, Canada). Roscovitine (2-(S)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine) was purchased from Calbiochem (La Jolla, CA, USA) and solubilized in dimethyl sulfoxide before freezing at  $-20^{\circ}\text{C}$  as a 10 mmol/l stock. In the majority of studies, roscovitine medium consisted of M199 with Earle's salts, 10% FBS (v/v),  $1 \times$  nucleosides, 2 mmol/l L-glutamine,  $1 \times$  non-essential amino acids, 0.1 mmol/l  $\beta$ -mercaptoethanol, 50 U/ml penicillin and 50  $\mu\text{g}/\text{ml}$  streptomycin. Media as required for *in vitro* production of embryos were prepared as previously described by Parrish *et al.* (1988); HEPES-Tyrodes abumin, lactate and pyruvate (TALP), fertilization-TALP and sperm-TALP, Biggers *et al.* (2000); modified potassium simplex optimized medium (KSOM) containing 0.5% (w/v) bovine serum albumin (BSA), 10 mmol/l glycine and 1 mmol/l glutamine) or as noted in later sections.

### *In vitro* maturation, fertilization and culture of embryos

*In vitro* maturation (IVM), fertilization (IVF) and embryo culture were performed as previously described (Lawrence *et al.* 2004) with a few exceptions. COC were collected from antral follicles (3–8 mm) and then cultured for 24 h (30–40 COC in each well of a Nunclon 4-well plate containing 500  $\mu\text{l}$  oocyte maturation medium (OMM; M199 with Earle's salts, 10% (v/v) FBS, 2 mmol/l L-glutamine, 0.2 mmol/l sodium pyruvate, 50  $\mu\text{g}/\text{ml}$  gentamicin, 0.3  $\mu\text{g}/\text{ml}$  LH and 5.0  $\mu\text{g}/\text{ml}$  FSH) at  $38.5^{\circ}\text{C}$  in 5%  $\text{CO}_2$  and humidified air. Oocytes, presumed mature, were fertilized with Percoll-prepared frozen-thawed semen (750 000 total sperm/ml). Eight to ten hours after IVF, putative zygotes were denuded of cumulus and associated spermatozoa by vortexing (4 min). Putative zygotes were sequentially cultured in KSOM containing  $1 \times$  non-essential amino acids for the first 3 days post-IVF, thereafter in KSOM containing  $1 \times$  non-essential and essential amino acids in an atmosphere of 5.5%  $\text{CO}_2$ , 7%  $\text{O}_2$  and 87.5%  $\text{N}_2$  at  $38.5^{\circ}\text{C}$  in humidified air. The ability of oocytes to cleave (assessed by recording the number of 1, 2, 4 and 8–16 cell embryos) and develop to the blastocyst stage was recorded on days 4 and 9 post-IVF. The total number of nuclei within each blastocyst was determined by staining the embryos with Hoechst 33342 (10  $\mu\text{g}/\text{ml}$ ; 20 min).

### Assessment of ZP hardening, nuclear stage and cortical granule types

ZP hardening was estimated by recording the time (s) required for 0.5% (w/v) pronase to solubilize the ZP of individual oocytes. Dissolution time was expressed as the time-interval after placement of the zona-intact oocyte into pronase until the ZP was no longer visible with a stereomicroscope (Coy *et al.* 2002). Nuclear stage and cortical granule types (type I, aggregates; type II, aggregates with some dispersion; type III, dispersion of

granules) were evaluated by staining oocytes after ZP removal as described by Long *et al.* (1994) and Hosoe & Shioya (1997). Oocytes were fixed in 3% (w/v) paraformaldehyde at room temperature for 1 h, and then washed five times (20 min each time) in blocking solution (Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS) but containing 1% BSA (w/v), 0.01 mol/l glycine and 0.05% (v/v) Triton X-100). Oocytes were stained with 20 µg/ml lens culinaris agglutinin conjugated with fluorescein isothiocyanate (FITC) in DPBS for 30 min in the dark. Stained oocytes were then washed in DPBS containing 1 mg/ml polyvinylpyrrolidone and mounted on glass slides with 50% glycerol in DPBS containing 0.5 µg/ml Hoechst 33342. Nuclear status and cortical granule type were assessed in individual oocytes by sequential exposure to ultraviolet light using 4',6-Diamidino-2-phenylindole (DAPI) (wavelength 330–380 nm) and FITC (wavelength 450–490 nm) filters respectively, at 200 × magnification.

#### Experiment 1: dose of S-roscovitine to maintain the germinal vesicle and prevent cumulus expansion

COC were cultured with 0, 12.5, 25 or 50 µmol/l S-roscovitine for 24 h in an atmosphere of 5.5% CO<sub>2</sub> and humidified air at 38.5 °C. After 24 h, oocytes were evaluated for cumulus expansion (compact or expanded) and then denuded. Oocytes were then stained with Hoechst 33342 (10 µg/ml; 20 min) to evaluate nuclear status. The number of oocytes at the GV stage was recorded. Experiment 1 was replicated on five different occasions. In one experimental replicate, 0.3 µg/ml LH and 5.0 µg/ml FSH

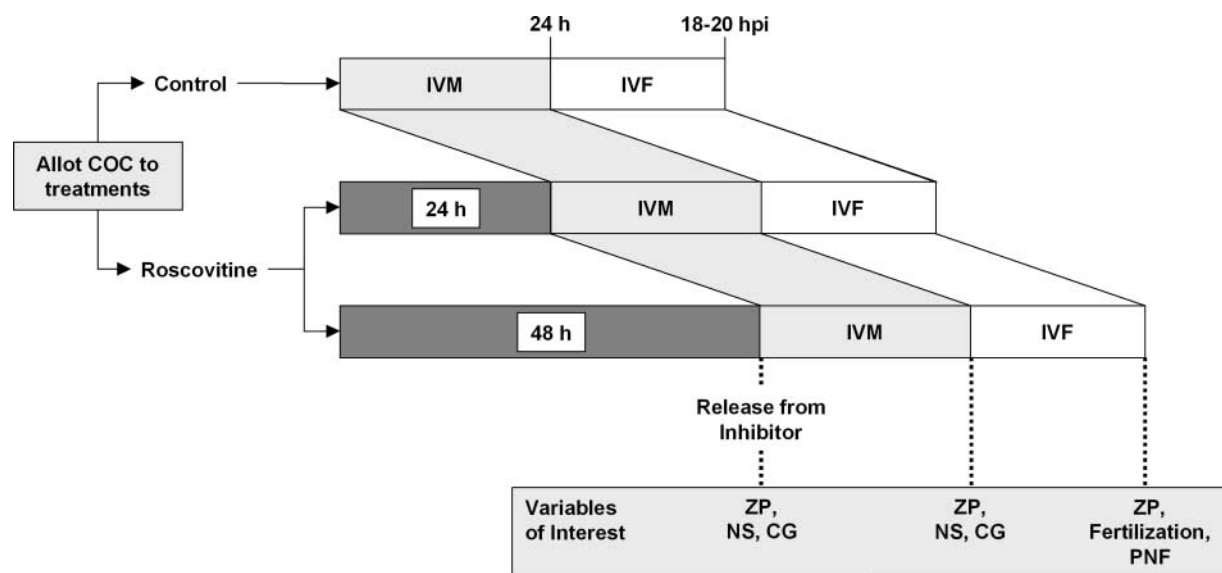
were added to the roscovitine medium to examine the extent to which S-roscovitine inhibited cumulus expansion. The total number of oocytes evaluated within each treatment ranged from 129 to 172.

#### Experiment 2: nuclear maturation of oocytes after culture in S-roscovitine for up to 66 h

COC were cultured with 50 µmol/l S-roscovitine for 21, 42 or 66 h in 5.5% (v/v) CO<sub>2</sub> and humidified air at 38.5 °C. After culture, oocytes were divided within treatment: nuclear stage was determined in approximately half while those remaining were removed from roscovitine medium and cultured for an additional 24 h in OMM. Nuclear stage of oocytes (GV, metaphase I or II (MI or MII)) was evaluated using Hoechst 33342 (10 µg/ml; 20 min). Experiment 2 was replicated on four different occasions.

#### Experiment 3: ZP hardening, nuclear stage, cortical granule type and fertilization of oocytes after culture in S-roscovitine for 0, 24 or 48 h

COC were placed in OMM (control) or in roscovitine medium (50 µmol/l). Oocytes were cultured in S-roscovitine for 24 or 48 h. Afterwards, oocytes were removed from the roscovitine medium and allowed to undergo IVM. Sperm were added to oocytes presumed to be mature. Groups of oocytes were evaluated before IVM (immediately after removal from antral follicles or after roscovitine culture; GV stage), after IVM or 18–20 h after IVF (schematic of experimental design; Fig. 1). ZP resistance to pronase digestion, nuclear stage and cortical



**Figure 1** Schematic of the experimental design and variables of interest for experiment 3. Immediately after removal from antral follicles, bovine oocytes were placed in OMM (containing 0.3 µg/ml LH and 5.0 µg/ml FSH) and allowed to undergo IVM or in 50 µmol/l S-roscovitine for 24 or 48 h before IVM. Thereafter, oocytes were removed from roscovitine medium and allowed to undergo IVM and IVF. ZP hardening (ZP), nuclear stage (NS) and cortical granule (CG) type were examined in oocytes immediately after removal from follicles or roscovitine culture for 24 or 48 h and after IVM. Eighteen to twenty hours after insemination (hpi), ZP hardening was assessed and oocytes were evaluated for fertilization and pronuclear formation (PNF).

granule type in individual oocytes were evaluated immediately after oocyte collection or roscovitine culture for 24 or 48 h, and after IVM. Eighteen to twenty hours after IVF, oocytes were evaluated for ZP hardening and fertilization using Hoechst 33342 (0.5 µg/ml). Variables of interest included penetration (oocytes that were penetrated by at least one spermatozoa), number of sperm per oocyte, pronuclear formation (oocytes containing at least two pronuclei), monospermy (oocytes with one pronucleus and one sperm head either condensed, swollen or as pronucleus) and putative embryos (monospermic oocytes showing pronuclear formation). Experiment 3 was replicated on three different occasions. The total number of oocytes examined in each treatment is described in the Tables.

**Experiment 4: embryo development after culture of oocytes in S-roscovitine for 0, 24 or 48 h**

COC were cultured in 0 or 50 µmol/l roscovitine at 38.5 °C in 5.5% (v/v) CO<sub>2</sub> and humidified air. After 24 or 48 h, oocytes were washed to remove roscovitine and cultured for an additional 24 h in OMM. Oocytes that were presumed to be mature were divided within the treatment; half were fertilized while the remaining half served as parthenogenetic controls. The ability of presumptive zygotes to cleave and develop to the blastocyst stage was recorded on days 4 and 9 post-IVF respectively. The total number of nuclei in blastocysts was evaluated. Experiment 4 was replicated on seven different occasions and included a total of 275-363 oocytes per treatment.

**Statistical analyses**

Data were analyzed as a randomized complete or incomplete block design (blocked on replicate) using mixed models of SAS (SAS Inc. 2001) with some exceptions noted below. Data were tested for normality (Shapiro–Wilk >0.90) and transformed when necessary. Data are expressed as a proportion of total for ease of comparison with those reported in the literature and are, for most experiments, presented as least square means ± S.E.M.

Differences among some means were tested using Fisher’s Exact test (*P* < 0.05) including penetration, pronuclear formation, monospermy and putative embryos.

**Results**

**Experiment 1: dose of S-roscovitine to maintain the germinal vesicle and prevent cumulus expansion**

Culture of bovine oocytes in 25 or 50 µmol/l S-roscovitine for 24 h after removal from 3-8 mm antral follicles maintained an intact GV in the majority of oocytes examined (Table 1; *P* < 0.005; S.E.M. = 4.5). These findings were consistent regardless of the presence or absence of gonadotropins in the roscovitine medium. Culture of oocytes with 50 µmol/l S-roscovitine inhibited cumulus expansion (Table 1; *P* < 0.0001; S.E.M. = 5.6).

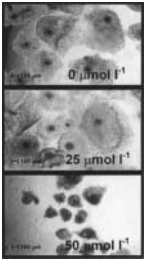
**Experiment 2: nuclear maturation of oocytes after culture in S-roscovitine for up to 66 h**

More than 90% of the oocytes cultured in 50 µmol/l S-roscovitine for 66 h had an intact GV (Table 2). After removal from the inhibition medium, approximately 70% of the oocytes cultured in roscovitine for 42 h progressed to MII after IVM. However, culture of oocytes in 50 µmol/l S-roscovitine for 66 h reduced the proportion that progressed to MII after IVM.

**Experiment 3: ZP hardening, nuclear stage, cortical granule type and fertilization of oocytes after culture in S-roscovitine for 0, 24 or 48 h**

Culture of COC in S-roscovitine for 24 or 48 h did not alter ZP hardening (185.7-233.1 s; S.E.M. = 24.5; *P* = 0.65) before or after IVM or after IVF. More than 97% of the oocytes cultured in 50 µmol/l S-roscovitine had an intact GV after 48 h (Table 3). Roscovitine culture for 48 h did not compromise nuclear maturation as progression of oocytes to MII was similar to those cultured in OMM. The proportion of GV-stage oocytes that had type III cortical granules (premature translocation to the oolemma)

**Table 1** The proportion of bovine oocytes that had an intact GV and expanded cumulus after culture in medium with (OMM) or without (0) gonadotropins at different concentrations of S-roscovitine.

| S-Roscovitine (µmol/l) | No. of oocytes | GV (%)            | Expanded cumulus (%) | Roscovitine with gonadotropins  |
|------------------------|----------------|-------------------|----------------------|---|
| 0 (OMM)                | 129            | 0 <sup>a</sup>    | 100 <sup>a</sup>     |  |
| 0                      | 171            | 10 <sup>a</sup>   | 88.9 <sup>a</sup>    |   |
| 12.5                   | 172            | 52 <sup>b</sup>   | 22.0 <sup>b</sup>    |   |
| 25                     | 167            | 87.6 <sup>c</sup> | 13.0 <sup>bc</sup>   |   |
| 50                     | 139            | 96.8 <sup>c</sup> | 1.8 <sup>c</sup>     |   |
| S.E.M.                 |                | 4.5               | 5.6                  |   |
| <i>P</i> value         |                | <0.005            | <0.0001              |   |

<sup>a,b,c</sup>Differ within column.

**Table 2** Nuclear maturation of bovine oocytes after culture in S-roscovitine (50 µmol/l) for 21, 42 or 66 h.

| Time in roscovitine (h) | No. of oocytes | Intact GV (%) | No. Matured | GV (%) | MI (%) | MII (%)           |
|-------------------------|----------------|---------------|-------------|--------|--------|-------------------|
| 21                      | 155            | 94.8          | 109         | 17.4   | 11.7   | 69.2 <sup>a</sup> |
| 42                      | 167            | 96.2          | 148         | 11.9   | 10.9   | 70.4 <sup>a</sup> |
| 66                      | 185            | 90.7          | 167         | 21.6   | 14.8   | 52.1 <sup>b</sup> |
| S.E.M.                  |                | 1.8           |             | 3.8    | 4.5    | 5.5               |
| P value                 |                | 0.14          |             | 0.20   | 0.80   | 0.04              |

No. matured, the number of oocytes removed from roscovitine medium and allowed to undergo *in vitro* maturation.

<sup>a,b</sup>Differ within column.

**Table 3** Nuclear stage and cortical granule types (CGI, II or III) after culture of bovine oocytes in S-roscovitine for 0, 24 or 48 h.

| Time in roscovitine (h) | No. of COC | Before IVM     |      |      |                   | After IVM      |        |      |       |                   |
|-------------------------|------------|----------------|------|------|-------------------|----------------|--------|------|-------|-------------------|
|                         |            | No. of COC (%) |      |      |                   | No. of COC (%) |        |      |       |                   |
|                         |            | GV             | CGI  | CGII | CGIII             | MII            | CGI    | CGII | CGIII |                   |
| 0                       | 111        | 98.2           | 23.9 | 65.1 | 11.3 <sup>a</sup> | 134            | 86.6   | 1.8  | 27.2  | 71.1 <sup>a</sup> |
| 24                      | 130        | 93.9           | 30.0 | 65.0 | 5.0 <sup>a</sup>  | 124            | 91.9   | 3.5  | 24.8  | 71.7 <sup>a</sup> |
| 48                      | 111        | 97.3           | 28.0 | 52.3 | 19.6 <sup>b</sup> | 116            | 86.2   | 0.0  | 14.4  | 85.6 <sup>b</sup> |
| P value                 |            | 0.1927         |      |      | 0.003             |                | 0.2980 |      |       | 0.007             |

<sup>a,b</sup>Differ within column.

was higher after roscovitine culture for 48 h (Table 3). The proportion of oocytes penetrated by a spermatozoa and the mean number of sperm within each oocyte were similar in the OMM control and roscovitine-treated oocytes (Table 4). The median number of sperm per oocyte was 1.00 for oocytes cultured in S-roscovitine for 0, 24 or 48 h ( $P > 0.29$ ). Pronuclear formation and proportion of monospermic and putative embryos derived from roscovitine-treated oocytes was similar to the OMM control.

#### Experiment 4: embryo development after culture of oocytes in S-roscovitine for 0, 24 or 48 h

Culture of oocytes in roscovitine medium for 48 h did not compromise cleavage or development to the 8–16 cell and blastocyst stages (Table 5) when compared with the OMM control. The proportion of oocytes that cleaved ( $P < 0.04$ ) and developed to the blastocyst ( $P < 0.02$ ) stage was higher after roscovitine culture for 24 h (Table 5)

**Table 4** Fertilization of bovine oocytes cultured in S-roscovitine for 0, 24 or 48 h.

| Time in roscovitine (h) | No. of COC | PEN (%) | S/O* | PNF* (%) | MONO* (%) | PE† (%) |
|-------------------------|------------|---------|------|----------|-----------|---------|
| 0                       | 111        | 78.4    | 1.5  | 85.1     | 65.5      | 51.4    |
| 24                      | 128        | 71.9    | 1.6  | 70.7     | 67.4      | 48.4    |
| 48                      | 115        | 80.9    | 1.6  | 77.4     | 64.5      | 52.2    |
| P value                 |            | 0.23    | 0.77 | 0.07     | 0.92      | 0.84    |

PEN, penetration; S/O, number of spermatozoa per oocyte; PNF, male pronuclear formation; MONO, monospermy; PE, putative embryos.

\* Based on penetrated oocytes.

† PE = proportion of monospermic oocytes having pronuclei.

compared with those cultured in roscovitine for 0 (OMM) or 48 h. The number of nuclei comprising blastocyst stage embryos was similar regardless of treatment origin (98.6, 97.6 and 90.6 for blastocysts derived from oocytes cultured in roscovitine for 0, 24 or 48 h respectively; S.E.M. = 5.7;  $P < 0.2$ ). Roscovitine culture did not increase parthenogenetic development of oocytes (6.1, 2.4 and 6.5% cleaved for 0, 24 and 48 h with roscovitine respectively; S.E.M. = 2.1).

#### Discussion

Numerous studies have described the effectiveness of the R-enantiomer of roscovitine to meiotically inhibit bovine oocytes (Mermillod *et al.* 2000, Kasinathan *et al.* 2001, Lagutina *et al.* 2002, Ponderato *et al.* 2002, Vigneron *et al.* 2003). However, reported differences in the potency of the R- versus the S-enantiomer of roscovitine to inhibit p34<sup>cdc2</sup> kinase activity (De Azevedo *et al.* 1997, Havlicek *et al.* 1997, reviewed by Meijer & Raymond 2003) along with recent unpublished observations (L Meijer, unpublished observations) showing increased selectivity towards other protein kinases, warrant further investigation of the S-enantiomer of roscovitine.

Results showed that 50 µmol/l S-roscovitine inhibited gonadotropin-induced cumulus expansion and maintained >90% of bovine oocytes from antral follicles at the GV stage for 48 h without compromising nuclear maturation, fertilization or early embryo development. Static culture with roscovitine for longer time-periods (66 h) was not without consequence, however, as oocyte development was compromised. Furthermore, culture of bovine oocytes

**Table 5** Embryo development after oocyte culture in S-roscovitine for 0, 24 or 48 h.

| Time in roscovitine (h) | No. | Cleaved (%)       | 8–16 cell (% of PZ) | 8–16 cell (% of cleaved) | Blastocysts (% of PZ) | Blastocysts (% of cleaved) |
|-------------------------|-----|-------------------|---------------------|--------------------------|-----------------------|----------------------------|
| 0                       | 363 | 55.8 <sup>a</sup> | 49.6 <sup>a</sup>   | 89.6                     | 18.4 <sup>a</sup>     | 33.1                       |
| 24                      | 275 | 81.4 <sup>b</sup> | 73.8 <sup>b</sup>   | 91.1                     | 31.1 <sup>b</sup>     | 38.1                       |
| 48                      | 348 | 61.0 <sup>a</sup> | 55.9 <sup>a</sup>   | 91.5                     | 17.6 <sup>a</sup>     | 29.6                       |
| S.E.M.                  | —   | 6.1               | 5.6                 | 2.7                      | 3.0                   | 3.1                        |
| P value                 | —   | 0.04              | 0.04                | 0.87                     | 0.02                  | 0.11                       |

PZ, putative zygote.

<sup>a,b</sup>Differ within column.

with S-roscovitine for 24 h was coincident with higher cleavage and blastocyst development.

The studies described are the first of which we are aware to characterize the time-period that bovine oocytes may be maintained at the GV stage in a static culture with roscovitine. The findings are significant as delineation of the time-period that bovine oocytes can be meiotically inhibited, without compromising continued development, is a necessary step towards refinement of two-step culture systems aimed at allowing additional time *in vitro* for the oocyte to acquire full developmental competence. Reduced development after culture of oocytes in 50 µmol/l S-roscovitine for 66 h (fewer oocytes underwent nuclear maturation) was likely multifactorial. For instance, nutrients become depleted during static culture and roscovitine activity may have diminished over time, similar to other meiotic inhibitors (Farin & Yang 1994).

The disparity of the results described (i.e. oocyte development was improved after roscovitine culture for 24 h) versus those reported by others (comparable development for control and roscovitine-cultured oocytes; Mermillod *et al.* 2000, Lagutina *et al.* 2002, Ponderato *et al.* 2002) is not necessarily related to the use of a different enantiomer as additional efforts by Edwards' laboratory have not shown improvements in oocyte development after culture for 24 h with S-roscovitine. The absence of a beneficial effect of roscovitine to increase oocyte development (Payton *et al.* 2004) was coincident with improved development of untreated controls (29.7% of oocytes developed to blastocyst) and omission of specific constituents comprising the roscovitine-containing medium. The roscovitine-containing medium used in our studies was M199 containing 10% FBS, 1 × nucleosides, 2 mmol/l L-glutamine, 1 × non-essential amino acids, 0.1 mmol/l β-mercaptoethanol, 50 U/ml penicillin and 50 µg/ml streptomycin. Even though M199 does not contain asparagine or cytidine, Payton *et al.* (2004) omitted 1 × nucleosides and 1 × non-essential amino acids because of concerns about potential carryover effects to alter the resumption of meiosis (Downs 2000). Certain culture medium constituents have been shown to improve the development of meiotically inhibited oocytes (Downs & Mastropolo 1997). Taken together, the observations imply that components important for DNA, RNA, protein synthesis and/or differentiation may be limiting. This may explain in

part why supplementation of inhibition media with hormones and growth factors does not necessarily improve oocyte development (Ponderato *et al.* 2002).

Premature translocation of the cortical granules to the oolemma during roscovitine culture for 48 h in our study raised concerns as this result suggests that not all of the events associated with resumption of meiosis are inhibited during prolonged culture. The effects of roscovitine culture for shorter time-periods include altered kinetics of nuclear maturation (i.e. GV breakdown, extrusion of the first polar body and progression to MII occurred 4–6 h earlier; Calder *et al.* 2001, Marchal *et al.* 2001, Lagutina *et al.* 2002) and slight changes in nuclear ultrastructure (roscovitine culture for 8 h in M199 containing 0.8% (w/v) BSA; Faerge *et al.* 2001). However, the significance of these effects remain unclear as fertilization, continued development to blastocyst (Mermillod *et al.* 2000, Duque *et al.* 2002, Lagutina *et al.* 2002; after 48 h in this study), and establishment and maintenance of pregnancy up to 120 days (Kasinathan *et al.* 2001) are at least comparable with untreated controls. Certainly, higher doses are likely to induce irreparable damage given that 125 µmol/l roscovitine (M199 + 3 mg/ml BSA) disrupted the integrity and expansion of surrounding cumulus cells, and induced swelling of mitochondrial cristae and cortical granule degeneration (Lonergan *et al.* 2003).

The solubility of the ZP was examined after prolonged culture of oocytes in roscovitine as aging murine oocytes *in vitro* induces ZP hardening (Gianfortoni & Gulyas 1985). In our study, roscovitine culture for 48 h did not affect the solubility of the ZP to pronase which has been used as an indirect measure of ZP hardening (Hosoe & Shioya 1997). Dissolution times were consistent with those reported previously (Hosoe & Shioya 1997). These results corroborate the idea that events occurring after IVF (i.e. the zona reaction) continue to differ from those occurring *in vivo* (Coy *et al.* 2002).

The use of 50 µmol/l S-roscovitine was necessary to inhibit gonadotropin-induced cumulus expansion. The disparity in the dose of roscovitine utilized by others (Mermillod *et al.* 2000, Calder *et al.* 2001, Duque *et al.* 2002, Lagutina *et al.* 2002, Vigneron *et al.* 2003) versus that required in our study may be due to differences in roscovitine activity related to source, use of a different enantiomer, differences in the composition of the roscovitine

medium or other unidentified factors. Dr Laurent Meijer has provided the roscovitine used in the majority of available studies (Mermillod *et al.* 2000, Lagutina *et al.* 2002, Lonergan *et al.* 2003, Vigneron *et al.* 2003). In contrast, we utilized a commercial source of the (S)-enantiomer. Differences in the R- versus the S-enantiomer to inhibit CDK activity have been described ( $IC_{50}$  = 650 and 800 nM respectively; Havlicek *et al.* 1997). In particular, De Azevedo *et al.* (1997) showed that the R-enantiomer of roscovitine was almost twice as potent at inhibiting cdc2/cyclin B kinase activity compared with the S-enantiomer. It is also important to note that the roscovitine medium used in our study was supplemented with 10% (v/v) FBS. Calder *et al.* (2001) reported that a lower dose of roscovitine could be used when the medium was supplemented with BSA versus serum.

The effectiveness of R-roscovitine to inhibit epidermal-growth factor (EGF)-induced cumulus expansion (Mermillod *et al.* 2000) in bovine oocytes has been previously described. Because roscovitine has also been shown to inhibit MAP kinases (Krischek & Meinecke 2001, Marchal *et al.* 2001), it is plausible that inhibition of EGF or gonadotropin-induced cumulus expansion may be the result of roscovitine-inhibited EGF (Keel *et al.* 1995) or FSH-dependent (Su *et al.* 2001) MAP kinase activity. An alternative hypothesis, however, is that roscovitine may prevent cumulus expansion by inhibiting gonadotropin-induced increases in transcription. In other cell types, dose-dependent reductions (5–50  $\mu$ mol/l) in  $^3$ H-uridine incorporation (Ljungman & Paulsen 2001) have been noted even when the effects of R-roscovitine to reduce nucleoside transport (Huang *et al.* 2003) were corrected (Sankrithi & Eskin 1999). Roscovitine-induced alterations in transcription have also been noted using DNA microarrays (Lam *et al.* 2001) and ribonuclease protection assays (Sankrithi & Eskin 1999). Most recently, Vigneron *et al.* (2003) reported global reductions in cox-2 mRNA after culture of bovine oocytes with 25  $\mu$ mol/l R-roscovitine. The reported effects of roscovitine to alter transcription warrants further investigation before implementation as the first of a two-step culture system designed to allow additional time *in vitro* for transcriptional-mediated events presumed necessary for differentiation.

A concern was raised during the review of this paper about potential contamination of our cultures with R-roscovitine as a result of enantiomeric interconversion. As there are no available studies to validate this concern, Dr Laurent Meijer (Meijer & Raymond 2003) and two additional organic chemists were consulted. The first possible means of contamination may occur during chemical synthesis of S-roscovitine. However, the potential for this is minimal, if at all (L Meijer, personal communication). Even so, because the biochemical effects of the S- versus the R-enantiomers are similar (Meijer & Raymond 2003) trace amounts of R-roscovitine during culture may not be biologically detectable. A second possibility for contamination of our cultures with R-roscovitine would be interconversion

(S to R) yet this is also highly unlikely because of the chemical stability of the roscovitine enantiomers (L Meijer & D Baker, personal communications). Enzyme-mediated interconversion after uptake by the COC cannot be ruled out; however, there is no available literature to support this concept. It should be noted that our experiments required twice as much S-roscovitine to effectively inhibit cumulus expansion when compared with that reported for the R-enantiomer (Mermillod *et al.* 2000). This agrees with De Azevedo *et al.* (1997) who showed that R-roscovitine was almost twice as potent as the S-enantiomer and would support the predominance of the S-enantiomer in our culture system.

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## References

- Avery B, Hay-Schmidt A, Hyttel P & Greve T 1998 Embryo development, oocyte morphology, and kinetics of meiotic maturation in bovine oocytes exposed to 6-dimethylaminopurine prior to *in vitro* maturation. *Molecular Reproduction and Development* **50** 334–344.
- Biggers JD, McGinnis LK & Raffin M 2000 Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biology of Reproduction* **63** 281–293.
- Calder MD, Antonello LL & Watson AJ 2001 Roscovitine-induced inhibition of bovine oocyte maturation is enhanced in BSA-supplemented media and progression to MII is accelerated following release from inhibition. *Biology of Reproduction* **64** 118 (Abstract).
- Coy P, Gadea J, Romar R, Matas C & Garcia E 2002 Effect of *in vitro* fertilization medium on the acrosome reaction, cortical reaction, zona pellucida hardening and *in vitro* development in pigs. *Reproduction* **124** 279–288.

- Coy P, Canovas S, Ruiz S, Matas C, Gadea J, Garcia E, Sansegundo M, Garcia F, Gumbao D & Romar R 2004 First pregnancies after transferring of *in vitro* produced pig embryos pre-cultured with roscovitine. *Human Reproduction* **19** 41–42 (Abstract).
- De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M & Kim SH 1997 Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. *European Journal of Biochemistry* **243** 518–526.
- Dode MAN & Adona PR 2001 Developmental capacity of *Bos indicus* oocytes after inhibition of meiotic resumption by 6-dimethylaminopurine. *Theriogenology* **65** 171–180.
- Downs SM 2000 Adenosine blocks hormone-induced meiotic maturation by suppressing purine *de novo* synthesis. *Molecular Reproduction and Development* **56** 172–179.
- Downs SM & Mastropolo AM 1997 Culture conditions affect meiotic regulation in cumulus cell-enclosed mouse oocytes. *Molecular Reproduction and Development* **46** 551–566.
- Duque P, Diez C, Royo L, Lorenzo PL, Carneiro G, Hidalgo CO, Facal N & Gomez E 2002 Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. *Human Reproduction* **17** 2706–2714.
- Faerge I, Mayes M, Hyttel P & Sirard MA 2001 Nuclear ultrastructure in bovine oocytes after inhibition of meiosis by chemical and biological inhibitors. *Molecular Reproduction and Development* **62** 375–386.
- Farin CE & Yang L 1994 Inhibition of germinal vesicle breakdown in bovine oocytes by 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB). *Molecular Reproduction and Development* **37** 284–292.
- Gianfortoni JG & Gulyas BJ 1985 The effects of short-term incubation (aging) of mouse oocytes on *in vitro* fertilization, zona solubility, and embryonic development. *Gamete Research* **11** 59–68.
- Hagemann LJ, Beaumont SE, Berg M, Donnison MJ, Ledgard A, Peterson AJ, Schurmann A & Tervit HR 1999 Development during single IVP of bovine oocytes from dissected follicles: interactive effects of estrous cycle stage, follicle size and atresia. *Molecular Reproduction and Development* **53** 451–458.
- Havlicek L, Hanus J, Vesely J, Leclerc S, Meijer L, Shaw G & Strnad M 1997 Cytokinin-derived cyclin-dependent kinase inhibitors: synthesis and cdc2 inhibitory activity of olomoucine and related compounds. *Journal of Medical Chemistry* **40** 408–412.
- Hosoe M & Shioya Y 1997 Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote* **5** 371–376.
- Huang M, Wang Y, Cogut SB, Mitchell BS & Graves LM 2003 Inhibition of nucleoside transport by protein kinase inhibitors. *Journal of Pharmacology and Experimental Therapeutics* **304** 753–760.
- Kasinathan P, Knott JG, Spell AR, King TL & Robl JM 2001 Effect of roscovitine delayed maturation on development of nuclear transplant embryos. *Theriogenology* **55** 273 (Abstract).
- Keel BA, Hildebrandt JM, May JV & Davis JS 1995 Effects of epidermal growth factor on the tyrosine phosphorylation of mitogen-activated protein kinases in monolayer cultures of porcine granulosa cells. *Endocrinology* **136** 1197–1204.
- Krischek C & Meinecke B 2001 Roscovitine, a specific inhibitor of cyclin-dependent protein kinases, reversibly inhibits chromatin condensation during *in vitro* maturation of porcine oocytes. *Zygote* **9** 309–316.
- Lagutina I, Ponderato N, Lazzari G & Galli C 2002 Kinetics of oocyte maturation and subsequent development of IVF, parthenogenetic, and NT bovine embryos after meiotic inhibition with roscovitine. *Cloning Stem Cells* **4** 113–119.
- Lam LT, Pickeral OK, Peng AC, Rosenwald A, Hurt EM, Giltane JM, Averett LM, Zhao H, Davis RE, Sathyamoorthy M *et al.* 2001 Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. *Genome Biology Research* **2** 0041.1–0041.11.
- Lawrence JL, Payton RR, Godkin JD, Saxton AM, Schrick FN & Edwards JL 2004 Retinol improves development of bovine oocytes compromised by heat stress during maturation. *Journal of Dairy Science* **87** 2449–2454.
- Ljungman M & Paulsen MT 2001 The cyclin-dependent kinase inhibitor roscovitine inhibits RNA synthesis and triggers nuclear accumulation of p53 that is unmodified at Ser15 and Lys382. *Molecular Pharmacology* **60** 785–789.
- Lonergan P, Khatir H, Carolan C & Mermillod P 1997 Bovine blastocyst production *in vitro* after inhibition of oocyte meiotic resumption for 24 h. *Journal of Reproduction and Fertility* **109** 355–365.
- Lonergan P, Faerge I, Hyttel PM, Boland M & Fair T 2003 Ultrastructural modifications in bovine oocytes maintained in meiotic arrest *in vitro* using roscovitine or butyrolactone. *Molecular Reproduction and Development* **64** 369–378.
- Long CR, Damiani P, Pinto-Correia C, MacLean RA, Duby RT & Robl JM 1994 Morphology and subsequent development in culture of bovine oocytes matured *in vitro* under various conditions of fertilization. *Journal of Reproduction and Fertility* **102** 361–369.
- Marchal R, Tomanek M, Terqui M & Mermillod P 2001 Effects of cell cycle dependent kinases inhibitor on nuclear and cytoplasmic maturation of porcine oocytes. *Molecular Reproduction and Development* **60** 65–73.
- Meijer L & Raymond E 2003 Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. *Accounts of Chemical Research* **36** 417–425.
- Mermillod P, Tomanek M, Marchal R & Meijer L 2000 High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 hours in culture by specific inhibition of MPF kinase activity. *Molecular Reproduction and Development* **55** 89–95.
- Motlik J, Pavlok A, Lapathitis G & Kubelka M 2000 Impact of two-step *in vitro* culture systems on developmental potency of oocytes. *Reproduction in Domestic Animals* **35** 267–271.
- Parrish JJ, Susko-Parrish J, Winer MA & First NL 1988 Capacitation of bovine sperm by heparin. *Biology of Reproduction* **38** 1171–1180.
- Payton RR, Romar R, Coy P, Saxton AM, Lawrence JL & Edwards JL 2004 Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress *in vitro*. *Biology of Reproduction* **71** 1303–1308.
- Pincus G & Enzmann EV 1935 The comparative behavior of mammalian eggs *in vivo* and *in vitro*. I. The activation of ovarian eggs. *Journal of Experimental Medicine* **62** 665–675.
- Ponderato N, Crotti G, Turini P, Duchi R, Galli C & Lazzari G 2002 Embryonic and foetal development of bovine oocytes treated with a combination of butyrolactone I and roscovitine in an enriched medium prior to IVF and IVF. *Molecular Reproduction and Development* **62** 513–518.
- Saeki K, Nagao Y, Kishi M & Nagai M 1997 Developmental capacity of bovine oocytes following inhibition of meiotic resumption by cycloheximide or 6-dimethylaminopurine. *Theriogenology* **48** 1161–1172.
- Sankrithi N & Eskin A 1999 Effects of cyclin-dependent kinase inhibitors on transcription and ocular circadian rhythm of Aplysia. *Journal of Neurochemistry* **72** 605–613.
- SAS Inc., 2001 In *SAS/STAT User's Guide*, Cary, NC: SAS Institute.
- Su Y-Q, Rubinstein S, Luria A, Lax Y & Breitbart H 2001 Involvement of MEK-mitogen-activated protein kinase pathway in follicle-stimulating hormone-induced but not spontaneous meiotic resumption of mouse oocytes. *Biology of Reproduction* **65** 358–365.
- Vigneron C, Nuttinck F, Perreau C, Reinaud P, Charpigny G & Mermillod P 2003 Effect of roscovitine, a cdk1 inhibitor, and of the presence of oocyte on bovine cumulus cell expansion and cyclooxygenase-2 expression. *Molecular Reproduction and Development* **65** 114–121.

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