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Differential Surface Ultrastructural Characteristics and Volumetric Dynamics of Bovine Oocytes during Maturation In Vivo Versus In Vitro

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Abstract: *Surface characteristics of the bovine cumulus-oocyte complex (COC) during maturation in vivo versus in vitro were compared by scanning electron microscopy (SEM). Oocyte diameter changes during maturation were also analyzed prior to oocyte fixation. In vivo matured oocytes were retrieved from live superovulated cows by transvaginal ultrasound-guided aspiration of all visible ovarian follicles (≥2 mm in diameter) 0, 12 and 24 h (n=85, 38 and 39, respectively) after hCG administration. In vitro matured (IVM) oocytes refer to those recovered from small antral follicles (2–6 mm in diameter) of slaughterhouse ovaries and then cultured in standard maturation conditions for 0 h (n=188), 12 h (n=138) or 24 h (n=228). SEM analysis showed that immature oocytes of both origins manifested compact COCs with smooth cumulus surface and minimal intercellular spaces among the cumulus cell mass. At 24 h of maturation, more IVM oocytes manifested cumulus expansion compared to in vivo matured oocytes (100%, n=228 vs 44%, n=39), but cumulus expansion was found much more prominently in the cumulus-expanded in vivo matured oocytes than in the IVM oocytes. The zona pellucida showed a fibrous, open mesh-like structure, irrespective of the maturation stage and the source of oocytes. The vitelline surface of the immature oocytes was characterized by distribution of large cellular tongue-shaped protrusions (TSPs) varying in density. These TSPs structures gradually changed to microvilli (MV)-predominant structures upon maturation of the oocytes. At 12 h of maturation, significantly more oocytes ($p<0.05$) manifested cumulus expansion and transition from TSPs-to MV-predominant vitelline surface structures in the in vitro group (100%, n=106) compared to only 11% (n=38) in the in vivo group. Oocyte diameter decreased gradually in the oocytes maturing in vitro between 0 and 24 h of incubation, being 127 ± 1, 122 ± 1 and 116 ± 1 μm at 0, 12 and 24 h of incubation, respectively. The corresponding values for the oocytes maturing in vivo were 121 ± 2, 129 ± 2, and 101 ± 1 μm, respectively. In conclusion, we found in our study conditions that initiation of oocyte maturation in vivo after hCG administration seemed to be slower than in vitro after maturation culture. But, at 24 h after the onset of maturation more prominent and complete cumulus expansion and more dramatic volumetric changes were observed in the in vivo matured oocytes than in the IVM oocytes.*

Key words: *Oocyte maturation, Oocyte diameter, Ultrastructure, Cattle.*

If cumulus-oocyte complexes (COCs) are liberated from the follicular environment and incubated in suitable culture media, the oocytes are able to complete nuclear maturation spontaneously *in vitro* [1]. The events of nuclear maturation *in vitro* seemed similar to those occurring *in vivo* [2–6], but fertilization and subsequent development of these oocytes have been known to differ from oocytes matured *in vivo* [5, 7–11]. These well-documented findings were confirmed by a recent study with transvaginal collection of *in vivo* matured and immature oocytes [12]. A high 60% blastocyst development was obtained for *in vivo* matured oocytes versus 35% blastocysts for *in vitro* immature oocytes cultured (*in vitro* matured). The difference in fertilization and developmental competence between the *in vitro* and *in vivo* matured oocytes suggest that oocytes need to undergo both nuclear and cytoplasmic maturation prior to normal fertilization and development. Although the evaluation of nuclear maturation is quite straightforward, the morphological characteristics of cytoplasmic maturation

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have not been well established. In our previous study, the surface characteristics of the cumulus cells, the zona pellucida (ZP) and the vitelline membrane of bovine oocytes were evaluated during their maturation *in vitro*. We have demonstrated profound patterns of ultrastructure and volumetric changes in the oocytes during different stages of maturation and after fertilization *in vitro* [13], but no direct comparisons of these parameters for oocytes matured *in vivo* versus *in vitro* have been made.

The objective of this study was to evaluate by SEM the ultrastructural changes in bovine oocytes during maturation *in vivo* versus *in vitro*. The diametric changes in oocytes during maturation *in vivo* versus *in vitro* were also compared in this study. Based on the previous finding that oocytes with homogeneous and heterogeneous ooplasm differ in maturation and fertilization development [14], the patterns of surface ultrastructures and the dynamic volumetric changes in the homogeneous and heterogeneous oocytes were also analyzed.

Materials and Methods

Collection of oocytes

COCs retrieved in vivo: Sixteen adult Holstein cows were superovulated for oocyte collections as described previously [15]. The FSH (FSH-P, Schering-Plough, Kenilworth, NJ) injection was started on the 10th day after estrus (estrus = day 0). A total of 40 mg of FSH-P was injected over 4 days twice daily and 25 mg of $PGF_2\alpha$ (Lutalyse, Upjohn, Kalamazoo, MI) was given twice together with the 7th and 8th injections of FSH-P. An injection of 2500 i.u. of hCG (Sigma) was given intravenously at approximately 36 h after the first $PGF_2\alpha$ injection to ensure the start of ovulation. The COCs were collected 0, 12 and 24 h after hCG (3, 3 and 4 cows, respectively) by transvaginal ultrasound-guided follicular aspiration. In addition, 6 superovulated cows were punctured at 36 h and 48 h (3 animals each) after the last FSH-P injection (corresponding to the 12 h and 24 h hCG groups) to serve as "time-lag" controls. These groups were found similar to the 0 h hCG group and the data were pooled in this report.

The oocytes recovered by the *in vivo* aspiration procedure were classified on the basis of the cumulus morphology, as reported by Looney *et al.* [16]. The oocytes covered with multilayers of cumulus cells (>4 or 3–4 cumulus layers) were classified as grades A and B, respectively, and the oocytes with a few or no cumulus cells were classified as grades C and D. Grade C and D oocytes were considered to be incompetent and were eliminated from SEM analysis. The oocytes with expanded cumulus investment (expanded COCs) were classified as grade E and were used for ultrastructural and volumetric analyses.

At 0 h after hCG, 59 COCs recovered from different cows were pooled and assigned to one of the following three groups: Cumulus-intact (n=10), zona pellucida intact (ZP-intact) (n=16) and ZP-free groups (n=33). At 12 h after hCG, 37 COCs recovered from individual cows were assigned to one of the following three groups: 13 cumulus-intact (including 4 expanded COCs), 12 ZP-intact and 12 ZP-free oocytes. At 24 h after hCG, 27 COCs recovered were processed and allocated to one of the three groups as described above with 8, 9, and 10 oocytes per group, respectively.

COCs retrieved post mortem: The COCs were collected by aspirating small antral follicles (2–6 mm in diameter) on ovaries obtained from a slaughterhouse. The oocytes with or without cumulus cells and/or ZP were processed for SEM immediately after collection (at 0 h, n=188), or after 12 h (n=138) or 24 h (n=228) of maturation incubation. The COCs were incubated in TCM 199 (Earle's salts, Gibco, Grand Island, NY) containing 25 mM Hepes and 7.5% fetal calf serum with the addition of 0.5 μg/ml ovine FSH (NIDDH), 5 μg/ml of ovine LH (NIDDH) and 1 μg/ml estradiol (Sigma, St. Louis, MO) at 39°C in 5% CO_2 and 95% air as reported previously [6].

Based on a recent report [14], two types of oocytes were identified microscopically after the removal of cumulus investment, based on the distribution of cytoplasmic granules within the ooplasm. Because these were categorized as evenly (homogeneous), and unevenly distributed (heterogenous) types, the ZP-free oocytes were divided into two groups depending upon the distribution of the cytoplasmic granules in the ooplasm at 0, 12 and 24 h of maturation. The oocytes with homogeneous and heterogeneous ooplasm were compared in terms of maturation rate, diameter of oocytes, and surface characteristics as shown by SEM.

Removal of cumulus cells and the zona pellucida

The cumulus cells were removed by pipetting COCs in medium containing 0.2% hyaluronidase. The ZP was removed by a procedure modified from that of Yang *et al.* [17] which has shown no detrimental effect on subsequent development, at least to the blastocyst stage (our unpublished observation). Oocytes were exposed to prewarmed acidified (pH = 2.5) Dulbecco's phosphate-buffered saline (DPBS, Gibco) containing 0.1% polyvinyl alcohol (PVA, Sigma) for 1 min and 0.5% pronase

(Sigma) for 3–5 min followed by gentle pipetting. The ZP-free oocytes were incubated for 20 min in calcium and magnesium-free DPBS at 39°C.

SEM observations

Oocytes with or without their investments in each group were processed according to the methods reported previously [13, 18]. The samples were fixed for 1 h in 3% glutaraldehyde and 0.5% paraformaldehyde in Hanks' balanced salt solution (HBSS) with 0.1% PVA. They were rinsed in three changes of HBSS and placed on small coverslips (6 × 6 mm) precoated with 0.1% poly-L-lysine solution (Sigma). The oocytes on the coverslips were postfixed in 1% osmium tetroxide in HBSS for 1 h. After rinsing, the samples were incubated in 2% tannic acid solution for 2 h, rinsed, and then reosmicated for 1 h in 1% osmium tetroxide in distilled water. The specimens were then dehydrated through or by a series of increasing concentrations of ethanol, critical point dried, and sputter-coated with gold or gold/palladium. Observations were performed with a Zeiss DSM-960 or a JOEL JSM-5300 scanning electron microscope at an accelerating voltage of 8 or 10 kV.

Diameter of oocytes

The diameters of the oocytes were measured after removal of cumulus investments (prior to fixation of the oocyte) and were expressed as the mean ± s.e.m. After a brief measurement of their diameters, the oocytes were randomly assigned to ZP-intact and ZP-free groups.

Statistical analyses

Data on the diameter of oocytes were subjected to a one-way analysis according to Bartlett's test for uniformity variances, and statistical differences among means were detected by Duncan's multiple range test [19]. Fisher's exact probability test and χ^2 test were used for proportions.

Results

Recovery of oocytes by in vivo follicular aspiration

It was very difficult to recover COCs *in vivo* from stimulated, fully grown follicles by follicular aspiration, because these follicles contained large expanded oocytes and very viscous fluid. Sometimes follicles ruptured when punctured with aspiration needles and follicular fluid containing COCs was not completely recovered. As a result, recovery rates of COCs from stimulated females were lower than from non-stimulated females (unpublished observation). The numbers and classifications of oocytes recovered are shown in Table 1. Significantly more cumulus-expanded oocytes were found in the 24 h group ($p<0.05$) than in the 0 and 12 h groups. The numbers of oocytes processed for SEM are shown in Table 2.

Ultrastructure of cumulus investment

The surface structures of the *in vitro* COCs before and after maturation were similar to those observed previously [13]. SEM analysis demonstrated that the cumulus cells of immature *in vitro* oocytes had a compact structure with minimal intercellular spaces (Fig. 1a), whereas during (12 h IVM) and after (24 h IVM) maturation cumulus cells became increasingly less compacted with wider intercellular spaces (compare Figs. 1a, 1b and 1d). Furthermore, the thread-like cell processes of the matured cumulus cells of *in vitro* matured COCs became well developed and thicker (Fig. 1d).

In vivo retrieved oocytes were covered with multilayers of cumulus cells either arranged compactly or expanded, probably depending upon the follicle size at recovery. Prior to hCG injection, almost all the oocytes collected (97%, 57/59) showed compact cumulus investments as shown in Table 1. At 12 h after hCG, 78% (33/42) of the oocytes recovered still had a compact cumulus, only 10% (4/42) had expanded cumulus

Table 1. Number and classifications of *in vivo* oocytes recovered by ultrasound-guided aspiration from all visible antral follicles

Time after hCG (h)	No. of cows	No. of oocytes	Number and classifications of oocytes		
			A/B grade	C/D grade	Expanded (%)
0 h*	9	85	57	26	2 (2)[a]
12 h	3	42	33	5	4 (10)[a]
24 h	4	39	10	12	17 (44)[b]

*, Three animals were used as controls, corresponding to 0, 12 and 24 h of hCG treatment. Data were pooled. [a,b]Values with different superscripts differ, $p<0.05$.

Table 2. Number of oocytes collected *in vitro* or *in vivo* and processed for SEM

Oocyte source	Hours of maturation	Number of oocytes		
		Cumulus-intact	ZP-intact	ZP-free
In vitro	0 h	49	55	84
	12 h	30	32	76
	24 h	51	53	124
*In vivo**	0 h	10	16	33
	12 h	9	12	12
		(4)	–	–
	24 h	3	3	4
		(5)	(6)	(6)

*, Figures show number of oocytes graded as A and B, and those in parenthesis show the numbers of oocytes with expanded cumulus.

layers, and the remainder (12%) were denuded (considered as degenerating/degenerated). In contrast, at 24 h after hCG only 26% (10/39) of the oocytes recovered had compact cumulus investments, 44% (17/39) displayed expanded cumulus layers, and 30% (12/39) were denuded. When COCs had compact cumulus layers irrespective of the time after hCG, SEM analysis showed that there were few intercellular spaces (Figs. 1c and 1e). The compactness of the cumulus mass of the *in vivo*-derived immature COCs were similar to those of immature *in vitro* COCs (just after recovery from slaughterhouse ovaries), although some cumulus cells were slightly elongated in *in vivo*-derived COCs (Fig. 1e). One notable difference was that the cumulus-expanded *in vivo* maturing and matured oocytes were more heavily mucified than the *in vitro* maturing and matured oocytes (compare 1c with 1b and 1f with 1d). This finding was also obtained by light microscopy for the two sources of matured oocytes (compare Figs. 2a with 2b), suggesting more active synthesis of hyaluronic acid matrix molecules *in vivo* than *in vitro*.

Ultrastructure of the ZP

The ZP surface was characterized by a fibrous network, with wide meshes and deep holes (Figs. 3a and 3b). Numerous residuals of the cytoplasmic processes were seen to be embedded in the holes of the wide mesh of the ZP (Fig. 3b). The surface of the ZP did not show remarkable changes during maturation either *in vivo* or *in vitro*. Corona radiata cells closely attached to the ZP were also elongated after maturation irrespective of the source of the oocytes (Fig. 3a).

Ultrastructure of the vitelline membrane

The vitelline surface of immature oocytes, regardless of their source, was covered with various sized cellular tongue-shaped protrusions (TSPs), sometimes mixed with few microvilli (MV). Based on the size and density of TSPs and the predominance of MV, oocytes were classified into three types: Type A with a dense distribution of cellular TSPs of various sizes (Figs. 4a and 5e), Type B with a mixed distribution of the small TSPs and MV (Figs. 4b and 5f) and Type C with predominant distribution of MV (Figs. 4c and 4d). The Type A pattern was frequently observed in cumulus-intact oocytes after 0 and 12 h of hCG (84%, n=19), whereas those of Type B or Type C were mainly noted after 24 h of hCG (88%, n=8) regardless of the expansion of the cumulus cells. The non-expanded cumulus oocytes in the latter group are primarily Type B suggesting that the gonadotropin treatments seem to affect the membrane surface ultrastructures of oocytes from small follicles.

When the *in vivo* oocytes with expanded cumulus cells were compared to those with compacted ones from the same animals, the vitelline surface of expanded COCs was obviously Type C, MV-predominant pattern (Figs. 4c and 4d) whereas the compacted ones primarily manifested the Type B morphology (Figs. 4b and 5f). The oocytes with expanded cumulus regardless of time after hCG had extruded the first polar body (100%, n=23).

As we observed previously [13] after maturation *in vitro*, the surface pattern of oocytes was changed from TSPs-predominant (Type A, Figs. 5e and 5f) to MV-predominant, where MV were evenly distributed on the surface (Type C, Figs. 5i and 5j). This change in the surface pattern was found not only in the oocytes with a first polar body, showing complete nuclear maturation, but also in oocytes without the first polar body and at 12 h of maturation, showing maturating oocytes (Figs. 5g and 5h).

Size of oocytes

Oocyte diameters prior to, during and after maturation in both *in vitro* and *in vivo* oocyte groups were measured and compared. The analysis of variance demonstrated a significant decline in the diameter of the oocytes during maturation *in vivo* and *in vitro* (Tables 3 and 4). For *in vivo* oocytes, the oocyte diameter decreased sharply between 12 h and 24 h of hCG (129 ± 2 vs 101 ± 1 μm, $p<0.05$), although it increased slightly between 0 h and 12 h (121 ± 1 vs 129 ± 2 μm, $p<0.05$). The size of the oocytes differed among individual ani-

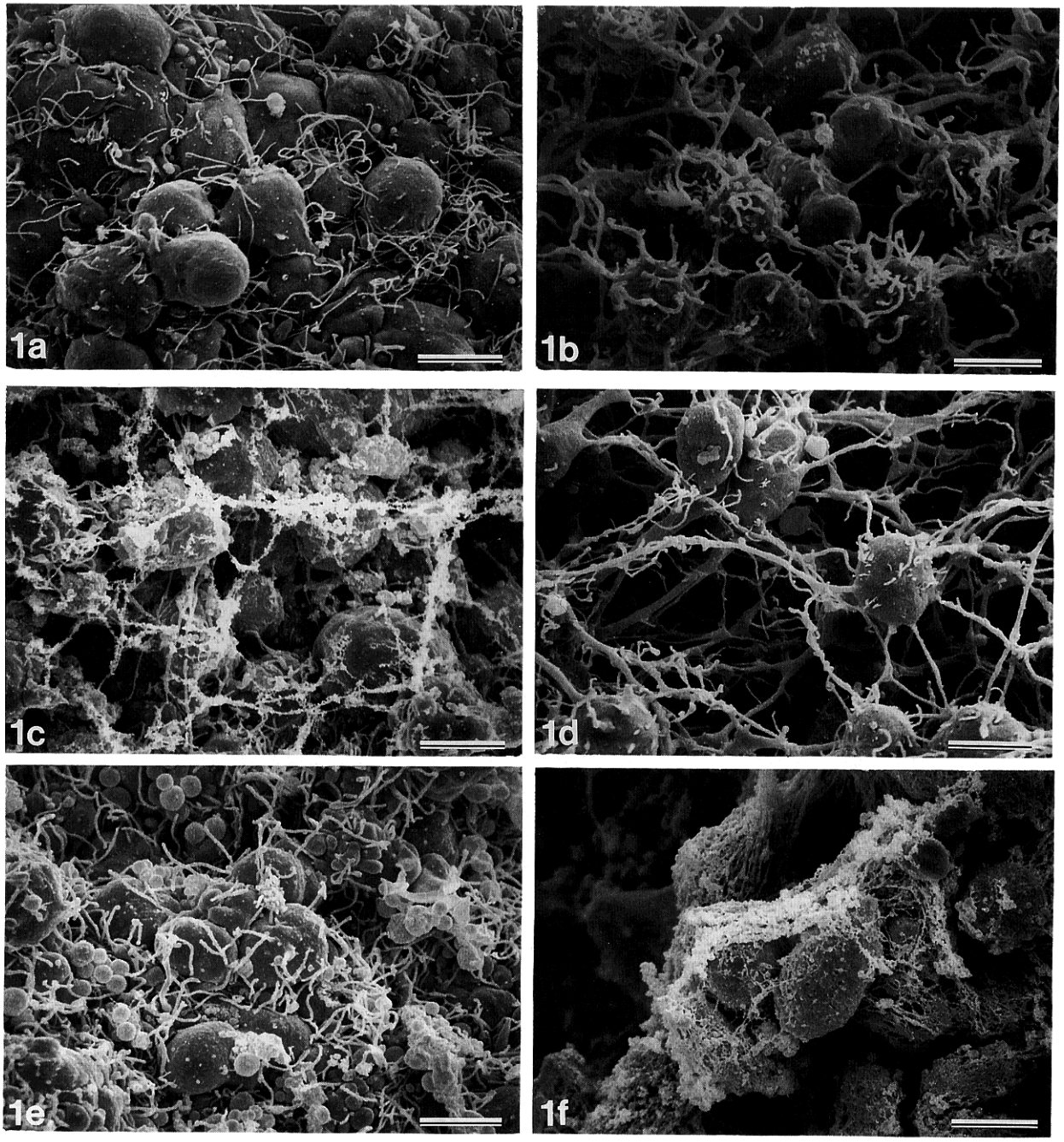

Fig. 1. Surface features of cumulus investments of the cumulus-oocyte complexes (COCs) during maturation *in vitro* and *in vivo*. 1a: An *in vitro* COC at 0 h of incubation. The cumulus cells had a smooth surface and an extensive network of thread-like cell processes was noted among them. Bar represents 10 μm. 1b: An *in vitro* COC at 12 h of incubation. The thread-like cell processes were thicker, and the intercellular spaces among the cumulus cells became wider than in the immature state (1a). Bar represents 10 μm. 1c: An *in vivo* COC 12 h after hCG. Intercellular spaces among cumulus cells were obvious. Note cell-to-cell communications among them. Bar represents 10 μm. 1d: An *in vitro* COC after 24 h of incubation. Note much wider intercellular spaces and much thicker cellular projections among cumulus cells. Bar represents 10 μm. 1e: An *in vivo* COC with compacted cumulus layers recovered 24 h after hCG. Note much smaller intercellular spaces among cumulus cells than 1f, but some of the cumulus cells were slightly elongated. Bar represents 10 μm. 1f: An *in vivo* COC with expanded cumulus layers recovered 24 h after hCG. Cumulus cells were elongated. Note much wider intercellular spaces among them, filled with a lot of extra cellular matrix. Bar represents 10 μm.

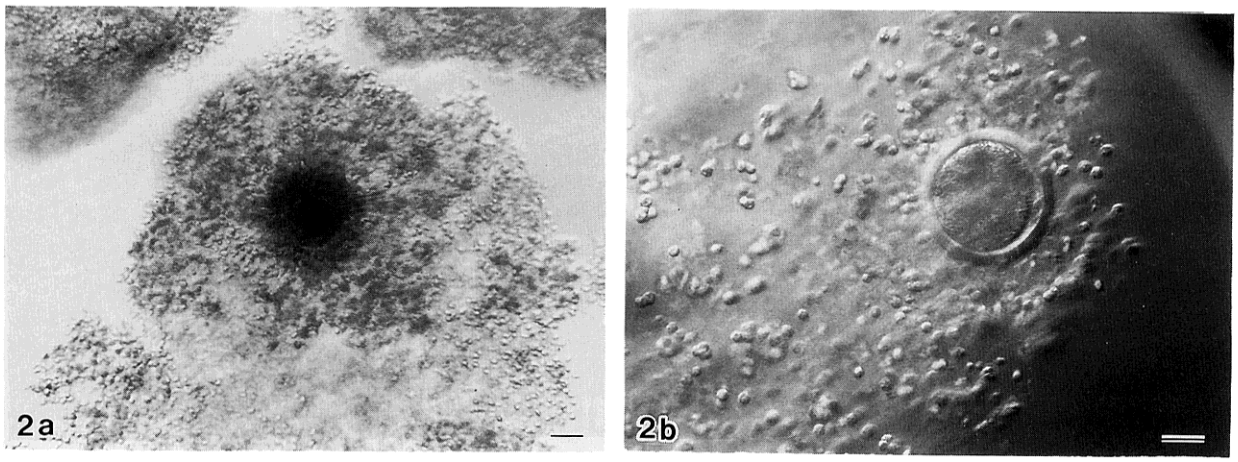

Fig. 2. Light micrographs of matured COCs from *in vitro* (2a) and *in vivo* (2b). Bar represents 50 μm. Much greater expansion of cumulus mass was noted in an *in vivo* matured COC (2b) than in an *in vitro* matured COC (2a).

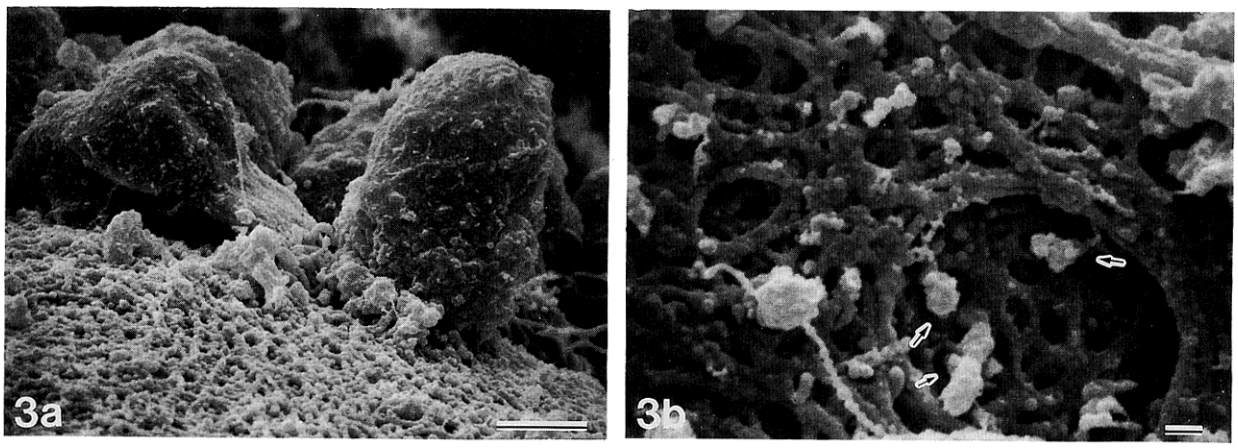

Fig. 3. Surface features of ZP of an *in vivo* oocyte with expanded COC 24 h after hCG. 3a. ZP surface with some corona radiata cells. Some expanded and elongated corona cells were noted. Bar represents 5 μm. 3b. Higher magnification of the ZP surface in 3a. The ZP showed a fibrous network, with numerous wide meshes. Some cellular debris (arrows) was observed in the deep, wider holes. Bar represents 1 μm.

mals 12 h after hCG treatment ($p<0.05$), and this may be responsible for the slight increase observed in oocyte diameter 12 h after hCG. However, this animal to animal difference was not found at 24 h after hCG, probably due to the general reduction in the volume of the oocytes at this point (Table 3).

In the immature oocytes recovered from slaughterhouse ovaries, there was no perivitelline space observed by light microscopy (Figs. 5a and 5c). After 12 h of incubation, formation of the perivitelline space was detectable even under a stereomicroscope in about 65% (n=49) of oocytes, and was obvious in all (n=49) the oocytes after 24 h of incubation (Figs. 5b and 5d). The decrease in diameter of the *in vitro* oocytes coincidently occurred with the appearance of the perivitelline space described above. The oocyte diameters were 127 ± 1 μm, 122 ± 1 μm and 116 ± 1 μm respectively at 0, 12 and 24 h of incubation *in vitro* (Table 4). There was no significant difference in diameter between the oocytes with and without a polar body after 24 h of incubation, suggesting that the oocyte diameter reduction is probably associated with the progression of oocyte maturation rather than do the release of the first polar body (Table 4).

The oocytes with homogeneous or heterogeneous ooplasm

When the oocytes were divided according to the dis-

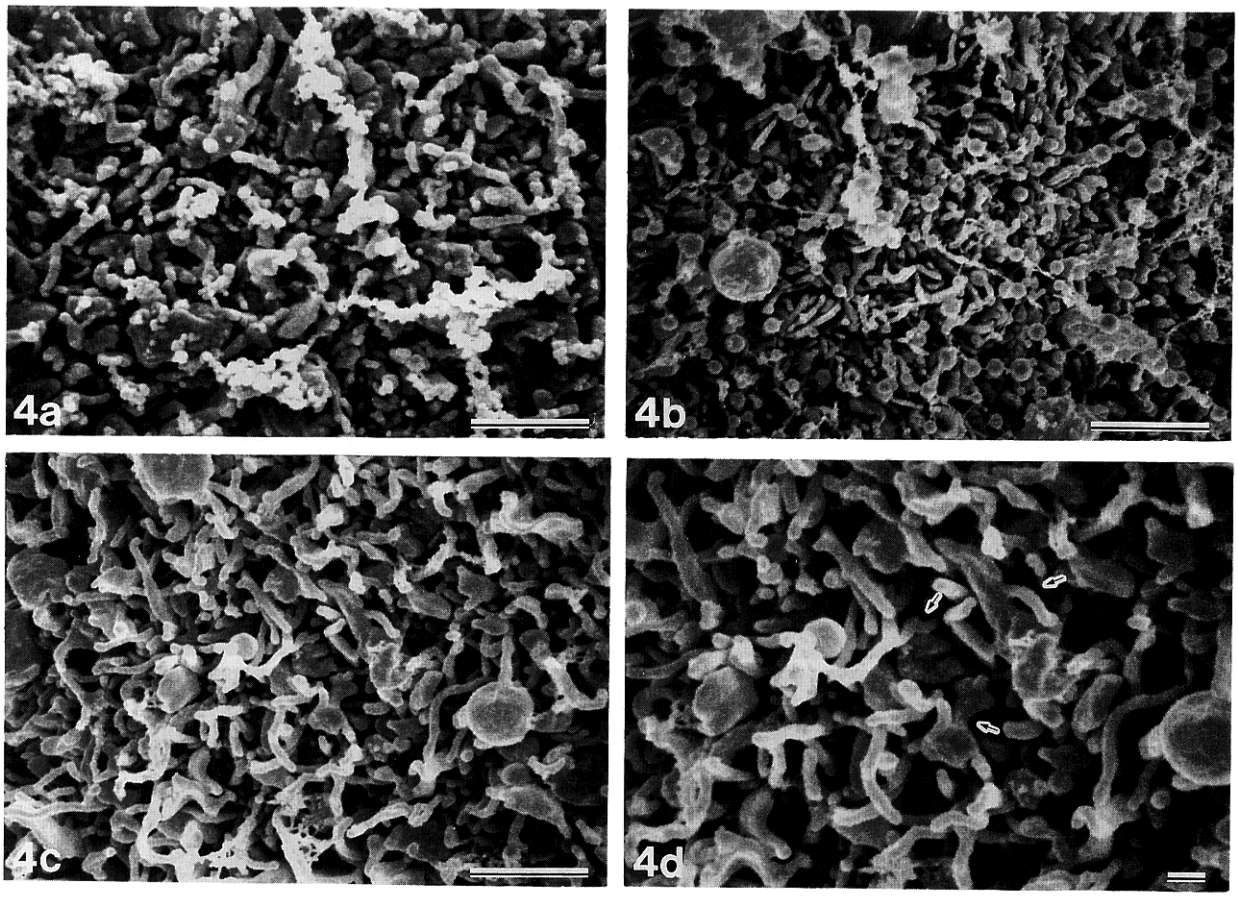

Fig. 4. Surface features of vitelline membrane of oocytes during maturation *in vivo*. 4a: Vitelline surface of an oocyte with compact cumulus 12 h after hCG. The surface was covered uniformly with cellular tongue-shaped protrusions (TSPs, relatively larger protrusions than MV) of various sizes. Bar represents 5 μm. 4b: Vitelline surface of an oocyte with compact cumulus 24 h after hCG. The surface was densely covered with small cellular TSPs and occasional MV. Bar represents 5 μm. 4c: Vitelline surface of an oocyte with expanded cumulus layers recovered 24 h after hCG. The surface was densely covered with MV. Bar represents 5μm. 4d: Higher magnification of the vitelline surface in 4c. Some TSPs had notched tips (arrows). Bar represents 1 μm.

tribution of cytoplasmic granules after maturation culture for 24 h (Figs. 5a-5d), the maturation rate (based on the extrusion of the first polar body) of the oocytes with heterogeneous ooplasm (Figs. 5c and 5d) appeared to be higher than that of the oocytes with homogeneous ooplasm (Figs. 5a and 5b) (22/23, 95.7% vs 24/32, 75.0%, p=0.085). The overall mean of the maturation rate *in vitro* was 82.7 ± 3.6% in 6 replicates (n=177).

There was no statistical difference in the diameters of homogeneous (135 ± 2 μm, n=12) and heterogeneous (132 ± 2 μm, n=19) oocytes prior to the start of incubation, but significant differences were noted between the diameters of homogeneous (126 ± 1 μm, n=22) and heterogeneous (122 ± 1 μm, n=27, p<0.05) oocytes after 12 h of incubation. At 24 h of incubation, there was no difference in the diameter between the two types of oocytes (117 ± 1 μm, n=24, for homogeneous and 115 ± 1 μm, n=22, for heterogeneous ones). And at 12 h of incubation, the perivitelline space was noted in only 11 out of 22 (50%) homogeneous oocytes, whereas it was found in 21 of 27 (78%) heterogeneous oocytes. This difference in proportion was found to be significant (χ^2 = 4.128, p<0.05).

SEM observations clearly showed structural differences in the vitelline surface between these two types of oocytes (homogeneous vs heterogeneous) at each time point. Immediately after recovery from the slaughterhouse ovary (immature state), TSPs-predominant pattern (Type A) appeared in 8 out of 10 homogeneous oocytes examined (Fig. 5e). The others were, one oocyte with very small, scattered TSPs; one oocyte with a mixed pattern of small TSPs and MV (Type B). In con-

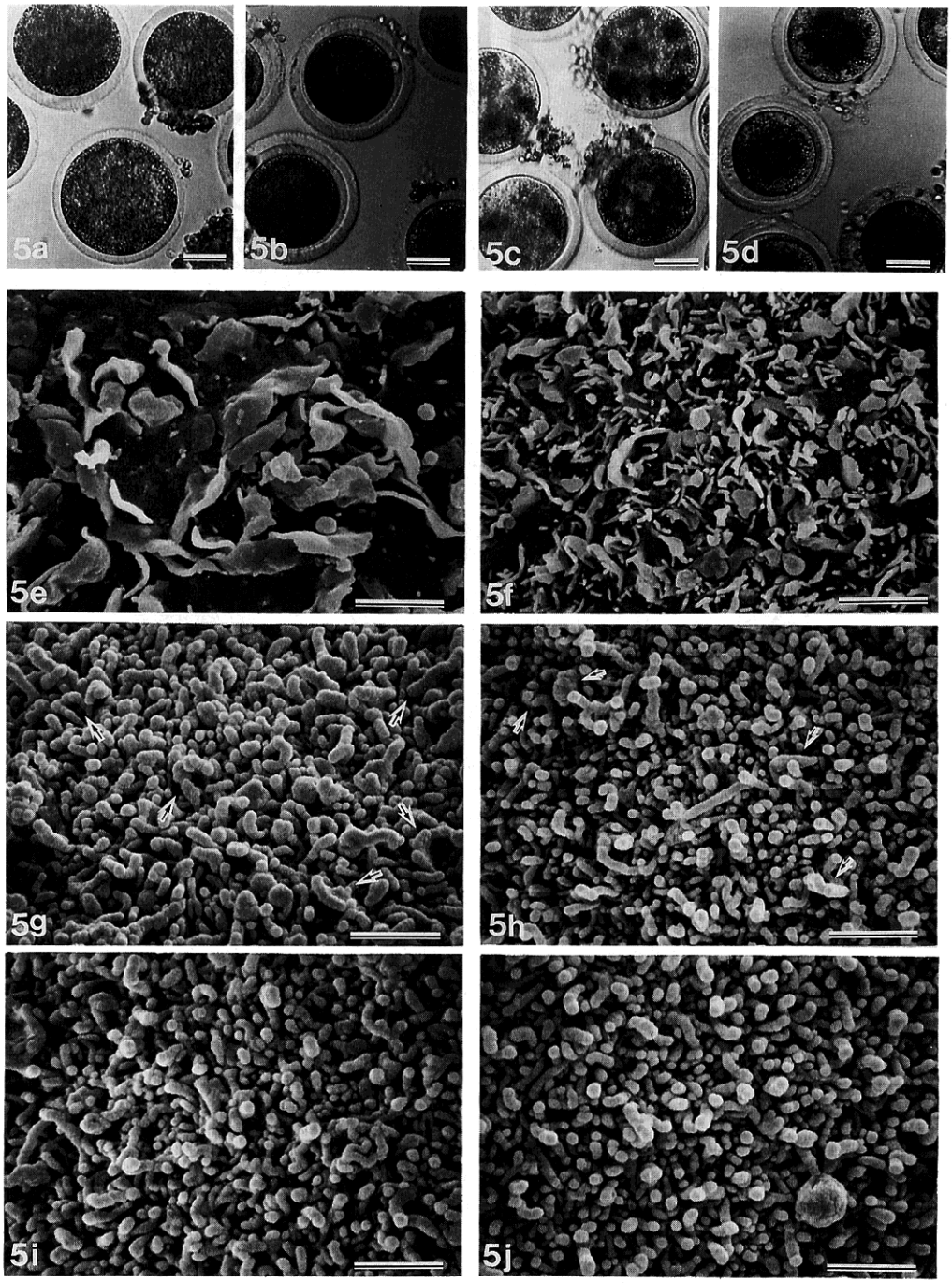

Fig. 5. Characteristics of *in vitro* oocytes recovered from slaughterhouse ovaries, and divided into homogeneous (a, b, e, g and i) and heterogeneous (c, d, f, h and j) oocytes. Bar represents 50 μm in 5a–5d, and 5 μm in 5e–5j. 5a, 5b: Light micrographs of *in vitro* oocytes with homogeneous ooplasm before (5a) and after 24-h maturation incubation (5b). 5c, 5d: Light micrographs of *in vitro* oocytes with heterogeneous ooplasm before (5c) and after 24-h maturation incubation (5d) . 5e, 5f: At 0 h of incubation, the surface of a homogeneous oocyte was covered with dense distribution of TSPs (5e), whereas that of a heterogeneous oocyte was covered with a moderate distribution of relatively small TSPs, with occasional MV (5f). 5g, 5h: At 12 h of incubation, the surface of a homogeneous oocyte was still covered with some TSPs, which became slightly smaller (5g, arrows). The surface of a heterogeneous oocyte had a MV-predominant pattern with occasional TSPs (5h, arrows). 5i, 5j: At 24 h of incubation, the surface of both a homogeneous oocyte (5i) and a heterogeneous oocyte (5j) had a MV-predominant pattern.

Table 3. Size variations (μm ± s.e.m.) of oocytes recovered at different times after FSH/hCG treatment

Hours after hCG	Cow ID	No. of oocytes	Condition of cumulus*	Oocyte diameters	Range of diameters
0 h	Pooled data	47	Compact	120.0 ± 1.0[a]	110.3–135.9
		2	Expanded	108.2 ± 5.6[b]	102.6–113.8
12 h	Cow 5276	8	Compact	120.1 ± 1.9[a]	112.8–129.7
	Cow 5242	14	Compact	135.4 ± 1.6[c]	128.2–148.7
	Cow 5295	2	Compact	124.5 ± 8.9[a]	115.4–133.3
24 h	Cow 5227	3	Compact	119.6 ± 1.7[a]	117.9–123.1
		2	Expanded	101.6 ± 1.0[b]	101.5–102.6
	Cow 5300	4	Expanded	100.7 ± 0.7[b]	100.0–102.6
	Cow 5350	2	Compact	118.0 ± 5.1[a]	112.8–123.1
		4	Expanded	99.4 ± 1.2[b]	97.4–102.6
	Cow 5230	2	Compact	120.5 ± 2.6[a]	117.9–123.1
		2	Expanded	103.6 ± 1.5[b]	101.5–105.1

*, 'Compact' shows oocytes with compact cumulus layers; 'Expanded' shows those with expanded cumulus. [a,b,c], Means with different superscripts differ, $p<0.05$.

Table 4. Overall diameters (μm ± s.e.m.) of oocytes collected *in vitro* or *in vivo*

Oocyte source	Hours of maturation	No. of oocytes	Oocyte diameter	Range of diameters
*In vitro**	0 h	139	127.2 ± 0.7[a]	105.1–149.7
	12 h	108	121.7 ± 0.6[a]	102.6–139.5
	24 h w/o PB	32	115.4 ± 1.2[b]	101.5–130.8
	24 h w PB	151	116.1 ± 0.5[b]	97.4–133.3
*In vivo***	0 h Compact	47	120.9 ± 0.9[a]	110.3–135.9
	0 h Expanded	2	108.2 ± 5.6[b]	102.6–113.8
	12 h Compact	24	129.3 ± 1.9[c]	112.8–148.7
	24 h Compact	7	119.4 ± 1.5[a]	112.8–123.1
	24 h Expanded	12	101.2 ± 0.7[b]	97.4–105.1

*, w/o PB, without a polar body; w PB, with a polar body. **, 'Compact' shows oocytes with compact cumulus layers; 'Expanded' shows those with expanded cumulus. [a,b,c], Means with different superscripts within the same source of oocytes are different ($p<0.05$).

trast, 9 out of 9 unevenly distributed heterogeneous oocytes manifested a mixed pattern of small TSPs and MV (Type B, Fig. 5f) indicating that these oocytes are more advanced in maturation than the homogeneous oocytes. At 12 h of incubation, 7 out of 13 homogeneous oocytes displayed nearly MV-predominant patterns (Type C), but the remaining 6 showed mixed patterns of TSPs and MV (advanced Type B, Fig. 5g). In heterogeneous oocytes, however, all 12 had the MV-predominant pattern (Type C, Fig. 5h). A similar tendency was observed at 24 h of incubation. In the homogeneous oocytes, 13 of 19 had changed to a nearly MV-predominant pattern (Type C) and the other 6 still showed scattered TSPs in MV (advanced Type B). On the other hand, 11 of 12 heterogeneous oocytes had already changed completely to the MV-predominant pattern (Type C, Fig. 5j). These observations suggest that during oocyte maturation TSPs degraded and changed to a MV-predominant pattern more quickly in heterogeneous oocytes than in homogeneous oocytes.

Discussion

Under physiological conditions, COCs in the follicles respond coordinately to a preovulatory surge of gonadotropins to acquire nuclear and cytoplasmic maturation [20]. Several previous studies, which compared the efficiency of IVF and embryonic development after IVM,

have shown that hormonal supplementation during IVM yielded improved rates of both fertilization and embryonic development [6, 21–24]. Although the medium used for IVM in this study included FSH, LH, and estradiol (E_2), the expansion of cumulus mass was found much less prominent in the *in vitro* matured oocytes than in the *in vivo* matured oocytes (see Figs. 1 and 2). This suggests that the standard *in vitro* maturation condition is still less suitable than the *in vivo* condition.

Transzonal cell communication between follicle cells and the oocyte via gap junctions exists throughout folliculogenesis [25–28] and is thought to play an important role in oocyte growth and maturation [20]. Recent studies have suggested that oocyte maturation involves modifications of the cumulus cell cytoskeleton that may regulate the onset, progression and completion of oocyte maturation [25] and cumulus expansion [29]. Previous studies have shown that rates of maturation and fertilization of bovine oocytes were lower in a cumulus-free culture condition than in the cumulus-enclosed condition [3, 30–32] suggesting a positive role of the cumulus investment in the developmental competence of oocytes. More evidence of the modifications of organelles and ultrastructural changes correlating with oocyte competence is reviewed by Hyttel *et al.* [33], but the differential dynamic surface ultrastructural and volumetric changes in oocytes during maturation *in vitro* versus *in vivo* reported in this study provided new insights into mammalian oocyte maturation.

Surface morphology of the oocytes

SEM features of cumulus investments, ZP and vitelline surfaces of *in vitro* oocytes were similar to those observed in our previous study [13]. Expansion of the cumulus after maturation was observed in COCs for both *in vivo* and *in vitro* oocytes although more extensive expansion was found during maturation *in vivo* than *in vitro* (Fig. 2a versus 2b). Cumulus expansion is believed to be induced by gonadotropins in cattle [6, 25] as well as other species [34, 35]. This morphological alteration is shown to depend on an increase in the synthesis of hyaluronic acid, glycosaminoglycans, and glycoproteins as reported in the mouse [36–40]. Our study demonstrated more extensive cumulus mucification in *in vivo* matured oocytes than in *in vitro* ones, suggesting more active synthesis of the above molecules during *in vivo* maturation. Changes in surface morphology of individual cumulus cells during maturation observed here were also generally in agreement with previous SEM reports on cattle [41], mice [42, 43], hamsters [44] and man [45, 46], and were in agreement with the transmission electron microscopic observations in cattle [27, 29]. Recently it has been shown that the volumetric expansion of cumulus oophorus is triggered by an assembly of cytoskeletal components (F-actin) in cumulus cells and that these cells, at all stages of cumulus expansion, are interconnected by an extensive network of functional gap junctions [29].

Ultrastructural and immunocytochemical studies have shown that the cumulus cell processes pass through the ZP with gap junctions contacting the surface of the ooplasm [25, 26, 28, 29, 47–50]. These projections maintain the biochemical communications between the oocyte and cumulus cells [29, 34, 48, 49, 51, 52]. TSPs observed here and in the previous study [13] are interpreted to represent the terminals of the transzonal cytoplasmic processes that were disconnected from the cumulus projections when the ZP was removed from the oocyte. Neverthless, it should be pointed out that the cumulus removal procedure itself does no harm to the oocytes because similar development was obtained in the cumulus-free-oocytes after activation treatment as compared to IVF oocytes after standard procedures [53].

Numerous ultrastructural studies have shown a substantial decrease in cell-to-cell communication during *in vivo* or *in vitro* maturation of COCs in cattle [27–29, 52, 54–56], sheep [49], pigs [57, 58] and mice [51, 59]. The authors of those studies suggested that LH could induce uncoupling of gap junctions between the cumulus and ooplasm, which may be related to the resumption of oocyte meiotic maturation. In addition, changes in locations of cytoplasmic organelles, such as mitochondria and some vesicles, occur a few hours after the final loss of gap junctions *in vivo* [54, 56] and *in vitro* [28, 55]. Such organelle migrations may be linked to the loss of intercellular communication between cumulus cells and the oocyte, as reported here, and the cytoskeletal changes in the cytoplasmic processes as reported by others [25, 28, 29]. It is reasonable to consider that such cytoskeletal alterations in the ooplasm could induce a surface change from TSPs-predominant to MV-predominant, because incubation with an actin polymerization inhibitor, dihydrocytochalasin B, inhibited the maintenance of a microvillous surface of matured bovine oocytes *in vitro* (Suzuki, Du and Yang, unpublished observation). Because volumetric reduction during maturation was observed prior to polar body release, it is possible that changes in the distribution of cytoskeletal components probably cause the volumetric changes in the oocytes.

Volumetric changes in oocytes

The present study, together with the previous report [13], clearly demonstrated that the size of oocytes decreased significantly during maturation both *in vivo* and *in vitro*, resulting in the formation and enlargement of the perivitelline space observed in the maturing and matured oocytes. The formation of a perivitelline space after oocyte maturation has been previously reported in cattle [56, 60, 61] and hamsters [62]. As pointed out previously [13], the increase in the perivitelline space (decrease in the volume of the ooplasm) may aid in disconnecting the junctions between the oocytes and the cytoplasmic projections of cumulus cells under physiological conditions. The volumetric decrease may also be related to the very dense distribution of MV on the vitelline surface observed as an oocyte matures as described in this study and in our previous report [13]. The decrease in the size of the maturing and matured oocytes was considered to be the result of physiological contraction of the ooplasm, probably due to changes in the distribution of cytoskeletal components as mentioned above. The discrepancy in oocyte volumes at 12 h of maturation found in the *in vivo* group but not the *in vitro* group suggests the asynchrony of oocyte populations and a delay in oocyte maturation *in vivo*. And the more profound reduction in oocyte volume in the *in vivo* group at 24 h of maturation may reflect the better cytoplasmic maturation of *in vivo* oocytes. Further studies are necessary for the elucidation of the relationships between morphological/volumetric changes in the oocyte and the redistribution of cytoskeletal components during maturation.

Distribution of cytoplasmic granules

As pointed out by Momozawa and Fukuda [14], two types of oocytes could be distinguished depending on the distribution of cytoplasmic granules: oocytes with homogeneous and heterogeneous ooplasm. The latter was superior to the former in the maturation rate *in vitro*. This was confirmed by morphological/volumetric observations in this study. SEM evaluations revealed faster changes in the heterogeneous oocytes from a TSPs-predominant pattern to a MV-predominant pattern, which showed a mature status. Formation and enlargement of the perivitelline space occurred in higher proportion with heterogeneous oocytes than with homogeneous oocytes. The faster maturation of the heterogenous oocyte may suggest more "readiness" for maturation of the heterogenous than the homogeneous oocytes. Further studies are needed to clarify the relationship between fertilization/development rate and the type of oocytes.

Conclusions

In this study the dynamic changes in the surface ultrastructures of the cumulus, and of the zona pellucida and vitelline membrane of oocytes during maturation *in vitro* versus *in vivo* were compared. The volumetric changes in oocytes during *in vitro* versus *in vivo* maturation were also measured. Further, the ultrastructural changes in oocytes with homogeneous versus heterogeneous cytoplasm were analyzed. Together, the following conclusions are drawn from this study.

1. The overall patterns of ultrastructural changes in oocytes during maturation *in vitro* are similar to those *in vivo*: increase in intercellular spaces between cumulus cells and transition of vitelline surface from TSP-predominant to MV-predominant structures. However the manifestation of ultrastructural and volumetric changes appeared earlier during maturation *in vitro* than *in vivo*. In contrast, the ultrastructural changes and the expansion of cumulus *in vivo* were more profound than those *in vitro*.

2. Oocyte maturation involves volumetric reduction regardless of whether maturation is *in vitro* or *in vivo*, but a greater reduction in size was found in *in vivo* matured oocytes (from 121 μm to 101 μm in diameter) than in *in vitro* matured oocytes (from 127 μm to 116 μm in diameter). These more dramatic changes in ultrastructure and oocyte volume may reflect the quality and completeness of the cytoplasmic maturation of oocytes.

3. Oocytes with heterogeneous cytoplasm seemed to mature faster and to have a higher maturation rate than oocytes with homogenous cytoplasm, but the subsequent development competence remains to be tested.

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