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Effect of astaxanthin addition to an individual culture system for *in vitro* maturation of bovine oocytes on accumulation of reactive oxygen species and mitochondrial activity

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Abstract

We investigated the effects of astaxanthin (Ax) on the accumulation of reactive oxygen spices (ROS) and mitochondrial activity in bovine oocytes during individual maturational culture. Oocytes were cultured individually for 22 h in multi-well plates with or without 500 ng/ml Ax. After maturational culture, ROS accumulation of oocytes was lower in Ax-treated oocytes than in non-treated oocytes ($P < 0.05$). Mitochondrial activity was higher in Ax-treated oocytes than in non-treated oocytes ($P < 0.05$). The development of blastocysts in the Ax-treated oocytes tended to be higher than in non-treated oocytes ($P = 0.06$). These results indicate that Ax treatment improves the developmental competence of bovine oocytes, maybe due to the reduction of ROS accumulation and the improvement of mitochondrial activity.

Key Words: IVM, mitochondria, ROS

An *in vitro* growth (IVG) culture system for bovine small-sized oocytes derived from early antral follicles (0.5–1.0 mm in diameter) has recently been developed[3,4,13,16)]. To evaluate the developmental competence of each bovine oocyte, an individual culture system using a 60-well multi-well (MW) plate, which enables oocytes to develop to a blastocyst stage comparable to a group culture system[9)], was used for *in vitro* maturation (IVM). To correctly evaluate the competence of oocytes derived from IVG, the development of oocytes after IVM, *in vitro* fertilization (IVF), and *in vitro* culture (IVC) need to be improved. When various antioxidants (quercetin, cysteamine, carnitine, vitamin C, or resveratrol) were supplemented for IVM medium,

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blastocyst development of bovine oocytes after IVF was improved, likely due to a decrease in reactive oxygen species (ROS) accumulation[14]. However, IVM supplemented with antioxidants was performed in droplets covered with mineral oil (approximately 20 oocytes per droplet)[14], and the effect of antioxidants on individual IVM culture has not been examined yet.

Astaxanthin (Ax) is a red-orange carotenoid pigment that is present in fishery products, such as salmon, shrimp, and crab[8], and exhibits a more powerful antioxidant activity than vitamin C, vitamin E, and β-carotene[12]. The antioxidant activity of Ax was 100- to 500-fold greater than vitamin E and 15-fold greater than those of other carotenoids[12]. Moreover, 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[6]. Additionally, it is soluble in lipids and, thus, is incorporated into cell membranes and reduces DNA damage[8]. It was reported that Ax supplementation (500 ng/ml) effectively promoted the maturation, fertilization, and development of oocytes exposed to heat stress during group IVM culture in pigs[1]. However, the effect of Ax supplementation on individual IVM culture for bovine oocytes has not been reported.

In the present study, we investigated the effect of Ax supplementation (500 ng/ml), which was reported to be an effective concentration for porcine IVM[1], to individual IVM culture using a MW plate[9] on ROS accumulation, and evaluated the development of bovine oocytes to blastocysts. Moreover, we also evaluated the effect of Ax supplementation on mitochondrial activity in oocytes after IVM, because mitochondria produce ROS and mitochondrial activity affects the developmental competence of bovine oocytes[7,10].

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless indicated otherwise. Bovine ovaries (Holstein breed) obtained from a local abattoir were stored in plastic bags at 20°C and transported to the laboratory within 6–10 h of their collection. After the ovaries were washed three times with physiological saline, cumulus–oocyte complexes (COCs) were aspirated from small antral follicles (2–8 mm in diameter) as previously described[11]. The COCs with brown-colored ooplasm[11] were washed twice in tissue culture medium 199 (TCM-199; Thermo Fisher Scientific, Roskilde, Denmark) supplemented with 0.1% polyvinyl alcohol, 25 mM HEPES, 10 mM sodium bicarbonate, and 50 μg/ml gentamicin sulfate (isolation medium, pH 7.4)[2] at 37°C. Before IVM culture, COCs were washed once in maturation medium, consisting of 25 mM HEPES-buffered TCM-199 supplemented with 10% fetal calf serum (FCS, Invitrogen), 0.02 units/ml follicle-stimulating hormone (from porcine pituitary), 1 μg/ml estradiol-17β, 0.2 mM sodium private, and 50 μg/ml gentamicin sulfate. Then COCs were individually cultured for 22 h under a humidified atmosphere of 5% CO_2 in air at 39°C using a MW plate (Nunc 163118, Thermo Fisher Scientific, Roskilde, Denmark) as previously described[9]. For investigating the effect of Ax on the developmental competence of oocytes, 500 ng/ml Ax (840 nM) was added to IVM medium and the group without Ax supplementation served as the control.

Before (immature) and after IVM, bovine oocytes were used for the evaluation of ROS accumulation (immature, Ax-treated, and control; n = 24, 26, and 16, respectively) or mitochondrial activity (immature, Ax-treated, and control; n = 15, 22, and 14, respectively) in oocytes as previously described[4]. Briefly, oocytes were denuded from cumulus investment by vortexing. Denuded oocytes with a polar body after IVM were used for experiments as mature oocytes. For evaluating mitochondrial activity, oocytes were incubated for 15 min in the dark at 37°C in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 1 μM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and 10% FCS. Membrane potentials of mitochondria in oocytes were calculated as the ratio of florescent intensity of activated

mitochondria, expressed as red fluorescence in JC-1 staining, to less activated mitochondria, expressed as green fluorescence in JC-1 staining ($\Delta\psi$m, red/green fluorescent intensity). For evaluating ROS accumulation, oocytes were incubated for 15 min in the dark at 37°C in DPBS supplemented with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCFHDA) and 10% FCS. ROS in oocytes were defined as the mean green fluorescent intensity of DCFHDA. All images were taken precisely at the same parameters for all groups using a digital fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) and the mean fluorescent intensity of the images was calculated using analysis software (BZ-H2A, Keyence).

IVF and IVC were also performed on the COCs for investigation of the developmental competence to the blastocyst stage using commercially available medium, BO-IVF, BO-SemenPrep, and BO-IVC (Bioscience, Cornwall, UK), in accordance with the manufacturer's instructions. Briefly, 450 μl BO-IVF medium prepared in a 4-well dish (Nunc 176740, Thermo Fisher Scientific) without an overlay of oil was incubated at 39°C in 5% CO_2 in air at least for 1 h before IVF. Frozen bovine semen collected from a Holstein bull was used for IVF. After thawing the semen in a 37°C water bath for 30 sec, the semen was layered on 4 ml BO-SemenPrep medium in a 15-ml conical tube and then washed by centrifugation at 300 × *g* for 5 min. The supernatant was discarded and the sedimented sperm were resuspended in BO-IVF medium at 20×10^6 sperm/ml. Finally, 50 μl of sperm suspension was mixed with 450 μl BO-IVF medium in a 4-well dish (final concentration: 2×10^6 sperm/ml) and 20–30 COCs were transferred to a well. The COCs and sperm were co-incubated for 22–24 h at 39°C in 5% CO_2 in humidified air. At the end of IVF, presumptive zygotes were denuded from cumulus investments by vortexing. Denuded presumptive zygotes were washed three times with BO-IVC medium and cultured for 6 days as groups of 20–30 zygotes in 30-μl droplets of the medium covered with paraffin oil at 39°C in humidified air of 5% CO_2, 5% O_2, and 90% N_2. After 2 and 7 days after IVF (44–48 and 168–170 h after IVF, respectively), the cleavage and the development to the blastocyst stage were assessed, respectively. All blastocysts were subjected to counting of the total number of cells by an air-drying method[15]. Mitochondrial activity and ROS accumulation in oocytes were analyzed by one-way ANOVA followed by Tukey-Kramer's honestly significant difference test. The data for embryonic developments were analyzed by Student's *t*-test. All analyses were performed using software JMP Pro version 12.0.1 (SAS Institute, Cary, NC, USA).

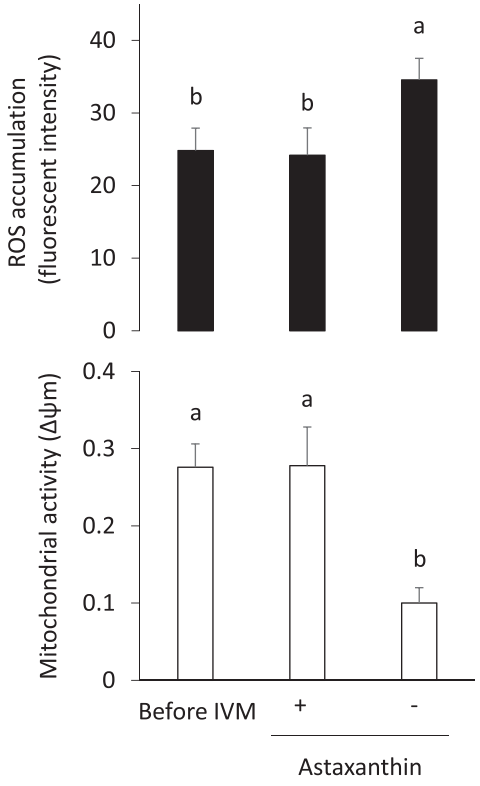

Fig. 1. ROS accumulation and mitochondrial activity before and after IVM with and without astaxanthin treatment. [ab] Different letters indicate the difference between groups ($P < 0.05$).

ROS accumulation of oocytes after IVM in the Ax-treated group was similar with that before IVM; however, ROS accumulation in the control group after IVM was higher than that before IVM and of the Ax-treated group ($P < 0.05$; Fig. 1). Mitochondrial activity in the Ax-treated group after IVM was similar to that

Table 1. Effect of astaxanthin during *in vitro* maturation on bovine embryo development

Astaxanthin	No. of oocytes (replicates)	% Cleavage	% Blastocyst	Mean no. of cells in blastocysts
+	159 (6)	80.5 ± 6.7	16.0 ± 5.4	160.3 ± 79.7 (26)
−	175 (6)	81.2 ± 8.7	12.3 ± 6.9	156.1 ± 73.7 (22)
P values		0.87	0.06	0.91

Values indicate the mean ± standard deviation.
Percentages of cleavage and blastocysts are based on oocytes cultured for *in vitro* maturation.

before IVM, but higher than that in the control group ($P < 0.05$; Fig. 1). These results indicate that Ax works as an antioxidant in oocytes and protects mitochondria from ROS produced by themselves[17]. As shown in Table 1, the cleavage rates were similar in the two groups regardless of Ax treatment; however, the blastocysts rate tended to be higher in the Ax-treated group than in the control ($P = 0.06$), although total cell numbers in blastocysts were similar between the two groups. These results indicate that reduction of ROS accumulation and improvement of mitochondrial activity enhance the developmental competence of bovine oocytes.

Recently, it was reported that Ax supplementation to IVM medium counteracted the heat shock-induced increase in ROS and restored oocyte developmental competency in bovine oocytes cultured in group[5], but also reported that high Ax concentrations (100, 200, and 500 μM) had a negative effect on the percentage of cleaved embryos that developed to the blastocyst stage[5]. In addition, under the heat-shock condition (41°C), 12.5 and 25 nM Ax rescued the deleterious effect of heat shock on the proportion of cleaved embryos that reached the blastocyst stage, but more than 50 nM Ax did not have any positive effects[5]. In the present study, we used only 840 nM Ax (500 ng/ml) under an individual IVM condition at 39°C (a preferable temperature for bovine IVM) and found positive effects of Ax on oocyte developmental competence. These findings suggest that Ax has different effects among different concentrations. In future, we should examine the appropriate concentration of Ax for individual IVM culture for bovine oocytes.

In conclusion, Ax supplementation to individual IVM culture using a 60-well MW plate improves the developmental competence of bovine oocytes due to the reduction of ROS accumulation and the improvement of mitochondrial activity in oocytes.

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