Effect of 0.5 mM Dibutyryl cAMP on Meiotic Maturation during Different Incubation Time and Embryonic Development Following *In Vitro* Fertilization or Parthenogenetic Activation in Porcine Oocytes

Il-jeoung Yu[*]

Department of Theriogenology & Reproductive Biotechnology, College of Veterinary Medicine & Bio-Safety Institute, Chonbuk National University, Jeonju 561-756, Korea

ABSTRACT

Presently, the effect of 0.5 mM dibutyryl cAMP (dbcAMP)-supplemented maturation medium during different incubation time on meiotic arrest (germinal vesicle) and resumption (metaphase II) of porcine oocytes and embryonic development of porcine oocytes following *in vitro* fertilization (IVF) or parthenogenetic activation (PA) was determined. Porcine cumulus oocyte complexes (COCs) were cultured in 0.5 mM dbcAMP for 17, 22, 27, or 42 h, and an additional 22 h without 0.5 mM dbcAMP. The nuclear status was examined at each time point. Oocytes cultured from 39~49 h displayed more than 80% meiotic resumption. More than 85 % of meiotic arrest was presented at 17~22 h. Oocytes were cultured for 22 h with 0.5 mM dbcAMP and additional 22 h without dbcAMP to assess developmental potential following IVF or PA. There were no significant differences in blastocyst rates among the dbcAMP-IVF, IVF, dbcAMP-PA, and PA groups, although cleavage rate of IVF group was significantly higher than those of dbcAMP-PA, and PA groups. In conclusion, 0.5 mM dbcAMP influenced meiotic maturation of porcine oocytes depending on incubation time of oocyte, although embryonic development was not improved in both IVF and PA.

(Key words : dibutyryl cAMP, porcine oocyte, meiotic arrest and resumption, embryonic development)

INTRODUCTION

In vitro matured oocytes have become dominant in porcine developmental technologies, replacing *in vivo*-derived oocytes. Maturation of oocytes may affect embryonic development after nuclear transfer, both in parthenogenetic activation (PA) as well as *in vitro*-fertilized (IVF) embryos (Nagashima *et al*., 2003). However, the developmental competence of oocytes collected from various sized follicles might be different (Yoshida *et al*., 1989; Funahashi and Day, 1993; Blondin and Sirard, 1994; Funahashi *et al*., 1997; Wang *et al*., 1998).

Synchronization of oocytes before maturation *in vitro* is essential to simultaneously complete DNA transcription and nuclear maturation (Mermillod *et al*., 2000; Sirad, 2001). Oocytes in arrest at the germinal vesicle (GV) during synchronization can obtain the time necessary to mature *in vitro* and for further developmental competence of embryos (Funahashi *et al*., 1997; Hashimoto *et al*., 2002; Ponderato *et al*., 2002; Wu *et al*., 2002).

Cyclic adenosine monophosphate (cAMP) is the principal regulator of the oocyte meiotic cell cycle (Taieb *et al*., 1997; Ferell, 1999; Bagg *et al*., 2006). *In vivo*, decreased cAMP content in oocytes is associated with the initiation of germinal vesicle breakdown (GVBD) (Mattioli *et al*., 1994). In other words, a drop of the oocyte cAMP level is involved in the resumption of meiosis. However, physical removal of oocytes from their follicles can induce a premature reduction of oocyte cAMP content during *in vitro* maturation. This adversely affects coordination of nuclear maturation and cytoplasmic maturation, and subsequently developmental potential (Bagg *et al*., 2006). High intracellular levels of cAMP maintain oocyte meiotic arrest at the GV stage by addition of an analogue, dibutyryl cyclic adenosine monophosphate (dbcAMP), into the medium for the *in vitro* maturation of oocytes (Cho *et al*., 1974; Bagg *et al*., 2006; Kim *et al*., 2008). An effective meiosis-inhibiting agent in pig oocytes is dbcAMP (Bagg *et al*., 2006; Kim *et al*., 2008). The presence of dbcAMP increases the synchrony of meiotic progression and the incidence of blastocyst formation following IVF (Funahashi *et al*., 1997; Somfai *et al*., 2003). However, a constant concentration of 1 mM has been used to arrest porcine oocytes, although dbcAMP is linked with oocyte maturation in studies that focused on the effect of dbcAMP on embryonic development by comparing oocytes treated with

---

[*] Correspondence : E-mail : iyu@jbnu.ac.kr

dbcAMP or dbcAMP-free oocytes (Funahashi *et al*., 1997; Miyoshi *et al*., 2002; Bagg *et al*., 2006; Kim *et al*., 2008). I demonstrated that 0.5 mM dbcAMP could be efficient concentration to arrest oocyte meiosis (data unpublished).

The present study was carried to determine the effect of 0.5 mM dbcAMP supplemented to maturation medium during different incubation time of oocytes on meiotic arrest and resumption of porcine oocytes and to assess the effect of 0.5 mM dbcAMP on subsequent developmental competence of embryos fertilized *in vitro* or activated parthenogenetically.

MATERIALS AND METHODS

1. Culture Media

Unless otherwise noted, all chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA). The medium used for collection and washing of porcine oocyte-cumulus complexes (COCs) was modified Tyrode's lactate-HEPES-polyvinyl alcohol (TL-HEPES-PVA) composed of 114 mM NaCl, 3.2 mM KCl, 0.4 mM $Na_2H_2PO_4$, 2 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 5 mM $NaHCO_3$, 20 mM HEPES, 16.6 mM sodium lactate (60% syrup), 0.5% (w/v) PVA, 10 IU/ml penicillin, and 10 μg/ml streptomycin. Oocyte maturation was accomplished in North Carolina State University 23 medium (NCSU-23; Gil *et al*., 2007) supplemented with 10% porcine follicular fluid (PFF), 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml pregnant mare serum gonadotropin (PMSG), and 10 IU/ml human chorionic gonadotropin (HCG). PFF was collected from ovarian follicles that ranged from 3~6 mm in diameter by centrifugation at 1,600 ×g for 30 min at room temperature and filtration through a 1.2 μm syringe filter. The final product was stored at −20℃ until use. Fertilization medium, based on modified Tris-buffered medium (mTBM; 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (Trizma Base), 11 mM D-glucose, and 5 mM sodium pyruvate), was supplemented with 2 mM caffeine and 0.2% bovine serum albumin (BSA). *In vitro* culture 1 (IVC 1) medium was composed of D-glucose-free NCSU-23 supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate (60% syrup), and 0.4% BSA (A-6003). *In vitro* culture 2 (IVC 2) medium was NCSU-23 containing 0.4% BSA.

2. *In Vitro* Maturation

Porcine ovaries were collected from a local abattoir and were transported to the laboratory at 34~36℃ in 0.9% saline su-

pplemented with 100 IU/ml penicillin G and 100 μg/ml streptomycin. COCs were aspirated through an 18 gauge needle. Oocytes with a compact cumulus mass and a dark, evenly graduated cytoplasm were washed three times in TL-HEPES-PVA medium and maturation medium, respectively. Oocytes (n=20~25) were cultured in a 100 μl droplet of maturation medium supplemented with 10 IU/ml PMSG and 10 IU/ml HCG for 22 h, and for a further 22 h in hormone-free maturation medium. Oocyte maturation was carried out under sterile mineral oil in a humidified atmosphere of 5 % CO_2 in air at 39℃.

3. *In vitro* Fertilization Procedure

Extended spermatozoa supplied by Irae Yangdon (Jeonju, Korea) were maintained at 17℃ for 2 days. Percoll solutions and gradients were prepared as described previously (Yu *et al*., 2002). A two-layer discontinuous gradient was formed by layering 1 ml of a 45% Percoll solution on top of 1 ml of a 90% Percoll solution in a 15 ml conical tube. Aliquants of extended semen (3 ml) were layered onto Percoll gradient and centrifuged for 20 min at 850 × g at room temperature. The pellet recovered after aspiration of the supernatant was washed two times by centrifugation with 5 ml Dulbecco's phosphate buffered saline (D-PBS) supplemented with 0.1% BSA, 10 IU/ml penicillin, and 10 μg/ml streptomycin at 350 × g for 3 min. After the supernatant was discarded, motile spermatozoa were collected. Sperm concentration (spermatozoa/ml) was adjusted to 10×10^5 by dilution with mTBM. Oocytes with expanded cumulus cells were treated with 1 mg/ml hyaluronidase in TL-HEPES-PVA and were denuded by gentle aspiration with a small bore glass pipette. The denuded oocytes were washed three times with mTBM and oocytes were transferred to a mTBM insemination drop (45 μl) overlaid with mineral oil (20~25 oocytes per drop). A 5 μl volume of spermatozoa was added to each insemination drop to give a final concentration of 1×10^5 spermatozoa/ml. Oocytes and spermatozoa were co-cultured for 6 h in a humidified atmosphere of 5 % CO_2 in air at 39℃.

4. Pathenogenetic Procedure

Oocytes were parthenogenetically activated by incubation in IVC 1 medium containing 5 μM ionomycin for 5 min, washing twice, and incubation for 3 h in NCSU-23 medium supplemented with 2.0 mM 6 D-methyl amino purine (6 D-MAP).

5. *In Vitro* Culture

Following either parthenogenic activation (PA) or *in vitro*

fertilization (IVF), presumptive zygotes were washed three times, transferred to 50 μl droplets (15~20 presumptive zygotes per drop) of IVC 1 medium, and incubated for 2 days at 39℃ in a humidified atmosphere of 5 % CO_2 in air. After 2 days of embryo culture, the cleavage rate was assessed. Embryos were then washed twice, transferred to 50 μl droplets of IVC 2 medium, and incubated for 6 days, after which the blastocyst rate and cell numbers in blastocyst were determined.

5. Assessment of Meiotic Stage

Oocytes denuded by vigorous shaking in 3% sodium citrate solution were fixed in a 4-well culture dish (Nunc, Rochester, NY, USA) containing 500 μl of ethanol:acetic acid (3:1 v/v) for 72 h. They were then dispensed onto a slide to which a cover slip was added. Each sample was stained with aceto-orcein [1% (w/v) orcein in 45% (v/v) acetic acid] and destained with glycerol:acetic acid:distilled water (1:1:3, v/v). Nuclear maturation of oocytes was evaluated under a light microscope at 400 × magnification. Meiotic stages were classified as previously described (Romar and Funahashi, 2006) as being at germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI), or metaphase Ⅱ (MII).

6. Assessment of Cell Numbers in Blastocyst

Blastocysts were stained in 500 μl of Hoechst 33342 (5 μg/ ml) in TL-HEPES-PVA for 15 min at room temperature, mounted on slides, and covered with cover slips. Cell numbers in blastocyst were determined using a Nikon TE2000 fluorescent inverted microscope (Nikon, Tokyo, Japan) at 400 × magnification.

7. Statistical Analysis

Each experiment was replicated 6~7 times. All percentage data was subjected to arcsine transformation. All data and data sets are presented as the mean ± SE. Maturation of oocytes and development of porcine embryos following IVF or PA were analyzed by Duncan's multiple range test using the Statistical Analysis System ver. 8× (SAS, Cary, NC, USA). $P<0.05$ was considered to be statistically significant.

8. Experimental Design

Two series of experiment were used. The first experimental design assessed the effect of 0.5 mM dbcAMP-supplemented oocyte maturation medium during different incubation time on meiotic arrest and resumption of porcine oocyte. The oocytes

were cultured in maturation medium supplemented with 0.5 mM dbcAMP and hormones for 17, 22, 27, or 42 h, and then were cultured in dbcAMP- and hormone-free maturation medium for another 22 h. A proportion of oocytes from each group were removed, denuded, and fixed at 17, 22, 27, or 42 h; the remaining oocytes were fixed at 39, 44, 49, or 64 h after denuding cumulus cells. The meiotic stages were assessed in the populations.

The second experimental design assessed the effect of 0.5 mM dbcAMP-supplemented oocyte maturation medium on the developmental competence of embryos following PA or IVF. Oocytes were cultured in maturation medium supplemented with 0.5 mM dbcAMP and hormones for 22 h, and were then cultured in dbcAMP- and hormone-free maturation medium for another 22 h. A portion of the oocytes were denuded and used for PA, with the remainder being denuded and used for IVF. The rates of cleavage, and blastocyst, and the cell numbers in blastocyst were determined.

RESULTS AND DISCUSSION

1. Effect of 0.5 mM dbcAMP-supplemented Oocyte Maturation Medium during Different Incubation Time on Meiotic Arrest and Resumption of Porcine Oocyte

The effect of dbcAMP on reproduction in pigs has been well-studied (Funahashi et al., 1997; Miyoshi et al., 2002; Bagg et al., 2006; Kim et al., 2008). However, only 1 mM dbcAMP has been used to assess the meiosis arrest of oocytes. In previous study, I sought to more decisively determine the effective concentration of dbcAMP for meiotic arrest and resumption in porcine oocytes. I demonstrated that lower concentration of 0.5 mM dbcAMP were also effective on meiotic arrest of oocytes (data unpublished).

The proportion of oocytes arrested at the GV was significantly decreased ($P<0.05$) in a time-dependent manner (Fig. 1a). Culture time-dependent decreases in the GV rate have been reported in porcine oocytes treated with 1 mM dbcAMP (Miyoshi et al., 2002; Bagg et al., 2006). These results are consistent with these findings that the rate of meiotic arrest decreased as culture time increased. However, GV rate at 42 h decreased to 40% in this study was contrast with less than 10% GV rate at 40 h presented by Bagg et al (2006). On the other hand, Miyoshi et al (2002) indicated that GV rate at 42 h decreased to 40%. It is in agreement with results from our study. I suggested that 0.5 mM concentration of dbcAMP du-

ring longer culture period could be effective to arrest meiosis of porcine oocytes.

The proportions of oocytes in the MI and MII stages were significantly increased at 42 h ($P<0.05$). However, the proportion of oocytes in the GVBD stage was decreased at 42 h.

Meiotic resumption of oocytes incubated for 22 h in dbcAMP-free maturation medium was assessed (Fig. 1b). The proportions of MII stage oocytes at 39, 44, and 49 h were significantly higher than that of MII oocytes at 64 h ($P<0.05$). The proportions of GV and MI stages were significantly increased at 64 h ($P<0.05$). During a longer period of culture, 30% of oocytes cultured in dbcAMP for 42 h were matured to the MII
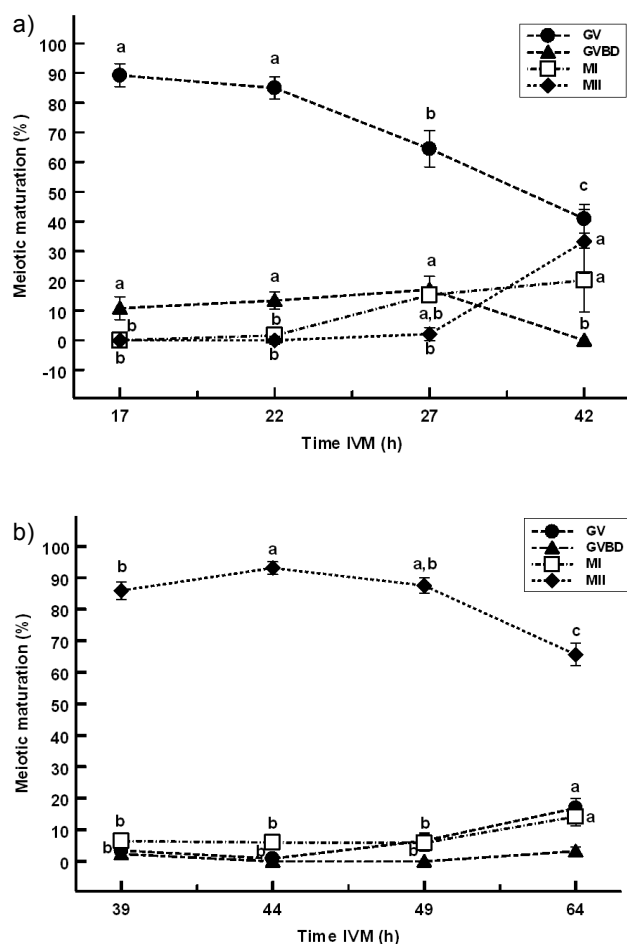

Fig. 1. Maturation of porcine oocytes. (a) Maturation of oocytes cultured in maturation medium supplemented with 0.5 mM dbcAMP for 17, 22, 27 or 42 h. (b) Maturation of oocytes cultured in maturation medium for additional 22 h after removal of dbcAMP. Different superscripts [a–c] indicate significant differences in meiotic progression at a particular time point ($P<0.05$).

stage and 35% of the remaining oocytes progressed to the MII stage even after additional 22 h culture (a total of 64 h). These results indicate that the appropriate culture time for meiosis arrest by 0.5 mM dbcAMP is between 17 and 22 h. As to the effect of subsequent culture, the highest MII rate (90%) was shown after 22 h of culture with dbcAMP and after an additional 22 h culture without dbcAMP (a total of 44 h). This result is not different with that in other studies (Miyoshi *et al*., 2002; Bagg *et al*., 2005). However, these previous studies did not determine whether culturing for more than 44 h affected meiosis resumption of oocytes. In the present study, I demonstrated that more than 80% of oocytes proceeded to MII after 27 h culture with dbcAMP and an additional 22 h culture (a total of 49 h culture).

2. Effect of 0.5 mM dbcAMP-supplemented in Oocyte Maturation Medium on Developmental Competence of PA or IVF Embryos

To determine whether the meiotic arrest of oocytes treated with 0.5 mM dbcAMP might contribute to improve embryo development, COCs were cultured with 0.5 mM dbcAMP for 22 h, cultured for an additional 22 h without dbcAMP, used for IVF or PA, and further cultured *in vitro*. The rates of cleaved embryos and blastocysts were assessed. In addition, cell numbers in blastocyst were counted. The effect of dbcAMP on embryonic development was not different regardless of dbcAMP treatment, although cleavage rate of IVF embryos was significantly higher than that of parthenogenetic embryos (72~74% vs. 55~59%, $P<0.05$). The blastocyst formation rate and mean cell numbers in blastocyst among groups were not significantly different, although blastocyst rate was the highest in dbcAMP-PA (29.2%; Table 1). As a result, 0.5 mM dbcAMP treatment of oocytes was not effective on improving the developmental competence of embryos following either parthenogenetic activation or *in vitro* fertilization. This result was contrasts with studies that 1 mM dbcAMP-mediated arrest of meiotic resumption improved the rate of development to the blastocyst stage of *in vitro* matured and fertilized porcine oocytes (Funahashi *et al*., 1997; Kim *et al*., 2008). However, Miyoshi *et al* (2002) reported that 1 mM dbcAMP treatment of porcine oocytes did not improve the development of embryos reconstructed by nuclear transfer. On the other hand, Bagg *et al* (2006) reported that 1 mM dbcAMP treatment increased subsequent blastocyst rates of prepubertal oocytes following either parthenogenetic activation or *in vitro* fertilization, whereas blastocyst rates of adult oocytes remained unchanged. I cannot explain the diffe-

Table 1. Effect of 0.5 mM dbcAMP on development of porcine embryos following IVF or PA

Groups	No. of oocytes	Cleaved (%)	Blastocyst (%)	Cell numbers of blastocysts (N)
IVF[*]	160	74.9 ± 4.9^a	20.3 ± 4.5	31.0 ± 2.3
dbcAMP-IVF	160	72.2 ± 4.9^{ab}	16.3 ± 4.3	27.8 ± 5.8
PA[**]	165	59.4 ± 2.4^b	15.0 ± 3.9	23.2 ± 2.6
dbcAMP-PA	165	55.6 ± 3.6^b	29.2 ± 6.6	25.4 ± 2.4

IVF[*]: *In vitro* fertilization; PA[**]: Parthenogenetic activation.

rence among previous studies. However, I suggest that cytoplasmic maturation can affect embryo development. Oocyte maturation includes two phenomena: nuclear maturation, involving reinitiation and completion of the first meiotic division, and cytoplasmic maturation that enables normal fertilization and further embryonic development (Marchal *et al.*, 2001). In the future study, the assay of cytoplasmic maturation using measurement of glutathione contents should be considered to determine the effect of dbcAMP on cytoplasmic maturation.

In conclusion, I have shown that 0.5 mM dbcAMP influenced meiotic maturation of porcine oocytes depending on incubation time of oocyte, although embryonic development including blastocyst formation was not improved in both IVF and PA. In addition, I suggest that the appropriate culture duration to retain the GV stage of porcine oocyte in 0.5 mM-supplemented maturation medium is between 17 and 22 h and sufficient culture time to develop to MII is 39~49 h in oocytes exposed to 0.5 mM dbcAMP.

REFERENCES

Bagg MA, Nottle MB, Grupen CG and Armstrong DT. 2006. Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of *in vitro* matured pre-pubertal and adult pig oocytes. Mol. Reprod. Dev. 73: 1326-1332.

Blodin P and Sirard MA. 1994. The influence of oocyte and follicular morphology on developmental competence in superovulated heifers. Theriogenology 41:164.

Cho WK, Stern S and Biggers JD. 1974. Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation *in vitro*. J. Exp. Zool. 187:383-386.

Ferell JE Jr. 1999. Xenopus oocyte maturation: new lessons from a good egg. Bioassays 21:833-842.

Funahashi H, Cantley TC and Day BN. 1997. Synchronization of meiosis in porcine oocytes by exposure to dibutyryl cyclic adenosine monophosphate improves developmental competence following *in vitro* fertilization. Biol. Reprod. 57:49-53.

Funahashi H and Day BN. 1993. Effect of the duration of exposure to supplemental hormones on cytoplasmic maturation of pig oocytes *in vitro*. J. Reprod. Fertil. 98:179-185.

Gil MA, Alminana C, Cuello C, Parrilla I, Roca J, Vazquez JM and Martinez EA. 2007. Brief coincubation of gametes *in vitro* fertilization: Role of sperm:oocyte ratio and post-coincubation medium. Theriogenology 67:620-626.

Hashimoto S, Minami N, Takakura R and Imai H. 2002. Bovine immature oocytes acquire developmental competence during meiotic arrest *in vitro*. Biol. Reprod. 66:1696-1701.

Kim JS, Cho YS, Song BS, Wee G, Park JS, Cho YK, Yu K, Lee KK, Han YM and Koo DB. 2008. Exogeous dibutyryl cAMP affects meiotic maturation via protein kinase A activation; it stimulates further embryonic development including blastocyst quality in pigs. Theriogenology 69:290-301.

Marchal R, Tomanek M, Terqui M and Mermillod P. 2001. Effects of cell cycle dependent kinases inhibitor on nuclear and cytoplasmic maturation of porcine oocytes. Mol. Repod. Dev. 60:65-73.

Mattioli M, Galeati G, Barboni B and Seren E. 1994. Concentration of cyclic AMP during the maturation of pig oocytes *in vivo* and *in vitro*. J. Reprod. Fertil. 100:403-409.

Mermillod P, Tomanek M, Marchal R and Meijer L. 2000. High developmental competence of cattle oocytes maintained at the germinal vesicle stage for 24 h in culture by specific inhibition of MPF kinase activity. Mol. Reprod. Dev. 55: 89-95.

Miyoshi K, Rzucidlo SJ, Pratt SL and Stice SL. 2002. Utility of rapidly matured oocytes as recipients for production of cloned embryos from somatic cells in the pig. Biol. Reprod. 67:540-545.

Nagashima H, Fujimura T, Takahagi Y, Kurome M, Wako N, Ochiai T, Esaki R, Kano K, Saito S, Okabe M and Murakami H. 2003. Development of efficient strategies for the production of genetically modified pigs. Theriogenology 59: 95-106.

Ponderato N, Crotti G, Turini P, Duchi R, Galli C and Lazzari G. 2002. Embryonic and fetal development of bovine oocytes treated with a combination of butyrolactone I and roscovitine in an enriched medium prior to IVM and IVF. Mol. Reprod. Dev. 62:513-518.

Romar R and Funahashi H. 2006. *In vitro* maturation and fertilization of porcine oocytes after a 48 h culture in roscovitine, an inhibitor of p34cdc2/cyclin B kinase. Anim. Reprod. Sci. 92:321-333.

Sirad MA. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. Theriogenology 55:1241-1254.

Somfai T, Kikuchi K, Onishi A, Iwamoto M, Fuchimoto D, Papp AB, Sato E and Nagai T. 2003. Meiotic arrest maintained by cAMP during the initiation of maturation enhances meiotic potential and developmental competence and reduces polyspermy of IVM/IVF porcine oocytes. Zygote 11:199-206.

Taieb F, Thibier C and Jessus C. 1997. On cyclins, oocytes, and eggs. Mol. Reprod. Dev. 48:397-411.

Yoshida M, Bamba K and Kojima Y. 1989. Effects of gonadotropins and estradiol-17β on the timing of nuclear maturation and cumulus mass expansions in pig oocytes cultured *in vitro*. Jpn. J. Anim. Reprod. 35:86-91.

Yu I, Songsasen N, Godke RA and Leibo SP. 2002. Differences among dogs in response of their spermatozoa to cryopreservation using various cooling and warming rates. Cryobiology 44:62-78.

Wang WH, Abeydeera LR, Prather RS and Day BN. 1998. Morphologic comparison of ovulated and *in vitro*-matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. Mol. Reprod. Dev. 49:308-316.

Wu GM, Sun QY, Mao J, Lai L, McCauley TC, Park KW, Prather RS, Didion BA and Day BN. 2002. High developmental competence of pig oocytes after meiotic inhibition with a specific m-phase promoting factor kinase inhibitor, butylactone I. Biol. Reprod. 67:170-177.