***In Vitro* Fertilization and Cortical Granule Distribution of Bovine Oocytes Having Heterogeneous Ooplasm with Dark Clusters**

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ABSTRACT. *In vitro* maturation, fertilization and subsequent development of oocytes with homogeneous (category 1), or heterogeneous ooplasm (category 2) were investigated. No significant differences were observed in the nuclear maturation and total fertilization rates between the two categories. However, category 2 oocytes showed a higher normal fertilization rate due to their lower incidence of polyspermy as compared to category 1 oocytes. Electron microscopic study revealed that all category 2 oocytes had cortical granules lined up next to the plasma membrane, and that some category 1 oocytes still had small clusters of cortical granules after maturation. Although the proportion of cleaved zygotes was higher in category 2, the percentages of cleaved zygotes that developed to the blastocyst stage did not differ between the two categories. These results demonstrate that oocytes with heterogeneous ooplasm have a higher capacity for normal fertilization due to the reduction in polyspermy. This can be attributed to the normal distribution of cortical granules in category 2 oocytes after maturation. —KEY WORDS: bovine, cortical granule, fertilization, morphology.

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Bovine oocytes from small antral follicles exhibit a wide variety of morphological characteristics [8, 11]. It has been widely accepted that oocytes with homogeneous ooplasm and compact multilayered cumulus investment are suitable for *in vitro* maturation [11]. The number of oocytes with heterogeneous ooplasm having dark clusters collected from the ovary is more than double (approximately 25%) that of the oocytes with homogeneous ooplasm [8]. Recent studies have suggested that the oocytes with heterogeneous ooplasm with dark clusters have a similar or higher capacity for *in vitro* maturation [4, 5], fertilization [8, 12] and development [2, 7, 8] than the oocytes with homogeneous ooplasm. However, the previous results were inconsistent and fragmental, since *in vitro* maturation, fertilization, or subsequent development were separately examined by different groups under the different culture conditions. Moreover, the relationship between the developmental capacity and the ultrastructural features of the oocytes with various morphologies have not been examined.

In this study, therefore, we compared the *in vitro* maturation, fertilization and subsequent developmental competence of oocytes with homogeneous ooplasm and the oocytes having heterogeneous ooplasm with dark clusters. The relationship between developmental capacity and ultrastructural features was also evaluated using an electron microscope.

MATERIALS AND METHODS

Oocyte classification: The cumulus-oocyte complexes (COCs) were aspirated from small antral follicles of slaughterhouse-obtained bovine ovaries as described previously [15]. They were washed two times in Hepes-buffered Tyrode's medium (TALP-Hepes) [1] supplemented with 3 mg/ml bovine serum albumin (BSA, fraction V,

Sigma Chemical Co., St. Louis, U.S.A.), 0.2 mM sodium pyruvate (Sigma) and 50 µg/ml gentamicin sulfate (Sigma). During the washing procedure, oocytes were examined, selected and classified under a stereomicroscope by the following criteria. Category 1 represented oocytes with homogeneous ooplasm and multilayered cumulus investment. Category 2 represented oocytes having heterogeneous ooplasm with dark clusters, and multilayered cumulus investment.

***In vitro* maturation, fertilization and culture:** *In vitro* maturation, fertilization and culture were carried out using the procedure described previously [15]. Briefly, the COCs were washed once in the maturation medium; 25 mM Hepes-buffered TCM 199 supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, NY, U.S.A.), 0.02 units/ml follicle-stimulating hormone (Sigma), 1 µg/ml estradiol-17β (Sigma) and 0.2 mM sodium pyruvate and 50 µg/ml gentamicin sulfate. The oocytes in each category were cultured separately for 22 hr in a 50 µl droplet of maturation medium (10 or 11 oocytes per droplet) covered with paraffin oil at 39°C in a humidified atmosphere of 5% CO₂ in air. Frozen bovine semen collected from a Holstein bull was used for *in vitro* fertilization. After thawing the semen in a 37°C water bath for 30 sec, the motile sperm were separated using a discontinuous gradient with 45 and 90% Percoll (Pharmacia BioProcess, Uppsala, Sweden) by centrifugation at 700 × g for 20 min. The sperm were subsequently washed with a modified Brackett and Oliphant (mBO) medium [3] by centrifugation at 500 × g for 5 min. The mature oocytes were co-incubated with the sperm at the concentration of 5 × 10⁶ sperm/ml in a 100 µl droplet of mBO medium containing 3 mg/ml fatty-acid-free BSA (Sigma) and 2.5 mM theophylline (Sigma) covered with paraffin oil for 20 hr at 39°C in a humidified atmosphere of 5% CO₂ in air. After insemination, presumptive zygotes were stripped of

cumulus cells by vortexing in TALP-Hepes. They were washed three times with culture medium, a modified synthetic oviduct fluid supplemented with 20 amino acids and insulin (mSOFai) [15]. Groups of 25 to 35 presumptive zygotes were cultured in 50 μ l droplets of mSOFai covered with paraffin oil at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

Experiment 1: The oocytes in each category were cultured separately for 12 or 22 hr in the maturation medium to clarify the difference in nuclear maturation between the two categories. At the end of culture, the COCs were vortexed and stripped of cumulus investment. The naked oocytes were fixed with ethanol:acetic acid (3:1), and stained with 1% aceto-orcein. Their nuclear status was examined under a phase-contrast microscope.

Experiment 2: The fertilization rate of oocytes in each category was evaluated at 20 hr post-insemination (hpi), after they were fixed and stained as described above. Oocytes were considered to be fertilized when they had an enlarged sperm head(s) or male pronucleus(ei) with the corresponding sperm tail(s). The normal fertilization was determined by the presence of a pair of pronuclei and a corresponding sperm tail.

Experiment 3: This experiment was conducted to evaluate the subsequent developmental capacity of oocytes in each category. After *in vitro* maturation and *in vitro* fertilization, presumptive zygotes derived from the oocytes in each category were first cultured for 52 hr in mSOFai. At 72 hpi, they were divided into 5 groups according to their developmental stages (1-cell, 2-cell, 3- to 4-cell, 5- to 7-cell and $\geq$ 8-cell stages). The embryos in each group were then separately cultured in fresh culture medium until 168–170 hpi to determine their development to the blastocyst stage. All of the blastocysts obtained were fixed and their total cell numbers were counted using the air-drying method described elsewhere [14].

Experiment 4: This experiment evaluated the difference in the ultrastructural features, especially cortical granule distribution, between the two categories of oocytes, by using a transmission electron microscope. Before and after 22-hr culture for maturation, the oocytes in each category were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline at pH 7.3, and post-fixed in 1.0% osmium tetroxide in the same buffer. They were then dehydrated in a graded series of ethanol, treated with propylene oxide, embedded in epoxy resin and sectioned. The semi-thin sections stained with 0.5% toluidine blue at pH 7.5 were observed under a light microscope. The ultra-thin sections stained with ethanolic uranyl acetate and lead citrate were examined under a transmission electron microscope (JEM-1210; JEOL, Tokyo, Japan). The evaluations of ultrastructural features in the semi-thin and ultra-thin sections were performed on sections of the equatorial plane of the oocyte.

Statistical analysis: The experimental data on maturation, fertilization and total cell number in blastocysts were analyzed by Student's *t*-test. The data on *in vitro* development were analyzed by the chi-square test.

RESULTS

Experiment 1: There was no significant difference in the nuclear status between the two categories at 12 and 22 hr after *in vitro* culture for maturation (Table 1).

Experiment 2: Although the total fertilization rates did not differ between the two categories, the normal fertilization rate in category 2 was higher than that of category 1 ($P < 0.05$, Table 2). The incidence of polyspermy in category 1 was significantly higher than that of category 2 ($P < 0.01$).

Experiment 3: The proportion of cleaved zygotes was significantly higher in category 2 than in category 1 at 72 hpi ($P < 0.01$, Table 3). However, there were no significant differences between the two categories in the proportion of embryos at each developmental stage (2-cell, 3- to 4-cell, 5- to 7-cell or $\geq$ 8-cell stage) based on the number of cleaved zygotes. The percentages of embryos that developed to the blastocyst stage and total cell numbers in the blastocysts were not different between the two categories.

Experiment 4: Before the culture for maturation, cortical granules were organized in large clusters (Fig. 1a) in all category 1 oocytes ($n=10$) and in 5 of 10 category 2 oocytes. The rest of the category 2 oocytes ($n=5$) presented cortical granules partly lined up next to the plasma membrane (Fig. 1b). The lipid droplets were more abundant and often larger in diameter in the category 2 oocytes (Fig. 1c).

After 22-hr culture for maturation, 7 of 15 category 2 oocytes had cortical granules located individually next to the plasma membrane. In the other category 2 oocytes ($n=8$), cortical granules were organized in small clusters and partly lined up next to the plasma membrane. In category 1, large clusters of cortical granules were still observed in 7 of 15 oocytes. Four category 1 oocytes had cortical granules located individually next to the plasma membrane. In the rest of the category 1 oocytes ($n=4$), cortical granules were organized in small clusters and partly

Table 1. Nuclear status of oocytes at 12 hr- and 22 hr-culture for maturation in two categories

Item	Oocyte category ^{a)}	
	1	2
12 hr after culture		
No. of oocytes cultured	70	63
(No. of replicates)	(3)	(3)
% of metaphase I	98.5 $\pm$ 2.6	98.3 $\pm$ 2.9
% of anaphase I and telophase I	1.5 $\pm$ 2.6	1.7 $\pm$ 2.9
22 hr after culture		
No. of oocytes cultured	67	71
(No. of replicates)	(3)	(3)
% of metaphase I	4.7 $\pm$ 4.6	1.4 $\pm$ 2.5
% of metaphase II	95.3 $\pm$ 4.6	98.7 $\pm$ 2.5

Values are means $\pm$ SD.

a) Category 1: oocytes with homogeneous ooplasm, Category 2: oocytes having heterogeneous ooplasm with dark clusters.

lined up next to the plasma membrane.

DISCUSSION

The present results agreed with previous findings [4, 5] that oocytes having heterogeneous ooplasm with dark clusters have potential for nuclear maturation *in vitro* comparable to that of oocytes with homogeneous ooplasm. However, the maturation rate of oocytes with heterogeneous ooplasm after 24-hr culture was reported to be significantly higher than that of oocytes with homogeneous ooplasm [12]. The discrepancy may be due to the low maturation rate (approximately 80%) after 24-hr culture of the oocytes with homogeneous ooplasm under 2% CO₂ in air in the previous report [12].

The normal fertilization and cleavage rate were higher in category 2 oocytes than in category 1 due to the low

incidence of polyspermy. In previous reports [8, 12], however, there was no significant difference in the proportions of normally fertilized oocytes between the two groups that were comparable to the present categories 1 and 2. The disagreement with our results may be due to the difference in the experimental design used for *in vitro* fertilization; co-culturing with somatic cells [8] or using calcium ionophore A23185 and several concentrations of sperm for insemination [12]. In the present study, the total fertilization rates were constant at about 95% among replicates in both categories, whereas the fertilization rate varied between 30 and 90% in both categories in the previous study [12].

Cortical granules are distributed throughout the entire inner surface of the sphere of the oocyte during final maturation [10, 17]. Exocytosis of cortical granules is the most common mechanism for the prevention of polyspermy. The clustering of the cortical granules after maturation is likely to be responsible for the frequently encountered abnormal cortical granule release, which may dispose the oocytes to polyspermic penetration [9]. In all category 2 oocytes, cortical granules lined up next to the plasma membrane after the culture for maturation, whereas some category 1 oocytes had small clusters of cortical granules. It is probable that category 1 oocytes could not acquire the ability to block polyspermy due to the improper distribution next to the plasma membrane.

It was shown that the difference in the cortical granule distribution was present in the oocytes with various morphologies before *in vitro* maturation [5], and was no longer observed after *in vitro* maturation [4]. However, the difference in the cortical granule distribution in each

Table 2. *In vitro* fertilization in two categories at 20 hr post-insemination

Item	Oocyte category ^{a)}	
	1	2
No. of oocytes inseminated (No. of replicates)	134 (5)	131 (5)
% of total fertilization	94.5 ± 3.4	96.0 ± 4.1
% of normal fertilization	86.2 ± 4.9 ^{b)}	93.8 ± 2.2 ^{c)}
% of polyspermy	8.3 ± 3.4 ^{d)}	2.2 ± 2.0 ^{e)}

Values are means ± SD.

a) see the footnote in Table 1.

b, c) and d, e) Values with different superscripts differ significantly (P<0.05 and P<0.01, respectively).

Table 3. Developmental stages at 72 hr post-insemination and development to blastocyst stage at 168–170 hr post-insemination derived from oocytes of each category

Item	Oocyte category ^{a)}	
	1	2
No. of zygotes cultured	254	256
No. (%) of cleaved zygotes ^{b)}	207 (81.9) ^{c)}	235 (91.6) ^{d)}
No. (%) of each cell stage/cleaved at 72 hpi		
2-cell stage	43 (20.8)	36 (15.3)
3–4-cell stage	43 (20.8)	44 (18.7)
5–7-cell stage	40 (19.3)	45 (19.1)
≥8-cell stage	81 (39.1)	110 (46.8)
Total no. (%) of blastocysts/cleaved zygotes	42 (20.3)	55 (23.4)
No. (%) of blastocysts derived from embryos at each stage at 72 hpi		
2-cell stage	0 (0.0)	0 (0.0)
3–4-cell stage	1 (2.3)	1 (2.3)
5–7-cell stage	7 (17.5)	9 (20.0)
≥8-cell stage	34 (42.0)	45 (40.9)
Total cell no. in blastocysts (mean ± SD)	129.5 ± 48.6	149.1 ± 57.9

Data were pooled for 6 replicates.

a) see the footnote in Table 1.

b) The embryos that developed to ≥2-cell stage were considered to be cleaved zygotes.

c, d) Values with different superscripts differ significantly (P<0.01).

Fig. 1. Ultrastructural features of bovine oocytes under a transmission electron microscope. a; Cortical granules are organized in a large cluster (arrowhead). b; Cortical granules lined up next to the plasma membrane (arrowhead). c; The lipid droplets (L) are abundant and appear to cluster with mitochondria (M). Bar = 1 μ m

category remained after maturation in the present study. One possible reason for this inconsistency might be the difference in the *in vitro* maturation system. Oocytes were previously co-cultured with granulosa cells in a large well without an oil covering [4].

In spite of the significant difference in the percentage of cleaved zygotes, the proportion of cleaved zygotes that developed to the blastocyst stage did not differ between the two categories. This finding agrees with previous reports [7, 8], and suggests that the developmental capacity of normally fertilized oocytes to the blastocyst stage does not differ between the two categories. However, the developmental rate of cleaved zygotes to the blastocyst stage in this study was lower than that obtained under our routine procedure (30–40%) [16]. The present culture conditions may be suboptimal, since we divided embryos into several groups according to their developmental stages at 72 hpi. This procedure reduces the embryo density in culture and decreases the embryonic developmental rate [2, 6, 13]. Therefore, we might have failed to detect differences in the developmental rate of the two categories. Thus, we need to conduct further studies under optimal conditions.

It is concluded that oocytes with heterogeneous ooplasm have a higher capacity for normal fertilization than oocytes with homogeneous ooplasm, probably due to the reduction in polyspermy. This can be attributed to the normal distribution of cortical granules in the oocytes with heterogeneous ooplasm.

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