Nuclear Maturation of Porcine Oocytes *in vitro*: Effect of the Cumulus-Oocyte Complexes Quality

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Abstract

The objective of this study was to examine the effect of the cumulus-oocyte complexes (COCs) quality on the ability of porcine oocytes to mature in vitro. Porcine COCs were collected from 2-6 mm follicles of slaughterhouse ovaries. The oocytes used for IVM were classified into three categories based on the compactness and transparency of the cumulus investment and homogeneity and transparency of the ooplasm. The oocytes were then matured in vitro for 44 h. At 22 of maturation culture, most of the oocytes in all groups were identified still at germinal vesicle (GV) stage and metaphase I (M-I) stage. After 44 h of culture, a greater proportion of Category I and II oocytes completed in vitro maturation through the second meiotic as compared with that of Category III oocytes (P<0.05). The proportion of oocytes remaining at M-I stage and the degenerative oocytes in Category III oocytes were significantly higher than those of oocytes in other groups (P<0.05). These data indicate that porcine oocytes with high quality cytoplasm and a cumulus cell complement have a much greater chance of maturing in vitro than that lower quality oocytes. The morphological grading of immature oocytes is an appropriate selection criterion for their developmental ability.

Key words: porcine oocyte, cumulus-oocyte complexes, maturation in vitro, Metaphase- II

Introduction

The concept of in vitro maturation (IVM) of oocytes in domestic animals is of particular importance, because new technologies for producing transgenic and cloned animals give us the potential for very rapid genetic manipulation and dissemination. Especially the production of transgenic and cloned pigs has been intensively studied, because pigs are physiologically very close to humans and so there has been profound interest in using transgenic and cloned pigs as organ donors for xenotransplantation (Prather, 2000; Prather *et al.*, 2003).

Since the number of in vivo matured oocytes that can be obtained surgically from sows or gilts are so limited that there is an urgent requirement for the establishment of reliable IVM and in vitro fertilization methods for porcine oocytes collected from large number of ovaries obtained at a slaughterhouse. Despite the establishment of several systems to generate porcine embryos in vitro has been reported (Nagai *et al.*, 1990; Abeydeera *et al.*, 1998; Kikuchi *et al.*, 2002), the efficiency is still poor and the quality of the embryos inferior compared with their

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in vivo counterparts. Various factors, such as culture media, oxygen concentration, embryo density, and kind of energy substrate during in vitro production of embryos (IVP) may affect the rate of preimplantation embryo development (Bavister, 1995). In some cases, the low developmental competence of IVM oocytes is related to their quality at the beginning of maturation. The mammalian ovary contains a huge number of a small follicle of various sizes, and each follicle encloses a small oocyte with a high degree of variation in follicle-oocyte quality. During routine harvest from whole ovaries, we are selecting heterogeneous populations of oocytes and a significant number of these oocytes may be at least partially atretic and are incapable of maturation in vitro. Thus selecting oocytes that are most likely to develop is crucial.

For routine IVM, follicular oocytes are commonly selected on the basis of the assessment of morphological features such as cumulus thickness and compactness and the homogeneity of the ooplasm. The morphological quality of cumulus-oocyte complexes (COCs) before culture have been reported affects the maturation of oocytes in bovine (Shioya *et al.*, 1888; Khurana and Niemann, 2000), domestic cat (Wood and Wildt, 1997), or ewe and lamb (Kelly *et al.*, 2007) in vitro, therefore for the successful in vitro maturation of porcine oocytes, it would be important to observe COCs before culture and collect morphologically intact COCs. This study was conducted to evaluate the effect of the cumulus-oocyte complexes quality on the ability of porcine oocytes to mature in vitro. The oocytes used for IVM were classified into three categories based on compactness and transparency of the cumulus investment and homogeneity and transparency of the ooplasm.

Materials and Methods
In vitro maturation (IVM)

Porcine ovaries were obtained from a slaughterhouse and were transported to the laboratory in 0.9% physiological saline at 35°C. Oocytes were aspirated by an 18-gauge needle into a disposable 10-mL syringe from follicles of 2-6 mm in diameter and were collected in modified phosphate-buffered saline (m-PBS) supplemented with 100 IU/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate. In vitro maturation of oocytes was carried out according to the method described by Karja *et al.*, (2004) with minor modificarions. Collected cumulus-oocyte complexes (COCs) were graded into three morphological categories according to the following criteria: oocytes having a homogenous evenly granulated cytoplasm surrounded by a compact cumulus oophorous with more than five layers were classified as Category I (Figure 1A); oocytes with fewer than five layers of compact cumulus cells investment and having a homogenous evenly granulated cytoplasm were classified as Category II (Figure 1B); and oocytes surrounded by less compact cumulus cells investment and having irregular ooplasm with dark cluster were put in Category III (Figure 1C).
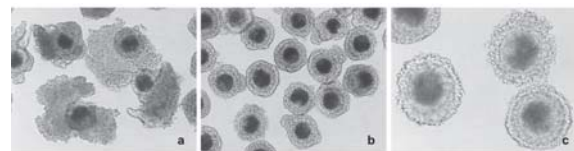

Figure 1. Grades of various cumulus-oocyte complexes harvested from porcine ovaries at collection. (a) Category I: oocytes have a uniform, dark cytoplasm combined with a full complement of five or more layers of tightly compacted cumulus oophorus cells. (b) Category II: oocytes have a uniform, dark cytoplasm with complete complements of corona radiate cells, but fewer than five layers of cumulus oophorus cells. (c) Category II: Oocytes lack uniformaly which is expressed as mosaic transparency of the cytoplasm, oocytes have nearly a full complements of corona radiate and some cumulus oophorus, but are not as tightly compacted as higher grade. (Magnification 100x).

The oocytes in each group were cultured for 22 h in 500 µL of maturation medium, a modified North Carolina State University (NCSU)-23 solution (Petters and Wells, 1993) supplemented with 10% (v:v) porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma), 50 µM â-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 10 IU/mL pregnant mare serum gonadotropin, 10 IU/mL human chorionic gonadotropin, and 50 mg/mL gentamicin (Sigma). They were subsequently cultured in the NCSU-23 solution without dbcAMP and hormones for 22 h. All culture was performed at 38.5 °C and 5% CO_2 under 5% O_2.

Assessment of Meiotic Status of Oocytes

To determine the rate of oocyte maturation, at 22 h and 44 h of maturation culture, oocytes were denuded manually with a small-bore pipette in PBS supplemented with 1 mg/mL hyaluronidase. Denuded oocytes were then fixed with acetic acid: ethanol (1:3 v:v) for 48 to 72 h. The fixed oocytes were stained with acetic-orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. The precise maturational stage of each oocyte was determined, based on the changes in its chromosome configuration and nuclear membrane. Chromosomal configurations (Figure 2) were categories as described by Johnston and Wildt (1989) and Mandelbaum (2000)
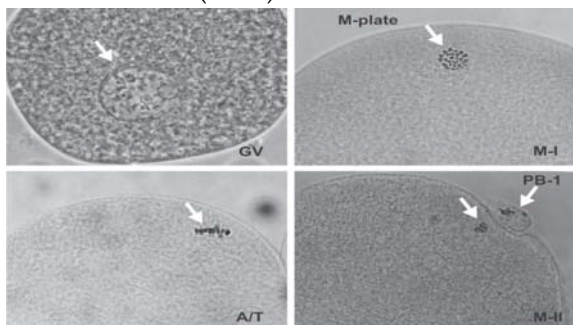

Figure 2. Chromatin configurations in porcine oocytes. GV: Germinal vesicle with a round-shaped nucleus; M-I: metaphase I with M-plate; A/T: Anaphase/Thelophase; M-II: metaphase II with polar body (PB-1). (Magnification 400x).

The germinal vesicle (GV) stage consisted of a round-shaped nucleus containing a prominent nucleolus and fine filament of chromatin. Metaphase I (M-I) was identified by the appearance of paired chromosomes. Anaphase/Telophase (A/T) was characterized by chromosomal separation and movement to separate poles or by the appearance of two equally spread groups of chromosomes. Metaphase II (M-II) was identified by the appearance of two groups of chromosomes, one spread and the other clustered and comprising the developing polar body-1. Oocytes that had no identifiable chromosomal configuration or fragmented or distinctly irregular in shape were classified as degenerate

Statistical analysis

Data are expressed as means ± SEM. The percentages of oocytes reaching each stage of meiosis were subjected to arcsine transformation before analysis variance (ANOVA). The transformed data were tested by ANOVA followed by post hoc testing with Fisher's protected least significant difference test (PLSD test) using Statview program (Abacus concepts, Inc., Berkeley, CA, USA). Differences with a probability value (P) of 0.05 or less were considered significant.

Results

The three morphologically different groups of COCs were subjected to IVM in this study. The results are presented in table 1 and 2.

At 22 of maturation culture, most of the oocytes in all groups were identified at germinal vesicle (GV) stage and metaphase I

COCs grade	No. of oocytes matured	Mean ± SEM (n) of oocytes at each stage**				
		GV	M-I	A/T	M-II	Deg.
Category I	23	52.5 ± 7.2 (11)	44.7 ± 5.0 (11)[a]	0 (0)	0 (0)	2.8 ± 2.8 (1)
Category II	96	39.1 ± 5.0 (36)	56.3 ± 4.3 (55)[a-b]	0 (0)	0 (0)	4.7 ± 1.9 (5)
Category III	115	33.4 ± 6.3 (42)	64.2 ± 7.5 (70)[b]	0 (0)	0.4 ± 0.4 (1)	2.1 ± 1,6 (2)

*four-five replicate trials were carried out. Percentages are expressed as means ± SEM.
**GV: germinal vesicle, M-I: metaphase I, A-T: anaphase I and telophase I, M-II: metaphase II.
[a-b] Value with different superscript letters are significantly different (P< 0.05).

Table2. Nuclear status of porcine oocytes after 44 h of maturation culture in vitro*

	No. of	Mean ± SEM (n) of oocytes at each stage**				
COCs grade	oocytes matured	GV	M-I	A/T	M-II	Deg.
Category I	129	2.8 ± 1.2 (4)	5.1 ± 2.0 (6)ᵃ	0 (0)	87.9 ± 2.8 (114)ᵃ	4.1 ± 1.7 (5)ᵃ
Category II	170	0.9 ± 0.9 (1)	8.3 ± 2.5 (11)ᵃ	0 (0)	86.7 ± 2.8 (150)ᵃ	4.1 ± 1.3 (8)ᵃ
Category III	160	2.3 ± 1.3 (5)	17.9 ± 3.7 (25)ᵇ	0.8 ± 0.8 (1)	69.6 ± 3.1 (112)ᵇ	9.3 ± 1.2 (17)ᵇ

*seven to eight replicate trials were carried out. Percentages are expressed as means ± SEM.
**GV: germinal vesicle, M-I: metaphase I, A-T: anaphase I and telophase I, M-II: metaphase II.
ᵃ⁻ᵇ Value with different superscript letters are significantly different (P< 0.05).

stage. These data indicate that these oocytes are still in progressing to resume meiosis at and was incomplete until these oocytes were cultured an additional 22 h. Further analysis demonstrated that between 22 h and 44 h, the proportion of oocytes in GV and M-I stages progressively decline and were replaced by an increase in M-II chromatin. After 44 h of culture, a greater proportion of Category I and II oocytes completed in vitro maturation through the second meiotic metaphase (M-II) as compared with that of Category III oocytes (P<0.05). The proportion of oocytes in M-II between Category I and Category II oocytes, however was not different (P> 0.05). The proportion of oocytes remaining at M-I stage and degenerative oocytes in Category III oocytes were significantly higher than those of oocytes in other groups (P<0.05).

Discussion

Oocyte maturation remains an enigmatic process that is generally understood to span the time from when messages initiate germinal vesicle breakdown (GVBD) to completion of the nuclear changes resulting in expulsion of the first polar body. The process of maturation encompasses a complex series of molecular and structural events, culminating in the arrest of the oocyte chromosomes on the metaphase II plate in anticipation of sperm penetration and activation for fertilization. This maturation involves nuclear and cytoplasmic maturation. Nuclear maturation refers to the resumption of meiosis and progression to the metaphase II (M-II) stage, whereas cytoplasmic maturation encompasses other, poorly

understood, a series of cytoplasmic events (morphological, functional, and biochemical) necessary for fertilization and early embryonic development. These processes are believed to progress in parallel to one another, and synchronization of nuclear and cytoplasmic maturation is essential for establishing optimal oocyte developmental potential.

The results of this study demonstrated that good quality (Category I and II) oocytes that have compact cumulus cells investment and a homogenous evenly granulated cytoplasm support completion of nuclear maturation better than Category III oocytes.

Moreover, at the end of maturation culture more poor quality of COCs (Category III) were identified as degenerative oocytes compared than other groups and more than 17% of these oocytes remaining at M-I stage, indicating that oocytes surrounded by less compact cumulus cells investment may be at least partially atretic and incapable to mature in vitro. Thereby, our findings indicate that attachment of compact cumulus cells to oocytes may have a critical role for maturation of porcine oocytes in vitro. Similar to our finding, other worker reported the beneficial effect of the quality of oocyte and cellular investment surrounding on its developmental ability (Eppig, 1982; Madison et al., 1992; Lonergan et al., 1994; Wood and Wildt, 1997; Khurana and Niemann, 2000; Kelly et al., 2007). These authors demonstrated that the layers of cumulus cells surrounding the oocyte are an important element for both nuclear and cytoplasmic maturation of the oocyte. These cells act as a 'go-between' between the oocyte and the follicular or culture environments. Intracellular communication between the oocyte and the cumulus cells takes place via gap junctions which are facilitated by means of microfilamentar transzonal projections of the cumulus cells directly in contact with the zona pellucida, i.e. corona radiata cells

(Allworth and Albertini, 1993). These processes transverse the zona pellucida and the oocyte plasma membrane (oolemma), thereby establishing a route of communication by which direct transfer of substances important for oocyte growth and maintenance of meiotic arrest can take place (Tanghe, 2002).

According to Moor *et al.* (1990), the cellular relationship between the oocyte and somatic follicular cells is fundamental to oocyte maturation. Follicular cells play a critical role in the regulation of oocyte meiotic arrest and the resumption of meiosis, in addition to providing nutrients. Studies on the maturation of pig oocytes *in vitro* by co-culture with either follicular cells or follicular fluid (Niwa, 1993; Sirard *et al.*, 1993) indicate that follicular cells secrete factors that play a crucial role in supporting oocyte cytoplasmic maturation. The cumulus cells may produce glycosaminoglycans, steroid hormones and other factor that support cytoplasmic maturation, which is responsible for the capacity to undergo male pronucleus formation, monospermic fertilization, and early embryonic development (Danforth, 1955; Yamauchi and Nagai, 1999; Dode and Graves, 2002). A major advantage of the persistent interaction between cumulus cells and oocyte is the acquisition of the factors in the ooplasm required to decondense penetrated spermatozoa and form male pronuclei. Inability to perform this function has often been the major abnormality of oocytes matured *in vitro* (Mattioli *et al.*, 1988; Larsen and Wert, 1988). Therefore, it is accepted that cumulus cells support the maturation of oocytes to the metaphase II stage and greatly enhance cytoplasmic maturation, which is responsible for the capacity to undergo normal fertilization and subsequent embryonic development. In addition, cumulus cells not only support oocyte maturation associated with developmental competence, but also

act as a scavengers, removing materials from the culture medium (Khurana and Niemann, 2000).

The cumulus cells play a critical role in protective the oocytes against oxidative stress by enhancing the gluthatione content in the oocytes so assist that the oocytes to ovoid undergoing DNA fragmentation (Tatemono *et al.*, 2000; Wongsrikeao *et al.*, 2005). Although, nuclear maturation is easily supported by the in vitro system; nevertheless, the cytoplasmic component of oocyte maturation that essential for an optimal fertilizability and an adequate ability to support early development is not completely satisfactory. Therefore, further morphological analysis or by monitoring the developmental ability of the IVM oocytes in an in vitro fertilization and embryo culture system are required.

In conclusion, oocytes with high quality cytoplasm and a cumulus cell complement have a much greater chance of maturing in vitro that do lower quality oocytes. Morphological grading of immature oocytes is an appropriate selection criterion for their developmental ability. By using highly selected population of good quality, it is possible to increase efficiency of IVP in pig.

References

Abeydeera, L.R., Wang, W.H., Cantley, T.C., Prather, R.S., Day, B.N. 1998. Presence of â-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. *Theriogenology,* **50,** 747-756.

Allworth, A.E. and Albertini, D.F. 1993. Meiotic maturation in cultured bovine oocytes is accompanied by remodeling of the cumulus cell cytoskeleton. *Dev. Biol.*, **158,** 101-112.

Bavister, B.D. 1995. Culture of preimplantation embryos: facts and

artifacts. *Hum. Reprod. Update,* **1,** 91-148.

Danforth, D.R. 1995. Endocrine and paracrine control of oocyte development. *Am. J. Obstet. Gynecol.,* **172,** 747-752.

Dode, M.A. and Graves, C. 2002. Involvement of steroids hormones on in vitro maturation of pig oocytes. *Theriogenology,* **57,** 811-821.

Eppig, J.J. 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation and cumulus expansion. *Dev. Biol.,* **89,** 268-272.

Johnston, L.A., O'Brien, S.J., Wildt, D.E. 1989. In-vitro maturation and fertilization of domestic cat follicular oocytes. *Gamete Res.,* **24,** 343-356.

Karja, N.W.K., Wongsrikeao, P., Murakami, M., Agung, B., Fahrudin, M., Nagai, T., Otoi, T. 2004. Effects of oxygen tension on the development and quality of porcine in vitro fertilized embryos. *Theriogenology,* **62,** 1585-1595.

Kelly, J.M., Kleemann, D.O., Rudiger, S.R., Walke, S.K. 2007. Effects of grade of oocyte–cumulus complex and the interactions between grades on the production of blastocysts in the cow, ewe and lamb. *Reprod. Dom. Anim.,* **42**, 577-582.

Khurana, N. K. and Niemann, H. 2000. Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology,* **54,** 741-756.

Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita, T., Nagai, T. 2002. Successful piglet production after transfer of blastocysts produced by a modified in vitro system. *Biol. Reprod.,* **66,** 1033-1041.

Larsen, W.J. and Wert, S.E. 1988. Role of cell junctions in gametogenesis and in early embryonic development. *Tissue Cell*, **20,** 809-848.

Lonergan, P., Monaghan, P., Rizos, D., Boland, M. P., Gordon, I.**.** 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. Mol. Reprod. Dev., **37**, 48-53.

Mandelbaum, J. 2000. Oocytes. *Hum. Reprod.,* **15,** (Suppl. 4), 11-18.

Madison, V., Avery, B., Greve, T. 1992. Selection of immature bovine oocytes for developmental potential in vitro. *Anim Reprod. Sci.,* 27, 1-11.

Mattioli, M., Galeati, G., Seren, E. 1988. Effect of follicle somatic cells during pig oocyte maturation on egg penetration and male pronuclear formation. *Gamete Res.,* **20,** 177-183.

Moor, R.M., Mattioli, M., Ding, J., Nagai, T. 1990 Maturation of pig oocytes *in vivo* and *in vitro.* *J. Reprod. Fertil. Suppl.,* **40,** 197-210.

Nagai, T. and Moor, R.M. 1990. Effect of oviduct cells on the incidence of polyspermy in pig eggs fertilized in vitro. *Mol. Reprod. Dev.,* **26,** 377-382.

Niwa, K. 1993. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. *J. Reprod. Fertil. Suppl.,* **48,** 49-59.

Petters, R.M. and Wells, K.D. 1993. Culture of pig embryos. *J. Reprod. Fertil. Suppl.,* **48,** 61-73.

Wongsrikeao, P., Otoi, T., Murakami, M., Karja, N.W.K., Budiyanto, A., Nii, M., Suzuki, T. 2005. Relationship between DNA fragmentation and nuclear status of in vitro-matured porcine oocytes: role of cumulus cells. *Reprod. Fertil. Dev.,* **16,** 773-780.

Prather, R.S. 2000. Pigs is pigs. *Science,* **289,** 1886-1887.

Prather, R.S., Hawley, R.J., Carter, D.B., Lai, L., Greenstein, J.L. 2003. Transgenic swine for biomedicine and agriculture. *Theriogenology*, **59,** 115-23.

Shioya, Y., Kuwayama, M., Fukushima, M., Iwasaki, S., Hanada, A. 1988. in vitro fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured in vitro. *Theriogenology*, **30,** 489-496.

Sirard, M.A., Dubuc, A., Bolamba, D., Zheng, Y., Coenen K. 1993. Follicle-oocyte-sperm interactions *in vivo* and *in vitro* in pigs. *J. Reprod. Fertil. Suppl.,* **48,** 3-16.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., De Kruif, A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol. Reprod. Dev.,* **61,** 414-424.

Tatemoto, H., Sakurai, N., Muto, N. 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. *Biol. Reprod.,* **63,** 805-810.

Wood, T.C. and D.E. Wildt. 1997. Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in-vitro. *J. Reprod. Fertil.,* **110,** 355-360.

Yamauchi, N. and Nagai, T. 1999. Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. *Biol. Reprod.,* **61,** 823-833.