Astaxanthin improves the developmental competence of *in vitro* grown oocytes and modifies the steroidogenesis of granulosa cells derived from bovine early antral follicles

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Abstract

We investigated the influence of Astaxanthin (Ax), which exhibits strong antioxidant activity, during *in vitro* growth (IVG) on the developmental competence of oocytes and steroidogenesis of granulosa cells derived from early antral follicles. Bovine oocyte-cumulus-granulosa complexes collected from early antral follicles were cultured for 12 days in the presence (500 μM) or absence (control) of Ax. Viability of oocytes and antrum formation in granulosa cell layer during IVG culture were greater in the presence of Ax than in its absence ($P<0.05$). Regardless of Ax treatment, estradiol-17β production increased during IVG culture; however, progesterone production was significantly lower in the presence of Ax than in its absence ($P<0.05$). Reactive oxygen species levels were lower in Ax-treated oocytes than in controls after IVG ($P<0.05$). Although the nuclear maturation and cleavage rates did not differ regardless of Ax treatment, Ax treatment led to weaker cathepsin B activity in oocytes and better blastocyst rates than those in controls ($P<0.05$). Accordingly, Ax treatment during IVG increased total cell numbers in blastocysts ($P<0.05$). These results indicate that Ax supplementation to IVG medium improves the quality of bovine oocytes due to its antioxidative effects on growing oocytes and its suppression of the luteinization of granulosa cells.

Additional Keywords: astaxanthin, cathepsin B, early antral follicle, IVG, ROS generation

Introduction

Assisted reproductive technologies (ARTs) including *in vitro* maturation (IVM), the *in vitro* fertilization (IVF) of oocytes, and *in vitro* culture (IVC) for the production of transferable embryos are uniquely desirable for improving the breeding of animals and also for the perpetuation programs of wild and endangered species. Moreover, the isolation and IVC of ovarian follicles to obtain fertilizable oocytes and further embryo development have been regarded as a propitious strategy to strive against infertility issues (Hansen 2014; Szamatowicz 2016; Kushnir *et al.* 2017).

The follicle features a fitting microenvironment that is responsible for confirming the production of oocytes with high quality and integrity, allowing its final growth, capacitation, and the nuclear and cytoplasmic maturation needs of the female gamete until it is released for fertilization into the uterine tubes (Hennet and Combelles 2012). In growing follicles, the concentration of estradiol-17β (E_2) increases in conjunction with follicular development, while elevations in progesterone (P_4) concentrations are accompanied by follicular degeneration (Kruip and Dieleman 1985). However, oocyte-cumulus-granulosa complexes (OCGCs) have been shown to produce large amounts of P_4 during *in vitro* growth (IVG) cultures (Yang *et al.* 2016; Sakaguchi *et al.* 2017), similar to degenerating bovine follicles (Kruip and Dieleman 1985). Previous studies have documented and focused on the establishment of a suitable system for the IVG of bovine OCGCs obtained from early antral follicles or mimicking *in vivo* development of bovine follicles; however, success has been limited (Makita and Miyano

2015; Huang *et al.* 2016; Makita *et al.* 2016; Sakaguchi *et al.* 2017). Therefore, further research is needed in order to improve IVG systems.

Astaxanthin (Ax) is a red-orange carotenoid pigment that is present in fishery products such as salmon, shrimp, and crab (Kuraji *et al.* 2016). Previous studies reported that Ax possesses a wide range of biological functions including the control of lipid peroxidation, anti-inflammatory and anti-tumor activities, the scavenging of reactive oxygen species (ROS), and as a hydroxyl radical antioxidant (Namekawa *et al.* 2010; Fassett and Coombes 2011; Kuraji *et al.* 2016). Moreover, Ax feeding was beneficial to improve chicken egg yolk color; egg quality during storage and it also improved the carcass traits and meat quality of pigs (Yang *et al.* 2006). Besides, there is an increase in the number of corpora lutea (CL), implantation sites and fetuses, and a decrease in the percentage of stillborn kits among minks fed Ax (Hansen *et al.* 2014). Furthermore, Ax exhibits more powerful antioxidant activity than vitamin C, vitamin E, and β-carotene; the antioxidant activity of Ax was shown to be 100- to 500-fold greater than that α-tocopherol and 15-fold greater than those of other carotenoids (Naguib 2000). The antioxidant effects of Ax on the developmental competence of *in vitro*-produced bovine embryos have been attributed to the induction of antioxidant genes and suppression of apoptotic genes (Jang *et al.* 2010). Additionally, it is soluble in lipids and, thus, is incorporated into cell membranes and reduces DNA damage (Kuraji *et al.* 2016). Ax added to the IVC medium of embryos improved bovine embryonic development impaired by heat stress (Namekawa *et al.* 2010), and its supplementation effectively promoted the maturation, fertilization, and development of oocytes exposed to heat stress during IVM in pigs (Do *et al.* 2015). Therefore, Ax is also assumed to be beneficial during

IVG; however, the effects of Ax on bovine OCGCs obtained from early antral follicles during IVG culture currently remain unknown. A previous study reported that oxidative stress induced apoptosis in mouse granulosa cells (Weng *et al.* 2016). Therefore, the antioxidative effects of Ax are expected to improve the steroid hormone environment for OCGCs.

Cathepsin B (CTSB) is a lysosomal cysteine protease found in many types of cells such as bovine oocytes (Balboula *et al.* 2010). A relationship has been reported between CTSB activity and apoptosis, in which CTSB was found to induce the apoptotic pathway by activating caspases, and the inhibition of CTSB during IVM significantly improved the developmental competence of bovine COCs as well as the quality of their embryos (Balboula *et al.* 2010). Moreover, the activity of CTSB inversely correlated with the developmental competence of bovine oocytes, and, thus, CTSB activity may be a useful marker for oocytes and embryos of inferior quality (Balboula *et al.* 2013). Although the role of CTSB activity has been elucidated in bovine oocytes, its activity in bovine oocytes derived from IVG and the effect of Ax on CTSB activity currently remains unclear.

In the present study, the effects of Ax supplementation during IVG on the growth parameters of OCGCs (survivability, antrum formation in the granulosa cell layer, and diameter of oocytes), the quality of oocytes after IVM (CTSB activity), maturation, the further embryonic development of bovine oocytes obtained from early antral follicles, and the production of steroid hormones from granulosa cells were investigated.

Materials and methods

Chemicals

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

Collection of OCGCs from early antral follicles and IVG

Bovine ovaries (n=158) were obtained from a local abattoir. They were transported to the laboratory within 6 to 10 hours of collection in a plastic bag at 20°C. After three washes in physiological sterile saline, sliced ovarian cortex tissues (<1 mm thick) were prepared using a surgical blade (No. 11) and stored in TCM199 (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 elsewhere (Huang *et al.* 2013; Huang 2014). Under a stereomicroscope, early antral follicles (0.5-1 mm in diameter) were dissected from the sliced ovarian tissues using a surgical blade (No. 20) **(**Fig. 1A**)**. OCGCs were isolated from follicles using a pair of fine forceps and those with a normal appearance were individually cultured in 96-well culture plates (Falcon 353872, Becton Dickinson, Franklin Lakes, NJ, USA) with 200 µL of growth medium for 12 days at 39°C in humidified air with 5% CO_2. Growth medium consisted of HEPES-buffered TCM199 (Invitrogen) supplemented with 0.91 mM sodium pyruvate, 10 ng/mL androstenedione, 5% fetal calf serum (FCS; Invitrogen), 4 mM hypoxanthine, 4% polyvinylpyrrolidone (PVP; MW 360,000), and 50 mg/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 CCD camera (Moticam 2000, Shimadzu Rika

129 Corporation, Tokyo, Japan), and the diameters of oocytes (excluding the zona pellucida) were
130 assessed using software (Motic Images Plus 2.2s, Shimadzu) **(**Fig. 1B**)**. During the IVG
131 culture, half (100 µL) of the growth medium was replaced by the same amount of fresh
132 medium every 4 days. In the Ax-treated group, 500 µM of Ax was added to IVG medium.
133 IVG medium without Ax was used as a control. The dose of Ax was selected according to a
134 previous study, in which 500 µM Ax achieved the highest developmental competence in
135 bovine IVF embryos (Jang *et al.* 2010).
136
137 *Evaluation of OCGC growth*
138 The viability of OCGCs, antrum formation in the granulosa cell layer, and diameter of
139 oocytes were employed as OCGC growth parameters in the present study. OCGC growth
140 parameters were measured before and after the IVG culture. The survivability of OCGCs was
141 evaluated by their morphological appearance according to previously reported criteria
142 (Huang *et al.* 2013). Oocytes were considered to be viable when completely enclosed by a
143 healthy granulosa cell layer at the end of IVG, and isolated oocytes had a cytoplasm with a
144 normal appearance and several layers of cumulus cells **(**Fig. 1C**)**. OCGCs were considered to
145 be abnormal and/or dead when oocytes were denuded by a scattering cumulus and granulosa
146 cells and/or had an abnormal appearance **(**Fig. 1D**)**.
147
148 *IVM of in vivo-grown and IVG oocytes*
149 *In vivo*-grown oocytes (approximately 120 µm in diameter) were collected from antral
150 follicles (2–8 mm in diameter) as described previously (Huang *et al*., 2013; Huang 2014) and

submitted them to IVM. Briefly, cumulus-oocyte complexes (COCs) were incubated in 50-μL droplets of IVM medium (approximately 10 COCs per droplet) and were then covered with paraffin oil for 22 h at 39°C in a humidified atmosphere with 5% CO_2 (Takahashi and Kanagawa 1998a). COCs derived from OCGCs after IVG were cultured individually in microwell plates (Mini Trays 163118; NUNC, Roskilde, Denmark) filled with 6 mL of maturation medium (Nagano *et al.* 2013). Maturation medium consisted of HEPES-buffered TCM199 supplemented with 0.2 mM sodium pyruvate, 0.02 units/mL FSH (from the porcine pituitary), 1 mg/mL E_2, 10% FCS, and 50 mg/mL gentamicin sulfate at 39°C for 22 h under 5% CO_2 in air.

Evaluation of oocyte nuclear maturation

Following IVM, oocytes were denuded from cumulus cells by vortexing and were then stained with 1% aceto-orcein. In order to evaluate nuclear maturation, their nuclear status was classified as germinal vesicle (GV; Fig. 2A), germinal vesicle breakdown (GVBD), metaphase I (M I), and metaphase II (M II; Fig. 2B). based on observations under a phase-contrast microscope (Nagano *et al.* 2006). Before fixation after IVM, the diameter of each denuded oocyte was measured and regarded as the oocyte diameter after the IVG culture.

IVF and IVC

IVF using frozen semen was performed according to a previously described procedure (Takahashi and Kanagawa 1998a) with slight modifications. Briefly, motile sperm (5×10^6 sperm/mL) separated by a Percoll gradient (45% and 90%) were incubated with COCs in a

100-μL droplet (approximately 10 COCs per droplet) of modified Brackett and Oliphant isotonic medium (Brackett and Oliphant 1975) containing 3 mg/mL fatty acid-free BSA and 2.5 mM theophylline (Takahashi and First 1992) at 39°C for 18 h in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. IVC of inseminated oocytes (presumptive zygotes) was performed as previously described (Takahashi and Kanagawa 1998b). Briefly, after an incubation with sperm, presumptive zygotes were freed from cumulus cells by vortexing and washing three times in culture medium. Cumulus-free zygotes were cultured for 6 days in 30-μL droplets (approximately 20 zygotes per droplet) of culture medium at 39 °C under 5% CO_2, 5% O_2, and 90% N_2. Culture medium consisted of modified synthetic oviduct fluid containing 1 mM glutamine, 12 essential amino acids for basal medium Eagle, seven non-essential amino acids for minimum essential medium, 10 μg/mL insulin, and 5 mM glycine, 5 mM taurine, 1 mM glucose, and 3 mg/mL fatty acid-free BSA. Cleavage (Fig. 2C) and blastocyst (Fig. 2D) rates were assessed after 2 days (approximately 30 h) and 6 days (approximately 150 h) of IVC, respectively. The total cell numbers of blastocysts obtained after 6 days of IVC were counted using an air-drying method (Takahashi and First 1992).

Evaluation of ROS generation after IVG oocytes

After 12 days of the IVG culture, COCs derived from OCGCs were transferred and incubated in Petri dishes at 37 °C for 15 min in the dark in 500 μL Dulbecco's phosphate-buffered saline (DPBS) supplemented with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) and 10% FCS, as described previously (Huang *et al.* 2016). Hoechst 33342 was added at a concentration of 25 μg/ml to detect nuclei, and incubated under the same culture

conditions for a further 10 min. After washing three times in DPBS, stained oocytes were mounted on glass slides with coverslips and examined under a fluorescence microscope using an excitation filter of 495 nm to observe ROS generation (Fig. 3A) and an excitation filter of 365 nm to detect nuclei (DMi8, LEICA Co., Wetzlar, Germany). In order to compare the fluorescent intensity of the captured images of COCs (area of oocytes), the average of total fluorescence emissions (pixels) was examined by the image analyzing software ImageJ 1.38e (LISTSERV, NIH, MD, USA). All images were taken precisely at the same parameters for all groups.

Evaluation of CTSB activity after IVM oocytes

The detection of CTSB activity in COCs derived from OCGCs was performed using the Magic red CTSB detection kit (P 6133; Immunochemistry Technologies LLC, Bloomington, MN, USA) according to the manufacturer's instructions and as previously described (Balboula *et al.* 2010). Briefly, COCs after IVM were incubated in 500 μL DPBS with 2 μL of the reaction mix in a 4-well dish (176740 Nunc, Thermo Fisher Scientific, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 at 38.5 °C for 20 min. Hoechst 33342 was added at a concentration of 25 μg/ml to detect nuclei, and incubated under the same culture conditions for a further 10 min. After rinsing in DPBS containing 3 mg/ml PVP, stained fresh COCs were mounted onto a glass slide with a coverslip, and examined under the fluorescence microscope (LEICA). An excitation filter of 365 nm was used to detect nuclei, while an excitation filter of 550 nm was applied to observe CTSB activity. CTSB activity images of

oocytes were captured and analyzed by ImageJ software (NIH) (Fig. 3A). All images were taken precisely at the same parameters for all groups.

Hormonal assay

The culture medium (100 µL) at 4, 8, and 12 days of the IVG culture was collected and frozen at −30 °C until the P_4 and E_2 assays using a competitive double antibody enzyme immunoassay, as previously described (Yanagawa *et al.* 2015). Briefly, samples (n=61 and 36 for the Ax and control groups, respectively) were subjected to 2- to 2000-fold serial dilutions with assay buffer (145 mM NaCl, 40 mM Na_2HPO_4, and 0.1% BSA (w/v), pH 7.2). Diluted samples (20 µL) were incubated with the primary antisera and HRP-labeled hormone (100 µL each) in the wells of a 96-well microplate (Costar 3590, Corning, NY, USA) coated with the secondary antiserum at 4 °C for 16-18 h. The primary antisera used for the E_2 and P_4 assays were anti-estradiol-17β-6-CMO-BSA (FKA 204, Cosmo Bio, Tokyo, Japan) and anti-progesterone 3-CMO-BSA (KZ-HS-P13, Cosmo Bio, Tokyo, Japan), respectively. Goat anti-rabbit serum (111-005-003, Jackson Immune Research, PA, USA) was used as secondary antiserum. After washing all wells four times with more than 300 µL of washing buffer (0.05% Tween 80), 150 µL of TMB solution (5 mM citric acid, 50 mM Na_2HPO_4, 500 mM UHP, 1 mM TMB, and 2% DMSO) was added to each well and incubated at 37 °C for 40 min. The absorbance of the solution in the wells was measured at 450 nm using a microplate reader (Model 550, Bio-Rad Laboratories, Tokyo, Japan) after stopping the chromogenic reaction with 50 µL of 4 N H_2SO_4. All samples were assayed in triplicate. Assay

sensitivities were 7.1 pg/well for E_2 and 11.2 pg/well for P_4. The inter- and intra-assay coefficients of variation were 5.1 and 4.0% for E_2 and 10.1 and 3.9% for P_4, respectively.

E_2 and P_4 production during each period (days 4-8 and days 8-12) was calculated using the following formula (Sakaguchi *et al*., 2017): Steroid hormone production (ng) = 0.2 (mL) × concentration at the end of the period (ng/mL) - 0.1 (mL) × concentration at the start of the period (ng/mL).

Experimental design

Experiment 1

A total of 692 OCGCs (14 replicates) were used to evaluate the effects of the presence or absence of Ax on oocyte viability and antrum formation after 12 days of IVG. Oocyte diameters were measured before and after the IVG culture; measurements of cumulus-denuded oocytes (6-7 replicates, n = 110 for Ax and n = 76 for control) derived from 296 OCGCs (10 to 20 oocytes per replicate) after IVM were used as the diameters after IVG. In addition, a total of 51 oocyte derived from 89 OCGCs (6 replicates) were used to evaluate the effect of Ax addition to IVG medium on the nuclear status immediately after IVG.

Experiment 2

In order to evaluate the effects of Ax on ROS generation by IVG oocytes, 44 viable IVG oocytes derived from 65 OCGCs were used to examine ROS generation (3 replicates; 5-8 oocytes per replicate). In order to evaluate the quality of oocytes after IVM, CTSB activity

(3 replicates) was investigated using 49 IVG oocytes derived from 82 OCGCs (6 to 8 oocytes per replicate).

Experiment 3

In order to investigate oocyte developmental competence, IVF was performed using 154 IVG oocytes derived from 249 OCGCs (4 replicates; 20 oocytes per replicate).

Experiment 4

In order to investigate steroidogenesis in granulosa cells during the IVG culture, E_2 and P_4 production during each period (days 0-4, 4-8, and 8-12) was calculated in the Ax-treated and control groups.

Statistical analysis

All statistical analyses were performed using JMP software version 11.0.0 (SAS Institute, Cary, NC, USA). The effects of Ax on oocyte diameters, cleavage and blastocyst rates, and blastocyst cell numbers were analyzed using a one-way ANOVA followed by Turkey-Kramer's HSD as a post hoc test. The effects of Ax on the viability and antrum formation of OCGCs, and the oocyte nuclear status were analyzed by the chi-squared test. E_2 and P_4 production in the Ax and control groups was compared by a one-way ANOVA followed by Turkey-Kramer's HSD as a post hoc test. Differences of $P < 0.05$ were considered to be significant.

Results

Effects of the Ax treatment on OCGC growth parameters after IVG.

The results for the growth parameters of OCGCs before and after the IVG culture in the presence and absence of the Ax treatment are shown in Fig. 4. OCGC viability decreased in a day-dependent manner in both groups. However, OCGC viability during the IVG culture was significantly higher in the Ax-treated group than in the control ($P < 0.05$). The percentage of antrum-forming OCGCs increased in a day-dependent manner in both groups and was significantly higher in the Ax-treated group than in the control ($P < 0.05$). The diameters of oocytes were significantly larger ($P < 0.05$) than those before the IVG culture in both groups; however, no significant difference was observed in oocyte diameters between the Ax and control groups ($P > 0.05$).

Effects of the Ax treatment on ROS and CTSB activities

ROS and CTSB activities in IVG oocytes were greater in the control group than in the Ax-treated group ($P < 0.01$; Fig. 3B).

Effects of the Ax treatment before and after IVG on the nuclear maturation of IVG oocytes and their subsequent development

No significant differences were noted between meiotic stages before IVM (Table 1). Most of IVG oocytes (approximately 90%) were arrested at GV stage regardless of Ax addition. However, after IVM, the percentage of oocytes at the M II stage was slightly higher in the

Ax group than in the control group ($P = 0.05$) and the percentage of oocytes progressed beyond GVBD stage were higher in Ax-treated group than the control group ($P < 0.05$; Table 2). *In vivo*-grown oocytes showed higher maturation rate than both group of IVG oocytes (Table 2). No significant differences were noted in cleavage rates after IVF between the groups ($P > 0.05$; Table 3), but cleavages rate in both IVG groups were lower than that in *in vivo*-grown oocytes. The blastocyst rate based on inseminated oocytes was significantly higher in the Ax-treated group than in the control group ($P < 0.05$). The mean cell number in blastocysts was significantly larger in the Ax-treated group than in the control group ($P < 0.05$). However, these values were higher in *in vivo*-grown oocytes than in both IVG groups.

Hormone production

The results of steroidogenesis by OCGCs were shown in Fig. 5. Throughout the IVG culture period, the production of P_4 was greater in the control group than in the Ax-treated group ($P < 0.05$); however, the production of E_2 between days 0-4 was higher in the Ax-treated group than in the control group ($P < 0.05$). E_2 production was lower in the Ax-treated group than in the control group between days 4-8 ($P < 0.05$), but was similar between days 8-12.

Discussion

The supplementation of culture medium with antioxidants has been shown to protect oocytes and embryos from the detrimental effects of heat and oxidative stress (Jang *et al.* 2010). Oxidative stress is identified as an imbalance between the production and neutralization of

ROS that may occur because of excess ROS production and/or a deficiency in antioxidant mechanisms (Combelles *et al.* 2009). The generation of ROS in *in vitro* culture media has harmful effects on oocytes, embryo quality, the post-fertilization development of embryos, and assisted reproduction outcomes (Das *et al.* 2006). ROS are highly reactive with complex cellular molecules including lipids, proteins, and DNA, and produce significant malfunctions such as enzyme inactivation, the loss of membrane integrity, mitochondrial abnormalities, and DNA fragmentation (Agarwal A 2005). ROS also induce development blocks and the retardation of mammalian preimplantation embryos (Guérin *et al.* 2001). The present study showed that the Ax treatment during IVG significantly decreased ROS production by oocytes after 12 days of IVG to lower than that in IVG media without Ax. This result indicates that supplementation with Ax mitigates the deleterious effects of IVG long incubation-induced ROS on the growth parameters and subsequent development of bovine embryos.

Ax elicits strong antioxidant effects on cellular, lipid peroxidation, and embryonic development (Jang *et al.* 2010). Combelles *et al*. (2009) reported that in order for an embryo to acquire developmental competence, it is vitally important for antioxidants to be stored in oocytes (as mRNA transcripts or proteins) through their growth and maturation stages. Furthermore, Ax is soluble in lipids, so it is incorporated into the cell membrane and reduces DNA damage (Kuraji *et al.* 2016) as demonstrated by decreased ROS production after IVG and improved developmental competence of oocytes. Therefore, Ax-improving effects during IVG may be attributed to its antioxidant activity. Moreover, the supplementation of Ax to maturation medium improved oocyte maturation, fertilization, and developmental competence after fertilization under normal or heat stress conditions (Do *et al.* 2015).

347 Namekawa *et al*. (2010) reported that the improving effects of Ax appeared to be due to its
348 ability to alter the expression of stress-related genes.

349 Our results showed that the number of oocytes at the M II stage was slightly higher in the
350 IVG group treated with Ax than in the control group, and the blastocyst rate and cell numbers
351 in blastocysts were significantly higher in the IVG group treated with Ax. These results
352 suggest that Ax improved the quality of oocytes during IVG, as reflected by enhanced
353 cytoplasmic maturation and developmental competence. The reduction observed in CTSB
354 activity in the Ax-treated group in the present study indicated an improvement in cytoplasmic
355 maturation. Successful IVM requires not only nuclear, but also cytoplasmic maturation
356 (Combelles *et al.* 2009). Success has been achieved in terms of nuclear maturation *in vitro*;
357 however, cytoplasmic maturation is delayed, reflecting asynchrony between nuclear and
358 cytoplasmic maturation (Combelles *et al.* 2009). The accomplishment of nuclear changes to
359 produce a M II oocyte is not recognized as developmental competence and does not reflect
360 the molecular and structural maturity of an oocyte, which is sometimes termed cytoplasmic
361 maturation (Trounson *et al.* 2001). CTSB is a lysosomal cysteine protease of the papain
362 enzyme family that is involved in the induction of apoptosis, degradation of the extracellular
363 matrix, and catabolism of intracellular proteins (Balboula *et al.* 2010). Previous studies
364 indicated that an inverse relationship exists between CTSB activity and the quality of bovine
365 oocytes and embryos (Balboula *et al.* 2010). The artificial inhibition of CTSB with the
366 specific inhibitor E-64 improved the developmental competence of preimplantation embryos
367 and increased the total number of good quality embryos by attenuating apoptosis (Balboula
368 *et al.* 2013). The present study showed that CTSB activity was significantly weaker in MII

oocytes treated with Ax during IVG than in those in the control group, indicating that Ax supplementation has the ability to improve the quality of oocytes. This result suggests that the significant increases observed in the blastocyst rate and embryonic quality are due to the promotion of oocyte cytoplasmic maturation.

The results obtained between days 4 to 8 and days 8 to 12 showed that P_4 production was significantly lower when Ax was added, indicating that the production of P_4 by granulosa cells was inhibited by the supplementation of IVG media with Ax. Although E_2 production decreased between days 4 to 8 compared with days 0 to 4 regardless of Ax treatment, E_2 production in Ax-treated group significantly increased from days 8-12 as OCGCs grew during IVG similar to the *in vivo* development of dominant follicles (Kruip and Dieleman 1985). P_4 is generally regarded as a suppressor of follicle growth and inhibits mitosis in small granulosa cells as well as follicle development (Peluso and Pappalardo 1998), as observed by the positive correlation reported between circulating P_4 levels and delayed antral follicle development in rats (Buffler and Roser 1974) and monkeys (Ting *et al.* 2015). Additionally, in atretic follicles, granulosa cells luteinized and their P_4 production increased (Jolly *et al.* 1994). Moreover, OCGCs that produced mature oocytes secreted slightly larger amounts of E_2 and less P_4 than OCGCs that produced immature oocytes, and OCGCs with antrums produced more E_2 and less P_4 than those without antrums, similar to follicles that grew *in vivo* (Sakaguchi *et al.* 2017). Endo *et al*. (2013) suggested that E_2 secretion was closely related to OCGC developmental competence, that E_2 itself induced OCGCs to secrete E_2, and that OCGCs forming antrums exhibited similar levels of gene expression to healthy follicles that grew *in vivo.* Sakaguchi *et al*. (2017) reported that although antrum formation in the

granulosa cell layer was related to the steroidogenesis of OCGCs, a relationship did not exist between oocyte maturation and antrum formation in the granulosa cell layer. On the other hand, the synthesis of P_4 is a feature of the OCGC luteinization process (Murphy 2000). Based on these findings, Ax was shown to promote antrum formation and E_2 production with the suppression of P_4 production in the present study, suggesting its effects on anti-luteinization and subsequent steroidogenesis, similar to healthily growing follicles *in vivo*. In our previous study (Sakaguchi *et al*., 2017), E_2 production decreased between days 8 and 12, but increased in the present study even though we used basically the same IVG medium. In the previous study, we used 50 μg/ml ascorbic acid 2-glucoside as an antioxidant instead of Ax. Since ascorbic acid 2-glucoside produces glucose during cultures, we speculate that the glucose produced may have exerted harmful effects on E_2 production by granulosa cells. Recently, Kamada *et al*. (2017) reported that low concentration (0.1 to 10 nM) of Ax increased the P_4 production of luteal cells, but high concentrations (1,000 nM) of Ax suppressed P_4 production of luteal cells. And they speculated that the effect of Ax on P_4 production was not caused by antioxidative function. We used high concentration (500 μM) of Ax for IVG and did not examine the function of Ax in the present study. In further study, we should examine the mechanism of enhanced E_2 production and suppressed P_4 production by Ax in detail.

In conclusion, the present results demonstrated that Ax supplementation improved the growth parameters and developmental competence of bovine oocytes derived from early antral follicles after IVG by suppressing ROS generation during IVG. Lower CTSB activity in IVG oocytes after IVM may indicate improvements in cytoplasmic maturation.

413 Furthermore, the inhibition of P_4 production without the suppression of E_2 production
414 between days 8 to 12 of IVG culture suggests that the luteinization of granulosa cells during
415 IVG cultures is inhibited by the antioxidative effects of Ax. Further investigation is needed
416 to clarify the function of Ax on steroidogenesis during IVG.
417
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420
421 **References**
422 Agarwal, A., and Sharma R. (2005). Oxidative stress and its implications in female infertility
423 - a clinician's perspective. *Reprod. Biomed. Online* **11**, 641-650.

424
425 Balboula, A.Z., Yamanaka, K., Sakatani, M., Hegab, A.O., Zaabel, S.M., and Takahashi, M.
426 (2010). Intracellular cathepsin B activity is inversely correlated with the quality and
427 developmental competence of bovine preimplantation embryos. *Mole. Reprod. Dev.* **77**,
428 1031-1039.

429
430 Balboula, A.Z., Yamanaka, K., Sakatani, M., Kawahara, M., Hegab, A.O., Zaabel, S.M., and
431 Takahashi, M. (2013). Cathepsin B activity has a crucial role in the developmental
432 competence of bovine cumulus–oocyte complexes exposed to heat shock during *in vitro*
433 maturation. *Reproduction* **146**, 407-417.

Brackett, B.G., and Oliphant, G. (1975). Capacitation of Rabbit Spermatozoa *in vitro*. *Biol. Reprod.* **12**, 260-274.

Buffler, G., and Roser, S. (1974). New data concerning the role played by progesterone in the control of follicular growth in the rat. *Acta Endocrinol. (Copenh)* **75**, 569-578.

Combelles, C.M.H., Gupta, S., and Agarwal, A. (2009). Could oxidative stress influence the in-vitro maturation of oocytes? *Reprod. Biom. Online* **18**, 864-880.

Das, S., Chattopadhyay, R., Ghosh, S., Goswami, S.K., Chakravarty, B.N., and Chaudhury, K. (2006). Reactive oxygen species level in follicular fluid—embryo quality marker in IVF? *Hum. Reprod.* **21**, 2403-2407.

Do, L.T.K., Luu, V.V., Morita, Y., Taniguchi, M., Nii, M., Peter, A.T., and Otoi, T. (2015). Astaxanthin present in the maturation medium reduces negative effects of heat shock on the developmental competence of porcine oocytes. *Reprod. Biol.* **15**, 86-93.

452 Endo, M., Kawahara, M. R., Cao, F., Kimura, K., Kuwayama, T., Monji, Y., and Iwata, H.
453 (2013). Estradiol supports *in vitro* development of bovine early antral follicles. *Reproduction*
454 **145**, 85-96.
455
456 Fassett, R.G., and Coombes, J.S. (2011). Astaxanthin: A Potential Therapeutic Agent in
457 Cardiovascular Disease. *Marine Drugs* **9**, 447-465.
458
459 Guérin, P., El Mouatassim, S., and Ménézo, Y. (2001). Oxidative stress and protection
460 against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum.*
461 *Reprod. Update* **7**, 175-189.
462
463 Hansen, P.J. (2014). Current and future assisted reproductive technologies for mammalian
464 farm animals. *Adv. Exp. Med. Biol.* **752**, 1-22.
465
466 Hennet, M. L., and Combelles, C.M.H. (2012). The antral follicle: a microenvironment for
467 oocyte differentiation. *Int. J. Dev. Biol.* **56**, 819 - 831.
468
469 Huang, W. (2014). 'Studies on *in vitro* maturation/fertilization/development and
470 mitochondrial activity of *in vitro*-grown bovine oocytes derived from early antral follicles.'

(Doctoral dissertation). Hokkaido University Collection of Scholarly and Academic Papers, https://eprints.lib.hokudai.ac.jp/dspace/bitstream/2115/58152/1/Huang_Weiping.pdf

Huang, W., Kang, S.-S., Nagai, K., Yanagawa, Y., Takahashi, Y., and Nagano, M. (2016). Mitochondrial activity during pre-maturational culture in *in vitro*-grown bovine oocytes is related to maturational and developmental competences. *Reprod. Fertil. Dev.* **28**, 349-356.

Huang, W., Nagano, M., Kang, S.-S., Yanagawa, Y., and Takahashi, Y. (2013). Effects of *in vitro* growth culture duration and prematuration culture on maturational and developmental competences of bovine oocytes derived from early antral follicles. *Theriogenology* **80**, 793-799.

Jang, H., Ji, S., Kim, Y., Lee, H., Shin, J., Cheong, H., Kim, J., Park, I., Kong, H., and Park, C. (2010). Antioxidative Effects of Astaxanthin against Nitric Oxide-Induced Oxidative Stress on Cell Viability and Gene Expression in Bovine Oviduct Epithelial Cell and the Developmental Competence of Bovine IVM/IVF Embryos. *Reprod. Domest. Anim.* **45**, 967-974.

Jolly, P.D., Tisdall, D.J., Heath, D.A., Lun, S., and McNatty, K.P. (1994). Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate

491 response to follicle-stimulating hormone and luteinizing hormone, and follicular atresia. *Biol.*
492 *Reprod.* **51**, 934-44.

493

494 Kamada, H., Akagi, S., and Watanabe, S. (2017). Astaxanthin increases progesterone
495 production in cultured bovine luteal cells. *J. Vet. Med. Sci.* **79**, 1103-1109.

496

497 Kruip, T.A., and Dieleman, S.J. (1985). Steroid hormone concentrations in the fluid of bovine
498 follicles relative to size, quality and stage of the oestrus cycle. *Theriogenology* **24**, 395-408.

499

500 Kuraji, M., Matsuno, T., and Satoh, T. (2016). Astaxanthin affects oxidative stress and
501 hyposalivation in aging mice. *J. Clin. Bioch. Nut.* **59**, 79-85.

502

503 Kushnir, V.A., Barad, D.H., Albertini, D.F., Darmon, S.K., and Gleicher, N. (2017).
504 Systematic review of worldwide trends in assisted reproductive technology. *Reprod. Biol.*
505 *End.* **15**, 1-9.

506

507 Makita, M., and Miyano, T. (2015). Androgens promote the acquisition of maturation
508 competence in bovine oocytes. *J. Reprod. Dev.* **61**, 211-217.

509

510 Makita, M., Ueda, M., and Miyano, T. (2016). The fertilization ability and developmental
511 competence of bovine oocytes grown *in vitro*. *J. Reprod. Dev.* **62**, 379-384.

512

513 Murphy, B.D. (2000). Models of Luteinization. *Biol. Reprod.* **63**, 2-11.

514

515 Nagano, M., Kang, S.-S., Koyama, K., Huang, W., Yanagawa, Y., and Takahashi, Y. (2013).
516 *In vitro* maturation system for individual culture of bovine oocytes using micro-volume
517 multi-well plate. *Jap. J.Vet. Res.* **61**, 149-154.

518

519 Nagano, M., Katagiri, S., and Takahashi, Y. (2006). ATP content and
520 maturational/developmental ability of bovine oocytes with various cytoplasmic
521 morphologies. *Zygote* **14**, 299-304.

522

523 Naguib, Y.M.A. (2000). Antioxidant Activities of Astaxanthin and Related Carotenoids. *J.*
524 *Agr. F. Chem.* **48**, 1150-1154.

525

526 Namekawa, T., Ikeda, S., Sugimoto, M., and Kume, S. (2010). Effects of Astaxanthin-
527 containing Oil on Development and Stress-related Gene Expression of Bovine Embryos
528 Exposed to Heat Stress. *Reprod. dom. anim.* **45**, 387-391.

529

Peluso, J.J., and Pappalardo, A. (1998). Progesterone mediates its anti-mitogenic and anti-apoptotic actions in rat granulosa cells through a progesterone-binding protein with gamma aminobutyric acidA receptor-like features. *Biol. Reprod.* **58**, 1131-1137.

Sakaguchi, K., Huang, W., Yang, Y., Yanagawa, Y., and Nagano, M. (2017). Relationship between *in vitro* growth of bovine oocytes and steroidogenesis of granulosa cells cultured in medium supplemented with bone morphogenetic protein-4 and follicle stimulating hormone. *Theriogenology* **97**, 113-123.

Szamatowicz, M. (2016). Assisted reproductive technology in reproductive medicine - possibilities and limitations. *Ginekol. Pol.* **87**, 820-823.

Takahashi, Y., and First, N.L. (1992). *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**, 963-978.

Takahashi, Y., and Kanagawa, H. (1998a). Effect of oxygen concentration in the gas atmosphere during *in vitro* insemination of bovine oocytes on the subsequent embryonic development *in vitro*. *J.Vet. Med. Sc.* **60**, 365-367.

550 Takahashi, Y., and Kanagawa, H. (1998b). Effects of Glutamine, Glycine and Taurine on the
551 development of *in vitro* fertilized bovine zygotes in a chemically defined medium. *J. Vet.*
552 *Med. Sc.* **60**, 433-437.

553

554 Ting, A.Y., Xu, J., and Stouffer, R.L. (2015). Differential effects of estrogen and
555 progesterone on development of primate secondary follicles in a steroid-depleted milieu *in*
556 *vitro*. *Hum. Reprod.* **30**, 1907-1917.

557

558 Trounson, A., Anderiesz, C., and Jones, G. (2001). Maturation of human oocytes *in vitro* and
559 their developmental competence. *Reproduction* **121**, 51-75.

560

561 Weng, Q., Liu, Z., Li, B., Liu, K., Wu, W., and Liu, H. (2016). Oxidative Stress Induces
562 Mouse Follicular Granulosa Cells Apoptosis via JNK/FoxO1 Pathway. *PLoS ONE* **11**,
563 e0167869.

564

565 Yanagawa, Y., Matsuura, Y., Suzuki, M., Saga, S., Okuyama, H., Fukui, D., Bando, G.,
566 Nagano, M., Katagiri, S., Takahashi, Y., and Tsubota, T. (2015). Accessory corpora lutea
567 formation in pregnant Hokkaido sika deer (Cervus nippon yesoensis) investigated by
568 examination of ovarian dynamics and steroid hormone concentrations. *J. Reprod. Dev.* **61**,
569 61-6.

570 Yang, Y. X., Kim, Y. J., Jin, Z., Lohakare, J. D., Kim, C. H., Ohh, S. H., Lee, S. H., Choi .
571 J. Y., and Chae B. J. (2006). Effects of dietary supplementation of astaxanthin on production
572 performance, egg quality in layers and meat quality in finishing pigs. *Asian. Aust. J. Anim.*
573 *Sci.* **19**, 1019-1025.
574
575 Yang, Y., Kanno, C., Huang, W., Kang, S.-S., Yanagawa, Y., and Nagano, M. (2016). Effect
576 of bone morphogenetic protein-4 on *in vitro* growth, steroidogenesis and subsequent
577 developmental competence of the oocyte-granulosa cell complex derived from bovine early
578 antral follicles. *Reprod. Biol. End.* **14**, 1-8.
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582 **Figure Legends**

583

584 **Figure 1:** Collected early antral follicle and *in vitro* culture of Oocyte-cumulus-granulosa
585 complexes (OCGCs).
586 (A) Early antral follicle (white arrow) under 1 mm in diameter was collected under
587 stereomicroscope. Follicular diameter was measured by grid line (1 mm) on the bottom of a
588 petri dish; (B) viable OCGC enclosed by several layers of healthy granulosa cells. Oocyte
589 diameter was calculated as the mean value of longest and shortest diameter using software
590 (Motic Images Plus 2.2s, Shimadzu). The OCGC formed several antra after 4, 8 and 12 days
591 of IVG (white arrowhead); (C) viable OCGC enclosed by several layers of healthy granulosa
592 cells without antra after 12 days of IVG; (D) a degenerated oocyte and dispersed granulosa
593 cells after 12 days of IVG. Scale bars indicate 1 mm (A) and 100 μm (B, C, D), respectively.

594

595 **Figure 2.** Oocyte nuclear status before and after IVM and embryo development after IVF.
596 (A) Immature oocyte having germinal vesicle (white arrow) before IVM; (B) mature oocyte
597 having metaphase II plate (white arrow) and a polar body (arrowhead) after IVM; (C) cleaved
598 embryos after 2 days of IVF; (D) blastocysts after 7 days of IVF.

599

600 **Figure 3:** Effects of the addition of astaxanthin (Ax) to IVG medium on reactive oxygen
601 species (ROS) and cathepsin B (CTSB) activities before and after IVM in cumulus-oocyte
602 complexes (COCs).

(A) Quantification of the relative fluorescence intensity of ROS and CTSB activity. (B) ROS and CTSB were detected as green and red fluorescence, respectively. The relative fluorescence intensities of ROS and CTSB were measured using 44 and 49 COCs (3 replicates each), respectively.

** Asterisk indicates a significant difference between the groups ($P < 0.01$).

Figure 4: Viabilities, antrum formation by oocyte-cumulus-granulosa complexes (OCGCs) during IVG cultures, and diameters of IVG oocytes obtained from early antral follicles in the presence or absence of astaxanthin (Ax).

Percentages of viability and antrum formation were calculated based on the pooled data of all replicates.

* Asterisk indicates a significant difference between experimental groups on the same days ($P < 0.05$).

^{a,b,c} Different letters indicate a significant difference between the days of the IVG culture in the control group ($P < 0.05$).

^{x,y,z} Different letters indicate a significant difference between the days of the IVG culture in the group treated with Ax ($P < 0.05$).

Figure 5: Production of progesterone (P_4) and estradiol-17β (E_2) from OCGCs in the presence or absence of astaxanthin (Ax) during the IVG culture.

* Asterisk indicates a significant difference between the experimental groups ($P < 0.05$).

624 [a,b] Different letters indicate a significant difference between the days of the IVG culture in
625 the control group ($P < 0.05$).
626 [x,y,z] Different letters indicate a significant difference between the days of the IVG culture in
627 the Ax-treated group ($P < 0.05$).
628

Table 1

Nuclear status of bovine *in vitro*-grown oocytes obtained from early antral follicles before maturation.

Treatment	No. of oocytes (replicates)	% meiotic stage					
		GV	GVBD	M I	M II	Deg	GVBD-M II
Control	27 (3)	92.6	0	0	0	7.4	0
Ax	24 (3)	95.8	0	0	0	4.2	0

GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II, Deg; degeneration.

Table 2

Effects of astaxanthin (Ax) during IVG culture on meiotic resumption after IVG in oocytes obtained from early antral follicles.

Oocyte	Ax	No. of oocytes (replicates)	% meiotic stage					
			GV	GVBD	M I	M II	Deg	GVBD-M II
In vivo-grown	-	66 (3)	4.5	1.5[a]	7.6[a]	84.8[a]	1.5[a]	93.9[a]
IVG	0 µM	76 (6)	9.2	9.2[b]	30.3[b]	38.2[b]	13.2[b]	77.6[a]
	500 µM	110 (7)	3.6	6.4[b]	30.9[b]	52.7[b]	6.4[b]	90.0[b]

GV, germinal vesicle; GVBD, germinal vesicle breakdown; M I, metaphase I; M II, metaphase II, Deg; degeneration.

[ab] Different superscripts within a column indicate a significant difference between groups ($P < 0.05$).

Table 3

Effects of astaxanthin (Ax) during IVG culture on cleavage and blastocyst production rates and cell numbers in blastocysts derived from IVG oocytes.

Oocyte	Ax	No. of oocytes (replicates)	% cleavage	% blastocyst	Cell numbers in blastocysts (n)
In vivo-grown	-	87 (4)	80.8 ± 3.5[a]	42.9 ± 10.0[a]	154.8 ± 14.0[a] (37)
IVG	0 μM	73 (4)	58.9 ± 13.1[b]	16.4 ± 4.1[c]	107.4 ± 7.1[c] (12)
	500 μM	81 (4)	65.9 ± 9.4[b]	23.6 ± 2 .2[b]	126.1 ± 11.1[b] (19)

Values are mean ± S.D.

Percentages of blastocysts are based on inseminated oocytes.

[abc] Different superscripts within a column indicate a significant difference between groups ($P < 0.05$).

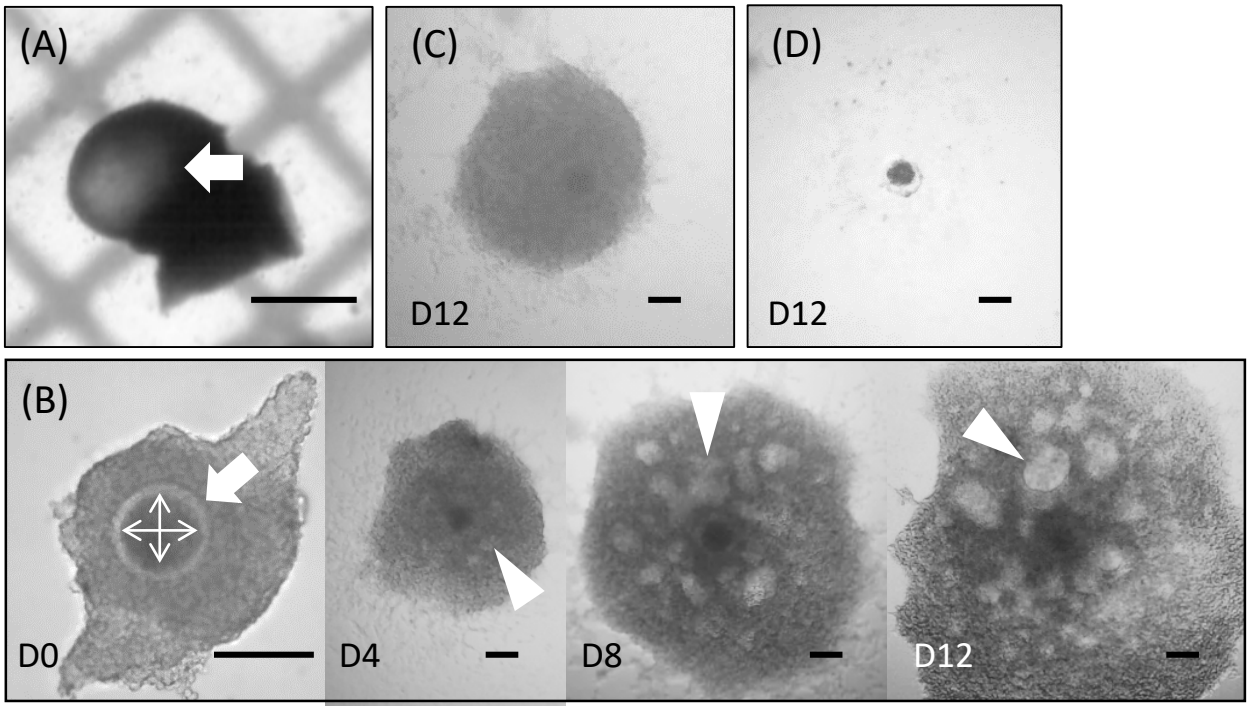

Figure 1

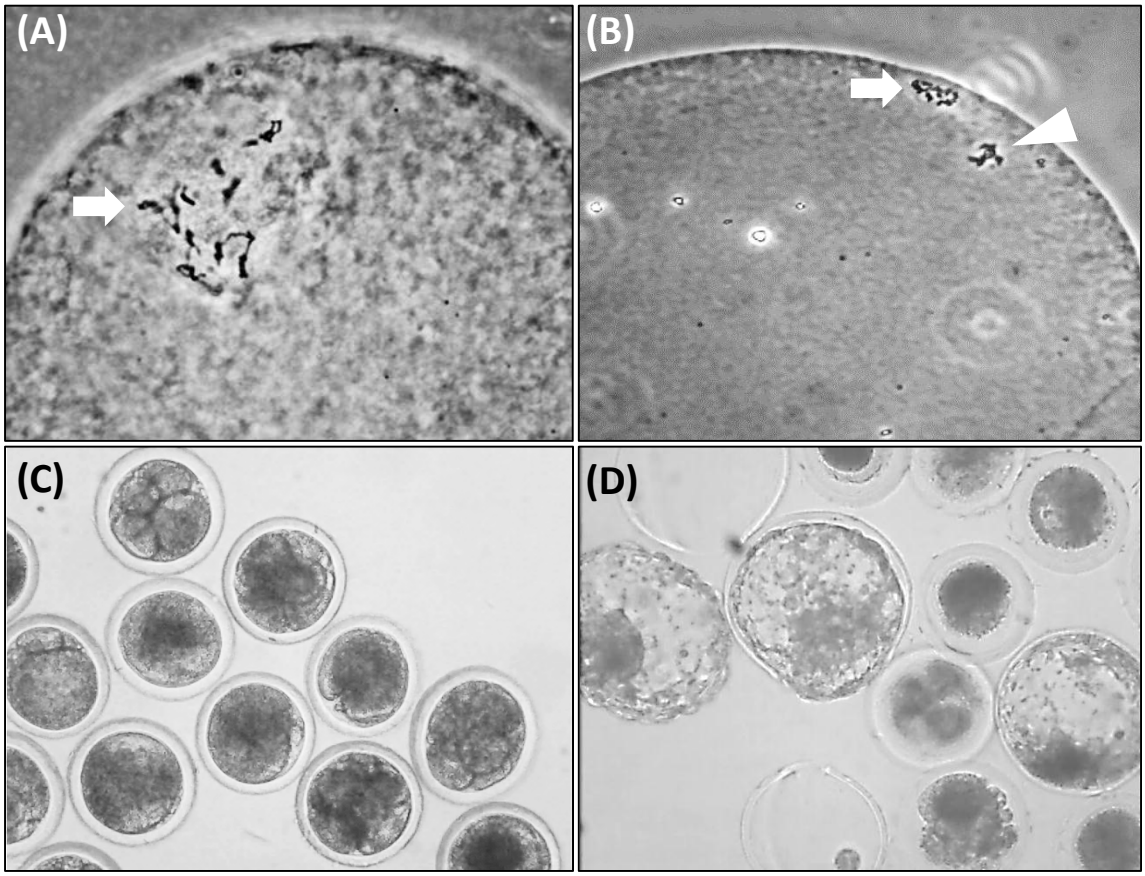

Figure 2

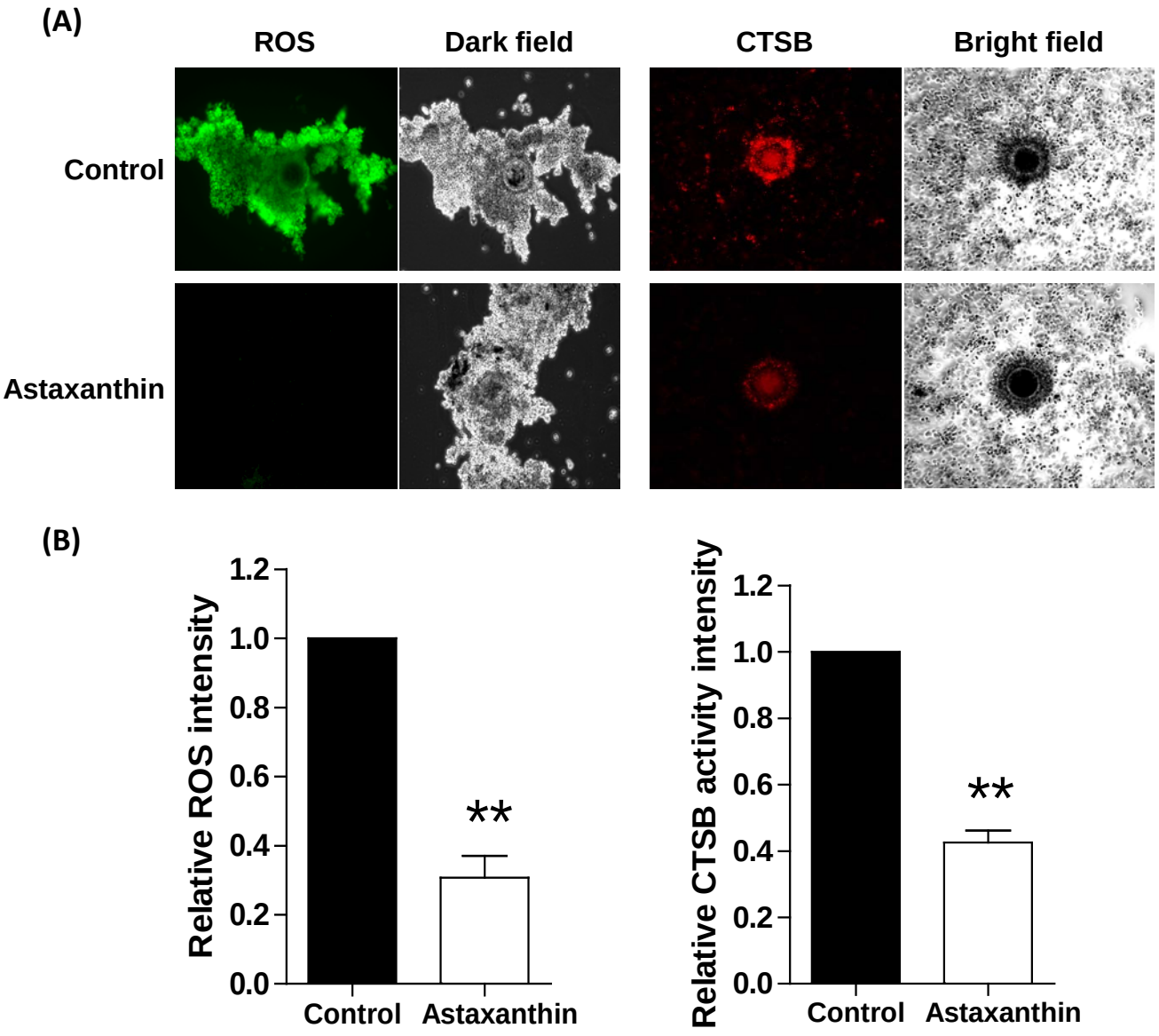
(A)
ROS
Dark field
CTSB
Bright field
Control
Astaxanthin
(B)
Relative ROS intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
Astaxanthin
Relative CTSB activity intensity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Control
Astaxanthin

Figure 3

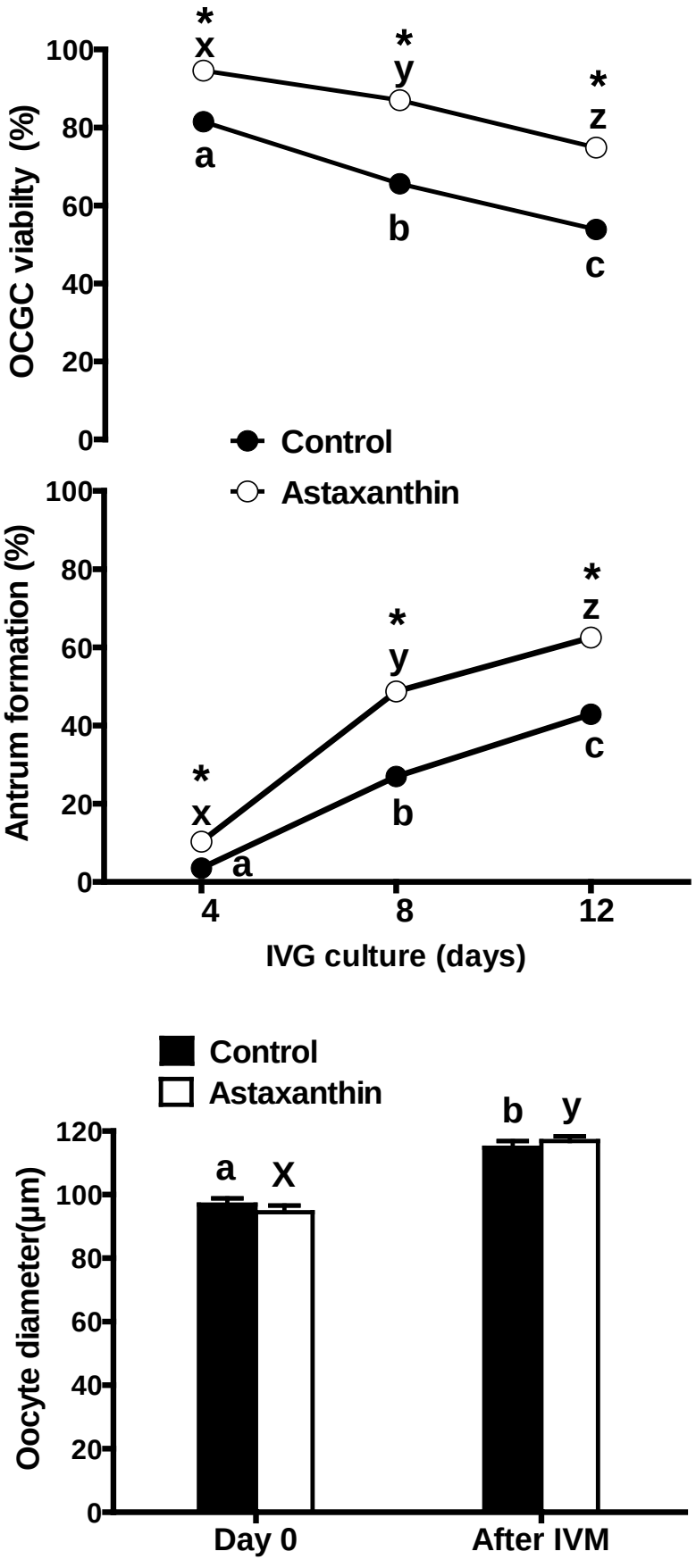
OCGC viability (%)
100
80
60
40
20
0
*
x
*
y
*
z
a
b
c
Control
Astaxanthin
Antrum formation (%)
100
80
60
40
20
0
*
z
*
y
*
x
c
b
a
4
8
12
IVG culture (days)
Control
Astaxanthin
Oocyte diameter(μm)
120
100
80
60
40
20
0
a
X
b
y
Day 0
After IVM

Figure 4

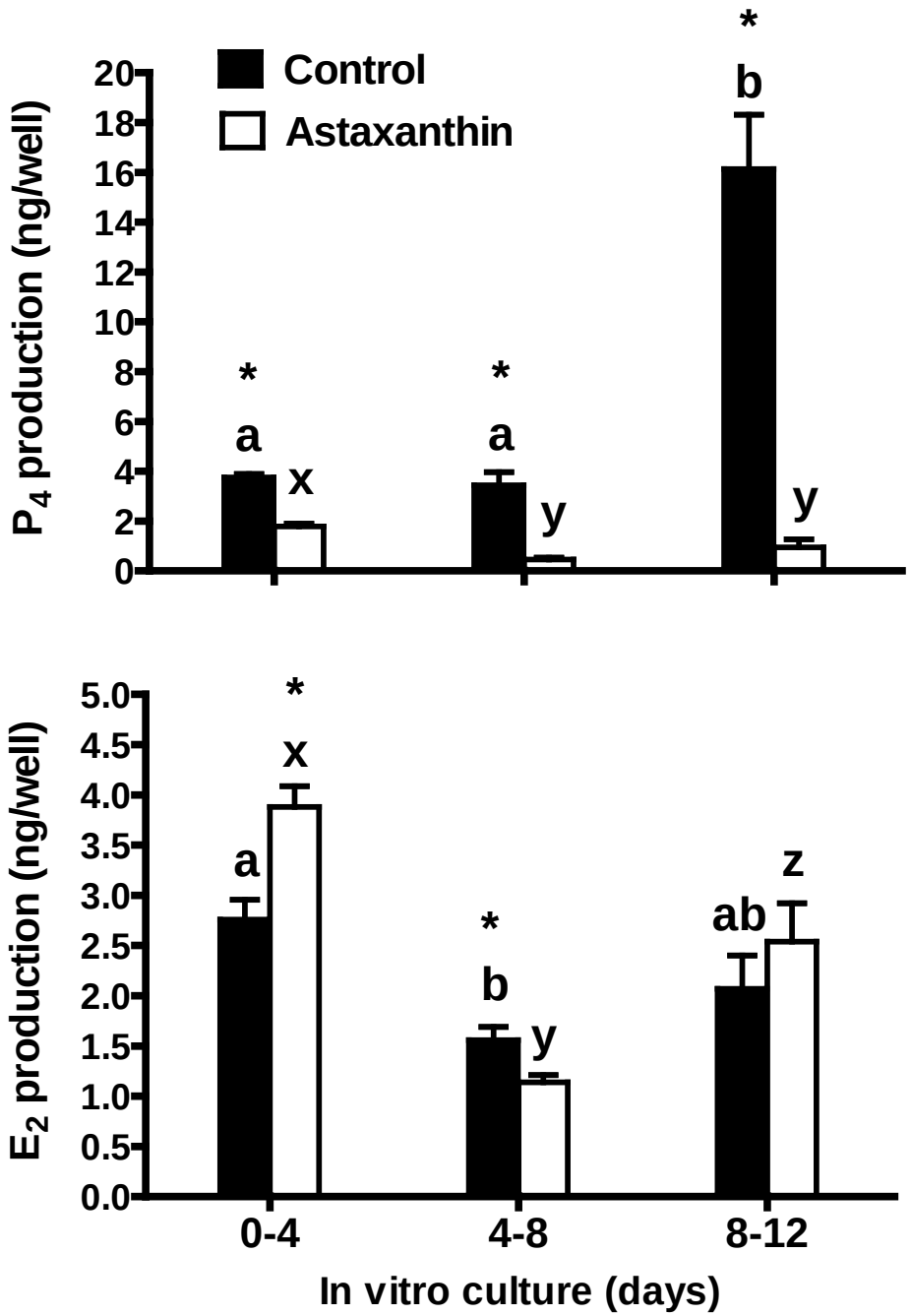
Control
Astaxanthin
P_4 production (ng/well)
20
18
16
14
12
10
8
6
4
2
0
*
a
x
*
a
y
*
b
y
E_2 production (ng/well)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
x
a
*
b
y
z
ab
0-4
4-8
8-12
In vitro culture (days)

Figure 5