Cytoskeletal and Mitochondrial Distributions in Porcine Oocytes at Different Germinal Vesicle Stages

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Abstract: *To clarify the cytological characteristics of the porcine oocytes having meiotic competence, the oocytes retrieved from small (1–2 mm in diameter) and large follicles (3–6 mm) were compared. Cumulus-lacking oocytes were also examined to distinguish the characteristics between growing and atretic follicular oocytes. Distributions of cytoskeleton and mitochondria were observed by fluorescence staining in relation to the germinal vesicle (GV) stages, classified as GV 0 and GV I - GV IV. Oocyte size (107.2 ± 0.6 μm vs. 116.9 ± 0.7 μm, p<0.05) and maturation rate (9 vs. 87%, p<0.01) were lower in the small follicular oocytes than the large follicular oocytes. GV 0 oocytes were more frequently found in the small follicles than in the large follicles (67 vs. 9%, p<0.01). Most of the GV 0 oocytes from small follicles were likely forming the cytoplasmic microtubules and microfilaments. In contrast, most of the GV oocytes from large follicles showed an even distribution of fibrous microtubules in the ooplasm. In some of the small follicular oocytes beyond the GV II stage, fluorescence intensities of microtubules and mitochondria decreased. These features were similar to those observed in the cumulus-lacking oocytes. The results suggest that most of the GV 0 oocytes may be developing cytoplasmic cytoskeleton, and consequently are unable to complete meiosis. Also, advanced GV stage oocytes with low density of cytoskeleton and mitochondria, especially those from small follicles, may be derived from atretic follicles.*

Key words: *Cytoskeleton, Maturation, Mitochondria, Pig Oocyte*

Introduction

Heterogeneity in the quality and developmental competence of follicular oocytes obtained from slaughterhouse ovaries is one of the limiting factors in current animal biotechnology. Traditional criteria have relied on the size of follicles and the morphological assessment of cumulus-oocyte complexes (COCs). For *in vitro* maturation (IVM) in pigs, the oocytes are usually recovered from antral follicles of 3–6 mm in diameter. However, porcine follicular oocytes vary remarkably in chromatin configurations at recovery and in subsequent developmental competence [1–4]. A clear relationship was noted between the oocyte diameter and the meiotic competence in pigs [1], as well as in cattle [5]. The small follicular oocytes were reported to have less ability to undergo both nuclear and cytoplasmic maturation during IVM [6, 7]. It is important, therefore, to clarify the cytological characteristics of the oocyte having meiotic competence. In the present study, the distribution of cytoskeleton and mitochondria in oocytes retrieved from large follicles (3–6 mm in diameter) and small follicles (1–2 mm in diameter) were examined in relation to the germinal vesicle (GV) configuration. Furthermore, there is a possibility that oocytes derived from stimulated and atretic follicles may be collected together when aspirated from antral follicles. Thus, we also examined the oocytes without a cumulus layer at recovery, presumed atretic oocytes, since the majority of cumulus-lacking oocytes are considered to be oocytes derived from atretic follicles [8].

Materials and Methods

Oocyte collection and maturation

Ovaries were collected from prepubertal gilts at a

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local abattoir. Follicles present in the cortex of the ovaries were categorized into two groups according to their size, small (1–2 mm in diameter) and large (3–6 mm in diameter), from which COCs were aspirated with 24- and 18-gauge needles fixed to disposable syringes, respectively. The oocytes enclosed by compact cumulus cells were selected for small and large follicular groups. Some cumulus-lacking oocytes from the large follicles were also examined, because they were presumed to be oocytes retrieved from atretic follicles [8]. After being washed with Dulbecco's phosphate buffered saline (DPBS) containing 0.1% polyvinyl alcohol (Sigma Chemical Co., St. Louis, MO, USA), cumulus cells were removed from COCs by vigorous vortexing in a 1-ml solution of 0.1% hyaluronidase (Sigma) in calcium-free DPBS for 1–2 min. The ooplasm diameter was then measured under an inverted microscope with a micrometer eyepiece (Olympus, Tokyo, Japan).

Some of the cumulus-intact or cumulus-lacking oocytes were cultured for 24 h in NCSU23 medium supplemented with 10% (v/v) porcine follicular fluid, 0.57 mM L-cysteine (Sigma), 10 ng/ml epidermal growth factor (Sigma), 10 i.u./ml eCG (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) and 10 i.u./ml hCG (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan). They were then incubated in NCSU23 without hormonal supplements for an additional 20 h in an atmosphere of 5% CO_2 at 39°C, as reported previously [9, 10]. The oocytes were then processed for fluorescence observation as follows.

Fluorescence observation

Active mitochondria were stained with rhodamine 123 (Rh123, 10 μg/ml, Molecular Probes, Eugene, OR, USA) for 15 min in NCSU23, washed 3 times in NCSU23, mounted on slide glasses, and imaged immediately after labeling [11]. To assess the distribution of microtubules and microfilaments, the oocytes were processed as reported previously [9,10]. After fixation in a microtubule stabilization fixative, the samples were exposed to anti-β tubulin primary antibodies (1:200; Sigma) at 37°C for 2 h, and incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200; Sigma) at 37°C for 1 h. After rinsing, the samples were stained with rhodamine-phalloidin (1:1,000; Molecular Probes) for microfilaments for 1 h, and then stained for DNA with Hoechst 33342 (10 μg/ml, Sigma) in mounting medium containing DPBS and glycerol (1:1).

The samples were viewed on a fluorescence microscope (BX-FLA, Olympus). A U-MNIBA filter set (Olympus) was used for Rh123 and FITC, a U-MWIB set (Olympus) was used for rhodamine, and a U-MWU set (Olympus) for Hoechst. A CCD digital camera system (DP70, Olympus) was used to obtain images on a computer, and color adjustment and image analysis were performed by DP Manager (Olympus) and ImageJ 1.36b (Wayne Rasband, NIH, USA). Follicular oocytes were classified into GV 0 to GV IV stages [1, 12], and distributions of the microtubules, microfilaments, and mitochondria of the ooplasm were observed.

Statistical analysis

Proportional data were analyzed by the χ^2 test or Fisher's exact probability test. Oocyte size and fluorescence intensity were subjected to one-way analysis of variance and differences between the means were determined by using Tukey's multiple range test. Each experiment was repeated at least three times, and data are presented as the mean ± SEM.

Results

Oocyte size and nuclear maturation

Oocytes recovered from the small follicles were significantly smaller in diameter than those from the large follicles (107.2 ± 0.6 μm, n=93 vs. 116.9 ± 0.7 μm, n=84, $p<0.05$), although no difference was found in thickness of the zona pellucida (14.4 ± 0.5 vs. 16.2 ± 0.4 μm for the corresponding groups, respectively). Maturational stages of the oocytes before (0 h) and after culture (44 h) are shown in Table 1. GV 0-stage oocytes were more frequently found in the small follicular group than in the large follicular group (67 vs. 9%, $p<0.01$), although no GV 0 oocytes appeared in the cumulus-lacking group. The majority (66%) of the cumulus-lacking oocytes were at the GV II - GV IV stages at recovery. In cumulus intact groups, the maturation rate was lower in the small follicular oocytes than in the large follicular oocytes (9 vs. 87%, $p<0.01$). Cumulus-lacking oocytes matured at a significantly lower rate compared to the cumulus-intact oocytes (4 vs. 87%, $p<0.01$). After culture, oocytes with an abnormal chromatin configuration or without chromatin, which were considered to have degenerated, had increased in the cumulus-intact small follicular group, and in the cumulus-lacking large follicular group (39 and 47%, respectively, Table 1).

Table 1. Nuclear maturation of the porcine oocytes retrieved from small and large follicles

Follicular size	Time of culture(h)	No. oocytes examined	No. (%) oocytes at each maturational stage of§:						No. (%) oocytes degenerated
			GV0	GVI	GVII-IV	ProM	MI	MII	
Cumulus intact									
Small	0	350	233 (67)^{A}	98 (28)^{B}	19 (5)^{Da}				
(1–2 mm in diameter)	44	138		7 (5)^{D}	32 (23)^{C}		32 (23)^{A}	13 (9)^{B}	54 (39)^{A}
Large	0	304	28(9)^{B}	157 (52)^{A}	118 (39)^{B}	1 (1)			
(3–6 mm in diameter)	44	139		3 (2)^{D}	2 (1)^{b}		10 (7)^{B}	121 (87)^{A}	3 (2)^{B}
Cumulus lacking									
Large	0	219		58 (27)^{B}	145 (66)^{A}	1 (1)	4 (2)^{C}		11 (5)^{B}
(3–6 mm in diameter)	44	129		19 (15)^{C}	25 (19)^{C}	1 (1)	18 (14)^{AB}	5 (4)^{B}	61 (47)^{A}

§, GV, germinal vesicle; ProM, prometaphase; MI, metaphase-I; MII, metaphase-II. Values with different superscripts in the same column differ significantly ($^{A–D}$, $p<0.01$; $^{a,\,b}$, $p<0.05$).

Cytoskeletal distribution in the oocyte

Typical fluorescence micrographs of the cytoskeleton in the porcine GV oocytes are shown in Fig. 1. Some oocytes showed non-fibrous microtubular signals in the ooplasm, which was referred to as ‘forming’ (Fig. 1a, a’). The others contained large amounts of fibrous microtubules in the ooplasm, varying from an intermediately formed (Fig. 1b, b’) to an evenly formed pattern (Fig. 1c, c’). More GV oocytes showed an evenly formed pattern of fibrous microtubules throughout the ooplasm in the large follicular group (Fig. 1d, e) than in the small follicular group (Table 2).

The porcine GV oocytes were characterized by the cortical microfilaments stained intensely and the cytoplasmic microfilaments stained moderately (Fig. 1, right column). However, about half (48–57%) of the oocytes from small follicles showed incomplete formation of cortical microfilaments (Fig. 1b”, c”) and/or uneven distribution of microfilaments in the ooplasm (Fig. 1b”, c”). Although uneven distribution of microfilaments was also observed in the large follicular group (Fig. 1d” vs. 1e”), the proportion of the oocytes showing even distribution of microfilaments was higher in the large follicular group than in the small follicular group (Table 3).

Fluorescence intensities of microtubules (Table 2) and microfilaments (Table 3) were significantly lower in the small follicular group than in the large follicular group, when the mean intensities of the cumulus-intact GV I oocytes were calculated as 100%. Interestingly, in the small follicular group, fluorescence intensity of microtubules was lower in the GV II-IV oocytes than in the GV I oocytes. In other words, the advanced stage oocytes from the small follicles possessed cytoplasmic microtubules with low density. This tendency was also noted in the signals of microtubules and microfilaments (Tables 2 and 3) of the cumulus-lacking oocytes, which included oocytes derived from the atretic follicles [8].

Mitochondrial distribution in the oocytes

Typical fluorescence micrographs of the mitochondria in the pig oocytes are shown in Fig. 2. The small follicular GV oocytes showed a peripheral distribution of mitochondria (Fig. 2a) more frequently than the large follicular oocytes (10/62 vs. 4/81, $p<0.05$). The remaining GV oocytes in the small and large follicles of the cumulus intact group showed an even distribution of the mitochondria in the ooplasm (Fig. 2a, c). Table 4 shows relative intensities of mitochondrial fluorescence relative to the mean intensity of the cumulus-intact GV I oocytes from large follicles represented as 100%. The fluorescence intensity of mitochondria did not differ between the small and large follicular groups, but there was a tendency for the oocytes beyond the GV II stage to possess less dense distributions of active mitochondria than the GV I oocytes, irrespective of the follicular size and the condition of the cumulus layer.

Discussion

The results of the present study indicate that oocytes retrieved from small follicles are smaller in size and lower in maturation ability than those from large follicles. The diameter of the oocytes from large follicles (3–6 mm in diameter) reached their fully-grown size of 116.9 ± 0.7 μm. Our results are supported by the observations of Lucas *et al.* [4], who showed a positive correlation between oocyte size (101–117 μm) and nuclear maturation. They also found a higher proportion of GV 0 oocytes among small-sized oocytes. In the present study, about half (41–67%) of small follicular oocytes were at the GV 0 stage, while only 9% of the

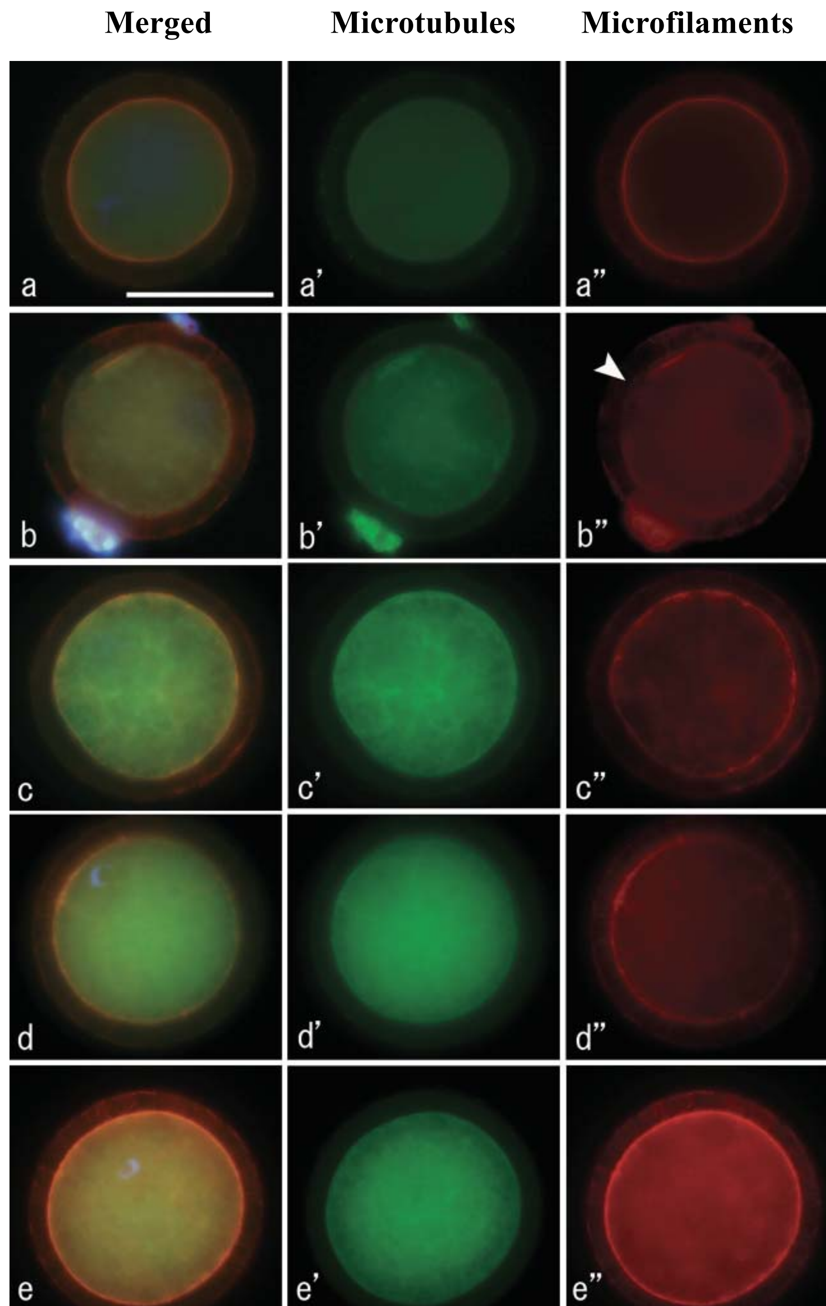

Fig. 1. Fluorescence micrographs showing distributions of chromatin and cytoskeleton of porcine GV oocytes from small (a-c) and large follicles (d,e). Left column (a-e) shows merged images; middle column (a'-e') shows microtubule (MT) labeling; and right column (a"-e") shows microfilament (MF) labeling of the same oocyte. MT are green, MF are red, and chromatin is blue; yellow shows the distribution of MT and MF. The bar in (a) represents 100 μm for all micrographs. A GV 0 oocyte (a) showing amorphous and non-fibrous appearances of MT signals in the ooplasm (a'). Cortical MF have been completely formed, but cytoplasmic MF are very low in density (a"). A GV 0 oocyte (b) showing an intermediate (partial fibrous) distribution of MT in the ooplasm (b'). Cortical MF are partially interrupted (arrow head), but cytoplasmic MF are high in density (b"). A GV 0 oocyte (c) showing a fibrous distribution of MT in the ooplasm (c'). Cortical MF are not evenly formed yet, and cytoplasmic MF are also scattered (c"). A GV I oocyte (d) showing a fibrous distribution of MT in the ooplasm (d'). Cortical and cytoplasmic MF are not evenly distributed (d"). A GV I oocyte (e) showing a fibrous distribution of MT in the ooplasm (e'). Cortical and cytoplasmic MF are evenly distributed (e").

Table 2. Distribution of microtubules in porcine GV oocytes retrieved from small and large follicles

Follicular size	No. oocytes examined	No. oocytes at GV stage	No. (%) oocytes showing distribution pattern of: Forming*	Intermediate	Evenly formed	Fluorescence intensities of cytoplasmic microtubules**
Cumulus intact						
Small	168	GV0 52	19 (37)	11 (21)	22 (42)^{C}	77 ± 4^{B}
(1–2 mm in diameter)		GVI 56	8 (14)	16 (29)	32(57)^{B}	78 ± 4^{Ba}
		GVII-IV 60	9 (15)	20 (33)	31 (52)^{Bd}	69 ± 3^{Bb}
Large	103	GV0 8	1 (12)		7 (88)	94 ± 9
(3–6 mm in diameter)		GVI 68	7 (10)	11 (16)	50 (74)^{ABbc}	100 ± 3^{A}
		GVII-IV 27		2 (7)	25 (93)^{Aa}	109 ± 3^{A}
Cumulus lacking						
Large	98	GV0 0	–	–	–	–
(3–6 mm in diameter)		GVI 26		4 (15)	22 (85)^{Aab}	92 ± 4
		GVII-IV 72			72 (100)	72 ± 3^{B}

Different superscripts show significant difference ($^{A–C}$, p<0.01; $^{a–d}$, *p*<0.05). *, 'Forming' shows a non-fibrous distribution pattern of microtubular signals in the ooplasm. **, Values were calculated relative to the mean intensities of the cumulus-intact GVI oocytes from large follicles, 100%.

Table 3. Distribution of microfilaments in porcine GV oocytes retrieved from small and large follicles

Follicular size	No. oocytes examined	No. oocytes at GV stage	No. (%) oocytes showing distribution pattern of: Uneven	Even	Fluorescence intensities of cytoplasmic microfilaments*
Cumulus intact					
Small	168	GV0 52	29 (56)	23 (44)^{B}	71 ± 4^{B}
(1–2 mm in diameter)		GVI 56	27 (48)	29 (52)^{b}	71 ± 4^{B}
		GVII-IV 60	39 (65)	21 (35)^{B}	70 ± 4^{B}
Large	103	GV0 8	4 (50)	4 (50)	103 ± 10^{a}
(3–6 mm in diameter)		GVI 68	21 (31)	47 (69)^{Aa}	100 ± 3^{A}
		GVII-IV 27	8 (30)	19 (70)^{A}	92 ± 6^{a}
Cumulus lacking					
Large	98	GV0 0	–	–	–
(3–6 mm in diameter)		GVI 26	6 (23)	20 (77)^{Aa}	74 ± 4^{B}
		GVII-IV 72	23 (31)	49 (69)^{A}	63 ± 5^{b}

Different superscripts show significant difference ($^{A, B}$, *p*<0.01; $^{a, b}$, *p*<0.05). *, Values were calculated relative to the mean intensities of the cumulus-intact GVI oocytes from large follicles, 100%.

cumulus-intact oocytes from large follicles had a GV 0 nucleus. A similar tendency has been described in ruminants [5, 13].

The present study was conducted to clarify the cytological characteristics of the oocytes having developmental ability. Several cytological and molecular events which are closely linked to nuclear maturation are associated with cytoplasmic maturation in mammalian oocytes [14]. Cytoplasmic changes involve redistribution of chromosomes and organelles through reorganization of the cytoskeleton [8, 11, 15]. The present observations suggest that the GV 0 oocytes were in the process of polymerizing microtubules and microfilaments in the ooplasm. Such incomplete organization of the cytoskeleton in the small follicular oocytes may account for their low rates of maturation after IVM. Another reason for low meiotic competence may be the peripheral distribution of mitochondria in small follicular oocytes as opposed to the even distribution of mitochondria in large follicular oocytes.

We previously reported the condition of the cumulus layer covering the oocyte affected cytoskeletal reorganization and mitochondrial distribution during oocyte maturation in pigs [8]. Since the mitochondrial organization is related to the ability to regulate intracellular homeostasis and cytoplasmic maturation [8, 11, 15, 16], it may be important to establish the mechanism of mitochondrial translocation mediated by cytoskeletal reorganization. Our recent study

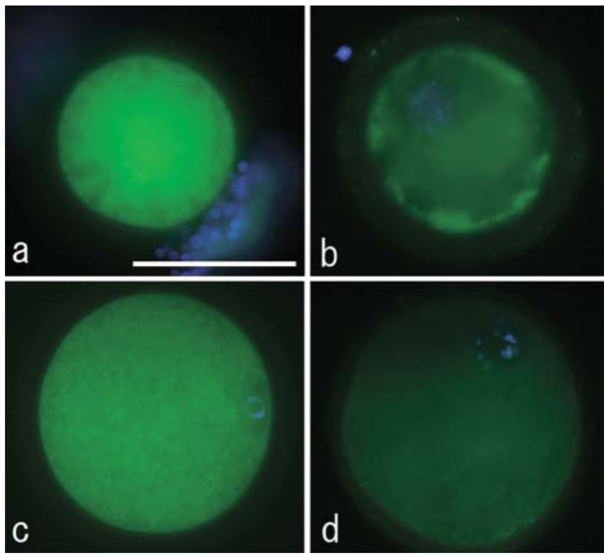

Fig. 2. Fluorescence micrographs showing the distributions of mitochondria and chromatin of the porcine GV oocytes from small (a, b) and large follicles of the cumulus-intact group (c, d). Mitochondria are green and chromatin is blue. The bar in (a) represents 100 μm for all micrographs. Two types of mitochondrial distributions observed in GV 0 oocytes from small follicles: (a) an even distribution and (b) a peripheral distribution in the ooplasm. A GV I oocyte (c) and a GV III oocyte (d) from large follicles: (c) shows an even distribution of mitochondria, whereas (d) shows decreased density of mitochondria. The relative fluorescence intensities of mitochondria (see text) are 147% in (a), 84% in (b), 98% in (c) and 60% in (d).

demonstrated that microtubules function as anchors of mitochondria in the perinuclear region, while microfilaments play a role as anchors of mitochondria in the peripheral region [17]. Therefore, establishment of the cytoskeletal system of mammalian oocytes through cytoplasmic maturation may be a prerequisite for the acquisition of developmental competence. The present observations reveal that the cytoskeletal and mitochondrial distributions differ between oocytes from small and large follicles, and that the majority of oocytes at the GV 0 stage did not gain the ability to complete meiosis until they became larger than 117 μm in diameter. The culture condition applied in this study may have been unsuitable for the subsequent growth and development of the small oocytes retrieved from small follicles. Therefore, the development of a new culture system, such as an *in vitro* growth technique for oocyte-granulosa cell complexes [18], will be necessary to grow such small oocytes to gain competence.

Table 4. Fluorescence intensities of mitochondria in porcine GV oocytes retrieved from small and large follicles

Follicular size	No. oocytes examined	No. oocytes at each GV stage		Fluorescence intensity**
Cumulus intact				
Small	62	GV0	21	96 ± 5
(1–2 mm		GVI	24	104 ± 5
in diameter)		GVII-GVIII	17	97 ± 8
Large	76	GV0	5	97 ± 9
(3–6 mm		GVI	41	100 ± 3[a]
in diameter)		GVII-GVIV	30	90 ± 5[b]
Cumulus lacking				
Large	66	GV0	0	–
(3–6 mm		GVI	26	97 ± 5
in diameter)		GVII-GVIV	40	92 ± 4

*, GV IV oocytes were not recovered from the small follicles. **, Values were calculated relative to the mean intensities of the cumulus-intact GVI oocytes from large follicles, 100%. [a, b], Values with different superscripts in the same follicular group differ significantly (p<0.05).

It is noteworthy that there was a tendency for more advanced GV stage oocytes retrieved from small follicles to show less dense microtubules and mitochondria as well as in the cumulus-lacking group. This observation suggests that decreased intensities of microtubules and mitochondria may be associated with atresia of the follicles, which probably occurs frequently in follicles stimulated before oocyte aspiration. Therefore, in addition to GV configuration, a combined evaluation of cytoskeletal distribution/intensity and mitochondrial activity at recovery may be a useful criterion for distinguishing between atretic and non-atretic follicular oocytes. The present results suggest that the small follicles in the porcine ovary may include both growing oocytes with developing cytoskeletal architecture and atretic oocytes with degrading cytoskeleton.

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References

1) Christmann, L., Jung, T. and Moor, R.M. (1994): MPF components and meiotic competence in growing pig oocytes. Mol. Reprod. Dev., 38, 85–90.
2) Hirao, Y., Tsuji, Y., Miyano, T., Okano, K., Miyake, M., Kato, S. and Moor, R.M. (1995): Association between p34^{cdc2} levels and meiotic arrest in pig oocytes during early growth. Zygote, 3, 325–332.
3) Grupen, C.G., Nagashima, H. and Nottle, M.B. (1997): Asynchronous meiotic progression in porcine oocytes matured *in vitro*: a cause of polyspermic fertilization? Reprod. Fertil. Dev., 9, 187–191.
4) Lucas, X., Martinez, E.A., Roca, J., Vazquez, J.M., Gil, M.A., Pastor, L.M. and Alabart, J.L. (2002): Relationship between antral follicle size, oocyte diameters and nuclear maturation of immature oocytes in pigs. Theriogenology, 58, 871–885.
5) Fair, T., Hyttel, P. and Greve, T. (1995): Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol. Reprod. Dev., 42, 437–442.
6) Sun, Q.Y., Lai, L., Bonk, A., Prather, R.S. and Schatten, H. (2001): Cytoplasmic changes in relation to nuclear maturation and early embryo developmental potential of porcine oocytes: Effects of gonadotropins, cumulus cells, follicular size, and protein synthesis inhibition. Mol. Reprod. Dev., 59, 192–198.
7) Liu, R-H., Li, Y-H., Jiao, L-H., Wang, X-N., Wang, H. and Wang, W-H. (2002): Extracellular and intracellular factors affecting nuclear and cytoplasmic maturation of porcine oocytes collected from different sizes of follicles. Zygote, 10, 253–260.
8) Suzuki, H. and Saito, Y. (2006): Cumulus cells affect distribution and function of the cytoskeleton and organelles in the porcine oocytes. Reprod. Med. Biol., 5, 183–194.
9) Suzuki, H., Jeong, B-S. and Yang, X. (2000): Dynamic changes of cumulus-oocyte cell communication during in vitro maturation of the porcine oocytes. Biol. Reprod., 63, 723–729.
10) Suzuki, H., Takashima, Y. and Toyokawa, K. (2001): Influence of incubation temperature on meiotic progression of porcine oocytes matured in vitro. J. Mamm. Ova Res., 18, 8–13.
11) Suzuki, H., Satoh, M. and Toyokawa, K. (2005): Changes in distribution of active mitochondria during oocyte maturation and fertilization in the hamster. J. Mamm. Ova Res., 22, 163–169.
12) Motlik, J. and Fulka, J. (1976): Breakdown of the germinal vesicle in pig oocytes in vivo and in vitro. J. Exp. Zool., 198, 155–162.
13) Han, Z-B., Lan, G-C., Wu, Y-G., Han, D., Feng, W-G., Wang, J-Z. and Tan, J-H. (2006): Interactive effects of granulosa cell apoptosis, follicle size, cumulus-oocyte complex morphology, and cumulus expansion on the developmental competence of goat oocytes: a study using the well-in-drop culture system. Reproduction, 132, 749–758.
14) Eppig, J.J. (1996): Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. Reprod. Fertil. Dev., 8, 485–489.
15) Suzuki, H., Saito, Y., Kagawa, N. and Yang, X. (2003): In vitro fertilization and polyspermy in the pig: Factors affecting fertilization and cytoskeletal organization of the oocytes. Microsc. Res. Tech., 61, 327–334.
16) Sun, Q.Y., Wu, G.M., Lai, L., Park, K.W., Cabot, R., Cheong, H.T., Day, B.N., Prather, R.S. and Schatten, H. (2001): Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. Reproduction, 122, 155–163.
17) Kabashima, K., Matsuzaki, M. and Suzuki, H. (2007): Both microtubules and microfilaments mutually control mitochondrial distribution in two cell embryos of golden hamsters. J. Mamm. Ova Res., 24, 120–125.
18) Hirao, Y., Itoh, T., Shimizu, M., Iga, K., Aoyagi, K., Kobayashi, M., Kacchi, M., Hoshi, H. and Takenouchi, N. (2004): In vitro growth and development of bovine oocyte-granulosa cell complexes on the flat substratum: Effects of high polyvinylpyrrolidone concentration in culture medium. Biol. Reprod., 70, 83–91.