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Bovine oocytes in early antral follicles grow in serum-free media: effect of hypoxanthine on follicular morphology and oocyte growth

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Summary

Some culture systems have been shown to support oocyte growth in mice, although there has been little success in applying these systems to other species. In the present study, we compared three culture conditions for growing bovine oocytes and examined the effect of hypoxanthine on oocyte growth. In the first experiment, early antral follicles, 0.4–0.7 mm in diameter were collected, and oocyte–cumulus–granulosa cell complexes (OCGs) and oocyte–cumulus cell complexes (OCs) were dissected from the follicles. Follicles (Fs), OCGs and OCs were embedded in collagen gels and cultured in serum-supplemented medium for 16 days. In the Fs, OCGs and OCs cultured in hypoxanthine-free medium, 21%, 9% and 4% of the oocytes showed normal morphology, respectively, and hypoxanthine (4 mM) increased the percentages in all the groups (Fs, 37%; OCGs, 29%; OCs, 10%). In the second experiment, Fs were cultured in serum-free medium with or without hypoxanthine for 16 days. Histological examination demonstrated that hypoxanthine maintained the integrity of the follicular basement membrane. After a growth culture, 91% of the oocytes showed normal morphology, and 87% of the oocytes were at the germinal vesicle stage in serum-free, hypoxanthine-supplemented medium. The mean diameters of the oocytes were significantly larger (117.6 ± 5.7 μm) than they were in the other groups and than they had been before the culture (approximately 95 μm). After a subsequent maturation culture of the oocytes, 85% underwent germinal vesicle breakdown and 23% reached the second metaphase. These results demonstrate that growing bovine oocytes from early antral follicles grow efficiently in follicles cultured in serum-free, hypoxanthine-supplemented medium and acquire meiotic competence.

Keywords: Cow, Growing oocyte, Hypoxanthine, *In vitro* culture, Serum-free media

Introduction

Eppig & O'Brien (1996) demonstrated that mouse oocytes in primordial follicles grew to the final size, underwent fertilisation *in vitro*, and developed to live young. Approximately 120 000 small non-growing and growing oocytes are contained in a cow's ovaries (Erickson, 1966). Many attempts have been made to utilise the small oocytes as a source of mature eggs. Yamamoto *et al.* (1999) have successfully produced a calf from growing bovine oocytes collected from early antral follicles. This result demonstrated the potential of small oocytes to act as a source of mature eggs, although the efficiency of the procedure was quite low. These researchers collected theca cell-free oocyte–cumulus–granulosa cell complexes from early antral follicles and cultured them in medium containing serum and hypoxanthine for 14 days. Gutierrez *et al.* (2000) cultured bovine preantral follicles containing theca cells for 28 days in serum-free medium. The cultured follicles developed an antrum, demonstrating that the follicles survived. Theca cells have been reported to secrete the growth factors that stimulate granulosa cells to proliferate and differentiate in a paracrine manner and consequently to promote

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follicular development (Skinner & Coffey, 1988; Parrott & Skinner, 1998). Serum is an extremely complex mixture of many small and large biomolecules with physiologically balanced growth-promoting and growth-inhibiting activities (Maurer, 1986). Some of the components have been found to maintain the viability and stimulate the growth of many kinds of mammalian cells in culture. Serum may stimulate the proliferation and differentiation of oocyte-surrounding somatic cells, granulosa cells and theca cells. However, relatively large lot-to-lot variations in sera may induce different effects on oocyte growth and the proliferation and differentiation of surrounding somatic cells. It is still not well understood whether theca cells and serum are required for oocyte growth *in vitro*.

Hypoxanthine has been reported to maintain the normality of oocyte morphology and to promote oocyte growth *in vitro*. It has promoted the continued association of mouse oocytes with companion granulosa cells during a 12 day culture period, and therefore had a beneficial effect on oocyte growth (Eppig & Downs, 1987). The addition of hypoxanthine to the culture medium increased the number of granulosa-cell-enclosed bovine oocytes after culture (Harada *et al.*, 1997). The beneficial effect of hypoxanthine on follicular morphology has also been shown in bovine preantral follicles that were cultured for 5 days (Figueiredo *et al.*, 1994). The morphological examination conducted by these researchers demonstrated that cross-sections of follicles cultured in the presence of hypoxanthine were similar to those of uncultured follicles. However, how hypoxanthine affects oocytes and the surrounding somatic cells is not understood.

In the first experiment in this study, we collected growing bovine oocytes from early antral follicles 0.4–0.7 mm in diameter, and cultured them in three different conditions of surrounding somatic components. We cultured intact early antral follicles, oocyte–cumulus–granulosa cell complexes and oocyte–cumulus cell complexes in serum-supplemented medium with or without hypoxanthine for 16 days based on the previously reported collagen gel-embedding method (Harada *et al.*, 1997). In the second experiment, we cultured intact follicles in serum-free medium with or without hypoxanthine and examined the follicular morphology, oocyte growth and nuclear morphology of the oocytes. Some oocytes that recovered after the growth culture were subjected to a maturation culture and their meiotic competence was examined.

Materials and methods

Collection of bovine early antral follicles

Ovaries were obtained from pure-bred Japanese Black and cross-bred cows slaughtered at a local abattoir. Following three washes in Dulbecco's phosphate-buffered saline, early antral follicles were dissected from the ovarian cortex while immersed in TCM199 (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% polyvinylalcohol, 0.85 mg/ml $NaHCO_3$, 0.08 mg/ml kanamycin and 25 mM HEPES. Follicles 0.4–0.7 mm in diameter were defined as early antral follicles in this study. Connective tissue surrounding the follicles was stripped off with fine forceps, and only the early antral follicles, in which no granulosa cells were detached from the follicle walls, were selected under a dissection microscope (×40).

Growth culture of oocytes

Our culture method was based on that described by Harada *et al.* (1997). From the early antral follicles (Fs), oocyte–cumulus complexes with parietal granulosa cells (OCGs) or oocyte–cumulus cell complexes (OCs) were collected with fine forceps. Groups of 10–15 Fs, OCGs or OCs were embedded in collagen gels following two washes in HEPES-buffered TCM199. A collagen mixture was made by mixing a 0.3% acid collagen solution (Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan), 10× concentrated TCM199, and 0.05 N sodium hydroxide solution containing 22 mg/ml $NaHCO_3$ and 47.7 mg/ml HEPES, at a ratio of 8:1:1 (v:v:v). A 1.0 ml aliquot of the mixture was placed in a Petri dish (#1008, Falcon, Lincoln Park, USA), and Fs, OCGs or OCs were put into the mixture with a small volume of medium. Another 0.5 ml of the collagen mixture was poured over them. The dishes were then placed in an incubator at 38.5 °C for 20 min. After gelatinisation, 3 ml of culture medium was poured onto the gels, and they were cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 and 95% air. The basic culture medium was TCM199 containing 2.2 mg/ml $NaHCO_3$, 0.08 mg/ml kanamycin and 0.1 mg/ml sodium pyruvate.

In the first experiment, Fs, OCGs and OCs were cultured in the basic culture medium containing 10% fetal calf serum (FCS; Dainippon Pharmaceutical, Osaka, Japan) with or without 4 mM hypoxanthine (Sigma, St Louis, MO) for 16 days. In the second experiment, only Fs were used. They were embedded in collagen gels and cultured in serum-supplemented or serum-free medium with or without 4 mM hypoxanthine for 16 days. FCS was substituted by 3 mg/ml bovine serum albumin (BSA; International Regents Corporation, Kobe, Japan) in the serum-free medium. In both experiments, half the volume of the culture medium was exchanged for fresh medium every 4 days.

After the growth culture, the gels and follicles were torn with fine forceps and the oocytes were recovered. Some oocytes had been denuded, and others were enclosed by granulosa cells. The oocytes enclosed by

granulosa cells were completely denuded by pipetting. Oocytes with deformed and disintegrated ooplasm were considered to be degenerating oocytes and were excluded from further analysis. Oocytes showing normal morphology were considered surviving oocytes. The diameters of the oocytes (excluding zona pellucida) were measured to the nearest 1 μm with an ocular micrometer attached to an inverted phase-contrast microscope. After the diameters had been measured, the oocytes were mounted onto slides, fixed in acetic ethanol (1:3), stained with 1% aceto-orcein, and examined under a differential interference microscope at ×400 magnification. The nuclear stages of the oocytes were classified according to the chromatin configuration. Germinal vesicle stage (GV) was classified into three categories: the filamentous chromatin stage (FC) and stringy chromatin stage (SC) as defined for small growing oocytes by Hirao *et al.* (1995), and the germinal vesicle I–III stage (GVI–III) as defined for fully grown oocytes by Motlik *et al.* (1978). Oocytes that underwent germinal vesicle breakdown (GVBD) were classified into diakinesis (D), the first metaphase (MI) and the second metaphase (MII). Oocytes that had normal cytoplasm with an abnormally condensed nucleus or with scattering chromosomes were classified as abnormal oocytes. For controls, oocytes were collected from ovarian early antral (0.4–0.7 mm) or late antral (3–5 mm) follicles. Their diameters were measured, and their nuclear morphology was then examined in the same manner as described above.

Maturation culture

Early antral follicles were collected, embedded in collagen gels, and cultured for 16 days as described above. After the growth culture, denuded and granulosa-cell-enclosed oocytes were washed in HEPES-buffered TCM199 and further cultured individually in 10 μl drops of the maturation medium, which was the basic culture medium, for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 and 95% air. After the maturation culture, the oocytes were denuded, stained, and examined as described above. Oocytes with abnormal configurations of condensed chromosomes were classified as degenerating oocytes. As controls, OCs collected from early antral (0.4–0.7 mm) or late antral (3–5 mm) follicles were cultured in maturation medium for 24 h, and their nuclear morphology was then examined.

Histological examination

Five or six follicles in each experimental group were fixed in 3% formaldehyde in phosphate-buffered saline for histological examination. Fixed follicles were dehydrated and embedded in K4M (Polyscience, Warrington, USA). They were serially sectioned at 1.25 μm and stained with toluidine blue. The morphologies of the follicles after 8 days of the growth culture were examined under a microscope and compared with those observed before the culture.

Statistical analysis

Six independent experiments were conducted, and the results from all replicates were pooled and analysed. Statistical differences in the mean diameters of the oocytes were analysed by Student's *t*-test. Other values were analysed by the chi-square with Yates' correction for continuity. A probability of less than 0.05 was considered significant.

Results

Oocyte growth in Fs, OCGs and OCs cultured in serum-supplemented medium

After 1 day of culture, OCGs became spherical structures in which the oocytes were enclosed by cumulus and parietal granulosa cells. After 4 days of culture, the spherical structure of the OCs had collapsed, and the cumulus cells had grown into the gels from the periphery of the oocytes (Fig. 1*a*). On the other hand, more than half the OCGs formed antrum-like structures (Fig. 1*b*), but none of the structures were maintained by the 16th day of culture. The structures of Fs with follicular antra were maintained after 8 days. Thereafter the antra became invisible with the shrinking of the collagen gels.

After 16 days, the oocytes were recovered. In Fs, OCGs and OCs cultured in hypoxanthine-free medium, 21%, 9% and 4% of oocytes showed normal morphology, respectively (Table 1), and hypoxanthine increased the percentages in all groups (Fs, 37%; OCGs, 29%; OCs, 10%). Two types of oocytes were collected: denuded oocytes and granulosa-cell-enclosed oocytes. Oocytes attached to granulosa cells were recovered from Fs and OCGs cultured in hypoxanthine-supplemented medium, and all the oocytes showed normal morphology. The mean diameters of the surviving oocytes in all groups were significantly higher than the diameters of the oocytes from the early antral follicles before culture (Table 2).

The nuclear configuration of the surviving oocytes was examined (Table 2). All oocytes collected from early antral follicles before culture had a germinal vesicle in which decondensed filamentous chromatin (FC) or stringy chromatin (SC) was distributed. After 16 days of culture, more than 60% of the surviving oocytes in OCGs and OCs underwent germinal vesicle breakdown (GVBD). In contrast, 59% and 84% of the

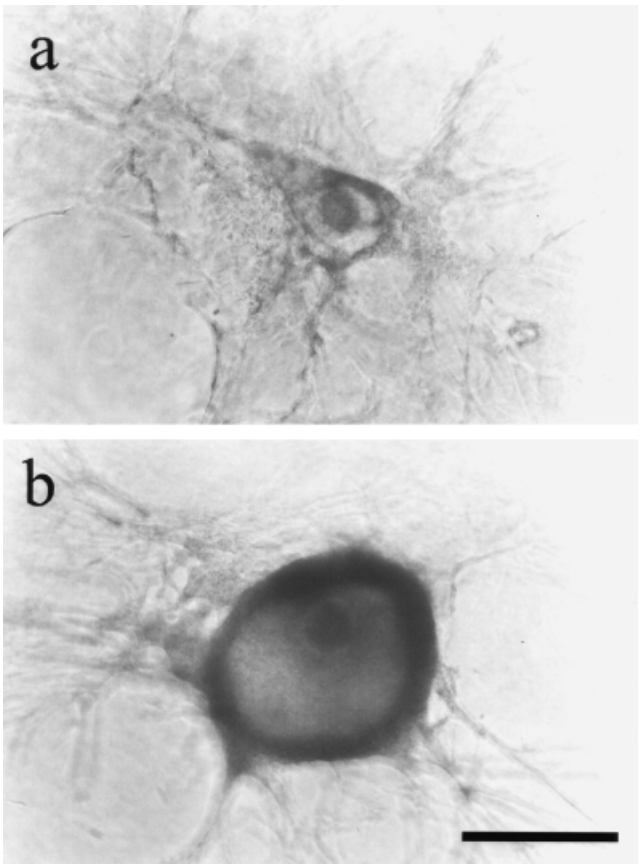

Figure 1 Morphologies of an oocyte–cumulus cell complex (OC) and an oocyte–cumulus–granulosa cell complex (OCG) from bovine early antral follicles during culture in collagen gels. (*a*) After 4 days of culture, the cumulus cells in the OC grew into the collagen gel from the periphery of the oocyte. (*b*) The OCG formed an antrum-like structure after 4 days of culture. Scale bar represents 400 μm.

oocytes from Fs cultured in medium with and without hypoxanthine, respectively, had a germinal vesicle.

Oocyte growth in Fs cultured in serum-free medium and their meiotic competence

In serum-free medium, follicular cells were suppressed from growing into collagen gels. Follicular cavities in Fs cultured in serum-free, hypoxanthine-supplemented medium were observed after 16 days of the culture. We compared the histological sections of Fs cultured in hypoxanthine-supplemented and hypoxanthine-free media after 8 days. In Fs cultured in hypoxanthine-free medium, the thecal layer and basement membrane had disappeared (Fig. 2*b*), and it was difficult to distinguish theca cells from granulosa cells. Antral cavities were not visible in Fs cultured in hypoxanthine-free medium, while they were observed in those cultured in hypoxanthine-supplemented medium (Fig. 2*c*). In hypoxanthine-supplemented medium, oocytes were surrounded by a few layers of cuboidal cumulus granulosa cells closely attached to the zona pellucida. The outermost layer of mural granulosa cells formed a well-organised lining along the basement membrane.

Cumulus-cell-enclosed oocytes were recovered from Fs cultured in hypoxanthine-supplemented medium, although all the recovered oocytes had been denuded in hypoxanthine-free medium (Table 3). The number of morphologically normal oocytes was significantly higher in serum-free medium, and hypoxanthine significantly increased the number of morphologically normal oocytes in the medium to 91% ($p < 0.05$). The mean diameter of oocytes cultured in serum-free, hypoxanthine-supplemented medium (117.6 ± 5.7 μm) was significantly higher than the mean diameter of those in other groups (Table 4). The oocytes grew to

Table 1 Effect of hypoxanthine on the morphology of bovine oocytes in Fs, OCGs and OCs cultured for 16 days

Culture methods*		No. of oocytes cultured	No. (%) of surviving oocytes			No. (%) of oocytes degenerating
Complexes	Hypoxanthine		Total	Denuded	GC enclosed	
Fs	–	80	17 (21)^{a,b}	17 (21)^{a}	0	63 (79)^{a,b}
Fs	+	52	19 (37)^{a}	3 (6)^{b,c}	16 (31)	33 (63)^{b}
OCGs	–	88	8 (9)^{c}	8 (9)^{b,c}	0	80 (91)^{c}
OCGs	+	52	15 (29)^{a}	8 (15)^{a,c}	7 (13)	37 (71)^{b}
OCs	–	92	4 (4)^{c}	4 (4)^{b}	0	88 (96)^{c}
OCs	+	52	5 (10)^{b,c}	5 (10)^{a,b}	0	47 (90)^{a,c}

GC, granulosa cells.
*Intact early antral follicles (Fs), oocyte–cumulus–granulosa cell complexes (OCGs) and oocyte–cumulus cell complexes (OCs) were embedded in collagen gels, and cultured in the medium containing 10% fetal calf serum, 0.1 mg/ml sodium pyruvate and 0 (–) or 4 mM (+) hypoxanthine for 16 days.
$^{a–c}$Values with different superscripts in the same column differ significantly ($p < 0.05$).

Table 2 *In vitro* growth and nuclear morphology of bovine oocytes in Fs, OCGs and OCs cultured for 16 days

Culture methods*		No. of oocytes for growth culture	No. of surviving oocytes	Mean diameter of oocytes (μm ± SD)	Nuclear morphology of surviving oocytes† (%)					
					GV					
Complexes	Hypoxanthine				Total	FC	SC	GVI–III	Abnormal	GVBD
Fs	–	80	17	109.2 ± 5.9^{a,b}	10 (59)^{a,b}	4 (24)^{a}	0	6 (35)	0	7 (41)^{a,b}
Fs	+	52	19	106.6 ± 2.9^{a}	16 (84)^{a}	9 (47)^{a}	4 (21)^{a}	3 (16)	0	3 (16)^{a}
OCGs	–	88	8	112.6 ± 7.0^{b,c}	3 (38)^{b}	0	1 (13)^{a,b}	2 (25)	0	5 (62)^{b}
OCGs	+	52	15	116.2 ± 6.4^{c}	3 (20)^{b}	0	1 (7)^{a,b}	2 (13)	0	12 (80)^{b}
OCs	–	92	4	112.5 ± 4.5^{b,c}	1 (25)^{a,b}	0	1 (25)^{a,b}	0	0	3 (75)^{a,b}
OCs	+	52	5	113.6 ± 6.2^{b,c}	1 (20)^{b}	0	0	1 (20)	0	4 (80)^{b}
Before culture‡			69	95.0 ± 2.2^{d}	69 (100)	68 (99)^{b}	1 (1)^{b}	0	0	0

*Intact early antral follicles (Fs), oocyte–cumulus–granulosa cell complexes (OCGs) and oocyte–cumulus cell complexes (OCs) were embedded in collagen gels, and cultured in medium containing 10% fetal calf serum, 0.1 mg/ml sodium pyruvate and 0 (–) or 4 mM (+) hypoxanthine for 16 days.

†Germinal vesicle (GV) stage is classified into FC, SC, GVI–III and Abnormal. FC, filamentous chromatin stage; SC, stringy chromatin stage as defined by Hirao *et al*. (1995); GVI–III stage, as defined by Motlik *et al*. (1978). Abnormal, abnormally condensed nucleus. GVBD, germinal vesicle breakdown.

‡Oocytes were collected from follicles 0.4–0.7 mm in diameter.

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

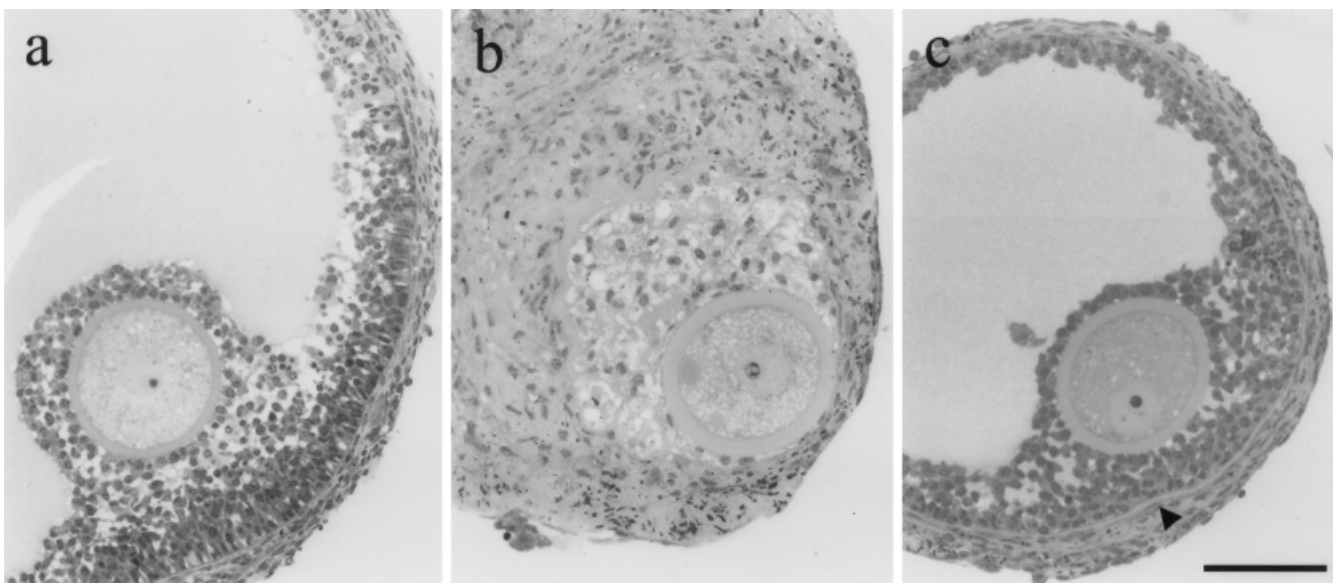

Figure 2 Histological sections of bovine early antral follicles cultured for 8 days. (*a*) An early antral follicle collected from a bovine ovary before culture. (*b*) A follicle cultured in serum-free medium without hypoxanthine. (*c*) A follicle cultured in serum-free medium containing hypoxanthine. Note that the basement membrane disappeared in the follicle in (*b*), while it was maintained in the follicle in (*c*) (arrowhead). Scale bar represents 100 μm.

a size similar to that of fully grown oocytes from late antral follicles.

The nuclear configurations of oocytes after the growth culture were examined (Table 4). In the oocytes collected from early antral follicles before the culture, decondensed filamentous or stringy chromatin was distributed throughout the germinal vesicle as described in the first experiment. In contrast, a nucleolus was surrounded by condensing chromatin in the germinal vesicle in most of the oocytes from late antral follicles. Of the surviving oocytes cultured in serum-free, hypoxanthine-supplemented medium, 84% were at GVI–III. The distribution of the nuclear morphology of the oocytes was similar to that of the oocytes collected from late antral follicles.

When oocytes collected from early antral follicles

Table 3 Effect of hypoxanthine on the morphology of bovine oocytes in early antral follicles cultured in serum-free medium for 16 days

Culture methods*		No. of oocytes cultured	No. (%) of surviving oocytes			No. (%) of oocytes degenerating
Serum	Hypoxanthine		Total	Denuded	GC enclosed	
+	–	35	6 (17)[a]	6 (17)[a]	0	29 (83)[a]
+	+	67	16 (24)[a]	4 (6)[a]	12 (18)[a]	51 (76)[a]
–	–	35	19 (54)[b]	19 (54)[b]	0	16 (46)[b]
–	+	67	61 (91)[c]	6 (9)[a]	55 (82)[b]	6 (9)[c]

GC, granulosa cells.
*Early antral follicles (0.4–0.7 mm) were embedded in collagen gels, and cultured in serum-supplemented (+) or serum-free (–) medium containing 0.1 mg/ml sodium pyruvate and 0 (–) or 4 mM (+) hypoxanthine for 16 days. Fetal calf serum (10%) was substituted by 3 mg/ml bovine serum albumin in serum-free medium.
[a–c]Values with different superscripts in the same column differ significantly ($p < 0.05$).

Table 4 *In vitro* growth and nuclear morphology of bovine oocytes cultured in serum-free medium for 16 days

Culture methods*		No. of oocytes for growth culture	No. of surviving oocytes	Mean diameter of oocytes (μm ± SD)	Nuclear morphology of surviving oocytes† (%)					
						GV				
Serum	Hypoxanthine				Total	FC	SC	GVI–III	Abnormal	GVBD
+	–	35	6	103.3 ± 3.1[a]	6 (100)	1 (17)[a,b]	3 (50)[a,c]	1 (17)[a]	1 (17)	0
+	+	67	16	107.4 ± 4.6[a]	12 (75)[a]	5 (31)[a]	7 (44)[a]	0	0	4 (25)[a]
–	–	35	19	111.7 ± 5.1[b]	4 (21)[b]	3 (16)[a,b]	0	0	1 (5)	15 (79)[b]
–	+	67	61	117.6 ± 5.7[c]	53 (87)[a,c]	1 (2)[b]	1 (2)[b]	51 (84)[b]	0	8 (13)[a,c]
In vivo (0.4–0.7 mm)‡		93	93	95.2 ± 4.2[d]	93 (100)	82 (88)[c]	9 (10)[b,d]	0	2 (2)	0
In vivo (3–5 mm)‡		52	52	120.4 ± 4.3[c]	50 (96)[c]	0	8 (15)[c,d]	41 (79)[b]	1 (2)	2 (4)[c]

*Early antral follicles (0.4–0.7 mm) were embedded in collagen gels, and cultured in serum-supplemented (+) or serum-free (–) medium containing 0.1 mg/ml sodium pyruvate and 0 (–) or 4 mM (+) hypoxanthine for 16 days. Fetal calf serum (10%) was substituted by 3 mg/ml bovine serum albumin in serum-free medium.
†Germinal vesicle (GV) stage is classified into FC, SC, GVI–III and Abnormal. FC, filamentous chromatin stage; SC, stringy chromatin stage as defined by Hirao *et al.* (1995); GVI–III stage, as defined by Motlik *et al.* (1978). Abnormal, abnormally condensed nucleus. GVBD, germinal vesicle breakdown.
‡Oocytes were collected from follicles 0.4–0.7 mm and 3–5 mm in diameter.
[a–d]Values with different superscripts in the same column differ significantly ($p < 0.05$).

were subjected to a maturation culture, 95% of them remained at the germinal vesicle stage (Table 5). On the other hand, 88% of the oocytes collected from late antral follicles underwent GVBD, and 72% of them reached the second metaphase (MII). Oocyte maturing to MII were obtained only from the follicles cultured in serum-free, hypoxanthine-supplemented medium. Overall, 63% (25/40) of the oocytes reached MI or beyond, and 23% of them matured to MII.

Discussion

We collected bovine early antral follicles by micro-dissection using forceps. This method allows the isolation of morphologically normal follicles with an intact basement membrane and the maintenance of theca–granulosa–oocyte interactions (van den Hurk *et al.*, 1998). Cross-sections of the micro-dissected follicles showed that theca cells and mural granulosa cells formed well-organised linings along the basement membrane. Oocytes in the follicles survived efficiently after the growth culture and suppressed spontaneous maturation. Theca cells have been reported to secrete growth factors which control follicular development. Within the bovine follicle, transforming growth factor α is produced by theca cells, which can subsequently play a paracrine role in regulating granulosa cell proliferation (Skinner & Coffey, 1988). Theca-cell-derived keratinocyte growth factor and hepatocyte growth factor stimulate granulosa-cell-derived kit ligand expression and promote the differentiation of granulosa cells (Parrott & Skinner,

Table 5 Maturation of *in vitro*-grown bovine oocytes cultured in serum-free medium for 16 days

Culture methods*		No. of oocytes for growth culture	No. of oocytes for maturation culture	Maturational stage of oocytes† (%)						No. (%) of oocytes degenerating
					GVBD					
Serum	Hypoxanthine			GV	Total	D	MI	MII	Abnormal	
+	–	39	10	2 (20)^{a}	2 (20)^{a,b}	1 (10)	0	0	1 (10)^{a,b}	6 (60)^{a}
+	+	45	19	1 (5)^{a,b}	4 (21)^{b}	2 (11)	1 (5)^{a}	0	1 (5)^{b,c}	14 (74)^{a}
–	–	38	24	1 (4)^{a,b}	18 (75)^{c}	2 (8)	6 (25)^{a,b}	0	10 (42)^{a}	5 (21)^{a}
–	+	45	40	1 (3)^{a,b}	34 (85)^{c}	3 (8)	16 (40)^{b}	9 (23)^{a}	6 (15)^{b,c}	5 (13)^{b}
In vivo (0.4–0.7 mm)‡			87	83 (95)^{c}	4 (5)^{a}	4 (5)	0	0	0	0
In vivo (3–5 mm)‡			112	1 (1)^{b}	99 (88)^{c}	7 (6)	13 (12)^{a}	81 (72)^{b}	0	12 (11)^{b}

*Early antral follicles (0.4–0.7 mm) were embedded in collagen gels, and cultured in serum-supplemented (+) or serum-free (–) medium containing 0.1 mg/ml sodium pyruvate and 0 (–) or 4 mM (+) hypoxanthine for 16 days. Fetal calf serum (10%) was substituted by 3 mg/ml bovine serum albumin in serum-free medium. After growth culture, surviving oocytes were collected, and were cultured in maturation medium containing 0.1 mg/ml sodium pyruvate and 10% fetal calf serum for 24 h and examined.

†GV, germinal vesicle stage; GVBD, germinal vesicle breakdown. GVBD is classified into D, MI, MII and Abnormal; D, diakinesis stage; MI, metaphase I stage; MII, metaphase II; Abnormal, chromosomes scattered in cytoplasm.

‡Oocytes were collected from follicles 0.4–0.7 mm and 3–5 mm in diameter and then cultured for 24 h.

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

1998). Furthermore, theca cells are essential for antral formation in the mouse (Telfer *et al.*, 1990; Nayudu & Osborn, 1992; Boland *et al.*, 1993). However, theca-cell-free bovine oocyte–cumulus– granulosa cell complexes collected from early antral follicles reorganise antrum-like structures, in which the oocytes grow (Harada *et al.*, 1997). Alternatively, antrum, rather than theca cells, may be important for oocyte survival and growth by maintaining the intrafollicular microenvironment and sustaining the integrity of the follicles. Serum may affect the integrity of follicles by stimulating the proliferation of the two kinds of somatic cells in the follicles in two different ways. In the present study, culturing Fs in serum-free medium prevented the excessive proliferation of follicular cells by retaining the integrity of the follicles more efficiently than in Fs cultured in serum-supplemented medium. Paracrine factors secreted by follicular somatic cells and oocytes regulate many important aspects of ovarian follicular development in mammals (van den Hurk *et al.*, 1997; Albertini *et al.*, 2001). In follicles, locally acting factors have been identified that appear to exert important effects on oocyte growth and somatic cell division and differentiation. It is thought that these local interactions between oocyte and somatic cells are sufficient for oocyte growth and for maintaining the follicular integrity without serum factors.

Hypoxanthine has been reported to maintain the association between cultured oocytes and the surrounding granulosa cells in the mouse (Eppig *et al.*, 1985) and cow (Harada *et al.*, 1997), and to promote oocyte growth. Hypoxanthine more effectively maintained the association in serum-free medium than in serum-supplemented medium. There is little information regarding the effect of hypoxanthine on follicular morphology with the exception of the study of Figueiredo and colleagues (1994), who demonstrated that the number of cells per square millimetre in the granulosa layer was lower in bovine preantral follicles cultured in the absence of hypoxanthine than in those cultured in the presence of hypoxanthine. In the present study, oocytes surrounded by cumulus cells were recovered only from follicles cultured in hypoxanthine-supplemented medium. The histological sections of the follicles cultured in hypoxanthine-supplemented medium maintained the basement membrane and a clear antral cavity with a cumulus–granulosa-cell surrounded oocyte. On the other hand, theca cells changed to irregular shapes and the basement membrane disappeared in the follicles cultured in hypoxanthine-free medium. It has been speculated that hypoxanthine has several effects on the morphologies and functions of follicular somatic cells. Since hypoxanthine is a naturally occurring cAMP-phosphodiesterase inhibitor in follicular fluid, it is thought that hypoxanthine in the culture medium maintained the cAMP level in surrounding somatic cells as well as in oocytes and maintained their functions, such as the association between oocytes and granulosa cells, and the maintenance of the integrity of the basement membrane. The exact mechanism by which hypoxanthine maintains the basement membrane was not shown in the present study, but it is known that the basement membrane supports the integrity of the follicles and the interactions between oocytes and granulosa cells.

Oocytes acquire meiotic competence during the growth phase in the ovaries, and the meiotically competent oocytes approaching their final size resume meiosis spontaneously without hormonal stimulation when they are liberated from the antral follicles. It is thought that oocytes grown *in vitro* resume meiosis spontaneously. In fact, some of the *in vitro*-grown bovine oocytes underwent GVBD even in the hypoxanthine-supplemented medium. However, the oocytes recovered from follicles cultured in serum-free, hypoxanthine-supplemented medium were arrested at the GV stage. The presence of gap junctions between granulosa cells and oocytes is well known (Anderson & Albertini, 1976). These junctions permit the two-way transfer of molecules between somatic cells and oocytes (Heller *et al*., 1981; Brower & Shultz; 1982). This intercellular communication would permit the distribution of factors such as hypoxanthine and cAMP, which could regulate the maintenance of oocytes in meiotic arrest (Eppig & Downs, 1987). Bovine oocytes have been reported to achieve complete nuclear maturation to MII with a diameter of 110 μm *in vivo* (Fair *et al*., 1995). In the present study, oocytes from Fs in serum-free, hypoxanthine-supplemented medium reached 110 μm or more in diameter, and some of them matured to the second metaphase after the subsequent maturation culture. Since oocytes did not undergo GVBD before the growth culture, the results suggest that oocytes acquired meiotic competence during the growth culture. In fact, the distribution of the nuclear morphology of the oocytes after the growth culture was similar to that of the oocytes collected from late antral follicles, except that a few oocytes that grew *in vitro* matured to MII after the maturation culture. It seems likely that substances essential for oocyte maturation were not sufficiently accumulated in *in vitro*-grown oocytes during the growth culture period.

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