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Effects of Preservation of Porcine Oocytes by Dibutyryl Cyclic AMP on *in vitro* Maturation, Fertilization and Development

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Abstract

In this study, the effects of dibutyryl cyclic AMP (dbcAMP) on the preservation of porcine oocytes were investigated. Oocytes were preserved for 24 h or 48 h in modified NCSU37 supplemented with or without 1 mM dbcAMP. After the preservation, 98.3% and 95.2% of oocytes incubated with dbcAMP for 24 h and 48 h, respectively, were at the germinal vesicle stage, whereas about a half of the oocytes incubated without dbcAMP underwent germinal vesicle break down. These preserved oocytes were cultured for 46 h. The *in vitro* maturation (IVM) rates of oocytes preserved with dbcAMP for 24 h (84.0%) and 48 h (55.3%) were significantly higher than those of oocytes preserved without dbcAMP (48.0% and 21.6%, respectively). IVM oocytes with a visible first polar body were selected, fertilized *in vitro* (IVF), and cultured for five days. The rate of oocytes preserved for 24 h with dbcAMP and developed to the blastocyst stage after IVF was 21.4% and did not differ from that of oocytes without preservation (23.8%); however, the blastocyst development rate significantly decreased for oocytes preserved with dbcAMP for 48 h (5.2%). These results indicate that dbcAMP is effective for the preservation of porcine oocytes for 24 h without decreasing their developmental ability after IVF.

Discipline: Animal industry
Additional key words: *In vitro* production, Porcine

Introduction

Recently, a large number of oocytes have been used to produce cloned or transgenic animals[11]. Although *in vivo* matured oocytes were used in the first reported successful production of somatic cell nuclear transfer (SCNT) in pigs[12], it was reported that *in vitro* matured (IVM) oocytes are also useful for SCNT in pigs[3]. Consequently, more and more IVM oocytes have been required not only for the production of SCNT embryos but also for research towards the production of manipulated zygotes with a high quality[11]. In addition, there is a demand for the production of normal IVM/*in vitro* fertilization (IVF) oocytes to be used for comparative studies with manipulated ones. However, the production of IVM oocytes for SCNT takes much time and requires much labor. Furthermore, it has the disadvantage of limiting the flexibility in designing and scheduling experiments because the starting time of IVM must be restricted by the business hours of slaughterhouses, where ovaries, the source of oocytes, can be obtained. If a method to preserve ovaries or oocytes were established, it would be possible to produce a large number of embryos at once using a large number of oocytes obtained by proper scheduling of oocyte collection and the start of IVM.

Previous studies have shown that the preservation of oocytes with dibutyryl cyclic AMP (dbcAMP), an inhibitor of meiotic resumption, is useful for the production of either SCNT or parthenogenetically activated (PA) embryos in pigs[5]. Mammalian oocytes, arrested at the meiotic prophase (GV stage), can resume meiosis spontaneously *in vitro* upon their release from antral follicles followed by a drop in intracellular cAMP levels[15]. The addition of dbcAMP, a membrane-permeable analogue of cAMP, to preservation media can prevent this spontaneous meiotic resumption in porcine oocytes[4]. However, it remains unknown if this method can be applied to por-

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cine IVM/IVF because there have been no studies dealing with IVF of oocytes preserved by this method. Thus, in this study, we examined whether the oocyte preservation method using dbcAMP can be adapted to porcine IVM, and the adaption of the preservation method to IVF was then examined.

Materials and methods

1. Basic medium for preservation, *in vitro* maturation (IVM) and fertilization (IVF) of oocytes, and subsequent *in vitro* culture (IVC) of embryos

Modified NCSU-37 medium[4,8] containing 0.6 mM cysteine (Sigma Chemical Co., St. Louis, MO, USA) with 100 IU/mL penicillin (Sigma), 50 μg/mL streptomycin (Sigma) and 10% porcine follicular fluid (pFF) was used as the basic medium (mNCSU-37) for preservation and IVM of oocytes. The pFF was prepared by aspirating follicles (3–8 mm in diameter) located in the outer layer of prepubertal porcine ovaries preserved at 4ºC, and stored at -30ºC until just before use. For the IVF medium, pigFM[17] supplemented with 2 mM caffeine (Sigma) and 3 mg/mL BSA (fraction V, Sigma) was used. PZM-5 (Research Institute for the Functional Peptides Co., Ltd., Yamagata, Japan) medium was used for IVC of embryos.

2. Preparation and preservation of oocytes

Porcine ovaries were obtained from prepubertal crossbred gilts at a local slaughterhouse and transported to the laboratory at 30–35ºC within 1 h of slaughter. Cumulus-oocyte complexes (COCs) were collected from follicles (3–5 mm in diameter) in the outer layer of the ovaries by aspiration with an 18-gauge needle into a 10 mL disposable syringe. COCs surrounded by not less than three layers of cumulus cells were selected and washed three times with TALP-HEPES (Research Institute for the Functional Peptides), and then washed three times in mNCSU-37. COCs were cultured in 100 μL preservation medium; mNCSU-37 supplemented with or without 1 mM dbcAMP (Sigma) in a Reproplate (Research Institute for the Functional Peptides), covered with liquid paraffin (light mineral oil; Nakarai Tesque, Kyoto, Japan) for 0, 24, or 48 h in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 39ºC. Each droplet contained 10–20 COCs.

3. IVM of oocytes

IVM of oocytes was performed as previously described[8]. Briefly, subsequent to preservation, about 15 COCs were cultured in 100 μL of mNCSU-37 supplemented with 10 IU hCG (Sankyo, Tokyo, Japan), 10 IU PMSG (Sankyo), and 1 mM dbcAMP, covered with liquid paraffin (light mineral oil; Nakarai Tesque) for 20 h, and then subsequently cultured in mNCSU-37 with neither dbcAMP nor gonadotrophin for 24 h at 39ºC in an atmosphere of 5% CO_2 and 5% O_2 in N_2 gas. After the maturation culture, COCs were transferred into TALP-HEPES supplemented with 150 IU/mL hyaluronidase, and the oocytes were then freed from the cumulus cells mechanically by repeated pipetting using a fine glass pipette.

4. Developmental competence of preserved oocytes after IVM, IVF, and IVC

Frozen-thawed spermatozoa (1 x 10^8 cells/mL) were pre-incubated for 15 min at 37ºC in an M199 medium (Invitrogen, Carlsbad, California, USA), supplemented with 10% fetal calf serum (v/v) (Funakoshi, Tokyo, Japan), and adjusted to pH 7.8[10]. A portion (10 μL) of the pre-incubated spermatozoa was introduced into 90 μL of Pig-FM containing about 10 PB oocytes; PB oocytes were co-cultured with spermatozoa at a final concentration of 2 x 10^5 cells/mL for 3 h at 39ºC in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. The putative zygotes were then transferred to a 100 μL droplet of PZM-5 and cultured for five days at 39ºC in an atmosphere of 5% CO_2 and 5% O_2 in N_2.

5. Experimental design

In Experiment 1, COCs were cultured in mNCSU-37 supplemented with or without 1 mmol/L dbcAMP (Sigma) for 0, 24, or 48 h, and then their nuclear status was observed. Then they were matured *in vitro,* and their nuclear maturation was assessed by their nuclear phase and the extrusion of PB after fixation. The oocytes after preservation culture and/or maturation culture were fixed with acetic acid:ethanol (1:3 v/v) for 48–72 h, and stained with 1% (v/v) orcein; the oocytes with a first polar body and a metaphase plate (the second meiotic metaphase: M-II) were classified as matured oocytes.

In Experiment 2, to investigate the ability of the PB oocytes to develop to the blastocyst stage *in vitro* after IVF, the inseminated oocytes were cultured *in vitro* (Fig. 1). The denuded oocytes were transferred to hyperosmolarity (320 mOsm/kg) TALP-HEPES supplemented with 20 mM sorbitol (WAKO, Tokyo, Japan) and examined to determine whether they extruded the first polar body (PB oocytes) under a stereoscopic microscope at 37ºC. The selected PB oocytes were used for IVF. At the end of the insemination for 10 h, putative zygotes were fixed with acetic acid:ethanol (1:3 v/v) for 48–72 h, and then stained with 1% (v/v) orcein. To examine the incidence of oocyte penetration, the rates of oocytes with male and female pronuclei were assessed. Although PB oocytes were considered to be matured, it should be noted that a difference in the maturation rate between the two methods was re-

ported[7]; 94% of oocytes with a visible first polar body under a stereomicroscope were found to be at the metaphase II stage after fixation and staining.

6. Statistical analysis

Data were analyzed using analysis of the chi-square test. A *p* value of <0.05 was considered to be statistically significant.

Results

1. Experiment 1. Effect of dbcAMP on meiotic arrest at germinal vesicle (GV) stage and subsequent nuclear maturation during IVM

Almost all of the oocytes preserved for 0 h, which did not undergo preservation, stayed at the GV stage (99.0%), and after 24 h and 48 h of preservation incubation, the majority of oocytes preserved with dbcAMP remained at the GV stage (98.3% and 95.7%, respectively; Table 1), whereas approximately half of the oocytes preserved without dbcAMP remained at the GV stage (58.0% and 46.0%, respectively; Table1), and the rest underwent germinal vesicle breakdown (GVBD). The maturation rates, defined as rates of oocytes with a metaphase plate and one polar body, for oocytes preserved with dbcAMP for 24 h (84.0%) and 48 h (55.3%) were significantly lower than those for oocytes without preservation (0 h, 95.3%), but significantly higher than those for oocytes preserved without dbcAMP (48.0% and 21.6%, respectively; Table 1) ($p < 0.05$).

2. Experiment 2. Developmental competence of preserved oocytes after IVM and IVF

When PB oocytes from 0 h, 24 h, and 48 h preserved oocytes, respectively, were fertilized and cultured *in vitro*, the penetration and monospermic penetration rates of the PB oocytes significantly decreased at 24 h or 48 h of preservation (53.0% at 0 h preservation, 37.2% at 24 h, and 32.4% at 48 h for penetration rate, and 36.5% at 0 h preservation, 27.1% at 24 h, and 26.4% at 48 h for monospermic penetration rate, respectively; Table 2). In contrast, the rates of PB oocytes that developed to the blastocyst stage were not different between oocytes preserved for 0 h (23.8%) and 24 h (21.4%); however, the rate was significantly lower in PB oocytes preserved for 48 h (5.2%) (Table 3) .

Discussion

In this study, we demonstrated that the meiotic arrest of porcine oocytes can be successfully maintained in a medium supplemented with dbcAMP and pFF for at least 48 h, and at least for 24 h while keeping the ability of the preserved oocytes to develop into blastocysts after IVM, IVF, and IVC at a similar rate as oocytes without preservation. In addition, it has already been reported that dbcAMP could maintain meiotic arrest and developmental potential of porcine oocytes after parthenogenetic

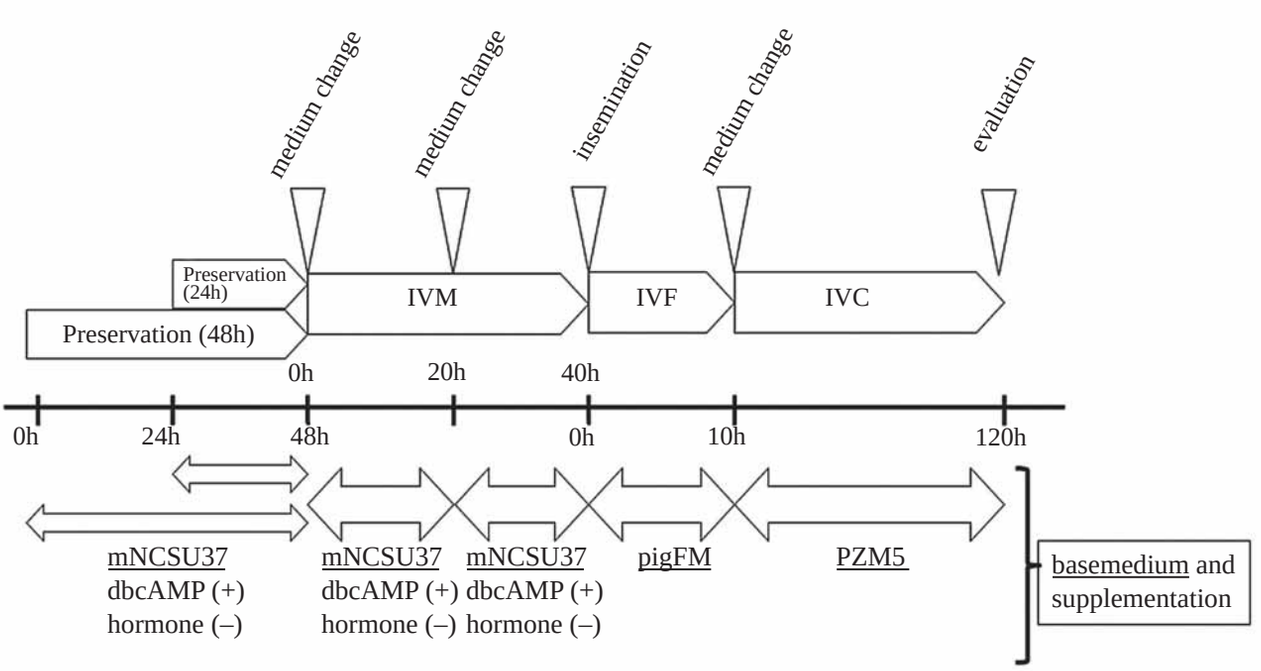

Fig. 1. The scheme of Experiment 2 showing the time duration and supplementation (dbcAMP, hormones) of preservation and *in vitro* maturation media

Table 1. Effect of dbcAMP on the preservation* (meiotic arrest) of oocytes at the germinal vesicle (GV) stage and their maturation rate after *in vitro* maturation**

Preservation period (h)	dbcAMP	No. of oocytes remaining at GV stage / examined (%)		No. of oocytes matured*** / examined (%)	
0		100/101	(99.0)[a]	102/107	(95.3)[c]
24	+	113/115	(98.3)[a]	100/119	(84.0)[d]
24	-	65/112	(58.0)[b]	49/102	(48.0)[e]
48	+	110/117	(94.0)[a]	57/103	(55.3)[f]
48	-	52/113	(46.0)[b]	22/102	(21.6)[g]

* Oocytes were preserved in NCSU-37 medium supplemented with 10% follicular fluid, and with or without 1 mM dbcAMP.
** Preserved oocytes were matured in modified NCSU37 supplemented with 1 mM dbcAMP and gonadotrophins for 22 h, and subsequently cultured in the same maturation medium but without dbcAMP or gonadotrophins for an additional 24 h.
*** Oocytes with a first polar body and metaphase plate were considered as mature. The experiments were repeated 3-5 times.
[a-g] Values with different superscripts in the same column differ significantly ($p<0.05$).

Table 2. Effect of the preservation* period of porcine oocytes on *in vitro* fertilization (IVF)

Preservation period (h)	No. of oocytes used for IVF	No. of oocytes penetrated (%)		No. of oocytes with monosperm / inseminated(%)		No. of oocytes with monosperm / penetrated (%)
0	115	61	(53.0)[a]	42	(36.5)[c]	(68.9)[e]
24	129	48	(37.2)[b]	35	(27.1)[d]	(72.9)[e]
48	68	22	(32.4)[b]	18	(26.4)[d]	(81.8)[e]

The experiments were repeated 3-5 times.
* Oocytes were preserved in NCSU-37 medium supplemented with 10% follicular fluid and 1 mM dbcAMP.
** IVM oocytes with the visible first polar body were selected as mature oocytes, used for IVF, and cultured in PZM-5 for 10 h to assess their penetration rate.
[a-e] Values with different superscripts in the same column differ significantly ($p<0.05$).

Table 3. Effect of the preservation*period of porcine oocytes on the subsequent development to the blastocyst stage after *in vitro* maturation (IVM) and fertilization (IVF)

Preservation period (h)	No. of oocytes used for IVC	No. of oocytes developed to blastocyst (%)	
0	585	139	(23.8)[a]
24	543	116	(21.4)[ab]
48	210	11	(5.2)[b]

The experiments were repeated 3-5 times.
* Oocytes were preserved in NCSU-37 medium supplemented with 10% follicular fluid and 1 mM dbcAMP.
** IVM oocytes with the visible first polar body were selected as mature oocytes, used for IVF, and cultured in PZM-5 for five days to assess their development to the blastocyst stage.
[a-b] Values with different superscripts in the same column differ significantly ($p < 0.05$).

activation (at least for 48 h) and somatic cell nuclear transfer (at least for 96 h)[5]. Thus, these findings suggest that porcine oocytes can be preserved in the medium supplemented with dbcAMP for 24 h without meiotic resumption and keeping the ability to develop to the blastocyst stage.

When oocytes were preserved with or without dbcAMP, their maturation rate to M-II was lower in comparison with the oocytes without preservation. However, a significantly higher maturation rate was obtained in oocytes preserved for 24 and 48 h with dbcAMP than those in those preserved without dbcAMP (control group). After preservation culture, the nuclear morphology of the oocytes was different from that of the oocytes without preservation. The nuclear morphology is classified into four categories: GV-I, GV-II, GV-III, and GV-IV in order of their developmental stage by the shape of chromatin, the presence of nucleolus, and the status of the nucleoplasm[9]. After preservation culture, the oocytes beyond the GV-II (66.2% of oocytes preserved with dbcAMP for

24h) appeared after preservation, whereas almost all the oocytes without preservation were at the GV-I stage (96.5%). It has been suggested that the synchronization of the oocytes at the GV-II stage at 20 h after the start of the maturation culture is related to efficient *in vitro* production of IVM/IVF embryos[4].

It is an important issue to clarify the reason for the low maturation rate in the control group (without dbcAMP). Considering that approximately half of the oocytes preserved without dbcAMP remained at the GV stage and the rest underwent germinal vesicle breakdown (GVBD) after preservation for both 24 and 48 h, the meiotic resumption was induced without gonadotrophins in half of the preserved oocytes during the first 24 h of preservation without dbcAMP. In fact, when oocytes were preserved for 24 h without dbcAMP, 5.8% and 32.6% of them matured to the first and second meiotic metaphases, respectively, which are nearly equal to those of oocytes preserved for 48 h without dbcAMP (17.4% and 34.2%, respectively). When the oocytes preserved without dbcAMP for 24 h were cultured in the maturation medium, their maturation rate to M-II (48.0%) was close to that of the oocytes that underwent GVBD during 24 h of preservation. Thus, it can be suggested that in the case of preservation for 24 h the oocytes observed as mature after IVM were derived from oocytes that underwent GVBD at the end of the preservation period. On the other hand, in the case of preservation for 48 h, the rate of oocytes matured to M-II after the preservation (34.2%) was higher than that for the preserved oocytes after IVM culture (21.6%). Thus, it can be suggested that the preserved oocytes underwent degenerative progression during IVM culture, and the oocytes observed as immature were derived from the oocytes that remained at the GV stage after the preservation period. From these results it can be suggested that the properties of oocytes concerned with meiotic resumption or GVBD were altered during the preservation without dbcAMP, and oocytes could not perform GVBD and were arrested at the GV stage, regardless of the conditions for oocyte maturation. Thus, the low maturation rate in the oocytes preserved without dbcAMP may be explained by the failure of meiotic resumption of the oocytes preserved without dbcAMP that remained at the GV stage and by the presence of the degenerated oocytes that underwent GVBD.

When preserved for 24 h, the rate of PB oocyte penetrated became significantly lower than that of PB oocytes without preservation. Cumulus cells have been known to affect several events around fertilization, which include the attachment of sperm to oocytes, acrosome reaction, and the penetration of sperm into zona pellucida[1]. Indeed, it was reported that the morphological change of cumulus-oocyte complex occurred during preservation and IVM[5]. Furthermore, a moderate number of follicle cells could facilitate sperm penetration of porcine follicular oocytes and male pronuclear formation[6]. Thus, in this study, we performed IVF using denuded (cumulus cell free) oocytes in order to compare only the quality of the oocytes including zona pellucida, excluding the influence of cumulus cells on IVF. However, even without cumulus cells during insemination, the preservation of COCs for 24 h by exposure to dbcAMP had detrimental effects on the sperm penetration of the preserved oocytes in this study. The alterations in the morphology of zona pellucida were reported to occur during IVM, and it was accompanied by functional changes such as sperm-binding patterns[14]. Thus, the detrimental effects of oocyte preservation on sperm penetration may be explained by the reduced ability of zona pellucida to promote acrosome reaction or sperm attachment during preservation with dbcAMP. However, the effect was neutralized during IVC of IVF oocytes for five days, resulting in the same rates of development to the blastocyst stage between oocytes with or without preservation. The reason for the neutralization is not clear. However, an explanation may be that during preservation oocytes exposed to cysteine in the medium consequently obtained factors related to developmental competence restricted to fertilized oocytes such as glutathione[1,13]. Furthermore, it was reported that an exposure of oocytes from pre-pubertal gilts to dbcAMP improved their development to the blastocyst stage through the accumulation of cAMP[2,4]. Thus, it can be suggested that the developmental ability of oocytes preserved with dbcAMP was improved as a consequence of the longtime exposure to dbcAMP.

A major issue with porcine IVF technique is that many embryos have aberrant ploidy, mainly because of polyspermic penetration, and this phenomenon is partly caused by the fertilization of first meiotic metaphase I stage oocytes, which are arrested at the metaphase of 1st meiosis[16]. Thus, in this study, we used PB oocytes for IVF after checking the maturation status by confirming the existence of the first polar body in the perivitelline space of the oocytes.

Conclusion

The present results indicate that this preservation method using dbcAMP for porcine oocytes can be applied not only to produce SCNT embryos but also IVF embryos. In particular, it is very effective when large quantities of oocytes are needed, and it also enables flexible schedules to produce the embryos *in vitro*. However, it should be noted that it is necessary to investigate wheth-

er this preservation method can be applied to produce IVF piglets by transferring IVP embryos, obtained from oocytes preserved with dbcAMP, to recipients.

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References

1. Abeydeera, LR. (2001) *In vitro* fertilization and embryo development in pigs. *Reprod. Suppl.*, **58**, 159–173.
2. Bagg et al. (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**, 1326–32.
3. Betthauser J. et al. (2000) Production of cloned pigs from *in vitro* systems. *Nature Biotech.*, **18**, 1055–1059
4. Funahashi, H., Cantley, TC. & 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.
5. Kawakami, M., Kato, Y. & Tsunoda, Y. (2005) Maintenance of meiotic arrest and developmental potential of porcine oocytes after parthenogenetic activation and somatic cell nuclear transfer. *Cloning Stem Cells*, **7**, 167–177.
6. Kikuchi, K. et al. (1993) Effect of follicle cells on *in vitro* fertilization of pig follicular oocytes. *Theriogenology*, **39**, 593–9.
7. Kikuchi, K. et al. (1999) Cytoplasmic maturation for activation of pig follicular oocytes cultured and arrested at metaphase I. *J. Reprod. Fertil.*, **116**, 143–156.
8. Kikuchi, K. et al. (2002) Successful piglet production after transfer of blastocysts produced by a modified *in vitro* system. *Biol. Reprod.*, **66**, 1033–1041.
9. Motlik, J. & Fulka, J. (1976) Breakdown of the germinal vesicle in pig oocytes *in vivo* and *in vitro*. *J. Exp. zool.,* **198**, 155–62.
10. Nagai, T. et al. (1988) *In-vitro* fertilization of pig oocytes by frozen boar spermatozoa. *J. Reprod. Fertil.*, **84**, 585–591.
11. Nagai, T. et al. (2006) Up date of *in vitro* production of porcine embryos. *Frontiers in Bioscience*, **11**, 2565–2573.
12. Onishi, A. (2000) Pig cloning by microinjection of fetal fibroblast nuclei. *Science*, **289**, 1188–90.
13. Petters, RM. & Wells, KD. (1993) Culture of pig embryos. *J. Reprod. Fertil Suppl.*, **48**, 61–73.
14. Rath, D. et al. (2005) Zona pellucida characteristics and sperm-binding patterns of *in vivo* and *in vitro* produced porcine oocytes inseminated with differently prepared spermatozoa. *Theriogenology*, **63**, 352–62.
15. Shimada, M. et al. (2003) Phosphatidylinositol 3-kinase in cumulus cells is responsible for both suppression of spontaneous maturation and induction of gonadotropin-stimulated maturation of porcine oocytes. *J. Endocrinol.*, **179**, 25–34.
16. Somfai, T. et al. (2005) Development to the blastocyst stage of immature pig oocytes arrested before the metaphase-II stage and fertilized *in vitro*. *Anim. Reprod. Sci.*, **90**, 307–28.
17. Suzuki, K. et al. (2000) Effect of hyaluronan on monospermic penetration of porcine oocytes fertilized *in vitro*. *Int. J. Androl.*, **23**, 13–21.