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Effect of increased oxygen availability and astaxanthin supplementation on the growth, maturation and developmental competence of bovine oocytes derived from early antral follicles

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ABSTRACT

In vitro growth (IVG) culture of bovine oocyte-cumulus-granulosa complexes (OCGCs) is generally carried out for 12 or 14 days using conventional gas impermeable culture devices. The culture duration may be longer compared to follicular development *in vivo*. During follicular development, follicles receive oxygen from micro vessels; however, oxygen supply is limited under the culture using conventional gas impermeable devices. The purpose of this study was to investigate the effect of increasing dissolved oxygen availability using a gas permeable (GP) culture device with or without antioxidant (astaxanthin, Ax) supplementation on 8-day IVG culture systems for bovine OCGCs derived from early antral follicles. We cultured OCGCs in GP, GP supplemented with Ax (GP + Ax), and a conventional gas impermeable device (control) for 8 or 12 days. OCGC viability were significantly higher when cultured for 8 days than 12 days ($p < 0.001$) in all culture condition, but significant difference was not observed between groups ($p > 0.05$). Antrum formation rates of OCGCs were higher after 12 days than 8 days of culture in all culture condition ($p < 0.001$) and were significantly higher in the control than GP groups regardless of Ax supplementation ($p < 0.05$). Oocyte diameters were similar among day-8 GP + Ax, day-8 control and day-12 control groups ($p > 0.05$). Nuclear maturation rates of oocytes grown *in vitro* for 8 days were significantly higher in the GP + Ax group than in the control and the GP groups ($p < 0.05$) and similar to oocytes grown for 12 days regardless of the culture conditions ($p > 0.05$). The generation of reactive oxygen species in OCGCs on day 8 of IVG culture was significantly lower in the GP + Ax group than those of the GP and control groups ($p < 0.05$). IVG oocytes after eight days of culture developed into blastocysts, and the cleavage and blastocyst rates were similar in all treatment groups. However, *in vivo*-grown oocytes had significantly higher ($p < 0.05$) cleavage and blastocyst rates than the IVG oocytes in all groups. The present study demonstrates that increased oxygen availability using a GP culture device with Ax supplementation promotes oocyte growth and maturation competence but inhibits proliferation of granulosa cells and antrum formation compared with a conventional gas impermeable culture device, and that OCGCs can attain developmental competence after 8 days of IVG culture.

1. Introduction

The current methods for *in vitro* production of embryos are dependent upon the supply of developmentally competent oocytes, which are only present in the ovary in relatively small numbers [1]. Moreover, the success rate and quality of embryos produced by

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in vitro maturation (IVM) vary due to the poor quality of oocytes that are selected for maturation [2,3], since these are taken from a heterogenous group of morphologically indistinguishable healthy or degenerating antral follicles [4]. The use of oocytes derived from smaller early antral follicles offers a potential solution, and this may be applied in animal production systems, conservation of rare animals [5], and treatment for infertility in humans [6]. Since many of the early antral follicles are lost during their growth phase [7,8], the development of an *in vitro* growth (IVG) culture system that allows growth and acquisition of oocyte developmental competence while preventing follicle degeneration *in vitro* is necessary.

Increasing the survival rates of oocyte-cumulus-granulosa complexes (OCGCs) during IVG culture is the major challenge of the current IVG culture systems in cattle. Previous studies have shown that survival rates of OCGCs during IVG culture for 10 days [9] or longer [9–12] markedly decreases, especially from day 8 of culture as the number of degenerating OCGCs increases with the duration of culture. Interestingly, the longer the OCGCs were cultured, the more progesterone was secreted than estrogen, suggesting luteinization of granulosa cells [12,13], which is a typical finding in degenerating follicles *in vivo* [14]. These findings suggest that the duration of current IVG systems is too long to support the viability of OCGCs *in vitro*. Further evidence comes from the finding that *in vivo* bovine follicles take about 6.8 days to grow from 0.68 mm to 3.67 mm in diameter [15]; this is a suitable diameter to produce mature oocytes after IVM. This means that the optimal culture condition will support oocyte development and oocyte acquisition of developmental competence within an 8-day culture. The present study investigated an 8-day IVG culture system designed to mimic the growth of early antral follicles *in vivo*. However, previous studies demonstrated that OCGCs grown for 10 days had lower maturation rates than those grown for 12 days [9]. Follicles receive a supply of nutrients, oxygen, and hormonal support from capillary vessels in theca cell layer during their development [16–19]. Therefore, we speculated that the possible reasons for OCGC degeneration after 8-day culture are the increased thickness of the granulosa cell layer and insufficient supply of oxygen from the bottom of the culture device.

The availability of oxygen at the bottom of the culture well decreases with the depth of the culture media, poor solubility of oxygen, and the presence of serum in the culture media [20]. The use of a gas permeable (GP) culture device has been proposed to improve oxygen availability during cell culture [20]. In some studies, a GP culture device improved the survival rates of bovine follicles cultured in 20% oxygen due to increased oxygenation at the bottom of the culture well [21,22]. However, the effect of such a device on the survival and growth of bovine oocytes derived from early antral follicles has not been evaluated. Moreover, increased oxygenation carries a risk to cells of oxidative stress [23]; therefore, an IVG culture system using such a device must suppress excessive production of reactive oxygen species (ROS) by OCGCs. Astaxanthin (Ax) is a xanthophyll carotenoid which is found in various microorganisms and marine animals [24]. It has biological activities including antioxidant, anti-lipid peroxidation, and anti-inflammation effects [25]. The positive effects of Ax supplementation during *in vitro* embryo production in domestic animals has received great attention [26–28]. Recently, we demonstrated that Ax supplementation during 12 days of IVG culture using a conventional 96-well plate increased the survival rates of bovine oocytes derived from early antral follicles by suppressing the effects of ROS and inhibiting the luteinization of granulosa cells [29].

In the present study, we evaluated the effectiveness of an IVG culture system using a GP culture device with Ax supplementation on the survival, growth, and developmental competence of *in vitro* grown bovine oocytes after eight days of IVG culture.

2. Materials and methods

2.1. Chemicals

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

2.2. Isolation of OCGCs and IVG culture

Bovine ovaries obtained from a local abattoir were transported to the laboratory within 6–10 h after collection. Thin ovarian cortex tissues were prepared using a surgical blade (No. 11) and stored in tissue culture medium (TCM-199; Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol, 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethane-sulfonic acid (HEPES), 10 mM sodium bicarbonate, and 50 μg/mL gentamicin sulfate (isolation medium; pH 7.4), as described previously [13]. Early antral follicles (0.5–1 mm in diameter) were dissected from sliced ovarian tissues in a 90-mm Petri dish that had a 1-mm scale on its bottom (Fig. 1A). After opening the follicles using forceps and a blade, OCGCs were isolated from follicles (Fig. 1B). Morphologically normal OCGCs were individually cultured in 96-well plates. OCGCs that had a homogeneous cytoplasm and more than three compact layers of cumulus and granulosa cells were defined as normal. In the present study, we used two types of 96-well plates; a conventional device (control: Primaria 353 872, Corning, NY, USA) and a GP device (GP: Lumox 94.6120.096, Sarstedt, Numbrecht, Germany) at 39 °C in humidified air with 5% CO_2. To each well, 200 μL of growth media was added. The growth medium was HEPES-buffered TCM-199 supplemented with 0.91 mM sodium pyruvate, 10 ng/ml androstenedione, 5% fetal calf serum (FCS; Invitrogen, Grand Island, NY, USA), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (PVP; molecular weight 360 000), 10 μg/ml amphotericin B, and 50 μg/ml gentamicin sulfate. At the onset of the IVG culture, OCGCs were photographed under an inverted microscope (CK 40; Olympus, Tokyo, Japan) attached to a charge coupled device camera (Moticam 2000; Shimadzu Rika Corporation, Tokyo, Japan) and the day 0 diameters (excluding the zona pellucida) were assessed using Motic Images Plus 2.2s software (Shimadzu) as previously described [9]. During culture, half the growth medium (100 μL) was replaced by the same amount of fresh medium every four days.

2.3. Evaluation of OCGC viability and antrum formation

The morphological appearance of the OCGCs was evaluated on days 4, 8, and 12 of IVG culture under an inverted microscope. OCGCs with an evenly granulated ooplasm and completely enclosed by several layers of healthy cumulus and granulosa cells with or without cavities (Fig. 2A and B, respectively) were defined

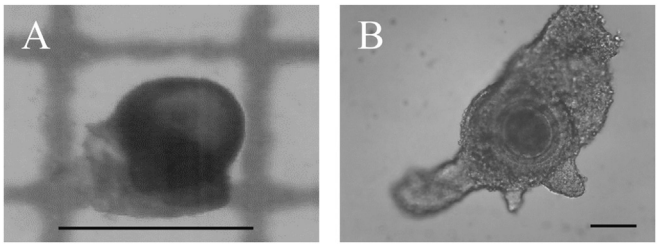

Fig. 1. Representative isolated early antral follicle (A) and collected oocyte-cumulus-granulosa complex (OCGC) (B). A: follicle of <1 mm in diameter was identified by grid line on the bottom of a petri dish. Scale bar = 1 mm. B: healthy OCGC before IVG culture was surrounded by multiple layers of granulosa cells. Scale bar = 100 μm.

as normal. OCGCs with scattered cumulus and granulosa cells or denuded oocytes were defined as abnormal (Fig. 2C).

2.4. Evaluation of dissolved oxygen in the spent culture medium

Dissolved oxygen concentrations in the spent media (approximately 100 μL) of morphologically normal OCGCs cultured in GP or the control devices were evaluated by the I-STAT system ($G3^+$ cartridge, Abbot Point of Care Inc., Princeton, NJ, USA) on days 4, 8, and 12 of culture.

2.5. Evaluation of granulosa cell proliferation and quality

The total number, viability, and diameters of granulosa cells after growth culture were evaluated from morphologically normal OCGCs on days 4, 8, and 12 by an acridine orange/propidium iodide cell viability kit together with a cell counter (F23001 and L20001, respectively; Logos Biosystems, Gyunggi, Republic of Korea) as previously described [10,13]. Briefly, the culture medium in the well of each viable OCGC was removed and replaced by 80 μL of Dulbecco's phosphate-buffered saline without calcium or magnesium (DPBS) supplemented with 0.125% trypsin and 0.05% EDTA to prepare the granulosa cells for counting. After 10 min of trypsinization and pipetting several times, 20 μL of FCS were added to stop the digestion. Denuded oocytes were removed from the well and discarded.

2.6. Evaluation of oocyte growth and nuclear maturation of IVG oocytes after IVM culture

After IVG culture, oocytes were subjected to IVM as previously described [30]. Cumulus-oocyte complexes (COCs) were individually transferred to the microwell plates (Mini Trays 163 118; NUNC, Roskilde, Denmark) filled with 6 mL of IVM medium and cultured for 22 h at 39 °C under 5% CO_2 in air. The maturation medium consisted of HEPES-buffered TCM-199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/mL FSH (from porcine pituitary), 1 mg/mL estradiol-17β, 10% FCS, and 50 μg/mL gentamicin sulfate. Immediately after IVM, oocytes were treated with 500 IU/mL of hyaluronidase in DPBS for 10 min, then denuded from their expanded cumulus cells by repeated pipetting using a fine pippete as described elsewhere [31]. After complete denudation, the denuded oocytes were photographed, and their diameters were measured under an inverted microscope equipped with CCD camera as described above. Then evaluation of nuclear maturation status was done as described elsewhere [32]. Briefly, the denuded oocytes were fixed using a mixture of ethanol and acetic acid (3 : 1) overnight and were later stained with 1% aceto-orcein. The nuclear status was examined under a phase contrast microscope. Oocytes at metaphase II with a polar body were defined as mature, while oocytes with other nuclear statuses were defined as immature.

2.7. Evaluation of ROS generation in day 8 IVG OCGCs

Intracellular ROS generation in the granulosa cell layer was detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA) as previously reported elsewhere [33] with minor modifications. Briefly, after cell uptake, DCHFDA is deacetylated by cellular esterases to a nonfluorescent compound, which is later oxidized by ROS into 2′,7′-dichlorofluorescein (DCF) [34,35]. The culture medium in the well of each viable OCGC was removed and replaced with 200 μL of DPBS to wash the wells. After washing, DPBS was replaced and incubated with 200 μL of DPBS including 100 μM DCHFDA in 5% CO_2 in air at 39 °C for 30 min. After incubation, the DCHFDA solution was again replaced by 200 μL of DPBS. The DCHFDA-loaded OCGCs were placed in a multimode microplate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany) with temperature maintained at 37 °C. The excitation filter was set at 485 nm and the emission filter was set at 530 nm. To evaluate the concentration of hydrogen peroxide (H_2O_2) produced by the OCGCs, a standard curve was determined using 200 μL of DPBS including 100 μM DCF by the addition of different concentrations of H_2O_2 and the DCF fluorescence was determined using the plate reader as described above.

2.8. Developmental competence of day-8 IVG oocytes

All media used in this experiment were purchased from IVF Bioscience (Cornwall, UK). After IVG culture, COCs were subjected to IVM as follows: 10 to 15 oocytes washed two times in BO-wash media and then once in the BO-IVM media before being cultured in 50 μL BO-IVM droplets covered with paraffin oil for 20–24 h at 39 °C in 5% CO_2.

Motile sperm (2×10^6 cells/mL) separated by centrifugation two times (300×*g*) for 5 min in BO-SemenPrep were coincubated with IVG oocytes in 500 μL of BO-IVF medium in 4-well dish without an oil overlay for 18 h at 39 °C in 5% CO_2 in air. After 18–24 h of IVF, the inseminated oocytes were denuded of their surrounding cumulus cells and excess spermatozoa were removed by vortexing. The presumptive zygotes were washed three times in 100 μL BO-IVC media and cultured in a 30-μL droplet (approximately 15 zygotes/droplet) with an overlay of paraffin oil at 5% O_2, 5% CO_2, and 90% N_2 at 39 °C for 150 h. Cleavage and blastocyst rates were examined at 48 h and 168 h after IVF, respectively, under an inverted microscope.

2.9. Experimental design

2.9.1. Experiment 1: difference in dissolved oxygen availability and the effects of Ax supplementation on OCGC morphology, oocyte growth and maturation competence during 12 days of IVG culture

To determine the effects of the culture conditions, *i.e.*, culture in the gas permeable device (GP) versus gas permeable device with 10 μg/mL astaxanthin supplementation (GP + Ax) versus

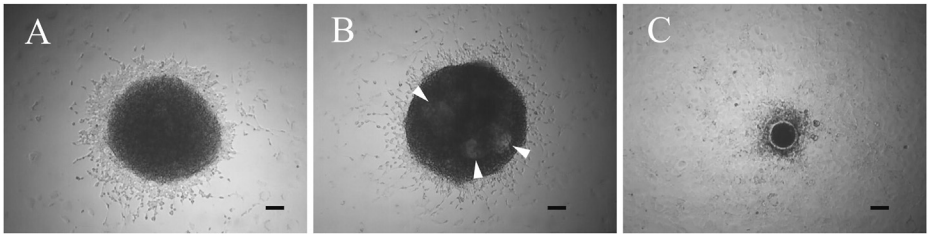

Fig. 2. The morphological appearance of OCGCs during *in vitro* growth culture. A: viable OCGC with no antrum. B: viable OCGC with antrum formation (white arrowhead). C: degenerated OCGC with apparent loss of surrounding granulosa cells. Scale bar = 100 μm.

conventional gas impermeable culture device (control) and the duration of culture (8 days versus 12 days) on bovine IVG culture, a 3 × 2 factorial experimental design was used. The concentration of Ax was determined according to previous studies [26,29]. A total of 1068 OCGCs were cultured under 5% CO_2 in air with the following sample distribution: 406 in GP, 371 in GP + Ax, and 291 in the control groups. Dissolved oxygen in the spent culture media (n = 35) was compared between the GP and control groups as well as granulosa cell properties (n = 240) in GP, GP + Ax, and control groups on days 4, 8, and 12 of IVG culture. OCGC viability (n = 1068), antrum formation (n = 1068), oocyte growth (n = 240), and oocyte nuclear maturation were evaluated at days 8 and 12 of culture. However, 12 oocytes accidently collapsed during the denudation of cumulus cells.

2.9.2. Experiment 2: reactive oxygen species generation of day-8 grown OCGCs and developmental competence of oocytes derived from them

Since experiment 1 revealed superior viability rates during 8-day than 12-day IVG culture, experiment 2 focused on ROS generation and developmental competence of day-8 IVG oocytes. A total of 318 OCGCs were used to evaluate ROS generation (n = 42) and developmental competence (n = 276) of day-8 IVG oocytes. *In vivo*–derived COCs collected from antral follicles (measuring 2–8 mm in diameter: n = 78) were also subjected to IVM/IVF/IVC and served as an *in vivo* control for blastocyst development.

2.10. Statistical analysis

All statistical analyses were performed using JMP software version 14.0.0 (SAS Institute, Gary, NC, USA). All numerical variables were analyzed for normality using the Shapiro-Wilk test. Linear regression analysis was performed on variables with binary outcomes, *i.e.*, OCGC viability, OCGC antrum formation, and oocyte nuclear maturation competence as independent variables, while culture conditions (*i.e.*, GP vs. GP + Ax vs. control groups) and duration of culture (*i.e.*, day 8 vs. day 12) as independent variables. The mean difference between groups and durations of culture were compared using Tukey-Kramer's HSD test [36].

Dissolved oxygen, oocyte diameter, and granulosa cell properties (*i.e.*, cell number, diameter, and viability) were analyzed using two-way ANOVA by including culture conditions, duration of culture, and interaction of culture conditions and duration of culture as dependent variables in the model. The differences in means between culture condition and duration of culture were compared using Tukey-Kramer's HSD test. The mean difference in ROS generation, cleavage rate, and blastocyst rate at day 8 of culture were compared using Tukey-Kramer's HSD test. P- values of less than 0.05 were considered significant.

3. Results

3.1. Experiment 1

Dissolved oxygen concentrations, expressed as oxygen partial pressure (mmHg) in media did not differ between GP and conventional device on days 4, 8, and 12 of IVG culture (Fig. 3).

Viability rates of OCGCs were significantly higher in OCGCs cultured for 8 days than those cultured for 12 days in all experimental groups ($p < 0.001$, Fig. 4A). There was no significant difference in viability rates during 8 or 12 days of culture within GP, GP + Ax, and the control groups ($p > 0.05$). Antrum formation rates of OCGCs increased when the duration of culture was extended ($p < 0.001$, Fig. 4B). The antrum formation rate of OCGCs in the control group was higher than those in the GP and GP + Ax groups ($p < 0.05$).

There was no significant difference in granulosa cell number between the first 4 days of culture in all culture conditions ($p > 0.05$; Table 1). The granulosa cell number was significantly higher in the control than in GP and GP + Ax on days 8 and 12 of culture ($p < 0.05$). There was no difference in granulosa cell viability and diameter among experimental groups during IVG culture.

In oocyte diameter, there was a significant interaction between culture conditions and duration of culture ($p < 0.001$), then we compared six groups (3 culture conditions × 2 duration of culture) as described in Fig. 5. On day 8 of culture, oocyte diameter was larger in the GP + Ax but was statistically similar to the control group ($p > 0.05$; Fig. 5). Oocyte diameter on day 12 of culture was significantly larger in the control group ($p < 0.05$) but was similar to the day-8 GP + Ax and control groups ($p > 0.05$).

In nuclear maturation rates, there was a significant interaction between culture conditions and duration of culture ($p < 0.001$), then we compared six groups (3 culture conditions × 2 duration of culture) as described in Fig. 6. Nuclear maturation rate at day 8 was significantly higher in the GP + Ax group than the control and the GP groups ($p < 0.05$). Nuclear maturation rates at day 12 were similar amongst the groups ($p > 0.05$) and also statistically similar to that of GP + Ax group at day 8 ($p > 0.05$).

3.2. Experiment 2

As shown in Table 2, the generation of ROS, expressed as the average concentration of H_2O_2 in OCGCs on day 8 of IVG culture, was significantly lower in the GP + Ax group than those of the GP and control groups ($p < 0.05$). Since the granulosa cell numbers on day 8 of culture were significantly higher in the control than in the GP and GP + Ax groups, the average concentration per a granulosa cell was calculated and further compared. The concentration of H_2O_2 per cell in the GP group tended to be higher than in the GP + Ax and control groups ($p = 0.07$ and $p = 0.09$, respectively), and there were no differences between the GP + Ax and control groups.

As shown in Fig. 7, IVG oocytes cultured for eight days developed into blastocysts. The cleavage and blastocyst rates in IVG oocytes were similar in all treatment groups ($p > 0.05$; Table 3). However, *in vivo*-grown oocytes had significantly higher cleavage and blastocyst rates than the IVG oocytes in all groups ($p < 0.05$).

4. Discussion

Dissolved oxygen is one of the most important factors affecting

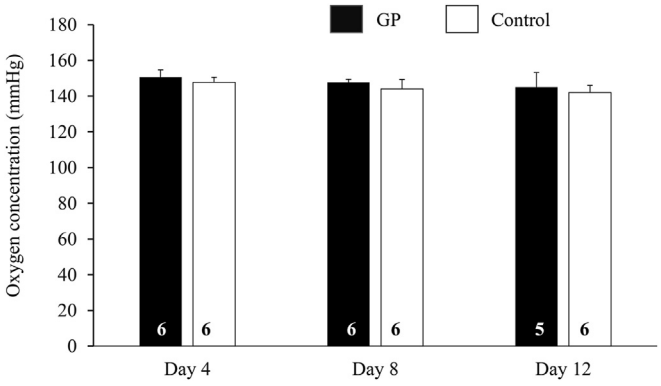

Fig. 3. Dissolved oxygen concentration in media used for culture of OCGCs in the gas permeable (GP) and conventional (control) culture devices on days 4, 8 and 12 of *in vitro* growth culture. Error bar indicates standard error of mean.

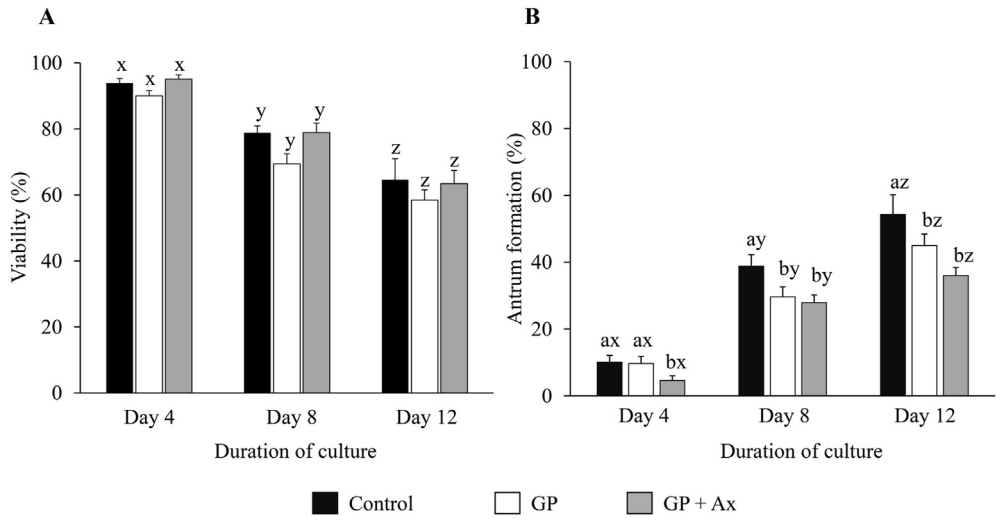

Fig. 4. Effects of gas permeable culture device and astaxanthin supplementation on the viability (A) and antrum formation (B) of OCGCs during *in vitro* growth culture. The numbers of cultured OCGCs were 406, 371, and 296 in GP, GP + Ax, and control groups, respectively. [ab] Different letters indicate significant difference among culture conditions on the same day of culture ($p < 0.05$). [xyz] Different letters indicate a significant difference between the duration of culture in the same group ($p < 0.001$).Error bar indicates standard error of mean.

the outcome of cell culture. Generally, conventional monolayer cultures are often reported to be hypoxic when incubated in air *i.e.*, a 5% CO_2 atmosphere, because the plastic material used is almost gas-impermeable, and cells are supplied with O_2 only by one-dimensional diffusion through the culture medium [37]. The height of the culture media, poor solubility of oxygen in it, and presence of serum in the culture media affect pericellular oxygen availability to cells at the bottom of culture well [20]. Unlike a conventional 96-well gas impermeable device, gas permeable culture devices allow direct diffusion of oxygen from the bottom of the wells, bypassing the diffusion barrier created by the medium above [20]. Using mathematical analysis of dissolved oxygen diffusion to cells with different oxygen consumption rates (*i.e.*, liver cells and alveolar macrophages), Jensen et al. [38] concluded that cells with high respiration rates on highly permeable devices will not deplete their pericellular PO_2 and will not accumulate high pericellular PCO_2; therefore, the pericellular environment of cultures on highly permeable devices can be accurately controlled by the incubator atmosphere. However, we found no significant difference in dissolved oxygen concentrations between the GP and control culture devices in the present study. In a previous study where dissolved oxygen concentration was compared between the conventional gas impermeable culture device and the gas permeable device [21], dissolved oxygen was measured from the bottom of the well under two different heights of media (5 mL vs. 2.5 mL in the same size of wells) and was found to be higher in 2.5 mL than in 5 mL. In the present study, we collected 100 μL spent medium from the middle depth of the well containing 200 μL of culture medium. We speculate that superficial medium containing a high concentration of oxygen in the gas impermeable culture device may have mixed up with the deeper less oxygenated parts, resulting in no significant difference in dissolved oxygen concentrations between the GP and control groups. Increased oxygen levels are known to induce cellular ROS production [39] and the finding that ROS production per cell tended to be higher in GP than GP + Ax and control groups may further indicate that dissolved oxygen at the bottom of the GP culture device might have been higher in gas permeable than conventional gas impermeable culture device.

Maintaining viability rates for bovine OCGCs grown *in vitro* presents a major challenge for current IVG culture systems. In the present study, culture of OCGCs for 8 days resulted in higher viability rates than culture for 12 days regardless of the culture conditions employed. This is consistence with previous studies that demonstrated that extending the duration of culture from 10 or 12 days–14 days [9] or from 12 days to 16 days [10] significantly

Table 1
Effect of the gas permeable culture device and astaxanthin supplementation on the granulosa cell proliferation, viability and diameter during *in vitro* growth culture.

Days of culture	Culture condition	No. of oocytes	Characteristics of granulosa cells		
			No. ($\times 10^4$ cells)	Viability (%)	Diameter (μm)
4	GP	27	12.6 ± 6.8^{x}	96.8 ± 4.2	12.1 ± 1.3
	GP + Ax	19	9.6 ± 3.5^{x}	94.2 ± 6.5	11.9 ± 1.0
	Control	25	13.1 ± 7.9^{x}	93.4 ± 17.5	11.9 ± 2.7
8	GP	33	14.7 ± 8.5^{x}	95.7 ± 5.6	11.5 ± 1.5
	GP + Ax	23	12.5 ± 5.1^{x}	95.4 ± 6.7	12.0 ± 2.1
	Control	38	19.6 ± 9.5^{x}*	95.8 ± 4.6	11.4 ± 1.2
12	GP	29	25.7 ± 11.6^{y}	96.4 ± 3.1	12.3 ± 1.2
	GP + Ax	20	20.9 ± 6.6^{y}	96.8 ± 3.8	11.2 ± 0.9
	Control	26	28.4 ± 10.1^{y}*	98.0 ± 2.7	11.6 ± 1.3

Values are means ± standard deviation.
* An asterisk indicates a significant difference between control group and other groups ($p < 0.05$).
[xy] Different superscripts indicate a significant difference between the duration of culture in the same treatment group ($p < 0.05$).
GP: Gas permeable device, Ax: Astaxanthin supplementation.

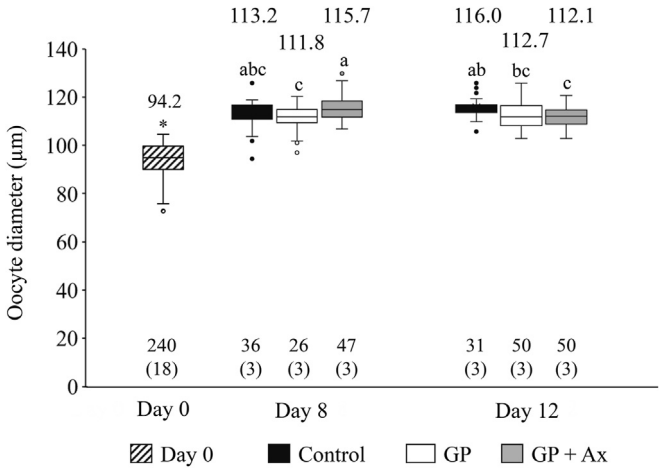

Fig. 5. Oocyte diameters after 8 and 12 days of culture in the control, GP, and GP + Ax groups. Lines on the boxes delineate the 25th, 50th, and 75th percentiles, whereas the whiskers depict the 10th and 90th percentiles. Values above boxes indicate the mean diameters (μm) of oocytes. Values under boxes indicate the numbers of oocytes examined and the number of replicates is shown in parentheses. * Asterisk indicates a significant difference between the values measured before and after IVG in all groups after 8 or 12 days of culture ($p < 0.05$).[abc] Different letters indicated significant difference among six groups (3 culture conditions × 2 duration of culture) ($p < 0.05$).

reduced OCGC viability, suggesting that OCGCs do not survive well under longer culture systems using conventional gas impermeable culture devices. Granulosa cell proliferation increases as the duration of culture is extended. In the present study, granulosa cell number in the conventional gas impermeable device was higher than in the GP devices regardless of astaxanthin supplementation. We speculate that, during more than 8 days of IVG culture of OCGCs using a conventional gas impermeable culture plate, the increase in granulosa cell density at the bottom of the culture well creates a potentially hypoxic pericellular environment that cause luteinization of granulosa cells [40]. Further, high granulosa cell densities are also associated with differentiation of cultured granulosa cells resulting in down-regulation of key genes of folliculogenesis like CYP19A1 and FSH receptors [41], a situation that is similar to early

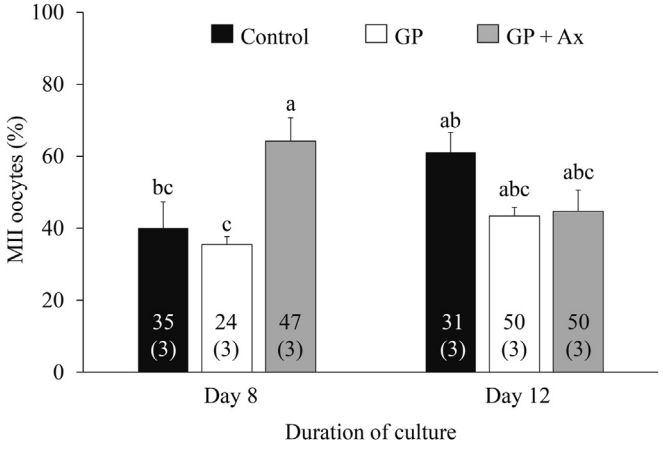

Fig. 6. Maturation competence of oocytes grown in the control, GP, and GP + Ax after 8 and 12 days of IVG culture. Maturation competence is defined by the percentage of oocytes at metaphase II (MII) stage of meiosis after culture for *in vitro* maturation. Values inside the bars indicate the numbers of oocytes examined and the number of replicates is shown in parentheses.[abc] Different letters indicated significant difference among six groups (3 culture conditions × 2 duration of culture) ($p < 0.05$).Error bar indicates standard error of mean.

post-luteinizing hormone surge conditions *in vivo*. Some studies have evaluated the effect of a GP culture device in optimizing the pericellular oxygen availability to support the viability of cultured mouse [22] and bovine [21] follicles, and demonstrated higher follicle survival rates in the GP culture device. However, our results, where OCGCs were used rather than ovarian follicles revealed no significant difference in viability rates of OCGCs grown in GP, GP + Ax, and the conventional gas impermeable culture device whether after 8 or 12 days of culture. The available dissolved oxygen in both culture devices could have easily passed through the granulosa and cumulus cell compartments to reach the oocyte thanks to the absence of the theca cell compartment in the OCGCs; but this raises questions regarding the specific quantities of dissolved oxygen that are detrimental or necessary for OCGC survival during IVG culture.

Antrum formation is one of the important processes during folliculogenesis *in vivo*. Granulosa cells contribute in antrum formation process by producing hyaluronan and the chondroitin sulfate proteoglycans that generate an osmotic gradient [42]. Further, granulosa cells have aquaporins that are actively involved in the transportation of water into the follicle and the ability of granulosa cells to move relative to each other to allow fluid to accumulate during antrum formation [42]. In the present study, OCGCs grown in the GP device, regardless of astaxanthin supplementation, showed significantly lower antrum formation rates than that in the conventional gas impermeable culture device. Lower granulosa cell numbers observed in OCGCs grown in GP and GP + Ax could result in lower concentration of hyaluronan, the chondroitin sulfate proteoglycan and aquaporins that are necessary for antrum formation compared to OCGCs grown in the control device.

The rate of oocyte growth is directly related to the proliferation of the surrounding granulosa cells and the establishment of bidirectional communication between the granulosa cells and the oocyte *in vivo* [43]. It is believed that rapid proliferation of granulosa cells during follicular development occurs under low oxygen conditions [44] similar to some types of tumor cells that rapidly proliferate under lower oxygen concentrations [45]. In the present study, granulosa cell proliferation was highest in the OCGCs cultured in the conventional gas impermeable culture device than those cultured in the GP device regardless of astaxanthin supplementation on both days 8 and 12 of culture. Lower granulosa cell proliferation in GP could be a result of higher ROS production, which is known to induce apoptosis of granulosa cells [46]. Meanwhile, one study showed that astaxanthin inhibited proliferation and induced apoptosis of mice H22 hepatoma cells [47]. Astaxanthin supplementation in the GP device could have the same effect during *in vitro* growth of OCGCs, resulting in lower granulosa cell counts observed in the GP + Ax group as well in the present study.

Bovine follicles take approximately 6.8 days to grow from 0.68 mm to 3.67 mm in diameter *in vivo* [15], and the diameter of the oocyte reaches approximately 116 μm [48]. Fair et al. [48] reported a positive correlation between bovine oocyte diameter and nuclear maturation competence. Further, Huang et al. [9] showed that growing bovine oocytes for 10 days and 12 days in a conventional gas impermeable culture device yielded oocytes with diameters of 109.8 μm and 115.5 μm, respectively, corresponding to maturation competences of about 36.1% and 53.6%, respectively. This suggests that the longer the duration of culture *in vitro*, the greater the oocyte diameter and maturation competence. However, OCGC grown in the GP + Ax and the control groups had achieved favorable growth diameters (115.7 μm and 113.2 μm, respectively) with only 8 days of culture, statistically similar to the control group (116.0 μm) after 12 days of culture in the present study. Furthermore, nuclear maturation competence of IVG oocytes after 8 days of

Table 2
Effect of astaxanthin on the intracellular levels of hydrogen peroxide (H_2O_2) in OCGCs at day 8 of IVG culture.

Culture condition	No. of OCGCs (replicates)	H_2O_2 accumulation (μM)	H_2O_2/cell (pcM)*
GP	13 (2)	73.4 ± 8.5[a]	485.9 ± 56.5†
GP + Ax	16 (2)	54.0 ± 8.7[b]	429.7 ± 69.3
Control	13 (2)	82.9 ± 14.1[a]	429.3 ± 73.0

Values are means ± standard deviation.
[a,b] Different superscripts indicate significant differences in levels of hydrogen peroxide generation between treatment groups ($p < 0.05$).
*Day 8 granulosa cell numbers (Table 1) were used to calculate H_2O_2/cell.
† Hydrogen peroxide generation per cell tended to be higher in GP than in GP + Ax and control groups ($p = 0.07$ and 0.09 respectively).

culture was the best in the GP + Ax group, statistically similar to the control group after 12 days of IVG culture. Astaxanthin supplementation has been shown to promote oocyte maturation during porcine IVM due to its antioxidant effect in one study [26], but there was no significant difference in maturation competence when astaxanthin was supplemented in a 12-day bovine IVG culture system using gas impermeable culture device in another study [29]. Significant interactions of duration of culture and culture conditions on the outcomes of oocyte diameter and maturation competence were observed in the present study, suggesting that the combination of GP + Ax culture condition and 8 days of IVG culture was more favorable for oocyte growth and the attainment of maturation competence. A previous report highlighted that growth of oocytes *in vitro* is slower than their *in vivo* counterparts [10] partly due to suboptimal culture conditions. Our findings support the notion that IVG culture of bovine oocytes beyond eight days could be too long, owing to the significant loss of viability associated with extended IVG culture despite the possibility of obtaining mature oocytes after 8 days. Interestingly, the control group also had a large oocyte diameter (113.2 μm) and a similar nuclear maturation rate (40.1%) compared with our previous results after 10-day IVG culture (109.8 μm and 36.1%, respectively) [9]. In the present study, we added androstenedione instead of estradiol-17β to IVG culture medium. We speculate that the difference in these results may have been caused by the addition of androstenedione

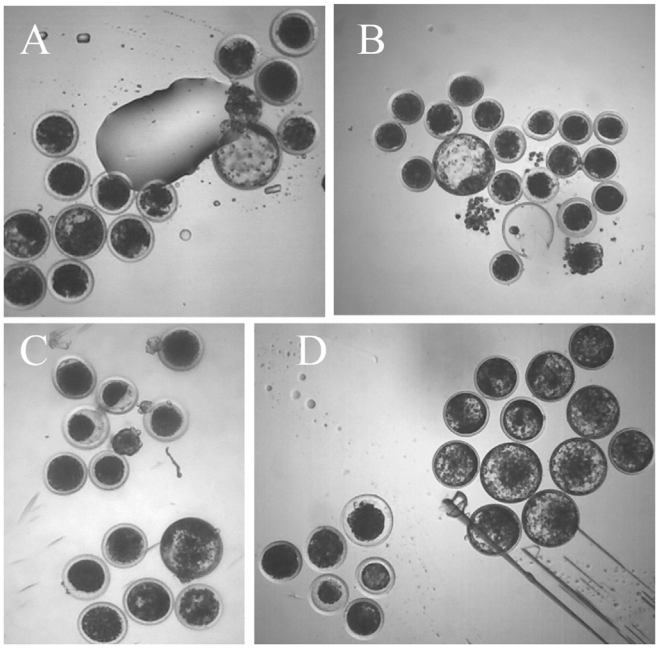

Fig. 7. Development to blastocyst stage of IVG oocytes derived from OCGCs cultured for eight days. Blastocysts from GP (A), GP + Ax (B), control (C), and *in vivo*-grown oocytes (D).

because androgen receptors are expressed in bovine oocytes and granulosa cells of early antral follicles [49]. In addition, several reports demonstrated that an androgen (*i.e.*, testosterone), promoted the transition from primary to secondary follicles in ovarian cortex fragments cultured for 10 days [50]. In another report, follicular growth and differentiation previously inhibited by an anti-androgen antibody were reversed by the addition of androstenedione in mouse preantral follicles cultured for six days [51]. Further, it is also reported that androgens promoted ovarian follicle growth after shorter exposures during early follicle development in mammals [52]. With respect to IVG culture of bovine OCGCs derived from early antral follicles, it has been reported that adding androstenedione to the IVG medium promoted the acquisition of oocyte meiotic competence [49,53]. However, these studies involved the culture of OCGCs for 14 days and the oocyte growth and competence of nuclear maturation after a shorter culture period of androgen exposure were not examined. Further studies should examine the effect of androstenedione on the development of OCGCs in detail.

Oocyte development competence is progressively acquired during the period of oocyte growth accompanying the follicular development stage [54]. Our previous study [9] showed that the development competence of bovine IVG oocytes derived from early antral follicles decreased as the culture duration was extended from 12 to 14 days in a conventional gas impermeable culture device. Superior oocyte growth and maturational competence were observed when using a GP culture device with Ax supplementation for eight days in the present study and we were able to obtain blastocysts. However, the developmental competences in all groups were similar and lower than those of *in vivo*-grown counterparts. During follicular development *in vivo*, theca cells support the follicular structure and provide androgen to granulosa cells for the synthesis of estrogen [55]. Furthermore, follicular development is regulated by theca cell-secreted growth factors [56]. In the present study, we added androstenedione, but no growth factors that theca cells supply. In a future study, we will develop a culture system including theca cell-derived growth factors or a co-culture system of OCGCs with theca cells [57].

Taken together, our findings show that 8-day culture of OCGCs is generally more favorable than 12-day culture regardless of other culture conditions adopted during IVG, owing to the ability of day-8 grown OCGCs to achieve higher viability rates and the ability of some of day-8 IVG oocytes to attain developmental competence to a degree that was unable to be achieved before. Further, the present study has also revealed that culture in gas permeable culture device with astaxanthin supplementation and in the conventional gas impermeable device presents both strengths and weaknesses during 8 days of culture; GP + Ax promoted oocyte growth and maturation competence better, while the conventional gas impermeable culture device promoted antrum formation and granulosa cell proliferation better. In future studies, an IVG culture system that can promote antrum formation, granulosa cell proliferation, oocyte diameter, oocyte maturation competence, and development

Table 3
Effect of the gas permeable culture device and astaxanthin supplementation on the cleavage and blastocysts rates after 8 days of *in vitro* growth culture.

Oocytes*	Culture condition	No. of oocytes (replicates)	Cleavage rate (%)	Blastocyst rate (%)
In vitro-grown	GP	85 (5)	28.1 ± 10.6[a]	13.9 ± 9.6[a]
	GP + Ax	80 (4)	28.5 ± 7.2[a]	10.0 ± 3.0[a]
	Control	33 (3)	34.5 ± 7.4[a]	13.7 ± 2.5[a]
In vivo-grown	–	78 (3)	83.3 ± 2.5[b]	50.7 ± 13.4[b]

Values are means ± standard deviation.
[ab] Different superscripts indicate significant differences between groups ($p < 0.05$).
*Oocytes derived from antral follicles of 2-8 mm in diameter were used as an *in vivo*-grown control.

competence after 8 days of culture should be explored. Further, other factors such as addition of different growth factors not included in the present IVG culture systems should be evaluated to assess how well they would interact with the gas permeable culture device or the conventional culture device during 8 days of IVG culture.

In conclusion, IVG culture systems using a gas permeable culture device with Ax supplementation promoted oocyte growth and maturational competence of bovine oocytes with only eight days of culture but inhibited granulosa cell proliferation and antrum formation of OCGCs compared with a conventional gas impermeable culture devise. However, the developmental competence of IVG oocytes after eight days of culture was still lower than the *in vivo*-grown oocytes after conventional IVF. Further studies on other factors affecting oocyte and follicular development should be carried out to establish an *in vitro* model of bovine oocyte and follicular growth.

CRediT authorship contribution statement

Madalitso Chelenga: Formal analysis, Investigation, Writing - original draft. **Kenichiro Sakaguchi:** Formal analysis, Investigation, Writing - review & editing. **Mohammed A. Abdel-Ghani:** Investigation. **Yojiro Yanagawa:** Methodology. **Seiji Katagiri:** Writing - review & editing. **Masashi Nagano:** Supervision, Writing - review & editing, Project administration, Funding acquisition.

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