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Relationship between bovine oocyte morphology and *in vitro* developmental potential

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Summary

We investigated the relationship between the morphology of oocytes collected from small antral follicles and their developmental capacity. Immature oocytes were classified into seven groups and cultured *in vitro* for maturation (IVM), fertilization (IVF) and development to blastocysts (IVC). After IVF, sperm penetration and normal fertilization rates were higher in the oocytes whose cytoplasm appeared brown. The rate of polyspermy was highest in the oocytes whose cytoplasm was black. After IVC, the rates of cleavage and of development to the blastocyst stage were also higher in the brown oocytes. Although the oocytes with dark clusters in a pale cytoplasm showed lower cleavage rates, cleaved zygotes had high developmental rates the same as the oocytes with a brown cytoplasm. Transmission electron microscopy showed that the oocytes with a pale or black cytoplasm had organelles arranged differently from other oocytes before IVM. Most of the oocytes with a brown and homogeneous cytoplasm or small diameter had the characteristics of immature cytoplasm (large clusters of cortical granules) even after IVM. On the other hand, the brown oocytes with a dark zone at the periphery or with dark clusters showed the same arrangement of organelles as *in vivo* matured oocytes. The oocytes with a pale or black cytoplasm appeared to be degenerating and/or ageing. In conclusion, a dark ooplasm indicates an accumulation of lipids and good developmental potential, while a light-coloured ooplasm indicates a low density of organelles and poor developmental potential. A black ooplasm indicates ageing and low developmental potential.

Keywords: Developmental ability, Electron microscopy, *In vitro* culture, Oocyte morphology, Pseudomaturation

Introduction

Many calves are produced by oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and embryo transfer. However, the production of morulae and blastocysts from *in vitro* matured oocytes shows a modest success rate compared with that from *in vivo* matured oocytes (Leibfried-Rutledge *et al*., 1987). One reason for this is the suboptimal maturational culture conditions for the acquisition of developmental capacity. In fact, we cannot achieve a good enough cytoplasmic maturation of bovine oocytes in the IVM system (Hyttel *et al*., 1986) and the mRNA expression of *in vitro* matured oocytes was different from that of *in vivo* matured oocytes (Lonergan *et al*., 2003), although nuclear maturation is easily achieved *in vitro* (Hyttel *et al*., 1986; Nagano *et al*., 1999). Another possible reason for the low developmental rates of bovine *in vitro* matured oocytes may be degeneration. The cow is a mono-ovulator, therefore most of the oocytes used for *in vitro* embryo production (IVP) are destined to degenerate. If we correctly estimate their capacity and destiny before IVM culture, we can select only those oocytes capable of developing into blastocysts for IVP calves. Moreover, if we can recognize the mechanism of follicular selection during the estrous cycle and the relationship between follicular growth/degeneration and oocyte developmental capacity, we will develop a new method for IVP of cattle such as the culture of preantral and antral follicles *in vitro*.

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Bovine oocytes derived from small antral follicles vary in appearance, cytoplasmic features and/or cumulus attachment (Leibfried & First, 1979). It is well known that cumulus cells play important roles in the maturation of oocytes and are essential for the cytoplasmic maturation *in vitro*. Many researchers have focused on the cumulus conditions of oocytes, and it was reported that oocytes with a multilayered cumulus showed higher nuclear maturation, fertilization and developmental rates than denuded oocytes and oocytes with corona radiata alone (Xu *et al*., 1986; Shioya *et al*., 1988; Yang & Lu, 1990; Madison *et al*., 1992; Lonergan *et al*., 1994; Momozawa & Fukuda, 1995; Stojkovic *et al*., 2001). Some researchers have focused on the relationship between the cytoplasmic appearance of oocytes and their competence for IVM (Younis *et al*., 1989; Momozawa & Fukuda, 1995; Nagano *et al*., 1999), fertilization (Younis *et al*., 1989; Momozawa & Fukuda, 1995; Nagano *et al*., 1999) and subsequent development (Hawk & Wall, 1994; Blondin & Sirard, 1995; Hazeleger *et al*., 1995; Nagano *et al*., 1999). Their results suggested that denuded oocytes and oocytes with corona radiata alone showed less developmental capacity, and that the appearance of the ooplasm reflected the *in vitro* developmental capacity of the oocyte. However, no reports have investigated the relationship between the ultrastructure of bovine oocytes and their developmental potential. If the relationship with bovine oocyte morphology and developmental potential can be elucidated, the use of oocytes to produce *in vitro* derived embryos will be encouraged by developing a non-invasive method of selecting developmentally competent oocytes. In addition, increasing interest in biotechnological uses for oocytes matured *in vitro* further increases the demand for oocytes selected on the basis of developmental potential. Therefore, we investigated the developmental competence of oocytes and examined the morphological characteristics of the ooplasm.

Materials and methods

Oocyte recovery

The ovaries of cattle (mostly of the Holstein breed) were obtained at a local abattoir, kept in normal saline at 37 °C and delivered to the laboratory within 4 h after their collection. Cumulus–oocyte complexes (COCs) were collected from small antral follicles (2–8 mm in diameter) as described previously (Nagano *et al*., 1999). All COCs were washed twice in HEPES-buffered Tyrode's medium (TALP-HEPES) (Bavister *et al*., 1983) supplemented with 3 mg/ml of bovine serum albumin (BSA, fraction V, Sigma Chemical, St Louis, MO), 0.2 mM sodium pyruvate and 50 μg/ml of gentamicin sulfate (Sigma).

Table 1 Cytoplasmic appearance of oocytes in the seven groups

	Cytoplasmic characteristics	
Oocyte group	Colour	Appearance
1	Brown	Homogeneous
2	Brown	Homogeneous with a dark zone around the periphery
3	Brown	Heterogeneous with dark clusters
4	Pale	Homogeneous
5	Pale	Heterogeneous with dark clusters
6	Black	Homogeneous
7	Variable cytoplasmic features and a diameter of <115 μm	

All oocytes have several layers of unexpanded cumulus cells. Oocytes of groups 1–6 are ≥115 μm in diameter.

Oocyte classification

The COCs were examined under a stereomicroscope (×15–50) and divided into seven groups according to their appearances as described in Table 1 and Fig. 1

In vitro maturation and fertilization

The COCs were subjected to IVM and IVF as described previously (Takahashi *et al*., 1996). Briefly, the COCs were washed once in the maturation medium, which was 25 mM HEPES-buffered TCM 199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), 0.02 units/ml of follicle stimulating hormone (Sigma), 1 μg/ml of estradiol-17β (Sigma), 0.2 mM sodium pyruvate and 50 μg/ml of gentamicin sulfate. They were then cultured for about 22 h in 50 μl droplets of maturation medium (10 COCs/droplet) covered with paraffin oil at 39 °C in a humidified atmosphere of 5% CO_2 in air. After IVM, about 15 COCs were co-incubated with motile sperm (5×10^6 sperm/ml) in a 100 μl droplet of modified Brackett & Oliphant (mBO) medium (1975) for 20 h at 39 C in a humidified atmosphere of 5% CO_2 in air (day 0). The droplets of mBO medium contained 3 mg/ml of fatty-acid free BSA (Sigma) and 2.5 mM theophylline (Sigma) and were covered with paraffin oil. The motile sperm from frozen semen were separated using 45% and 90% Percoll (Pharmacia BioProcess, Uppsala, Sweden) gradients. At the end of IVF culture, presumptive zygotes were transferred to 1.5 ml tubes and vortexed for 4 min in TALP-HEPES to strip cumulus investments.

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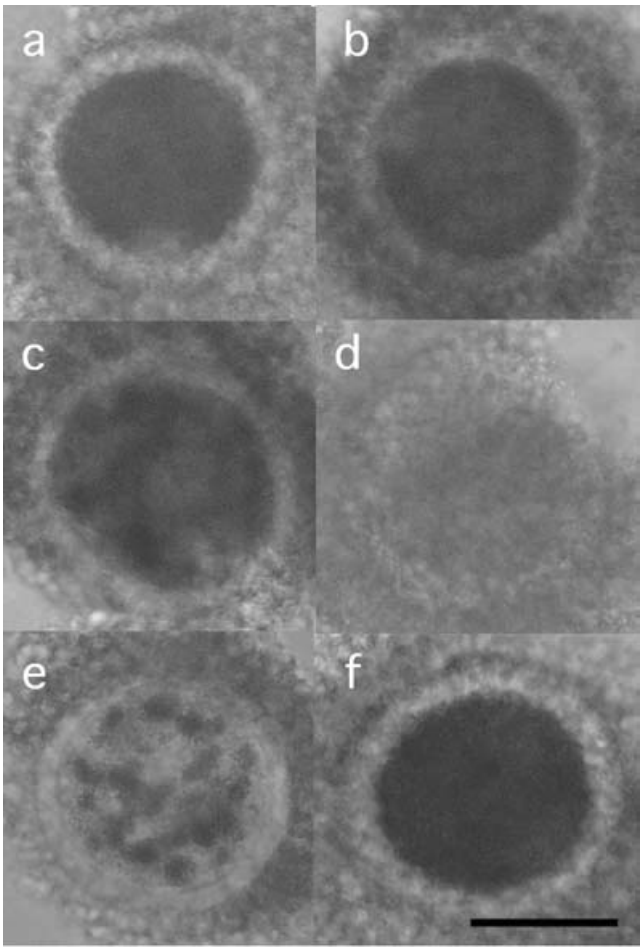


Figure 1 Oocyte morphology under a stereomicroscope. The figure shows typical images of groups 1–6 (*a–f*, respectively). An oocyte of group 7 is not illustrated since these are classified by diameter (<115 μm) regardless of the appearance of the cytoplasm. See Table 1 for the classification of oocyte groups. Scale bar represents 100 μm.

Evaluation of fertilization

After IVF, the presumptive zygotes were fixed with ethanol:acetic acid (3:1) and stained with 1% aceto-orcein to evaluate oocyte fertilizability. They were then examined for normal fertilization as indicated by the presence of two pronuclei and the corresponding sperm tail under a phase-contrast microscope.

In vitro culture and evaluation of subsequent embryonic development

After removal of the cumulus cells, the presumptive zygotes were washed three times with culture medium. The culture medium was a modified synthetic oviduct fluid (mSOFai) (Takahashi *et al*., 1996) with 3 mg/ml of BSA instead of polyvinyl alcohol. All presumptive zygotes were cultured for 168–170 h in groups of about 30 embryos per 50 μl droplet of mSOFai covered with paraffin oil at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Cleavage and development to the blastocyst stage of inseminated oocytes were evaluated at 168–170 h (day 7) after insemination. All blastocysts were fixed and the number of live cells was counted using an air-drying method reported previously (Takahashi & First, 1992).

Examination of ooplasmic structure by light and transmission electron microscopy

The COCs were fixed with 2.5% glutaraldehyde in 0.1 M Sörensen's phosphate buffer solution at pH 7.3, post-fixed with 1.0% osmium tetroxide in the same buffer, dehydrated in a graded series of ethanol, treated with propylene oxide, embedded in epoxy resin and sectioned. The semi-thin sections were stained with 0.5% toluidine blue at pH 7.5 and observed under a light microscope. The ultra-thin sections were contrasted with ethanolic uranyl acetate and lead citrate, and observed under a transmission electron microscope (JEM-1210, JEOL, Tokyo, Japan). The evaluation of semi-thin and ultra-thin sections was performed using sections of the equatorial plane of the oocytes, whereas the nuclear morphology was studied with other relevant sections.

Experimental design

Experiment 1
The COCs in seven groups were subjected to IVM/IVF and their fertilizability examined.

Experiment 2
After IVF, presumptive zygotes in seven groups were cultured and the cleavage, development to the blastocyst stage and the numbers of cells in blastocysts were examined.

Experiment 3
The morphological features (nuclear status, lipid accumulation and organelle-free area) of COCs immediately after collection (immature, $n = 5$) and COCs after maturational culture (mature, $n = 10$) in seven groups were examined. The number of cortical granules (CGs) in a cluster and the number of CG clusters were counted. A minimum of 1000 μm of the cell border was searched per oocyte. Cumulus cell process endings (CCPEs) were classified as deep, intermediate or non-penetrating according to a previous study (de Loos *et al*., 1991). Briefly, if ≥75% of the total area was located in the cortex of the oocyte, the CCPE was considered to be deeply penetrating. It was

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denoted as non-penetrating when ≤25% of the total area was located in the cortex. The remaining CCPEs were denoted as intermediate. From an oocyte in each group, 100 CCPEs were scored.

Statistical analysis

All data on fertilizability and developmental potential, and numbers of CG clusters and CCPE penetration, were analysed using a one-way analysis of variance followed by Tukey–Kramer's honestly significant difference as a *post hoc* test. The number of CG clusters before and after IVM in each group was compared by Student's *t*-test. All analyses were performed using SAS software (JMP version 5, SAS Institute, Cary, NC).

Results

Experiments 1

As shown in Table 2, the oocytes with brown ooplasm (groups 1–3) showed higher rate of normal fertilization than the other groups. Among oocytes with brown ooplasm, those with dark clusters (group 3) showed the highest rate of normal fertilization. The rate of polyspermy was not significantly different among all groups, but tended to be higher in oocytes with black ooplasm (group 6) than oocytes in group 3 ($p = 0.07$).

Table 2 *In vitro* fertilization of the oocytes

Oocyte group	No. of replicates (oocytes)	% of oocytes	
		Normally fertilized	Polyspermy
1	7 (108)	66.3 ± 15.7^{ab}	6.4 ± 6.3
2	8 (148)	70.1 ± 17.8^{ab}	4.3 ± 3.9
3	8 (143)	78.9 ± 11.8^{a}	1.2 ± 2.3
4	8 (94)	55.0 ± 8.1^{b}	3.0 ± 6.5
5	6 (81)	54.4 ± 16.5^{b}	6.8 ± 7.8
6	6 (62)	46.7 ± 14.8^{b}	11.5 ± 9.3
7	8 (121)	49.2 ± 13.6^{b}	7.0 ± 7.3

Values are the mean ± SD.
[ab]Values with different superscripts differ significantly ($p < 0.05$).

Experiment 2

As shown in Table 3, the oocytes with brown ooplasm (groups 1–3) showed a higher rate of cleavage and development to blastocyst stage than the other groups. Among oocytes with brown ooplasm, those with dark clusters (group 3) showed the highest rate of cleavage and development to the blastocyst stage. Although the oocytes with pale ooplasm with dark clusters (group 5) showed a low cleavage rate, they showed high development to blastocyst stage (per cleaved oocytes) and large cell numbers of blastocysts the same as the oocytes in groups 1–3.

Experiment 3

As shown in Table 4, light microscopic and ultramicroscopic features showed the germinal vesicle (GV) was located at a peripheral position in the cytoplasm in all groups before IVM. The oocytes in groups 1 and 7 had a round GV (Fig. 2*a*); however, the GV of some oocytes in groups 2–6 showed undulation of the nuclear membrane (Fig. 2*b*). Two out of 5 oocytes in group 6 showed chromosomal condensation (Fig. 2*c*). After IVM, most oocytes in all groups showed GV breakdown, but only 1 oocyte of group 6 had a GV. One oocyte in group 4 and 1 oocyte in group 5 had degenerated, and most of the oocytes in groups 1–6 reached the metaphase II stage (8/10, 8/10, 9/10, 6/9, 9/9 and 7/10, respectively). Few oocytes were at the metaphase II stage in group 7 (3/10). Before IVM, all oocytes in groups 3, 5 and 6 had a large number of lipid droplets in their cytoplasm and these droplets were sometimes associated with mitochondria. After IVM, the lipid droplets were not accumulated in the

Table 3 Subsequent development of the oocytes

Oocyte group	No. of replicates (oocytes)	% of oocytes developed to		
		≥2-cell	Blastocysts*	Cell no. of blastocysts (*n*)
1	5 (111)	72.2 ± 6.1^{ab}	22.6 ± 9.2^{ab}	119.9 ± 52.8^{b} (18)
2	5 (142)	74.2 ± 7.4^{ab}	24.4 ± 9.3^{ab}	158.8 ± 68.5^{ab} (25)
3	7 (197)	84.8 ± 7.9^{a}	29.4 ± 5.1^{a}	178.8 ± 54.2^{a} (48)
4	5 (108)	47.3 ± 14.6^{c}	9.2 ± 7.2^{b}	98.5 ± 53.6^{ab} (4)
5	6 (172)	54.6 ± 13.5^{bc}	24.0 ± 12.8^{ab}	184.6 ± 54.8^{a} (23)
6	5 (89)	38.4 ± 17.7^{c}	7.5 ± 11.2^{b}	163.7 ± 46.3^{ab} (3)
7	4 (92)	43.5 ± 12.6^{c}	14.0 ± 19.7^{ab}	154.5 ± 57.7^{ab} (6)

Values are the mean ± SD.
[abc]Values with different superscripts differ significantly ($p < 0.05$).
*Blastocysts/≥2-cell embryos.

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Table 4 Structural characteristics of the oocytes before and after *in vitro* maturation

Maturational culture (No. of oocytes examined)	Characteristics	Oocyte group 1	2	3	4	5	6	7
Before (5)	Germinal vesicle at periphery	5/5	5/5	5/5	5/5	5/5	3/3*	5/5
	Undulating nuclear membrane	0/5	2/5	3/5	3/5	4/5	3/3*	0/5
	Large number of lipid droplets	0/5	1/5	5/5	0/5	5/5	5/5	0/5
	Mitochondria with lipid droplets	1/5	3/5	5/5	2/5	4/5	5/5	2/5
	Organelle-free areas	0/5	0/5	0/5	1/5	1/5	2/5	1/5
After (10)	Large number of lipid droplets	8/10	9/10	9/10	0/9**	9/9**	10/10	6/10
	Mitochondria with lipid droplets	10/10	10/10	10/10	4/9**	9/9**	10/10	10/10
	Organelle-free areas	10/10	10/10	10/10	9/9**	9/9**	10/10	10/10

*Two oocytes indicated germinal vesicle breakdown.
**One oocyte degenerated and eliminated from evaluation.

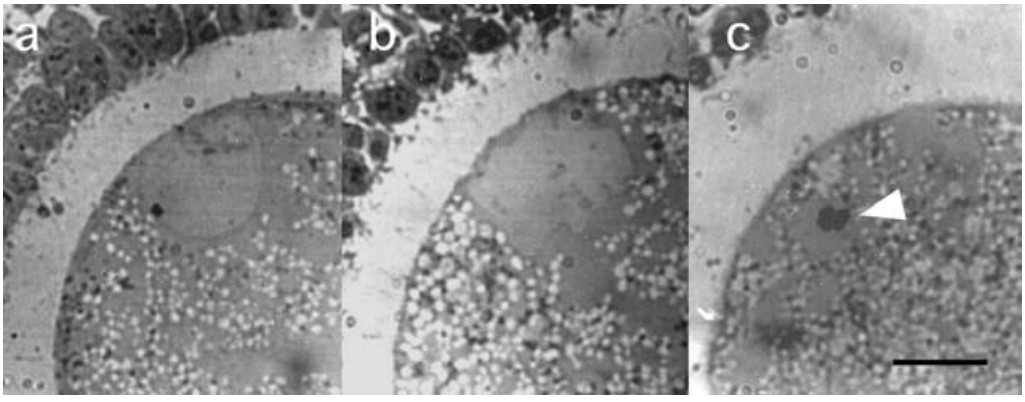


Figure 2 Nuclear status before maturational culture. (*a*) The oocyte had a round germinal vesicle (GV) (group 1). (*b*) The oocyte had a GV with an undulating nuclear membrane (group 2). (*c*) The oocyte showed chromosomal condensation (arrowhead) (group 6). Scale bar represents 10 μm.

oocytes of group 4. Some oocytes in groups 4–7 had an organelle-free area in the periphery of the cytoplasm before IVM. All oocytes in all groups exhibited an organelle-free area in the periphery of the cytoplasm after IVM.

As shown in Table 5, before IVM, most of the oocytes in all groups had clustered CGs (Fig. 3*a*); however, some oocytes in groups 3–7 showed partially dispersed CGs (Fig. 3*b*), and one oocyte in group 6 had no clustered CGs. After IVM, more than half of the oocytes in groups 3, 5 and 6 had no clustered CGs. All oocytes in groups 1, 2 and 7 had clustered CGs, although the numbers were decreased after IVM compared with before IVM ($p < 0.05$).

As shown in Table 6, the oocytes in groups 3, 5 and 6 (heterogeneous and black oocytes) had a smaller number of CCPEs that deeply penetrated their cytoplasm (Fig. 4*a*) before IVM. Although most of the non-penetrating CCPEs in groups 6 lost the gap junction with ooplasm before IVM, the gap junction was maintained in the oocytes in groups 3 and 5 (Fig. 4*b*). After IVM, all CCPEs were extruded from the cytoplasm and the gap junctions disappeared in all oocytes in all groups.

Discussion

Immature oocytes were grouped based on colour, namely brown, pale and black. Light and electron microscopy revealed that immature pale oocytes

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Table 5 The number of cortical granule (CG) clusters in the oocytes* before and after *in vitro* maturation (IVM)

	No. of oocytes having					
	Solitary CGs		CG clusters		No. of CG clusters	
Oocyte group	Before IVM	After IVM	Before IVM	After IVM	Before IVM	After IVM
1	0/5	9/10	5/5	10/10	40.2 ± 18.1^{abA}	9.6 ± 6.3^{aB}
2	0/5	10/10	5/5	10/10	38.4 ± 18.7^{abA}	7.5 ± 4.4^{abB}
3	5/5	10/10	5/5	4/10	33.2 ± 14.4^{abA}	1.5 ± 2.5^{bcB}
4	1/5	9/9**	5/5	8/9**	64.2 ± 26.1^{aA}	9.0 ± 5.9^{aB}
5	5/5	9/9**	5/5	3/9**	35.2 ± 13.3^{abA}	2.4 ± 4.1^{bcB}
6	5/5	10/10	4/5	2/10	20.4 ± 17.2^{bA}	0.4 ± 1.0^{cB}
7	3/5	9/10	5/5	10/10	58.0 ± 19.6^{aA}	7.1 ± 4.7^{abB}

Values are mean ± SD.
^{abc}Different superscripts indicate a significant difference in the same columns ($p < 0.05$).
^{AB}Different superscripts indicate a significant difference in the same lines ($p < 0.05$).
*A minimum of 1000 μm of the cell border was searched per oocyte.
**One oocyte had degenerated after IVM.

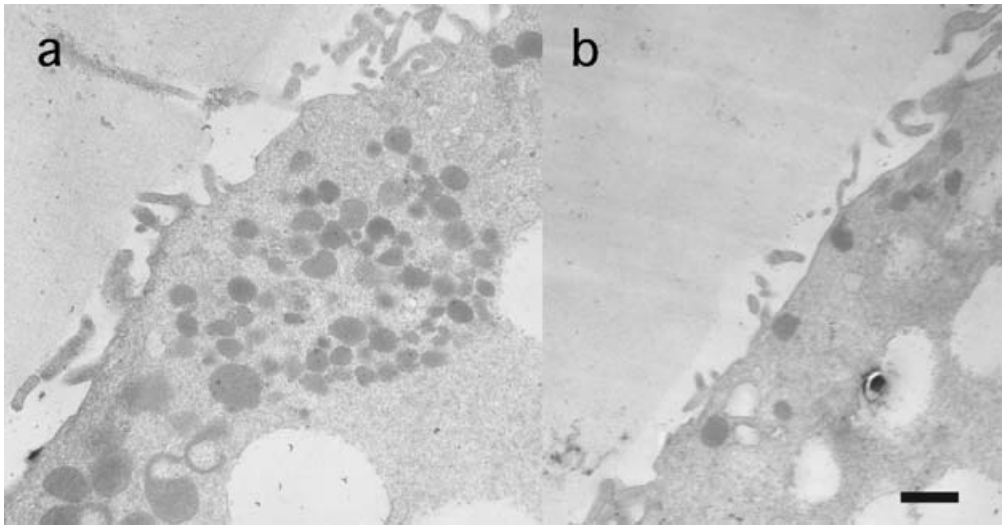


Figure 3 Cortical granule (CG) distribution pattern in the ooplasm. (*a*) CGs clustered near the oocyte membrane (group 1). (*b*) CGs were in a solitary position near the oocyte membrane (group 3). Scale bar represents 500 nm.

(groups 4 and 5) had organelle-free areas in their cytoplasm. Therefore a pale colour indicates a low density of organelles in the ooplasm. Black oocytes (group 6) had many lipid droplets in their cytoplasm, i.e., black colour indicates an accumulation of lipids. The oocytes with a heterogeneous cytoplasm with a dark zone at their periphery (group 2) or dark clusters in their cytoplasm (groups 3 and 5) also had many lipid droplets. It is possible that these dark regions reflect the accumulation of lipids.

Most of the oocytes in groups 1–6 reached the metaphase II stage after IVM, but not those in group 7 (small oocytes). The diameter of bovine oocytes corresponded to the nuclear maturational ability (Sato *et al*., 1990; Fair *et al*., 1995). This indicates that most of the oocytes in group 7 are developing and have not yet acquired maturational ability. Before the LH surge, bovine oocytes in a dominant follicle had a nucleus with a smooth membrane *in vivo* (Kruip *et al*., 1983). Most of the oocytes in groups 1 and 7 had nuclei with a smooth nuclear membrane before IVM, indicating that they were not affected by the signal starting nuclear maturation. The oocytes in groups 2–6 had nuclei with an undulating membrane before IVM. After the LH surge, the nuclear membrane of the oocyte in a dominant follicle undulated and broke down *in vivo* (Kruip *et al*., 1983). Therefore undulation of the nuclear membrane indicates a resumption of meiosis. However,

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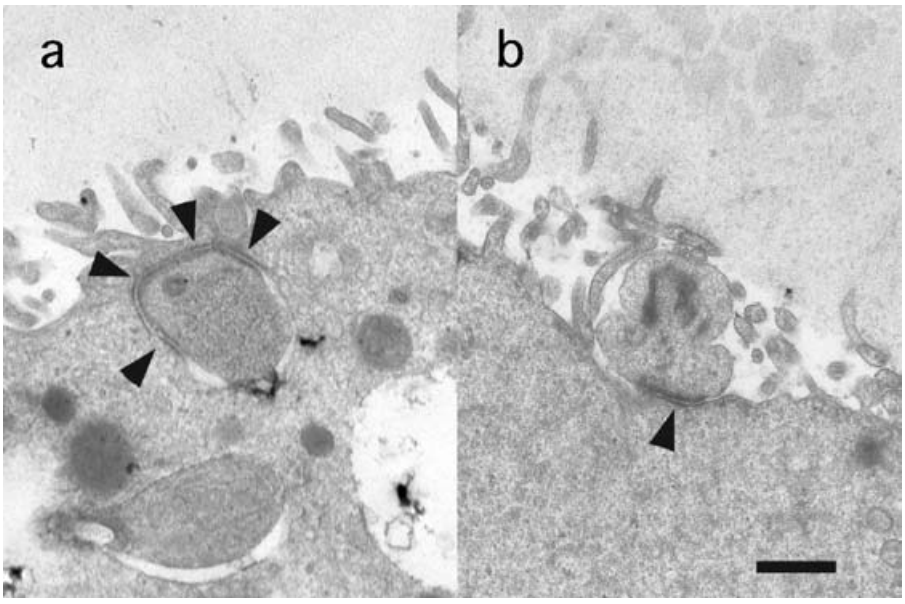


Figure 4 Cumulus cell process ending (CCPE) before maturational culture. (*a*) CCPE completely penetrated the cytoplasm (group 2). (*b*) CCPE extruded from the cytoplasm (group 3). Arrowheads show gap junctions. Scale bar represents 500 nm.

Table 6 Percentage of each type of cumulus cell process ending (CCPE) before maturational culture

Oocyte group	CCPE penetration into cytoplasm*		
	Deeply penetrating	Intermediate	Non-penetrating
1	84.1 ± 9.2^{a}	12.8 ± 10.1	3.0 ± 2.4^{d}
2	67.3 ± 6.1^{ab}	18.8 ± 3.7	13.0 ± 6.0^{cd}
3	18.6 ± 10.7^{cd}	13.9 ± 5.9	67.4 ± 10.7^{ab}
4	54.8 ± 15.3^{ab}	14.2 ± 6.8	31.0 ± 16.5^{cd}
5	40.1 ± 21.6^{bc}	16.8 ± 5.6	43.1 ± 20.3^{bc}
6	6.6 ± 12.4^{d}	9.5 ± 11.9	83.9 ± 23.7^{a}
7	53.5 ± 21.2^{b}	18.2 ± 8.8	28.2 ± 17.2^{cd}

Values are the mean ± SD.
[abc]Different superscripts indicate a significant difference in the same columns ($p < 0.05$).
*A minimum of 1000 μm of the cell border was searched per oocyte.

undulation of the nuclear membrane was observed in degenerating oocytes (de Loos *et al*., 1992; Assey *et al*., 1994). The nuclear morphology and arrangement of the organelles in degenerating oocytes are similar to those in maturing oocytes (Byskov, 1978; Hyttel *et al*., 1986), a phenomenon called pseudomaturation. We used the oocytes from small follicles (2–8 mm) for IVM, therefore the oocytes with an undulating nuclear membrane may enter the pseudomaturation phase. Some oocytes in groups 4–7 already had an organelle-free area in their peripheral region, which generally appeared 12–18 h after maturational culture (Hyttel *et al*., 1986). These results also indicate that some oocytes in groups 4–7 may enter the pseudomaturation phase.

In group 6, most of the CCPEs were extruded from the ooplasm, and the gap junction between CCPEs and the ooplasm had been lost. Cumulus cells arrest the meiosis of oocytes through the gap junction (Tsafriri *et al*., 1982; Hyttel, 1987; Downs, 1993). Therefore some oocytes in group 6 showed GV breakdown before IVM. In contrast, the gap junctions were maintained in groups 3 and 5, in spite of the fact that most CCPEs were extruded from the ooplasm. During oocyte maturation, penetrating CCPEs were extruded from the ooplasm and the gap junction between the oocyte and cumulus cells collapsed (Kruip *et al*., 1983; Hyttel *et al*., 1986). However, the collapse of gap junctions between the oocyte and cumulus cells also occurs during ageing or degeneration of the oocyte (Assey *et al*., 1994). Therefore, the oocytes in group 6 seemed to be starting to degenerate. These morphological characteristics may correspond to the follicular environment surrounding the oocytes. In future studies the correlation between the follicular phase of an estrous cycle and the developmental ability of oocytes must be examined and this understanding would be useful to develop a follicle culture system *in vitro*.

After IVF, the polyspermy rates tended to be higher in group 6 than group 3. The distribution of CGs is correlated with normal fertilization. Before the LH surge, CGs form large clusters in the periphery of bovine oocytes *in vivo*, and distribute throughout the

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inner surface of the spherical oocytes after the LH surge (Kruip *et al*., 1983). For normal fertilization, CGs must disperse throughout the periphery of oocytes. Exocytosis of CGs is the most common mechanism for the prevention of polyspermy. The clustering of CGs after IVM is likely to be responsible for the frequently encountered abnormal CG release (Kruip *et al*., 1983; Thibault *et al*., 1987), that may dispose the oocytes to polyspermic penetration (Hyttel *et al*., 1988). *In vivo* aged mouse oocytes could not undergo normal exocytosis (Ducibella *et al*., 1990). Some oocytes in groups 3–7 showed a dispersed distribution of CGs before IVM. On the other hand, most of the oocytes in groups 1, 2, 4 and 7 had clustered CGs even after IVM. Although the oocytes in group 6 showed high rates of polyspermy, most had no clustered CGs. These results indicate that the polyspermy of oocytes in groups 1, 2, 4 and 7 might be caused by an insufficient distribution of CGs, and that the polyspermy of oocytes in groups 5 and 6 might be caused by ageing. The distribution of CGs during cytoplasmic maturation correlates with microfilament function (Sun, 2003). An extensive microfilament network is also observed in the bovine oocyte cortex (Kim *et al*., 2000).

In the present study, the rate of cleavage and of the development of cleaved zygotes to the blastocyst stage were higher in the brown-coloured oocytes (groups 1–3) than in the pale, black and small oocytes (groups 4, 6 and 7, respectively). In spite of the lower cleavage rate, the development of cleaved zygotes originating from oocytes with dark clusters and a pale ooplasm (group 5) was similar to that in groups 1–3. Some oocytes in group 5 may possess a relatively high developmental capacity the same as the oocytes in groups 1–3. The oocytes in groups 3 and 5 had dark clusters in their ooplasm and the ultrastructures of these oocytes were similar. The group 3 oocytes may be at the stage just before degeneration, while some oocytes in group 5 may be starting the degeneration process. Therefore some oocytes in group 5 might have completely degenerated after IVM and did not cleave after IVF. Before IVM, the oocytes in groups 3 and 5 had a large number of lipid droplets. Electron microscopy indicated that these lipid droplets were associated with mitochondria and the smooth endoplasmic reticulum. In view of the likely functional relation between these three elements, such clusters have been termed metabolic units (Kruip *et al*., 1983). The association of these three elements might indicate the conversion of lipids and carbohydrates as sources of energy for the synthesis of ATP by mitochondria, necessary for oocyte maturation and subsequent development after fertilization. The good developmental ability of oocytes may correlate with their ATP production. Blastocysts originating from the oocytes in groups 3 and 5 had higher cell numbers than those in other groups. The higher cell number may also be due to the high level of ATP production. The production of ATP in these oocytes should be examined.

In conclusion, the developmental potential of oocytes collected from small follicles has already been decided before IVM, and is reflected in the morphological appearance of the oocyte. A dark ooplasm indicates an accumulation of lipids and good developmental potential, while a light-coloured ooplasm indicates a low density of organelles and poor developmental potential. A black ooplasm indicates ageing and low developmental potential. This study clarified that oocytes that had entered the pseudomaturation phase had good potential to develop to the blastocyst stage *in vitro*; however, the full-term developmental ability of these oocytes was not clear. We plan to investigate the full-term developmental ability of oocytes whose organelle arrangements show pseudomaturation.

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