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Effect of Follicle Size on Cumulus-Expansion, *In Vitro* Fertilization and Development of Porcine Follicular Oocytes

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Abstract. The aim of the present experiment was to investigate the effect of follicular size on oocyte cumulus-expansion, *in vitro* fertilization and subsequent developmental competence. After maturation culture, the rates of porcine oocytes with expanded cumulus derived from antral follicles ≥ 5 mm, 4–4.9 mm, 3–3.9 mm and 2–2.9 mm in diameter were 90.5%, 89.7%, 85.4% and 67.4%, respectively. After *in vitro* fertilization, the developmental competence of oocytes dependently increased with follicular size. Higher cleavage rates and higher proportions of 3–4-cell embryos were obtained from ≥ 4 mm follicles compared to 2–2.9 mm follicles ($P < 0.05$). Although the proportions of 6–8-cell and 12–16-cell embryos obtained from ≥ 5 mm, 4–4.9 mm, 3–3.9 mm and 2–2.9 mm follicles showed no significant differences, embryos obtained from 2–2.9 mm follicles showed a complete failure to develop beyond the 8-cell stage, embryos obtained from 3–3.9 mm follicles failed to develop beyond the 16-cell stage and none of embryos developed beyond the early stage of morula. The percentages of expanded cumulus-oocyte complexes (COCs) and 2-cell cleavage rates of oocytes derived from 2–2.9 mm follicles were not significantly different when the oocytes were matured for 36 h, 42 h and 48 h *in vitro*. The exposure to 5% or 15% porcine follicular fluid (PFF) from the large follicles during *in vitro* maturation (IVM) had no significant effect on the cleavage and subsequent developmental rates of porcine oocytes compared to PFF from small follicles.

Key words: Porcine oocytes, Cumulus-expansion, *In vitro* fertilization, *In vitro* development, Follicular size

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In mammals, meiotic division of the oocyte is initiated during fetal life and is arrested at the diplotene stage of prophase I shortly before or after the birth. The oocyte remains under meiotic arrest until the ovulatory luteinizing hormone (LH) surge, which stimulates the resumption of meiosis in the Graafian follicle. It is also known that

mammalian follicular oocytes undergo spontaneous meiotic maturation when liberated from follicles and cultured in an appropriate medium [1].

Recently, it has been described that the oocyte from the dominant follicle underwent further ultrastructural modifications and attained full developmental competence through a process that might be termed “capacitation” [2]. Ding *et al.* [3]

reported that follicular maturation is paralleled by, and functionally related to, the maturation of the oocyte inside the follicle. The results confirmed that diversity of follicular development may affect the oocyte quality and that oocytes would be at different developmental stages when derived from different sized follicles. The developmental competence of follicular oocytes *in vitro* may be affected by the follicular sizes [4]. Some researchers investigated the effect of follicular size on oocyte quality and developmental competence following *in vitro* maturation (IVM), fertilization (IVF), and culture (IVC) in bovine and goat [5–8]. It is well recognized that the characteristics of follicles could be an important parameter to determine the developmental competence of oocytes fertilized *in vitro*.

Porcine oocytes from antral follicles (2–6 mm in diameter) on the surface of slaughtered ovaries have been collected for *in vitro* production of porcine embryos [9–11]. However, there is a large variation in reported IVM-IVF results among laboratories [12]. Prather and Day [12] reported that even though only cumulus-oocyte complexes (COCs) with uniform ooplasm and compact cumulus cell mass were usually selected for oocyte maturation, there was a large variation in the dictyate stage of the first meiotic prophase among oocytes collected from follicles of different sizes. Therefore, the quality of oocytes collected from different follicular sizes, or from different ages might be a major source of variation in IVM results. Furthermore, following IVM and IVF only 3% of the embryos developed into blastocysts after *in vitro* culture [11, 13]. From these data, it is clear that only a small proportion of porcine oocytes selected for IVM can complete full cytoplasmic maturation, which confers the ability to support embryonic development. So it is necessary to further investigate the effects of differences in the follicular environment on oocyte quality.

The objectives of the present study were 1) to investigate the effect of follicular size on the type of porcine oocytes obtained for IVM and on the ability of such oocytes to be fertilized and undergo cleavage and early embryonic development *in vitro*; and 2) to assess the effect of using different proportions of porcine follicular fluid (PFF) from follicles of different sizes in the IVM medium on cumulus-expansion and *in vitro* development (IVD) of porcine oocytes.

Materials and Methods

Collection of follicular oocytes

Ovaries were obtained from perpubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in saline (9 g NaCl/l, 100 IU penicillin GK/ml, 100 IU streptomycin sulphate/ml) at 37–38 °C. Ovaries were immediately freed from their hilus and connective tissues, and washed 2–3 times in saline at 37 °C. COCs were aspirated with a 10-ml syringe equipped with a 19-gauge needle. Only oocytes with compact cumulus cells were selected. They were washed 3 times in Dulbecco's-PBS (D-PBS) with 5% newborn calf serum (NCS: Gibco; Cat. No. 16010–159) (pH: 7.48) for the experiments.

In-vitro maturation (IVM)

COCs were washed 3 times with IVM medium, which consisted of TCM199 (Gibco; Cat. No. 31100–035), 10% fetal calf serum (FCS: Gibco; Cat. No. 16000–036), 10 IU/ml eCG (Ninbo, China; Cat. No. 981018), 10 IU/ml hCG (Ninbo, China; Cat. No. 980630), 1 µg/ml estradiol –17β (Fluka, Switzerland; EEC No. 2000238), 100 mg/l sodium pyruvate, 900 mg/l calcium lactate, 550 mg/l D-glucose, 5958 mg/l Hepes, and 100 IU/ml penicillin GK, 100 IU/ml streptomycin sulphate (pH: 7.38), then transferred to a droplet of IVM medium (10–15 oocytes/100 µl) under mineral oil (Sigma; Lot. 103H10455) in a polystyrene dish (35 mm: Nunclon, Denmark; Cat. No. 153066), and cultured for 36 h at 39 °C under 5% CO₂ in a CO₂ incubator (Asahi, Japan; Model 4020). After culture, oocytes with expanded cumulus mass were selected and subjected to insemination.

Sperm preparation

The sperm preparation was carried out using a modification of the method described by Nagai *et al.* [14]. The epididymides of the boar (Landrace × Meishan × Yorkshire, 8 months old) were removed at the slaughterhouse and returned to the laboratory in saline at 37 °C within 15 min. The epididymal spermatozoa were extruded from the distal portion of the cauda by pressure with a 20-ml syringe, then diluted with BF-3 solution (1:3) (BF-3 solution: 40 g/l lactose, 20 g/l casein, 20 g/l Tris, 10 g/l citric acid monohydrate, 10 g/l sucrose) and kept for 2 h at 4 °C. Next the solution was diluted

with 4% glycerol (1:1) and balanced for 2 h at 4 C. After balance, the sperm suspensions were frozen in 0.1 ml pellets and stored in liquid nitrogen until required for IVF.

Frozen spermatozoa (3 pellets) were thawed in 10 ml D-PBS containing 5% NCS, 2 mg/ml BSA-V (Böchringer, Germany) (pH: 7.48) at 37 C. Immediately after thawing, the suspensions were centrifuged for 4 min at 200 g, the supernatant was discarded and the spermatozoa were subsequently diluted to $2-4 \times 10^8$ cells/ml and preincubated in sperm preincubation medium which consisted of TCM199 with Earle's salts, 12% FCS, 0.9 mg/l calcium lactate, 0.1 g/l sodium pyruvate, 0.55 g/l D-glucose, 5.958 g/l Hepes, 100 IU/ml penicillin GK, and 100 IU/ml streptomycin sulfate (pH: 7.80), at 37 C under 5% CO₂ in air for 1 h. After preincubation, the proportion of spermatozoa with progressive forward motility was more than 60%.

In-vitro fertilization(IVF), examination of oocytes and embryos culture

The oocytes with expanded cumulus mass were washed three times and transferred to the fertilization medium, BO solution containing 10 mg/ml BSA-V, and 2 mM caffeine (pH: 7.48). A portion of the preincubated sperm suspension was introduced into the medium so that the final concentration for IVF was $2-4 \times 10^7$ cells/ml.

After incubation for 12–16 h, the oocytes were removed from the surrounding cumulus mass and spermatozoa by agitation using a narrow-bore glass pipette and washed twice with an embryo culture medium which consisted of TCM199 with Earle's salts, 10% FCS, 3.7 ml/l sodium lactate (60% syrup), 40 mg/l sodium pyruvate, 5958 mg/l Hepes, 100 IU/ml penicillin GK, and 100 IU/ml streptomycin sulfate (pH: 7.40). Then 10–15 oocytes were transferred to a droplet of the embryo culture medium (100 μ l) containing granulosa cells prepared by the method described below.

To determine the rate of fertilization, some embryos were randomly selected after insemination and fixed for 3–4 days with acetic alcohol (methanol and acetic acid, 3:1, v/v) at 4 C, stained with 1% aceto-orcein and examined under a phase-contrast microscope (Olympus, Japan). Oocytes having enlarged sperm heads and/or male pronuclei with sperm tails were regarded as fertilized. Among the fertilized oocytes, fertilization was considered normal fertilization if

a sperm-tail closed to a male pronucleus in the cytoplasm observed.

Preparation of granulosa cells

The oocytes with expanded cumulus mass were treated with 0.1% hyaluronidase (Sigma; H6254), and the suspended granulosa cells were washed 2–3 times with the embryo culture medium. Then, the cells were cultured at a concentration of 1×10^5 cells/ml in the same medium until confluency and used for the co-cultivation with the embryos.

Experimental design and statistical analysis

In Experiment 1, the effects of follicular size on porcine oocytes obtained for IVM and on the ability of such oocytes to develop *in vitro* following IVM/IVF/IVC were examined. On recovery, oocytes were classified into four groups according to the size of the follicles (mm in diameter) from which the oocytes were derived (group 1: 2–2.9 mm; group 2: 3–3.9 mm; group 3: 4–4.9 mm; group 4: ≥ 5 mm).

In Experiment 2, the effects of different culture times (36 h, 42 h and 48 h) during *in vitro* maturation on cumulus-expansion and the developmental competence of oocytes derived from 2–2.9 mm follicles were tested.

In Experiment 3, the effects of different concentrations (5% and 15%) of porcine follicular fluid (PFF) from follicles of different sizes (group 1: 2–2.9 mm; group 2: 3–3.9 mm; group 3: 4–4.9 mm; group 4: ≥ 5 mm) during IVM on cumulus-expansion and the developmental competence of porcine oocytes from > 2 mm follicles were investigated. Each PFF sample aspirated from different sized follicles was centrifuged at 1,000 g for 20 minutes at room temperature to remove blood cells and debris. The supernatant was transferred to a sterile centrifuge tube and stored at -20 C until use. PFF was passed through 0.22- μ m membrane filters before use.

Data were analyzed with ANOVA, χ^2 -test and differences were considered to be significant at $P < 0.05$.

Results

Experiment 1

The results of Experiment 1 are presented in Tables 1 and 2. After COCs were cultured for 36 h,

Table 1. Effect of follicular size on cumulus-expansion of porcine oocytes

Group	No. of oocytes cultured	No. of oocytes with expanded cumulus (%)
1	141	95(67.4) ^a
2	164	140(85.4) ^b
3	78	70(89.7) ^b
4	63	57(90.5) ^b

^{a, b} Values in the same column with different superscripts differ significantly ($p < 0.05$).

Table 2. Effect of follicular size on *in vitro* development of porcine oocytes after IVF

Group	No. of oocytes used for IVF	<i>In vitro</i> development to (%)					
		2-cell	3–4-cell	6–8-cell	12–16-cell	Early morula	Beyond morula
1	92	13 (14.1) ^a	2 (15.4) ^d	1 (7.7)	0	0	0
2	134	28 (20.9) ^{ab}	11 (39.3) ^{de}	6 (21.4)	1 (3.6)	0	0
3	63	21 (33.3) ^{cb}	11 (52.4) ^e	6 (28.6)	2 (9.5)	1 (4.8)	0
4	54	26 (48.1) ^c	17 (65.4) ^e	9 (34.6)	2 (7.7)	1 (3.8)	0

^{a, b, c, d, e} Values in the same column with different superscripts differ significantly ($p < 0.05$).

the COCs with expanded cumulus mass were selected for IVF. The rates of oocytes with expanded cumulus from the four different groups were 67.4%, 85.4%, 89.7%, and 90.5%, respectively. A significant difference between groups 1 vs. 2, 3 and 4 was calculated ($P < 0.05$), while the difference among groups 2, 3 and 4 was not significant (Table 1).

To examine the fertilization rate, oocytes after incubation for 12–16 h with spermatozoa were randomly selected for fixing and staining. The percentage of oocytes penetrated was 75.6% (34/45), the rate of polyspermy was 23.5% (8/34) and the normal fertilization rate was 61.8% (21/34).

The oocytes used for IVF were co-cultured with granulosa cell monolayers. The cleavage rates and the proportions of 3–4-cell, 6–8-cell embryos increased progressively with follicular sizes. The cleavage rates and the proportions of 3–4-cell embryos in groups 3 and 4 were significantly higher ($P < 0.05$) than those in group 1. Although the proportions of 6–8-cell and 12–16-cell embryos showed no significant differences among groups, embryos in group 1 did not develop beyond the 8-cell stage. Also, embryos in group 2 failed to develop beyond the 16-cell stage and all embryos did not develop beyond the early stage of morula (Table 2).

Experiment 2

The rates of COCs with expanded cumulus derived from 2–2.9 mm follicles were 67.4%, 61.9% and 71.7% for 36 h, 42 h and 48 h culture, respectively, and the cleavage rates of 2-cell embryos were 14.1%, 17.4% and 11.4% without significant differences among them (Table 3).

Experiment 3

The results of Experiment 3 are presented in Tables 4 and 5. Neither the rates of COCs with expanded cumulus nor the cleavage rates differed significantly between treatments. With regard to the cleavage rate, supplementation of 5% and 15% PFF from large follicles into the IVM medium resulted in higher cleavage rate than those cultured in the medium supplemented with PFF from small follicles, but there was no significant difference between treatments.

Discussion

It is well recognized that oocytes recovered from slaughterhouse materials for *in vitro* embryo production are extremely heterogeneous in terms of quality and developmental competence [15]. The widespread use of *in vitro*-produced porcine embryos has emphasized the necessity for non-

Table 3. Effect of culture time on cumulus-expansion and IVD of porcine oocytes derived from 2–2.9 mm follicles

Culture time (h)	No. of oocytes cultured	No. of oocytes with expanded cumulus(%)	No. of oocytes used for IVF	In-vitro development to (%)			
				2-cell	3–4-cell	6–8-cell	>8-cell
36	141	95 (67.4)	92	13 (14.1)	2 (15.4)	1 (7.7)	0
42	42	26 (61.9)	23	4 (17.4)	0	0	0
48	53	38 (71.7)	35	4 (11.4)	1 (25.0)	0	0

Table 4. Effect of different concentrations of porcine follicular fluid (PFF) from different follicle sizes during IVM on cumulus-expansion of porcine oocytes

PFF from group	PFF conc.	No. of oocytes cultured	No. of oocytes with expanded cumulus (%)
1	15%	81	67(82.7)
	5%	85	69 (81.2)
2	15%	91	75 (82.4)
	5%	91	73 (80.2)
3	15%	70	62(88.6)
	5%	90	74(82.2)
4	15%	68	61(89.7)
	5%	90	75 (83.3)
	0%	91	74 (81.3)

Table 5. Effect of different concentrations of porcine follicular fluid (PFF) from different follicle sizes during IVM on IVD of oocytes

PFF from group	PFF conc.	No. of oocytes used for IVF	In vitro development to (%)					
			2-cell	3–4-cell	6–8-cell	12–16-cell	Early morula	Beyond morula
1	15%	67	30 (44.8)	11 (36.7)	4 (13.3)	1 (3.3)	0	0
	5%	69	25 (36.2)	6 (24.0)	2 (8.0)	0	0	0
2	15%	75	33 (44.0)	12 (36.4)	6 (18.2)	1 (3.0)	0	0
	5%	73	30 (41.1)	9 (30.0)	4 (13.3)	0	0	0
3	15%	62	26 (41.9)	11 (42.3)	7 (26.9)	2 (7.7)	0	0
	5%	74	30 (40.5)	12 (40.0)	6 (20.0)	2 (6.6)	0	0
4	15%	61	29 (47.5)	13 (44.8)	8 (27.6)	3 (10.3)	2 (10.3)	0
	5%	75	34 (45.3)	14 (41.2)	9 (26.5)	3 (8.8)	1 (2.9)	0
	0%	73	26 (35.6)	12 (46.2)	7 (26.9)	2 (7.7)	1 (3.8)	0

invasive methods for the selection of oocytes competent for *in vitro* development. Such selection may be exerted at various points along the developmental axis and thus may be based on one or more of several parameters, including the characteristics of the enclosing follicle.

Procházka *et al.* [16] demonstrated that IVM rate and cumulus-expansion of porcine COCs were affected by the sizes of the donor follicles, but they did not further investigate the relationship between the follicular size and the developmental competence of oocytes fertilized *in vitro*. To our knowledge, the present study is the first to

demonstrate a clear relationship between follicular size and cumulus-expansion, IVF and IVD in porcine oocytes.

As reported in porcine, meiotic competence is acquired progressively during follicular growth [17]. Full maturation involves both nuclear and cytoplasmic events that confer on the oocyte capacity for supporting normal fertilization and early embryonic development [12]. In agreement with the findings from goat and bovine, with increase of the follicular size, the developmental competence of porcine oocytes fertilized *in vitro* increased gradually in the present study. As

pointed out by Sirard *et al.* [18], oocytes accumulate a very stable form of RNA that is translated during maturation, fertilization, and early embryonic development. The RNA accumulation could be well influenced by the quality of the follicular microenvironment. Fluctuations in this microenvironment may result in variable developmental potential following fertilization [19]. Tan and Lu [20] and McCaffrey *et al.* [21] concluded that a factor inherent in oocytes derived from smaller follicles limited their further development in bovine. Barnes *et al.* also observed that follicle size can greatly affect oocyte developmental competence and concluded that the factors required for improved development are to be found within the cytoplasm of the oocytes derived from the larger follicles [5]. It is well known that nuclear events within oocytes derived from >2 mm follicles are at a minimum. The transcripts produced during this period of low transcriptional activity are critical for development and the improved developmental competence is due to cytoplasmic maturation of the oocytes during the final phases of folliculogenesis. Such follicles, although not preovulatory, are presumably among those competing for dominance and thus may provide an environment more conducive to proper cytoplasmic maturation, giving their oocytes greater capacity for embryonic development. The results reported here suggest that the competence to undergo cytoplasmic maturation is also acquired progressively by porcine oocytes during follicular growth. Therefore possible explanations for the relationship between follicular size and oocyte quality may be: 1) a factor inherent in the oocytes derived from the smaller follicles limits their further development; 2) the cytoplasm of oocytes derived from the larger follicles may have some factors required for improved development; and 3) the larger follicles may provide a better environment to induce cytoplasmic maturation for supporting subsequent embryonic development.

In an effort to determine if some special ingredient was contained in the follicular environment of the oocytes from large follicles, PFF from the different sized follicles was used in the IVM medium. The results indicate that supplementation of PFF from large follicles into the IVM medium is likely favorable to the acquisition of developmental competence by immature

oocytes.

For some reason, culture time for maturation may affect IVM, IVF and IVD of oocytes. Especially, oocytes derived from the smaller follicles may require a longer culture time for maturation *in vitro*. In order to examine the effects of culture time on IVM and IVD of COCs, oocytes derived from 2–2.9 mm follicles were cultured *in vitro* for 36 h, 42 h and 48 h. The results indicated that cumulus-expansion and the development competence of oocytes in the three groups were not significantly different, suggesting that before being isolated from follicles, the oocytes had already grown beyond some point for synthesizing some specific RNAs and proteins, then acquired the full competence to support the development of oocytes fertilized *in vitro*. Those changes and accumulation in the oocytes did not occur *in vitro*, even though the culture time *in vitro* was prolonged, thus the developmental competence may be related to the different developmental stages of oocytes before culture *in vitro* [22].

The embryo culture medium used in the present study was the same medium reported by Yoshida *et al.* [11]. Yoshida *et al.* [11] demonstrated that this culture medium may not have been suitable for embryos beyond the 2-cell stage due to lactate and pyruvate added to the medium. The detrimental effect of lactate and pyruvate on pig embryo development has been reported by Davis and Day [23] and Davis [24]. The present results showed that some embryos could develop beyond the 4-cell stage when co-cultured with granulosa cell monolayers. To some extent, the presence of somatic cells can overcome the detrimental effects of lactate and pyruvate on pig embryo development *in vitro*. Our study supports the hypothesis that the co-culture system could remove some toxic compounds from the culture medium. Generally, most of porcine embryos developing beyond the 4-cell stage can develop to the blastocyst stage. However, in the present study, “Developmental Block” seemed to exist at each cleavage stage and more than 10% of embryos lost their viability at each cleavage stage when cultured *in vitro*. Possible explanations for this are: 1) the viability of embryos generated *in vitro*, and 2) the culture conditions following *in vitro* fertilization are not appropriate for porcine zygotes. It is well known that different metabolic requirements are required at the different developmental stages of

embryos during *in vitro* culture [25, 26].

In summary, the following conclusions can be drawn from the present study: 1) there is a clear relationship between follicular size and porcine oocyte quality in terms of cumulus-expansion, IVF and IVD; 2) the developmental competence is acquired progressively by the porcine oocyte during follicular growth. 3) the developmental competence of oocytes derived from 2–2.9 mm follicles cultured for 36 h, 42 h and 48 h was not significantly different. 4) supplementation of the IVM medium with PFF from large follicles is to some extent favorable to the acquisition of developmental competence by immature oocytes; and 5) porcine embryos can overcome

“Developmental Block” when co-cultured with granulosa cell monolayers, and different culture conditions based on the different stages of porcine embryo development are required to optimize an *in vitro* culture system.

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