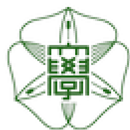
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The relationship between antral follicle count in a bovine ovary and developmental competence of *in vitro*-grown oocytes derived from early antral follicles

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ABSTRACT

To clarify the relationship between ovarian reserve and the developmental competence of bovine oocytes, oocyte-granulosa complexes (OGCs) collected from early antral follicles (≤1 mm) in ovaries with high (≥25) and low (<25) antral follicle counts (AFCs) were used. OGCs derived from different AFC groups were cultured for growth followed by maturation, fertilization and blastocyst formation. Viability of OGCs during growth culture was similar between groups; however, OGCs in the high-AFC group had a larger number of granulosa cells than the low-AFC group at 12 days of growth. The proportion of matured oocytes in the high-AFC group was higher than that in the low-AFC group. Mitochondrial activity of oocytes before maturation in the high-AFC group was higher than that in the low-AFC group; however, accumulation of reactive oxygen species was similar between groups. Cleavage rate in the high-AFC group tended to be higher than that in the low-AFC group, although blastocyst development was similar between groups. In conclusion, oocytes derived from ovaries with high AFC have higher maturational ability and fertilizability than those from low AFC. The difference may be caused by high proliferation of granulosa cells from ovaries with high AFC.

Major functions of ovaries are producing fertilizable oocytes having developmental competence that result in successive conception and secreting sex steroid hormones that induce the estrous cycle and sustain pregnancy. Ovarian reserve is defined as potential ability of these functions (38), and is proposed as a factor of the fertility for mammalian female animals including humans (6) and cattle (22). The number of small antral follicles (antral follicle count; AFC) in a pair of ovaries detected by ultrasonography is considered as an indicator of ovarian reserve (7), because it correlates with the number of primordial follicles (23) and is stable in individual animals (1, 10).

During the long lifetime of women, ovarian reserve decreases as aging. Therefore, the relationship between oocyte quality and ovarian reserve in aged women is well investigated, and it is reported that aged women with small ovarian reserve had low quality oocytes (12, 32). In addition, the time of onset of menopause is closely correlated with ovarian reserve; small reserve resulted in earlier menopause (11). The size of the ovarian reserve varies between individuals of the same species or strain, but the relationship between oocyte quality and small ovarian reserve of young women is not known. Therefore, we need to investigate the relationship between oocyte quality and ovarian reserve of young animals.

It is reported that dairy cattle can be used as a novel experimental model for women reproduction; because cattle are single-ovulating species that have

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two or three well characterized FSH-induced waves of growth and atresia of antral follicles during their reproductive cycles (16) similar to women (2). Cows with low AFC, estimating small ovarian reserve, tend to have low fertility; for example, a long open period (27), low steroidogenic capability (25) and poor responsiveness to superstimulatory treatments (21) even though they were in the same age class. In the previous study (28), we used the high- and low-AFC cows (6.9 ± 1.8 and 6.6 ± 2.6 years old, respectively) and showed that oocytes collected by ovum pick-up from the high-AFC cows had higher fertilizability than those from the low-AFC cows at 3 to 4 days interval of ovum pick-up. However, only in the high-AFC cows, the quality of oocytes was impaired by extending interval of ovum pick-up to 7 days, possibly due to the earlier degradation of follicles compared to the low-AFC cows. From these results we have speculated that the growth rate of follicles is different between the high- and low-AFC cows, and the difference affects on the quality of oocytes.

Dairy cows are commonly culled before ending their fertile life, and we can collect their ovaries at an abattoir. Ministry of Agriculture, Forestry and Fisheries in Japan reported that mean parity of culled Holstein cows was 3.5 in 2012. From the mean parity, the age at culling was estimated around 7 years old. It means that we can investigate the relationship between oocyte quality and ovarian reserve of young animals. However, it is difficult to use the conventional *in vitro* maturation, fertilization and culture system for clarifying the relationship between ovarian reserve and oocyte quality. Because oocytes derived from antral follicles of 2–8 mm in diameter, which are commonly used in the conventional culture system, are already at various stage of degradation (14, 30) and have various maturational and developmental competences depending on their degradation stages (13, 26, 29). For investigating oocyte quality correctly, we should use oocytes derived from follicles before degradation. Recently, we reported that *in vitro*-grown bovine oocytes derived from early antral follicles, just before recruitment to follicular waves (0.5–1 mm in diameter), achieved good development to blastocysts corresponding to the development of *in vivo*-grown oocytes (19, 20).

In the present study, to investigate the relationship between ovarian reserve estimated with AFC and oocyte quality, we cultured bovine oocyte-granulosa complexes (OGCs) collected from early antral follicles in ovaries with high and low AFCs. During culture for growth, we examined viabilities of OGCs and the proliferation of granulosa cells. Nuclear maturation, mitochondrial activity and the accumulation of reactive oxygen species (ROS) in oocytes after growth culture were also evaluated. Moreover, the embryonic development of oocytes derived from different AFC ovaries were investigated after *in vitro* fertilization.

MATERIALS AND METHODS

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

Collection of early antral follicles and growth culture of oocytes. Ovaries from Holstein cows obtained at a local abattoir were kept at 20°C and were transported to the laboratory within 6 to 10 h after collection. After 3 washes in physiological saline, sliced ovarian cortex tissues (<1 mm in thickness) were prepared using a surgical blade (No. 11) and stored in TCM-199 (Invitrogen, Grand Island, NY, USA) supplemented with 0.1% polyvinyl alcohol, 25 mM HEPES, 10 mM sodium bicarbonate, and 50 mg/mL gentamicin sulfate (isolation medium, pH 7.4, at 37°C), as described by Huang *et al.* (19). Under a stereomicroscope, early antral follicles (0.5–1.0 mm in diameter) were dissected from the sliced ovarian tissues using a surgical blade (No. 20). OGCs were isolated from follicles using a pair of fine forceps and those with normal appearance were individually cultured in 96-well culture plates (Falcon 353872; Becton Dickinson, Franklin Lakes, NJ, USA) with 200 µL of the growth medium for 12 days at 39°C in humidified air with 5% CO_2. The growth medium consisted of HEPES-buffered TCM-199 (Invitrogen) supplemented with 0.91 mM sodium pyruvate, 1 µg/mL estradiol-17β (E_2), 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (MW 360,000), and 50 µg/mL gentamicin sulfate. At the onset of growth culture, OGCs were photographed under an inverted microscope (CK 40; Olympus, Tokyo, Japan) attached with a CCD camera (Moticam 2000; Shimadzu Rika Corporation, Tokyo, Japan), and viability of OGCs was determined by their morphologies as previously reported (19). During growth culture, half (100 µL) of the growth medium was replaced with the same amount of fresh medium every 4 days.

Evaluation of granulosa cell proliferation during growth culture. The OGCs with morphologically normal appearance at 8 and 12 days of growth cul-

ture were used for granulosa cell count. The number of granulosa cells in a well was measured by a cell counter (Luna FL Dual Fluorescence Cell Counter; Logos Biosystems, Virginia, USA). Briefly, after removing cumulus-oocyte complexes and 175 μL of culture media from a well, 100 μL of Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS(−); Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.125% trypsin and 0.05% EDTA was added to a well. After 10 min of trypsinization and pipetting several times, 25 μL of FCS was added to stop the digestion. Then 18 μL of cell suspensions was mixed with 2 μL of acridine orange/propidium iodide cell viability kit (F23001; Logos Biosystems), and the numbers of live and dead granulosa cells were counted.

Prematurational and maturational culture of in vitro-grown oocytes. Before maturational culture, the cumulus-oocyte complexes recovered from morphologically normal OGCs were cultured individually in a well in microwell plates (Mini Trays 163118; NUNC, Roskilde, Denmark) that contained 6 mL of prematuration medium as described previously (20). The prematuration medium consisted of HEPES buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 2×10^{-6} units/mL FSH (from porcine pituitary), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μg/mL E_2, 10% FCS, and 50 μg/mL gentamicin sulfate at 39°C in humidified air with 5% CO_2 for 10 h. Some of cumulus-oocyte complexes were subsequently cultured for another 22 h at 39°C in humidified air with 5% CO_2 for maturation. The maturation medium consisted of HEPES buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/mL FSH, 1 μg/mL E_2, 10% FCS, and 50 μg/mL gentamicin sulfate, and each cumulus-oocyte complex was cultured individually in a well in microwell plates (31).

Evaluation of oocyte nuclear status before and after maturation. The cumulus-oocyte complexes before and after maturational culture were denuded from cumulus cells by vortexing, fixed with fixative solution (75% ethanol and 25% acetic acid), and stained with 1% aceto-orcein solution as described previously (29). Nuclear status was examined under a phase-contrast microscope as follows: germinal vesicle, metaphase I, anaphase I/telophase I, and metaphase II. Oocytes at metaphase II stage were defined as matured.

Evaluation of mitochondrial activity and ROS accumulation in oocytes before and after maturation. Evaluation of mitochondrial activity and ROS accumulation in oocytes were performed as described previously (20). For evaluation of mitochondrial activity, oocytes before and after maturational culture were treated with 500 IU/mL of hyaluronidase in DPBS(−) for 10 min, then oocytes were denuded from cumulus cells by repeat pipetting using a fine pipette. Denuded oocytes were incubated for 15 min in the dark at 37°C in DPBS(−) supplemented with 1 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1, Mitochondrial Membrane Potential Detection Kit JC100; Cell Technology Inc., Fremont, CA, USA), 1 μg/mL Hoechst 33342 and 10% FCS. Images of oocytes were acquired using a digital fluorescent microscope (BZ-9000; Keyence, Osaka, Japan) and the mean fluorescence intensity of the images was calculated using analysis software (BZ-H2A; Keyence). Nuclear statuses of oocytes were evaluated by blue fluorescence of Hoechst 33342, and oocytes having germinal vesicle before maturational culture and oocytes progressed to metaphase II stage after maturational culture were subjected to the evaluation of mitochondrial activity. Membrane potentials of mitochondria in oocytes were calculated as the ratio of florescent intensity of activated mitochondria, expressed as red fluorescence of JC-1 staining, to less activated mitochondria, expressed as green fluorescence of JC-1 staining ($\Delta\psi$m, red/green fluorescent intensity).

Oocytes before and after maturational culture were denuded from cumulus cells by vortexing. Denuded oocytes were incubated for 15 min in the dark at 37°C in DPBS(−) supplemented with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA), 1 μg/mL Hoechst 33342 and 10% FCS. Oocytes at germinal vesicle stage before maturational culture and at metaphase II stage after maturational culture were subjected to the evaluation of ROS accumulation, and images of them were acquired using a digital fluorescent microscope. The mean fluorescent intensity of the images was calculated using analysis software, and ROS accumulation in oocytes was defined as the mean fluorescent intensity of oocytes.

In vitro fertilization and culture of oocytes. After maturational culture, some oocytes were co-cultured with spermatozoa (5×10^6 cells/mL) in 100-μL droplets (5–13 oocytes/droplet) of fertilization medium covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2, 5% O_2, and 90% N_2 for 18 h (29). Fertilization medium was modified Brackett and Oliphant isotonic medium (5) supplemented

with 2.5 mM theophylline and 3 mg/mL fatty acid-free bovine serum albumin (BSA). After the thawing of frozen semen from a Holstein bull, motile sperm were separated using a Percoll (GE Healthcare, Pittsburgh, PA, USA) gradient (45% and 90%). Then inseminated oocytes (presumptive zygotes) were cultured as previously described (37). Briefly, after co-incubation with spermatozoa, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured in 30-μL droplets (5–26 zygotes/droplet) of culture medium covered with mineral oil in a humidified atmosphere at 39°C with 5% CO_2, 5% O_2, and 90% N_2 for 6 days. The culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, 7 nonessential amino acids for minimum essential medium, 10 mg/mL insulin, and 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/mL fatty acid-free BSA. Cleavage and blastocyst rates were determined after 2 days (approximately 30 h) and 6 days (approximately 150 h) of culture, respectively. Total cell numbers in a blastocyst obtained after 6 days of culture were counted using an air-drying method (36).

Experimental group. AFC in an ovary was determined by the number of antral follicles of ≥2 mm in diameter. Usually, AFC means the number of follicle in pair of ovaries, but we could not collect the pair of ovaries from a cow at the abattoir. For adapting the criteria of AFC (high: ≥25, intermediate: 16-24, and low: ≤15) described by Ireland *et al*. (22), we compared the AFC in an ovary detected by ultrasonography to the AFC detected by the direct inspection. In the preliminary study, the number of follicles in an ovary detected by direct inspection (15.5 ± 2.4, $n = 4$) after ovariectomizing was approximately double compared to the number of follicles detected by ultrasonography (7.0 ± 1.4, $n = 4$). Cushman *et al.* (10) reported positive correlation between the number of antral follicles in one ovary and that in the contralateral ovary. Therefore, we allocated the ovary having more than 25 follicles of ≥2 mm in diameter as the high-AFC group and others as the low-AFC group. Then 189 and 342 ovaries were used as the high- and low-AFC groups, respectively.

Experimental design. During OGC collection, the number of early antral follicles and the number of collected OGCs in the high- and low-AFC groups were counted. OGCs having normal appearance were also counted and submitted to growth culture. The viability of cultured OGCs was examined every 4 days. In the present study, some of OGCs were used for the evaluation of proliferation of granulosa cells, and nuclear maturation, mitochondrial activity and ROS accumulation of oocytes.

Statistical analysis. The numbers of follicles and OGCs in high- and low-AFC ovaries were compared by Student's *t*-test. The viability of OGCs during growth culture was compared by Tukey-Kramer's HSD. The number of granulosa cells at 8 and 12 days of growth culture, mitochondrial activity and ROS accumulation in oocytes were compared by Student's *t*-test. The data of nuclear status was analyzed by chi-square test. Percentage data of cleavage and blastocyst rates were subjected to an arcsine square-root transformation, and analyzed by Student's *t*-test. Cell numbers in blastocysts were also analyzed by Student's *t*-test. All analyses were performed using software (JMP Pro 10.6; SAS Institute Inc., Cary, NC, USA). Values were considered significantly different at $P < 0.05$.

RESULTS

As shown in Table 1, the mean number of early antral follicles dissected from one ovary in the high-AFC group was larger than that from the low-AFC groups ($P < 0.01$). The mean numbers of OGCs collected and OGCs having normal appearance in the high-group were larger than those in the low-AFC group. However, the proportion of OGCs having normal appearance based on total OGCs was similar between the high- and low-AFC groups (66.0 ± 0.1% and 67.4 ± 0.2%, respectively).

As shown in Fig. 1, the proportions of live OGCs in both groups were similar and decreased until 8 days of growth culture. As shown in Table 2, there was no difference in the mean number of granulosa cells in a well between the high- and low-AFC groups at 8 days of growth culture; however, the proportion of live cells in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$). Numbers of granulosa cells in both groups increased from 8 to 12 days of growth culture. Although the high-AFC group had larger number of cells than the low-AFC group at 12 days of growth culture ($P < 0.05$), the proportions of live cells were similar between groups.

As shown in Table 3, the proportions of oocytes at germinal vesicle stage before maturational culture were similar between the high- and low-AFC groups. However, the proportion of oocytes at metaphase II

Table 1 *Relationship between AFC, numbers of early antral follicles and oocyte-cumulus-granulosa complexes (OGCs)*

AFC group	No. of ovaries (replicates)	AFC* (range)	No. of early antral follicles collected (*n*)	No. of OGCs collected (*n*)	No. of OGCs with normal appearance (*n*)
High (≥25)	189 (72)	37.4 ± 10.2^a (25–80)	19.5 ± 7.0^a (3687)	14.8 ± 5.6^a (2796)	10.0 ± 3.5^a (1806)
Low (<25)	342 (69)	16.7 ± 4.7^b (9–24)	9.5 ± 3.9^b (3259)	6.9 ± 3.4^b (2372)	4.8 ± 1.5^b (1563)

Values are mean ± SD.
* Antral follicle count: number of antral follicles of ≥2 mm in diameter.
[a, b] Different superscripts indicate a significant difference between groups ($P < 0.01$).

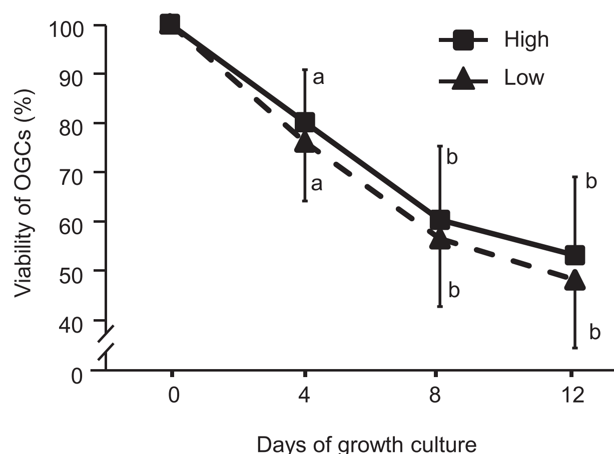

Fig. 1 Effects of antral follicle count (AFC) on viability of oocyte-granulosa complexes (OGCs) during *in vitro* growth culture. The numbers of cultured OGCs in the high- and low-AFC groups were 1,701 (68 replicates) and 1,490 (65 replicates), respectively. Solid line shows viability of OGCs in the high-AFC group and dashed line shows it in the-low AFC group. [a, b] Different letters indicate a significant difference between days of growth culture in each group.

stage after maturational culture in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$).

As shown in Fig. 2, before maturational culture (at germinal vesicle stage), the mitochondrial activity of oocytes in the high-AFC group was higher than that in the low-AFC group ($P < 0.05$). After maturational culture (at metaphase II stage), the mitochondrial activities of oocytes in both groups increased compared to those before maturational culture ($P < 0.05$) and were similar between the high- and low-AFC groups. As shown in Fig. 3, before and after maturational culture, ROS accumulations of oocytes in both AFC groups were similar; however, ROS accumulation decreased after maturational culture compared to before maturational culture in both groups ($P < 0.05$).

The results of embryonic development of oocytes were described in Table 4. Cleavage rate in the high-AFC group tended to be higher than that in the low-AFC group ($P = 0.09$). There were no significant differences in both blastocyst rates based on inseminated and cleaved oocytes, although values of standard deviation were large. The numbers of cells in blastocysts in both groups were similar between groups.

DISCUSSION

In the present study, the high-AFC group had larger number of early antral follicles (≤1 mm) in an ovary than the low-AFC group, and the proportion of oocytes having normal appearance was similar between groups. Our results correspond to the previous report that AFC in an ovary was positively correlated to number of follicles at various stages, and the proportion of healthy follicles was similar between the high- and low-AFC groups (23). However, the number of granulosa cells at 12 days of growth culture in the high-AFC group was larger than those in the low-AFC group. The high viability of granulosa cells at 8 days of growth culture in the high-AFC group might contribute to larger number of granulosa cells at 12 days of growth culture. These results seem to indicate that the function of granulosa cells is different depending on their origin of ovaries with different AFC. In the present study, oocytes in the high-AFC group also showed higher competence of nuclear maturation than that in the low-AFC group. Granulosa cells regulate the meiotic resumption of oocytes through gap junction (35). It suggests that granulosa cells derived from the high-AFC cows are able to regulate the functions of meiosis more properly. Scheetz *et al*. (34) reported that the cultured granulosa cells derived from the low-AFC cows showed lower secretion of estradiol, which is essential for granulosa cell proliferation (17). In the present study, we added the much concentration (1 μg/

Table 2 *Relationship between AFC, granulosa cell proliferation and survivability during in vitro growth culture*

Duration of growth culture	AFC group	No. of oocytes (replicates)	Number of granulosa cells (×10^3)		Proportion (%) of live cells
			total	live	
8	High	54 (5)	37.0 ± 22.9	33.1 ± 22.4	88.7 ± 15.4[a]
	Low	45 (5)	32.5 ± 23.7	28.0 ± 23.7	79.9 ± 24.1[b]
12	High	78 (9)	54.5 ± 32.1[a]	39.3 ± 32.3[a]	69.3 ± 28.8
	Low	72 (9)	37.9 ± 20.7[b]	24.9 ± 16.0[b]	71.1 ± 27.3

Values are mean ± SD.
[a, b] Different superscripts indicate a significant difference ($P < 0.05$).

Table 3 *Nuclear status of oocytes derived from ovaries with high and low AFCs before and after in vitro maturation*

In vitro maturation	AFC group	No. of oocytes (replicates)	Proportion (%) of oocytes at			
			GV	M I	A I / T I	M II
Before	High	70 (5)	90.0	8.6	2.3	0
	Low	65 (5)	83.1	17.0	0	0
After	High	157 (9)	0	15.9	1.9	82.1[a]
	Low	118 (9)	0	22.9	7.6	69.5[b]

GV: germinal vesicle, M I: metaphase I, A I/T I: anaphase I or telophase I, M II: metaphase II.
[a, b] Different superscripts indicate a significant difference ($P < 0.05$).

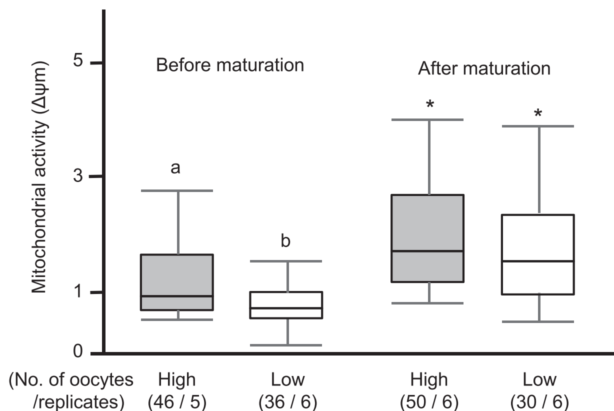

Fig. 2 Mitochondrial activity (Δψm) in ooplasm of oocytes before and after maturational culture in the high- and low-AFC groups evaluated by the ratio of red to green fluorescence intensity stained with JC-1. Oocytes at germinal vesicle stage and at metaphase II stage were used for evaluation of mitochondrial activity before and after *in vitro* maturation, respectively. Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. [a, b] Different letters indicate a significant difference between groups. * Asterisk indicates a significant difference before and after maturation in same group.

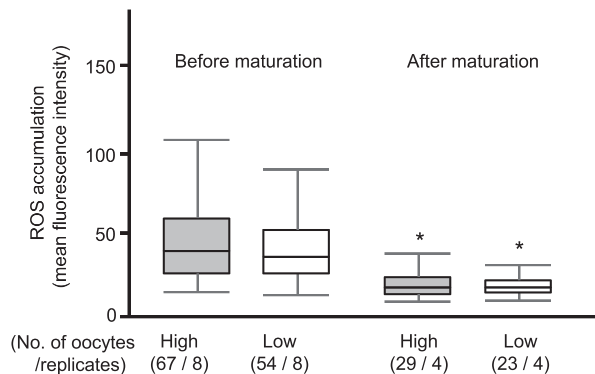

Fig. 3 Accumulation of reactive oxygen species (ROS) in ooplasm of oocytes before and after maturational culture in the high- and low-AFC groups evaluated by mean green fluorescence intensity stained with DCHFDA. Oocytes at germinal vesicle stage and those at metaphase II stage were used for evaluation of ROS accumulation before and after *in vitro* maturation, respectively. Lines of the boxes delineate the 25th, 50th and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. * Asterisk indicates a significant difference before and after maturation in same group.

mL) of estradiol to growth medium compared to that in the bovine dominant follicle of 10.5 mm in diameter (around 700 ng/mL) (3). It means that the estradiol is enough for proliferation of granulosa cells. However, Scheetz *et al*. (34) also reported that the granulosa cells derived from the low-AFC cows secreted more progesterone than that from the high-AFC cows. For developing follicles healthily, the ratio of estradiol to progesterone is very important (3). In further study, we should examine the function of

***Table 4** Fertilizability and developmental competence of oocytes derived from ovaries with high and low AFCs*

AFC group	No. of oocytes (replicates)	Cleavage rate (*n*)	Blastocyst rate (%) based on		Cell number in blastocysts (*n*)
			inseminated oocytes	cleaved oocytes	
High	127 (9)	65.3 ± 11.7 (84)	11.4 ± 10.0	19.9 ± 20.3	81.7 ± 34.4 (13)
Low	80 (8)	45.0 ± 30.6 (42)	8.4 ± 15.9	13.7 ± 26.1	86.6 ± 31.8 (5)

Values are mean ± SD.

steroidogenesis of granulosa cells derived from different AFC cows. Another possibility of the cause of difference in the proliferation of granulosa cells is the difference of oocyte function between groups. Differentiation and proliferation of granulosa cells are regulated by oocyte secreted factors (OSFs) such as GDF-9 or BMP-15 (15). Therefore, the secretion of OSFs from oocytes may be different between the high- and low-AFC groups. In the previous study, Scheetz *et al*. (34) cultured only granulosa cells and examined the function of granulosa cells. For the correct evaluation of granulosa cell function of different AFC groups, the expression levels of OSFs in oocytes should be examined by co-culture with oocyte and granulosa cells.

Also the mitochondrial activity in oocytes before maturational culture was higher in the high-AFC group than in low-AFC group. Mitochondrial activity is one of indicators of cytoplasmic maturation of oocytes (4). Huang *et al*. (19, 20) reported that *in vitro*-grown oocytes acquired high mitochondrial activity after 10-h prematurational culture, and showed higher maturation and development competences compared to oocytes without prematurational culture. Jeseta *et al*. (24) reported that the kinetics of mitochondrial activity and adenosine triphosphate (ATP) production during maturational culture were influenced by the atretic grade of granulosa cells. This indicates the importance of the correlation between oocyte and granulosa cells. Low mitochondrial activity of oocytes may be affected by lower number and also lower function of granulosa cells in the low-AFC group. In the ROS accumulation, there was no difference between the high- and low-AFC groups by the measurement of mean fluorescent intensity in ooplasm; nevertheless there was difference in the mitochondrial activity before maturational culture. ROS in ooplasm is considered to be a byproduct of electron transport chain activity in mitochondria when producing ATP (33). Our results suggest that the activity of anti-oxidant enzyme in the high-AFC group is higher than that in the low-AFC group. Expression patterns of mRNA coding anti-oxidant enzymes were reported to change in according to days from follicular emergence (39), follicular size and their status (9) in cattle. It was also reported that mRNA expression level of glutathione peroxidase between dominant and subordinate follicles was different and that glutathione peroxidase could prevent cell apoptosis from oxidative stress during high steroidogenic period (18). Thus, evaluation of anti-oxidant enzyme expression in oocytes and granulosa cells derived from different AFC cows is necessary.

There was no obvious difference in blastocyst development after *in vitro* fertilization between the high- and low-AFC groups; however, the cleavage rate tended to be high in the high-AFC group. The result corresponds to the high fertilizability of *in vivo*-grown oocytes in the high-AFC group as we previously reported (28). There was no significant difference but the standard deviation of cleavage and blastocyst development in the low-AFC group was large, although the cell numbers in blastocysts were similar between groups. These results may indicate that some of oocytes derived from the low-AFC cows have relatively high developmental potential the same as those from the high-AFC cows. In the present study, we used 5–26 oocytes per replicate for evaluation of the embryonic development. It is well known that the number of oocytes cultured simultaneously affects the outcome of blastocyst production (8, 40). The low-AFC cows basically provide a low number of oocytes; therefore we should develop the culture system for a few numbers of oocytes for evaluating the developmental competence of oocytes and for producing offspring effectively from cows with low AFC but having high potential as a domestic animal. These researches will contribute the artificial reproductive technologies for infertile women with low AFC (small ovarian reserve).

In conclusion, oocyte-granulosa complexes derived from early antral follicles in ovaries with high AFC have higher maturational ability, and it is assumed

to have higher fertilizability than those from ovaries with low AFC. Higher proliferation of granulosa cells during growth culture and higher mitochondrial activity in ooplasm before maturational culture may support both nuclear and cytoplasmic maturation of *in vitro*-grown oocytes from ovaries with high AFC. In further study, we need to investigate factors and mechanisms of proliferation of granulosa cells and activation of mitochondria in oocytes derived from different AFC ovaries.

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