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Effects of the presence of a large follicle and a corpus luteum in bovine ovaries on nuclear maturation of oocytes and steroidogenesis of granulosa cells cultured as oocyte-cumulus-granulosa complexes derived from early antral follicles

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Abstract

The presence of a dominant follicle (DF) and a corpus luteum (CL) in bovine ovaries is known to promote blood flow to each other and to antral follicles (6–6.9 mm). However, effects of the presence of DF and CL on the growth of smaller follicles are unknown. In the present study, we investigated effects of the presence of a large follicle (LF) and CL in bovine ovaries on the *in vitro* growth of oocyte-cumulus-granulosa complexes (OCGCs) derived from early antral follicles (0.5–1.0 mm). Slaughterhouse-derived bovine ovaries were divided into 4 groups (LF+CL, LF, CL, and Devoid (without LF and CL)) based on the presence of LF (≥8 mm) and CL (≥10 mm). OCGCs were collected from early antral follicles derived from each ovary and cultured 12 days. After that, surviving OCGCs were subjected to *in vitro* maturation. Estradiol-17β (E_2) and progesterone (P_4) productions were evaluated using waste culture media every 4 days. Recovery rates of OCGCs were higher in Devoid groups than those in LF and CL groups ($P < 0.05$). Nuclear maturation rates were higher in Devoid group ($P < 0.05$) and tended to be higher in LF+CL group ($P = 0.08$) than in CL group. The ratio of E_2/P_4 in LF+CL group was higher than those of others ($P < 0.05$). In conclusion, OCGCs derived from ovaries having both LF and CL have high maturational competence of oocytes, which also have healthier steroidogenesis of granulosa cells than other ovarian patterns in cattle.

Key Words: Bovine oocytes, Corpus luteum, Dominant follicle, Early antral follicle, *In vitro* growth

Introduction

Due to the strong demand of superior animals, the number of bovine embryos produced by *in vitro* embryo production is dramatically increasing, and that has already exceeded the number of embryos produced by *in vivo* embryo production at the global level[31)]. Ovaries from slaughterhouses are

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important sources of oocytes for *in vitro* embryo production. However, oocytes from slaughterhouse-derived ovaries originate from follicles at various stages of follicular development[8,16,19,22], which can be affected by estrous cycles.

The presence of a corpus luteum (CL) and a dominant follicle (DF) in ovaries reflects the estrous cycles of the animal, and can be divided into 4 patterns in mono-ovular species; DF+CL (an ovary with both DF and CL), DF (an ovary with DF alone), CL (an ovary with CL alone), and Devoid (an ovary with neither DF nor CL) [9,10]. These patterns are known to affect the blood flow of ovarian artery, and that were higher in DF+CL, CL, DF, and Devoid patterns in that order[11]. Further, blood flows of DF, CL, and antral follicles (6–6.9 mm) in an ovary are reported to be higher in DF+CL pattern than the other patterns[6]. In addition, it is suggested that odds ratio to obtain higher quality cumulus-oocyte complexes (COCs) from follicles with perifollicular blood flow (47.1%) was 3.3 (1.1–9.6) fold higher than those from follicles without perifollicular blood flow (14.6%)[23]. These reports indicate that the presence of DF and CL can affect growth of smaller antral follicles and developmental competence of oocytes derived from those follicles.

Although some researchers investigated effects of the presence of DF and CL in ovaries on collection rate of COCs and developmental competence of oocytes[5,24,30], no consensus exists on the relationship between the ovarian patterns and the quality of oocytes. Early antral follicle stage (0.4–1.0 mm) is follicular growth stage where oocytes acquire maturational and developmental competence. Oocytes from early antral follicles do not resume meiosis even after *in vitro* maturation (IVM), but they can be matured and acquired full-term developmental competence to a calf after 12 to 14 days of *in vitro* growth (IVG) culture[13,14,32]. If we can clarify the relationship between the growth and maturational competence of oocytes derived from early antral follicles and the presence of DF and CL in bovine ovaries, it can be utilized to select ovaries for IVG culture effectively. In the present study, we investigated whether the presence of a large follicle (LF) and CL in an ovary at early antral follicle stage can affect maturational competence of oocytes and steroidogenesis (productions of estradiole-17β (E_2) and progesterone (P_4), the ratio of E_2/P_4) of granulosa cells using bovine IVG culture of oocyte-cumulus-granulosa complexes (OCGCs). Also, we analyzed the relationship between steroidogenesis of granulosa cells and antrum formation in granulosa cell layers, which was related to a high ability for E_2 production[7,27], in each ovarian pattern.

Materials and Methods

Chemicals

All the chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Collection of OCGCs and the IVG culture

Ovaries of Holstein cows obtained from a local abattoir were stored in plastic bags at 20°C and transported to the laboratory within 6–10 hours of their collection. After the ovaries were washed three times with physiological saline, slices of ovarian cortical tissues (thickness <1 mm) were prepared using a surgical blade (no. 11) and stored in tissue culture medium 199 (TCM-199; Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol, 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 10 mM sodium bicarbonate, and 50 μg/ml gentamicin sulfate (isolation medium, pH 7.4) at 37°C, as described elsewhere[12]. Under a stereomicroscope, early antral follicles (0.5–1.0 mm in diameter) were dissected from sliced ovarian tissues using a surgical blade (no. 20) and fine forceps in a 90-mm petri dish that had a 1-mm scale on its bottom (FLAT Co., Ltd., Chiba, Japan). OCGCs were isolated from early antral follicles using a pair of fine forceps and subjected to IVG as previously

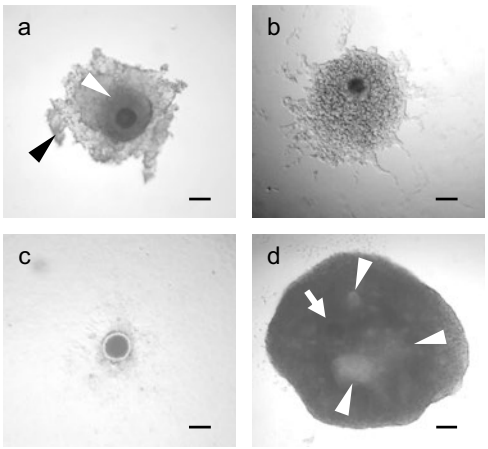

Fig. 1. Oocyte-cumulus-granulosa complexes (OCGCs) before and after day 12 of *in vitro* growth culture (IVG)
a: An OCGC before IVG. The OCGC has an oocyte surrounded by a cumulus investment and attached mural granulosa-cell layer. The white arrowhead indicates the cumulus investment. The black arrowhead indicates the mural granulosa-cell layer. b: A surviving OCGC without antrum formation in the granulosa cell layer after a 12-day IVG. OCGCs having oocytes with an evenly granulated ooplasm and enclosed by several layers of healthy granulosa cells were defined as surviving. c: A dead OCGC having a degenerated oocyte. d: A surviving OCGC with the formation of antra (white arrowheads) in the granulosa cell layer. The white arrow indicates an oocyte. Scale bars indicate 100 μm.

described[27]. Growth medium was HEPES-buffered TCM-199 supplemented with 0.91 mM sodium pyruvate, 5% (v/v) fetal calf serum (FCS; Invitrogen, Grand Island, NY, USA), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (MW 360,000), 50 μg/ml ascorbic acid 2-glucoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 55 μg/ml cysteine, 50 μg/ml gentamicin sulfate, and 10 ng/ml androstenedione as a precursor for E_2. Healthy OCGCs with oocytes surrounded by a cumulus investment and attached mural granulosa-cell layer (Fig. 1a) were cultured individually in a 96-well culture plate (Primaria 353872, Corning Life Sciences, Tewksbury, MA, USA) with 200 μl of growth medium at 39°C for 12 days in humidified air with 5% CO_2. At the onset of the IVG culture, OCGCs were photographed under an inverted microscope (CK 40, Olympus, Tokyo, Japan) with an attached CCD camera (Moticam 2000, Shimadzu Rika Corporation, Tokyo, Japan). The diameters of the oocytes without a zona pellucida were assessed using software (Motic Images Plus 2.2s, Shimadzu). Every 4 days of the IVG culture, half (100 μl) of the growth medium was replaced with the same amount of fresh medium. Spent media collected on days 4, 8, and 12 of the culture were stored at -30°C until steroid hormone assays were conducted.

Evaluation of OCGC morphologies

Every 4 days of the IVG culture, the viability of OCGCs was assessed by their morphological appearance[27]; *i.e.*, OCGCs having an evenly granulated ooplasm that was completely enclosed by several layers of a healthy cumulus and granulosa cells were defined as surviving (Fig. 1b). OCGCs having oocytes with an abnormal appearance and/or denuded by a scattering cumulus and granulosa cells were defined as dead (Fig. 1c). Simultaneous antrum formation in granulosa cell layers (Fig. 1d) was also recorded.

E_2 and P_4 assays

Spent media (100 μl) out of 200 μl of growth media was assayed to assess E_2 and P_4 concentrations using a competitive double antibody enzyme immunoassay, as previously described[28]. Assay sensitivities were 7.1 pg/well for E_2 and 11.2 pg/well for P_4. The inter- and intra-assay coefficients of variations were 16.9 and 4.0% for E_2 and 7.0 and 3.9% for P_4, respectively.

Evaluation of the growth and nuclear maturation of oocytes

After 12 days of the IVG culture, IVM was performed as previously described[20]. Briefly, oocytes surrounded by several layers of cumulus cells (COCs) from surviving OCGCs were washed with IVM medium, which consisted of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 20 μg/ml follicle-stimulating hormone (FSH), 1 μg/ml E_2, 10% FCS, and 50 μg/ml gentamicin sulfate. COCs were cultured in each well of micro-well plates (Mini Trays 163118, NUNC, Roskilde, Denmark) filled

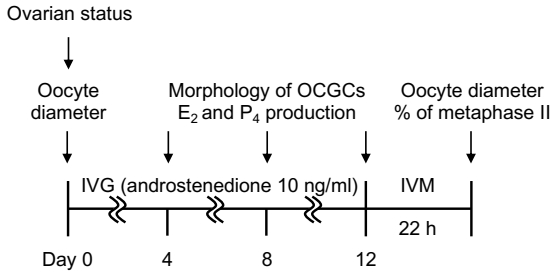

Fig. 2. Schematic of the experimental design
On day 0, the presence of large follicles (LFs) and corpora lutea (CLs) in ovaries from the local abattoir was confirmed, and the diameters of the largest follicles and CLs were measured. The ovaries were divided into 4 groups (LF+CL, LF, CL, and Devoid) based on the presence of LF at least 8 mm and CL at least 10 mm. In addition, we also recorded the surface area, the volume of ovaries, the number of antral follicles (≥2 mm), the density of antral follicles (≥2 mm), the number of isolated early antral follicles (0.5–1 mm), the number of collected healthy oocyte-cumulus-granulosa complexes (OCGCs), and the collection rate of healthy OCGCs. OCGCs from each ovary were cultured for 12 days in an *in vitro* growth (IVG) culture. The diameter of oocytes was evaluated on day 0 of IVG. On every 4 days of IVG, the morphology of OCGCs (viability of OCGCs and antrum formation in granulosa cell-layers) was evaluated. After 12 days of IVG, cumulus-oocyte complexes (COCs) derived from surviving OCGCs were subjected to *in vitro* maturation (IVM). The concentrations of estradiol-17β (E_2) and progesterone (P_4) in the IVG media of some of these OCGCs at each period of IVG every 4 days (days 0, 4, 8, and 12) were evaluated. After IVM, the diameter and nuclear status of oocytes were evaluated.

with 6 ml of IVM medium at 39°C under 5% CO_2 in air for 22 hours. After IVM, oocytes were denuded from cumulus cells by individual pipetting, photographed, and their diameters were measured. Oocytes were mounted individually on a slide glass and fixed with a mixture of acetic acid and ethanol (1:3) overnight. After fixation, oocytes were stained with 1% (w/v) aceto-orcein and the statuses of their nuclei were examined under a phase contrast microscope, as described elsewhere[21]. Oocytes at metaphase II and having a polar body were defined as mature; oocytes at other nuclear statuses were defined as immature.

Experimental design

A schematic of the experimental design was shown in Fig. 2. The presence of LFs and CLs in ovaries from the local abattoir was confirmed, and the diameters of the largest follicles and CLs were measured using a digital caliper (DN-150, Niigata seiki Co.,Ltd., Sanjyo, Japan). The ovaries were divided into 4 groups (LF+CL, LF, CL, and Devoid) based on the presence of LF at least 8 mm and CL at least 10 mm. It was reported that follicles at least 8 mm express luteinizing hormone receptors[1], and CLs at least 10 mm are acceptable for embryo transfer in cattle[18] although there is a possibility that CLs may be regressing. Because we measured the diameters of LFs and CLs on the surfaces of ovaries, the actual diameter of them could be larger than the measured values. In addition, we also measured the length, width, and height of each ovary using the digital caliper. Based on those values, the surface area and the volume of each ovary were calculated as those of ellipsoids using following formulas.

Surface area of ovaries (mm^2, approximation) $= 4\pi ((a^p b^p + a^p c^p + b^p c^p)/3)^{1/p}$

Volume of ovaries (mm^3) $= 4/3\pi abc$

(a = length, b = width, and c = height, p = 1.6075)

We compared the status of ovaries (the surface area and the volume, the number of antral follicles (≥2 mm), the density of antral follicles (≥2 mm), the number of isolated early antral follicles (0.5–1.0 mm), the number of collected healthy OCGCs, and the collection rate of healthy OCGCs among the groups. Follicles between larger than 1 mm and smaller than 2 mm in diameter were not counted because these follicles are generally not used for *in vitro* production of bovine embryos[2,17,19]. We used 96 ovaries (LF+CL: 16, LF: 27, CL: 20, and Devoid: 33,), and 357 OCGCs (LF+CL: 58, LF: 79, CL: 89, and Devoid: 131) were subjected to IVG for 12 days. The total culture replicates were 12. After IVG, 205 OCGCs judged as surviving (LF+CL: 33, LF: 43, CL: 49, and Devoid: 80) were subjected to IVM for 22 hours. E_2 and P_4 concentrations were measured in spent media used for the IVG culture of 162 OCGCs (LF+CL: 22, LF: 38, CL: 44, and Devoid: 58), which were subjected to IVM from 10 culture replicates. The viability of OCGCs was calculated based on all cultured OCGCs. Steroid hormone production during each period (days 0

Table 1. Relationship between the status of ovaries and the presence of a large follicle (LF) and corpus luteum (CL)

Parameters	Groups (No. of ovaries)			
	LF+CL (16)	Devoid (33)	LF (27)	CL (20)
Surface area of ovaries (cm^2)	27.5 ± 8.4^{ab}	21.1 ± 6.6^{c}	21.9 ± 6.8^{bc}	28.1 ± 7.2^{a}
Volume of ovaries (cm^3)	12.8 ± 5.7^{ab}	8.4 ± 4.1^{c}	9.2 ± 4.6^{bc}	13.1 ± 5.5^{a}
No. of antral follicle*	22.4 ± 16.9	16.8 ± 10.8	18.7 ± 8.8	24.5 ± 18.8
Density of antral follicles* ($/cm^2$)	0.77 ± 0.46	0.78 ± 0.41	0.84 ± 0.24	0.81 ± 0.47
No. of isolated early antral follicles (0.5-1 mm)	8.6 ± 7.7	8.4 ± 7.8	7.8 ± 5.5	12.0 ± 10.3
No. of collected healthy OCGCs**	3.7 ± 3.6	4.0 ± 3.9	3.0 ± 2.2	4.5 ± 3.3
Collection rate of healthy OCGCs*** (%)	43.1^{ab}	47.5^{a}	37.9^{b}	37.5^{b}

* Follicles with ≥2 mm in diameter were counted.
** Oocyte-cumulus-granulosa complexes.
*** Collection rate of healthy OCGCs was based on the number of collected healthy OCGCs/the number of isolated early antral follicles.
[a-c] Different superscripts indicate significant differences between each group ($P < 0.05$).
Values, except for collection rate of healthy OCGCs, are described as mean ± standard deviation.

to 4, 4 to 8, and 8 to 12) was calculated using the following formula.

Steroid hormone production (ng) = 0.2 (ml) × Concentration at the end of the period (ng/ml)

− 0.1 (ml) × Concentration at the start of the period (ng/ml)

The percentage of antrum formation in the granulosa cell layer was calculated based on OCGCs judged as surviving on day 12. We compared differences in the viability of OCGCs, the antrum formation rate in the granulosa cell layer, productions of E_2 and P_4, the E_2/P_4 ratio, the diameter, and the nuclear maturation rate of oocytes among groups.

Based on the results of antrum formation in the granulosa cell layer, productions of E_2 and P_4, and the E_2/P_4 ratio were compared between OCGCs without antrum (Antrum-) and OCGCs with antrum (Antrum+) in each group.

Statistical analysis

All statistical analyses were performed using software (StatView 4.51, Abacus Concepts, Inc., Calabasas, CA, USA or JMP Pro 14, SAS Institute, Cary, NC, USA). Data on the collection rate, the viability, and antrum formation of OCGCs, and the nuclear maturation rate were analyzed by logistic regression analysis. The initial models contained groups (LF+CL, LF, CL, and Devoid), days for IVG (days 4, 8, and 12), and their interactions as categorical explanatory variables for the viability, and antrum formation of OCGCs, and groups (LF+CL, LF, CL, and Devoid) for the collection rate and the nuclear maturation rate. Explanatory variables for the final models were determined using a backward elimination procedure. All variables with $P > 0.10$ were removed from the initial models. The final models contained days for IVG for the viability, days for IVG and the interaction between days for IVG and groups for antrum formation of OCGCs, and groups for the collection rate and the nuclear maturation rate. Odds ratios were estimated in the final models and Wald test was used to analyze differences among days for IVG and groups. $P < 0.05$ was considered to a significant difference. Data on the E_2/P_4 ratio was converted to natural logarithm before analyses. Other data were analyzed using a one-way analysis of variance (ANOVA) or a two-way repeated-measurement ANOVA. We analyzed the effects of groups (LF+CL, LF, CL, and Devoid or Antrum- and Antrum+), days for IVG (days 4, 8, and 12), and their interactions. To compare result among three or more groups (LF+CL, LF, CL, and Devoid for the diameter of oocytes and steroidogenesis or days 4, 8, and 12 for the steroidogenesis), Tukey-Kramer's honestly significant difference test was used as post-hoc

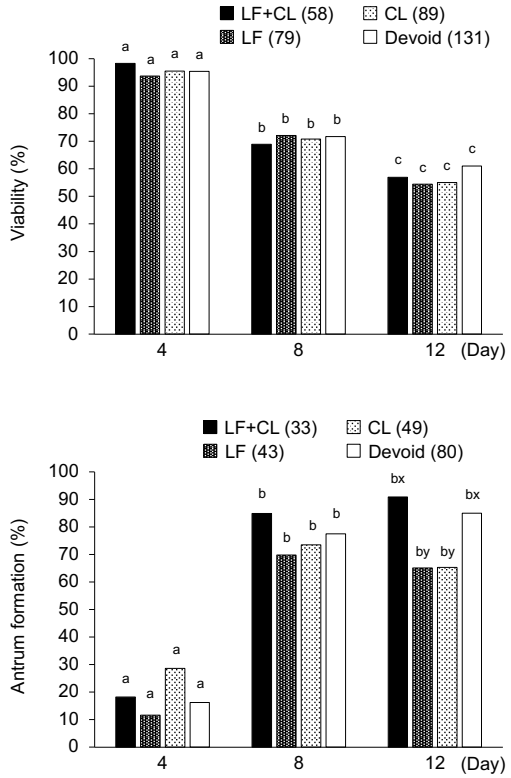

Fig. 3.
Effects of the presence of a large follicle (LF) and a corpus luteum (CL) on viability and antrum formation in granulosa cell-layer of oocyte-cumulus-granulosa complexes (OCGCs)
Numbers in parentheses indicate the number of OCGCs cultured.
The number of OCGCs with antrum formation was evaluated by those surviving on day 12.
[a-c] Different letters indicate significant differences among different culture periods in the same group ($P < 0.05$).
[x, y] Different letters indicate significant differences among the groups on the same day ($P < 0.05$).

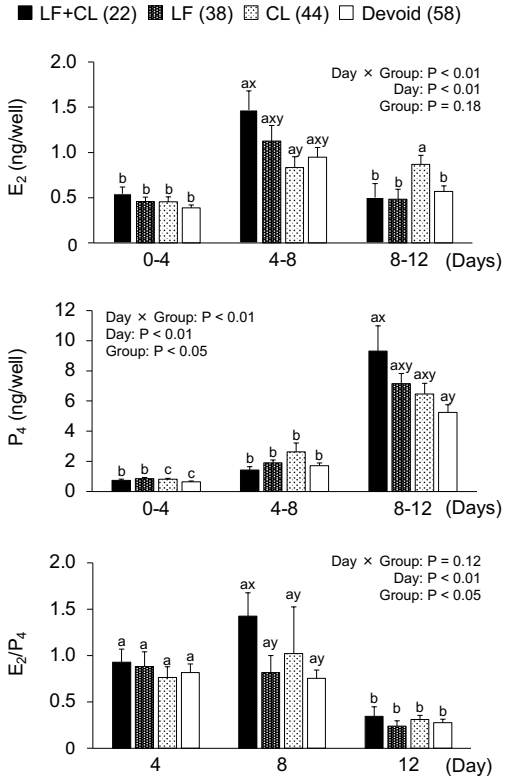

Fig. 4.
Effects of the presence of a large follicle (LF) and a corpus luteum (CL) on the production of estradiol-17β (E_2) and progesterone (P_4) by oocyte-cumulus-granulosa complexes (OCGCs), and the E_2/P_4 ratio in culture media
The results of an analysis by a two-way ANOVA were shown above each panel.
[a-c] Different letters indicate significant differences among different culture periods in the same group ($P < 0.05$).
[x, y] Different letters indicate significant differences among the groups on the same day ($P < 0.05$).
Numbers in parentheses indicate the number of OCGCs used for hormone measurement.
Error bars indicate standard error of the mean.

test. To compare results between two groups (days 0 and 12 for the diameter of oocytes or Antrum- and Antrum for the steroidogenesis), Student's *t*-test was used.

Results

Effects of the presence of LF and CL on ovarian status

As shown in Table 1, the surface area and the volume of ovaries were larger in LF+CL and CL groups than in Devoid group ($P < 0.05$). The number of antral follicles (≥2 mm) and the density of antral follicles (≥2 mm) did not significantly differ among the 4 groups. The number of isolated early antral follicles and the number of collected healthy OCGCs were also similar among the 4 groups, while the collection rate of healthy OCGCs was higher in Devoid group than in LF and CL groups ($P < 0.05$).

Effects of the presence of LF and CL on viability and antrum formation of OCGCs

As shown in Fig. 3, the viabilities of OCGCs in all groups decreased throughout the culture period

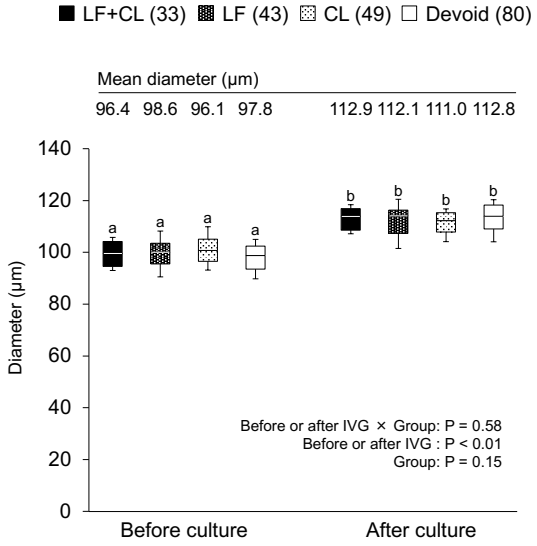

Fig. 5.
Effects of the presence of a large follicle (LF) and a corpus luteum (CL) on oocyte growth
Lines on the boxes in the box-and-whisker plot delineate the 25th, 50th, and 75th percentiles, while the whiskers depict the 10th and 90th percentiles.
Values above boxes in the box-and-whisker plot indicate the mean diameters (μm) of oocytes.
Numbers in parentheses indicate the number of oocytes submitted to *in vitro* maturation.
The results of an analysis by a two-way ANOVA were shown bottom right in the panel.
[a, b] Different letters indicate significant differences between before and after IVG ($P < 0.05$).

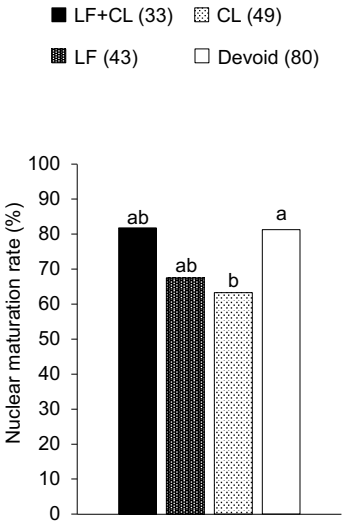

Fig. 6.
Effects of the presence of a large follicle (LF) and a corpus luteum (CL) on oocyte maturation
Numbers in parentheses indicate the number of oocytes submitted to *in vitro* maturation.
[a, b] Different letters indicate significant differences between the groups ($P < 0.05$).

($P < 0.05$) and did not significantly differ among the 4 groups. The percentages of OCGCs having antra increased throughout the culture period, and became higher in LF+CL and Devoid groups than in LF and CL groups ($P < 0.05$) on day 12.

Effects of the presence of LF and CL on the steroidogenesis of granulosa cells

As shown in Fig. 4, the two-way ANOVA showed that the interactions between the groups and the days of IVG on E_2 and P_4 production ($P < 0.01$). E_2 production and the E_2/P_4 ratio affected by the day of IVG ($P < 0.01$), while P_4 production and the E_2/P_4 ratio were affected by the groups ($P < 0.05$) and the day of IVG ($P < 0.01$). E_2 production from days 4 to 8 showed the highest values in all culture periods, and E_2 production from days 4 to 8 was higher in LF+CL group than in CL group ($P < 0.05$). P_4 production increased with the extension of the culture period ($P < 0.05$), and P_4 production from days 8 to 12 was higher in LF+CL group than in Devoid group ($P < 0.05$). The E_2/P_4 ratio was maintained approximately one until day 8, but decreased on day 12 in all groups ($P < 0.05$). The E_2/P_4 ratio of LF+CL group was higher than those of other 3 groups on day 8 ($P < 0.05$).

Effects of the presence of LF and CL on growth and maturation of oocytes

As shown in Fig. 5, the diameter of oocytes became larger after than before IVG in each group ($P < 0.05$), but there was no difference in the diameter among the groups.

As shown in Fig. 6, the nuclear maturation rate was higher in Devoid group (81.3%) than in CL group (63.3%) ($P < 0.05$), and tended to be higher ($P = 0.09$) than in LF group (67.4%). The nuclear maturation of LF+CL group (81.8%) was

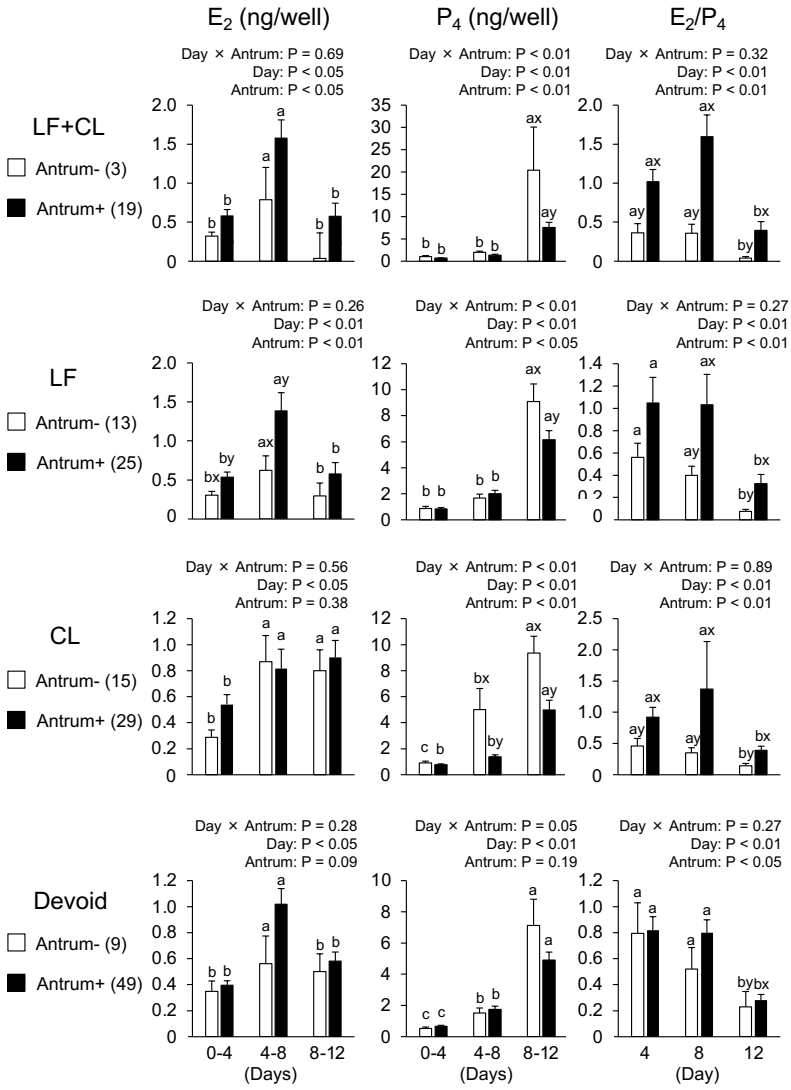

Fig. 7.
Relationship between steroidogenesis of granulosa cells and antrum formation in granulosa cell layers in each ovarian pattern
The results of an analysis by a two-way ANOVA were shown above each panel.
[a-c] Different letters indicate significant differences among different culture periods in the same group ($P < 0.05$).
[x, y] Different letters indicate significant differences between oocyte-cumulus-granulosa complexes (OCGCs) without antrum formation (Antrum-) and with antrum formation (Antrum+) in granulosa cell layers on the same day ($P < 0.05$).
Numbers in parentheses indicate the number of OCGCs used for hormone measurement.
Error bars indicate standard error of the mean.

comparable with that of Devoid group, and tended to be higher than that of CL group ($P = 0.08$).

Relationship between the relationship between steroidogenesis of granulosa cells and antrum formation in granulosa cell layers in each ovarian pattern

As shown in Fig. 7, E_2 production was higher in Antrum+ than in Antrum- in LF+CL and LF groups ($P < 0.05$). P_4 production was higher in Antrum- than in Antrum+ from days 4 to 12 in CL group ($P < 0.05$), and from days 8 to 12 in LF+CL and LF groups ($P < 0.05$). The E_2/P_4 ratio was higher in Antrum+ than Antrum- in all groups ($P < 0.05$).

Discussion

To the best of our knowledge, the present study is the first report showing that the presence of LF and CL in ovaries affects the maturational competence and steroidogenesis of *in vitro* growing OCGCs derived from early antral follicles. There are several reports focusing on the effect of the presence of LF and CL on oocyte competence derived from antral follicles (≥2 mm), these results are controversial. Some reports showed no effect of presence of LF and CL on developmental competence of oocytes to blastocysts[5,30], while another report suggested higher developmental competence in oocytes from Devoid ovaries than in those derived from ovaries with LF and CL[24]. As the stage of early antral follicle is the critical stage to determine the developmental competence of oocytes[13], our results may suggest that the presence of LF and CL during the stage of early antral follicle can be more important that during the stage of larger antral follicles (≥2 mm).

In the present study, the nuclear maturational rate of oocytes in Devoid group was higher than that of CL group and tended to be higher than that of LF group, while LF+CL group showed comparable nuclear maturation rate with Devoid group, which tended to be higher than that of CL group. Blood flow of ovarian artery is known to higher DF+CL, CL, DF, and Devoid patterns in that order[11]. In addition, follicles with perifollicular blood flow is more likely to produce good quality of COCs than follicles without perifollicular blood flow[23]. Due to the presence of both LF and CL, blood flow may become high in entire ovaries in LF+CL group, which may cause higher maturational competence of oocytes. In addition, both DF and CL are known to induce vascularization in ovaries[3,25]. Because blood supply can concentrate to LF and CL, there can be an area with low blood supply in ovaries with LF or CL alone. Although blood flow into ovarian artery is lower in Devoid ovaries than ovaries with LF+CL and LF or CL alone, blood flow in Devoid ovaries can be equally distributed. Therefore, we speculate that the proportion of cultured OCGCs with good quality would be higher in Devoid pattern than in LF and CL patterns, resulting the higher percentage of matured oocytes in Devoid pattern than in LF and CL patterns.

The antrum formation rate on day 12 was higher in LF+CL and Devoid groups, which showed better morphological quality before culture and higher maturational competence, than in LF and CL groups in the present study. We previously reported that OCGCs with antrum formation in granulosa cell layers and having oocyte with higher maturational competence produce more E_2 and less P_4[27]. Consistently, LF+CL group, which had higher antrum formation and nuclear maturational rates, showed higher E_2/P_4 ratio on day 8 and higher E_2 production from day 4 to day 8, while P_4 production from day 8 to 12 was higher than some other groups. Higher E_2 production and higher quality of OCGCs in LF+CL group may be linked to the vasodilatory effect of E_2[4,15]. On the other hand, Devoid group, which also had higher antrum formation and nuclear maturational rates, showed comparable E_2 and P_4 productions with LF and CL group. Pancarcı *et al.*[23] reported that E_2 and P_4 concentrations and E_2/P_4 ratio was similar between follicles with or without perifollicular blood flow, while the collection rate of COCs with good quality was higher from follicles with perifollicular blood flow than without perifollicular blood flow. In addition, they suggested that nitric oxide (NO), which also has a vasodilatory effect[26], was higher in follicles with perifollicular blood flow than without perifollicular blood flow. In bovine *in vitro* maturation of oocytes, inhibition of nitric oxide synthase impaired nuclear maturation of oocytes and subsequent embryo quality[29]. Further studies are needed to investigate the relationship among NO, the presence of LF and CL in ovaries, and growth of OCGCs.

In the present study, E_2 production was similar between Antrum- and Antrum+ in CL and Devoid groups. On the on the hand, the E_2/P_4 ratio was higher in Antrum+ than Antrum- in all groups. It is reported that antrum formation rate

in granulosa cell layers increased by the addition E_2 regardless of its concentration (0.1–10 μg/ml), and OCGCs with antrum formation produced more E_2 than OCGCs without antrum formation in the presence of androstenedione (1–10 μg/ml)[7]. In addition, we previously reported that OCGC with antrum formation produced more E_2 and less P_4 in the presence of androstenedione (10 ng/ml)[27]. These results suggest that the E_2/P_4 ratio can be more important for antrum formation rather than E_2 productivity.

In conclusion, the quality of OCGCs was better in LF+CL and Devoid groups than in the groups with LF or CL alone in terms of morphology before IVG culture, antrum formation in granulosa cell layers, and maturational competence of oocytes after IVG culture. In addition, OCGCs in LF+CL group, which showed high maturational competence and healthy steroidogenesis compared to other groups. These results clearly suggest that the presence of LF and CL in ovaries affects *in vitro* growth of OCGCs, which can be utilized to select good ovaries with good quality OCGCs prior to their collection. In addition, there is a possibility that the OCGC quality in early antral follicle affects the subsequent follicular development and oocyte developmental competence *in vivo*. It is necessary to investigate the linkage between the presence of LF and/or CL in ovaries at early antral follicle stage and future developmental competence and fertility of oocytes derived from those follicles in further study.

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