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In vitro maturation and fertilization of porcine oocytes after a 48 h culture in roscovitine, an inhibitor of p34^{cdc2}/cyclin B kinase

Raquel Romar [a,b], Hiroaki Funahashi [a,*]

[a] *Department of Animal Science and Technology, The Graduate School of Natural Science and Technology, Okayama University, Tsushima-naka, Okayama 700-8530, Japan*

[b] *Department of Physiology (Veterinary Physiology), Faculty of Veterinary Medicine, Murcia University, 30071 Murcia, Spain*

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Abstract

Maintaining oocytes at the germinal vesicle (GV) stage in vitro may permit enhanced acquisition of the developmental competence. The objective of the current study was to evaluate the nuclear and cytoplasmic maturation in vitro of porcine oocytes after pretreatment with *S*-roscovitine (ROS). Cumulus oocyte complexes (COC) were treated with 50 μM ROS for 48 h and then matured for various lengths of time in a conventional step-wise in vitro maturation (IVM) system by using dibutyryl cyclic AMP. The COC that were matured in the same system for 44 h without pretreatment with ROS were used as the control group. At various periods after the start of IVM, oocytes were assessed for the meiotic stages and subjected to in vitro fertilization (IVF) with fresh spermatozoa. The ROS treatment inhibited GV breakdown of 94.4% oocytes, with the majority arrested at the GV-I stage (67.4%). Maximum maturation rate to the metaphase-II stage after ROS treatment was achieved by 44 h of IVM (92.1%) and no differences were observed with control oocytes (95.0%). Penetration rate was correlated to the maturation rate. The duration of IVM had no effects on polyspermy and male pronuclear (MPN) formation rates at 8 h post insemination (hpi), whereas both rates increased at 22 hpi. Direct comparison with controls assessed at 22 hpi confirmed a lesser MPN formation in ROS-treated oocytes (73.7% compared with 53.6%). Glutathione (GSH) concentrations were less in oocytes treated with ROS than in control oocytes (5 compared with 7.7 pmol/oocyte) as well as blastocyst rate (22.0% compared with 38.1%, respectively). These results demonstrate that cytoplasmic maturation

* Corresponding author. Tel.: +81 86 251 8329; fax: +81 86 251 8388.
E-mail address: hirofun@cc.okayama-u.ac.jp (H. Funahashi).

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in porcine oocytes pretreated with ROS for 48 h did not equal that of control oocytes in the current IVM system.

Keywords: Pig–porcine; Roscovitine; In vitro maturation; In vitro fertilization; Glutathione

1. Introduction

For in vitro production of porcine embryos, the cumulus-oocyte complexes (COC) are collected from ovarian follicles that are 3–6 mm in diameter, meaning that the COC have not undergone the stage of dominant follicular growth before ovulation. A lesser developmental competence of in vitro matured oocytes following in vitro fertilization (IVF) appears to be due to a heterogeneous population of oocytes at the start of in vitro maturation (IVM) and/or the absence of the final phase of oocyte growth (Funahashi and Day, 1997; Coy and Romar, 2002). Temporal inhibition of meiotic resumption by using dibutyryl cyclicAMP in a step-wise IVM system has been successfully used to synchronize meiotic progress of porcine oocytes and to increase the developmental competence after IVM–IVF (Funahashi et al., 1997). For that reason, this step-wise IVM system has been widely adopted by many researchers and regularly employed for in vitro production of porcine embryos (reviewed by Abeydeera, 2001; Coy and Romar, 2002). There are other chemicals that have been known to reversibly maintain porcine oocytes at the germinal vesicle (GV) stage. Especially, in the presence of cyclin dependent kinase (cdk) inhibitors, such as butyrolactone-I at 50 μM (Le Beux et al., 2003) and roscovitine (Krischek and Meinecke, 2001; Marchal et al., 2001 at 50 and 25 μM, respectively), it has been suspected that transcripts and proteins are synthesized and stored in a stable form during the inhibitory phase (Mermillod et al., 2000). This may permit accumulation or storage of mRNAs and proteins essential for the acquisition of developmental competence in the oocyte and consequently may improve the oocyte quality associated with cytoplasmic maturation. With this hypothesis, effect of roscovitine on nuclear maturation of porcine oocytes has been widely studied and shown to be effective to maintain the meiotic arrest in a reversible manner when used at 50 μM for 44 h in oocytes from 2 to 3.5 mm follicle (Schoevers et al., 2005); and for at least 44–48 h in follicles from 3 to 6 mm in diameter (50 μM, Krischek and Meinecke, 2001; 25 μM, Marchal et al., 2001; 80 μM, Ju et al., 2003). However, some controversy still remains as to whether oocyte nuclear kinetics is accelerated (Marchal et al., 2001) or delayed (Krischek and Meinecke, 2001) when oocytes are matured after chemical removal and there have been no studies that have focused on the most suitable IVM period for roscovitine-treated oocytes. Unfortunately, the further employment of these inhibited oocytes for IVF has not been an area of emphasis and only porcine oocytes treated with 25 μM roscovitine for 22 h have been used for IVF (Marchal et al., 2001) and successful piglet production (50 μM, Coy et al., 2005b). There are no data assessing possible beneficial/detrimental effects of longer treatment of oocytes with roscovitine on IVF. Furthermore, 125 μM roscovitine for 24 h caused degeneration of the bovine cortical granules (Lonergan et al., 2003) and some extended cytoskeleton alterations in porcine oocytes at 80 μM for 44 h (Ju et al., 2003). Intracellular glutathione (GSH) content and the male pronuclear (MPN) formation

are variables that are widely used to assess porcine cytoplasmic maturation (Funahashi et al., 1995; Coy et al., 1999) but information about how roscovitine may affect these variables in porcine oocytes is not available.

Therefore, the objective in the present study was to assess the nuclear maturation and IVF of oocytes after treatment with roscovitine for 48 h. With a suitable maturation period, the effect of roscovitine arrest on cytoplasmic maturation, as determined by MPN formation, intracellular GSH content and further embryo development, was also examined.

2. Material and methods

2.1. Culture media

The medium used for COC collection and washing was modified Tyrode lactate–Hepes–polyvinyl alcohol (TL–HEPES–PVA) medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO3, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2{\cdot}6H_2O$, 2 mM $CaCl_2{\cdot}2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 μg/ml gentamicin and 65 μg/ml potassium penicillin G (Funahashi et al., 1997). The basic maturation medium used was BSA-free North Carolina State University 37 medium (Petters and Wells, 1993) supplemented with 0.6 mM cysteine, 5 μg/ml insulin and 10% (v/v) porcine follicular fluid (OMM37; Funahashi et al., 1997). The basic medium for IVF was modified Medium199 (mM199), which was Medium199 with Earle's salts (GIBCO; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 3.05 mM glucose, 2.92 mM hemi-calcium lactate, 0.91 mM sodium pyruvate, 12 mM sorbitol, 25 μg/ml gentamicin, 65 μg/ml potassium penicillin G, and 0.4% (w/v) BSA (Sigma; A4378). The medium used as semen diluent was modified Modena solution composed of 152.64 mM glucose, 23.46 mM sodium citrate, 11.9 mM $NaHCO_3$, 6.99 mM EDTA-2Na, 46.66 mM Tris, 15.10 mM citric acid, and 10 mg/l gentamicin sulfate. All media without modified TL–HEPES–PVA and modified Modena solution were equilibrated at 39 °C in an atmosphere of 5% CO_2 in air overnight prior to incubation with gametes. Porcine follicular fluid was prepared from antral follicles (3–6 mm in diameter) as described previously (Funahashi et al., 1994a).

2.2. Collection of cumulus oocyte complexes

Ovaries were collected from prepubertal gilts at a local abattoir. The time elapsed from animal slaughter to oocyte recovery did not exceed 4 h. The COC were aspirated from antral follicles (3–6 mm diameter) with an 18-gauge needle attached to a 10-ml disposable syringe. After pooling follicular contents in a 50-ml conical tube, supernatant was replaced three times in modified TL–HEPES–PVA medium at room temperature. The COC with uniform ooplasm and a compact cumulus cell mass were collected for experiments.

2.3. Roscovitine treatment and in vitro maturation

The roscovitine [2-(*S*)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] (Calbiochem, La Jolla, CA, USA) was solubilized in dimethyl sulfoxide (DMSO)

before freezing at −20 °C as a 10 mM stock as previously described without observing problems in DMSO concentration (Payton et al., 2004; Coy et al., 2005a). Inhibitory medium consisted of OMM37 with a final concentration of 50 μM roscovitine based on previous studies working with same enantiomer of this drug (Coy et al., 2005a). Fifty to sixty COC were cultured in 500 μl medium containing roscovitine (but not covered with paraffin oil) for 48 h at 39 °C in an atmosphere of 5% CO_2 in air. After the treatment, COC were washed extensively three times in roscovitine-free OMM37 and allocated in a conventional IVM system.

For IVM, groups of 50–60 COC were cultured in 500 μL of OMM37 supplemented with 1 mM dibutyryl cAMP, 10 IU/ml eCG and 10 IU/ml hCG covered with paraffin oil at 39 °C in an atmosphere of 5% CO_2 in air. At 22 h of culture, COC were washed three times with fresh OMM37 (dibutyryl cyclicAMP- and gonadotropins-free) and continued to culture until the end of the desired period of IVM (Funahashi et al., 1997). After culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and used for further treatments or fixation.

Meiotic progress of some oocytes were determined at 200× and 400× magnification following fixation (3:1, ethanol:acetic) for 24 h and staining with 1% (w/v) orcein. Oocytes were classified according to morphological criteria for characterization of meiotic stages by Ye et al. (2002). Briefly, oocytes were scored as GV-I (distinct nuclear envelop, a nucleolus, nucleoplasm of a finer texture than ooplasm and chromatin only stained around the nucleolus); GV-II (a ring of chromatin around nucleolus as GV-I, but with two to eight well-stained spots); GV-III (an irregular network of filamentous bivalents and no nucleolus); GV-IV (a cluster of chromatin with a less distinctive nuclear membrane); germinal vesicle breakdown (GVBD; included diakinesis and prometaphase I; no visible nuclear membrane); metaphase I (Met-I; orderly arrangement of the chromosomes on the equator of spindle); anaphase I (Ana-I; distinctive spindle with the chromosomes migrating towards the poles); telophase I (distinctive spindle with the chromosomes reaching two polar regions of the spindle); and metaphase II (Met-II; configuration of chromosomes as in Met I, but with first polar body) stages.

2.4. Preparation of boar spermatozoa and in vitro fertilization

Sperm rich fractions (30–50 ml) were collected by the gloved-hand method from a Birkshire boar, diluted four times with modified Modena solution and kept in darkness at room temperature until use within 24 h. Immediately preceding initiation of the IVF process, spermatozoa were washed three times in a 15-ml centrifuge tube with modified TL–HEPES–PVA by centrifugation at 750 × *g* for 3 min. The pellet was resuspended in caffeine-free mM199 and diluted to 1×10^6 cells/ml with the same medium. Oocytes were fertilized in an IVF system that has been reported to improve monospermic penetration in porcine oocytes (Funahashi and Romar, 2004). Briefly, 50 μl of the diluted sperm suspension were inseminated in the same volume droplet of mM199 containing 10 mM caffeine (final sperm concentration 5×10^5 cells/ml in mM199 containing 5 mM caffeine) with 30–35 denuded oocytes under paraffin oil. Gametes were co-cultured for 10 min at 39 °C in an atmosphere of 5% CO_2 in air. Then, oocytes with attached spermatozoa were passed trough caffeine-free mM199 one time and transferred to a 100-μl droplet of the same medium

under paraffin oil. Depending on the experimental requirements, at 8 and/or 22 h hpi, the cultured eggs were fixed, stained with 1% (w/v) orcein, and examined at 200× and 400× magnification (Funahashi and Nagai, 2001). Oocytes were designated as penetrated when they had at least one sperm head, a decondensed sperm nucleus, or a male pronucleus with corresponding sperm tail in the vitellus.

2.5. Assay of glutathione content

Intracellular content of glutathione was measured as described previously (Funahashi et al., 1994b). Briefly, COC were denuded by pipetting in modified TL–HEPES–PVA medium and washed three times in a buffer solution. Five microliters of buffer containing 30 denuded oocytes were transferred to a 1.5-ml microfuge tube and 5 μl of 1.25 M phosphoric acid added. Samples were frozen immediately (−80 °C) and kept in the freezer until assayed. The GSH content in the oocytes was determined by the DTNB-GSSG reductase recycling assay (Anderson, 1985). The total amount of GSH measured was divided by the number of oocytes in the sample to obtain the content per oocyte (pmol/oocyte).

2.6. Experimental design and statistical analysis

Experiment 1 was designed to determine a suitable IVM period for oocytes treated with roscovitine for 48 h. Nuclear progression, sperm penetration and MPN formation were assessed after a conventional porcine IVM/IVF system. To make this assessment, COC were treated with 50 μM roscovitine for 48 h and then subjected to a step-wise IVM program. Immediately after roscovitine treatment (ROS group) and at 22 h (ROS-IVM22 group), 30 h (ROS-IVM30 group), 38 h (ROS-IVM38 group) and 44 h (ROS-IVM44 group) of IVM culture, an aliquot of oocytes was processed to assess the meiotic stage and the other oocytes were subsequently inseminated. Oocytes cultured for 44 h in the same step-wise IVM system, but without roscovitine pretreatment, were utilized as the experimental control (IVM44 group). At 8 hpi, oocytes from the different groups were assessed for sperm penetration and MPN formation. Oocytes from ROS-IVM44 h group were divided with half being fixed 8 hpi and the other half 22 hpi. The experiment was replicated four times.

After the optimal IVM period was chosen, three additional experiments were conducted. In the second experiment, the effect of roscovitine pretreatment for 48 h on further IVF was examined in a direct comparison with non-treated controls by assessing sperm penetration and MPN formation results at 22 hpi. As described previously, oocytes were treated with roscovitine and cultured in a step-wise IVM system for 44 h (ROS-IVM44 group). Another batch of COC were cultured in the same IVM system for 44 h without roscovitine-pretreatment and used as controls (IVM44 group). This allowed for oocytes from both groups to be inseminated under the same conditions (5×10^5 cells/ml) avoiding fertilization variability. Four replicates of this experiment were conducted.

In experiment 3, the effect of roscovitine treatment on intracellular GSH content was evaluated. Immediately after collection, an aliquot of oocytes was taken to determine GSH (before culture group). From the remaining COC, one third was cultured in a step-wise IVM system for 44 h and subsequently processed for GSH content (IVM44 group). The other

COC were subjected to pretreatment with 50 μM roscovitine for 48 h. After the pretreatment processes, one group of COC was assessed for GSH (ROS group) and culture in the IVM system was continued for 44 h with the other group and subsequently processed for GSH content (ROS-IVM44 group). This experiment was replicated six times.

In experiment 4, the effect of roscovitine treatment on early embryo development was studied. Oocytes matured for 44 h with (ROS group) or without (control group) pretreatment of 48 h with 50 μM were fertilized as described in experiment 2. At 8 h after insemination, the presumptive zygotes from both groups were moved to modified NCSU-37 medium supplemented with 0.4% (w/v) BSA, 0.6 mM cysteine and 5 μg/ml insulin and cultured in an atmosphere of 5% CO_2 in air for 7 days. Forty eight hours after insemination, cleavage was evaluated under a stereomicroscope and the number of two- to four-cell stage embryos was recorded (cleaved embryos). Blastocysts were assessed on day 7 by observation of a clear blastocoele under the stereomicroscope. There were four replicates conducted in this experiment.

2.7. Statistical analysis

Data are presented as mean ± S.E.M. Data for all rates were modeled according to the binomial model of variables. The variables in all the experiments were analyzed by one-way ANOVA. These variables were, in experiment 1 – the nuclear stage (GV-I to Met II); in experiment 2, variables were – penetration rate, number of sperm cells per penetrated oocyte, male pronuclear formation and monospermy rate; in experiment 3, the variable was – GSH content; and in experiment 4, variables were – cleavage and blastocyst rates. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Effect of IVM duration after roscovitine treatment on further nuclear progression, sperm penetration and male pronuclear formation (experiment 1)

Immediately after roscovitine treatment, oocytes had a compact cumulus cells mass without signs of expansion and nuclear staining. There were 94.4% of the oocytes arrested at the GV stage (Table 1), mainly exhibiting a nucleus without any signs of chromatin condensation (GV-I; 67.4 ± 5.0%) or with two to eight well-stained spots (GV-II; 15.7 ± 3.9%). After the second half of IVM (after removing dibutyryl cyclic AMP and gonadotropins from IVM media), oocytes from all groups resumed meiosis but a significant effect of IVM duration was observed for all nuclear stages except for the GV-IV, GVBD and Ana-I. The greatest rate of nuclearly matured oocytes that developed to the Met-II stage was achieved when cultured in a step-wise IVM system for 44 h after pretreatment with ROS (92.1 ± 2.9%) and no differences were found with control oocytes (88.6 ± 2.4%). No oocytes from ROS-IVM22 group reached the Met-II stage of development and only 18.9 ± 4.2% did in the ROS-IVM30 group, although this rate increased up to 65.9 ± 5.0% in oocytes cultured for 38 h after ROS treatment. After IVM, cumulus cells of roscovitine-treated COC did not

Table 1
Nuclear progression of porcine oocytes subjected to meiotic inhibition with 50 μM roscovitine for 48 h (ROS) and subsequently cultured in a step-wise IVM system for 22 h (ROS-IVM 22), 30 h (ROS-IVM 30), 38 (ROS-IVM 38) and 44 h (ROS-IVM 44)

	ROS $N=89$	ROS-IVM22 $N=92$	ROS-IVM30 $N=90$	ROS-IVM38 $N=91$	ROS-IVM44 $N=88$	IVM44 $N=161$
GV-I	67.4 ± 5.0^{a}	10.9 ± 3.3^{b}	0^{c}	0^{c}	0^{c}	0^{c}
GV-II	15.7 ± 3.9^{a}	62.0 ± 5.1^{b}	1.11 ± 1.11^{c}	0^{c}	0^{c}	0^{c}
GV-III	7.9 ± 2.9^{a}	20.7 ± 4.2^{b}	2.2 ± 1.6^{a}	0^{a}	2.3 ± 1.6^{a}	0^{a}
GV-IV	3.4 ± 1.9	0	5.6 ± 2.4	3.3 ± 1.9	0	1.5 ± 0.9
GVBD	5.6 ± 2.4	5.4 ± 2.4	2.2 ± 1.56	0	1.1 ± 1.1	1.4 ± 0.8
Met-I	0^{a}	1.1 ± 1.1^{a}	68.9 ± 4.9^{b}	18.7 ± 4.1^{c}	2.3 ± 1.6^{a}	8.4 ± 1.5^{a}
Ana-I	0	0	0	0	2.3 ± 1.6	0
Tel-I	0^{a}	0^{a}	1.1 ± 1.1^{a}	12.1 ± 3.4^{b}	0^{a}	0^{a}
Met-II	0^{a}	0^{a}	18.9 ± 4.2^{b}	$65.9 + 5.0^{c}$	92.1 ± 2.9^{d}	88.6 ± 2.4^{d}

GV, germinal vesicle; GVBD, germinal vesicle breakdown; Met, metaphase; Ana, anaphase; and Tel, telophase. *N*, number of oocytes. Percentages with different superscripts (a, b, c, d) within rows are significantly different ($P<0.01$).

show as full an expansion as those from the control group and cumulus cells appeared to be darker (data not shown).

As shown in data included in Table 2, IVF assessment at 8 hpi indicated that there had been an increase in penetrability as the IVM period progressed. Oocytes from ROS-IVM44 group had the greatest penetration rate ($59.2 \pm 3.9\%$) and the mean number of spermatozoa in a penetrated oocyte (1.3 ± 0.1 cells). No effects of IVM duration were observed on male pronuclear formation (ranged from 0 to 15%) and monospermy rates (ranging from 82.8 to 100%).

When sperm penetration and MPN formation of oocytes from ROS-IVM44 group were assessed at 22 hpi a similar penetration rate and mean number of sperm per oocyte was observed, but rates of male pronuclear formation and polyspermy was significantly increased (Table 2).

Table 2
Sperm penetration and male pronuclear (MPN) formation at 8 hpi in porcine oocytes pretreated in 50 μM roscovitine for 48 h before IVM for different periods

Treatment	*N*	Percent penetrated	Number of sperm/ penetrated oocyte	Percent MPN formation[a]	Percent monospermy[a]
ROS-IVM22	155	12.2 ± 2.6 a (19/155)	1.0 a	0 (0/19)	100 (19/19)
ROS-IVM30	156	18.6 ± 3.1 ab (29/156)	1.0 ± 0.1 a	3.5 ± 3.5 a (1/29)	96.6 ± 3.5 a (28/29)
ROS-IVM38	154	25.3 ± 3.5 b (39/154)	1.2 ± 0.1 ab	10.3 ± 4.9 a (4/39)	84.6 ± 5.9 a (33/39)
ROS-IVM44	157	59.2 ± 3.9 c (93/157)	1.3 ± 0.1 bc	15.0 ± 3.7 a (14/93)	82.8 ± 3.9 a (77/93)
ROS-IVM44[b]	156	64.1 ± 3.8 c (100/156)	1.7 ± 0.1 cd	57.0 ± 5.0 b (57/100)	57.0 ± 5.0 b (57/100)

N, number of oocytes. Values with different letters (a, b, c, d) within columns are significantly different ($P<0.05$).
[a] Percentage from penetrated oocytes.
[b] Oocytes assessed at 22 hpi.

Table 3
Sperm penetration and male pronuclear formation at 22 hpi in porcine oocytes cultured in an IVM system for 44 h with or without pretreatment with 50 μM roscovitine for 48 h (ROS-IVM44 and IVM44, respectively)

Treatment	*N*	Percent penetrated	Number of sperm/ penetrated oocyte	Percent MPN formation[a]	Percent monospermy[a]
ROS-IVM44	169	57.4 ± 3.8 (97/169)	1.4 ± 0.1	53.6 ± 5.0 a (52/97)	57.7 ± 5.0 (56/97)
IVM44	173	59.5 ± 3.7 (103/173)	1.4 ± 0.1	73.7 ± 4.3 b (76/103)	63.1 ± 4.7 (65/103)

N, number of oocytes. Percentages with different letters (a, b, c, d) within column are significantly different ($P < 0.05$).
[a] Percentage from penetrated oocytes.

3.2. Effect of roscovitine on IVF (experiment 2)

A 48 h period of roscovitine treatment did not affect oocyte penetrability nor monospermy rate because no differences were observed between ROS-IVM44 and IVM44 groups (Table 3). However, male pronuclear formation rate was greater in the control group than in roscovitine-pretreated oocytes (73.7 ± 4.3 and 53.6 ± 5.0, respectively) at 22 hpi.

3.3. Effect of roscovitine on intracellular GSH content (experiment 3)

Intracellular GSH content of oocytes before (just after collection) and after pretreatment with ROS for 48 h was similar (Fig. 1; 4.8 ± 0.6 and 3.9 ± 0.1 pmol/oocyte, respectively). After IVM culture for 44 h, oocytes treated with roscovitine had a lesser intracellular amount

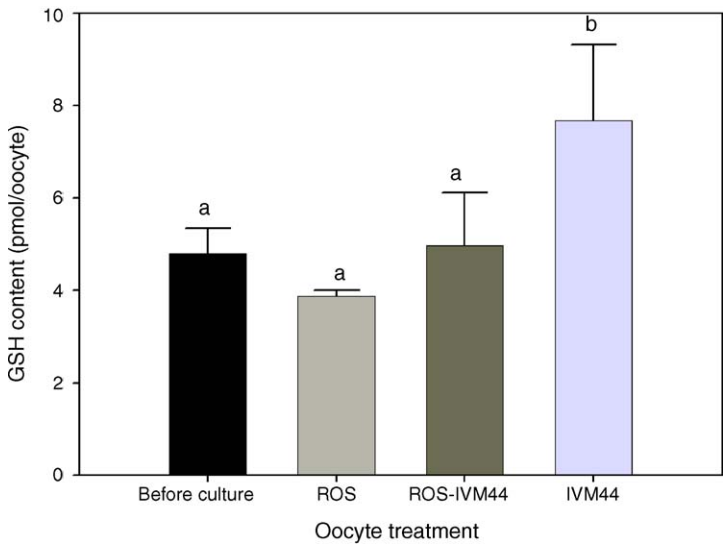

Fig. 1. Intracellular glutathione (GSH) content in porcine oocytes before (before culture) or after pretreatment with 50 μM roscovitine for 48 h (ROS), and after IVM for 44 h with or without the ROS-pretreatment (ROS-IVM44 and IVM44, respectively).

Table 4
Early development in vitro of porcine IVM–IVF oocytes that were or were not pretreated with 50 μM roscovitine for 48 h

Treatment	*N*	Percent cleaved	Percent of IVM–IVF embryos developed to:	
			Morula	Blastocyst
ROS-IVM44	173	70.9 ± 6.2	29.4 ± 5.2[a]	22.0 ± 6.0[a]
IVM44	184	87.0 ± 3.1	47.4 ± 1.2[b]	38.1 ± 1.0[b]

N, number of oocytes. Percentages with different superscripts (a, b) within columns are significantly different ($P < 0.05$).

of GSH than controls (5.0 ± 1.1 and 7.7 ± 1.6 pmol/oocyte, respectively). Control oocytes that were not treated with roscovitine had increased GSH content during IVM, but this was not observed in roscovitine-treated oocytes.

3.4. Effect of roscovitine on embryo development (experiment 4)

Roscovitine-treated oocytes tended to have developmental competence to the blastocyst stage, but this was less than for control oocytes (22.0 ± 6.0% and 38.1 ± 1.0%, respectively). Cleavage rate was not affected ($P = 0.06$) by the roscovitine treatment (87.0 ± 3.1% and 70.9 ± 6.2% for control and ROS groups, respectively) Table 4.

4. Discussion

Among protein kinases activated during entry into the metaphase, the cyclin dependent kinase CDK1/cyclin B was initially identified as a universal metaphase promoting factor. The first inhibitors to be discovered were 6-DMAP and isopentenyladenine, from which more potent and selective inhibitors were optimized, such as olomoucine and roscovitine (reviewed by Meijer and Raymond, 2003). The effect of roscovitine on the maintenance of oocyte meiotic arrest has been previously assessed in pigs using different concentrations, inhibitory periods, and sources of oocytes (Krischek and Meinecke, 2001; Marchal et al., 2001; Ju et al., 2003; Schoevers et al., 2005). Roscovitine can maintain the meiotic arrest in porcine oocytes for 48 h in a fully reversible manner because nearly 95% of oocytes did not show any sign of GVBD after inhibition and over 90% of oocytes reached the Met-II stage of development after culture in a step-wise system for 44 h. These results are consistent with other groups using the same inhibitory concentration of roscovitine for shorter periods (Krischek and Meinecke, 2001; Marchal et al., 2001; Coy et al., 2005b). In the present study, the first detailed nuclear stage analysis was evaluated after an inhibition for longer than 30 h, showing that during blocking of GVBD with roscovitine, a majority of oocytes (>80%) were maintained in the GV-I and GV-II stages of development. This result may suggest a complete and functional inhibition of oocytes by roscovitine because the nucleolus is the site for rRNA transcription and synthesis of ribosomal components.

Kinetics after roscovitine treatment in porcine oocytes has been reported both to be accelerated (Marchal et al., 2001) and delayed (Krischek and Meinecke, 2001). In the present

study after roscovitine treatment, 1.1 and 68.9% of oocytes cultured in an IVM system for 22 and 30 h, respectively, were at the Met-I stage of development. This progression would be similar to the porcine oocyte development under roscovitine-free in vitro conditions (Wehrend and Meinecke, 2001), because oocytes remained in the GV stage after 23.4 h and 33.9 h of culture with a majority of oocytes still being at the Met-I stage. Nuclear maturation kinetics of oocytes treated with roscovitine for 48 h and subsequently matured in a step-wise system does not differ from that of non-inhibited oocytes. Marchal et al. (2001) observed that no oocytes from the control group but 63% of oocytes precultured for 44 h in roscovitine reached the Met-II stage of development after 22 h of IVM culture. The inconsistency of the results in the present study may be explained by the presence of dibutyryl cyclicAMP in the present study during first 22 h of IVM. This chemical is a common additive in porcine IVM because it increases the homogeneity of oocyte nuclear maturation (Funahashi et al., 1997). Dibutyryl cyclicAMP prevents the GVBD by arresting oocytes at the GV-II stage of development, explaining why oocytes in the present experiment did not resume meiosis until 22 h after the start of IVM culture and why nuclear maturation did not occur as rapidly as that in dibutyryl cyclicAMP-free IVM system (Marchal et al., 2001). Also the lesser roscovitine concentration used in the previous study (25 μM) may explain this difference. In a step-wise system, the oocyte nucleus reaches the Met-II stage of development after 44 h of IVM (Funahashi et al., 1997). This is consistent with the current results using roscovitine-treated oocytes because the rate of oocytes reaching to the Met-II stage was similar to that of oocytes treated with or without roscovitine. The IVF data assessed at 8 hpi showed that oocytes cultured in an IVM system for 44 h had a greater penetrability by spermatozoa than those cultured for shorter periods, whereas no differences were observed in the MPN formation and monospermy rates. According to these results, therefore, 44 h would be the most desirable period for oocytes treated with roscovitine for 48 h to be cultured in the present step-wise IVM system.

The post-insemination assessment time was chosen according to previous results considering that in the presence of caffeine, MPN formation is almost completed at 6 h after insemination (Funahashi and Nagai, 2001; Matás et al., 2003). The increase in MPN formation observed at 22 hpi could, therefore, be due to a delay in pronucleus formation of roscovitine-treated oocytes. These results were confirmed in the second experiment of the present study when simultaneous fertilization of roscovitine-treated and control oocytes revealed similar penetrability and monospermy rate but a lesser MPN formation with roscovitine-treated oocytes. The extent of penetrability of porcine oocytes treated with roscovitine has still not been resolved. Penetration rate has been reported to be less in porcine oocytes in which maturation has been inhibited with roscovitine for 22 h (Marchal et al., 2001), although Coy et al. (2005b) did not observe any reduction in the penetrability of roscovitine-treated oocytes.

Regarding the lesser MPN formation, the cumulus expansion and oocyte GSH content was the focus in the present study. Cumulus cells have a key role in the provision of energy substrates, amino acids (including cysteine as a precursor of GSH) and nucleotides to the oocyte (Sutton et al., 2003). The cumulus expansion in the present study was completely inhibited with roscovitine treatment and the COC did not achieve the extent of expansion as those in the control group during IVM, confirmed previous observations (Marchal et al., 2001; Ju et al., 2003). Lonergan et al. (2003) observed that inhibition of develop-

ment of bovine oocytes with roscovitine for 24 h caused a disruption in the integrity of the surrounding cumulus cells and affected subsequent expansion of the cumulus cells during IVM. Also, more recently Schoevers et al. (2005) described a delayed cumulus expansion after a 44 h roscovitine treatment of preantral porcine oocytes. However, Vigneron et al. (2003) suggested that roscovitine treatment for 24 h allowed for the continued functionality of the surrounding somatic cells. The possible detrimental effect of roscovitine on cumulus cells may need further study.

As widely known, intracellular GSH content and MPN formation are strongly correlated (Yoshida et al., 1993; Sutovsky and Schatten, 1997). In porcine oocytes matured for 44 in NCSU media, intracellular GSH content has been reported to be about 7.5 pmol/oocyte (Brad et al., 2003; Coy et al., 2005b) what is consistent with that of results from the present study. However, in the present study, the ROS-IVM oocytes had lesser concentrations of intracellular GSH than those from the non-treated control group, which may explain the observed lesser MPN formation. Coy et al. (2005b) recently showed that porcine oocytes inhibited with roscovitine for 22 h and matured in a step-wise system for 44 h showed similar intracellular amounts of GSH as oocytes in a control group. Ju et al. (2003) also observed cytoskeleton alterations in porcine roscovitine-treated oocytes after 44 h of culture.

Embryo development has been evaluated both in porcine and bovine oocytes arrested with roscovitine for 22–24 h and although development is not compromised, an improvement in development has not been clearly demonstrated (Marchal et al., 2001; Coy et al., 2005b). However, in the present study, cytoplasmic maturation of oocytes treated with roscovitine for 48 h was compromised because there was a lesser developmental capacity of the treated oocytes as compared with those in the control group although blastocyst formation was observed in both groups. The treatment period of oocytes with roscovitine may be an important factor to take into account when assessing cytoplasmic maturation. McClue et al. (2002) observed that maximum effects of roscovitine occurred between 8 and 24 h of culture, so replacement of inhibitory medium every 22 to 24 h may be effective as a further strategy for prolonging the GV stage of oocytes.

In conclusion, the present results demonstrated that roscovitine treatment for 48 h reversibly maintained porcine oocytes at the GV stage and did not affect the nuclear maturation and fertilizability of these oocytes. However, MPN formation rate, intracellular GSH content and blastocyst formation rate of ROS-treated oocytes were less than non-treated control oocytes. After relatively longer treatment with roscovitine, cytoplasmic maturation of porcine oocytes does not seem to be achieved when using the current IVM conditions.

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