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—Original Article—

In vitro growth of bovine oocytes in oocyte-cumulus cell complexes and the effect of follicle stimulating hormone on the growth of oocytes

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Abstract. Several successful *in vitro* culture experiments have used oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) from early antral follicles (0.5–0.7 mm) for the growth of bovine oocytes. However, in studies related to *in vitro* oocyte maturation and *in vitro* embryo production, oocyte-cumulus cell complexes (OCCs) that have no mural granulosa cells have been widely used instead of OCGCs. The purpose of this study was to determine whether cumulus cells alone support oocyte growth. First, OCCs and OCGCs were cultured *in vitro* for 14 days to compare the integrity of the complexes as well as antrum formation. After 14 days, the diameter and meiotic competence of oocytes in OCCs and OCGCs were examined. Oocytes in OCCs grew fully and acquired meiotic competence similar to OCGCs, whereas antrum formation occurred later in OCCs as compared to OCGCs. Subsequently, the effects of follicle stimulating hormone (FSH) on *in vitro* growth of OCCs were examined for 14 days. When FSH was added to the culture medium, OCCs formed antrum-like structures one day earlier than those cultured without FSH. Oocytes cultured with 1 mIU/ml FSH grew fully and acquired meiotic competence. In contrast, when oocytes were cultured in media containing high concentrations of FSH, some of the OCCs collapsed and the number of degenerated oocytes increased. In conclusion, bovine oocytes in OCCs grow and acquire meiotic competence similar to OCGCs and, 1 mIU/ml FSH supports the development of OCCs and oocyte growth as observed in our culture system.

Key words: Bovine oocyte, Cumulus cell, Follicle stimulating hormone (FSH), *In vitro* growth

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A wide range of approaches have been developed towards *in vitro* growth of mammalian oocytes. Previous studies have reported that calves were produced from *in vitro* grown bovine oocytes [1–3]. In these studies, oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) collected from early antral follicles were used for *in vitro* growth culture experiments.

It has been demonstrated that bidirectional interaction between oocytes and granulosa cells is essential for oocyte development. To maintain connections among these cells, Hirao *et al*. [2] proposed a novel culture system in which oocyte-granulosa cell complexes were cultured with a high concentration of polyvinylpyrrolidone (PVP; molecular weight of 360,000) on the insert membrane. In this system, cultured complexes formed dome-like structures similar to follicular antra [2, 4, 5]. These antrum-like structures are thought to create a suitable microenvironment for oocyte growth [4, 6]. Makita and Miyano [4] demonstrated that a combination of 17β-estradiol and androstenedione maintained the structure of the complexes and promoted complete growth and acquisition of meiotic competence in more than half of the cultured bovine oocytes.

In studies related to *in vitro* growth of bovine oocytes, OCGCs have been used as culture materials to prevent migration of somatic cells away from the oocytes and to maintain the proper association between oocytes and somatic cells during long-term growth culture period, especially on a flat substratum [2, 6]. However, in studies regarding *in vitro* oocyte maturation and *in vitro* embryo production, oocyte-cumulus cell complexes (OCCs) that have no mural granulosa cells have been widely used instead of OCGCs. Since the culture systems for oocyte growth have been improved as mentioned above, we examined the possible use of OCCs as culture materials for oocyte growth. In the first experiment of the present study, we aimed to determine whether cumulus cells alone would support bovine oocyte growth. The integrity of OCGCs and OCCs as well as antrum formation were compared for 14 days of *in vitro* growth culture. After 14 days, the diameter and meiotic competence of oocytes cultured in these complexes were examined. In the first experiment, while oocytes in OCCs grew and acquired meiotic competence similar to OCGCs, antrum formation occurred later in OCCs as compared to OCGCs. Therefore, in the second experiment, we assessed the effect of follicle stimulating hormone (FSH) on OCCs. FSH promotes granulosa cell proliferation *in vivo* and *in vitro* [7–10]. OCCs were cultured with various concentrations of FSH for 14 days, and subsequently, the integrity and antrum formation by OCCs were compared. After 14 days, the diameter and meiotic competence of oocytes in cultured OCCs were examined.

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Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned.

Collection of OCGCs and OCCs

OCGCs were collected from bovine early antral follicles as described previously [4], and OCCs were prepared from OCGCs. Briefly, bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory. Ovaries were washed once with 0.2% (w/v) cetyltrimethylammonium bromide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and three times with Dulbecco's PBS containing 0.1% (w/v) polyvinyl alcohol (PBS-PVA). Ovarian cortical slices were collected using a surgical blade (No.21; ELP, Akiyama-seisakusyo, Tokyo, Japan) and forceps. Early antral follicles (0.5–0.7 mm in diameter) were dissected from ovarian cortical slices in 25 mM HEPES-buffered medium 199 (HEPES-199; Dojindo Laboratories, Kumamoto, Japan) containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate (FUJIFILM Wako Pure Chemical Corporation), and 0.08 mg/ml kanamycin sulfate. Follicles were opened using a surgical blade (No.10; Feather Safety Razor, Tokyo, Japan) and forceps to collect OCGCs containing growing oocytes. Some of the OCGCs were used for *in vitro* growth culture, and the remainder were used to prepare OCCs. Mural granulosa cells were removed from the OCGCs as much as possible using a narrow pipette. The resulting complexes, composed of growing oocytes surrounded by two or three layers of cumulus cells, are referred to as OCCs hereafter. The diameter of oocytes (excluding the zona pellucida) in both types of complexes was measured to the nearest 1 μm with an ocular micrometer (Olympus, Tokyo, Japan) attached to an inverted microscope. OCGCs and OCCs containing oocytes with diameters of 90–105 μm were selected.

To collect OCGCs containing fully grown oocytes, OCGCs from antral follicles (4–6 mm in diameter) were aspirated with follicular fluid using a syringe and a needle (18 ga: Terumo, Tokyo, Japan), and the diameter of oocytes was measured. OCGCs collected from antral follicles served as an *in vivo* fully grown control.

In vitro growth culture of OCGCs and OCCs

In vitro growth culture was performed as described previously [11] with some modifications. Briefly, groups of 5–15 OCGCs or OCCs collected from at least 5 biological replicates were cultured for 14 days on Millicell inserts (cell culture inserts 0.4 μm, 30 mm diameter; Merck Millipore, Darmstadt, Germany) placed in Petri dishes (Falcon No. 351008; Becton Dickinson and Co., Franklin Lakes, NJ, USA) at 38.5ºC under a controlled humidified atmosphere of 5% O_2, 5% CO_2, and 90% N_2 for 7 days, followed by an atmosphere of 5% CO_2 in air for 7 days [12]. The basic culture medium was α-minimum essential medium (αMEM, Cat. No. 11900-024; Invitrogen, Tokyo, Japan) supplemented with 5% (v/v) fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 50 μg/ml ascorbic acid 2-glucoside (Hayashibara Biochemical Laboratories, Okayama, Japan), 55 μg/ml cysteine, 0.05 μM dexamethasone, 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (molecular weight 360,000), 2.2 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate [2]. Based on a previous report [4], the culture medium was supplemented with 10 ng/ml 17β-estradiol and 10 ng/ml androstenedione (Tokyo Chemical Industry, Tokyo, Japan). Using this medium as a control, OCCs were cultured in the medium supplemented with 1, 5, 10, and 50 mIU/ml recombinant human follicle stimulating hormone (rhFSH; MSD KK, Tokyo, Japan) in the second experiment. These concentrations were selected based on previous reports [13]. The day on which OCGCs and OCCs were collected was designated as Day 0, and half of the culture medium was replaced with fresh medium every other day after Day 4.

Antrum formation by OCGCs and OCCs was observed every day by identifying visible spaces surrounded by granulosa cells or cumulus cells. Complexes with cytoplasmic degenerative oocytes, detachment of granulosa cells or cumulus cells from the zona pellucida, and collapsed complexes were classified as disintegrated complexes; all others were regarded as complexes that maintained their integrity.

After *in vitro* growth culture, the diameter of oocytes was measured as described above. Some of the oocytes were denuded mechanically, fixed with acetic acid-ethanol (1:3), and stained with 1% (w/v) aceto-orcein (FUJIFILM Wako Pure Chemical Corporation). The stages of meiotic division were assessed using Nomarski interference microscopy. Oocytes were classified based on the morphology of chromatin and the nuclear envelope [14, 15]. The stages of oocytes before meiotic resumption were classified as filamentous chromatin (FC), stringy chromatin (SC), and germinal vesicle I–IV (GV I–IV). After resumption of meiosis, the stages were classified as early diakinesis (ED), late diakinesis (LD), metaphase I (MI), anaphase I and telophase I (AI-TI), and metaphase II (MII). Oocytes showing cytoplasmic or nuclear abnormalities were regarded as degenerated oocytes.

In vitro maturation culture of OCGCs and OCCs

OCGCs and OCCs that maintained their integrity after 14 days of *in vitro* growth culture were further used for *in vitro* maturation which was performed as described previously [11, 16] with some modifications. OCGCs collected from early antral follicles and antral follicles were also subjected to maturation and served as *in vivo* controls. Briefly, OCGCs and OCCs were cultured in 50 μl microdrops of maturation medium covered with paraffin oil at 38.5ºC under a controlled atmosphere (5% CO_2 in air) for 22 h. Each microdrop contained 4–5 OCGCs or OCCs collected from at least 5 biological replicates. The maturation medium was TCM-199 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) FBS, 0.1 mg/ml sodium pyruvate (Nacalai Tesque, Kyoto, Japan), 2.2 mg/ml sodium bicarbonate, 0.08 mg/ml kanamycin sulfate, and 0.1 IU/ml human menopausal gonadotropin (Aska Pharmaceutical, Tokyo, Japan).

After 22 h, oocytes were denuded mechanically using 0.1% (w/v) hyaluronidase and a narrow pipette. They were fixed with acetic acid-ethanol (1:3), and stained with 1% (w/v) aceto-orcein to assess the stage of oocyte maturation.

Preparation of histological sections

Histological sections of cultured OCCs were prepared as described previously [17] with some modifications. Briefly, after *in vitro* growth culture, some of the complexes on Millicell inserts were washed once

with PBS-PVA and fixed in 4% (w/v) paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) overnight. The next day, the complexes were washed three times with PBS-PVA and dehydrated using a series of increasing concentrations of ethanol (50% for 30 min, followed by 70, 80, 90, 100 and 100% for 20 min each). Complexes maintaining their integrity were infiltrated and embedded in JB-4 resin (Polysciences, Warrington, PA, USA). A rotary microtome (HM 335 E; MICROM International GmbH, Walldorf, Germany) was used to make 7 µm sections from the specimens. Sections were stained with Mayer's hematoxylin solution (FUJIFILM Wako Pure Chemical Corporation) and 1% (w/v) eosin Y (FUJIFILM Wako Pure Chemical Corporation). EUKITT mounting medium (O. Kindler, Freiburg, Germany) was used to mount the sections.

Experimental design

Experiment 1: OCGCs and OCCs collected from early antral follicles were subjected to 14 days of *in vitro* growth culture. The integrity of OCGCs and OCCs was examined throughout the growth culture period. Half of the OCGCs and OCCs, which maintained their integrity after 14 days of growth culture, were used to examine the meiotic stage of oocytes. The remaining half of the OCGCs and OCCs were subjected to 22 h of *in vitro* maturation culture, and the meiotic competence of oocytes was determined. Antrum formation by the complexes was examined using other groups of OCGCs and OCCs throughout the growth culture period of 14 days. After 14 days of growth culture, the diameters of oocytes in OCGCs and OCCs that maintained their integrity were determined.

Experiment 2: We assessed the effect of FSH on oocyte growth using OCCs. OCCs collected from early antral follicles were cultured with different concentrations of FSH (1–50 mIU/ml) for 14 days. The integrity of OCCs as well as antrum formation were examined during the *in vitro* growth culture period of 14 days. After the growth culture period, the diameters of oocytes in OCCs were determined, and half of the OCCs that maintained their integrity were used to examine the meiotic stage of oocytes. The remaining half of the OCCs were subjected to *in vitro* maturation culture to determine the meiotic competence of oocytes.

Statistical analysis

Differences among mean (± SEM) diameters of oocytes were analyzed by one-way ANOVA followed by Tukey-Kramer multiple range test (Excel software with the add-in Ekuseru-Toukei 2010; Social Survey Research Information, Tokyo, Japan). All other experimental data were analyzed by a Chi-square test. Results with P value < 0.05 were considered as significant.

Results

Oocyte growth in OCGCs and OCCs

Typical morphologies of the developing OCGCs and OCCs during growth culture are shown in Fig. 1. OCCs had two or three layers of cumulus cells including an oocyte at the center (Fig. 1, B0). OCGCs

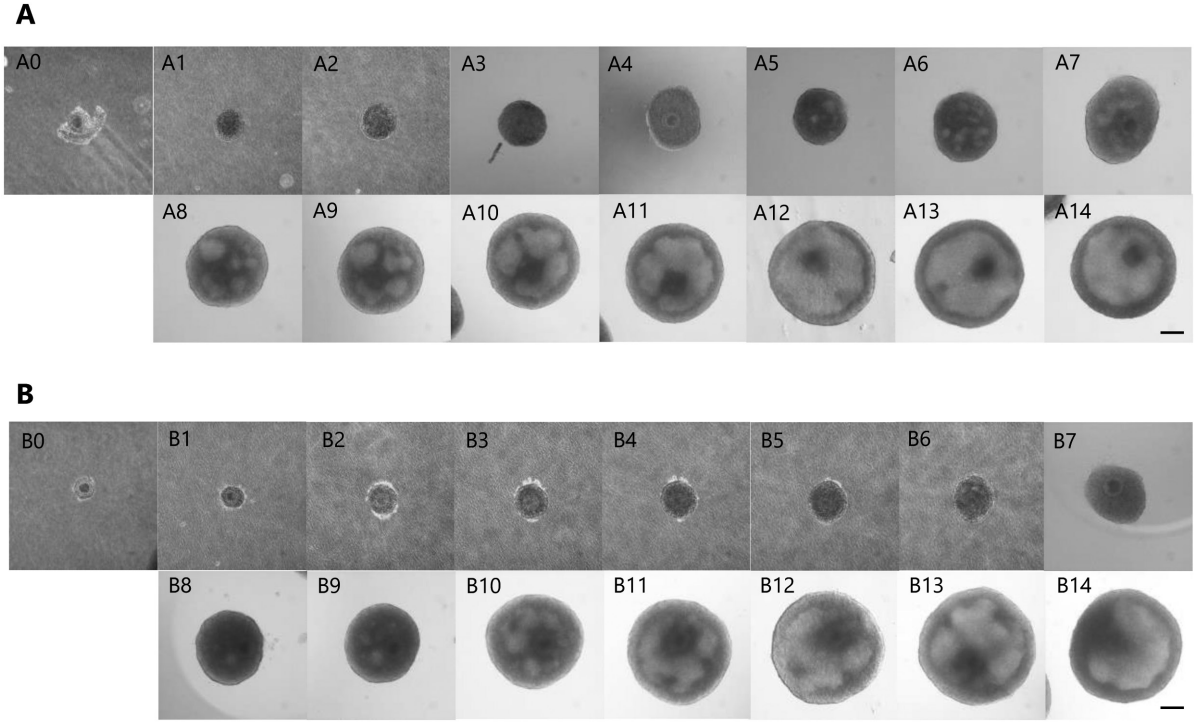

Fig. 1. Typical morphologies of bovine oocyte-cumulus cell-mural granulosa cell complexes (OCGCs; A) and oocyte-cumulus cell complexes (OCCs; B) during *in vitro* growth culture. OCGCs and OCCs were cultured for 14 days. A0–A14 and B0–B14 represent Days 0–14, respectively. After OCGCs and OCCs attached onto a membrane sheet, they increased in size, and some of them formed antrum-like structures. The scale bars represent 200 µm.

had further layers of mural granulosa cells attached to OCCs (Fig. 1, A0). After attaching onto a membrane sheet, the sizes of the OCGCs and OCCs were increased.

The integrity of OCGCs and OCCs during growth culture is shown in Fig. 2A. On Day 7, 96% of OCGCs and 99% of OCCs maintained spherical structures containing an oocyte in the center surrounded by cumulus cells and mural granulosa cells in OCGCs, and cumulus cells in OCCs (Fig. 1). After Day 7, some of the structures of OCGCs and OCCs collapsed, and the oocytes were denuded. On Day 14, OCGCs showed higher integrity than OCCs (84 and 63%, respectively).

As the complexes developed, some of them formed antrum-like structures (Fig. 1, A5–14, B8–14). OCGCs started forming antrum-like structures on Day 4, three days earlier than OCCs (Fig. 2B). Almost all antrum-like structures formed by OCGCs and OCCs were maintained until Day 14.

The mean diameters of oocytes in OCGCs and OCCs were significantly increased after growth culture compared to oocytes before culture (Fig. 3). After 14 days, the mean diameters of oocytes in OCGCs and OCCs were 120 μm or more.

After 14 days of *in vitro* growth culture, more than 80% of the oocytes in OCGCs and OCCs were at the GV stage (85 and 84%, respectively) (Table 1). Growing oocytes collected from early antral follicles (0.5–0.7 mm) were at the FC or SC stages, while all *in vivo* fully grown oocytes from antral follicles (4–6 mm) were at the GV stage.

In the subsequent maturation culture, more than 80% of the oocytes grown in OCGCs and OCCs reached MII (81 and 88%, respectively) (Table 2). Growing oocytes collected from early antral follicles (0.5–0.7 mm) remained at FC, SC, and GV stages (14, 75 and 11%, respectively) after 22 h of maturation culture, while 74% of fully grown oocytes from antral follicles (4–6 mm) reached MII.

Effect of FSH on OCC development and oocyte growth

In the first experiment, oocytes in OCCs grew and acquired meiotic competence similar to OCGCs. However, OCCs formed antrum-like structures three days later than OCGCs. Therefore, we next examined the effect of FSH, which stimulates the proliferation of granulosa cells, on antrum formation and oocyte growth using OCCs.

Typical morphologies of OCCs during growth culture are shown in Fig. 4A. During growth culture, the size of OCCs increased in all treatment groups. However, some of the OCCs cultured with 10 and 50 mIU/ml FSH were small, and some of them detached from the membrane sheet and floated in the medium.

The integrity of OCCs during growth culture is shown in Fig. 5A. On Day 7, 90% or more of OCCs maintained the structures that contained an oocyte surrounded by cumulus cells. After Day 7, the structures of some OCCs collapsed, and the oocytes were denuded. On Day 14, all OCCs cultured with 1 and 5 mIU/ml FSH maintained their structures. The integrity of OCCs cultured without FSH and, with 10 and 50 mIU/ml FSH were 95, 84 and 78%, respectively.

When FSH was added to the culture medium, OCCs formed antrum-like structures one day earlier than those cultured without FSH (Fig. 5B). On Day 14, the antrum formation rates of OCCs cultured without FSH and, with 1 and 5 mIU/ml FSH were high (93, 93 and 95%, respectively). In contrast, when OCCs were cultured with 10 and 50 mIU/ml FSH, some of the antrum-like structures collapsed during growth culture and in which structures all the oocytes degenerated (Fig. 4B, 50 mIU/ml FSH). Therefore, the antrum formation rates of the OCCs were decreased to 79% and 63% upon treatment with 10 and 50 mIU/ml FSH, respectively, on Day 14 (Fig. 5B). After growth culture, some of the OCCs that included degenerated oocytes were sticky.

After growth culture, the mean diameters of the oocytes in OCCs

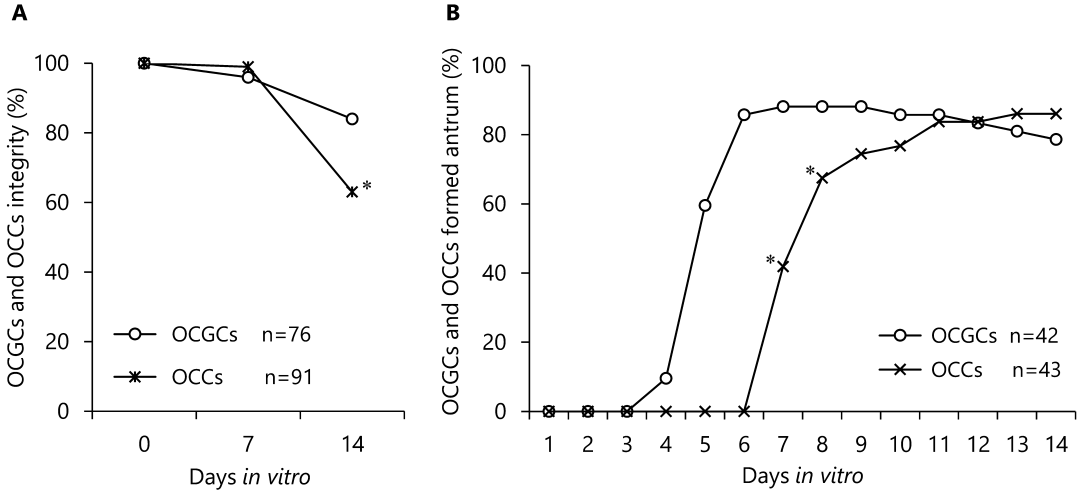

Fig. 2. Integrity of bovine oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) and oocyte-cumulus cell complexes (OCCs) (A) and formation of antrum-like structures by OCGCs and OCCs (B) during *in vitro* growth culture. Complexes with cytoplasmic degenerative oocytes, detachment of granulosa cells or cumulus cells from the zona pellucida, and collapsed complexes were classified as disintegrated complexes; all others were regarded as complexes maintaining their integrity. Antrum formation by OCGCs and OCCs was observed every day by identifying visible spaces surrounded by granulosa cells or cumulus cells. The number of complexes (n) used in each group is shown in each graph (A and B). * Values are significantly different from those of OCGCs ($P < 0.05$).

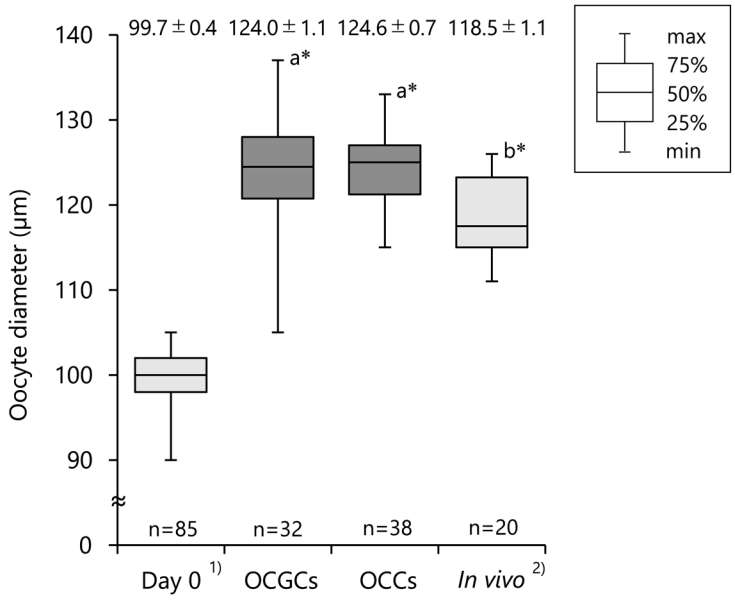

Fig. 3. Diameters of bovine oocytes in oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) and oocyte-cumulus cell complexes (OCCs) after *in vitro* growth culture. The number of oocytes (n) used in each group is shown at the bottom of each box. The mean (± SEM) diameters of oocytes are shown at the top of each box. [1)] The diameters of oocytes collected from early antral follicles (0.5–0.7 mm) before culture. [2)] The diameters of fully grown oocytes collected from antral follicles (4–6 mm). * Values are significantly different from those of oocytes before culture (Day 0) ($P < 0.05$). [a, b] Different alphabets denote significantly different values ($P < 0.05$).

Table 1. Meiotic stages of *in vitro*-grown bovine oocytes in oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) and oocyte-cumulus cell complexes (OCCs)

In vitro growth (day)	Types of complexes [1)]	Number of oocytes used	Number (%) of oocytes at each stage [4)]								
			FC	SC	GV	ED	LD	MI	AI-TI	MII	DG
0 [2)]	–	27	4 (15)	23 (85)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	OCGCs	27	0 (0)	0 (0)	23 (85)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (15)
	OCCs	32	0 (0)	0 (0)	27 (84)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (16)
In vivo [3)]	–	20	0 (0)	0 (0)	20 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[1)] OCGCs and OCCs collected from early antral follicles (0.5–0.7 mm) were cultured for 14 days. [2)] Oocytes were collected from early antral follicles before culture. [3)] Fully grown oocytes collected from antral follicles (4–6 mm). [4)] FC, filamentous chromatin; SC, stringy chromatin; GV, germinal vesicle I–IV; ED, early diakinesis; LD, late diakinesis; MI, metaphase I; AI-TI, anaphase I and telophase I; MII, metaphase II; DG, degeneration.

Table 2. Meiotic competence of *in vitro*-grown bovine oocytes in oocyte-cumulus cell-mural granulosa cell complexes (OCGCs) and oocyte-cumulus cell complexes (OCCs) after *in vitro* maturation

In vitro growth (day)	Types of complexes [1)]	Number of oocytes used	Number (%) of oocytes at each stage [4)]								
			FC	SC	GV	ED	LD	MI	AI-TI	MII	DG
0 [2)]	–	28	4 (14)	21 (75)	3 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	OCGCs	31	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (16)	1 (3)	25 (81)	0 (0)
	OCCs	32	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (6)	1 (3)	28 (88)	1 (3)
In vivo [3)]	–	35	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9 (26)	0 (0)	26 (74)	0 (0)

[1)] OCGCs and OCCs collected from early antral follicles (0.5–0.7 mm) were subjected to *in vitro* maturation culture after 14 days of *in vitro* growth culture. [2)–4)] See the footnotes in Table 1.

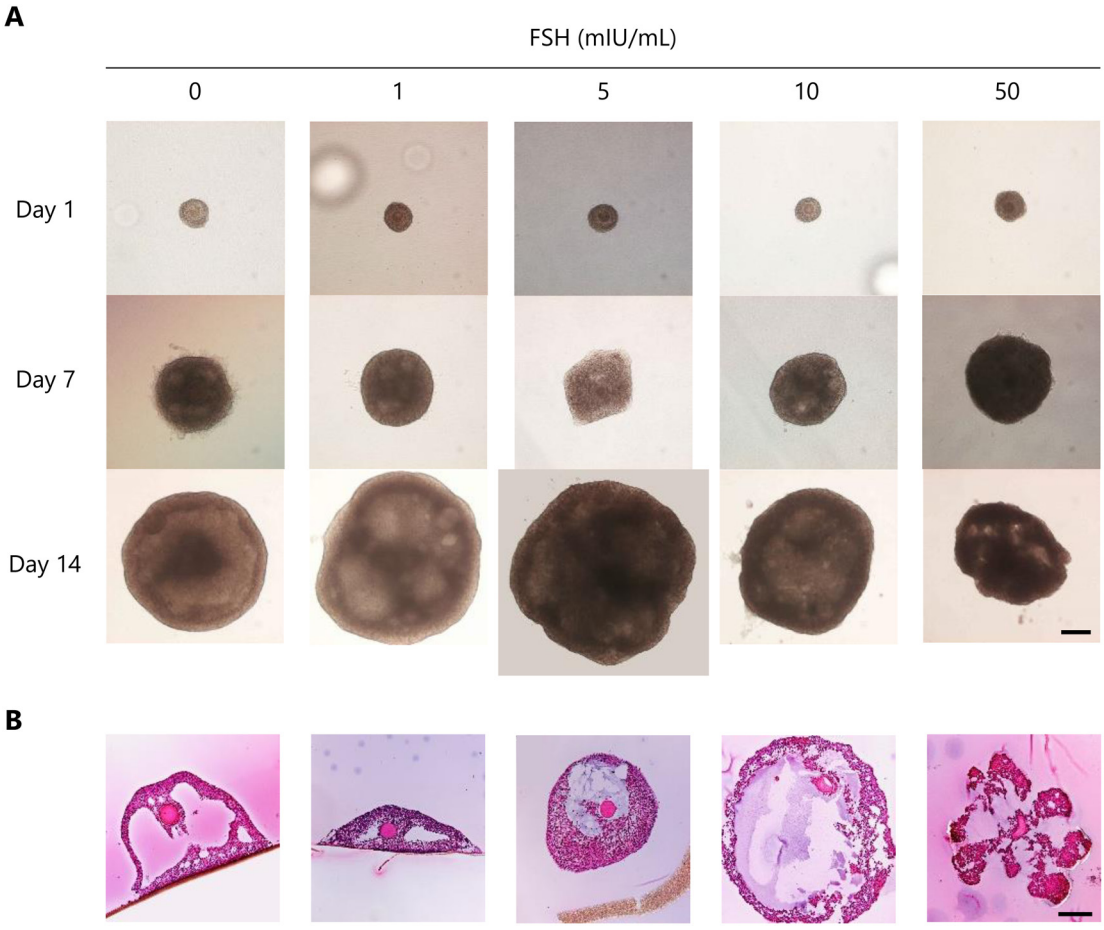

Fig. 4. Typical morphologies of bovine oocyte-cumulus cell complexes (OCCs) during *in vitro* growth culture with follicle stimulating hormone (FSH) (A) and representative images of histological sections of OCCs after 14 days of culture (B). OCCs were cultured for 14 days with varying concentrations of FSH ranging between 1–50 mIU/ml. The pictures represent OCCs on Days 1, 7 and 14 (A). After OCCs attached onto a membrane sheet, they increased in size, and some of the OCCs formed antrum-like structures (A). After 14 days of culture, antrum-like structures were observed inside the complexes (B). When OCCs were cultured with 10 or 50 mIU/ml FSH, some of the antrum-like structures collapsed before the end of the culture period. OCCs cultured with higher concentrations of FSH included degenerated oocytes. The scale bars represent 200 µm.

were significantly increased in all treatment groups compared to that of the oocytes before culture (Fig. 6). Oocytes cultured without FSH and with 1 mIU/ml FSH for 14 days grew to the same size as *in vivo* fully grown oocytes collected from 4–6 mm sized antral follicles (122.2 ± 0.9, 118.8 ± 1.3 and 123.0 ± 0.6 µm, respectively).

When the OCCs were cultured with 1 mIU/ml FSH, 86% of the oocytes were at the GV stage, which was equivalent to *in vivo* fully grown oocytes (Table 3). In contrast, when the OCCs were cultured with 5–50 mIU/ml FSH, the percentage of the oocytes in the GV stage was low, and the rate of degenerated oocytes was increased. Some of the oocytes in OCCs cultured with 5 mIU/ml FSH degenerated even though the antrum-like structures were maintained until the end of growth culture.

In the subsequent maturation culture experiments, 86% of the oocytes in OCCs cultured with 1 mIU/ml FSH reached MII stage (Table 4). In contrast, the maturation rates of oocytes cultured with 5–50 mIU/ml FSH were low, and the rates of oocytes in LD, MI, and AI-TI stages and degenerated oocytes were increased.

Discussion

The results of the first experiment indicated that bovine oocytes in OCCs grow and acquire meiotic competence similar to OCGCs. In other words, cumulus cells alone support oocyte growth *in vitro*. Oocytes and cumulus cells communicate bilaterally, thereby promoting oocyte growth. Previous studies have suggested that this communication is mediated by paracrine signals and gap junctions existing at the end of transzonal projections (TZPs) as well as extracellular vesicles [18–20]. Thus, it is assumed that the connections between oocytes and cumulus cells are maintained during *in vitro* growth culture.

After 14 days of *in vitro* growth culture, more than 80% of the oocytes in OCCs and OCGCs were at the GV stage. The nuclear stage of the oocytes reaches the GV stage from the FC and SC stages during the process of oocyte growth [15]. Therefore, oocytes

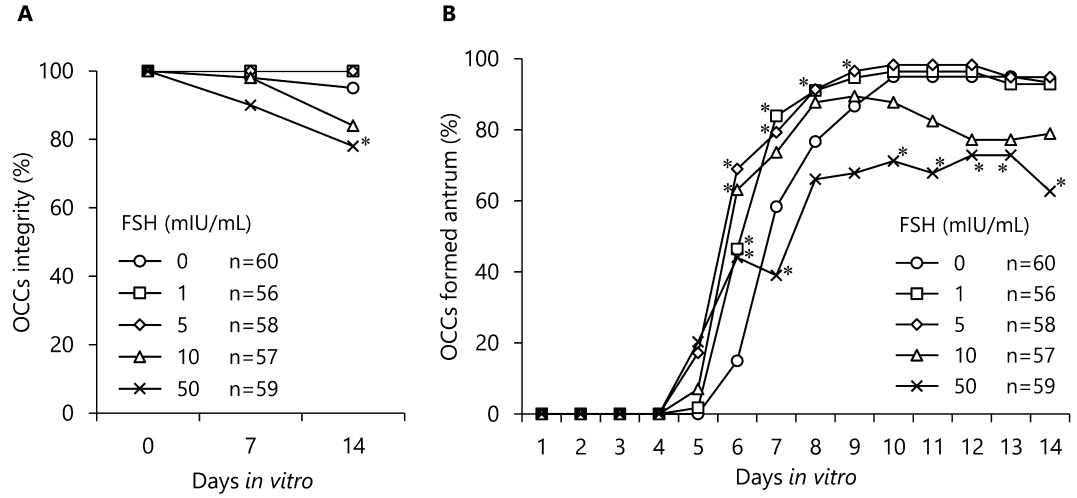

Fig. 5. Integrity of bovine oocyte-cumulus cell complexes (OCCs) (A) and formation of antrum-like structures by OCCs (B) during *in vitro* growth culture with follicle stimulating hormone (FSH). Complexes with cytoplasmic degenerative oocytes, detachment of cumulus cells from the zona pellucida, and collapsed complexes were classified as disintegrated complexes; all others were regarded as complexes maintaining their integrity. Antrum formation by OCCs was observed every day by identifying visible spaces surrounded by cumulus cells. The number of complexes (n) used in each group is shown in each graph (A and B). * Values are significantly different from those of OCCs cultured without FSH (0 mIU/ml) ($P < 0.05$).

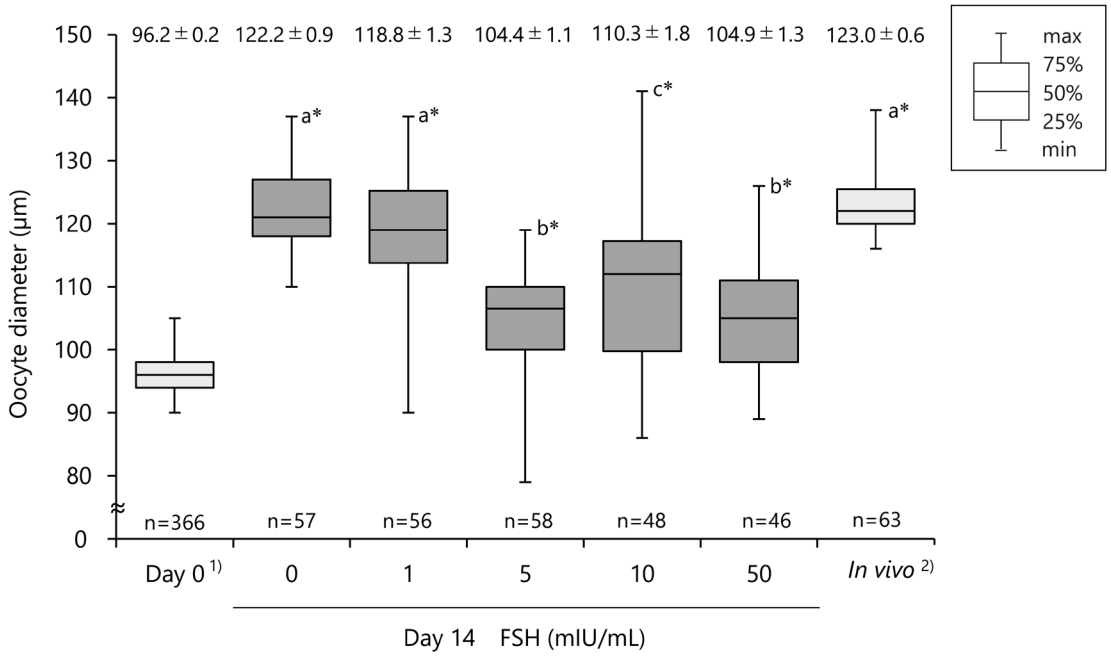

Fig. 6. Diameters of bovine oocytes in oocyte-cumulus cell complexes (OCCs) after *in vitro* growth culture with follicle stimulating hormone (FSH). The number of oocytes (n) used in each group is shown at the bottom of each box. The mean (± SEM) diameters of oocytes are shown at the top of each box. 1) The diameter of oocytes collected from early antral follicles (0.5–0.7 mm) before culture. 2) The diameter of fully grown oocytes collected from antral follicles (4–6 mm). * Values are significantly different from those of oocytes before culture (Day 0) ($P < 0.05$). a–c Different alphabets denote significantly different values ($P < 0.05$).

cultured in OCCs also progressed to the nuclear stage adequately. In addition, the mean diameters of oocytes in OCCs and OCGCs increased from approximately 100 to 120 μm, which is equivalent to the diameters of *in vivo* fully grown oocytes. When these grown oocytes were subjected to *in vitro* maturation, more than 80% of them matured to MII. It has been demonstrated that bovine oocytes with diameters greater than 110 μm acquired meiotic competence [21]. These results suggest that bovine oocytes in OCCs grow and

Table 3. Meiotic stages of *in vitro*-grown bovine oocytes in oocyte-cumulus cell complexes (OCCs) with follicle stimulating hormone (FSH)

In vitro growth (day)	FSH (mIU/ml) [1)]	Number of oocytes used	Number (%) of oocytes at each stage [4)]								
			FC	SC	GV	ED	LD	MI	AI-TI	MII	DG
0 [2)]	–	48	47 (98)	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	0	29	2 (7)	2 (7)	23 (79) [a]	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (7)
	1	28	1 (4)	1 (4)	24 (86) [ac]	0 (0)	2 (7)	0 (0)	0 (0)	0 (0)	0 (0)
	5	28	4 (14)	1 (4)	9 (32) [b]	6 (21)	4 (14)	0 (0)	0 (0)	0 (0)	4 (14)
	10	24	2 (8)	2 (8)	10 (42) [b]	0 (0)	1 (4)	2 (8)	0 (0)	0 (0)	7 (29)
	50	24	1 (4)	1 (4)	4 (17) [b]	2 (8)	2 (8)	0 (0)	0 (0)	0 (0)	14 (58)
In vivo [3)]	–	33	0 (0)	0 (0)	32 (97) [c]	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)

[1)] OCCs collected from early antral follicles (0.5–0.7 mm) were cultured with varying concentrations of FSH (1–50 mIU/ml) for 14 days. [2) – 4)] See the footnotes in Table 1. [a–c] Different alphabets denote significantly different values ($P < 0.05$).

Table 4. Meiotic competence of *in vitro*-grown bovine oocytes in oocyte-cumulus cell complexes (OCCs) with follicle stimulating hormone (FSH) after *in vitro* maturation

In vitro growth (day)	FSH (mIU/ml) [1)]	Number of oocytes used	Number (%) of oocytes at each stage [4)]								
			FC	SC	GV	ED	LD	MI	AI-TI	MII	DG
0 [2)]	–	28	25 (89)	0 (0)	3 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
14	0	28	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (14)	0 (0)	24 (86) [a]	0 (0)
	1	28	0 (0)	0 (0)	0 (0)	1 (3)	2 (7)	1 (3)	0 (0)	24 (86) [a]	0 (0)
	5	30	0 (0)	1 (3)	0 (0)	0 (0)	6 (20)	9 (30)	0 (0)	2 (7) [b]	12 (40)
	10	24	0 (0)	0 (0)	1 (4)	0 (0)	1 (4)	11 (46)	2 (8)	3 (13) [b]	6 (25)
	50	22	0 (0)	0 (0)	0 (0)	0 (0)	2 (9)	8 (36)	0 (0)	3 (14) [b]	9 (41)
In vivo [3)]	–	30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	30 (100)	0 (0)

[1)] OCCs collected from early antral follicles (0.5–0.7 mm) were subjected to *in vitro* maturation culture after 14 days of *in vitro* growth culture with 1–50 mIU/ml FSH. [2) – 4)] See the footnotes in Table 1. [a, b] Different alphabets denote significantly different values ($P < 0.05$).

acquire meiotic competence as observed in our culture system.

During culture experiments, while OCCs formed antrum-like structures similar to OCGCs, the formation by OCCs occurred later as compared to OCGCs. This delay seems to be attributable to the fact that OCCs are smaller than OCGCs before the start of culture. It is worth noting that cumulus cells alone were able to form antrum-like structures, although a small number of mural granulosa cells could have been mixed in the OCCs. Antrum formation *in vivo* is accompanied by the differentiation of granulosa cells in preantral follicles to mural granulosa cells and cumulus cells [22]. In the present study, cumulus cells in OCCs were assumed to differentiate into mural granulosa cell-like cells for the formation of antrum-like structures. It is not clear whether antrum and antrum-like structures *in vitro* are the same structures. However, the formation of an antrum-like structure promotes oocyte growth and acquisition of meiotic competence [2, 4]. This structure has been thought to prevent the diffusion of growth factors and create a microenvironment for supporting oocyte growth [6, 12]. Hence, delayed formation of antrum is a defect of OCCs. To address this issue, we next examined the effect of FSH on the development of OCCs.

In the second experiment, OCCs cultured with FSH formed antrum-like structures one day earlier than those cultured without FSH. It has been suggested that FSH stimulates the proliferation of bovine granulosa cells [10] and it is involved in the development of secondary follicles to antral follicles [23, 24]. When FSH was added to the medium, granulosa cells in the preantral follicles proliferated [7], the diameter of the follicles increased [8] and antrum-like structures were formed [25, 26]. The most appropriate concentration of FSH was 1 mIU/ml in our culture system since all OCCs maintained the structures and oocytes grew fully and acquired meiotic competence at a higher rate at this concentration. In contrast, when OCCs were cultured with 10 and 50 mIU/ml FSH, some of the antrum-like structures collapsed before the end of the culture period and all the oocytes degenerated in the collapsed structures. After growth culture, some OCCs that included degenerated oocytes were sticky. Cumulus cells seemingly degenerated due to their exposure to high concentrations of FSH. Since cumulus cells provide nutrients and physiologically active substances to oocytes, it is considered that deterioration of cumulus cells retards oocyte growth and causes degeneration of the oocytes. Recently, Alam *et al*. [17] suggested that growth differentiation factor 9 (GDF9) derived from oocytes is involved in the formation of antrum-like structures by bovine oocyte-granulosa cell complexes. Since the degenerated oocytes do not provide GDF9 to cumulus cells, antrum-like structures were not maintained and collapsed in the medium containing a high concentration of FSH. As all oocytes degenerated in the collapsed structures and, some of the oocytes degenerated even in those that maintained structural integrity when cultured with 5 mIU/ml FSH,

we assumed that degeneration of oocytes preceded cumulus cell degeneration in OCCs. In the present study, it cannot be denied that FSH in FBS affected the formation of antrum-like structures. However, supplementation with FSH at all examined concentrations promoted antrum formation as observed in this study. In addition, bovine oocytes did not grow and degenerate in the absence of FBS in our culture conditions, even when FSH was added to FBS-free medium.

In conclusion, bovine oocytes in OCCs grow and acquire meiotic competence similar to OCGCs, and 1 mIU/ml FSH supports the development of OCCs and oocyte growth in our experimental system.

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