Delay of nuclear maturation and reduction in developmental competence of pig oocytes after mineral oil overlay of *in vitro* maturation media

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Steroid hormones, such as progesterone, oestrogen, androgen and meiosis activating sterols, are secreted from cumulus cells that are stimulated by gonadotrophins during maturation of oocytes *in vitro*. These steroid hormones may be absorbed by mineral oil or paraffin oil; however, *in vitro* maturation of pig oocytes is commonly performed using medium covered by oil. In this study, high concentrations of progesterone, oestradiol and testosterone were detected in the culture medium after pig cumulus–oocyte complexes (COCs) were cultured with FSH and LH for 44 h in medium without an oil overlay. However, high concentrations of these steroid hormones were not detected in medium when COCs were cultured with the mineral oil overlay. When high concentrations of these steroid hormones were

secreted by COCs, germinal vesicle breakdown (GVBD) and the activation of p34^{cdc2} kinase and mitogen-activated protein (MAP) kinase in oocytes occurred earlier in comparison with oocytes cultured in medium covered with mineral oil. Moreover, a decrease in p34^{cdc2} kinase activity during meiotic progression beyond metaphase I was observed in oocytes cultured in conditions under which high concentrations of steroid hormones were secreted by COCs. In addition, the rate of development to the blastocyst stage after IVF was higher in oocytes matured in medium without an oil overlay. These adverse effects of oil may be explained by absorption by the oil of cumulus-secreted steroids or by the release of toxic compounds into the medium.

Introduction

Ovarian folliculogenesis in mammals is mainly under the endocrine control of the pituitary gonadotrophins FSH and LH. Stimulation of both LH and FSH leads to an increase in oestrogen concentration in the follicular fluid during preovulatory growth and development of ovarian follicles (Ainsworth *et al.*, 1980; Kumar *et al.*, 1992). After the LH surge, the oestrogen concentration of the follicular fluid of preovulatory follicles decreases; this is followed by a decrease in androgen and an increase in progesterone concentration (Ainsworth *et al.*, 1980). Osborn *et al.* (1986) studied the effects of inhibitors of follicular steroidogenesis on biological changes that occur in oocytes during meiotic maturation. This study showed that addition of a progesterone synthesis inhibitor reduced the proportion of oocytes that reached the MII stage and that addition of an aromatase inhibitor altered protein synthesis in oocytes. During meiotic maturation of cumulus–oocyte complexes (COCs) *in vitro*, progesterone is also produced by cumulus cells; the concentration of progesterone is increased by stimulation with LH, FSH or forskolin in pigs (Xia *et al.*, 1994; Coskun *et al.*, 1995), rats (Zhang and Armstrong, 1989) and cattle (Armstrong *et al.*, 1996). When rat COCs were cultured *in vitro* with FSH and a cytochrome

P450_{scc} inhibitor, progesterone secretion was almost completely inhibited and a low rate of oocyte fertilization was observed (Zhang and Armstrong, 1989). Moreover, the addition of oestrogen or testosterone to the maturation medium improved the developmental competence of bovine oocytes after IVF (Younis *et al.*, 1989; Silva and Knight, 2000). These observations imply that the steroid hormones play an important role within the oocyte during meiotic maturation of mammalian oocytes *in vivo* and *in vitro*.

Many dynamic morphological and biochemical changes occur to ensure the successful meiotic division during mammalian oocyte maturation. The maturation-phase promoting factor (MPF) is activated after the resumption of meiotic division (Masui and Markert, 1971). MPF is then transiently inactivated, and reactivated again to induce meiosis II (MII) in mouse (Choi *et al.*, 1991), pig (Naito and Toyoda, 1991) and bovine oocytes (Tatemoto and Terada, 1996; Wu *et al.*, 1997). After germinal vesicle breakdown (GVBD), mitogen-activated protein (MAP) kinase is also activated, and activated MAP kinase is required for a further increase in MPF activity and meiotic progression beyond the MI stage (Inoue *et al.*, 1995; Shimada and Terada, 2001; Shimada *et al.*, 2001a). Naito *et al.* (1992) reported that high MPF activity in pig oocytes at MII stage is essential for the development of the male pronucleus. Shimada and Terada (2002a) also showed that both MAP kinase and MPF were

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activated in pig oocytes during meiotic progression to the MII stage, followed by the decrease in cAMP content of oocytes. The decrease in cAMP content of oocytes was induced by binding of progesterone to its receptor in cumulus cells through the reduction of gap junction communication (Shimada and Terada, 2002b). Therefore, it is possible that the secreted steroid hormones, such as progesterone, regulate both cytoplasmic maturation and meiotic cell cycle via activation of both MPF and MAP kinase in pig oocytes.

In vitro maturation (IVM) of pig oocytes is commonly performed using medium covered by paraffin oil or mineral oil (Funahashi *et al.*, 1994; Abeydeera and Day, 1997; Ka *et al.*, 1997; Shimada *et al.*, 2001a). Owing to the high absorbing capacity of paraffin oil or mineral oil, the concentration of oestradiol or progesterone was reduced (Funahashi *et al.*, 1994), indicating that steroid hormones secreted by cumulus cells during IVM might also be absorbed by the paraffin oil or mineral oil. Thus, the IVM system used for pig oocytes, the culture of COCs in the medium covered by paraffin oil or mineral oil, may limit the developmental competence of pig oocytes matured *in vitro*. However, information about the biological and biochemical differences in oocytes cultured in the medium covered by mineral oil or not covered by mineral oil is very limited. The present study investigated the production of steroid hormones by COCs in maturation medium, the time course of meiotic maturation, p34^{cdc2} kinase activity and MAP kinase activity, and the developmental competence of pig oocytes cultured in medium covered or not covered by mineral oil overlay.

Materials and Methods

Isolation and culture of pig COCs

Pig COCs were isolated as reported by Shimada and Terada (2001). In brief, ovaries from 5- to 7-month-old prepubertal gilts were collected from a local abattoir. Oocytes were collected with a surgical blade from the surfaces of intact healthy antral follicles that measured 3–8 mm in diameter. Oocytes that had evenly granulated cytoplasm with at least four layers of unexpanded cumulus oophorus cells were selected and washed three times with the maturation medium mNCSU37 (Petters and Reed, 1991) which contained 20 ng pig FSH ml⁻¹ (NHPP, Torrance, CA), 0.5 µg pig LH ml⁻¹ (NHPP), 10% (v/v) fetal calf serum (Gibco BRL, Grand Island, NY) and 7 mmol taurine l⁻¹ (Sigma Chemical Co, St Louis, MO).

Twenty COCs were cultured in each well of a four-well multidish (Nunc, Roskilde) containing 500 µl of culture medium covered with or without 1 ml of mineral oil (M-8410, Lot no. 21K0039, Sigma) at 39°C in a humidified atmosphere of 5% CO₂ in air. Control experiments demonstrated that the osmolarity of cultures maintained under each condition varied by < 1% after 44 h of culture.

Quantification of progesterone, testosterone and oestradiol in medium by high-performance liquid chromatography (HPLC)-UV analysis

Quantification of steroid hormones by HPLC-UV was based on the procedures reported by Shimada and Terada (2002b). In brief, the medium in which COCs had been cultured for 44 h was collected into plastic tubes and centrifuged at 10 000 *g* for 20 min. The resulting supernatant was stored at -80°C until use. Before analysis, 0.5 ml of the medium sample was saponified by the addition of 0.3 ml of 0.3 mol NaOH l⁻¹ (Nakalai, Osaka), and progesterone was extracted from the saponified sample after mixing with 10 ml dichloromethane (Nakalai) for 5 min. After centrifugation, 10 ml dichloromethane fraction was collected into a disposal tube and the solvent from this fraction was removed by vacuum extraction for 120 min at 5°C. Samples were reconstituted in 100 µl of 50% (v/v) methanol solution.

The samples were separated using a reverse-phase CAPCELL PAK column (2.0 mm × 100 mm) (Shiseido, Tokyo). The solvent delivery system (TSK CCPD, TOSOH) contained 50% (v/v) methanol solution and the flow rate was adjusted to 200 µl min⁻¹. Samples (100 µl) were injected on to columns using an auto sampler (AS-8020, TOSOH). Progesterone, testosterone and oestradiol were detected at 240, 225 or 225 nm, respectively, using a UV detector (UV 8020, TOSOH); peak heights were measured using a computer integrator (Sic chromatocorder 11, TOSOH).

The standard curves for progesterone, testosterone and oestradiol were linear, from 0 to 800 ng ml⁻¹. The intra-assay coefficients of variation in medium with 100 ng ml⁻¹ of progesterone, testosterone or oestradiol were 4.15, 5.02 or 4.50%, respectively.

Assessment of nuclear maturation

At the end of culture, COCs were freed from cumulus cells, mounted on slides, fixed with acetic acid:ethanol (1:3) for 48 h and stained with aceto-lacmoid before examination under a phase-contrast microscope (× 400) for evaluation of their chromatin configuration.

Extract preparation for in vitro kinase assay

Oocytes were lysed according to the technique used by Shimada and Terada (2001). In brief, oocytes were washed several times in PBS and transferred into plastic tubes containing 5 µl cell lysis buffer (20.0 mmol Tris l⁻¹ (pH 7.5), 150.0 mmol NaCl l⁻¹, 1.0 mmol EDTA l⁻¹, 1.0 mmol EGTA l⁻¹, 1% (v/v) Triton-X100, 2.5 mmol sodium pyrophosphate l⁻¹, 1.0 mmol β-glycerophosphate l⁻¹, 1.0 mmol Na₃VO₄ l⁻¹, 1.0 µg leupeptin ml⁻¹ and 1.0 mmol phenylmethylsulphonyl fluoride (PMSF) l⁻¹) (Sigma). All chemicals except PMSF were purchased from New England Biolabs (Tozer Road, Beverly, MA). After suspension of the oocytes, the samples were frozen in liquid nitrogen and then sonicated

using an ultrasonic disruptor (UD-200, TOMY, Tokyo) fitted with CUP HORN (CH-0633, TOMY) three times for 25 s each at 1°C. The oocyte extracts were frozen and stored at -80°C just before use.

In vitro p34^{cdc2} kinase assay

The p34^{cdc2} kinase assay was performed according to the method described by Shimada and Terada (2001). Five microlitres of oocyte extract (containing ten oocytes) was mixed with 45 µl kinase assay buffer A composed of 25 mmol Hepes buffer I⁻¹ (pH 7.5) (MBL, Nagoya), 10 mmol MgCl₂ I⁻¹ (MBL), 10% (v/v) mouse vimentin peptide solution (SLYSSPGGAYC) (MBL) and 0.1 mmol ATP I⁻¹ (Sigma); the mixture was incubated for 30 min at 30°C. The reaction was terminated by the addition of 200 µl PBS containing 50 mmol EGTA I⁻¹ (MBL). The phosphorylation of mouse vimentin peptides was detected using an ELISA (MESACUP cdc2 kinase assay kit (MBL, code no. 5234)). Data were expressed as the fold strength of p34^{cdc2} kinase activity in oocytes just after collection from their follicles. Each independent experiment was repeated four times.

In vitro MAP kinase assay

A p44/42 MAP kinase assay kit (New England BioLabs) was used for measuring MAP kinase activity. The methods used for the MAP kinase assay were based on those reported by Anas *et al.* (2000). Five microlitres of oocyte extract (containing 20 oocytes) was mixed with 25 µl kinase assay buffer B (25 mmol Tris (pH 7.5) I⁻¹, 5 mmol β-glycerophosphate I⁻¹, 2 mmol dithiothreitol I⁻¹, 0.1 mmol Na₃VO₄ I⁻¹ and 10 mmol MgCl₂ I⁻¹), 0.1 mmol ATP I⁻¹ (Sigma) and 2 µg Elk 1 fusion protein, and the mixture was incubated for 30 min at 30°C. Chemicals except for ATP were purchased from New England Biolabs. The reaction was terminated by the addition of 10 µl of 4 × Laemmli sample buffer; the samples were boiled at 100°C for 5 min, and then subjected to 12.5% SDS-PAGE. The phosphorylation of Elk 1 fusion protein was detected by immunoblot analysis and chemiluminescence detection using anti-phospho-specific Elk 1 antibody. MAP kinase activity is expressed relative to a positive control; 5 ng active MAP kinase is defined as 100. Each independent experiment was repeated four times.

Cryopreservation of boar epididymal semen

A pair of testes and epididymides from a 2 year old boar (Gottingen miniature pig) was obtained at a local abattoir and transported to the laboratory at 25°C within 90 min. Luminal fluid containing spermatozoa was extruded from the distal portion of the cauda epididymidis by air pressure from a syringe. The fluid was diluted with 10 ml modified Hulsenberg VIII diluent (washing medium) according to the method described by Zeng *et al.* (2001) and rewarmed to 25°C. Sperm suspension was centrifuged in a 12 ml plastic tube at 800 g for 10 min and the supernatant discarded. The

precipitated spermatozoa were resuspended in 10 ml washing medium, and then maintained at 25°C for 2 h and centrifuged at 800 g for 5 min at 25°C to remove the washing medium. The sperm precipitates were diluted in modified BF5 extender (Zeng *et al.*, 2001) to a final concentration of 5 × 10⁸ spermatozoa ml⁻¹ and cooled to 5°C within 3 h. Spermatozoa resuspended in BF-5 extender were then mixed with an equal volume of BF-5 extender supplemented with 4% (v/v) glycerol as cryoprotectant. After equilibration in glycerol for 15 min, the sperm suspensions were frozen into pellets of 0.2 ml on solid CO₂. After 30 min, the pellets were transferred into liquid nitrogen for storage.

IVF

After 44 h of IVM of oocytes in the medium covered with or without mineral oil, cumulus cells were removed with 0.01% (w/v) hyaluronidase (Sigma) in the maturation medium and washed three times with the fertilization medium: modified Tris-buffered medium (mTBM) supplemented with 0.1% (w/v) BSA (Fraction V; Sigma A 7888) and 2 mmol caffeine I⁻¹ (Sigma). After washing, 20 oocytes were placed in 50 µl drops of the fertilization medium that had been covered with mineral oil in a 35 × 10 mm² polystyrene culture dish (Becton Dickinson and Co, Lincoln Park, NJ). The dishes were kept in the incubator for about 30 min until spermatozoa were added for fertilization. Frozen epididymal spermatozoa were thawed and washed by centrifugation at 700 g for 5 min in washing medium: mTBM supplemented with 0.1% (w/v) BSA (Fraction V; Sigma A 7888). The sperm pellet was resuspended and pre-cultured for 60 min in pre-culture medium: mTBM supplemented with 10% (v/v) fetal calf serum and 2 mmol caffeine I⁻¹. The concentration of spermatozoa during pre-culture was 2 × 10⁸ cells ml⁻¹. The pre-cultured spermatozoa were diluted to 2 × 10⁶ cells ml⁻¹ in the fertilization medium and 50 µl of this sperm suspension was added to 50 µl of the fertilization medium that contained 20 oocytes (final concentration of spermatozoa, 1 × 10⁶ cell ml⁻¹). Oocytes were co-cultured with spermatozoa for 6 h at 39°C in an atmosphere of 5% CO₂ in air. The mTBM used for IVF was essentially the same as that used by Abeydeera and Day (1997). As the pH of basic mTBM just after preparation is about 9.8–10.0, final IVF medium was kept in the incubator (an atmosphere of 5% CO₂ in air at 39°C) for 18–24 h to stabilize the pH to 7.2–7.3 before use.

In vitro production of embryos

After sperm–oocyte co-incubation, putative zygotes were washed three times in *in vitro* production medium and transferred to 100 µl drop of the same medium. The *in vitro* production medium was NCSU 37 containing 0.4% BSA (Fraction V, Sigma, A 8022). At 12, 48 and 144 h after IVF, the proportions of pronuclear formation, cleavage rate and blastocyst formation, respectively, were evaluated.

Table 1. Concentration of steroid hormones in the medium in which pig cumulus–oocyte complexes (COCs) were cultured for 44 h covered or not covered by mineral oil

Medium	Progesterone (ng ml ⁻¹)	Testosterone (ng ml ⁻¹)	Oestradiol (ng ml ⁻¹)
Covered by 1 ml mineral oil	2.5 ± 0.4	3.3 ± 0.8	0.7 ± 0.1
Not covered by mineral oil	64.2 ± 5.4*	5.3 ± 1.4	1.2 ± 0.2

Values are mean ± SEM of four replicates

*Significantly different from value for medium covered by mineral oil ($P < 0.05$).

Fig. 1. Time-dependent changes in nuclear status of pig oocytes cultured in medium either covered by 1 ml mineral oil or without mineral oil covering. Cumulus–oocyte complexes (COCs) cultured in medium (◆) with oil or (■) without oil that reached the germinal vesicle breakdown (GVBD) stage; COCs cultured in medium (▲) with oil or (●) without oil that reached the MII stage. *Significant difference compared with oocytes matured in the medium covered with oil ($P < 0.05$). Values are mean ± SEM of four replicates.

Statistical analysis

Statistical analyses of all data from three or four replicates were carried out by one-way ANOVA followed by Duncan's multiple ranges test using STATVIEW (Abacus Concepts, Inc, Berkeley, CA). All percentage data were subjected to arc-sine transformation before statistical analysis.

Results

The concentrations of progesterone, testosterone and oestradiol in media produced by cumulus cells surrounding oocytes during meiotic maturation

The concentration of progesterone in the medium in which COCs were cultured under mineral oil was significantly lower compared with the concentration in the medium in which COCs were cultured for 44 h without the oil overlay (Table 1). Both testosterone and oestradiol concentrations were slightly higher in the medium without the mineral oil overlay in comparison with that of medium covered by the oil, although these differences were not significant.

Time course of GVBD and meiotic progression reaching the MII stage of oocytes cultured in medium covered with or without mineral oil

A small proportion of the oocytes cultured in the medium without the mineral oil overlay underwent GVBD at 16 h of

culture, but this did not occur in the oocytes cultured in medium covered with mineral oil (Fig. 1). In oocytes cultured in medium covered with mineral oil, GVBD occurred at 20 h of culture. The proportion of oocytes that underwent GVBD after culture for 20, 24 and 28 h in medium covered with mineral oil was significantly lower than that of oocytes cultured in medium without the oil overlay ($P < 0.05$). However, at 32 h of culture, COCs produced a similar proportion of oocytes at the GVBD stage in each cultivation system.

At 40 h of culture, the proportion of oocytes that reached the MII stage in medium without the oil overlay was significantly higher ($P < 0.05$) than that of oocytes cultured in medium covered with oil; a further 4 h of culture (44 h) was required for a similar proportion of oocytes to reach the MII stage when cultured in medium covered with oil.

Time-dependent changes of p34^{cdc2} kinase activity and MAP kinase activity in oocytes cultured in medium covered with or without mineral oil

At 20 h of culture, p34^{cdc2} kinase activity was low in oocytes cultured in medium covered with mineral oil (Fig. 2); whereas a significant increase in kinase activity was observed, reaching a peak at 28 h of culture, in the oocytes cultured for 20 h without the oil overlay (Fig. 2). In oocytes cultured in medium without the oil overlay, the activity of p34^{cdc2} kinase significantly decreased ($P < 0.05$) at 36 h and then increased until it reached a second peak at 40–44 h of maturation (Fig. 2). In the oocytes cultured in medium covered with the mineral oil, p34^{cdc2} kinase activity was also increased at 24 h, reaching maximum activity at 28 h, and remained high for up to 44 h (Fig. 2). A significant difference in the activity of p34^{cdc2} kinase was observed between the oocytes cultured in medium covered with and without mineral oil only at 20, 24, 28 and 36 h of maturation (Fig. 2).

At 24 h of culture of COCs in medium covered with mineral oil, a low MAP kinase activity was detected in the oocytes (Fig. 3). However, a significant increase in this activity was observed in oocytes cultured at 24 h without the oil overlay (Fig. 3). MAP kinase activity of oocytes cultured in medium without the oil overlay was significantly higher compared with that in COCs cultured in medium covered with oil at 28 h or 32 h of culture (Fig. 3). MAP kinase activity of oocytes cultured in the medium covered

Fig. 2. Time-dependent changes of p34^{cdc2} kinase in pig oocytes cultured in medium covered by 1 ml mineral oil (■) or without the oil covering (□). Cumulus–oocyte complexes (COCs) were cultured in medium either covered by mineral oil or without mineral oil overlay. Data are expressed as fold strength of p34^{cdc2} kinase activity in oocytes immediately after collection from the follicles. *Significant difference compared with oocytes matured in the medium covered with oil ($P < 0.05$). Values are mean $\pm$ SEM of four replicates.

Fig. 3. Time-dependent changes of microtubule-associated protein (MAP) kinase activity in pig oocytes cultured in the medium covered by 1 ml mineral oil (■) or without the oil covering (□). Cumulus–oocyte complexes (COCs) were cultured in medium either covered by mineral oil or in the medium without mineral oil overlay. Activity is expressed as fold MAP kinase activity in which positive control (5 ng active MAP kinase activity) is defined as 100. *Significant difference compared with oocytes matured in the medium covered with oil ($P < 0.05$). Values are mean $\pm$ SEM of four replicates.

with mineral oil then gradually increased, and at 36 h of culture, MAP kinase activities of oocytes cultured in medium covered with and without mineral oil were not significantly different (Fig. 3).

Effects the mineral oil overlay during meiotic maturation on the pronuclear formation, cleavage rate and blastocyst formation after IVF

At 12 h after insemination, there was no difference in the incidence of maturation, penetration and monospermy

between oocytes matured in medium covered with mineral oil and those matured without the oil covering (Table 2). However, the proportion of male pronuclear formation in oocytes matured without the oil overlay was significantly higher than that in oocytes cultured in medium covered with oil. The number of spermatozoa in penetrated oocytes was slightly higher in oocytes matured in medium covered with mineral oil in comparison with that of oocytes matured without the oil covering; however, this increase was not significant. Moreover, the absence of mineral oil during meiotic maturation improved the proportion of cleaved

Table 2. Sperm penetration of pig oocytes matured in medium covered by 1 ml mineral oil (+) or not covered with mineral oil (–) for 44 h and then fertilized *in vitro*

Mineral oil	Number of oocytes examined	Percentage of oocytes				Number of spermatozoa per oocyte [‡]
		Matured	Penetrated*	Monospermy [†]	MPN formed [‡]	
+	60	83.3 ± 10.4	82.1 ± 4.7	58.1 ± 7.3	68.3 ± 2.7 ^a	1.78 ± 0.14
–	60	86.7 ± 2.9	86.7 ± 8.3	68.7 ± 5.6	88.8 ± 4.0 ^b	1.51 ± 0.18

Values are mean ± SEM of three replicates

^{ab}Values with no common superscript within columns are significantly different ($P < 0.05$).

*Percentage of oocytes matured.

[†]Percentage of matured oocytes that were penetrated.

[‡]Number of spermatozoa in penetrated oocytes.

MPN: male pronucleus.

Fig. 4. Early development *in vitro* of pig oocytes that were matured in the medium covered with (■) or without the mineral oil overlay (□) and then fertilized *in vitro*. Cumulus–oocyte complexes (COCs) were matured in medium covered by 1 ml mineral oil or in medium without mineral oil overlay. *Significant difference compared with oocytes matured in the medium covered with oil ($P < 0.05$). Values are mean ± SEM of four replicates. $n = 80$ oocytes per group.

oocytes at 2 days after insemination ($56.6 \pm 5.4\%$) and the proportion of oocytes reaching the blastocyst stage at 6 days of culture ($20.8 \pm 4.8\%$) in comparison with oocytes matured in the medium covered with oil ($35.0 \pm 4.2\%$ and $3.3 \pm 1.6\%$, respectively) (Fig. 4).

Discussion

During *in vitro* meiotic maturation of oocytes, progesterone and oestradiol are secreted by COCs of pigs (Xia *et al.*, 1994), rats (Zhang and Armstrong, 1989) and cattle (Armstrong *et al.*, 1996). In the present study, the increase in progesterone concentrations in the culture medium was produced by FSH- and LH-stimulated pig COCs; however, the high concentration of progesterone was not detected in the medium in which COCs were cultured with LH and FSH for 44 h under mineral oil. Both testosterone and oestradiol concentrations in the medium in which COCs were cultured without the oil covering were slightly higher than those in the medium covered with oil. Funahashi *et al.* (1994) reported that during *in vitro* culture of pig COCs exogenous oestradiol and progesterone were absorbed by

the paraffin oil which covered the medium. It is possible that the solubility of progesterone in mineral oil is higher than that of testosterone and oestradiol. However, Erbach *et al.* (1995) showed that zinc is a possible toxic contaminant of silicone oil in microdrop cultures of mouse embryos. A silicone oil overlay also affects bovine embryonic development (Van Soom *et al.*, 2001). Thus, the low secretion of steroid hormones by cumulus cells into the medium may be explained by the absorption of the hormones by the mineral oil or by the adverse effects of toxic compounds in mineral oil on the production of steroid hormones.

The high concentrations of steroid hormones secreted by COCs accelerated GVBD and activation of p34^{cdc2} kinase in oocytes cultured without the oil overlay in comparison with oocytes cultured under mineral oil. Osborn *et al.* (1986) reported that the addition of aminoglutethimide into gonadotrophin-containing medium produced low concentrations of progesterone and an increase in the proportion of oocytes arrested at the germinal vesicle stage in ovine oocytes. Shimada and Terada (2002b) showed that when pig COCs were cultured with LH, FSH and 0.5×10^{-3} mol aminoglutethimide l^{-1} , progesterone production and GVBD were almost completely inhibited, and the inhibition on GVBD was overcome by addition of progesterone. Moreover, the binding of progesterone that was secreted by LH- and FSH-stimulated cumulus cells to its receptor reduces connexin-43 content in cumulus cells (Shimada *et al.*, 2001b; Shimada and Terada, 2002b). The reduction of connexin-43 induces the reduction of gap junction communication which is necessary for GVBD in pig oocytes (Isobe *et al.*, 1998; Isobe and Terada, 2001). At this time, the concentration of progesterone secreted by FSH- and LH-stimulated cumulus cells is high (Shimada and Terada, 2002b). Thus, the high concentration of progesterone in the culture medium produced the reduction of gap junction communication in the outer layers of cumulus cells, resulting in acceleration of meiotic resumption in pig oocytes.

At the MI stage, high MAP kinase activity induces a further increase in p34^{cdc2} kinase activity in pig oocytes (Shimada *et al.*, 2001a). After GVBD in pig oocytes, a

further increase of MPF activity is required for meiotic progression beyond the MI stage (Naito *et al.*, 1992). Increasing MPF activity stimulates the ubiquitin proteolysis pathway which induces degradation of cyclin B, and triggers a decrease in MPF activity, leading to meiotic progression beyond the MI stage (Glotzer *et al.*, 1991; Hampl and Eppig, 1995). The present study showed that after GVBD, MAP kinase activation was accelerated, and p34^{cdc2} kinase activity was higher in oocytes cultured in the presence of a high concentration of steroid hormones in comparison with oocytes cultured under the oil overlay. Moreover, a decline in p34^{cdc2} kinase activity was detected in oocytes cultured for 36 h with a high concentration of steroid hormones. Asynchronous meiotic progression to the MII stage was observed in oocytes cultured in medium covered with mineral oil, which was also observed in other studies with pig oocytes (Motlik and Fulka, 1976; Funahashi and Day, 1993). Therefore, the decrease in mean activity of p34^{cdc2} kinase in the oocytes cultured under the mineral oil cannot be detected by *in vitro* kinase assay using 10 oocytes. These results support the hypothesis that high concentrations of the steroid hormones produce an earlier activation of MAP kinase after GVBD, which accelerates further increase of p34^{cdc2} kinase activity at the MI stage and induces more synchronous meiotic progression to the MII stage.

The secretion of progesterone by FSH- and LH-stimulated cumulus cells plays an important role in the acquisition of developmental competence in rat oocytes (Zhang and Armstrong, 1989). Moreover, meiosis-activating sterols which were purified from human follicular fluid (4,4-dimethyl-5 α -cholest-8,14,24-trien-3 β -ol) stimulate meiotic and cytoplasmic maturation of human and mouse oocytes (Byskov *et al.*, 1995; Cavilla *et al.*, 2001). This sterol was synthesized by COCs in response to FSH stimulation, and was also absorbed by the oil phase that covered the maturation medium (Byskov *et al.*, 1997). In the present study, absence of mineral oil that covers the maturation medium improves the rate of early embryonic development to the blastocyst stage after IVF, concomitantly with an increase of the concentration of progesterone, oestradiol and testosterone. These results indicate that substances absorbed by mineral oils, such as progesterone, oestradiol, testosterone and meiosis-activating sterols, which are secreted by cumulus cells and oocytes, play an important role in developmental competence of *in vitro* matured pig oocytes. However, further study is required to clarify the role of substances absorbed by mineral oil in cytoplasmic maturation of pig oocytes. It is thought that culture of pig COCs *in vitro* in the medium covered by mineral oil limits the developmental competence of oocytes.

In conclusion, the concentrations of progesterone, oestradiol and testosterone in medium was increased by FSH- and LH-stimulated pig COCs. However, high concentrations of these steroid hormones were not detected in the medium in which COCs were cultured with LH and FSH for 44 h in the presence of mineral oil. The high concentration

of the steroid hormones secreted by COCs accelerated GVBD and activated p34^{cdc2} kinase and MAP kinase in oocytes in comparison with those of oocytes cultured under the mineral oil. Moreover, a decrease in p34^{cdc2} kinase activity was observed during meiotic progression beyond the MI stage in oocytes cultured under the condition of high concentration of steroid hormones secreted by COCs. In addition, in the absence of mineral oil over the maturation medium the rate of early embryonic development to the blastocyst stage after IVF was improved. These results indicate that mineral oil overlay during *in vitro* meiotic maturation reduces fully maturation of pig oocytes.

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