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EFFECTS OF DIBUTYRYL CYCLIC ADENOSINE MONOPHOSPHATE AND HUMAN CHORIONIC GONADOTROPIN ON THE FORMATION OF ANTRAL FOLLICLE-LIKE STRUCTURES BY BOVINE CUMULUS–OOCYTE COMPLEXES

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This study evaluated the effect of dibutyryl cyclic adenosine monophosphate (dbcAMP) and human chorionic gonadotropin (hCG) on the formation of antral follicle-like structures (AFLSs) and on the meiotic status of bovine cumulus–oocyte complexes (COCs) embedded in collagen gel. Supplementation with dbcAMP increased the mean diameter of AFLSs during days 4–8 of culture compared with that of control COCs, irrespective of the concentration of dbcAMP used (0.5–2.0 mM). When the embedded COCs were cultured for 8 days with hCG, the diameters of AFLSs after 4 days of culture tended to be lower in the supplemented COCs than in the control COCs without hCG, irrespective of the concentration used (1–100 IU/mL). Supplementation with 10 IU/mL hCG increased the concentrations of anti-Müllerian hormone but not progesterone and oestradiol in the culture medium after 4 days of culture. Almost all oocytes collected from AFLSs had resumed meiosis by the end of culture, irrespective of supplementation of dbcAMP and hCG. These results indicate that although dbcAMP had a positive effect on AFLS formation and development, supplementation with hCG was detrimental. Moreover, hCG supplementation did not influence the luteinisation of granulosa cells in the AFLS for 4 days after the start of culture.

Key words: Antrum-like structures, cAMP, cumulus cells, gap junction, follicular formation

Three-dimensional (3-D) culture of cumulus–oocyte complexes (COCs) may provide a valuable approach for studying the regulation of follicular development. When the 3-D organisation of cumulus granulosa cells around the oocyte was maintained during oocyte culture, COCs formed an antral follicle-like structure (AFLS) and grew *in vitro* (Harada et al., 1997). The formation of an AFLS is dependent on the proliferation and differentiation of cumulus granulosa

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cells, which surround the oocyte and supply it with nutrients (Brower et al., 1981). Moreover, cumulus granulosa cells provide physical support for oocytes and mediate signals between the oocyte and endocrine hormones. Likewise, oocytes interact with cumulus granulosa cells by secreting paracrine factors to promote granulosa cell proliferation (Hirao, 2012). Therefore, it appears that the formation and maintenance of the AFLS requires interaction between the oocyte and cumulus granulosa cells.

Supplementation with an agent causing meiotic arrest has been suggested to be essential for 3-D culturing of small oocytes because oocytes in culture begin to degenerate upon resuming meiosis (Miyano, 2005). It has been demonstrated that meiosis resumes when the oocyte is removed from the inhibitory follicular environment (Pincus and Enzmann, 1935). The principal regulator of meiosis in the oocyte is cyclic adenosine monophosphate (cAMP): a high level of cAMP within the oocyte maintains meiotic arrest, whereas a decrease in cAMP level causes meiosis to resume (Francis and Corbin, 1994). Several molecules have been identified to be candidate regulators of intrafollicular meiotic inhibition, including hypoxanthine and dibutyryl cyclic adenosine monophosphate (dbcAMP), which prevent spontaneous maturation *in vitro* (Downs et al., 1985; Carroll et al., 1991).

The gonadotropic hormones follicle-stimulating hormone (FSH) and luteinising hormone (LH) have complementary effects: the combination of FSH and LH promotes the formation of the antral cavity in early pre-antral follicles and the acquisition of oocyte meiotic competence (Cortvrindt et al., 1998). LH promotes the production of androgens, which are aromatised in the granulosa layer under the influence of FSH (Evans et al., 1981). Moreover, FSH stimulates follicle survival and granulosa cell proliferation in culture of pre-antral follicles (Cortvrindt et al., 1998; Spears et al., 1998) and small oocytes from early antral follicles (Harada et al., 1997). FSH is thought to affect granulosa cells by activating adenylyl cyclase activity and promoting cAMP accumulation (Kolena and Channing, 1972). Shen et al. (1998) reported that the dbcAMP derivative of cAMP, rather than FSH, induces antrum formation in porcine COCs. However, little information concerning the effects of hormone supplementation and meiotic arresting substances on antrum formation and oocyte development is currently available in the bovine species because the studies of follicle and oocyte development *in vitro* have been conducted mainly in the mouse.

It is possible that more COCs aspirated from larger (1–5 mm) follicles may form follicular structures than those from smaller follicles. It would be more beneficial to culture oocytes from larger follicles because culture systems for COCs can easily be adapted to mimic the milieu of growth factors and hormones necessary for follicular development and oogenesis. However, very few studies using COCs derived from follicles 1–5 mm in diameter have been conducted to study antrum formation and oocyte development.

This study evaluated the effects of dbcAMP and human chorionic gonadotropin (hCG) supplementation during culture of bovine COCs embedded in collagen gels on AFLS formation and on the meiotic status of oocytes cultured for 8 days.

Materials and methods

Collection and collagen-gel embedding culture of bovine oocytes

Bovine oocytes were collected and cultured according to a previously reported method (Abakushina et al., 2011). Briefly, bovine ovaries were obtained from a slaughterhouse, and COCs were aspirated from follicles 1–5 mm in diameter using an 18-gauge needle connected to a 5-mL disposable syringe. COCs were collected in modified phosphate-buffered saline (m-PBS; Nippon Zenyaku Kogyo Co. Ltd., Fukushima, Japan) supplemented with 100 IU/mL penicillin G potassium (Meiji, Tokyo, Japan), 0.1 mg/mL streptomycin sulphate (Meiji) and 4 mM hypoxanthine (Kohjin Co., Ltd., Tokyo, Japan). Only COCs with uniform ooplasm and a compact cumulus cell mass were subsequently washed several times in TCM199 culture medium [TCM199 medium (Earle's salts) buffered with 25 mM HEPES (Invitrogen, Carlsbad, CA, USA)] supplemented with 5% (v/v) fetal bovine serum, 50 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO, USA), 4 mM hypoxanthine, 5 μg/mL insulin (Sigma-Aldrich) and 0.02 AU/mL follicle stimulating hormone (FSH; Kawasaki Seiyaku K.K., Kawasaki, Japan)]. The COCs were embedded in a collagen gel that was generated by mixing, at a ratio of 8:1:1 (v:v:v), a 0.3% acid collagen solution (Cellmatrix Type I; Nitta Gelatin Inc., Osaka, Japan), 10× concentrated TCM199 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), and a 0.05 N sodium hydroxide solution containing 22 mg/mL $NaHCO_3$ and 47.7 mg/mL HEPES. The embedded COCs were cultured in 600 μL of TCM199 culture medium at 38.5 °C in 5% CO_2 and 95% air for 8 days. During cultivation, half of the culture medium volume was replaced with freshly prepared medium 4 days after the start of culture.

Measurement of diameter of AFLS and meiotic status of oocytes

The diameter of the AFLSs of COCs embedded in collagen gels was measured every two days on a screen connected to a camera (Leica DFC; Leica Microsystems, Tokyo, Japan) on an inverted microscope (Fig. 1). The micrometer was calibrated with a stage micrometer, a 20× objective and a 10× eyepiece. The recorded diameter was the mean of two measurements made perpendicular to each other.

The meiotic status of oocytes cultured for 8 days was examined according to the method described by Abakushina et al. (2011). The AFLS formation relates to the proliferation of cumulus/granulosa cells surrounding oocytes and the

viability of oocytes (Harada et al., 1997). Therefore, only oocytes were recovered from the collagen gels with AFLS formation at the end of culture for 8 days. The recovered COCs were denuded, fixed and permeabilised in phosphate-buffered saline (PBS; Invitrogen) supplemented with 3.7% (w/v) paraformaldehyde and 1% (v/v) Triton X-100 (Sigma-Aldrich). The oocytes were then incubated overnight at 4 °C in a small drop containing PBS supplemented with 90% (v/v) glycerol (Wako Pure Chemical Industries Ltd., Osaka, Japan) and 1.9 μM bisbenzimide (Hoechst 33342; Sigma-Aldrich) on a slide. Fluorescent staining of nuclei was visualised with a fluorescence microscope (Nikon Optiphoto-2; Nikon Corp., Tokyo, Japan). The oocytes were classified according to their meiotic stage as follows: germinal vesicle (GV) – intact germinal vesicle (nuclear envelope); germinal vesicle breakdown (GVBD) – organisation and condensation of chromatin into chromosomes; metaphase I (MI) – alignment of chromosomes on meiotic spindle; anaphase I to telophase I (AT) – two distinct groups of chromosomes positioned on an elongated bipolar spindle with a spindle midzone; metaphase II (MII) – meiotic division resulted in chromosome number reduction and expulsion of first polar body (Roth and Hansen, 2005). Oocytes that were fragmented or distinctly irregular in shape were classified as degenerated.

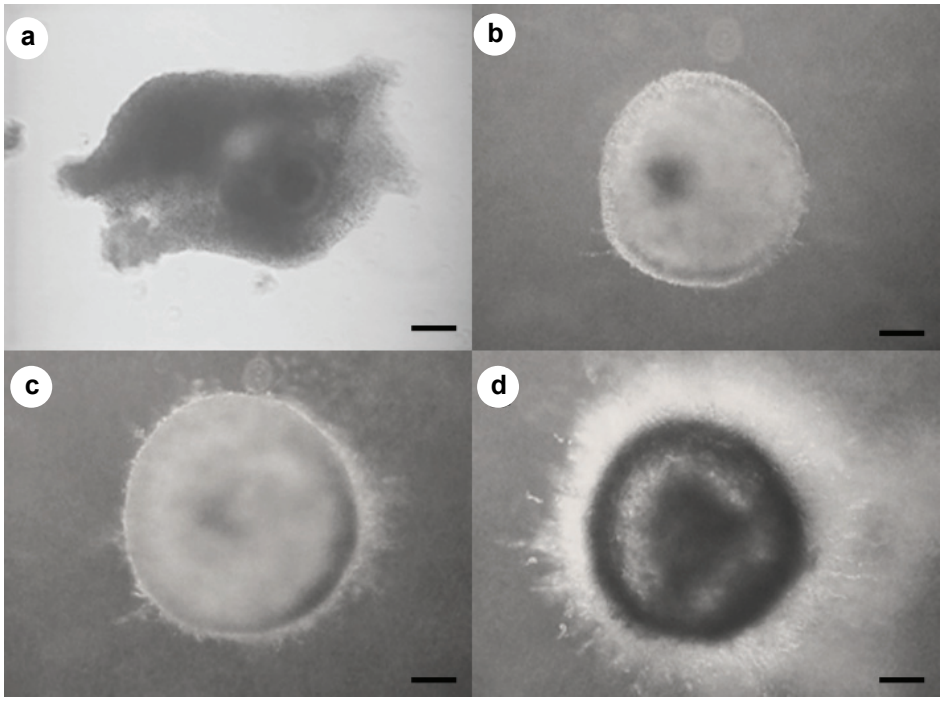

Fig. 1. Morphology of an antral follicle-like structure (AFLS) from bovine cumulus–oocyte complexes (a) embedded in collagen gel. The complexes were cultured for 2 days (b), 4 days (c) and 8 days (d). Scale bar represents 100 μm

Hormonal assessment

Concentrations of progesterone (P4), oestradiol (E2), and anti-Müllerian hormone (AMH) in the culture medium were measured using a commercially available ELISA kit (EIA-1561 for P4 and EIA-2693 for E2, DRG Diagnostic, Marburg, Germany, and AMH Gen II ELISA for AMH, Beckman Coulter Inc., CA, USA) according to the manufacturer's procedure. The sensitivity of each assay was 0.045 ng/mL for P4, 9.714 pg/mL for E2 and 0.08 ng/mL for AMH. The intra- and inter-experimental coefficients of variation were < 6.99% and < 9.96% for P4, < 6.81% and < 9.39% for E2, and < 5.4% and < 5.6% for AMH, respectively.

Experimental design

Experiment 1. The aim of this experiment was to evaluate the effect of dbcAMP on the formation of the AFLS and on the meiotic status of oocytes cultured for 8 days. In a previous study, we found that the mean diameters of AFLS in the group culture method were smaller than those in the individual culture method, but more COCs formed AFLS in the group culture method (Abakushina et al., 2011). Therefore, the COCs were embedded in collagen gels by the group culture method (3 COCs per gel) and then cultured in TCM199 culture medium supplemented with 0.5, 1.0 or 2.0 mM of dbcAMP (Sigma-Aldrich) for 8 days. As a control, the COCs were cultured in TCM199 culture medium without dbcAMP for the same length of time.

Experiment 2. The aim of this experiment was to evaluate the effect of hCG on the formation of AFLS during culture for 8 days. The COCs were cultured in TCM199 culture medium supplemented with 1.0 mM of dbcAMP because this concentration of dbcAMP was found most suitable for the formation of the AFLS in Experiment 1. The COCs were embedded in collagen gels (3 COCs per gel) and then cultured in TCM199 culture medium supplemented with 1, 10 or 100 IU/mL of hCG for 8 days. As a control, the COCs were cultured in TCM199 culture medium without hCG for the same length of time.

Experiment 3. To evaluate the relationship between the concentrations of supplemented hCG and hormones secreted from granulosa cells in the culture medium, the concentrations of progesterone, oestradiol and AMH in the culture medium containing COCs that had formed an AFLS after 4 days in culture were examined. In the second experiment, we found that the AFLS formed by COCs cultured with or without hCG reached the maximum diameter after 4 days of culture, irrespective of the concentrations of hCG. Therefore, the concentrations of progesterone, oestradiol and AMH on day 4 of culture were compared. The COCs were embedded in collagen gels (1 COC per gel) and then cultured in TCM199 culture medium supplemented with 0 (control), 1, 10 or 100 IU/mL of hCG.

Table 1

Formation of antral follicle-like structure (AFLS) and nuclear stage of bovine cumulus–oocyte complexes (COCs) cultured in medium containing dibutyryl cyclic adenosine monophosphate (dbcAMP)*

Concentration of dbcAMP (mM)	No. of COCs cultured	No. (%) of COCs with AFLS formation**	No. of oocytes examined***	No. (%) of oocytes at stage****					
				GV	GVBD	MI	AT	MII	Deg
0	53	21 (41.3 ± 6.8)[a]	21	2 (9.5)	6 (28.6)	6 (28.6)	1 (4.8)	0 (0)	6 (28.6)
0.5	50	21 (41.0 ± 3.1)[a]	19	0 (0)	6 (31.6)	4 (21.1)	0 (0)	2 (10.5)	7 (36.8)
1.0	56	31 (61.7 ± 8.3)[b]	30	1 (3.3)	8 (26.7)	7 (23.3)	2 (6.7)	0 (0)	12 (40.0)
2.0	54	28 (52.9 ± 4.1)[a, b]	27	0 (0)	8 (29.6)	4 (14.8)	1 (3.7)	3 (11.1)	11 (40.7)

*All experiments were repeated 7–9 times. **COCs formed and maintained the AFLS until the end of culture for 8 days. Percentages are expressed as the mean ± SEM. ***Oocytes were recovered from the collagen gels with AFLS formation at the end of culture for 8 days and then the meiotic status was examined. ****GV = germinal vesicle; GVBD = germinal vesicle breakdown; MI = metaphase I; AT = anaphase I and telophase I; MII = metaphase II; Deg = degenerated. [a–b]Values with different superscripts in the same column differ significantly ($P < 0.05$)

Statistical analysis

The percentages of COCs forming an AFLS, the AFLS diameters on each culture day, and the concentrations of progesterone, oestradiol and AMH in culture medium were tested by analysis of variance (ANOVA) with a subsequent Fisher's protected least significant difference (PLSD) test using the StatView program (Abacus Concepts Inc., Berkeley, CA, USA). The nuclear maturation rates were analysed using a chi-square analysis with Yates' correction. The differences with a probability value (P) of 0.05 or less were considered significant.

Results

As shown in Table 1, the rate of AFLS formation after 8 days of culture was significantly higher ($P < 0.05$) in COCs cultured with 1.0 mM dbcAMP than in COCs cultured with 0.5 mM dbcAMP and without dbcAMP. However, there were no significant differences among the groups in the meiotic status of oocytes recovered from AFLSs after 8 days of culture. The mean AFLS diameter during days 4–8 for COCs cultured with dbcAMP was significantly higher ($P < 0.05$) than those for control COCs cultured without dbcAMP, irrespective of the concentration of dbcAMP (Fig. 2).

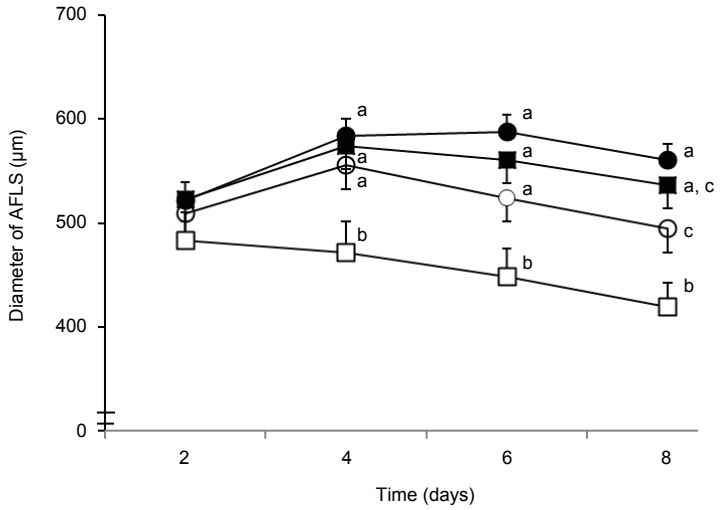

Fig. 2. Mean (± SEM) diameter of the antral follicle-like structure (AFLS) of bovine cumulus–oocyte complexes (COCs) during culture with dibutyryl cyclic adenosine monophosphate (dbcAMP) for 8 days. The COCs were embedded in collagen gels and cultured in medium containing 0 (□), 0.5 (○), 1.0 (■) and 2.0 (●) mM of dbcAMP. Only COCs (21–31 oocytes per group) that formed AFLS after culture for 4 days were selected and presented. Each bar represents the mean value ± SEM. Bars with different letters within each day differ significantly ($P < 0.05$)

Table 2

Formation of antral follicle-like structure (AFLS) and nuclear stage of bovine cumulus–oocyte complexes (COCs) cultured in medium containing human chorionic gonadotropin (hCG)*

Concentration of dbcAMP (mM)	No. of COCs cultured	No. (%) of COCs with AFLS formation**	No. of oocytes examined***	No. (%) of oocytes at stage****					
				GV	GVBD	MI	AT	MII	Deg
0	53	29 (56.6 ± 5.1)[a]	29	2 (6.9)	11 (37.9)	2 (6.9)	0 (0)	1 (3.4)	13 (44.8)
1	52	25 (50.9 ± 6.3)[a]	25	0 (0)	13 (52.0)	3 (12.0)	2 (8.0)	1 (4.0)	6 (24.0)
10	50	22 (49.3 ± 5.2)[a]	22	1 (0)	8 (36.4)	2 (9.1)	1 (4.5)	0 (0)	11 (50.0)
100	54	14 (25.4 ± 7.5)[b]	14	1 (7.1)	3 (21.4)	2 (14.3)	1 (7.1)	0 (0)	7 (50.0)

*All experiments were repeated 5–6 times. **COCs formed and maintained the AFLS until the end of culture for 8 days. Percentages are expressed as the mean ± SEM. ***Oocytes were recovered from the collagen gels with AFLS formation at the end of culture for 8 days and then the meiotic status was examined. ****GV = germinal vesicle; GVBD = germinal vesicle breakdown; MI = metaphase I; AT = anaphase I and telophase I; MII = metaphase II; Deg = degenerated. [a–b]Values with different superscripts in the same column differ significantly ($P < 0.05$)

When the COCs were cultured for 8 days in TCM199 culture medium supplemented with hCG, the rate of AFLS formation in cultures with 100 IU/mL hCG was significantly lower ($P < 0.05$) than in cultures supplemented with the other concentrations of hCG (Table 2). However, there were no significant differences among the groups in the meiotic status of oocytes recovered from AFLSs after 8 days of culture. The mean diameters of AFLS during days 4–8 for COCs cultured with 1.0 IU/mL hCG were significantly lower ($P < 0.05$) than those for control COCs cultured without hCG (Fig. 3). Moreover, the AFLS diameters beyond 4 days of culture tended to be lower in the COCs cultured with hCG than in the control COCs without hCG, irrespective of hCG concentration.

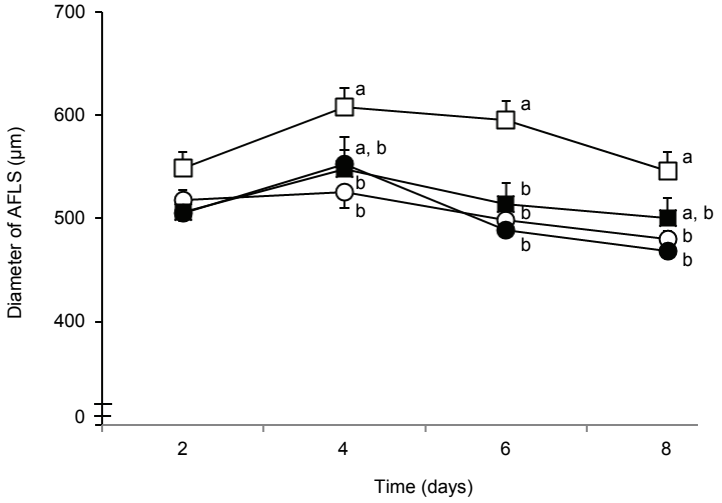

Fig. 3. Mean (± SEM) diameter of antral follicle-like structure (AFLS) of bovine cumulus–oocyte complexes (COCs) during culture with human chorionic gonadotropin (hCG) for 8 days. The COCs were embedded in collagen gels and cultured in medium containing 0 (□), 1 (○), 10 (■) or 100 (●) IU/mL of hCG. Only COCs (13–29 oocytes per group) that formed AFLS after culture for 4 days were selected and presented. Each bar represents the mean value ± SEM. Bars with different letters within each day differ significantly ($P < 0.05$)

The concentrations of progesterone and oestradiol in the medium of cultures where AFLS had formed after 4 days of culture did not differ among the groups (Fig. 4). However, the concentration of AMH in the culture medium supplemented with 10 IU/mL hCG was significantly higher than that of the culture medium supplemented with 100 IU/mL hCG and without hCG (control; $P < 0.05$, 1.81 ng/mL vs. 1.60 ng/mL and 1.57 ng/mL).

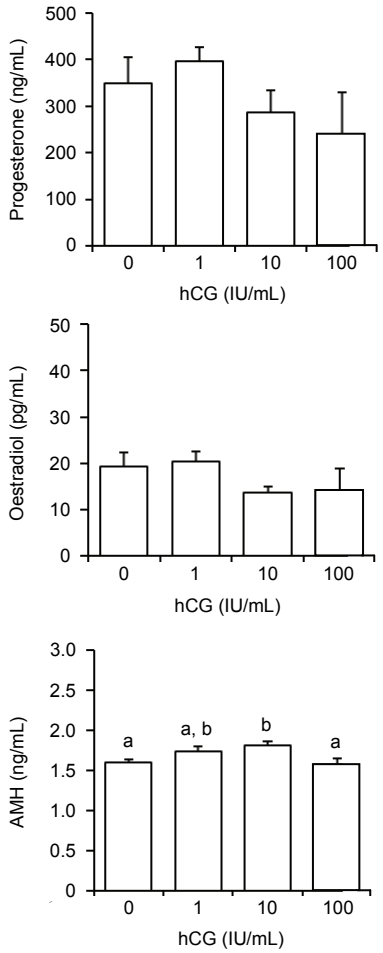

Fig. 4. Concentrations of progesterone, oestradiol and anti-Müllerian hormone (AMH) in the medium of cultures in which an antral follicle-like structure (AFLS) has been formed by bovine cumulus–oocyte complexes (COCs) following addition of human chorionic gonadotropin (hCG) for 4 days (5–7 samples per group). The COCs were embedded individually in collagen gels and cultured in medium containing 0, 1, 10 or 100 IU/mL of hCG. Each bar represents the mean value ± SEM. Bars with different letters differ significantly ($P < 0.05$)

Discussion

The collagen gel-embedded culture method provides a three-dimensional structure that maintains cell–cell connections and supports follicular development (Carroll et al., 1991). In the present study, we confirmed that the COCs derived from follicles 1–5 mm in diameter could form an AFLS due to the 3-D organisation of cumulus granulosa cells. Moreover, the addition of 1.0 mM dbcAMP

increased the formation rates and mean diameters of AFLS. The corona radiata cells, with numerous intracellular processes, penetrate through the zona pellucida and maintain their communication with the oocyte via gap junctions (Anderson and Albertini, 1976). It has been demonstrated that gap junction channels mediate meiotic arrest by allowing a factor, possibly cAMP, to pass from the granulosa cells to the oocyte (Dekel, 1988). Most of the intra-oocyte cAMP is synthesised in cumulus cells and enters the oocyte via gap junctions (Schultz et al., 1983). The dbcAMP used in the present study is a derivative of cAMP and easily passes through the cell membrane. After passing through the cell membrane, dbcAMP is metabolised to cAMP by enzymatic deacylation (Abell and Monahan, 1973). The dbcAMP has been shown to increase the number and/or maintenance of gap junctional communication between the oocyte and its surrounding cells (Carroll et al., 1991). In our findings, therefore, the increased formation rates of AFLS by supplementation with 1.0 mM dbcAMP might result in part from the enhancement of gap junctional communication between oocyte and cumulus cells. On the other hand, we used TCM199 culture medium containing 4 mM hypoxanthine as the basic medium for collagen gel-embedded COC culture. As a result, we observed that dbcAMP supplementation increased the mean diameters of AFLS of COCs during days 4–8 compared with the control COCs cultured without dbcAMP, irrespective of the concentrations of dbcAMP. These results may support the work of Hartshorne et al. (1994), who reported that dbcAMP stimulated antrum formation, whereas hypoxanthine also stimulated antrum formation but did not affect follicle growth.

Folliculogenesis is a complex developmental process that is regulated by various endocrine, paracrine and autocrine factors, as well as intraovarian cell–cell and cell-matrix interactions (Rodgers et al., 2003; Demeestere et al., 2005). Therefore, it is difficult to define the precise and individual roles that FSH and LH play in follicular growth and development. FSH has been reported to be crucial in follicle survival and granulosa cell proliferation in a culture of mouse preantral follicles (Spears et al., 1998). FSH supplementation in a 3-D culture system of bovine oocytes stimulates the proliferation of granulosa cells that maintain their morphological qualities, consequently allowing AFLS formation and the maintenance of gap junctional communication between oocyte and cumulus cells (Harada et al., 1997). The addition of a low concentration of LH during *in vitro* follicular culture enhances antral cavity formation and improves the meiotic competence of oocytes (Cortvrindt et al., 1998). In contrast, LH supplementation of FSH-containing medium has been shown to have no positive effect on the growth of mouse preantral follicles (Murray et al., 2008). In the present study, TCM199 culture medium containing FSH was used as a basic medium to examine the combined effects of FSH and hCG on AFLS formation in collagen-embedded bovine COCs. We found that 100 IU/mL hCG supplementation during 3-D culturing of COCs had a detrimental effect on AFLS formation. Moreover,

hCG supplementation decreased the mean diameter of AFLS during days 4–8 compared with mean AFLS diameter of COCs cultured without hCG. Xu et al. (2009) suggested that exposure of preantral follicles to LH before reaching an appropriate stage of development may disrupt signalling in the follicles. Therefore, our results indicate that supplementation with hCG might disrupt gap junctional communication between the oocyte and cumulus cells.

In a previous study, we observed that almost all oocytes had undergone the GVBD stage, irrespective of AFLS formation, when GV-stage oocytes with unknown oocyte diameter from follicles 1 to 5 mm in diameter were cultured for 14 days in collagen gel (Abakushina et al., 2011). In the present study, similarly, COCs aspirated from follicles 1–5 mm diameter were cultured for 8 days using the same culture method. We observed that almost all oocytes recovered from AFLSs had undergone the GVBD stage by the end of culture, and the meiotic status of oocytes was not influenced by the addition of dbcAMP and hCG. The GVBD-inhibiting factors prevent oocytes from undergoing GVBD by continuous transfer of the inhibitor through cumulus cells. It is predominantly gap junctional loss within the COCs that intervenes with oocyte meiotic resumption by blocking the conduction of meiosis inhibitory signals from outer cumulus cells to the oocyte (Tanghe et al., 2002). High intracellular levels of cAMP maintain oocyte meiotic arrest at the GV stage by activating cAMP-dependent protein kinase (Francis and Corbin, 1994), but intracellular concentrations of cAMP do not always correlate with the stage of nuclear arrest or progression (Crosby et al., 1985). In the present study, the mean diameters of AFLS gradually decreased concomitantly with the increase of the culture period, irrespective of the addition of dbcAMP. The 3-D structure is dependent on the cumulus granulosa cells surrounding oocytes in which oocytes receive nutrients from the cells (Brower et al., 1981). Therefore, it can be assumed that morphological changes of the AFLS during culture might be related to the loosened gap junctions between the oocyte and surrounding granulosa cells, resulting in high rates of meiotic resumption.

It has been suggested that high oestrogen levels can induce atresia in the developing follicles (Billig et al., 1993) and elevated progesterone is due to granulosa luteinisation or is a negative indicator for oocyte health (Fauser and Van Heusden, 1997). Xu et al. (2009) reported that exposure to FSH and LH during the 3-D culturing of preantral follicles did not influence the levels of E2 and P4 in the medium between days 7 and 14 of culture. In the present study, we found that the concentration of supplemental hCG did not affect the levels of E2 and P4 in the medium of cultures with formed AFLS after 4 days. However, the addition of 10 IU/mL hCG increased the levels of AMH in the culture medium. In the bovine ovary, AMH is localised in the granulosa cells of growing follicles, as well as in the cumulus cells (Monniaux et al., 2013). AMH concentrations are strongly diminished in atretic follicles and have a negative correlation with progesterone concentrations, in which low AMH concentrations in follicles are as-

sociated with luteinisation (Monniaux et al., 2008). Taken together, our results indicate that the granulosa cells in the AFLS proliferated for 4 days after the start of culture, and the supplementation of hCG during culture for 4 days did not stimulate granulosa luteinisation.

In conclusion, the results of this study demonstrate that supplementation with 1.0 mM dbcAMP had a positive effect on the formation and mean diameter of AFLS, whereas hCG supplementation was detrimental to AFLS development. At the end of culture, almost all oocytes collected from AFLSs had undergone the GVBD stage, and the addition of dbcAMP and hCG did not influence the meiotic status of oocytes. However, hCG supplementation did not promote the luteinisation of granulosa cells in the AFLS for 4 days following the start of culture.

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References

Abakushina, E. V., Morita, Y., Kaedei, Y., Tanihara, F., Namula, Z., Viet, V. L. and Otoi, T. (2011): Formation of an antral follicle-like structure of bovine cumulus–oocyte complexes embedded individually or in groups in collagen gels. Reprod. Domest. Anim. **46,** 423–427.

Abell, C. W. and Monahan, T. M. (1973): The role of adenosine 3',5'-cyclic monophosphate in the regulation of mammalian cell division. J. Cell. Biol. **59,** 549–558.

Anderson, E. and Albertini, D. F. (1976): Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J. Cell. Biol. **71,** 680–686.

Billig, H., Furuta, I. and Hsueh, A. J. (1993): Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology **133,** 2204–2212.

Brower, P. T., Gizang, E., Boreen, S. M. and Schultz, R. M. (1981): Biochemical studies of mammalian oogenesis: synthesis and stability of various classes of RNA during growth of the mouse oocyte *in vitro*. Dev. Biol. **86,** 373–383.

Carroll, J., Whittingham, D. G. and Wood, M. J. (1991): Effect of dibutyryl cyclic adenosine monophosphate on granulosa cell proliferation, oocyte growth and meiotic maturation in isolated mouse primary ovarian follicles cultured in collagen gels. J. Reprod. Fertil. **92,** 197–207.

Cortvrindt, R., Hu, Y. and Smitz, J. (1998): Recombinant luteinizing hormone as a survival and differentiation factor increases oocyte maturation in recombinant follicle stimulating hormone-supplemented mouse preantral follicle culture. Hum. Reprod. **13,** 1292–1302.

Crosby, I. M., Moor, R. M., Heslop, J. P. and Osborn, J. C. (1985): cAMP in ovine oocytes: localization of synthesis and its action on protein synthesis, phosphorylation, and meiosis. J. Exp. Zool. **234,** 307–318.

Dekel, N. (1988): Regulation of oocyte maturation. The role of cAMP. Ann. N. Y. Acad. Sci. **541,** 211–216.

Demeestere, I., Centner, J., Gervy, C., Englert, Y. and Delbaere, A. (2005): Impact of various endocrine and paracrine factors on *in vitro* culture of preantral follicles in rodents. Reproduction **130,** 147–156.

Downs, S. M., Coleman, D. L., Ward-Bailey, P. F. and Eppig, J. J. (1985): Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. Proc. Natl Acad. Sci. USA **82,** 454–458.

Evans, G., Dobias, M., King, G. J. and Armstrong, D. T. (1981): Estrogen, androgen, and progesterone biosynthesis by theca and granulosa of preovulatory follicles in the pig. Biol. Reprod. **25,** 673–682.

Fauser, B. C. and Van Heusden, A. M. (1997): Manipulation of human ovarian function: physiological concepts and clinical consequences. Endocr. Rev. **18,** 71–106.

Francis, S. H. and Corbin, J. D. (1994): Structure and function of cyclic nucleotide-dependent protein kinases. J. Rev. Physiol. **56,** 237–272.

Harada, M., Miyano, T., Matsumura, K., Osaki, S., Miyake, M. and Kato, S. (1997): Bovine oocytes from early antral follicles grow to meiotic competence *in vitro*: effect of FSH and hypoxanthine. Theriogenology **48,** 743–755.

Hartshorne, G. M., Sargent, I. L. and Barlow, D. H. (1994): Growth rates and antrum formation of mouse ovarian follicles *in vitro* in response to follicle-stimulating hormone, relaxin, cyclic AMP and hypoxanthine. Hum. Reprod. **9,** 1003–1012.

Hirao, Y. (2012): Isolation of ovarian components essential for growth and development of mammalian oocytes *in vitro*. J. Reprod. Dev. **58,** 167–174.

Kolena, J. and Channing, C. P. (1972): Stimulatory effects of LH, FSH and prostaglandins upon cyclic 3',5'-AMP levels in porcine granulosa cells. Endocrinology **90,** 1543–1550.

Miyano, T. (2005): JSAR Outstanding Research Award. *In vitro* growth of mammalian oocytes. J. Reprod. Dev. **51,** 169–176.

Monniaux, D., Clemente, N., Touze, J. L., Belville, C., Rico, C., Bontoux, M., Picard, J. Y. and Fabre, S. (2008): Intrafollicular steroids and anti-mullerian hormone during normal and cystic ovarian follicular development in the cow. Biol. Reprod. **79,** 387–396.

Monniaux, D., Drouilhet, L., Rico, C., Estienne, A., Jarrier, P., Touze, J. L., Sapa, J., Phocas, F., Dupont, J., Dalbies-Tran, R. and Fabre, S. (2013): Regulation of anti-Mullerian hormone production in domestic animals. Reprod. Fertil. Dev. **25,** 1–16.

Murray, A. A., Swales, A. K., Smith, R. E., Molinek, M. D., Hillier, S. G. and Spears, N. (2008): Follicular growth and oocyte competence in the *in vitro* cultured mouse follicle: effects of gonadotrophins and steroids. Mol. Hum. Reprod. **14,** 75–83.

Pincus, G. and Enzmann, E. V. (1935): The comparative behavior of mammalian eggs *in vivo* and *in vitro*: I. The activation of ovarian eggs. J. Exp. Med. **62,** 665–675.

Rodgers, R. J., Irving-Rodgers, H. F. and Russell, D. L. (2003): Extracellular matrix of the developing ovarian follicle. Reproduction **126,** 415–424.

Roth, Z. and Hansen, P. J. (2005): Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. Reproduction **129,** 235–244.

Schultz, R. M., Montgomery, R. R. and Belanoff, J. R. (1983): Regulation of mouse oocyte meiotic maturation: implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. Dev. Biol. **97,** 264–273.

Shen, X., Miyano, T. and Kato, S. (1998): Promotion of follicular antrum formation by pig oocytes *in vitro*. Zygote **6,** 47–54.

Spears, N., Murray, A. A., Allison, V., Boland, N. I. and Gosden, R. G. (1998): Role of gonadotrophins and ovarian steroids in the development of mouse follicles *in vitro.* J. Reprod. Fertil. **113,** 19–26.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M. and de Kruif, A. (2002): Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol. Reprod. Dev. **61,** 414–424.

Xu, M., West-Farrell, E. R., Stouffer, R. L., Shea, L. D., Woodruff, T. K. and Zelinski, M. B. (2009): Encapsulated three-dimensional culture supports development of nonhuman primate secondary follicles. Biol. Reprod. **81,** 587–594.