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—Original Article—

Bovine Oocytes in Secondary Follicles Grow in Medium Containing Bovine Plasma after Vitrification

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Abstract. There has been no culture system that supports the growth of bovine oocytes for more than 2 weeks. In the present study, bovine secondary follicles were cultured for 4 weeks, and the effects of supplemented protein components and FSH in the culture medium on the growth of the oocytes were examined. The effect of vitrification of secondary follicles on the subsequent oocyte growth was also examined. Secondary follicles (150 to 200 μm in diameter) containing growing oocytes (approximately 60 μm in diameter) were dissected from ovaries and cultured in a medium supplemented with FSH (0, 25 or 50 ng/ml) and one of the following four kinds of protein components: bovine serum albumin (BSA), bovine plasma (BPL), fetal calf serum (FCS) and bovine follicular fluid (BFF). In BSA- and BPL-supplemented media with 0 or 25 ng/ml FSH, more than 50% of follicles showed no degenerative signs during culture, and oocytes significantly increased in size after 4 weeks ($P<0.05$). Higher percentages of granulosa cell-enclosed oocytes were recovered from the follicles cultured in BPL-supplemented media with 0 and 25 ng/ml FSH, and the oocytes grew to 90 μm or more in diameter. In FCS- and BFF-supplemented media, FSH increased the numbers of degenerating follicles. Next, vitrified-warmed secondary follicles were cultured in BPL-supplemented medium. One third of the follicles showed no degenerative signs, and the oocytes increased in diameter to 88.8 ± 3.1 μm after 4 weeks of culture. These results suggest that a BPL-supplemented medium supports oocyte growth in bovine secondary follicles for 4 weeks, even after vitrification and warming of the follicles.

Key words: Bovine, Oocyte growth, Plasma, Secondary follicle, Vitrification

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In the mouse, non-growing oocytes in primordial follicles grow to a final size and acquire maturation, fertilization and development abilities in culture [1]. For livestock production, *in vitro* growth culture of ovarian small oocytes is a promising method of providing mature oocytes. However, there has been a little success in culture of non-growing and growing oocytes in domestic species because their follicles develop to a larger size and thus take longer than those in mice.

In the cow, it has been reported that growing oocytes (90–99 μm in diameter) collected from early antral follicles grow to a final size of 120 μm after 14-days culture [2, 3]. Smaller oocytes from follicles at an earlier stage, such as secondary and primary follicles, are thought to require more time in culture. Katska *et al*. [4] and Itoh *et al*. [5] cultured bovine preantral follicles, and the longest periods of culture were 23 days (no data concerning oocyte growth) and 13 days (oocyte diameter after culture: 72.2 ± 1.1 μm), respectively. There has been no culture system that supports the viability and growth of oocytes from bovine secondary follicles for more than 2 weeks.

Serum-supplemented medium has often been used for growth culture of bovine oocytes [2–4, 6]. In some experiments, bovine serum albumin (BSA)-supplemented medium has been used for culture [5, 7]. Besides these two media, we employed plasma- and follicular fluid-supplemented media in the present study. Plasma contains a lower concentration of platelet-derived growth factor, which stimulates cell proliferation of connective tissues, than serum [8]. The maturation medium is often supplemented with follicular fluid to support oocyte maturation [9]. Follicular fluid contains factors secreted by granulosa and theca cells, such as steroid hormones and growth factors (kit ligand and activin A) [10], which are thought to regulate follicle development, oocyte growth and maturation.

In addition to oocyte growth culture, cryopreservation of ovarian follicles is a promising method of animal production. Embryos have been cryopreserved by the vitrification method, which has been applied to preservation of oocytes and ovarian tissues. In a previous study in the mouse, vitrified-warmed preantral follicles were cultured, and mature oocytes were obtained [11]. Furthermore, we reported recently that vitrified-warmed bovine secondary follicles developed along with oocyte growth in grafts transplanted into immunodeficient mice [12].

In the present study, bovine secondary follicles were cultured for 4 weeks in a medium supplemented with FSH and one of four kinds of protein components, BSA, bovine plasma (BPL), fetal calf serum (FCS), and bovine follicular fluid (BFF), and the oocyte growth was examined. Furthermore, the effect of vitrification of secondary follicles on the subsequent oocyte growth was examined.

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Materials and Methods

Collection of bovine secondary follicles

Ovaries were obtained from purebred and crossbred Japanese Black cows slaughtered at a local abattoir and were washed in phosphate-buffered saline 3 times. Secondary follicles were dissected from the ovarian cortex in a laboratory at the abattoir and transported to a laboratory at Kobe University within 2 h while immersed in TCM199 (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (wt/vol) polyvinyl alcohol (Sigma, St. Louis, MO, USA), 10.1 mM $NaHCO_3$, 0.14 mM kanamycin sulphate (Sigma) and 25 mM HEPES (HEPES199) at 20 C.

Collection of secondary follicles was based on the method described by Senbon *et al*. [13]. Briefly, ovarian cortex tissues measuring approximately 0.5 × 0.5 × 0.5 mm and containing 1 secondary follicle of 150 to 200 μm in diameter were dissected using blades while immersed in HEPES199. The connective tissues surrounding the follicles were stripped off using forceps, and healthy secondary follicles that had a basement membrane with an intact contour, an oocyte localizing at the center of the follicles and no signs of degeneration of granulosa cells were selected under a dissection microscope. The diameters of the follicles (inside the basement membrane) were measured to the nearest 1 μm by an ocular micrometer (Olympus, Tokyo, Japan).

Before culture, some secondary follicles were torn with a needle (25G, Terumo, Tokyo, Japan) and fine forceps, and oocyte-granulosa cell complexes were collected. Oocytes were denuded by a small-bore pipette, transferred individually into 15 μl droplets of the culture medium covered with mineral oil (Nacalai Tesque, Kyoto, Japan, HR3-411), and their diameters (excluding the zona pellucida) were measured. These oocytes were mounted on slides, fixed in acetic ethanol (1:3), stained with 1% (wt/vol) aceto-orcein and examined for their nuclear morphology under a differential interference microscope. The nuclear stage was determined on the basis of changes in the configuration of chromatin and the nuclear envelope [14].

Culture of secondary follicles

After washing twice in HEPES199 and then twice in a basic culture medium, secondary follicles were embedded in collagen gels based on the methods described by Hirao *et al*. [15]. The collagen mixture consisted of 0.3% (wt/vol) acid collagen solution (Cellmatrix Type I; Nitta Gelatin, Osaka, Japan), 10 times-concentrated α-Minimum Essential Medium (αMEM; Invitrogen, Tokyo, Japan) and 0.05 N sodium hydroxide solution containing 0.26 M $NaHCO_3$ and 0.2 M HEPES at a ratio of 8:1:1. The 800 μl of mixture was placed on the bottom of the culture dish (No. 3001, BD Falcon, Franklin Lakes, NJ, USA) and a group of 8 to 12 secondary follicles were transferred into the mixture with a small volume of the medium. The gels were put in an incubator at 38.5 C for 20 min. After gelatinization, another 300 μl of collagen mixture was poured over the gel and gelatinized again to sandwich the follicles. After that, 4 ml of culture medium was poured on the gel, and the follicles were cultured at 38.5 C in a humidified atmosphere of 5% CO_2 and 95% air for 4 weeks. On every fourth day, half of the volume of the culture medium was exchanged for fresh medium.

The basic culture medium was αMEM containing 0.9 mM sodium pyruvate, 50 μM β-mercaptoethanol (Nacalai Tesque) and 0.14 mM kanamycin sulfate. The culture medium was supplemented with 3 mg/ml BSA (Wako Pure Chemical Industries, Osaka, Japan), 5% (vol/vol) BPL (Nippon Biotest Laboratories, Tokyo, Japan), 5% (vol/vol) FCS (Dainippon Pharmaceutical, Osaka, Japan) or 5% (vol/vol) BFF and FSH (Porcine, Batch No, 24021905; Biogenesis, Poole, UK) at a concentration of 0, 25 or 50 ng/ml. BFF was aspirated from antral follicles 2 to 7 mm in diameter. BPL, FCS and BFF were heat-inactivated at 56 C for 30 min. FCS was then centrifuged at 1,500 × *g* for 20 min, BPL and BFF were centrifuged at 9,000 × *g* for 20 min three times and the supernatants were collected. BPL, FCS and BFF were stored at –20 C before use.

Examination of follicle development and oocyte growth

After 1, 2, 3 and 4 weeks, follicle morphology was observed, and the diameters of follicles were measured. Follicles showing degenerative signs, such as breakdown of the follicle contour and degeneration of oocyte and/or granulosa cells, were classified as degenerative follicles and excluded from further examination. After 4 weeks, the gels and follicles were torn with a blade and fine forceps, and the oocyte-granulosa cell complexes were recovered from the follicles. When the oocyte-granulosa cell complexes were gently sucked in and out by a 200- to 300-μm wide-bore pipette, some of the oocytes became denuded. These oocytes were classified as 'denuded oocytes', while the oocytes enclosed by granulosa cells were classified as 'granulosa cell-enclosed oocytes'. Oocytes showing an oval shape and those having degenerated cytoplasm were considered abnormal oocytes and excluded from further examination. All of the oocytes were then denuded completely by a small-bore pipette and transferred into droplets of medium, and their diameters were measured. They were then fixed, stained and examined for nuclear morphology in the same manner as described above.

Culture of secondary follicles after vitrification

Secondary follicles were cultured in BPL-supplemented media after vitrification and warming. The vitrification method was based on the method of Kuwayama and Kato [16]. The basic solution (M-199) was TCM199 containing 10.5 mM HEPES (H-7523, Sigma), 9.5 mM Na-HEPES (H-8651, Sigma), 4.2 mM $NaHCO_3$ and 0.09 mM kanamycin sulphate. The equilibration solution was M-199 containing 7.5% (vol/vol) ethylene glycol (EG; Sigma), 7.5% (vol/vol) dimethyl sulfoxide (DMSO; Sigma) and 20% (vol/vol) FCS. The vitrification solution consisted of 15% EG, 15% DMSO, 20% FCS and 0.5 M sucrose in M-199. The warming solution was 20% FCS and 1 M sucrose in M-199, and the dilution solution was 20% FCS and 0.5 M sucrose in M-199. The washing solution was 20% FCS in M-199.

Secondary follicles collected from ovaries were randomly allocated into four groups. The first group of follicles were put into equilibration solution in a culture dish (No. 1008, BD Falcon) at room temperature for 15 min and then put into vitrification solution in a plastic dish (SH90-15, IWAKI, Japan) at room temperature for 1 min. Three to four follicles were put on a Cryotop (Kitazato

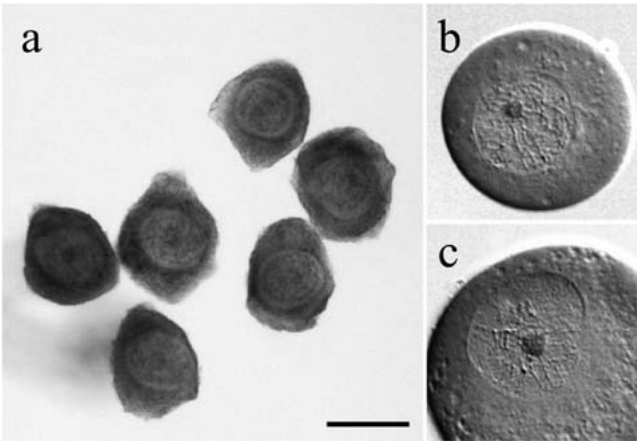

Fig. 1. Bovine secondary follicles collected from ovarian cortical tissues (a), and the nuclear morphology of the oocytes before (b) and after (c) 4 weeks of culture. The scale bar in Fig. 1a represents 200 μm.

BioPharma, Shizuoka, Japan) and plunged into LN_2 (–196 C) for storage. For warming, the vitrified follicles were directly put into warming solutions at 38.5 C. They were then immediately transferred into the dilution solution at room temperature for 5 min and transferred to the washing solution at room temperature. After washing twice in HEPES199 and then twice in a basic culture medium, the follicles were cultured in BPL-supplemented media for 4 weeks as described above. The second group of follicles was treated with vitrification and warming solutions, but without being plunged into LN_2, and then cultured in BPL-supplemented media. The third group consisted of fresh secondary follicles, which were directly cultured in BPL-supplemented media. From the fourth group of follicles, oocyte-granulosa cell complexes were collected. Oocytes were denuded, and their diameters were measured. The nuclear morphology of the oocytes was also examined in the same manner as described above.

After 1, 2, 3 and 4 weeks, follicle morphology was observed, and the diameters were measured as described above. After 4 weeks, follicles were opened, and oocyte-granulosa cell complexes were recovered from the follicles. Oocytes were denuded, and the diameters were measured. They were then fixed, stained and examined for the nuclear morphology in the same manner as described above.

Statistical analysis

The experiments were repeated five times. Statistical differences in the mean diameters of the follicles and oocytes were analyzed by the Student's *t*-test. Other values were analyzed by the χ^2 test with Yates' correction for continuity. A probability of less than 0.05 was considered significant.

Results

Follicle development and oocyte growth

Bovine secondary follicles measuring 150 to 200 μm in diameter were isolated from the surfaces of the ovaries (Fig. 1a) and cultured in collagen gels for 4 weeks. In the BSA-supplemented media, the morphology of the follicles did not change during the culture period regardless of the FSH concentration (Figs. 2a1–4, 2e1–4 and 2i1–4). More than 50% of the follicles showed no degenerative signs (Figs. 3a–c), and the mean diameters of the follicles did not increase after 4 weeks (Figs. 3d–f).

In the BPL-supplemented media, secondary follicles formed antra after 3 weeks of culture (Figs. 2b1–4, 2f1–4 and 2j1–4). More than 50% of the follicles showed no degenerative signs in the 0- and 25-ng/mL FSH-supplemented media (Figs. 3a and b), and the mean diameters of the follicles increased during culture (Figs. 3d and e). However, 64% of the follicles degenerated in the 50-ng/ml FSH-supplemented medium (Fig. 3c).

In the FCS-supplemented media, secondary follicles formed antra after 2 weeks of culture and increased in diameter (Figs. 2c1–4, 2g1–4 and 2k1–4). However, after 3 weeks, especially in the media supplemented with FSH, more than 60% of the follicles were degenerated (Figs. 3b and c).

In the BFF-supplemented media, secondary follicles formed antra after 2 weeks of culture (Figs. 2d1–4, 2h1–4 and 2l1–4), and the follicles expanded remarkably in the medium with 25 ng/ml FSH (Figs. 3d–f). However, the follicles degenerated in the media supplemented with FSH similarly to those in the FCS-supplemented media (Figs. 3a–c). In the FCS- and BPL-supplemented media, follicle cells outgrew and proliferated remarkably in the gels.

After culture, oocytes were recovered from the follicles. All oocytes obtained from the degenerated follicles were classified as degenerated. The mean diameters of the oocytes were significantly increased compared with those before culture (60.9 ± 0.7 μm, Table 1). In the BSA-supplemented media with 25 ng/ml FSH and BPL-supplemented media with 0 and 25 ng/ml FSH, about 50% of the recovered oocytes showed normal morphology with a spherical shape and homogeneous cytoplasm (surviving oocytes in Table 1). In the FCS- and BFF-supplemented media, the numbers of surviving oocytes were less than 20%. From the follicles cultured in the BPL-supplemented media with 0 and 25 ng/ml FSH, higher percentages of granulosa cell-enclosed oocytes were recovered compared with the follicles cultured in the BSA-supplemented media with 0 and 25 ng/ml FSH.

The oocytes were stained, and their nuclear morphology was examined. All of the oocytes collected from the secondary follicles before culture were at the germinal vesicle (GV) stage and showed a filamentous chromatin configuration (Fig. 1b). After culture, all of the oocytes stayed at the GV stage with filamentous chromatin (Fig. 1c).

Follicle development and oocyte growth after vitrification

None of the bovine secondary follicles showed degenerative signs after vitrification and warming. The vitrified-warmed secondary follicles were embedded in collagen gels and cultured in the BPL-supplemented medium for 4 weeks. During culture, some of the follicles formed antra after 2 weeks of culture and increased in diameter (Figs. 4a1–4); however, about half of the follicles were degenerated after 1 week, and the follicle structures were subse-

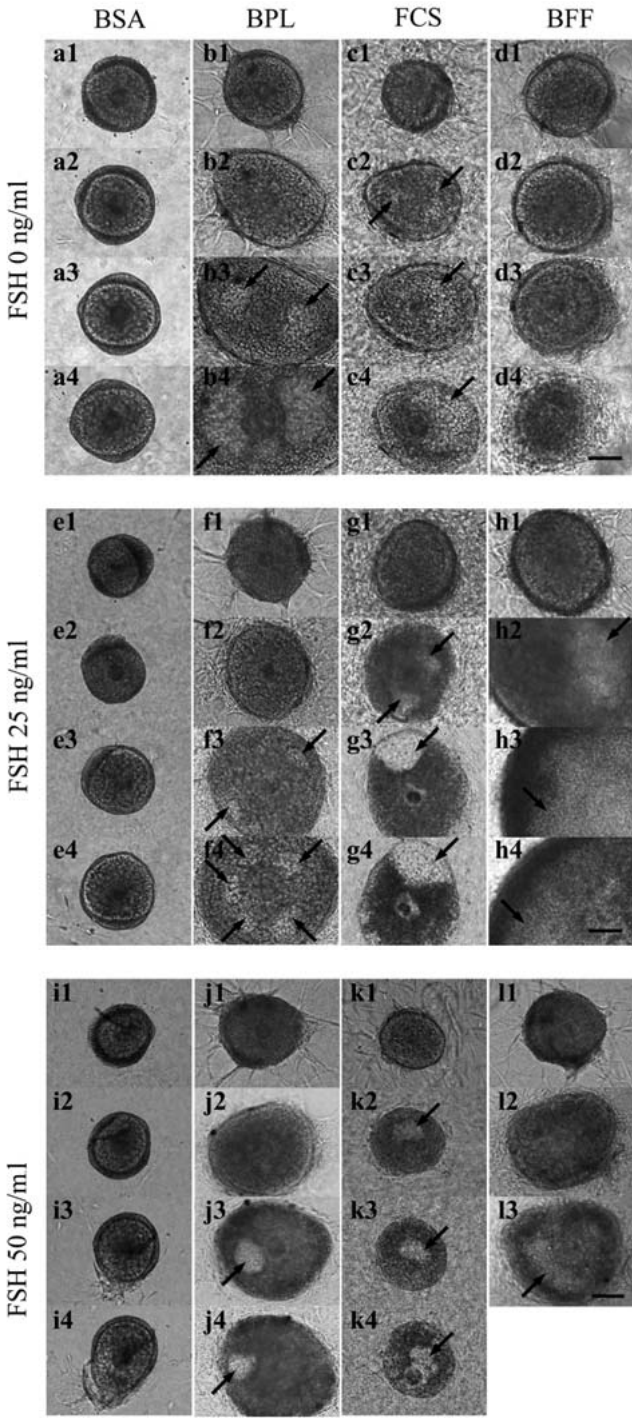

Fig. 2. Morphology of bovine secondary follicles cultured in the media supplemented with bovine serum albumin (BSA: a, e and i), bovine plasma (BPL: b, f and j), fetal calf serum (FCS: c, g and k) and bovine follicular fluid (BFF: d, h and l) without (a–d) or with 25 (e–h) or 50 (i–l) ng/ml FSH for 4 weeks. The follicles cultured with FCS and BFF degenerated during culture, and all of the follicles cultured in the BFF-supplemented media with 50 ng/ml FSH degenerated after 4 weeks. The scale bars represent 100 μm. Antrum-like structures are indicated by arrows.

quently broken (Figs. 4b1–3 and 5a). About 70% of the fresh secondary follicles and non-vitrified secondary follicles that were treated with vitrification solutions without being plunged into LN_2, showed no degenerative signs after 4 weeks (Fig. 5a). The mean diameter of the vitrified-warmed follicles increased to 322.4 ± 19.6 μm after 4 weeks (Fig. 5b). The diameter was not significantly different from the value for the fresh follicles (346.7 ± 14.0 μm).

All of the oocytes recovered from the non-degenerative follicles were enclosed by granulosa cells (Table 2). The mean diameters of the oocytes increased significantly in all groups, including the vitrified-warmed follicles, compared with the values before culture. In addition, no significant difference was found among the values in the three groups. All of the oocytes were at the GV stage with filamentous chromatin.

Discussion

In the present study, more than 50% of the bovine secondary follicles showed no degenerative signs by the end of 4 weeks of culture in BPL- or BSA-supplemented media, and most of the oocytes recovered from these follicles were morphologically normal and had a significantly increased diameter. The percentages of degenerated follicles were higher in other groups than in the BSA- and BPL-supplemented groups. Culture in BSA-supplemented media prevented the excessive proliferation of follicle cells and retained the integrity of the follicles. In the BPL-supplemented media, the integrity of the follicles was maintained while they developed. However, follicle cells proliferated remarkably in the gel in the FCS- and BFF-supplemented media, and the follicles degenerated during culture. FCS contains platelet-derived growth factor, which seems to stimulate excessive follicle cell proliferation [17]. BFF contains serum components and steroid hormones [18, 19]. It is thought that the hormones stimulate follicle cell proliferation [20] and antrum expansion [21] and that this induced excessive follicle expansion in the gels. Similar to the FCS, the follicle development caused by BFF might induce an insufficient nutrient and oxygen supply inside the follicles.

In the BSA-supplemented media, formation of an antrum-like structure was not induced by FSH. Some components of serum or follicular fluid, such as IGF-I and EGF, may contribute to antrum-structure formation [22]. In the FCS-, BPL- and BFF-supplemented media, antrum-like structures were formed, and development of the structures was promoted by FSH. FSH receptors are expressed in bovine secondary follicles [23], and follicle development is regulated by FSH [24]. However, in this study, FSH in the FCS- and BFF-supplemented media induced degeneration of the follicles and oocytes after 4 weeks of culture. This result is consistent with a report showing that the viability of bovine oocytes in early antral follicles was decreased by supplementation with a high concentration of FSH (100 ng/ml) [25]. Under our culture conditions, supplementation of a high concentration of FSH to the medium was unfavorable for oocyte growth due to excessive cell proliferation and expansion of the follicles.

Gap junctions between oocytes and granulosa cells are necessary for oocyte growth [26]. Granulosa cells provide amino acids, metabolites and nucleotides to the oocyte via the junctions [27]. In

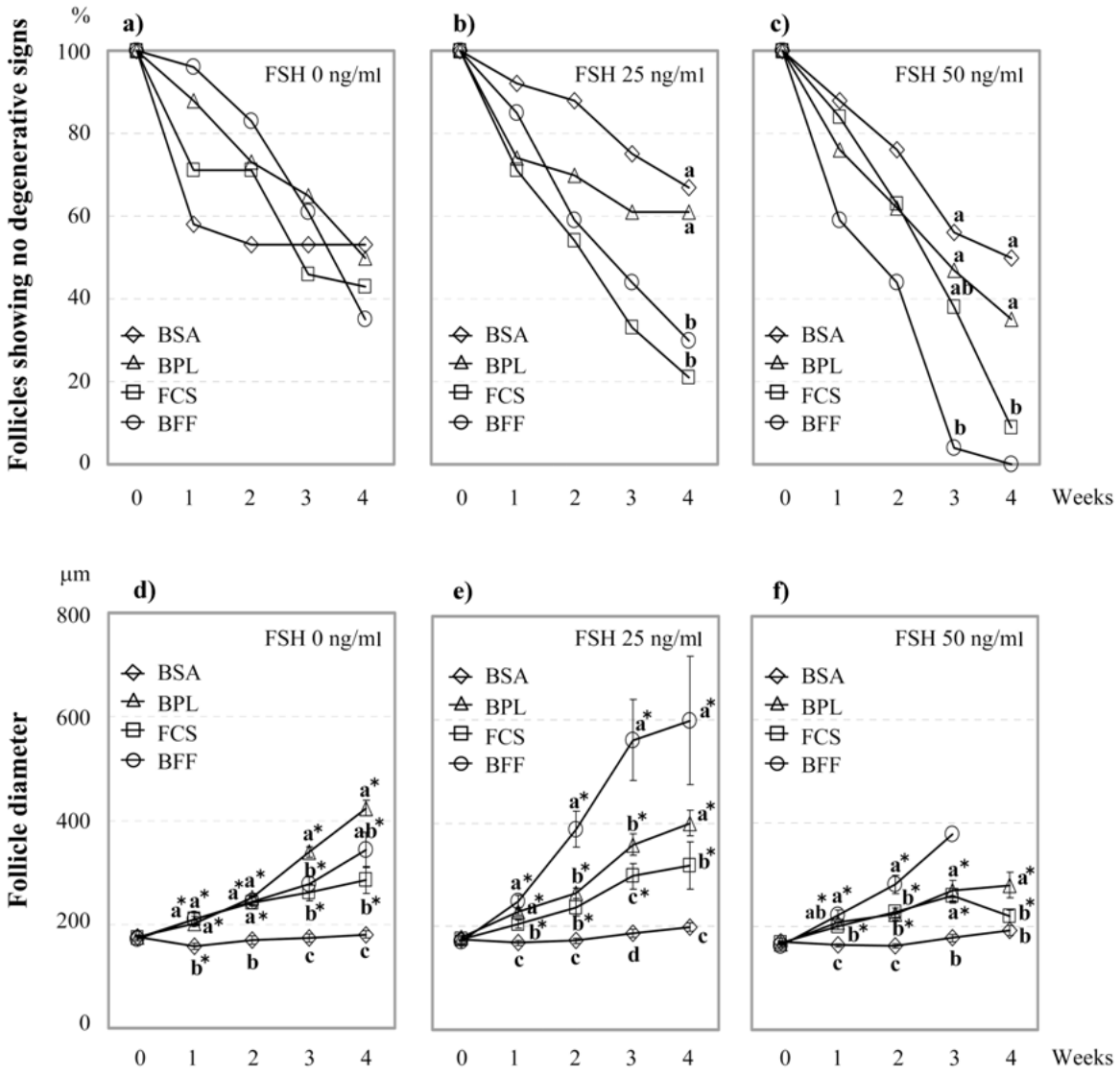

Fig. 3. Development of bovine secondary follicles cultured in the media supplemented with bovine serum albumin (BSA), bovine plasma (BPL), fetal calf serum (FCS) and bovine follicular fluid (BFF) with 0, 25 or 50 ng/ml FSH for 4 weeks. Figs. 3a–c show the percentage of follicles showing no degenerative signs, and Figs. 3d–f show the mean follicle diameters ± SEM. Values with different superscripts for the same culture period differ significantly ($P<0.05$). Asterisks indicate a significant difference from the value before culture ($P<0.05$).

the BPL-supplemented media with 0 and 25 ng/ml FSH, 35 and 48% of the oocytes were surrounded by granulosa cells after 4 weeks, respectively. BPL is therefore suggested to have a beneficial effect on the association between the oocyte and granulosa cells. On the other hand, in the BSA-supplemented media with 0 and 25 ng/ml FSH, lower percentages (11 and 25%, respectively) of oocytes were granulosa cell-enclosed, although the denuded oocytes survived and grew. It has been reported that bovine oocytes in early antral follicles grew in serum-free media for 2 weeks, although all the recovered oocytes were denuded [7]. The association between oocytes and granulosa cells seemed to be maintained during the culture period in the present study, although the junctions were loosened in the BSA-supplemented medium at the end of the culture period.

After culture, the oocytes grew to about 90 μm in diameter, although they showed the same filamentous chromatin configuration as the oocytes before culture. They were also not the size of fully-grown bovine oocytes (120–125 μm). Therefore, it is probable that they do not have maturational competence [25]. Although the oocytes around 60 μm in diameter in the secondary follicles grew to 90 μm after 4 weeks in the BPL-supplemented media in the present study, it is necessary to prolong the culture period and

Table 1. Oocyte growth in bovine secondary follicles cultured in the media supplemented with bovine serum albumin (BSA), bovine plasma (BPL), fetal calf serum (FCS) and bovine follicular fluid (BFF) for 4 weeks

Supplement	FSH (ng/ml)	No. of follicles cultured	No. (%) of surviving oocytes*	Mean diameter of oocytes (μm ± SEM)	No. (%) of oocytes	
					GC-enclosed**	Denuded
Before culture	–	–	23	60.9 ± 0.7 [a]	23 (100)	0 (0)
BSA	0	19	7 (37) [ab]	84.1 ± 3.9 [bc]	2 (11) [a]	5 (26) [ab]
	25	24	13 (54) [a]	74.7 ± 3.2 [bd]	6 (25) [a]	7 (29) [a]
	50	34	9 (26) [ab]	83.7 ± 3.0 [b]	7 (21) [ab]	2 (6) [b]
BPL	0	26	13 (50) [a]	97.2 ± 1.9 [c]	9 (35) [b]	4 (15) [ab]
	25	23	11 (48) [ab]	91.9 ± 2.5 [c]	11 (48) [b]	0 (0)
	50	34	7 (21) [b]	87.0 ± 2.0 [bc]	7 (21) [ab]	0 0)
FCS	0	28	5 (18) [b]	72.4 ± 3.0 [d]	3 (11) [a]	2 (7) [ab]
	25	24	2 (8) [b]	69.7 ± 1.5 [d]	2 (8) [a]	0 (0)
	50	32	0 (0)	—	0 (0)	0 (0)
BFF	0	23	4 (17) [ab]	100.0 ± 6.1 [c]	2 (9) [a]	2 (9) [ab]
	25	27	4 (15) [b]	80.8 ± 8.0 [b]	2 (7) [a]	2 (7) [ab]
	50	27	0 (0)	—	0 (0)	0 (0)

* Oocytes showing normal morphology with a spherical shape and homogeneous cytoplasm and without any degenerative signs after culture. ** GC: granulosa cells. [a–d] The values with different superscripts differ significantly ($P<0.05$).

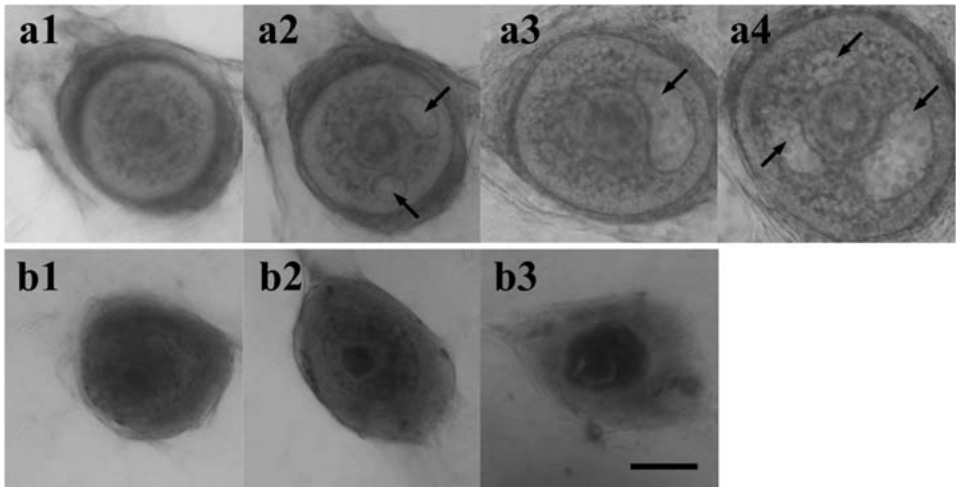

Fig. 4. Development and degeneration of vitrified and warmed bovine secondary follicles after 1 week (a1 and b1), 2 weeks (a2 and b2), 3 weeks (a3 and b3) and 4 weeks of culture (a4). The follicle in Figs. a1–4 formed an antrum-like structure after 2 weeks, and the diameter increased. Figs. b1–3 show the typical morphology of degenerated follicles. The follicle began to degenerate after 1 week, and the follicle structure then degraded. The scale bar represents 100 μm. Antrum-like structures are indicated by arrows.

improve the system for the oocytes to acquire maturational competence.

One third of the vitrified and warmed secondary follicles showed no degenerative signs after 4 weeks of culture in BPL-supplemented media. Although all of the follicles showed normal morphology just after vitrification and warming, half of the follicles degenerated after 1 week of culture. It has been reported that vitrification induces ultrastructural modifications in microvilli, mitochondria, vesicle formation and ooplasm of fully-grown bovine oocytes at the GV stage [28] and that embryos suffer considerable morphological and functional damage when cryopreserved by either freezing or vitrification [29]. In the present study, the vitrified-warmed follicles were thought to have such ultrastructural and functional damage, and the damage might have induced the degeneration of the follicles after culture. When oocytes and embryos are vitrified, the toxicity of the cryopro-

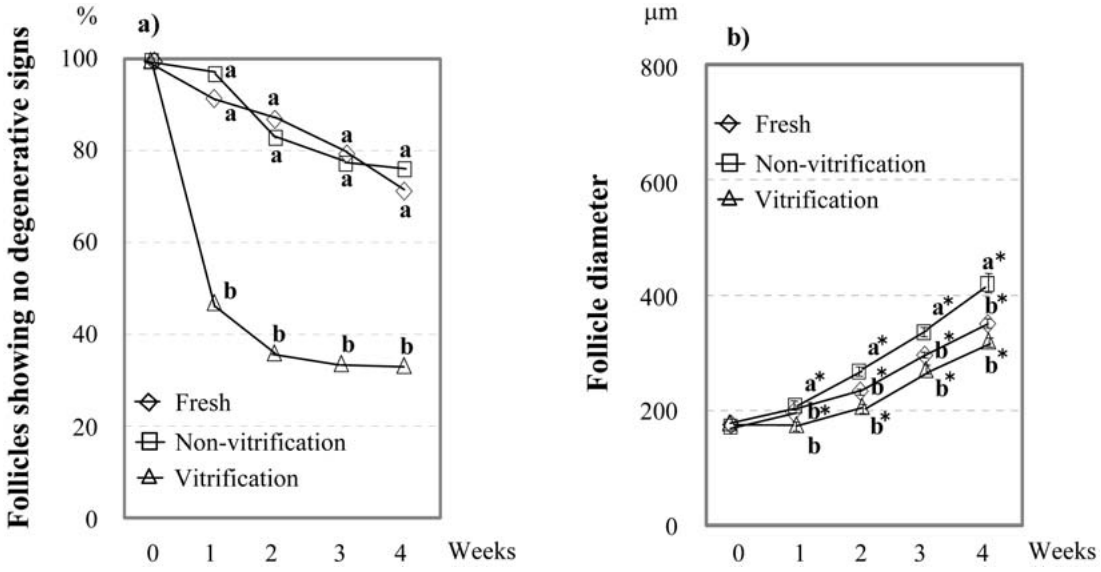

Fig. 5. Development of vitrified and warmed bovine secondary follicles cultured in the medium supplemented with bovine plasma for 4 weeks. Bovine secondary follicles after collection (Fresh), follicles treated with vitrification solutions without being plunged into LN_2 (Non-vitrification) and follicles after vitrification and warming (Vitrification) were cultured. Fig. 5a shows the percentage of follicles showing no degenerative signs, and Fig. 5b shows the mean follicle diameters ± SEM. Values with different superscripts for the same culture period differ significantly ($P<0.05$). Asterisks indicate a significant difference from the value before culture ($P<0.05$).

Table 2. Oocyte growth in bovine secondary follicles cultured in the medium supplemented with bovine plasma for 4 weeks after vitrification and warming

Treatment*	No. of follicles cultured	No. (%) of surviving oocytes**	Mean diameter of oocytes (μm ± SEM)	No. (%) of oocytes	
				GC-enclosed***	Denuded
Before culture	–	20	63.7 ± 1.9 [a]	20 (100)	0 (0)
Fresh	44	28 (64) [a]	95.0 ± 2.2 [b]	28 (64) [a]	0 (0)
Non-vitrification	39	29 (74) [a]	93.4 ± 1.6 [b]	29 (74) [a]	0 (0)
Vitrification	48	16 (33) [b]	88.8 ± 3.1 [b]	16 (33) [b]	0 (0)

* Fresh: Secondary follicles were cultured in bovine plasma-supplemented medium for 4 weeks. Non-vitrification: Secondary follicles were treated with vitrification solutions and cultured without being plunged into LN_2. Vitrification: Secondary follicles were cultured after vitrification and warming. ** Oocytes showing normal morphology with a spherical shape and homogeneous cytoplasm and without any degenerative signs after culture. *** GC: granulosa cells. [a,b] The values with different superscripts differ significantly ($P<0.05$).

tectants is critical. Without vitrification and warming, mouse embryos are injured by cryoprotectants [30]. However, under the present conditions, the bovine secondary follicles treated with the vitrification solutions without being plunged into LN_2 developed as well as the fresh control follicles. Furthermore, grown oocytes were recovered from the follicles after 4 weeks. This result indicates that the toxicity of the cryoprotectants used in the present study was not deleterious.

The mean diameters of the oocytes recovered from the vitrified and warmed follicles after 4 weeks of culture increased significantly to approximately 90 μm compared with the values before culture (about 60 μm). Bovine secondary follicles include oocytes, granulosa cells and theca cells. Under the vitrification and warming conditions used in the present study, all three types of cells, in one-third of the follicles at least, maintained the function for follicle development. Furthermore, it is thought that gap junctions between oocytes and granulosa cells were maintained in the secondary follicles during 4 weeks after vitrification and warming.

In summary, we have produced an *in vitro* culture system using a BPL-supplemented medium for bovine secondary follicles that supports oocyte growth for 4 weeks. In this system, oocytes in the vitrified-warmed secondary follicles grew for 4 weeks.

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