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Effect of caffeine on meiotic maturation of porcine oocytes

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Summary

This study was conducted to evaluate the effect of caffeine on the meiotic maturation of porcine oocytes. Oocyte–cumulus complexes were collected from slaughterhouse-derived ovaries and cultured for 24, 32 or 48 h in medium 199 supplemented with 10% fetal calf serum, 10 µg/ml FSH, 50 µg/ml sodium pyruvate and 50 µg/ml gentamicin in the presence or absence of 2.5 mM caffeine. Caffeine inhibited the meiotic resumption of pig oocytes effectively after 24 h of culture, and 95.5% of oocytes were arrested at the germinal vesicle (GV) stage (control 17.8%, $p < 0.05$). Prolonged culture with caffeine up to 32 h or 48 h, however, resulted in a significant decrease in the inhibitory effect (GV: 13.8% and 8.2%). The number of oocytes at metaphase II after 48 h of culture in the presence of caffeine was significantly lower than that in the control medium (65.3% vs 94.7%, $p < 0.05$). The withdrawal of caffeine after 24 h of culture resulted in the resumption of meiotic maturation, and the oocytes reached metaphase II after 48 h. However, the ability of caffeine-treated oocytes to develop to blastocysts after artificial activation was lower than that of the control (5.5% vs 9.1%, $p < 0.05$). Caffeine treatment significantly increased cAMP levels in the oocytes after 24 h of culture, while both Cdc2 kinase and MAP kinase activation were inhibited in the oocytes. These results suggest that caffeine, similarly to other purine derivatives, prolongs the meiotic arrest of porcine oocytes at the GV stage, perhaps by its action of increasing the cAMP level and by the suppression of Cdc2 kinase and MAP kinase activities in the oocytes.

Keywords: Caffeine, GV oocyte, Meiotic maturation, Pig

Introduction

An efficient exploitation of the female gamete pool and a shortening of the generation interval are important goals in modern animal husbandry. This requires maximizing the number of developmentally competent oocytes derived from a single female. To acquire developmental competence, oocytes have to undergo three different physiological stages in the ovaries: growth, ‘capacitation’ (acquisition of developmental competence: Mermillod *et al.*, 1999) and maturation. Oocyte growth occurs during the development of primary, secondary and preantral follicles, while oocyte

‘capacitation’ starts at the final stage of oocyte growth in the antral follicles, and maturation begins after the luteinizing hormone surge in domestic animals (Mermillod *et al.*, 1999). Under *in vitro* conditions, the process of oocyte maturation occurs spontaneously after the oocytes are removed from the follicular microenvironment (Pincus & Enzmann, 1935). The disappearance of the inhibitory factors present in the follicles induces the initiation of oocyte maturation even if the ‘capacitation’ process has not completely terminated. On this account, it is also likely that the termination of gene transcription, which is important for the regulation of the first cleavage and the onset of gene transcription in the early embryo, has not been completed in the oocytes. Such oocytes can be matured and fertilized but are not competent for development (Mermillod *et al.*, 1999). It is supposed that prolonged meiotic arrest *in vitro* may result in the termination of the ‘capacitation’ process, thus leading to an increase in developmental competence after subsequent fertilization. We investigated in the present study whether the developmental competence

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of porcine oocytes can be improved by a prolonged maturational course using a suitable inhibitor of the meiotic resumption.

Previous studies carried out in a wide variety of species have demonstrated that the meiotic arrest of oocytes is successfully maintained by cAMP analogues or phosphodiesterase inhibitors, which prevent the degradation of intracellular cAMP (Cho *et al.*, 1974; Jagiello *et al.*, 1981; Goren & Dekel, 1994; Mayes & Sirard, 2002). Moreover, purine derivatives were successfully used to maintain bovine oocytes at the germinal vesicle (GV) stage without affecting their developmental potential (Krischek & Meinecke, 2002), or to synchronize meiotic maturation in porcine oocytes (Funahashi *et al.*, 1997). In the present study, caffeine (1,3,7-trimethylxanthine), which is also a purine derivative (Butcher & Sutherland, 1962; Timson, 1977), was used to prolong the meiotic maturation of porcine oocytes.

In *in vitro* cultured mammalian oocytes, the effect of caffeine was first investigated in the mouse (Jagiello *et al.*, 1972). The authors found that doses of caffeine higher than 450 µg/ml in the culture medium inhibited oocyte maturation. Recently, similar inhibitory effects of caffeine were observed in hamster oocytes (Prather & Racowsky, 1992). These results suggest that caffeine, like other purine derivatives, probably inhibits cAMP phosphodiesterase, Cdc2 kinase activation (Racowsky, 1993; Chesnel *et al.*, 1994) and meiotic maturation. However, no study of the effect of caffeine on the meiotic process of porcine oocytes has been performed. The only available report revealed that this reagent delays the aging process of matured porcine oocytes (Kikuchi *et al.*, 2000). In the present study, we examined the effects of caffeine on meiotic maturation, cAMP level, and Cdc2 kinase and MAP kinase activities in porcine oocytes; their subsequent development to the blastocyst stage was also examined.

Materials and methods

Collection and maturation of oocytes

Porcine oocytes were obtained from the ovaries of prepubertal gilts at a local abattoir and transported to the laboratory at 38.5 °C. Oocyte–cumulus complexes were aspirated from antral follicles (2–6 mm), and only oocytes surrounded with several layers of cumulus cells were chosen for the following experiments. Oocytes were cultured in 4-well plates (Nunc, Denmark) in 500 µl of culture medium at 38.5 °C under 5% CO₂ in air. The culture medium was medium 199 (M4530; Sigma, CR) supplemented with 10% fetal calf serum (Gibco Invitrogen, UK), 10 µg/ml of follicle stimulating hormone (FSH) (from porcine pituitary;

Sigma), 50 µg/ml of sodium pyruvate and 50 µg/ml of gentamicin. All manipulation before the culture was done in M2 medium (M7167; Sigma) and was completed within 1.5 h.

The influence of caffeine on meiotic maturation was investigated by culturing porcine oocytes for 24, 32 or 48 h in medium supplemented with 2.5 mM caffeine (Sigma). To determine the reversibility of the effect, oocytes were first cultured for 24 h with 2.5 mM caffeine, then washed three times in M2 medium, and further cultured for 8 h or 24 h in culture medium without caffeine.

At the end of the culture, oocytes were mounted on slides, fixed with acetic alcohol (3:1, v/v) for at least 24 h, stained with 1% orcein, and examined under a phase-contrast microscope.

Parthenogenetic activation

Oocytes at metaphase II were activated after 48 h of maturational culture. Oocytes were denuded by using a fine pipette and 0.01% hyaluronidase (from bovine testis; Sigma) in M2 medium. Subsequently, the oocytes were treated with 5 µM ionomycin (Sigma) for 5 min and then cultured in 4 mM 6-dimethylaminopurine (6-DMAP; Sigma) for 4 h. After washing, the oocytes were cultured in NCSU 23 with 4 mg/ml bovine serum albumin (BSA) (A6003; Sigma), and embryo development evaluated under a phase-contrast microscope 7 days after activation.

cAMP level in the oocytes

The cAMP level of oocytes was determined by cAMP [¹²⁵I]SPA (scintillation proximity assay) (RPA538, Amersham BioSciences, UK). After incubation with or without caffeine, the oocytes were denuded and transferred into Dulbecco's phosphate-buffered saline (PBS) containing 1 mM 3-isobutyl-1-methylxanthine (Sigma). Thereafter, groups of 30 oocytes were lysed in polypropylene tubes containing 10 µl of 10% SDS. After checking the oocyte lysis under a microscope, the samples were frozen at –80 °C. Before the cAMP level was determined, each sample was thawed and mixed with 10 µl BSA (10 mg BSA/ml). The samples were extracted by 100 µl 6% trichloroacetic acid at 4 °C and centrifuged at 2000 g for 14 min at 4 °C. The supernatants were lyophilized and used for cAMP [¹²⁵I]SPA.

Cdc2 kinase and MAP kinase double assay

After the collection and maturation of oocytes as described above, sets of two oocytes from each experimental group were denuded, washed three times in PBS containing 0.1% polyvinyl alcohol (PBS-PVA) and transferred into polypropylene tubes

Table 1 Effect of caffeine on germinal vesicle breakdown of porcine oocytes

Time of culture period	Caffeine concentration	No. of oocytes examined	No. (%) of oocytes	
			GV	GVBD
24 h	0 mM	90	16 (17.8) ^b	74 (82.2) ^b
	2.5 mM	66	63 (95.5) ^a	3 (4.5) ^c
32 h	0 mM	48	0	48 (100.0)
	2.5 mM	58	8 (13.8) ^{b,c}	50 (86.2) ^{a,b}
	2.5 mM (24 h)*	60	3 (5.0) ^c	57 (95) ^a

GV, germinal vesicle stage; GVBD, germinal vesicle breakdown.

* Porcine oocytes were cultured for 24 h in culture medium supplemented with 2.5 mM caffeine and subsequently for 8 h in control medium.

^{a-c} Values with different superscripts in the same column differ significantly ($p < 0.05$).

with 1 μ l of PBS-PVA. Thereafter, 4 μ l of ice-cold extraction buffer was added and the mixture was stored at -80°C . The extraction buffer was composed of 80 mM β -glycerolphosphate, 25 mM HEPES (pH 7.2), 10 mM EGTA, 15 mM MgCl_2 , 1 mM dithiothreitol (DTT), 1 mM PMSF, 0.1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ aprotinin (Sigma) and 1 $\mu\text{g}/\text{ml}$ leupeptin (Sigma) (Nebreda *et al.*, 1995). Before the kinase assay, the mixture of oocytes and extraction buffer was thawed and centrifuged at 13000 g for 2 min. To measure Cdc2 kinase and MAP kinase activities, 5 μ l of substrate solution and 5 μ l kinase buffer were added and incubated for 10 min at 37°C . The kinase buffer was composed of 75 mM HEPES (pH 7.2), 75 mM β -glycerolphosphate, 0.1 mM EGTA, 75 mM MgCl_2 , 6 mM DTT, 10 mM ATP, 15 μM cAMP-dependent protein kinase inhibitor peptide (sequence: TTYADFIASGRTGRRNAIHD, Sigma) and 0.3 $\mu\text{Ci}/\mu\text{l}$ [γ - ^{32}P]ATP (250 $\mu\text{Ci}/25 \mu\text{l}$, Amersham Pharmacia Biotech, UK). The substrate solution contained 4.25 μl of histone H1 (5 mg/ml, from calf thymus; Boehringer, Tokyo, Japan) as a substrate of Cdc2 kinase, and 0.75 μl of myelin basic protein (MBP) (5 mg/ml, from bovine brain; Sigma) as a substrate of MAP kinase. Thereafter, the reaction was terminated by the addition of 5 μl of 4 $\times$ concentrated SDS sample buffer (Laemmli, 1970), and the samples were boiled for 5 min at 100°C . For the separation of labelled histone H1 and MBP 15% SDS-polyacrylamide gel was used. After being run, the gels were dried and autoradiographed.

Statistical analysis

The data in tables were compared using the chi-square test. The cAMP assay and the densitometric scan of double kinase assay bands were analysed using one-way ANOVA followed by the Newman-Keuls multiple comparison test. Every experiment was repeated at least four times. Differences at $p < 0.05$ were considered to be statistically significant.

Results

Effect of caffeine on the meiotic maturation of oocytes

Caffeine at a 2.5 mM concentration significantly inhibited the germinal vesicle breakdown (GVBD) of porcine oocytes over a 24 h period (Table 1). Treated oocytes remained at the GV stage with a nucleolus and ring- or horseshoe-shaped heterochromatin around the nucleolus (Fig. 1), while almost all of the control oocytes underwent GVBD. However, the inhibitory effect was reduced with prolonged culture, and after 32 h of the caffeine treatment the majority of oocytes (86.2%)

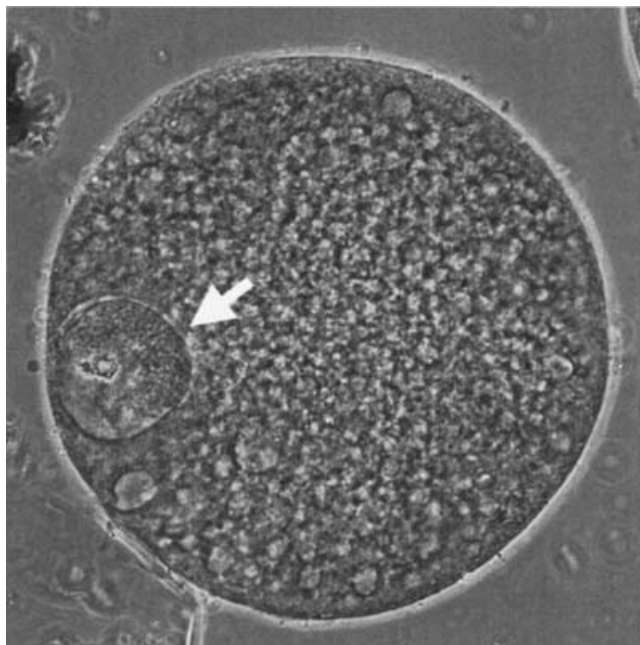


Figure 1 A porcine oocyte treated with 2.5 mM caffeine after 24 h of culture. The arrow shows a germinal vesicle with a nucleolus and ring-shaped heterochromatin around the nucleolus.

Table 2 Effect of caffeine on meiotic maturation of porcine oocytes after 48 h of culture

Treatment	No. of oocytes examined	No. (%) of oocytes		
		GV	MI	MII
Caffeine	49	4 (8.2)	13 (26.5) ^a	32 (65.3) ^b
Caffeine 24 h*	54	0	3 (5.6) ^b	51 (94.4) ^a
Control	57	0	3 (5.3) ^b	54 (94.7) ^a

GV, germinal vesicle stage; MI, the first metaphase; MII, the second metaphase.

* Porcine oocytes were cultured for 24 h in culture medium supplemented with 2.5 mM caffeine and subsequently for 24 h in control medium.

^{a,b} Values with different superscripts in the same column differ significantly ($p < 0.05$).

underwent GVBD. Nevertheless, when the caffeine treatment was prolonged to 48 h (Table 2), the number of oocytes at metaphase II (MII) was significantly lower than the number of control oocytes (65.3% vs control 94.7%, $p < 0.05$). On the other hand, the number of caffeine-treated oocytes that matured to MII was similar to the number of control oocytes that had done so after 48 h when the caffeine was withdrawn from the culture medium after 24 h (94.4%).

cAMP level in caffeine-treated oocytes

Caffeine treatment significantly increased cAMP levels in the oocytes (Fig. 2). Oocytes achieved the maximal level of cAMP after 24 h of culture with 2.5 mM caffeine. When the culture of oocytes in caffeine-

supplemented medium was prolonged to 32 h, the cAMP level decreased, and the level was comparable to that in untreated oocytes at the GV stage. Similar levels were observed in the oocytes when they were cultured with caffeine for 24 h and subsequently cultured in control medium for 8 h.

Cdc2 kinase and MAP kinase activity

As shown in Fig. 3, caffeine in the culture medium significantly inhibited the activation of both kinases in the oocytes after 24 h. However, when the culture was prolonged up to 32 h, Cdc2 kinase activity reached a level similar to that in the control, and MAP kinase was also activated. After 32 h or 48 h, both kinases showed high levels irrespective of the presence of caffeine in the culture medium during the first 24 h. Suppression of the activities of both kinases occurred at the same time that cAMP in the oocytes achieved the maximal level.

Parthenogenetic development

To evaluate the effect of caffeine on the developmental competence of porcine oocytes, we parthenogenetically activated the oocytes that had been cultured for 24 h in medium with 2.5 mM caffeine and then in normal culture medium for 24 h. The majority of the oocytes 19 h after ionomycin and 6-DMAP treatment had only one pronucleus and one polar body (46/51, data not shown), which indicates that the parthenogenetically activated embryos were diploid. The developmental competence of parthenogenetically activated oocytes is summarized in Table 3. The number of blastocysts

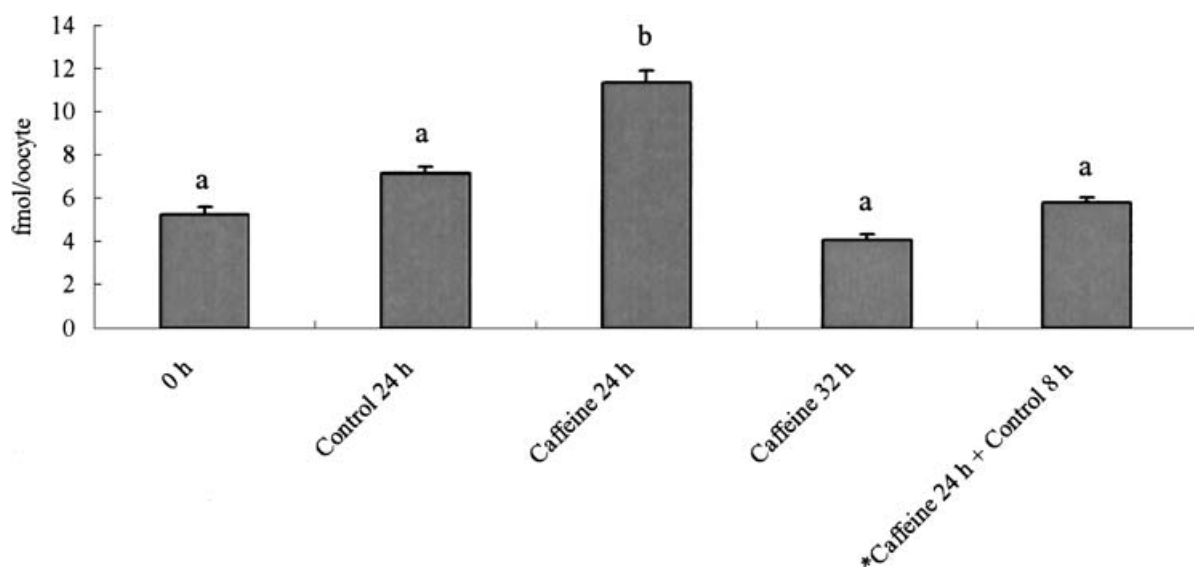


Figure 2 Effect of caffeine on cAMP levels in porcine oocytes. *Oocytes were cultured for 24 h in culture medium supplemented with 2.5 mM caffeine and subsequently for 8 h in control medium. Columns with different subscripts differ significantly ($p < 0.05$).

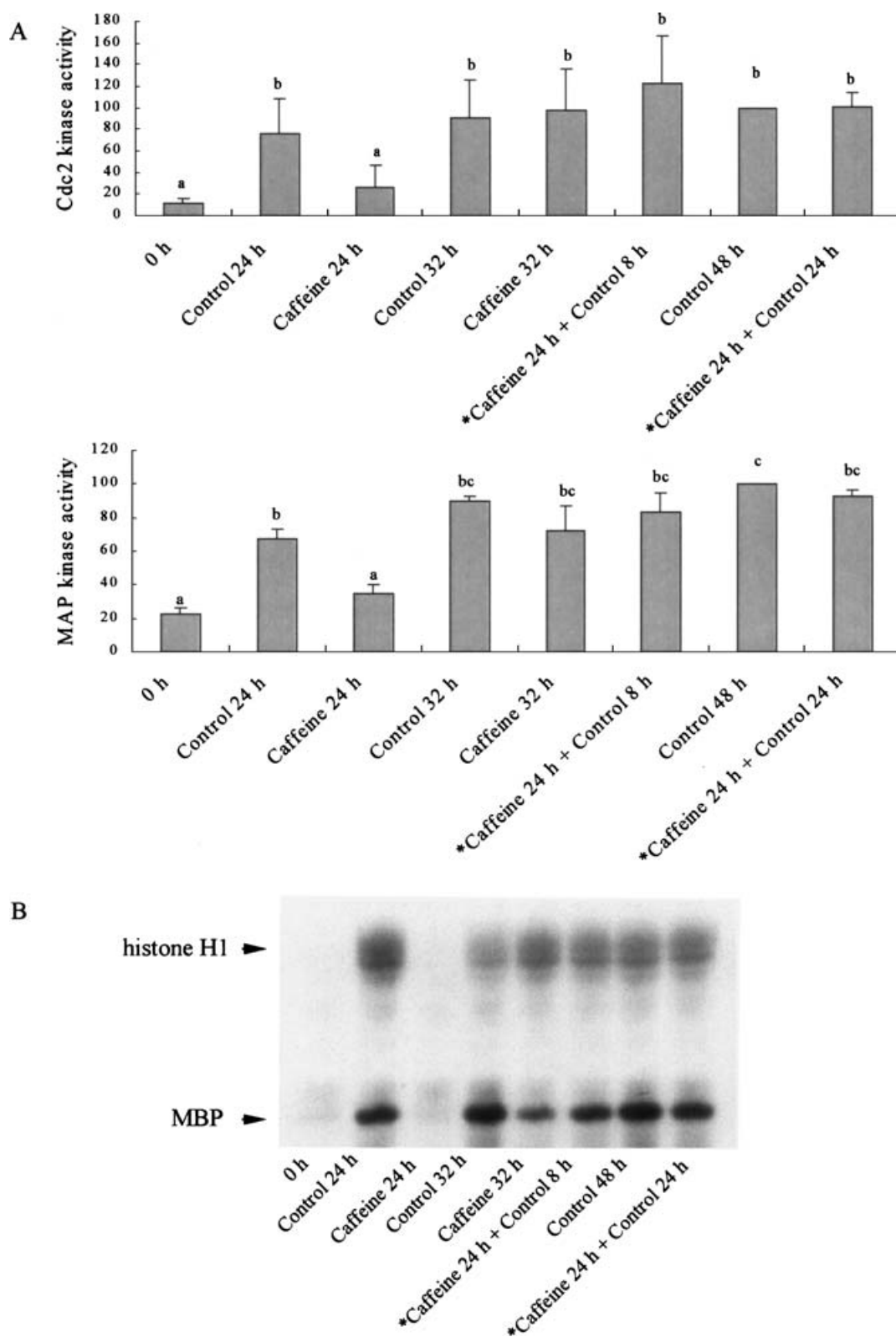


Figure 3 Effect of caffeine on Cdc2 kinase and MAP kinase activities in porcine oocytes. (A) The relative intensity of the bands as determined by scanning densitometry. The values of control 48 h were defined as 100% in each experiments. Columns with different subscripts differ significantly ($p < 0.05$). *Oocytes were cultured for 24 h in culture medium supplemented with 2.5 mM caffeine and subsequently for 8 h or 24 h in the control medium. (B) An autoradiograph showing Cdc2 kinase and MAP kinase activities in caffeine-treated oocytes. Changes in the activities of Cdc2 kinase and MAP kinase were detected by the phosphorylation of histone H1 and myelin basic protein (MBP) as their respective *in vitro* substrates.

Table 3 Development of caffeine treated oocytes after parthenogenetic activation

Treatment	Total no. of activated oocytes	No. (%) of blastocysts	No. (%) of degenerated oocytes
Caffeine*	400	22 (5.5) ^a	378 (94.5) ^a
Control	428	39 (9.1) ^b	389 (90.9) ^b

* Porcine oocytes were cultured for 24 h in culture medium supplemented with 2.5 mM caffeine and subsequently for 24 h in control medium.

^{a,b} Values with different superscripts in the same column differ significantly ($p < 0.05$).

derived from caffeine-treated oocytes was significantly lower than in the control group (5.5% vs control 9.1%, $p < 0.05$).

Discussion

The present study demonstrates that caffeine maintains GV arrest in porcine oocytes during the first 24 h of maturation culture, and thus effectively inhibits meiotic resumption. The majority of caffeine-treated oocytes had a germinal vesicle with a nucleolus surrounded by ring- or horseshoe-shaped chromatin. This result corresponds with the results of the synchronization of isolated porcine oocytes at the GV stage by another purine derivative, dbcAMP (Funahashi *et al.*, 1997; Mattioli *et al.*, 1994). In these earlier studies, the presence of dbcAMP in the medium completely inhibited the meiotic resumption of cumulus-enclosed porcine oocytes for 20 h, and its inhibitory effect was reversible. Similarly, we found that the inhibitory effect of caffeine was reversible, since the treated oocytes progressed normally to MII after the withdrawal of the caffeine. The findings support the hypothesis that the meiotic maturation of mammalian oocytes can be blocked reversibly by the introduction of membrane-permeable cAMP derivatives or phosphodiesterase inhibitors into the culture medium. Meiotic resumption of bovine and porcine oocytes was inhibited by the phosphodiesterase inhibitor IBMX (Luciano *et al.*, 1999) and the adenylate cyclase activator forskolin (Racowsky, 1985; Rose-Hellekant & Bavister, 1996). Moreover, our data also support the results of a study using hamster oocytes, where caffeine at the concentrations of 0.17–2 mM induced meiotic arrest dose-dependently (Prather & Racowsky, 1992).

Caffeine increased the cAMP level in porcine oocytes. After 24 h of culture in caffeine-supplemented medium, the intra-oocyte cAMP level was significantly higher than that in the control group; then the level

decreased after an additional 8 h culture in normal medium (total 32 h of culture). Although caffeine completely inhibited the meiotic maturation of oocytes during the first 24 h of culture, the inhibitory effect was reduced and the oocytes resumed their meiotic maturation in the prolonged culture. This finding suggests that the decrease in cAMP level correlates with the initiation of the meiotic maturation of porcine oocytes. Previous studies have found that oocyte meiotic resumption depends on a decreased concentration of cAMP followed by an inactivation of cAMP-dependent protein kinase (PKA) (Cho *et al.*, 1974; Kim & Menino, 1995; Fan *et al.*, 2002). On this account, we conclude that caffeine maintained the meiotic arrest of porcine oocytes by elevating the cAMP level.

The activation of PKA due to the elevation of the cAMP level in porcine oocytes is also likely to be the reason for the suppressed Cdc2 kinase activity after 24 h of culture in caffeine-supplemented medium. In previous studies it has been suggested that PKA prevents the activation of Cdc2 kinase by maintaining its catalytic subunit p34^{cdc2} in phosphorylated form (for review see Downs, 1995). In *Xenopus*, PKA is thought to phosphorylate some unidentified protein that directly or indirectly suppresses *c-mos* translation, which is required for the activation of MAP kinase (Matten *et al.*, 1994; Sagata, 1997). It has also been suggested that MAP kinase activity in porcine oocytes may be inhibited by the elevation of the cAMP level (Fan *et al.*, 2002; Shimada & Terada, 2002). In our experiments, MAP kinase activity was suppressed at the same time as Cdc2 kinase, when cAMP achieved a maximal level in the oocytes. In addition, MAP kinase activity in mammalian oocytes is supposed to be affected by phosphatidylinositol 3-kinase (PI-3K) (Shimada *et al.*, 1998; Shimada & Terada, 2002). The inactivation of PI-3K in cumulus cells efficiently suppresses MAP kinase activity and GVBD in porcine oocytes. Because caffeine is known to be an inhibitor of PI-3K in somatic cells (Foukas *et al.*, 2002), we assume that the activity of MAP kinase and GVBD were suppressed in porcine oocytes via the elevation of PKA activity as well as the inhibition of PI-3K in cumulus cells by caffeine.

The cAMP level in porcine oocytes significantly decreased after 32 h irrespective of the presence of caffeine in the culture medium. At the same time, Cdc2 kinase and MAP kinase activities increased. This result probably means that the activities of these kinases correlate with the cAMP levels. Shimada & Terada (2002) have suggested that cAMP is transported into porcine oocytes from cumulus cells via gap junctions, and the reduction in cAMP transfer results in a decrease of the oocyte cAMP level during meiotic maturation. Based on their findings we assume that the decrease of

the cAMP level after 32 h is due to a disruption of gap junctional communication between cumulus cells and oocytes.

A previous study using *in vitro* matured and fertilized porcine oocytes demonstrated that development to the blastocyst stage was almost double in the oocytes that were exposed to dbcAMP during the first 20 h of maturation culture as compared with the controls (Funahashi *et al.*, 1997). Since caffeine showed an effect on meiotic resumption that was comparable to that of dbcAMP, we examined the developmental ability of the caffeine-treated oocytes after artificial activation. In contrast to dbcAMP, the presence of caffeine in the maturation medium during the first 24 h resulted in a decrease in the number of blastocysts. This difference may result from an effect of caffeine other than phosphodiesterase inhibition, such as PI-3K inhibition, that affects the ability of porcine oocytes to develop to blastocysts.

In summary, the present study demonstrates that caffeine prolonged the meiotic arrest of porcine oocytes at the GV stage by increasing the cAMP level in the oocytes. We also proved that caffeine temporarily inhibits Cdc2 kinase and MAP kinase activity in the oocytes.

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