REPRODUCTION
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Birth of piglets after transferring of *in vitro*-produced embryos pre-matured with R-roscovitine

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Abstract

The objectives of this study were to evaluate: (1) the nuclear maturation, (2) the intracellular glutathione (GSH) content, (3) the normality of fertilization and (4) full development after transplantation of embryos derived from porcine oocytes pre-cultured with 50 μmol/l roscovitine (an inhibitor of $p34^{cdc2}$/cyclin B kinase) for 22 h. After treatment with roscovitine, the nuclear configuration of oocytes (Hoechst staining) was comparable with those examined just after collection: the majority of oocytes were arrested at the germinal vesicle (GV) 1 stage (63.2%). Roscovitine-treated oocytes progressed through meiosis to the metaphase II stage in a conventional step-wise *in vitro* maturation (IVM) program for 44 h in a proportion similar to control ones (>85.0%). When roscovitine-treated oocytes and non-treated oocytes were matured for 44 h and then co-cultured with fresh spermatozoa for 18 h, no differences were observed in oocyte penetrability, proportion of monospermic penetration and male pronuclear formation (>87%). Roscovitine increased the GSH synthesis in oocytes at 22 h, whereas, after 44 h, roscovitine-treated oocytes had similar amounts of GSH to non-treated oocytes. Finally, surgical transfer of zygotes at 22-24 h post-insemination, derived from roscovitine-treated oocytes, resulted in one pregnancy with 12 piglets born; control non-treated zygotes resulted in one pregnancy and 10 piglets born. The full-term developmental ability of mammalian oocytes pre-cultured with roscovitine prior to IVM is thereby demonstrated. This validation is important before the introduction of roscovitine into routine procedures.

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Introduction

Efforts to characterize oocytes within antral follicles not yet destined to ovulate have been hampered by an inability to maintain meiotic arrest in oocytes after removal from the follicle (reviewed by Picton & Gosden 1999). Whereas *in vivo*, meiotically competent oocytes are arrested at the germinal vesicle (GV) stage by the follicular environment until the preovulatory surge of gonadotropins, *in vitro*, oocytes resume meiosis spontaneously after removal from the follicular environment. Attempts to develop *in vitro* culture systems to maintain bovine or porcine oocytes at the GV stage using different meiotic inhibitors have been reported, and the results have been partially successful. The increasing interest in such studies is supported by several potential applications: (1) obtaining oocytes from different places (farms) that could be later sent to the laboratory under meiotic arrest for further *in vitro* maturation (IVM) and fertilization (IVF) – as an example, this method is being applied for commercial ovum pick up-in vitro embryo production (OPU-IVP) production of cattle embryos (P Mermillod, personal communication); (2) maintaining the oocyte's ability to synthesize and store molecules important for subsequent embryonic development during the inhibitory period, thus allowing *in vitro* production of competent oocytes (Kubelka *et al.* 2000, Mermillod *et al.* 2000, Motlik *et al.* 2000, Ponderato *et al.* 2001); (3) a improvement in the post-freezing survival rates of GV stage oocytes compared with those at the metaphase stage; (4) possible optimization of cell cycle synchrony and production of cells that respond more predictably to nuclear reprogramming (Gibbons *et al.* 2002); (5) prolonged and more flexible maturation schedules in the laboratory (Coy *et al.* 2004); (6) the development of *in vitro* models to study the effects of elevated temperature (heat stress) and other environmental factors on immature oocytes (Payton *et al.* 2004). Because of these applications, two-step culture systems (a pre-culture in a medium with inhibitor and a further culture in IVM medium) are proposed as useful tools in IVM programs (Motlik *et al.* 2000).

The resumption of meiosis is regulated by activation of the metaphase promoting factor (MPF; Wu *et al.* 1996,

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Motlik *et al.* 1998, Sirard *et al.* 1998). MPF is a cell division kinase (cdk) whose activation involves the formation of a complex between $p34^{cdc2}$ kinase (cdk1) and cyclin B. Once formed, this complex should be activated by dephosporylation of the threonine 14 and tyrosine 15 residues of the p34 subunit. The most common approach to block MPF activation and, consequently, the resumption of meiosis, has been the employment of cdk inhibitors. Roscovitine actively competes for the ATP binding sites in the cdk1 subunit of the MPF, and was first used to prevent germinal vesicle breakdown (GVBD) in cattle and pig oocytes (Mermillod *et al.* 2000, Krischek & Meinecke, 2001, Marchal *et al.* 2001, Ponderato *et al.* 2001, 2002). Specifically, the inhibitory effect of roscovitine on the cell cycle was discovered by Meijer (reviewed by Meijer & Raymond 2003). The results seem to demonstrate that it is more effective and produces less detrimental effects than other inhibitors such as cycloheximide (Faerge *et al.* 2001), butyrolactone I (Kitagawa *et al.* 1993, Meijer & Kim 1997, Kubelka *et al.* 2000, Fair *et al.* 2002) or 6-DMAP (Lonergan *et al.* 1997, Avery *et al.* 1998, Liu *et al.* 1998, Dode & Adona 2001).

Despite the numerous papers published in recent years, and even though studies in cattle have been more extensive than in pigs, no references are available showing the influence of pre-culture with roscovitine and further IVM on important parameters such as the nuclear stage during the maturation period, the intracellular glutathione (GSH) content of pre-cultured oocytes or male pronuclear formation after IVF of these gametes. Moreover, the birth of offspring has yet to be reported, and therefore also unreported is the competence to develop to term of embryos produced *in vitro* under the two-step IVM conditions. As a consequence, the overall objective of this experimental work was to assess the influence of roscovitine on the nuclear and cytoplasmic maturation of oocytes entering a two-step IVM system and to demonstrate its effectiveness to generate embryos with full-term developmental ability.

Materials and Methods

Culture media

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A. The R-enantiomer of roscovitine (2-(R)-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine; R-7772) was solubilized in dimethyl sulfoxide before freezing at −20 °C as a 10 mmol/l stock. The medium used for oocyte maturation was NCSU-37 (Petters & Wells 1993) supplemented with 0.57 mmol/l cysteine, 1 mmol/l dibutyryl cAMP, 5 μg/ml insulin, 50 μmol/l β-mercaptoethanol, 10 iu/ml equine chorionic gonadotropin (eCG) (Foligon; Intervet International B.V., Boxmeer, Holland), 10 iu/ml human CG (hCG) (Chorulon, Intervet International B.V.) and 10% porcine follicular fluid (v/v).

The basic medium used for IVF was essentially the same as that used by Rath *et al.* (1999). This medium, designated as TALP medium, consists of: 114.06 mmol/l NaCl, 3.2 mmol/l KCl, 8 mmol/l Ca-lactate·$5H_2O$, 0.5 mmol/l $MgCl_2 \cdot 6H_2O$, 0.35 mmol/l NaH_2PO_4, 25.07 mmol/l $NaHCO_3$, 10 ml/l Na-lactate, 1.1 mmol/l Na-pyruvate, 5 mmol/l glucose, 2 mmol/l caffeine, 3 mg/ml BSA (A-9647), 1 mg/ml polyvinyl alcohol (PVA) and 0.17 mmol/l kanamycin sulfate.

The embryo culture medium was NCSU-23 containing: 0.4% BSA (A-8022), 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate (Machaty *et al.* 1998).

Oocyte collection and in vitro maturation

Within 30 min of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline containing 100 μg/ml kanamycin sulfate at 38 °C, washed once in 0.04% cetrimide solution and twice in saline. Oocyte-cumulus cell complexes (COCs) were collected from antral follicles (3–6 mm diameter) by slicing, washed twice with Dulbecco's PBS supplemented with 1 mg/ml PVA and twice more in maturation medium previously equilibrated for a minimum of 3 h at 38.5 °C under 5% CO_2 in air. Only COCs with a complete and dense cumulus oophorus were used for the experiments (Coy *et al.* 2002). Groups of 50 COCs were cultured in 500 μl maturation medium for 22 h at 38.5 °C under 5% CO_2 in air. After culture, oocytes were washed twice in fresh maturation medium without dibutyryl cAMP, eCG and hCG and cultured for an additional 20–22 h (Funahashi & Day 1993). This IVM system is commonly employed in pigs for several years and is based upon the results from Funahashi *et al.* (1997).

Brilliant cresyl blue test

Immediately after collection, COCs were washed in PBS and exposed to brilliant cresyl blue (BCB; 13 μmol/l in PBS) for 90 min at 38.5 °C in a humidified air atmosphere. BCB staining determines the activity of glucose-6-phosphate dehydrogenase, an enzyme synthesized in growing oocytes but with less activity in grown oocytes (Roca *et al.* 1998, Rodríguez-González *et al.* 2002). After exposure to BCB, they were washed three times in PBS and observed under a stereomicroscope at × 20 and classified into two groups, depending on their cytoplasm coloration: oocytes showing blue cytoplasm or grown oocytes (BCB+) and oocytes without blue coloration or growing oocytes (BCB−).

Hoechst staining

Oocytes were fixed for 15 min (2% glutaraldehyde in PBS), stained for 15 min (1% Hoechst 33342 in PBS) and finally washed in PBS containing 1 mg/ml polyvinylpyrrolidone and mounted on glass slides. Oocytes were examined under an epifluorescence microscope at × 200 and

× 400 magnification and designated as GV-0, GV-I GV-II, GV-III, GV-IV (including diakinesis and prometaphase I), metaphase I (Met I), anaphase I (Ana I) and Met II stages according to the morphological criteria for characterization of meiotic stages by Funahashi *et al.* (1997).

GSH assay

The intracellular content of GSH was measured as described previously (Funahashi *et al.* 1995). Briefly, COCs were denuded by pipetting in PBS and washed three times in a buffer solution. Five microliters of buffer containing 30 oocytes per replicate and group were transferred to a 2 ml microfuge tube and 5 μl of 1.25 mol/l phosphoric acid added. Samples were frozen immediately (−20 °C) and kept in the freezer until assayed. The GSH content in the oocytes was determined by the dithionitrobenzonic acid–glutathione disulphide (DTNB–GSSG) reductase recycling assay (Anderson 1985). Briefly, 700 μl of 0.33 mg/ml NADPH in 0.2 mol/l sodium phosphate buffer containing 10 mmol/l EDTA (stock buffer, pH 7.2), 100 μl of 6 mmol/l 5,5′-dithiobis-(2-nitrobenzoic acid) in the stock buffer, and 190 μl of water were added into the microfuge tube. Ten microliters of 250 iu/ml glutathione reductase were added with mixing to initiate the reaction. The formation of 5-thio-2-nitrobenzoic acid was detected photometrically by a change of absorption at 412 nm. The total GSH content is calculated according to a standard curve. The total amount of GSH calculated was divided by the number of oocytes in the sample to obtain the content per oocyte (pmol/oocyte).

In vitro fertilization

COCs cultured for a total of 44 h in maturation medium were stripped of cumulus mechanically by gentle aspiration with a pipette. Oocytes were washed three times with TALP medium and groups of 30–35 oocytes were transferred into each well of a four-well multi-dish containing 250 μl IVF medium previously equilibrated at 38.5 °C under 5% CO_2. The sperm-rich fraction of semen from a mature, fertility-tested boar was collected by the gloved-hand method and immediately transported to the laboratory diluted 1:8 in Beltsville thawing solution. The semen samples were centrifuged (1200 ***g***, 3 min) and the resultant sperm pellets were diluted in TALP medium at the desired concentration (Matás *et al.* 2003). The sperm suspension (250 μl) was added to each fertilization well to obtain a final concentration of 3000 cells/oocyte. At 5 h post-insemination (hpi), oocytes were washed twice with fresh NCSU-23 by gentle aspiration through a glass pipette and allowed to continue in culture at 38.5 °C under 5% CO_2 until fixation or transfer.

At 18 hpi, a sample of oocytes was stained with Hoechst 33342 as described above and examined at × 400 magnification for evidence of sperm penetration and pronuclear formation under an epifluorescence microscope.

Embryo transfer

Multiparous (two pregnancies) crossbred sows with synchronized estrus cycles were used for surgical embryo transfer. Estrus was checked daily in the presence of a mature teaser boar. Occurrence of estrus was defined by the standing reflex in front of a boar (back-pressure test) and reddening and swelling of the vulva. Oviducts from animals which had showed heat 72 h before embryo transfer were exposed through mid-ventral incision under general anesthesia, as previously described (Coy *et al.* 1993). Over 100 potential embryos (22–24 hpi) were introduced in one oviduct by a Tom Cat Catheter (Kendall Co., Mansfield, MA, USA) connected to an insulin syringe containing PBS at 37 °C. The catheter was later observed under a stereomicroscope to check that all embryos had been transferred into the oviducts. The sows were kept under the usual farm conditions, and 25–28 days after transfer pregnancy diagnosis was carried out by ultrasonography (Echoscan T-100).

Experimental design

The experimental design is schematically represented in Fig. 1.

Before running the experiment, 831 oocytes in four replicates were stained just after collection with BCB to verify the growth stage and check if culture in roscovitine for 22 h inhibited the last step of growing. The numbers of oocytes examined were 264, 301 and 266 for before culture group, ROS group and A group respectively (see below for details).

Experiment 1. Nuclear status after 22 h culture in roscovitine

This experiment was carried out to establish whether culture of oocytes in NCSU-37 medium without dibutyryl cAMP, eCG and hCG and with 50 μmol/l roscovitine for the first 22 h of maturation (ROS group, $n = 152$) kept the nuclear stage at the same level as found in oocytes just after recovery (Before culture group, $n = 156$). As control groups, oocytes cultured for 22 h in supplemented NCSU-37 medium were used under usual IVM conditions, which included dibutyryl cAMP, eCG and hCG (A group, $n = 173$) and oocytes cultured for 22 h in the same NCSU-37 medium without dibutyryl cAMP, eCG and hCG (B group, $n = 164$). Therefore, it could be seen whether the inhibitory effect of roscovitine on the nuclear progression was similar, or dissimilar, to that demonstrated by dibutyryl cAMP, which only blocks oocytes at stages before the GV-II stage (Funahashi *et al.* 1997); the nuclear stage of inhibited oocytes (ROS and A groups) at 22 h and of those under supposed spontaneous resumption of meiosis (B group) could also be compared.

After culture, oocyte nuclear status (GV-0 to Met II) was recorded in all groups. This experiment was performed in four replicates.

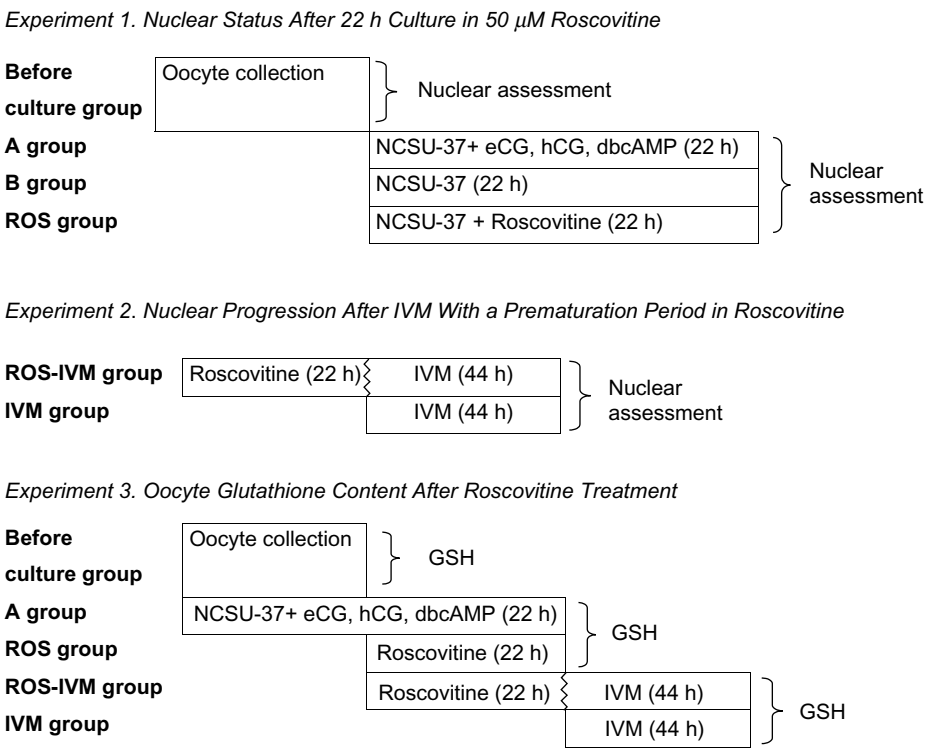

Figure 1 Experimental design. ET: Embryo transfer.

Experiment 2. Nuclear progression after IVM with a pre-maturation period in roscovitine

In order to assess the reversibility of the treatment with roscovitine, COCs were cultured for 44 h under permissive maturation conditions in each of three replicates with (ROS-IVM group, $n = 153$) or without (IVM group, $n = 161$) a previous culture period of 22 h in the presence of 50 μmol/l roscovitine. Nuclear stage was recorded by Hoechst staining.

Experiment 3. Oocyte GSH content after roscovitine treatment

This experiment was designed to examine the influence of roscovitine on the intracellular GSH content of oocytes just after collection, at 22 h of culture with or without roscovitine, and after maturation with or without a pre-culture in roscovitine. In seven replicates, 960 COCs were collected and allotted to five groups: (1) Before culture group (oocytes processed just after collection); (2) A group (oocytes cultured in supplemented NCSU-37 medium with dibutyryl cAMP, eCG and hCG); (3) ROS group (oocytes cultured for 22 h in supplemented NCSU-37 medium without dibutyryl cAMP, eCG and hCG and with 50 μmol/l roscovitine); (4) IVM group (oocytes cultured for 44 h under the described step-wise IVM system); (5) ROS-IVM group (oocytes cultured for 22 h as the ROS group and for a further 44 h as the IVM group). GSH content was measured as described above.

Experiment 4. IVF and full development of roscovitine-treated oocytes

Just after recovering, 631 oocytes (in four replicates) were cultured in the presence of 50 μmol/l roscovitine for 22 h. Following this period of time, oocytes were washed and allowed to mature for 44 h under permissive conditions (ROS-IVM group). Other 610 COCs were also collected and introduced in the IVM system at the same time as the ROS-IVM group. Matured oocytes from both groups were then fertilized and 18 hpi, samples of the potential embryos ($n = 133$ and $n = 127$ for ROS-IVM and IVM groups respectively) were processed to assess penetration and monospermy rates. The remaining cells (498 for ROS-IVM and 483 for ROS groups) were transferred into the oviduct of physiologically synchronized sows (one sow per group and replicate, eight sows in total). Pregnancies were assessed by ultrasonography 25-28 days after transfer.

Statistical analysis

Data are presented as means ± S.E.M. and all percentages were modeled according to the binomial model of variables. The variables in all the experiments were analyzed by one-way ANOVA. These variables were as follows: experiments 1 and 2, the nuclear stage (GV-0 to Met II); experiment 3, the GSH content; experiment 4, the percentage of penetration, the number of sperm cells per penetrated oocyte, the male pronuclear formation and the putative embryos (putative embryos were defined as the percentage of monospermic oocytes with two pronuclei with respect to the total number of penetrated oocytes). When ANOVA revealed a significant effect, values were compared using Tukey's test. P values < 0.05 were taken to denote statistical significance.

Results

After collection, 86.0 ± 2.1% of oocytes showed a blue cytoplasm coloration following BCB staining and were classified as grown oocytes. After 22 h of culture in medium with or without roscovitine, this proportion increased significantly to 95.0 ± 1.2 and 97.0 ± 1.0% respectively.

Nuclear status after 22 h in roscovitine

Roscovitine was effective in inhibiting the meiotic resumption after 22 h of culture, giving a higher percentage of GV-I stage oocytes and a lower percentage of GV-III stage oocytes in the ROS group than in A or B groups (Table 1). Just before culture and after treatment with roscovitine, oocytes were similar for both nuclear stages. The percentage of oocytes reaching the Met-I stage was higher in the B group than in the remaining three groups.

Nuclear progression after IVM with a 22-h pre-maturation period in roscovitine

After maturation for 44 h, oocytes from the ROS-IVM group resumed meiosis and reached Met II in the same proportions as in the IVM group, over 85% (Table 2).

Oocyte GSH content after roscovitine treatment

As shown in Fig. 2, intracellular GSH content increased after oocytes were cultured. When oocytes were kept for 22 h in culture, GSH content increased from 2.24 to 10.74 pmol/oocyte, and still higher when roscovitine was present during culture (15.23 pmol/oocyte). After IVM, no differences were observed independently of a 22 h pre-maturation period with (ROS-IVM) or without (IVM) roscovitine, being 7.02 and 7.48 pmol/oocyte respectively. However, both groups showed a lower amount of GSH than the ROS group.

IVF and full development of roscovitine-treated oocytes

No differences were observed in IVF results after insemination of oocytes treated (ROS-IVM) or not (IVM) with roscovitine. Total data from four replicates showed a penetration rate of 73.0 ± 3.9 and 78.7 ± 3.6% respectively for ROS-IVM and IVM groups, and from the penetrated oocytes 41.2 ± 5.0 and 37.0 ± 4.8% respectively were monospermic. Male pronuclear formation was >87% in both groups.

After embryo transfer, both ROS-IVM and IVM sows from the second replicate became pregnant and delivered 12 (four male/eight female) and ten (five male/five female) healthy piglets respectively (Table 3). The weight of animals (means ± S.E.M.), assessed 42 days after birth, was 8.3 ± 0.5 and 11.5 ± 0.6 kg respectively for ROS-IVM and IVM groups. Sows from replicates 1, 3 and 4 showed heat estrus 21–24 days after embryo transfer.

Discussion

A principal disadvantage of slaughterhouse material is the heterogeneity among batches of ovaries that could be responsible for variable results. In the present study, the growing stage of the oocytes was first assessed in four trials by BCB staining demonstrating that the selection of the follicles by slicing was appropriate, with the proportion of fully grown oocytes always >80%.

Table 1 Nuclear status of pig oocytes after 22 h culture in NCSU-37 with or without hormonal supplements, or with 50 μmol/l roscovitine.

Group	Before culture	A	B	ROS	P value
n	156	173	164	152	
GV-0	96 ± 2.4^{a}	0.6 ± 0.6^{b}	0^{b}	0^{b}	<0.001
GV-I	60.9 ± 3.9^{a}	37.0 ± 3.7^{b}	31.7 ± 3.6^{b}	63.2 ± 3.9^{a}	<0.001
GV-II	8.3 ± 2.2^{a}	37.0 ± 3.7^{b}	26.2 ± 3.5^{b}	29.6 ± 3.7^{b}	<0.001
GV-III	7.0 ± 2.1^{a}	23.7 ± 3.2^{b}	20.1 ± 3.1^{b}	7.2 ± 2.1^{a}	<0.001
GV-IV	7.0 ± 2.1^{a}	0^{b}	3.7 ± 1.5^{ab}	0^{b}	<0.001
Metaphase I	5.1 ± 1.8^{ab}	1.2 ± 0.8^{a}	13.4 ± 2.7^{b}	0^{a}	<0.001
Anaphase I	0	0	1.2 ± 0.9	0	0.12
Telophase I	0.6 ± 0.6	0	2.4 ± 1.2	0	0.06
Metaphase II	1.3 ± 0.9	0.6 ± 0.6	2.4 ± 1.2	0	0.53

Different superscript letters in the same row indicate that values are significantly different ($P < 0.05$). A; 22 h culture in NCSU-37 with hCG, eCG and dibutyryl cAMP. B; 22 h culture in NCSU-37 without hCG, eCG and dibutyryl camp. ROS; 22 h culture in NCSU-37 without hCG, eCG and dibutyryl cAMP, and with 50 μmol/l roscovitine.

Table 2 Nuclear status of pig oocytes after *in vitro* maturation with (ROS-IVM) or without (IVM) a prematuration period of 22 h in 50 μmol/l roscovitine.

Group	IVM	ROS-IVM	*P* value
n	161	153	
GV-0	0.6 ± 0.6	0	0.33
GV-I	6.2 ± 1.9	4.6 ± 1.7	0.52
GV-II	1.9 ± 1.1	0	0.09
GV-III	1.2 ± 0.9	1.3 ± 0.9	0.96
GV-IV	0[a]	6.5 ± 2.0[b]	<0.001
Metaphase I	1.9 ± 1.1	0.6 ± 0.6	0.34
Anaphase I	0	0	1
Telophase I	0.6 ± 0.6	1.3 ± 0.9	0.53
Metaphase II	87.6 ± 2.6	85.6 ± 2.8	0.61

Different superscript letters in the same row indicate that values are significantly different ($P < 0.05$).

Interestingly, the inhibitory effect of roscovitine on oocyte growth (measured as the activity of glucose-6-phosphate dehydrogenase) was not observed, since the proportion of BCB+ oocytes increased up to 95% after 22 h of culture. This could indicate that the two-step IVM system is useful for producing more developmentally competent oocytes. Although there are slight indications of a possible beneficial effect of pre-culture in roscovitine on further embryo development (Mermillod *et al.* 2000, Marchal *et al.* 2001, Coy *et al.* 2003, 2004), interpretation of these results must be cautious since an influence of roscovitine on the inhibition of RNA synthesis has also been demonstrated (Ljungman & Paulsen 2001). Moreover, recent studies have shown that roscovitine did not prevent most of the modifications of the protein phosphorylation pattern observed during maturation (Vigneron *et al.* 2004*a*) and that some pathways involved in the regulation of bovine oocyte maturation seem to be independent of MPF activity

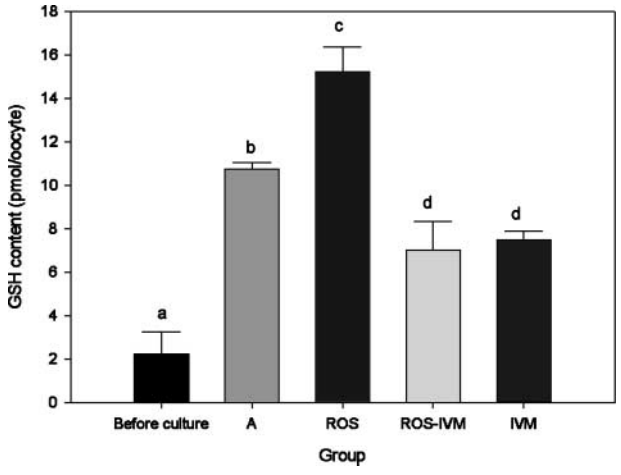

Figure 2 Intracellular GSH content in pig oocytes before culture, after 22 h culture in NCSU-37 with hCG, eCG and dibutyryl cAMP. A group; without hCG, eCG, dibutyryl cAMP. ROS group; without hCG, eCG, dibutyryl cAMP and with 50 μM roscovitine. IVM group; after a conventional IVM (22 h A + 22 h B). ROS-IVM group; after conventional IVM (22 h A +22 h B) with preculture in 50 μmol/l roscovitine. Different letters indicate significant differences ($P < 0.001$).

and meiotic resumption (Vigneron *et al.* 2004*b*). However, delaying meiotic resumption (without any aim of improving competence) may be of interest from a practical point of view for any of the mentioned reasons.

The effect of roscovitine on the resumption of meiosis has been shown previously in pigs. Ju *et al.* (2003) found that levels of 80–120 μmol/l roscovitine (Sigma, R-7772) were necessary to inhibit GVBD in 83–91% of oocytes. However, they assessed the nuclear stage of oocytes at 44 h from the beginning of culture in roscovitine, when the activity of the inhibitor could have been decreased in the medium (Meijer & Raymond 2003). In our study, doses of 50 μmol/l roscovitine were enough to block the GVBD in >90% of oocytes after 22 h of culture with similarities between control and ROS groups being evident for the GV-I and GV-III stages. In contrast, when the IVM medium without roscovitine was employed, oocytes started to resume meiosis spontaneously at this time. Moreover, when the NCSU-37 medium without dibutyryl cAMP was used, the progression to stages beyond GV-4 was faster, since the inhibitory effect of dibutyryl cAMP (Funahashi *et al.* 1997) was absent. These data first demonstrate that progression of porcine oocytes to the GV-III stage in the presence of 50 μmol/l roscovitine is inhibited in a different manner than in the presence of dibutyryl cAMP or in the absence of inhibitors, since they remain at similar nuclear stages to those in the follicles (as assessed just after recovery).

The reversibility of the roscovitine effect on the nuclear maturation has been corroborated in this study by the high proportions of Met II stage oocytes reached after 44 h of culture in the conventional IVM system following the 22 h of inhibition. However, it is well documented that nuclear and cytoplasmic maturation are not always correlated (Coy *et al.* 1999). The resumption of meiosis in oocytes after removal from roscovitine, presumably re-starting the activation of MPF, is not a prerequisite to ensure the cytoplasmic maturation of such oocytes. On the contrary, intracellular GSH content and the ability of oocytes to form a male pronucleus are commonly accepted parameters for assessing cytoplasmic maturation of porcine oocytes (Funahashi *et al.* 1995, Sawai *et al.* 1997, Coy *et al.* 1999). GSH is produced via the γ-glutamyl cycle and is dependent on the amount of cysteine available to the cell (Meister & Tate 1976). Cysteine is commonly added to pig oocyte maturation medium as a substrate for GSH synthesis to promote male pronuclear formation (Yoshida *et al.* 1993) at a 0.57 mmol/l concentration, and this was the case for NCSU-37. In this study, the GSH content of immature oocytes just after removal from follicles was lower than that found after 22 or 44 h of culture in IVM medium, the latter value being at the same baseline level as reported by Brad *et al.* (2003). The IVM system employed was presumably effective for oocyte GSH synthesis.

The GSH content was higher at 22 h of culture than at 44 h, in contrast to the findings of Yoshida *et al.* (1993)

Table 3 IVF results and pregnancies after surgical transfer of porcine embryo derived from *in vitro*-matured oocytes with (ROS-IVM) or without (IVM) a prematuration period of 22 h in 50 μmol/l roscovitine.

Group	Replicate	*N**	Penetration (%)	Spermatozoa/ oocyte	Male pronucleus (%)	Putative embryos** (%)	Number Transferred***	Pregnancy	Piglets born
IVM	1	30	90.0 ± 5.6	2.51 ± 0.3	100	33.3 ± 9.2	110	−	
ROS-IVM	1	25	60.0 ± 10.0	1.53 ± 0.2	86.7 ± 9.1	40.0 ± 13.1	110	−	
IVM	2	19	47.4 ± 11.8	1.56 ± 0.2	88.9 ± 11.1	44.4 ± 17.6	123	+	10
ROS-IVM	2	30	63.3 ± 8.9	1.47 ± 0.2	84.2 ± 8.6	57.9 ± 11.6	117	+	12
IVM	3	37	86.5 ± 5.7	2.53 ± 0.3	100	34.4 ± 8.5	110	−	
ROS-IVM	3	46	84.8 ± 5.3	2.54 ± 0.2	87.2 ± 5.4	23.1 ± 6.8	150	−	
IVM	4	41	78.0 ± 6.5	2.09 ± 0.3	87.1 ± 6.1	40.6 ± 8.8	140	−	
ROS-IVM	4	32	75.0 ± 7.8	1.54 ± 0.2	95.8 ± 4.2	58.3 ± 10.3	121	−	
ANOVA									
Roscovitine			0.38	0.05	0.20	0.38			
Replicate			0.0015	0.0018	0.73	0.06			
Rosc × repl.			0.48	0.24	0.13	0.37			

* Number of stained embryos 18 post-insemination.
** Proportion of zygotes with two visible pronuclei from the penetrated oocytes.
*** Number of transferred embryos 22–24 h post-insemination.

who have reported a continuous increase in GSH content during IVM. On the one hand, these authors employed TCM-199 for IVM, which is a rich culture medium with readily available GSH precursors (cysteine, cystine, glutamic acid, glutamine and glycine) and even glutathione. However, in our study NCSU-37 was used and was supplemented only with cysteine and glutamine (glutamine is the first cystine pre-cursor auto-oxidized, within 1 h, in the maturation medium (de Matos & Furnus 2000) that cannot be incorporated so easily as cysteine (Yoshida & Takahashi 1998)). On the other hand, it has been reported that GSH synthesis during initial and mid phases of porcine oocyte maturation is related to the acquisition of sperm nuclear decondensing ability (Yoshida 1993) and GSH synthesis during the initial stages of hamster oocyte maturation is responsible for the higher GSH levels in mature oocytes (Perreault *et al.* 1988). These observations suggest a strong GSH synthesis during the first half of oocyte maturation and would be in agreement with our results showing a GSH peak after 22 h of culture.

The present study also shows that oocytes pre-cultured in roscovitine for 22 h had a higher level of GSH than those cultured with dibutyryl cAMP for the same time. Cystine uptake activity is abolished by the mechanical disruption of gap junctional communication (Yoshida & Takahashi 1998), and GSH synthesis may be impaired due to the uncoupling of cumulus cells (de Matos *et al.* 1997). Since roscovitine-treated oocytes do not show cumulus expansion as shown by dibutyryl cAMP-treated oocytes (Marchal *et al.* 2001, Coy *et al.* 2004, and personal observations in the present experiments), the closest cooperation between cumulus cells and oocyte would be maintained for a longer time, explaining in part the higher GSH content observed in roscovitine-treated oocytes. Also, the inhibitory effect of roscovitine must be considered not only on the cdk but also on RNA synthesis (Ljungman & Paulsen 2001). Because GSH is involved in multiple functions, including DNA and protein synthesis (Lafleur *et al.* 1994), it could be expected that oocytes treated with roscovitine showed a higher GSH content than their counterparts, as was observed in our case since this functional activity of GSH was probably decreased.

Regarding male pronuclear formation, our data first show that roscovitine does not affect this parameter. This result was expected due to the similar levels of GSH found for *in vitro*-matured oocytes pre-cultured or not with roscovitine, since it has been reported that male pronuclear formation is related to intracellular GSH content (Yoshida *et al.* 1992, Funahashi *et al.* 1995). Moreover, no differences were found for any of the fertilization parameters assessed at 18 hpi, in agreement with most previous studies in bovine (Mermillod *et al.* 2000, Ponderato *et al.* 2001, Coy *et al.* 2005).

Finally, the key result in our study was the pregnancy of one recipient sow and the delivery of 12 healthy piglets when roscovitine-pre-cultured embryos were transferred. It is well documented that the *in vitro* production of pig embryos with the ability to develop to term is difficult due to polyspermy and deficient embryo culture media (Coy & Romar 2002, Funahashi 2003). Only 20–30% of transferred embryos survive despite considerable improvement in the techniques for *in vitro* production of porcine embryos (Abeydeera 2002), and approximately six piglets per sow after surgical embryo transfer are obtained (Abeydeera *et al.* 1998, Kikuchi *et al.* 1999). With these premises, and taking into account that about 37 and 41% of the transferred zygotes in IVM and ROS-IVM could be expected to develop to term, a high number of putative embryos was transferred at only 22–24 h of insemination and *in vitro* culture, in order to ensure a sufficient number of viable embryos in the oviduct of the synchronized sow. Thus, a final success of 25% for both IVM and ROS-IVM groups could be considered satisfactory and is among the normal rates reported in the literature.

In conclusion, this study demonstrates that pig oocytes cultured for 22 h with 50 μmol/l roscovitine remained at

similar nuclear stages to oocytes just after recovery. Cytoplasmic maturation assessed as intracellular GSH content and male pronuclear formation were not affected detrimentally by culture with roscovitine. Moreover, embryos obtained from this two-step IVM system can be developed to term showing the final competence of porcine oocytes pre-cultured with roscovitine, thus making its introduction desirable in assisted reproductive technologies programs.

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