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Relationship between the antral follicle count in bovine ovaries from a local abattoir and steroidogenesis of granulosa cells cultured as oocyte-cumulus-granulosa complexes

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Abstract. The antral follicle count (AFC) is used as an indicator of cow fertility. We herein investigated the relationship between AFC and the steroidogenesis of granulosa cells and confirmed the developmental competence of oocytes derived from early antral follicles (0.5–1.0 mm) using in vitro growth culture. Slaughterhouse-derived ovaries were divided into high (≥ 25) and low (< 25) AFC groups based on AFC (≥ 2.0 mm). Oocyte-cumulus-granulosa complexes (OCGCs) collected from early antral follicles were cultured for 12 days. The total number, viability, and diameter of granulosa cells and estradiol-17β and progesterone production during the culture were evaluated. Surviving oocytes on day 12 were subjected to in vitro maturation, and their volume and nuclear status were evaluated. Some oocytes were subjected to the evaluation of developmental competence to blastocysts. Although the total number and viability of granulosa cells did not differ between the groups, granulosa cell diameters were smaller in the high AFC group than in the low AFC group. The estradiol-17β and progesterone ratio on day 8 was higher in the high AFC group than in the low AFC group. Oocyte volumes and nuclear maturation rates were greater in the high AFC group than in the low AFC group. The development rate to blastocysts was 9.1% in the high AFC group, while no oocytes developed to blastocysts in the low AFC group. Therefore, estradiol-17β production by granulosa cells appears to be greater in high AFC cattle than in low AFC cattle, thereby promoting the acquisition of oocyte competence.

Key words: Antral follicle count, Bovine oocyte, In vitro growth, Steroidogenesis (J. Reprod. Dev. 64: 503–510, 2018)
and development to the blastocyst stage [10–12]. Consequently, oocyte-cumulus-granulosa complexes (OCGCs) derived from early antral follicles (0.5–1.0 mm in diameter) in the high AFC group with more than 25 follicles (≥ 2.0 mm in diameter) in an ovary collected at a slaughterhouse showed greater oocyte maturational competence and fertilizability as well as the greater proliferation of granulosa cells than those in the low AFC group (less than 25 follicles) [9]. However, in the previous study [9], we cultured OCGCs in medium containing a high concentration of estradiol-17β (E2, 1 µg/ml) to increase the E2/progesterone (P4) ratio, similar to a dominant follicle [13]; therefore, the relationships between AFC, the steroidogenesis of granulosa cells, and the developmental competence of oocytes have not yet been examined. In addition, we reported that granulosa cells surrounding in vitro-grown oocytes having higher maturational competence secreted slightly more E2 and less P4 than the granulosa cells surrounding less competent in vitro-grown oocytes using medium containing androstenedione (A4) instead of E2 [14].

In the present study, we investigated the relationship between AFC and the steroidogenesis of granulosa cells using a bovine IVG culture without the exogenous application of E2. In addition, we confirmed the oocyte competence of growth, maturation, and subsequent development to blastocysts.

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 h of their collection. After the ovaries were washed three times with physiological saline, slices of ovarian cortex 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 [15]. 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, Chiba, Japan). OCGCs were isolated from early antral follicles using a pair of fine forceps and subjected to IVG as previously described [14]. Growth medium was HEPES-buffered TCM-199 supplemented with 0.91 mM sodium pyruvate, 5% (v/v) fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% (w/v) polyvinylpyrrolidone (MW 360,000), 50 µg/ml ascorbic acid 2-glucoside (Wako Pure Chemical Industries, Osaka, Japan), 55 µg/ml cysteine, 50 µg/ml gentamicin sulfate, and 10 ng/ml A4 as a precursor for E2. OCGCs with oocytes surrounded by a cumulus investment and attached mural granulosa-cell layer were defined as surviving. C: A dead OCGC having a degenerated ooplasm and enclosed by several layers of healthy granulosa cells were defined as surviving. 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.

Evaluation of OCGC morphologies

Every 4 days of the IVG culture, the viability of OCGCs was assessed by their morphological appearance [14]; i.e., OCGCs having an evenly granulated ooplasm and enclosed by several layers of healthy 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), which was related to a high ability for E2 production [14, 16], was also recorded.

E2 and P4 assays

Spent media (100 µl) out of 200 µl of growth media was assayed to
assess E₂ and P₄ concentrations using a competitive double antibody enzyme immunoassay, as previously described [17]. Samples were subjected to 2- to 2000-fold serial dilutions with assay buffer (145 mM NaCl, 40 mM Na₂HPO₄, and 0.1% bovine serum albumin (BSA, w/v, pH 7.2)). Diluted samples (20 μl) were incubated with the primary antisera and horseradish peroxidase-labeled hormone (100 μl each) in the wells of a 96-well microplate (Costar 3590, Corning, NY, USA) coated with the secondary antiserum at 4°C for 16–18 h. The primary antiserus used for the E₂ and P₄ assays were anti-estradiol-17β-6-carboxymethylxolime (CMO)-BSA (FKA204, Cosmo Bio, Tokyo, Japan) and anti-progesterone-3-CMO-BSA (KZ-HS-P13, Cosmo Bio), respectively. Goat anti-rabbit serum (111-005-003, Jackson Immuno Research, West Gräve, PA, USA) was used as the secondary antiserum. After the washing of all wells four times with 300 μl of washing buffer (0.05% Tween 80), 150 μl of 3,3',5,5'-tetramethylbenzidine (TMB) solution (5 mM citric acid, 50 mM Na₂HPO₄, 500 mM urea hydrogen peroxide, 1 mM TMB, and 2% dimethyl sulfoxide) was added to each well and incubated at 37°C for 40 min. The absorbance of the solution in the wells was measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan) after stopping the chromogenic reaction with 50 μl of 4 N H₂SO₄. All samples were assayed in triplicate. Assay sensitivities were 7.1 pg/well for E₂ and 11.2 pg/well for P₄. The inter- and intra-assay coefficients of variations were 16.9 and 4.0% for E₂ and 7.0 and 3.9% for P₄, respectively.

Evaluation of granulosa cell characteristics
The total number, viability, and diameter of granulosa cells after the growth culture from morphologically normal OCGCs on days 8 and 12 were assessed using an acridine orange/propidium iodide cell viability kit together with a cell counter (F23001 and L2000, respectively; Logos Biosystems, Gyunggi, Republic of Korea) as previously described [18]. The culture medium in the well of each viable OCGC was removed and replaced with 80 μl of Dulbecco’s phosphate-buffered saline without calcium and magnesium (DPBS) supplemented with 0.125% (w/v) trypsin and 0.05% (w/v) EDTA to prepare the granulosa cells for counting. After 10 min of trypsinization and pipetting several times, 20 μl of FCS was added to stop digestion. The cell counter calculated the viability and gave a mean diameter of granulosa cells used 21 to 1123 cells/sample in the present study. The denuded oocyte was removed from the well and discarded.

Evaluation of the growth, nuclear maturation, and developmental competence of oocytes
After 12 days of the IVG culture, in vitro maturation (IVM) was performed as previously described [19]. Briefly, oocytes surrounded by several layers of cumulus cells (oocyte-cumulus complexes: 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 E₂, 10% FCS, and 50 μg/ml gentamicin sulfate. COCs were cultured in each well of micro-well plates filled with 6 ml of IVM medium at 39°C under 5% CO₂ in air for 22 h. After IVM, oocytes were denuded from cumulus cells by individual pipetting, photographed, and their diameters were measured. Oocyte volumes were calculated by their diameters. 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 [20]. Oocytes at metaphase II and having a polar body were defined as mature; oocytes at other nuclear statuses were defined as immature. After IVM, some COCs were subjected to in vitro fertilization (IVF) as previously described [21]. Briefly, frozen semen collected from a Holstein bull was used for IVF. After thawing the semen in a 37°C water bath for 40 sec, motile sperm (5 × 10⁶ sperm/ml) separated by a Percoll gradient (45% and 90%) were co-incubated with COCs in a 100-μl droplet (8 to 12 COCs per droplet) of modified Brackett and Oliphant isotonic medium [22] containing 3 mg/ml fatty acid–free BSA and 2.5 mM theophylline [23] for 18 h at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂. An in vitro culture (IVC) of inseedated oocytes (presumptive zygotes) was performed as previously described [21, 24]. Briefly, after a co-incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing for 5 min and washing three times in culture medium. Cumulus-free zygotes were cultured for 6 days in 30-μl droplets (8 to 12 zygotes per droplet) of culture medium at 39°C under 5% CO₂, 5% O₂, and 90% N₂. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, seven non-essential amino acids for minimum essential medium, 10 μg/ml insulin, 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/ml fatty acid–free BSA. During IVF and IVC, oocytes derived from different ovaries were pooled and cultured in a same droplet. Cleavage and blastocyst production rates were measured after 2 days (approximately 30 h) and 6 days (150 h) of IVC, respectively. The total live cell numbers of blastocysts obtained after 6 days of IVC were counted using an air-drying method [23].

Statistical analysis
All statistical analyses were performed using software (StatView 4.5.1, Abacus Concepts, Calabasas, CA, USA). Data on the viability of and antrum formation by OCGCs and the nuclear maturation rate were analyzed by the chi-squared test. Other data were analyzed using a two-way ANOVA followed by the Student’s t-test or Tukey-Kramer’s HSD test.

Experimental design
A schematic of the experimental design was shown in Fig. 2. AFC in ovaries from the local abattoir was assessed by the number of antral follicles (≥ 2 mm in diameter), and we allocated ovaries having 25 or more follicles to the high AFC group and others to the low AFC group as described elsewhere [9]. As shown in Fig. 3, we collected more OCGCs from a higher number of antral follicles (≥ 2 mm in diameter) in ovaries (P < 0.05, Tukey-Kramer’s HSD test). We used 186 ovaries as the low AFC group and 70 ovaries as the high AFC group. We collected 388 OCGCs in the low AFC group and 410 OCGCs in the high AFC group and subjected them to the same experiment. However, as shown in Table 1, 4 oocytes in the low AFC group and 4 in the high AFC group were accidentally lost after IVM during the pipetting of oocytes for the denudation of cumulus cells. Three oocytes in the low AFC group and 2 oocytes in the high AFC group were accidentally lost after IVF during the vortexing procedure.
As shown in Table 1, 193 OCGCs in the low AFC group and 225 in the high AFC group were subjected to IVG followed by IVM for the evaluation of oocyte volumes on day 0 of IVG. Granulosa cell characteristics were evaluated on every 4 days of IVG (days 0, 4, 8, and 12). 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, oocyte-cumulus complexes (COCs) derived from surviving OCGCs were subjected to in vitro maturation (IVM). The concentrations of estradiol-17β (E2) and progesterone (P4) 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 volume and nuclear status of some oocytes were evaluated. The remaining oocytes were subjected to in vitro fertilization (IVF) and an in vitro culture (IVC) for the evaluation of developmental competence to blastocysts.

Fig. 2. Schematic of the experimental design. AFC in an ovary from the local abattoir was assessed by the number of antral follicles (≥ 2 mm in diameter), and we allocated ovaries having 25 or more follicles to the high AFC group and others to the low AFC group. OCGCs from each ovary were cultured for 0, 4, 8, or 12 days in an in vitro growth (IVG) culture. Oocyte volume was evaluated on day 0 of IVG. Granulosa cell characteristics were evaluated on every 4 days of IVG (days 0, 4, 8, and 12). 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, oocyte-cumulus complexes (COCs) derived from surviving OCGCs were subjected to in vitro maturation (IVM). The concentrations of estradiol-17β (E2) and progesterone (P4) 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 volume and nuclear status of some oocytes were evaluated. The remaining oocytes were subjected to in vitro fertilization (IVF) and an in vitro culture (IVC) for the evaluation of developmental competence to blastocysts.

Fig. 3. Relationship between AFC and number of collected OCGCs in each ovary. A total of 186 ovaries as the low AFC group and 70 ovaries as the high AFC group were used, and 388 OCGCs in the low AFC group and 410 OCGCs in the high AFC group were collected. *-† Different letters indicate significant differences between different AFC (P < 0.05). Error bars indicate SEM.

As shown in Table 1, 193 OCGCs in the low AFC group and 225 in the high AFC group were subjected to IVG followed by IVM for the evaluation of oocyte volumes on day 0 of IVG and after IVM and the percentage of metaphase II oocytes after IVM. E2 and P4 concentrations were measured in spent media used for the IVG culture of 95 OCGCs in the low AFC group and 110 OCGCs in the high AFC group, which were subjected to IVM. Steroid hormone production during each period (days 0 to 4, 4 to 8, and 8 to 12) was calculated based on concentrations at the beginning and end of the period as previously described [14]. After IVM, 44 OCGCs in low AFC group and 44 OCGCs in high AFC group were subjected to IVF and IVC for the evaluation of developmental competence. As shown in Table 2, the remaining 151 OCGCs in the low AFC group and 141 OCGCs in the high AFC group were subjected to an evaluation of the characteristics of granulosa cells on days 0, 4, 8, and 12. The viability of OCGCs was calculated based on all cultured OCGCs. The percentage of antrum formation in the granulosa cell layer was calculated based on OCGCs judged as surviving on day 12.
AFC AND STEROIDOGENESIS IN CATTLE

Table 1. Number of oocyte-cumulus-granulosa complexes (OCGCs) cultured for the evaluation of nuclear maturation, production of estradiol-17β (E2) and progesterone (P4), and developmental competence

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of OCGCs used for oocyte volume and nuclear maturation</th>
<th>No. of OCGCs used for oocyte developmental competence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cultured</td>
<td>Survived</td>
</tr>
<tr>
<td>Low</td>
<td>193</td>
<td>111</td>
</tr>
<tr>
<td>High</td>
<td>225</td>
<td>137</td>
</tr>
</tbody>
</table>

<sup>a</sup> Four oocytes were lost during pipetting after in vitro maturation. <sup>b</sup> Three oocytes were lost during vortexing after in vitro fertilization. <sup>c</sup> Two oocytes were lost during vortexing after in vitro fertilization. The numbers in parentheses indicate rates of cleaved oocytes or oocytes developed to blastocysts after in vitro fertilization and in vitro culture. The cell numbers in blastocysts were 65 and 88 in the high AFC group.

Table 2. Number of oocyte-cumulus-granulosa complexes (OCGCs) cultured for the evaluation of granulosa cell characteristics

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total</th>
<th>Day 0</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cultured</td>
<td>Survived</td>
<td>Cultured</td>
<td>Survived</td>
</tr>
<tr>
<td>Low</td>
<td>151</td>
<td>26</td>
<td>34</td>
<td>32</td>
<td>38</td>
</tr>
<tr>
<td>High</td>
<td>141</td>
<td>27</td>
<td>27</td>
<td>23</td>
<td>37</td>
</tr>
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</table>

Relationship between AFC, viability, and antrum formation of OCGCs

As shown in Fig. 4, the viabilities of OCGCs in the high and low AFC groups decreased throughout the culture period (P < 0.05) and did not significantly differ between the two groups. The percentages of OCGCs having antrum increased throughout the culture period, and became higher in the high AFC (78.8%) group than in the low AFC group (67.7%, P < 0.05) on day 12.

Relationship between AFC and the steroidogenesis of granulosa cells

As shown in Fig. 5, the two-way ANOVA showed that the interactions between AFC and the culture duration on E2 production and the E2/P4 ratio (P < 0.01), and E2 production and the E2/P4 ratio were affected by AFC (P < 0.05) and the culture duration (P < 0.01). P4 production was affected by the culture duration, but not by AFC. E2 production from days 4 to 8 showed the highest values in all culture periods, and E2 production from days 4 to 8 was higher in the high AFC group than in the low AFC group (P < 0.05). P4 production increased with the extension of the culture period (P < 0.05), and did not significantly differ between the two groups. The E2/P4 ratio in the high AFC group did not decrease until day 8, and was higher than that in the low AFC group (P < 0.05) on day 8; however, the E2/P4 ratio decreased with the extension of the culture period (P < 0.05) in the low AFC group.

Discussion

In the present study, E2 production was greater in the high AFC
higher nuclear maturation rate and blastocyst development in the high AFC group. The present results on oocyte competence support our previous findings showing that oocytes derived from early antral follicles in ovaries with high AFC had a greater maturational ability, and were assumed to have greater fertilizability than ovaries with low AFC [9]. Moreover, the present study suggests that greater E₂ production and a higher E₂/P₄ ratio contributed to superior oocyte competence in the high AFC group.

Granulosa cell numbers and viabilities were similar between the two groups in the present study; however, the proliferation of granulosa cells was greater in the high AFC group than in the low AFC group. Furthermore, we confirmed a higher nuclear maturation rate and blastocyst development in the high AFC group. The present results on oocyte competence support our previous findings showing that oocytes derived from early antral follicles in ovaries with high AFC had a greater maturational ability, and were assumed to have greater fertilizability than ovaries with low AFC [9]. Moreover, the present study suggests that greater E₂ production and a higher E₂/P₄ ratio contributed to superior oocyte competence in the high AFC group.

Granulosa cell proliferation may be related to the addition of different steroid hormones to culture media in the two studies. We used a high concentration of E₂ in the previous study [9], whereas we used A₄ in the present study. Taketsuru et al. [25] reported that the viability of OCGCs was approximately 80% when OCGCs were cultured in E₂-supplemented medium for 14 days, but approximately 65% when OCGCs were cultured in A₄-supplemented medium. These findings suggest that the effects of A₄ on the enhancement of granulosa cell proliferation were weaker than those of E₂. The mean number of granulosa cells was 8.5 × 10⁴ cells after 12 days of IVG in our previous study using E₂-supplemented medium [18], but were 5.6 × 10⁴ and 6.6 × 10⁴ cells in the low and high AFC groups, respectively, in the present study. Although our previous study evaluating the effects of AFC on the granulosa cell number using E₂-supplemented medium [9] showed lower values (approximately 4.0–5.0 × 10⁴ cells) than the present results, we collected and counted granulosa cells after retrieving COCs [9], thereby reducing the number of remaining granulosa cells in a well.
The mean diameter of granulosa cells was larger in the low AFC group (11.3 ± 0.4 µm) than in the high AFC group (10.2 ± 0.3 µm) even before IVG. Previous studies reported that in vivo-grown large luteal cells (38.4 µm in diameter) originated from granulosa cells and in vitro-luteinized granulosa cells (38.4 µm) were larger than granulosa cells in preovulatory follicles (10.6 µm) [26, 27]. In addition, Scheetz et al. [28] suggested that P₄ production and the expression level of the oxytocin-neurophysin I precursor, both of which are markers of granulosa cell luteinization, were higher in granulosa cells from low AFC ovaries having 15 or fewer follicles in a pair of ovaries than from high AFC ovaries having 25 or greater follicles; however, they did not measure the diameter of granulosa cells. The difference in the diameter of granulosa cells may support luteinization or luteinization-like changes in the low AFC group, although P₄ production was not significantly different between both groups in the present study. Endo et al. [16] reported that E₂ promoted the growth and maturational competence of oocytes derived from early antral follicles using bovine IVG. Moreover, our previous findings [14] suggested that E₂ production by OCGCs producing matured oocytes after IVM was slightly higher than that of OCGCs producing immature oocytes after IVM. In the present study, the development rate to blastocyst was 9.1% in the high AFC group, and the developmental competence was similar to that of bovine oocytes with < 115 µm in diameter (11.9%) in our previous study [29]. On the other hand, we could not produce blastocysts from the oocytes in the low AFC group, although the cleavage rate after IVF was similar between both groups. The result may indicate the impaired developmental competence of oocytes in the low AFC group. In our previous study [9], some oocytes in the low AFC group developed to blastocysts when we used E₂ (1 µg/ml) for the growth medium instead of A₄ to increase the E₂/P₄ ratio like a dominant follicle [13]. E₂ addition may enhance the granulosa cell proliferation because the numbers of granulosa cells were relatively greater in the previous study (8.5 × 10⁴ cells/well at day 12) [18] compared to the present results, 5.6 and 6.6 × 10⁴ cells/well in the low and high AFC groups, respectively. These results suggest that the impaired E₂ production of granulosa cells in the low AFC group had a negative impact on oocyte growth, maturation, and developmental competence. In addition, we performed 10-h culture of in vivo-grown oocytes with low FSH containing medium before IVM culture (pre-IVM) in the previous study [9]. Also, we reported that pre-IVM improved the developmental competence of bovine oocytes derived from IVG [11] and oocytes with < 115 µm in diameter [29]. In further study, we should examine the effects of E₂/P₄ ratio during IVG culture and FSH treatment before IVM on the acquisition of developmental competence of in vitro-grown oocytes.

Scheetz et al. [28] also reported that FSH-mediated E₂ production was lower in granulosa cells from low AFC ovaries than from high AFC ovaries by culturing granulosa cells under serum-free conditions. However, we previously demonstrated that the addition of FSH did not enhance E₂ production and accelerated P₄ production [14], which may have been due to the addition of serum to the medium.
for the IVG culture. Granulosa cells cultured in media containing serum were found to have luteinized, compromised 
expression of oocyte competence. In future studies, we need to develop an IVG system that does not use serum, but enhances oocyte competence, or that inhibits the luteinization of granulosa cells even when growth medium contains serum.

In conclusion, E2 production by bovine granulosa cells cultured as OCGCs was greater in the high AFC group than in the low AFC group. These results indicate that granulosa cells in the low AFC group are starting luteinization, and the reduced production of E2 by granulosa cells in the low AFC group may impair the growth and meiotic competence of oocytes. In future studies, we need to identify possible factors inducing luteinization-like changes in the low AFC group.

Acknowledgements

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