# Porcine Oocytes with Meiotic Competence are Synchronized Early Stage of Germinal Vesicle (GV)

Kwang-Wook Park†

*Dept. of Animal Science & Technology, Sunchon National University, Suncheon 540-742, Korea*

## ABSTRACT

**Correlations between cumulus cells and germinal vesicle (GV) chromatin configuration were examined in porcine oocytes. Cumulus-oocyte complexes (COCs) were collected from 2~6 mm follicles and divided into three categories according to cumulus cell morphology. "A" group was compacted COCs with more than three cumulus cell layers. "B" group was COCs with less cumulus cell layers than "A" group. "C" group was COCs with one or less layer of cumulus cells. Cumulus cells were removed 0.1% hyaluronidase, and denuded oocytes were stained with Hoechst 33342. GV chromatin configuration was classified into GV-Con and GV-Dis. GV-Con meant that a nucleus was surrounded by condensed chromatin in a ring. GV-Dis meant that filamentous chromatin clumps were distributed in nucleus. The proportion (80.2%) of GV-Con in "A" group was significantly higher than "B" (62.0%) or "C" (44.9%). The proportion (55.1%) of GV-Dis in "C" group was significantly higher than "A" (19.8%) or "B" (38.0%). The meiotic competence of COCs was examined after 44 h culture. The proportion (90.0%) of oocytes reaching to metaphase II (M-II) in "A" group was significantly higher than "B" (76.5%) or "C" (45.5%). In conclusion, oocytes with good quality cumulus cell layers are synchronized early GV stage, and early GV stage is important for meiotic competence in pigs.**

**(Key words : Pig, Oocytes, Germinal vesicle, Cumulus cells, *In vitro* maturation)**

## INTRODUCTION

The development of reproductive technologies requires a better understanding about the factors that regulate meiotic maturation in oocytes. Although porcine *in vitro* fertilization (IVF) system has been developed, at best only 20% of oocytes can develop to the blastocyst stage (Funahashi *et al.*, 1997a; Funahashi *et al.*, 1997b; Grupen *et al.*, 1997; Long *et al.*, 1997). The specific reasons of this poor developmental competence are unclear, but incomplete cytoplasmic maturation of oocytes is one of the problems in porcine IVF (Funahashi and Day, 1997).

In pig maturing oocytes, the chromatin configuration of germinal vesicle (GV) was classified into four stages based on the chromatin changes, and on nucleolus and nuclear membrane disappearance (Motlik and Fulka, 1976). In GV-I, nuclear membrane and nucleolus are intact and chromatin forms a ring or horseshoe around the nucleolus. The GV-II is similar to the GV-I except that a few chromatin clumps can be detected near the membrane. In GV-III, chromatin clumps or strands are distributed throughout the nucleoplasm. In GV-IV, condensed chromatin clumps or strands are there but the nuclear membrane is not distinct and the nucleolus disappears completely.

Most of porcine oocytes derived from follicles are GV-I, and after *in vitro* culture, GV stage of oocytes was progressed to GV-IV or GV breakdown (GVBD) (Motlik and Fulka, 1976). The distribution of GV stage was different from follicle size. The oocytes derived from large follicles arrested GV-I stage (Nagai *et al.*, 1997; Sun *et al.*, 2004). Moreover higher percentage of oocytes from large follicles, which were supposed to be ovulated, was at the GV-I stage (Nagai *et al.*, 1997; Sun *et al.*, 2004).

Cumulus cells play important roles in maturation and subsequent development of oocytes *in vitro*. Several studies have shown that porcine oocytes with more cumulus cells had better chances to mature and develop *in vitro* (Tsafriri and Channing, 1975; Ozawa *et al.*, 2010). The quality of porcine oocytes is usually evaluated according to their number of cumulus cell layers and their cytoplasm before *in vitro* maturation. However, the correlation between cumulus cells and GV

* This work was supported by the Korea Research Foundation Grant funded by the Korean Government (NRF-2008-331-C00230) and partially by Sunchon National University Research Fund in 2008.

† Corresponding author : Phone: +82-61-750-3236, E-mail: parkkw@scnu.kr

stages of oocytes was not defined well.

The present study was conducted to confirm GV chromatin configuration and meiotic competence according to cumulus cell quality of oocytes.

## MATERIALS AND METHODS

### Preparation of Oocytes

Ovaries were collected from prepubertal gilts at a local abattoir and were transported to the laboratory in a 0.9% NaCl solution at 35℃. Cumulus-oocyte complexes (COCs) were aspirated from 2~6 mm diameter antral follicles with an 18-gauge needle fixed to a 10-ml disposable syringe. The COCs were divided into three categories according to cumulus cell morphology (Fig. 1). "A" group was compacted COCs with more than three cumulus cell layers. "B" group was COCs with less cumulus cell layers than "A" group. "C" group was COCs with one or less layer of cumulus cells. The COCs were washed three times in TCM 199 (31100-035, Gibco Grand Island, NY) supplemented with 0.1% polyvinylalcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml LH (L-5269, Sigma Chemical Co, St. Louis, MO), 0.5 μg/ml FSH (F-2293, Sigma), 10 ng/ml epidermal growth factor (E-4127, Sigma), 75 μg/ml penicillin G, and 50 μg/ml streptomycin. Approximately 20 COCs were transferred to 500 μl of the same medium which had been covered with mineral oil in a 4-well multidish (Nunc, Roskilde, Denmark) and equilibrated at 39℃ in an atmosphere of 5% $CO_2$ in air.

### Classification of GV Chromatin Configuration

In porcine oocytes, the chromatin configuration of germinal vesicle (GV) was divided into four stages based on the chromatin changes, and on nucleolus and nuclear membrane disappearance; GV-I, GV-II, GV-III, and GV-IV (Motlik and Fulka, 1976). In this paper, the GV stages were classified into GV-Con and GV-Dis (Fig. 2). GV-Con meant that most of nucleus was surrounded by condensed chromatin in a ring and it was analogous to GV-I or GV-II. Most of GV-Con was GV-I. GV-Dis meant that filamentous chromatin clumps were distributed in nucleus and it was analogous to GV-III or GV-IV.

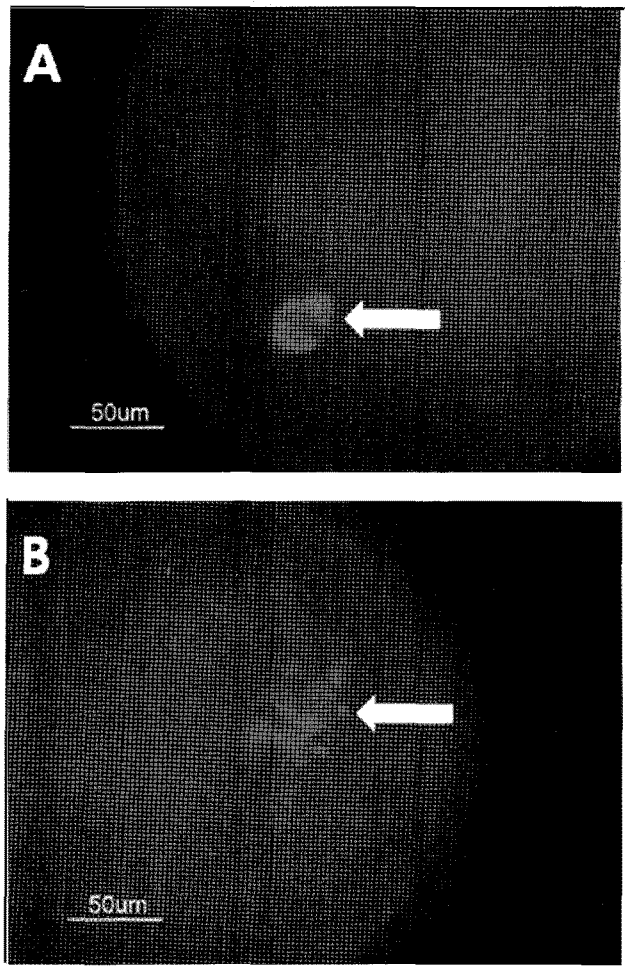


**Fig. 2. Germinal vesicle (GV) stages were classified into two groups according to chromatin status.** (A) GV-Con: a nucleus was surrounded by condensed chromatin (arrow) in a ring. B) GV-Dis: filamentous chromatin clumps (arrow) were distributed in nucleus.

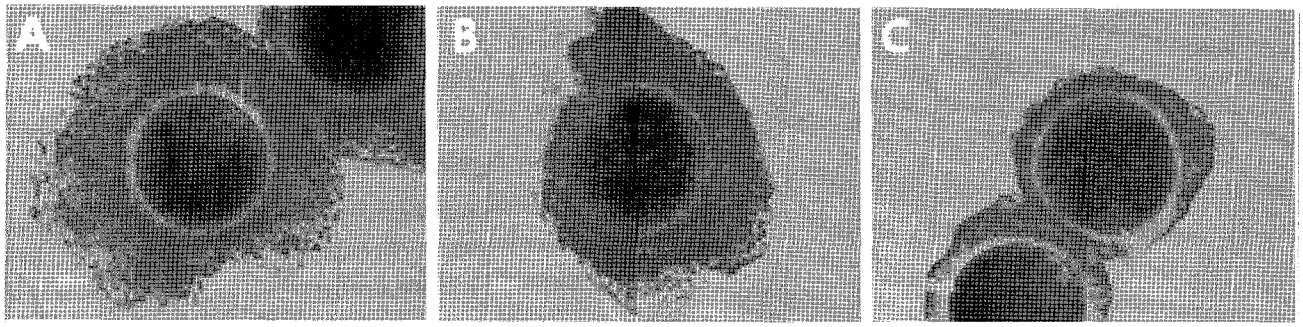


**Fig. 1. The cumulus-oocyte complexes (COCs) were classified into three categories.** (A) Cumulus cell layers were more than three and compacted. (B) Cumulus cell layers were less than "A" group but still compacted. (C) Cumulus cell layer was one or less.

**Experiment 1**

We confirmed the GV stages of oocytes derived from follicles. The COCs were divided into three categories according to cumulus cell morphology. The oocytes were freed from the cumulus cells by vigorously vortexing the cells for 4 min in TL-Hepes supplemented with 0.1% PVA and 0.1% hyaluronidase. Oocytes were stained with 5 μg/ml of bisbenzimide (Hoechst 33342) to identify the GVs by using an epifluorescent microscope.

**Experiment 2**

After 44 h culture, oocytes were freed from cumulus cells by vigorously vortexing the cells. Oocytes were stained with 5 μg/ml of bisbenzimide to examined nuclear maturation.

**Statistical Analysis**

We conducted four replicate trials for each treatment. Data were analyzed by analysis of variance and Duncan multiple-range test by using the general linear models in the Statistical Analysis System software to determine treatment differences. All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as the mean±SEM. A probability of $p<0.05$ was considered to be statistically significant.

## RESULTS

**GV Chromatin Configuration of Oocytes Derived From Follicles**

The COCs were divided into three categories according to cumulus cell morphology (Fig. 1). "A" group was compacted COCs with more than three cumulus cell layers. "B" group was COCs with less cumulus cell layers than "A" group. "C" group was COCs with one or less layer of cumulus cells. The proportion (80.2%) of GV-Con in "A" group was significantly higher than "B" (62.0%) or "C" (44.9%). The proportion (55.1%) of GV-Dis in "C" group was significantly higher than "A" (19.8 %) or "B" (38.0%). These results suggested that early GV stage predominated in the good quality oocytes with cumulus cells and intact cumulus cells might have functions to arrest progress of GVs in oocytes.

***In Vitro* Maturation of Oocytes**

The meiotic competence of COCs was examined after 44 h culture. The proportion (90.0%) of oocytes reaching to metaphase II (M-II) in "A" group was significantly higher than "B" (76.5%) or "C" (45.5%). These results suggested that the good quality oocytes with cumulus cells had high meiotic competence.

## DISCUSSION

In porcine oocytes, the chromatin configuration of germinal vesicle (GV) was divided into four stages (Motlik and Fulka, 1976). Most of porcine oocytes derived from follicles are GV-I, and after *in vitro* culture, GV stage of oocytes was progressed to GV-IV or GV breakdown (GVBD) (Motlik and Fulka, 1976). The distribution of GV stage was different from follicle size. The oocytes derived from large follicles arrested GV-I stage (Nagai *et al.*, 1997; Sun *et al.*, 2004). Moreover higher percentage of oocytes from large follicles, which were supposed to be ovulated, was at the GV-I stage (Nagai *et al.*, 1997; Sun *et al.*, 2004). Motlik and Fulka (1976) suggested that oocytes with GV of advanced stages could be from atretic follicles. These were consisted with other reports (Nagai *et al.*, 1997; Sun *et al.*, 2004). Therefore, advanced GV stage may be a sign of atretic oocytes in pigs.

Cumulus cells play important roles in maturation and subsequent development of oocytes *in vitro*. Several studies have shown that porcine oocytes with more cumulus cells had better chances to mature and develop *in vitro* (Tsafriri and Channing, 1975; Ozawa *et al.*, 2010). The ability of the oocyte to form male pronuclei (MPN) after sperm penetration depends on the presence of cumulus cells during maturation (Mattioli *et al.*, 1988a; Mattioli *et al.*, 1988b; Moor *et al.*, 1990) and fertilization (Kikuchi *et al.*, 1993; Ka *et al.*, 1997). The removal of cumulus cells during maturation induces a premature migration of cortical granules, leading to an increase in exocytotic events and a decrease in penetration ability (Galeati *et al.*, 1991). Cumulus cells secrete a meiosis-activating substance which induces the oocyte to undergo GVBD (Xia *et al.*, 2000). Therefore, the quality of porcine oocytes is usually evaluated according to their number of cumulus cell layers and their cytoplasm before *in vitro* maturation. However, the correlation between GV stages and cumulus cells was not defined well.

In this study we showed that oocytes with good quality cumulus cells (80.2%) were at the early GV stage, and had high meiotic competence after culture (90.0 %) (Table 1 and 2). These results indicate that cumulus cells may make oocytes arrest at early GV stages. Meanwhile, Sun *et al.* (2004) could not find any differences in GV chromatin configuration in oocytes with different quality of cumulus cells. The discrepancy might result from the differences of oocytes classification. They divided oocytes into two groups. One group had more than two layers of cumulus cells and the other one had less than one layer or no cumulus cells. We subdivided oocytes into three groups (Fig. 1). "A" group was compacted COCs with more than three cumulus cell layers. "B" group was COCs with less cumulus

**Table 1. Germinal vesicle (GV) chromatin configuration of porcine oocytes according to categories of cumulus-oocyte complexes**

| Categories of oocytes | No. of oocytes | % GV-Con (mean±SEM) | % GV-Dis (mean±SEM) |
|---|---|---|---|
| A | 75 | 80.2+3.0[a] | 19.8+3.0[a] |
| B | 73 | 62.0+4.6[b] | 38.0+4.6[b] |
| C | 76 | 44.9+3.7[c] | 55.1+3.7[c] |

[a~c] Within each column, values with different superscripts differ significantly, $p<0.05$ at least.

**Table 2. *In vitro* maturation of porcine oocytes according to categories of cumulus-oocyte complexes**

| Categories of oocytes | No. of oocytes | |
|---|---|---|
| | Examined | % Metaphase II (mean±SEM) |
| A | 81 | 90.0+3.6[a] |
| B | 80 | 76.5+3.1[b] |
| C | 79 | 45.5+7.1[c] |

[a~c] Within each column, values with different superscripts differ significantly, $p<0.05$ at least.

cell layers than "A" group. "C" grade was COCs with one or less layer of cumulus cells. In "C" group oocytes, we excluded oocytes without cumulus cells. Our results indicate that good quality oocytes with more than three cumulus cell layers are synchronized early GV stage, and early GV stage is important for meiotic competence in pigs.

In cattle, the GV configurations are very different from pigs (Park *et al.*, 1999; Chohen and Hunter, 2003). I previously reported that bovine oocytes from large follicles (2~6 mm in diameter) were at more advanced stages than porcine oocytes; GV-IV and GV-V (chromatin was condensed into thick clumps and the nuclear membrane was still visible) (Park *et al.*, 1999). These stages were just before GVBD. The differences of GV stage between pigs and cattle may come from the differences of species.

The oocytes with intact cumulus cells were arrested in early GV Stage (Table 1). These results indicate that cumulus cells have functions not to progress GV stage of oocytes directly or indirectly in antral follicles. We don't know how cumulus cells can make oocytes arrest early GV stages in antral follicles, but the beneficial effects of cumulus cells on oocyte maturation and development were well studied in pigs (Tsafriri and Channing, 1975; Ozawa *et al.*, 2010; Ka *et al.*, 1997) and cattle (Khurana and Niman, 2000; Geshi *et al.*, 2000; Chian and Sirard). Intact cumulus cells may send ooplasms the signals not to progress GV stages in antral follicles and if the signals are not enough, GV stages may be progressed and atretic oocytes may occur. In the next step, the mechanism how cumulus cells have oocytes arrest early GV stages should be examined.

In conclusion, oocytes with good quality cumulus cell layers are synchronized early GV stage, and early GV stage is important for meiotic competence in pigs.

## REFERENCES


1. Chian RC, Sirard MA (1995): Effects of cumulus cells and follicle-stimulating hormone during *in vitro* maturation on parthenogenetic activation of bovine oocytes. Mol Reprod Dev 42:425-431.
2. Chohan KR, Hunter AG (2003): Meiotic competence of bovine fetal oocytes following *in vitro* maturation. Anim Reprod Sci 76:43-51.
3. Funahashi H, Cantley TC, Day BN (1997a): Preincubation of cumulus-ooycte complexes before exposure to gonadotropins improves the developmental competence of porcine embryos matured and fertilized *in vitro*. Theriogenology 47:679-686.
4. Funahashi H, Cantley TC, Day BN (1997b): 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. Funahashi H, Day BN (1997): Advances in *in vitro* production of pig embryos. J Reprod Fertil Suppl. 52:271-283.
6. Galeati G, Modina S, Lauria A, Mattioli M (1991): Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution. Mol Reprod Dev 29:40-46.
7. Geshi M, Takenouchi N, Yamauchi N, Nagai T (2000): Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. Biol Reprod 63:1730-1734.
8. Grupen CG, Nagashima H, Nottle MB (1997): Asynchronous meiotic progression in porcine oocytes matured *in vitro*: a cause of polyspermic fertilization? Reprod Fertil Dev 9:187-191.
9. Ka HH, Sawai K, Wang WH, Im KS, Niwa K (1997): Amino acids in maturation medium and presence of cumulus cells at fertilization promote male pronuclear formation in porcine oocytes matured and penetrated *in vitro*. Biol Reprod 57:1478-1483.
10. Khurana NK, 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. Therioge-

nology 54:741-756.
11. Kikuchi K, Nagai T, Motlik J, Shioya Y, Izaike Y (1993): Effect of follicle cells on *in vitro* fertilization of pig follicular oocytes. Theriogenology 39:593-599.
12. Long CR, Dobrinsky JR, Johnson LA (1997): *In vitro* production of pig embryos. comparisons of culture media and boars: Theriogenology 51:1375-1390.
13. Mattioli M, Galeati G, Bacci ML, Seren E (1988a): Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte. Gamete Res 21:223-232.
14. Mattioli M, Galeati G, Seren E (1988b): Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronucleus formation. Gamete Res 20:177-183.
15. Moor RM, Mattioli M, Ding J, Nagai T (1990): Maturation of pig oocytes *in vivo* and *in vitro*. J Reprod Fertil Suppl 40:197-210.
16. MotliK J, Fulka J (1976): Breakdown of the germinal vesicle in pig oocytes *in vivo* and *in vitro*. J Exp Zool 198:155-162.
17. Nagai T, Ebihara M, Onishi A, Kubo M (1997): Germinal vesicle stages in pig follicular oocytes collected by different methods. J Reprod Deve 43:339-343.
18. Ozawa M, Nagai T, Somfai T, Nakai M, Maedomari N, Miyazaki H, Kaneko H, Noguchi J, Kikuchi K (2010): Cumulus cell-enclosed oocytes acquire a capacity to synthesize GSH by FSH stimulation during *in vitro* maturation in pigs. J Cell Physiol 222:294-301.
19. Park KW, Funahashi H, Niwa K (1999): Co-culture of cumulus-enclosed bovine oocytes with theca cells induces the meiotic arrest but does not inhibit germinal vesicle development. J Reprod Dev 45:223-231.
20. Sun XS, Liu Y, Yue KZ, Ma SF, Tan JH (2004): Changes in germinal vesicle (GV) chromatin configurations during growth and maturation of porcine oocytes. Mol Reprod Dev 69:228-234.
21. Tsafriri A, Channing CP (1975): Influence of follicular maturation and culture conditions on the meiosis of pig oocytes *in vitro*. J Reprod Fertil 43:149-152.
22. Xia GL, Kikuchi K, Noguchi J, Izaike Y (2000): Short time priming of pig cumulus-oocyte complexes with FSH and forskolin in the presence of hypoxanthine stimulates cumulus cells to secrete a meiosis-activating substance. Theriogenology 53:1807-1815.
(Received: 26 August 2010 / Accepted: 15 September 2010)