

Androgens promote the acquisition of maturation competence in bovine oocytes

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Abstract. Recent studies in mice suggest that androgens are important for normal follicle development. However, there have been few reports concerning the action of androgens in the growth of oocytes from large animals. The purpose of this study was to determine the roles of androgens in bovine oocyte growth *in vitro*. Oocyte-granulosa cell complexes (OGCs) collected from 0.4–0.7 mm early antral follicles were cultured for 14 days with 17 β -estradiol (E₂) and a non-aromatizable androgen, dihydrotestosterone (DHT). We also examined the ability of an androgen receptor (AR) inhibitor, hydroxyflutamide, to antagonize the effect of androgens on the oocytes. During growth culture, the OGC structures collapsed in the medium with DHT alone, while in the presence of E₂, the OGC structures were maintained. In the medium with both androgens and E₂, the mean diameter of oocytes was increased from 95 μ m to around 120 μ m, larger than those grown with E₂ alone (115 μ m). Also in the maturation culture, oocytes grown with androgens (A₄ or DHT) and E₂ showed higher percentages of metaphase II oocytes (63% or 69%, respectively) than those grown with E₂ alone (32%). Moreover, these maturation rates were decreased by hydroxyflutamide in a dose-dependent manner. Immunostaining showed that ARs were expressed in oocytes and granulosa cells in early antral follicles, and the nuclei of granulosa cells showed intense AR expression. In conclusion, although E₂ supports the OGC structure, additional androgens promote oocyte growth and their acquisition of meiotic competence via AR during *in vitro* growth culture.

Key words: Androgen, Cow, Oocyte growth, Oocyte maturation

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Female mammals are born with a huge number of oocytes in their ovaries, but only a small population of these oocytes begin to grow, and an even smaller number achieve the necessary growth and maturation to be ovulated [1]. In cows, less than 200 oocytes are ovulated over the reproductive life of an individual. Other oocytes stored in the ovaries degenerate before or during the growth phase. As these oocytes can be a potential source of fertilizable eggs, *in vitro* growth culture systems for oocytes are being developed in various mammalian species [2, 3].

Steroid hormones have crucial roles in folliculogenesis. Estrogens support follicle development by promoting granulosa cell proliferation and reducing atresia [4]. In contrast, androgens have been considered damaging for ovarian function [5–8]. However, recent studies using androgen receptor (AR)-knockout mice have revealed that the actions of androgens exerted through binding with ARs are important for normal follicle development [9–11]. Female AR-knockout mice showed decreased fertility, defective follicle development, a reduced ovulation rate, and premature ovarian failure. Androgen function has also been reported in *in vitro* culture of mouse ovarian tissues. Androgens increase the follicle diameter and enhance the development of preantral follicles [12–14]. Although there have been a few

reports concerning the maturation of *in vitro* grown oocytes from large animals, it has recently reported that androstenedione promotes the acquisition of meiotic competence in growing bovine oocytes *in vitro* [15, 16]. In these studies, however, whether androgens affect the acquisition of meiotic competence of oocytes directly is unclear because androstenedione can be aromatized to estrogens.

The objective of the present study was to determine the roles of androgens in bovine oocyte growth *in vitro*. We examined the effect of androgens, including a non-aromatizable androgen, dihydrotestosterone, on *in vitro* oocyte growth by culturing oocyte-granulosa cell complexes (OGCs) without theca cells. We also examined the expression of ARs in OGCs by the immunofluorescence staining and examined the ability of an AR inhibitor to antagonize the effect of androgens on the oocytes.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Collection of oocyte-granulosa cell complexes

Bovine ovaries were obtained from a local abattoir and transported to the laboratory. The ovaries were washed once in 0.2% (wt/vol) cetyltrimethylammonium bromide and three times in Dulbecco's phosphate-buffered saline containing 0.1% (wt/vol) polyvinyl alcohol (PBS-PVA). For collection of OGCs with fully grown oocytes, follicular fluids containing OGCs were drawn up from antral follicles (4–6 mm in diameter) using needles (18 ga; Terumo, Tokyo, Japan)

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and syringes; these OGCs served as the controls. For collection of OGCs with growing oocytes, ovarian cortical slices (1–1.5 mm) were made using a surgical blade (No. 10; Feather Safety Razor, Tokyo, Japan) and forceps. Under a dissecting microscope, early antral follicles (0.4–0.7 mm in diameter) were dissected from the cortices. The follicles were opened using forceps and a blade (No. 10) to isolate OGCs in 25 mM HEPES-buffered medium 199 (HEPES-199; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (wt/vol) PVA, 0.85 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin sulfate. After measuring the diameters of oocytes (excluding the zona pellucida) to the nearest 1 μm using an ocular micrometer attached to an inverted microscope, OGCs that contained oocytes of 90–100 μm in diameter were used for *in vitro* growth culture.

In vitro growth culture of oocytes

In vitro growth culture was performed according to a procedure described previously [16, 17] with slight modifications. Briefly, the OGCs with growing oocytes isolated from early antral follicles were individually cultured for 14 days in 0.2 ml of culture medium in 96-well culture plates (BioCoat Collagen I Cellware; BD Biosciences, San Jose, CA, USA) at 38.5 C under an atmosphere of 5% O₂, 5% CO₂ and 90% N₂ from day 0 to day 6, and an atmosphere of 5% CO₂ in humidified air from day 7 to day 14. The culture medium for oocyte growth was based on the medium used by Hirao *et al.* [17]. The basic medium was Minimum Essential Medium alpha medium (α -MEM; GIBCO, Invitrogen, Scotland, UK) supplemented with 5% (vol/vol) fetal bovine serum (FBS; ICN Biomedicals, Aurora, OH, USA), 4% (wt/vol) polyvinylpyrrolidone (molecular weight 360,000), 4 mM hypoxanthine, 50 $\mu\text{g}/\text{ml}$ ascorbic acid 2-glucoside (Hayashibara Biochemical Laboratories, Okayama, Japan), 55 $\mu\text{g}/\text{ml}$ cysteine, 0.05 μM dexamethasone, 1 mM sodium pyruvate, 2.2 mg/ml sodium bicarbonate and 0.08 mg/ml kanamycin sulfate. Using this medium as a control, two experiments were conducted. In the first experiment, 10 ng/ml of 17 β -estradiol (E₂; 37 nM), androstenedione (A₄; 35 nM; Tokyo Chemical Industry, Tokyo, Japan) or dihydrotestosterone (DHT; 34 nM) was added to the growth culture medium. The concentration was selected based on previous reports [15, 16]. In the second experiment, combinations of steroid hormones (0 or 10 ng/ml E₂, A₄, and DHT) and hydroxyflutamide (0, 1 or 5 $\mu\text{g}/\text{ml}$), which is an AR antagonist and is widely used as an AR inhibitor, were added to the growth culture medium.

In both experiments, the day of OGC isolation was designated day 0. The experiments were repeated at least three times. Seven to ten OGCs per group were cultured in each experiment, and 643 OGCs were used in total. Half of the culture medium was changed every other day after day 4. On days 0, 7 and 14, OGCs whose structures had collapsed—those, for example, that exhibited complete detachment of granulosa cells from oocytes and/or contained oocytes that showed cytoplasmic degeneration—were classified as degenerative OGCs. At the end of the 14-day culture, the diameters of oocytes that were enclosed by granulosa cells and showed no sign of degeneration were measured as described above, and subjected to further experiments.

Before and after *in vitro* growth culture, some oocytes were denuded, fixed with acetic acid-ethanol and then stained with 1% (wt/vol) aceto-orcein so that the stage of meiotic division could be assessed by Nomarski interference microscopy. The oocytes were

classified by the morphology of the chromatin and nuclear envelope according to the classifications of Motlik *et al.* [18] and Hirao *et al.* [19]. The stages for oocytes with an intact germinal vesicle were classified as filamentous chromatin (FC), stringy chromatin (SC), and germinal vesicle I–IV (GV). After germinal vesicle breakdown, the stages were classified as late diakinesis (LD), metaphase I (MI), anaphase I and telophase I (AI–TI), and metaphase II (MII).

In vitro maturation of oocytes

The OGCs with growing and fully grown oocytes collected from the early and large antral follicles, respectively, and those with surviving oocytes after 14 days of *in vitro* growth culture were further cultured in 50 μl microdrops of the maturation medium covered with paraffin oil at 38.5 C under an atmosphere of 5% CO₂ in humidified air for 22 h. Each microdrop contained 4–5 OGCs. The maturation medium was bicarbonate-buffered medium 199 supplemented with 10% FBS, 0.1 mg/ml sodium pyruvate, 0.1 IU/ml human menopausal gonadotropin (hMG; Asuka, Tokyo, Japan), 0.08 mg/ml kanamycin sulfate and 2.2 mg/ml sodium bicarbonate. After culture for 22 h, the oocytes were denuded mechanically using a small-bore pipette with the help of 0.1% (wt/vol) hyaluronidase. The oocytes were then fixed and stained to assess the stage of meiotic division, as described above.

Immunofluorescence staining for androgen receptors

The OGCs collected from early antral follicles were stained as follows. After being slightly denuded with a fine pipette, the oocytes with granulosa cells were washed twice in PBS–PVA for 15 min each and fixed in 4% (wt/vol) paraformaldehyde (PFA) in PBS–PVA containing 0.2% (vol/vol) Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 60 min. Fixed oocytes were washed three times in PBS–PVA for 15 min each and then blocked in PBS–PVA containing 1 mg/ml bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan) (PBS–PVA–BSA) at 4 C overnight. The oocytes were treated with a rabbit polyclonal anti-androgen receptor antibody diluted with PBS–PVA–BSA (1:5000, sc-816; Santa Cruz Biotechnology) for 2 h at room temperature. The binding specificity for ARs was determined by preadsorbing the antibody with a blocking peptide (10 $\mu\text{g}/\text{ml}$, sc-816P; Santa Cruz Biotechnology). After three 15-min washes in PBS–PVA–BSA, the oocytes were reacted with Alexa Fluor 488-labeled donkey anti-rabbit immunoglobulin G (IgG) (1:400, A21206; Molecular Probes, Invitrogen, Eugene, OR, USA) for 40 min at room temperature. The oocytes were then washed three times for 15 min each in PBS–PVA–BSA before being mounted on glass slides with ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (P36931; Molecular Probes, Invitrogen) and observed under a confocal laser scanning microscope (FV1000-KDM; Olympus, Tokyo, Japan).

The localization of androgen receptors *in vivo* was also determined immunohistochemically. Early antral follicles of 0.4–0.7 mm in diameter collected from bovine ovarian cortical slices were fixed in 4% PFA in PBS–PVA containing 0.2% Triton X-100 for 60 min. Fixed follicles were washed three times in PBS–PVA for 15 min each and then treated with PBS–PVA containing 5% (wt/vol) sucrose (PBS–PVA–Suc) for 30 min followed by 10%, 12.5%, 15%, and 20% PBS–PVA–Suc for 30 min each. Next, the follicles were embedded in a mixture of 33% (vol/vol) OCT compound (Sakura Finetek USA,

Torrance, CA, USA) in 20% PBS–PVA–Suc and frozen in liquid nitrogen. The embedded follicles were cut into 10 µm sections using a Microtome Cryostat (MICROM International, Walldorf, Germany). Follicular sections on APS-coated glass slides (Matsunami Glass Ind, Osaka, Japan) were refixed with 4% PFA in PBS–PVA containing 0.2% Triton X-100 for 15 min. After washing three times in PBS–PVA for 5 min each, they were blocked with PBS–PVA–BSA for 1 h and subsequently treated with the rabbit polyclonal anti-androgen receptor antibody (1:500, sc-816) diluted with PBS–PVA–BSA at 4 C overnight. After three 15-min washes in PBS–PVA–BSA, they were reacted with Alexa Fluor 488-labeled donkey anti-rabbit IgG (1:400, A21206) for 40 min at room temperature. Finally, they were washed three times for 15 min each in PBS–PVA–BSA, and treated with ProLong Gold Antifade Reagent with DAPI (P36931) and observed under a confocal laser scanning microscope.

Statistical analysis

For statistical analyses of the OGC integrity and the meiotic division of oocytes, data were subjected to one-way ANOVA followed by the Tukey-Kramer multiple range test (Excel software with the add-in Ekuseru-Toukei 2010; Social Survey Research Information, Tokyo, Japan). Differences among the mean (\pm SEM) diameters of *in vitro* and *in vivo* grown oocytes were analyzed using one-way ANOVA followed by the Tukey-Kramer multiple range test. Comparisons of the mean diameters of oocytes between the oocytes before culture and each *in vitro* cultured oocyte group were performed with an unpaired *t* test. Values of $P < 0.05$ were considered significant.

Results

In vitro growth of oocytes

Figure 1 shows the typical morphology of OGCs during growth culture. On day 1, each complex included an oocyte at the center with thin layers of surrounding granulosa cells (Fig. 1Aa1–d1 and Ba1–h1). The granulosa cells of each complex started to grow after day 1, and the complexes gradually developed. In the medium with E_2 or A_4 , the granulosa cells proliferated vigorously, and some complexes formed cavities in their granulosa cell layers (Fig. 1Ab2–3 and c2–3). However, in the medium without steroid hormones (none) or with DHT, proliferation of granulosa cells was less than in the medium with other steroid hormones, and oocytes gradually dissociated from granulosa cells by day 14 (Fig. 1Aa3 and d3). When cultured with combinations of E_2 and androgens (A_4 or DHT), the OGCs developed cavities in their granulosa cell layers and retained a dome-like structure throughout the culture period (Fig. 1Bc and f). In the medium containing hydroxyflutamide (OHF), OGCs also formed cavities, and the cavities further developed up to the end of culture (Fig. 1Bd, e, g and h).

The integrity of OGCs during growth culture is shown in Fig. 2. In the medium without steroid hormones (none), the OGC structure collapsed, and oocytes gradually became denuded between day 7 and day 14. Similarly, the integrity of OGCs cultured with DHT drastically declined between day 7 and day 14 (Fig. 2A). A higher integrity of OGCs was observed in the medium with E_2 or A_4 , and many OGCs in these media exhibited a sustained structure that contained viable oocytes enclosed by granulosa cells throughout the culture period.

Although DHT alone could not maintain the integrity of OGCs during culture, OGCs cultured with E_2 showed high integrity throughout the culture period regardless of the presence or absence of androgens (Fig. 2B). There were no effects of 1 µg/ml OHF (OHF1) on the morphology of OGCs during culture; however, high concentrations of OHF (OHF5) slightly decreased the OGC integrity (Fig. 2B).

The mean diameter of oocytes before and after growth culture is shown in Fig. 3. Oocytes became significantly larger in diameter after 14 days of growth culture in all culture media compared with the oocytes collected from 0.4–0.7 mm follicles (white box on the left in Fig. 3A and B). The increase in oocyte size was especially apparent in the medium with androgens (striped boxes in Fig. 3A), and the mean diameters of these oocytes were comparable to that of *in vivo* grown oocytes collected from 4–6 mm antral follicles (white box on the right in Fig. 3A). Similarly, oocytes cultured with both E_2 and androgens (dark gray boxes in Fig. 3B) grew to a larger size than those cultured with E_2 alone; the final size was comparable to that of *in vivo* grown oocytes. There were no significant differences between the mean diameters of oocytes grown in E_2+A_4 and E_2+A_4+OHF1 , or in E_2+DHT and $E_2+DHT+OHF1$; however, the oocyte size was smaller in the medium with 5 µg/ml OHF (dotted boxes in Fig. 3B).

Maturation competence of oocytes

Before maturation culture, growing oocytes collected from 0.4–0.7 mm early antral follicles ($n = 30$) were either at the FC (57% of oocytes) or SC (43%) stage, and *in vivo* grown oocytes collected from 4–6 mm follicles and *in vitro* grown oocytes cultured for 14 days were at the GV stage (29/29 and 62/67, respectively). After 22 h of maturation culture, growing oocytes collected from the early antral follicles remained in the germinal vesicle stages ($n = 62$, 35% FC and 65% SC), while *in vivo* grown oocytes (92/92) and almost all oocytes cultured for growth *in vitro* (318/325) underwent germinal vesicle breakdown, and some of these reached MII.

Figure 4 shows the percentages of MII oocytes to total oocytes that had been cultured for growth with steroid hormones and/or OHF. The maturation rate of oocytes cultured with E_2 (25%) was higher than that of oocytes grown without steroid hormones (3%) (Fig. 4A). Oocytes grown with A_4 matured to MII at a significantly higher rate (41%), although the maturation rate of MII oocytes cultured with DHT was low (7%) and was comparable to that of oocytes grown without steroid hormones. The percentages of MII oocytes to total oocytes used for growth culture with an AR inhibitor are shown in Fig. 4B. Oocytes grown with a combination of E_2 and A_4 showed a higher percentage of MII oocytes (63%) than those grown with E_2 alone (32%); this result is consistent with our previous work [16]. In this experiment, moreover, a high maturation rate (69%) was also found when oocytes were cultured for growth with a combination of E_2 and DHT. OHF decreased the rates of MII oocytes in a dose-dependent manner; 40% and 52% of oocytes grown with 1 µg/ml OHF matured to MII, while a few oocytes reached MII when cultured for growth with 5 µg/ml OHF. In addition, there were no significant differences between the maturation rates of oocytes grown with E_2 alone and those grown with steroid hormones plus 1 µg/ml OHF.

Although the percentages of MII oocytes out of the number of oocytes used for maturation culture were higher than those out of the number of oocytes used for growth culture, the effects of the

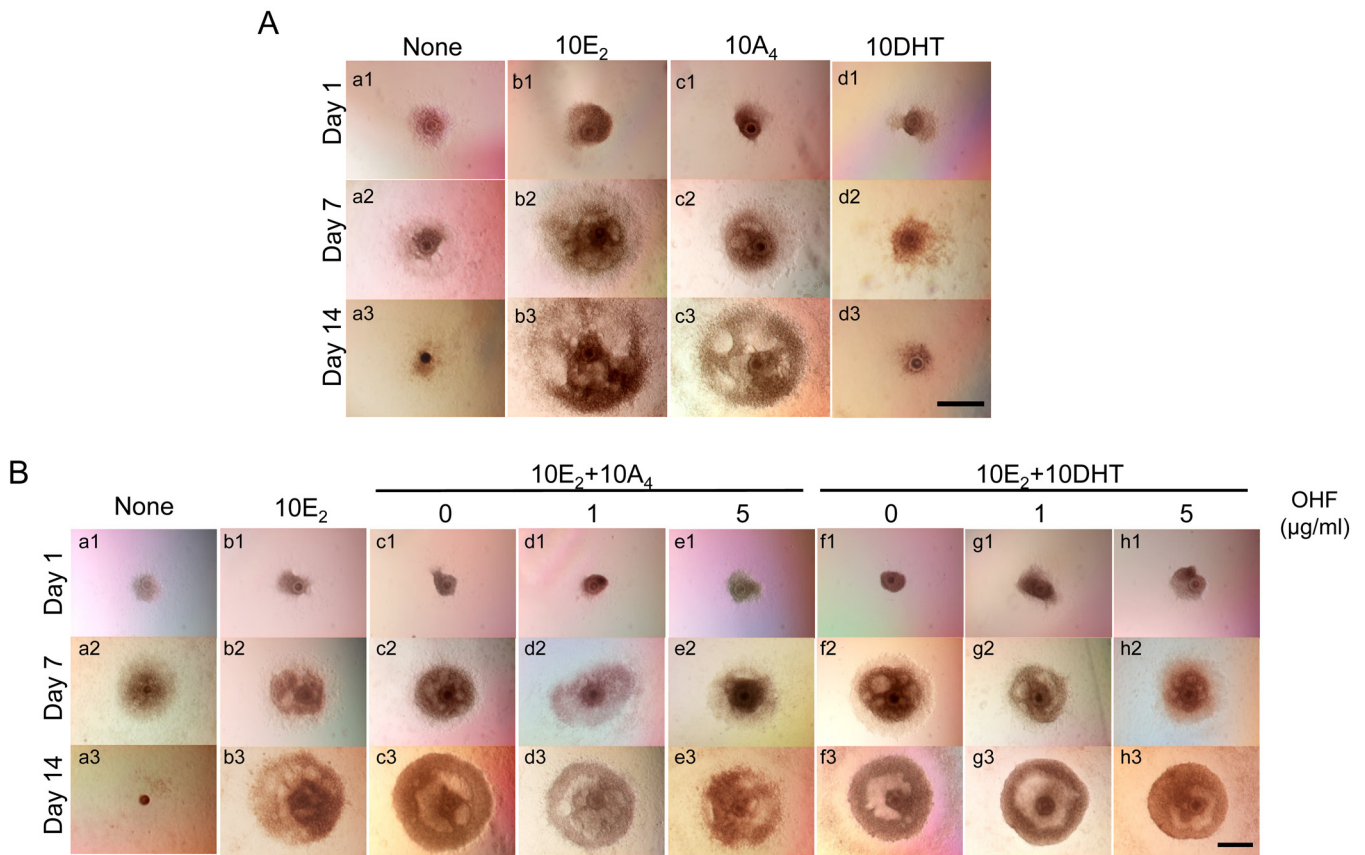


Fig. 1. Typical morphology of bovine oocyte-granulosa cell complexes (OGCs) during growth culture. OGCs were cultured for 14 days in the medium with steroid hormones alone (A) or with combinations of steroid hormones and OHF (B). E₂, 17β-estradiol; A₄, androstenedione; DHT, dihydrotestosterone; OHF, hydroxyflutamide; 10E₂, 10 ng/ml E₂; 10A₄, 10 ng/ml A₄; 10DHT, 10 ng/ml DHT; 10E₂ + 10A₄, 10 ng/ml E₂ plus 10 ng/ml A₄; and 10 E₂+10DHT, 10 ng/ml E₂ plus 10 ng/ml DHT. Scale bars represent 500 μm for each panel.

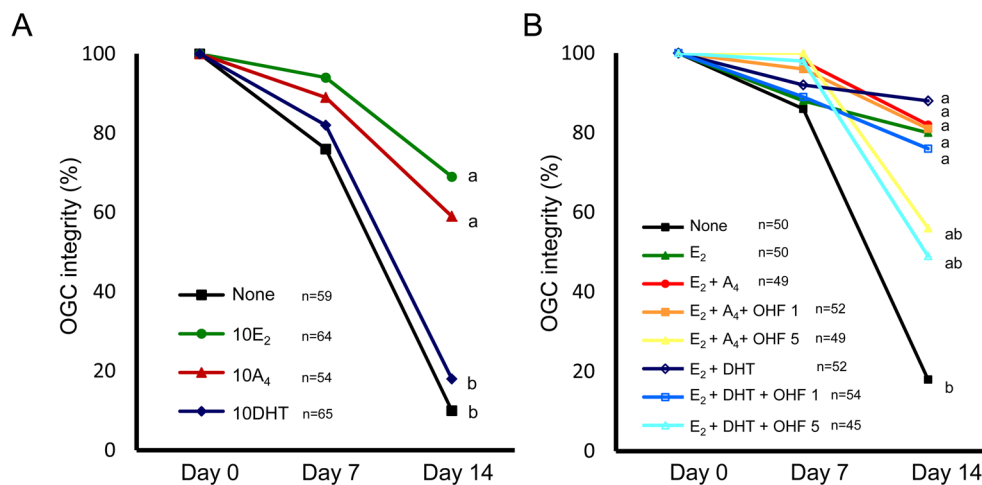


Fig. 2. Integrity of bovine OGCs during growth culture with steroid hormones alone (A) or with combinations of steroid hormones and OHF (B). On days 0, 7 and 14, OGCs that showed degenerative signs, such as cytoplasmic degeneration of oocytes and/or complete detachment of granulosa cells from oocytes, were classified as degenerative complexes. See the footnotes in Fig. 1 for abbreviations. ^{a,b} Values with different superscripts differ significantly ($P < 0.05$).

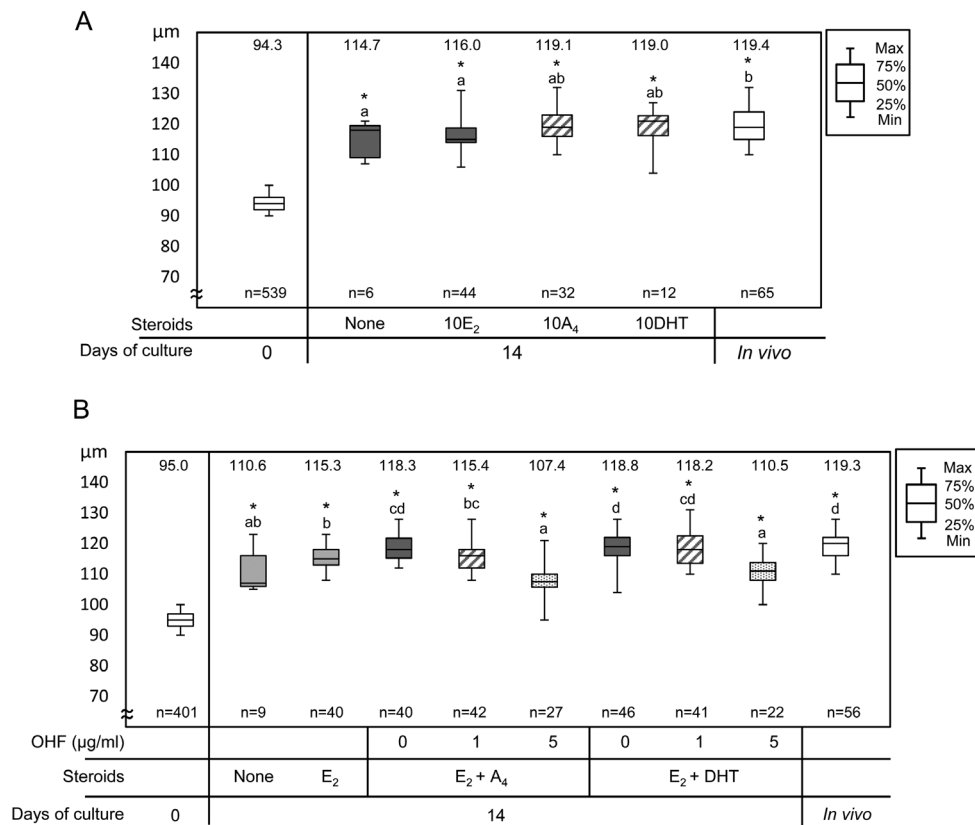


Fig. 3. Comparison of the diameters of bovine oocytes after 14 days of growth culture. For each panel, the white box on the left represents the diameter of oocytes isolated from early antral follicles (0.4–0.7 mm). The gray, striped and dotted boxes indicate the diameters of oocytes cultured for 14 days in the medium with or without steroid hormones and OHF. The white box on the right represents the diameter of oocytes obtained from antral follicles (4–6 mm). The numbers of oocytes examined are shown below each box (n), and the numbers above the boxes indicate the mean diameters of oocytes (µm). See the footnotes in Fig. 1 for the abbreviations. ^{a-d} Values with different superscripts differ significantly (P < 0.05). * Values were significantly different from those of oocytes before culture (white box on the left, P < 0.05).

steroid hormones and the inhibitor showed a similar pattern. Among the surviving oocytes on day 14, the maturation rate of oocytes cultured without steroid hormones was 33%, while 38%, 70% and 50% of oocytes matured to MII when they were cultured with E₂, A₄ and DHT, respectively. Oocytes grown with combinations of E₂ and androgens (E₂ + A₄ or E₂ + DHT) showed high maturation rates (78% or 78%), and the rates were comparable to that of *in vivo* grown oocytes (82%). When cultured with 1 µg/ml OHF, the percentages of MII oocytes out of the surviving oocytes used for maturation culture decreased to 50% (E₂ + A₄) or 68% (E₂ + DHT), and further decreased in the medium with 5 µg/ml OHF (0% for E₂ + A₄ or 14% for E₂ + DHT).

Expression of androgen receptors in early antral follicles and OGCs

Figure 5 shows the expression of ARs in 0.4–0.7 mm early antral follicles and OGCs collected from these follicles. In early antral follicles, AR expression was found in oocytes, granulosa cells and theca cells (Fig. 5A). Particularly intense AR immunosignals were observed in the nuclei of granulosa cells (Fig. 5Ac). In OGCs isolated from early antral follicles, AR expression in oocytes was

clearly visible (Fig. 5B). Oocytes showed overall AR staining in the cytoplasm and nuclei except in the nucleoli, whereas the nuclei of granulosa cells showed intense AR expression. Preadsorbing the AR antibody with the blocking peptide decreased the immunosignals of ARs in OGCs.

Discussion

We showed here that culturing bovine OGCs in media with combinations of E₂ and androgens resulted in better maturation rates for the oocytes than with E₂ alone. Moreover, oocytes cultured with E₂ and androgens grew to their full sizes; the diameters were similar to those of *in vivo* grown oocytes collected from 4–6 mm antral follicles. These results are consistent with our previous reports using A₄ for *in vitro* growth culture of oocytes [15, 16]; all of these findings suggest that androgens promote the growth and the acquisition of maturation competence of bovine oocytes. In the present study, oocytes grown with not only A₄ but also DHT in the presence of E₂ showed a significant increase in oocyte diameter and high maturation rates. Since DHT is not converted to estrogens, androgens added to the medium appeared to affect oocyte growth and maturation

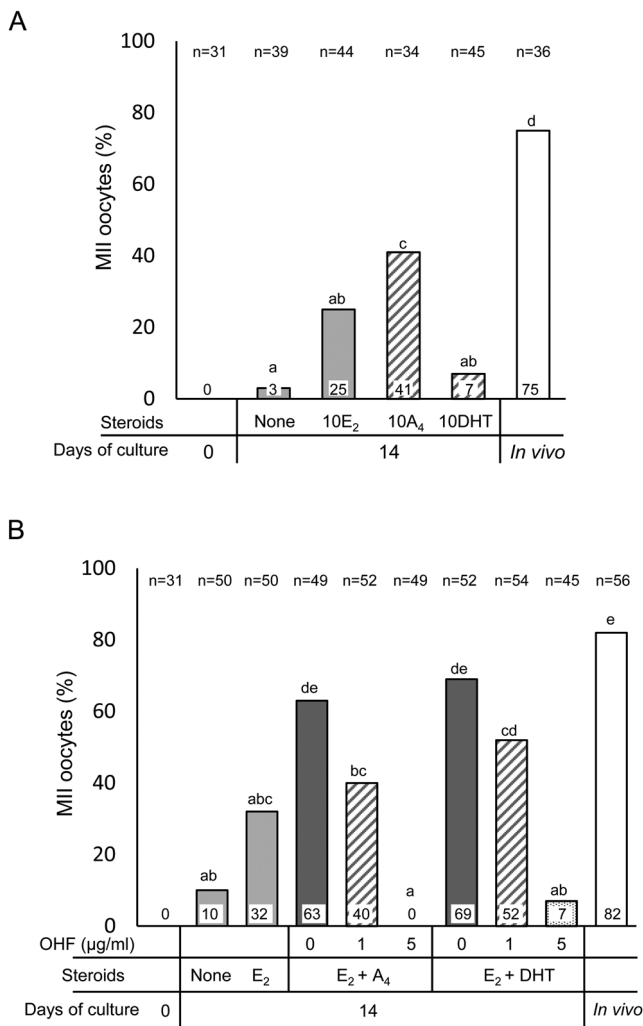


Fig. 4. Meiotic competence of bovine oocytes cultured for growth after *in vitro* maturation. The percentages of MII oocytes out of total oocytes initially used for *in vitro* growth culture are shown in both panels. For each panel, the white bars on the left and the right represent maturation rates of oocytes collected from early antral follicles (0.4–0.7 mm) and antral follicles (4–6 mm), respectively. The gray, striped and dotted bars indicate the maturation rates of oocytes used for growth culture. The numbers above the bars indicate the numbers of oocytes used for growth culture (n). The percentages of MII oocytes are shown at the bottom of each bar. ^{a-c} Values with different superscripts differ significantly ($P < 0.05$).

directly through the ARs.

To examine the ability of an AR inhibitor to antagonize the effect of androgens on oocyte growth and maturation *in vitro*, we employed a clinically used nonsteroidal AR antagonist, OHF. OHF has been widely used in the treatment of prostate cancer due to its selective blockade of androgen action and low side effect profile [20, 21]. In mice, OHF inhibits the acquisition of meiotic competence of oocytes during *in vitro* culture [22]. In the present study, addition of OHF to the culture medium containing androgens decreased the mean diameters of oocytes at day 14. In addition, OHF inhibited

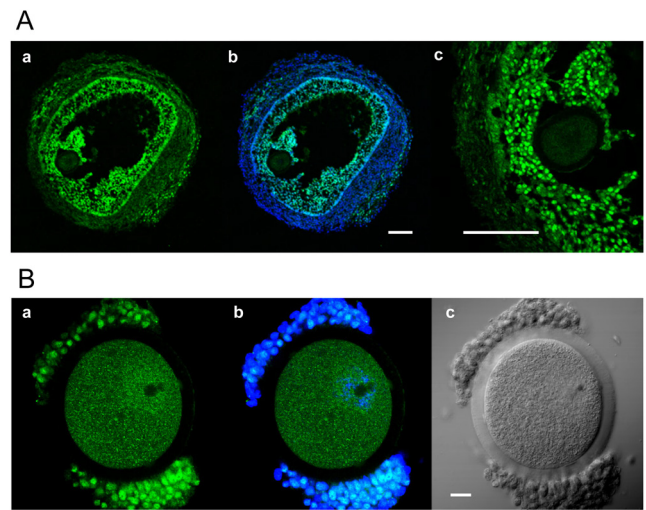


Fig. 5. Expression of androgen receptors in bovine early antral follicles and OGCs. (A) Cryosections of 0.4–0.7 mm follicles were treated with anti-androgen receptor antibody and Alexa Fluor 488-labeled immunoglobulin antibody. Alexa Fluor 488 staining marks androgen receptors in green (a–c). DAPI staining marks the chromatin in blue (b). A magnified image of the cumulus oophorus in (a) is shown in (c). Scale bars represent 100 μ m. (B) OGCs isolated from 0.4–0.7 mm follicles were stained. Alexa Fluor 488 staining marks androgen receptors in green (a, b). DAPI staining marks the chromatin in blue (b). A bright-field image is shown in (c). Scale bars represent 20 μ m.

the stimulatory effect of androgens on the acquisition of maturation competence in oocytes in a dose-dependent manner. These results indicate that the increase in oocyte diameter and oocyte maturation rate caused by androgens occur in part through the ARs. That is, androgens themselves, not converted estrogen, are able to promote oocyte growth and the acquisition of meiotic competence. With respect to the integrity of OGCs, however, we found that the OGC structure collapsed and that the oocytes were denuded during growth culture with only DHT. In the presence of E₂ and DHT, however, OGCs maintained high integrity throughout culture, suggesting that E₂ plays a role in oocyte survivability by preventing oocytes from being denuded. Androgens have been shown to upregulate FSH receptors in granulosa cells [23], and contribute to granulosa cell differentiation and follicle development [13, 14]. Also, insulin-like growth factor and its receptor, which play essential roles in folliculogenesis [24], are responsive to androgens [25–28]. Moreover, growth differentiation factor-9 requires androgens to promote follicle growth [28, 29]. These reports suggest that androgens, acting through ARs in granulosa cells, may regulate the expression and/or action of key ovarian growth factors and contribute to follicle or oocyte growth.

The AR is a member of the steroid hormone receptor superfamily and contains a DNA-binding domain and a hormone-binding domain. ARs are predominantly located within the cytoplasm, activated by binding with androgens and translocated into the nucleus [30, 31]. Studies across species have reported that ARs reside in granulosa cells and theca cells [32–35], and that they are also present in mouse [36], rat [37] and pig [38] oocytes. In the present study, the nuclei

of granulosa cells showed intense expressions of ARs in early antral follicles, indicating that functional ARs reside in granulosa cells. Since we also found AR expression in bovine oocytes, androgens have the potential to affect oocytes directly. There are a few reports about androgen action through ARs in oocytes; however, in mice, testosterone induces oocyte maturation through AR-mediated activation of mitogen-activated protein kinase and cyclin-dependent kinase 1 signaling [36].

In conclusion, E₂ maintained the structure of OGCs and supported oocyte viability and growth, while androgens in combination with E₂, but not alone, promoted the growth of bovine oocytes and their acquisition of meiotic competence during *in vitro* growth culture. The androgen-induced oocyte growth and maturation were suppressed by an AR inhibitor, indicating that androgens themselves promote the growth of oocytes and their acquisition of meiotic competence of oocytes, and that these stimulatory effects of androgens are mediated by the ARs in granulosa cells and/or oocytes.

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