



# Astaxanthin increases progesterone production in cultured bovine luteal cells

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**ABSTRACT.** Although astaxanthin (AST) is known to be a strong antioxidant, its effects on reproductive function in domestic animals have not yet been elucidated in detail. Therefore, we investigated the effects of AST on luteal cells, which produce progesterone (P4), an important hormone for maintaining pregnancy. Luteal cells were prepared by collagenase dispersion of the corpus luteum (CL). The addition of racemic AST at a low concentration (<10 nM) to cultured bovine luteal cells increased P4 in the culture medium ( $P<0.05$ ). This effect was attributed to an increase in the ability of luteal cells to produce P4 (P4/cell-DNA); however, the level of lipid peroxide (TBARS: thiobarbituric acid reactive substances) per cell did not decrease with the addition of AST, whose values were similar to that with the addition of luteinizing hormone. When optical isomers of AST (SS and RR types) were added to the culture medium, respectively, SS-AST was more effective in increasing P4 production than RR-AST. When 1 mg/kg-body weight of SS-AST derived from green algae was fed to cows for 2 weeks, its concentration in blood plasma was 10.9 nM on average, which was sufficient to expect an *in vitro* effect on the production of P4 in cows. These results suggested the potential of SS-AST supplements for cows to elevate luteal function.

**KEY WORDS:** astaxanthin, cow, luteal cells, progesterone, TBARS

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Astaxanthin (AST) is one of xanthophylls (oxygen-substituted carotenoids) that is known to be a pigment in crustaceans and salmonid fishes. These living organisms intake AST produced mainly by marine algae through the food chain [24]. The multiple functions of AST including its strong antioxidative effects have recently been elucidated. Therefore, its availability as an additive for foods, cosmetics and other products is increasing. AST was previously extracted from krill (*Euphausia superba*) [40]. A chemical synthetic method was developed subsequently. Green algae (*Haematococcus pluvialis*) or red yeast (*Phaffia rhodozyma*) cultivation methods have recently been used to produce chemically pure AST [1, 16]. AST has chiral carbons on two six-membered carbocyclic structures and consists of three different optical isomers. Chemically synthesized AST is a mixture of SS-type, RS-type and RR-type optical isomers. In nature, green algae produce esterified SS-AST. Whereas AST derived from red yeast is the non-esterified RR-type. Previous studies reported that these optical isomers have different absorption and tissue distribution characteristics [6, 30]. However, the difference in the physiological functions of these isomers has not yet been clarified.

Various biological activities of AST have been reported recently [11]. AST exhibits strong free radical scavenging [4, 25, 27] and singlet oxygen quenching activities [3, 42]. And, it exerts anti-inflammatory effects by blocking the expression of pro-inflammatory genes [19, 29]. It may also prevent heart disease [18, 35] and ameliorate diabetic symptoms [28, 45]. Furthermore, AST has shown the enhancement of immune function [32], inhibition of colon carcinogenesis [46] and attenuation of the promotion of hepatic metastasis [17]. It also exerts protective effects against retinal and lens damage [7]. However, limited information is available for the function of AST in animal reproduction. Hansen *et al.* [9] reported previously 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 AST. In human male patients, supplementation with AST improved sperm quality and function and increased spontaneous or intrauterine insemination-assisted conception rates [5].

On the other hand, the concentration of P4 during early pregnancy is known to have an important effect on the conception rate. Staples *et al.* [39] demonstrated that plasma P4 concentrations between 55 and 68 days postpartum were positively related to conception rate in cows. Mammalian embryo death in early gestation was attributed to the inadequate function of luteal cells [21]. A delay in the post-ovulatory increase in P4 in pregnancy has been shown to result in impaired embryo development [23]. We confirmed previously the need for an antioxidant (selenium) in maintaining bovine luteal function [13–15]. Therefore, the aim of the present study was to determine the effects of AST on the production of P4 in cultured bovine luteal cells.

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## MATERIALS AND METHODS

All procedures were approved by the Animal Care and Use Committee of the Institute of Livestock and Grassland Science, NARO.

### *Bovine luteal cell culture*

Luteal cells were prepared using the collagenase dispersion method [15]. Ovaries were collected at a slaughter house and transported to the laboratory. The CL used in this experiment was judged to be at mid-cycle according to the criteria of Ireland *et al.* [12]. Each CL was dissected aseptically and minced with scissors and a scalpel after the removal of its capsule. Minced tissues were digested with collagenase solution (1 mg/ml) (Yakult Pharmaceutical IND. Co., Ltd., Tokyo, Japan) at 37°C for 45 min in a spinner flask three times with the addition of fresh enzyme solution. The dispersed cells in the supernatant of the digesta were filtered through a nylon mesh (104  $\mu\text{m}$ ) and centrifuged at  $170 \times g$  for 5 min. Cell pellets were resuspended in fresh medium and loaded on a Percoll discontinuous gradient (60, 30 and 15%). After centrifugation at  $370 \times g$  for 15 min, purified cells in the 30% Percoll layer were washed with fresh medium before seeding into culture dishes. Viable cells were counted using a hemocytometer with the trypan blue exclusion method [43]. A total of 2 or  $5 \times 10^4$  viable cells were seeded per well in a 24- or 6-well dish in 1.0 or 2.0 ml of Medium 199 contained 10% fetal calf serum and antibiotics (streptomycin and penicillin) (Nakalai Tesque, INC., Kyoto, Japan). Cultures were incubated at 37°C in 5%  $\text{CO}_2$  / 95% air. The culture medium was removed after 36 hr, replaced with fresh medium containing 0.1 to 1,000 nM AST (racemic or isomers; chemically synthesized products) (Day 0) and subsequently changed every 48 hr using the same medium. Removed medium was stored at  $-20^\circ\text{C}$  until P4 analyses.

### *Determination of progesterone*

P4 concentrations in the condition medium were measured using a commercial ELISA kit (Kyoritsu, Tokyo, Japan) according to its attached protocol.

### *Measurement of thiobarbituric acid reactive substances*

Thiobarbituric acid reactive substances (TBARS; a general indicator of oxidative stress [44]) were measured using a TBARS Assay Kit (Cayman Chemical Co., Ann Arbor, MI, U.S.A.) according to its attached protocol.

### *Measurement of cell DNA*

Cell DNA was determined using a Qubit™ dsDNA BR Assay Kit (Invitrogen, Paisley, U.K.). Cells in each well were sonicated with 0.5 ml phosphate buffer over ice. Sonicated cell lysates were used to measure DNA contents. Standard solution (10  $\mu\text{l}$ ) or 20  $\mu\text{l}$  of the cell lysate was mixed with 190 or 180  $\mu\text{l}$ , respectively, of the working solution (made by diluting BR reagent 1:200 in BR buffer) in an assay tube. The final volume in each tube was 200  $\mu\text{l}$ . After being incubated at room temperature for 20 min, its fluorescent intensity was measured using a Qubit fluorometer.

### *Bioavailability of astaxanthin in cows*

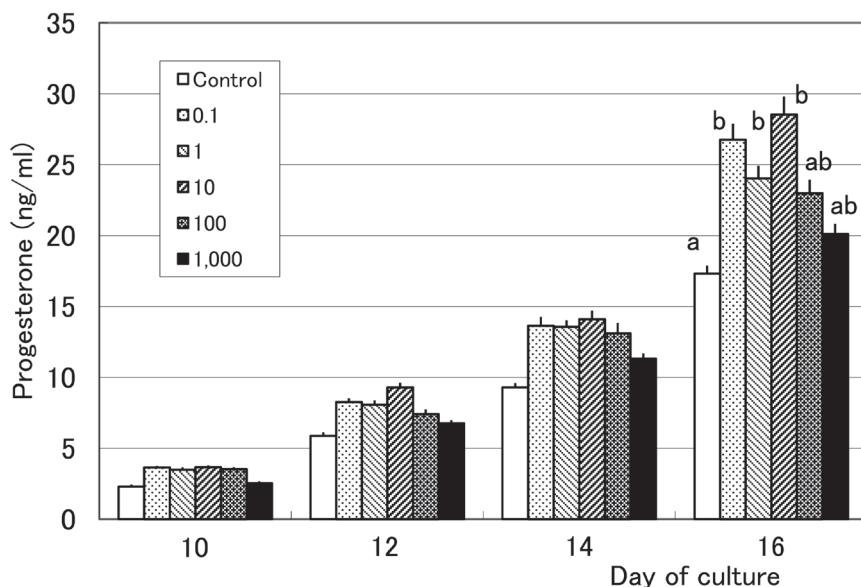
Six pregnant dairy cows (average body weight:  $616.3 \pm 13.5$  kg) were housed individually in stanchions provided with rubber comfort mats. Four cows were fed 1 mg/kg bodyweight of AST (SS-type) derived from *Haematococcus pluvialis* (Biogenic Co., Ltd., Tokyo, Japan) mixed with total mixed ration (TMR) every morning. Control cows ( $n=2$ ) were fed the same TMR without AST. TMR was composed of corn silage (20.7%), Italian ryegrass roll silage (12.3%), concentrate (39.2%), beet pulp (10.4%), alfalfa hay cube (9.2%) and Sudan grass silage (8.2%) at a DM base. The daily feed supply amount of TMR and Italian ryegrass roll silage were 10.1 to 11.2 kg·DM and 0.88 kg·DM, respectively. After a 2-week treatment, blood samples were collected from the jugular vein using heparinized tubes.

### *High-performance liquid chromatography analysis of astaxanthin in cow blood plasma*

AST in the blood plasma was measured by high-performance liquid chromatography (HPLC) (LC10, Shimadzu, Kyoto, Japan). Blood plasma (2 ml) was mixed with 0.1 ml acetone, 1.0 ml water and 1.0 ml ethanol containing 0.025% butylhydroxytoluene (BHT), and extracted with 5 ml n-hexane/dimethyleter (1:1) three times. After drying with nitrogen gas, the residue of the extract was resuspended in 0.1 ml ethanol/chloroform (9:1) containing 0.025% BHT. Reversed-phase HPLC was performed using a Symmetry C18 column (bead size: 5  $\mu\text{m}$ , diameter  $\times$  length: 4.6  $\times$  250 mm) (Waters, Milford, MA, U.S.A.) in methanol containing 0.04% phosphoric acid / t-butylmethyleter (83:17) at a flow rate of 0.5 ml/min. The absorbance wavelength was 474 nm. AST concentrations in samples were determined using a calibration curve made from the measured values of standard AST (racemic type) mixed with control plasma. This method cannot distinguish between isomers of AST.

### *Statistical analysis*

The data of P4 in culture medium were analyzed using the mixed method procedure [37]. Multiple comparisons analysis of mean values was performed using the Bonferroni method. Other data were subjected to ANOVA using the general linear models procedure. The results were considered significant at  $P<0.05$  and  $P<0.01$ .



**Fig. 1.** Effects of racemic astaxanthin on the production of progesterone into the culture medium by luteal cells in 10 to 16-day culture (triplicate data  $\times$  four corpora lutea). <sup>ab</sup>Different lowercase letters indicate statistically significant differences among treatments ( $P < 0.05$ ).

## RESULTS

### *Progesterone concentrations in the culture medium*

Figure 1 shows the effects of racemic AST (RR, RS and SS mixture) on the production of P4 into the culture medium by luteal cells for 2 days. P4 concentrations increased gradually with the culture time in all treatments. However, these increases were the greatest in treatments involving the addition of AST. On Day 16 of the culture, the addition of 0.1, 1.0 and 10 nM of AST significantly increased P4 concentrations in the culture medium more than the control ( $P = 0.0397$ ).

### *Progesterone production activity per cell*

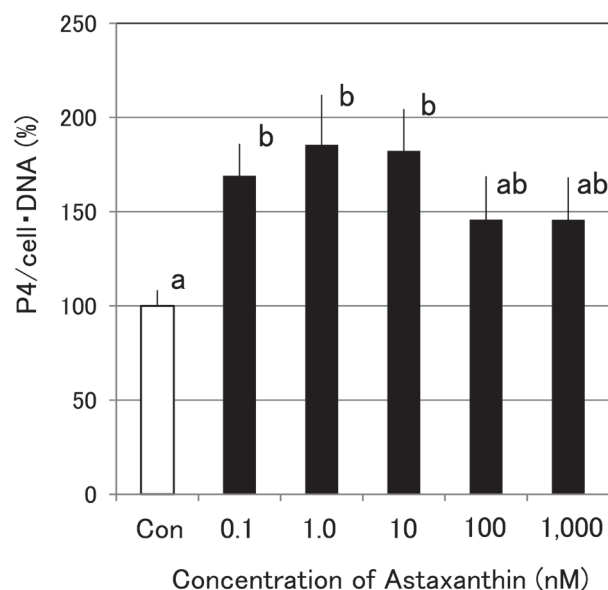
In order to estimate the effect of racemic AST on P4 production activity per cell, P4 produced in the last 2 days was divided by the total DNA content of cells in a well. P4 production activities (P4/cell·DNA) with the addition of 0.1 to 10 nM of AST were significantly greater than the control value ( $P = 0.034$ ) (Fig. 2).

### *Thiobarbituric acid reactive substances per cell*

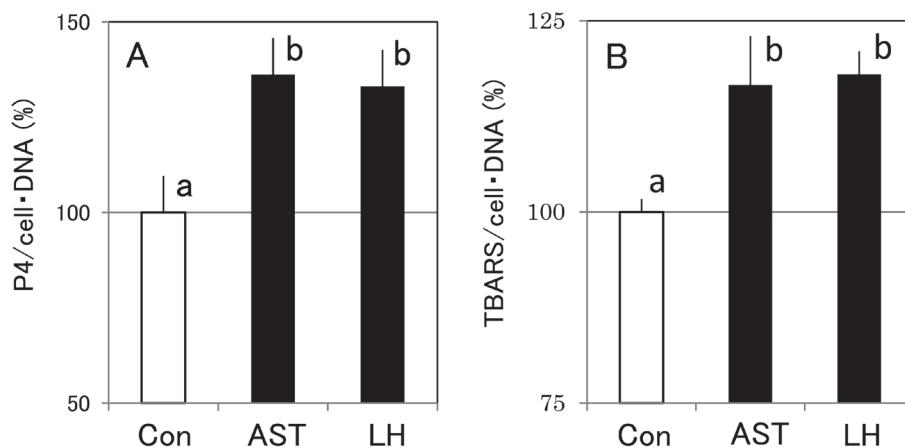
Figure 3 shows the effects of racemic AST on the level of lipid peroxide (TBARS) content in a cell. The addition of AST did not decrease TBARS/cell·DNA, rather it actually increased it. The addition of luteinizing hormone (LH) also increased TBARS/cell·DNA. When increases in the rates of P4/cell·DNA by the addition of AST or LH were similar, TBARS/cell·DNA was not different between the two treatments. Thus, AST did not decrease the production of lipid peroxides accompanying P4 synthesis in luteal cells.

### *Effect of astaxanthin optical isomers on progesterone production*

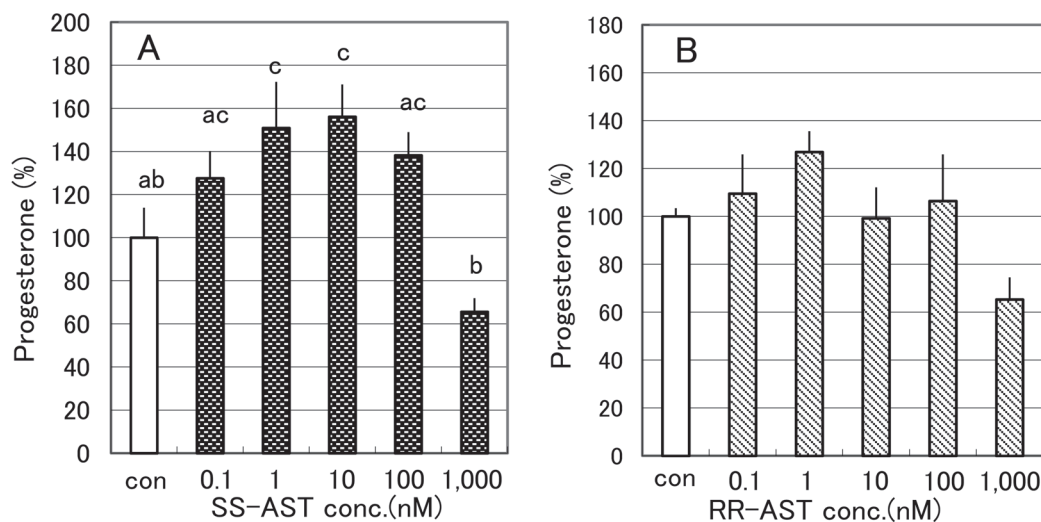
Chemically pure optical isomers (SS-type and RR-type) of AST were added to luteal cell cultures at concentrations of 0.1 to 1,000 nM, respectively. RR-AST showed small effect on the production of P4. On the other hand, SS-AST significantly increased its production ( $P = 0.003$ ) (Fig. 4). The relative values of P4 concentrations in the culture medium with the addition of 1 and 10 nM SS-AST on Day 10 were 151 and 156% of the control (no addition of AST), respectively.



**Fig. 2.** Effects of racemic astaxanthin on the progesterone production activity (progesterone production per cell·DNA) of luteal cells in a 16-day culture (quadruplicate data  $\times$  three corpora lutea). <sup>ab</sup>Different lowercase letters indicate statistically significant differences among treatments ( $P < 0.05$ ).



**Fig. 3.** Effects of racemic astaxanthin (10 nM) and luteinizing hormone (5 ng/ml) on lipid peroxide levels in a cell (A: progesterone production per cell·DNA, B: TBARS per cell·DNA) (triplicate data  $\times$  one corpus luteum). <sup>ab</sup>Different lowercase letters indicate statistically significant differences among treatments ( $P < 0.05$ ). Other replications ( $n = 2$ ) showed the same tendency.



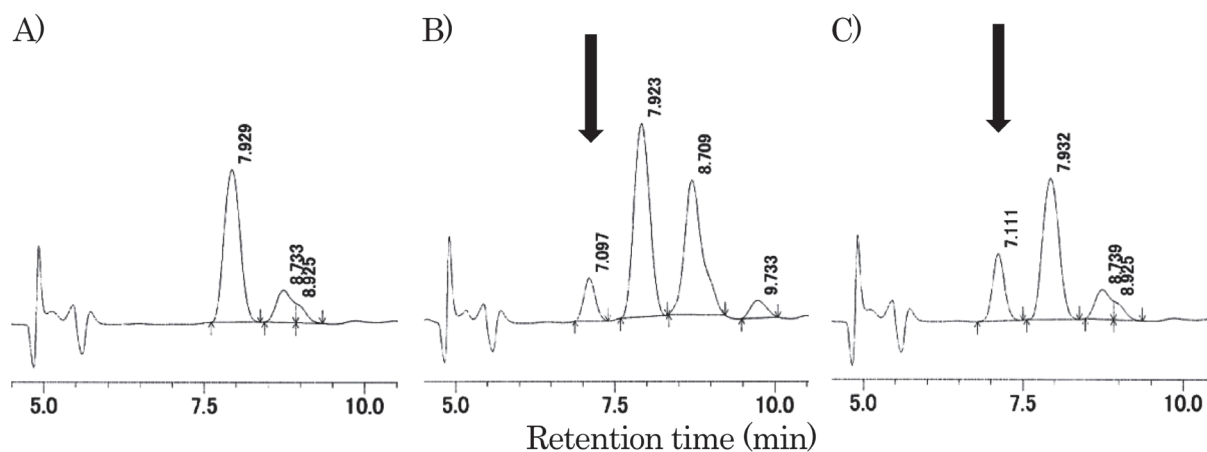
**Fig. 4.** Effects of astaxanthin optical isomers (RR type and SS type) on the production of progesterone into the culture medium by luteal cells (triplicate data  $\times$  one corpus luteum). <sup>ab</sup>Different lowercase letters indicate statistically significant differences among treatments ( $P < 0.05$ ). Other replications ( $n = 2$ ) showed the same tendency.

#### Detection of astaxanthin in blood plasma from cows

A representative HPLC chromatograph of plasma obtained from a control cow (A), a cow fed AST supplement (B) and control plasma spiked with standard AST (+4.47 ng) is shown in Fig. 5. AST was detected at a retention time of approximately 7.1 min on the HPLC profile (black arrow). The plasma concentration of AST in treated cows was calculated as  $6.53 \pm 0.23$  ng/ml ( $10.94 \pm 0.38$  nM) ( $n = 4$ ). When the volume of blood plasma in adult cows was estimated to be 3.9% of their body weight [34], approximately 0.025% of the total amount of AST (1.0 mg/body weight kg) fed for one day in this experiment was presented in the blood plasma. (AST absorption rate% =  $[\text{body weight (BW)} \times 0.039 \times 1,000] (\text{ml}) \times 6.53 (\text{ng/ml}) / [\text{BW} \times 1.0 (\text{mg})] \times 100 = 0.025$ ). An AST peak was not observed in the control cows.

## DISCUSSION

The CL is a tissue that inevitably accumulates peroxides because of its function (P4 production) [47]. Therefore, detoxification of accumulated peroxides by feeding antioxidative nutrients may enhance CL function. We have already shown that the addition of Se (an essential component of glutathione peroxidase identified as an antioxidant enzyme) to luteal cells increased P4 concentrations in the culture medium and decreased the amount of lipid peroxides in cells [15]. Furthermore, the feeding of Se to



**Fig. 5.** A representative HPLC chromatograph of plasma astaxanthin in cows. A) control cow, B) astaxanthin (SS-type)-fed cow, C) control plasma + standard astaxanthin (racemic type). Black arrows show astaxanthin peak (Retention time is about 7.1 min).

dairy cattle increased P4 concentrations in the estrus cycle [14]. In this study, AST also increased the P4 production of luteal cells. So, a similar effect to Se on P4 may be expected in cattle fed AST. AST is known to be strong antioxidant. However, this study showed that the effect of AST on P4 production is not caused by antioxidative function.

In this study, high concentrations of AST suppressed P4 production of luteal cells. Some *in vitro* investigations showed the adverse effect of high dose AST in another cell line [22, 33]. Nagaraj *et al.* [26] proposed that the mechanism of toxicity of high AST is an increase of membrane permeability and a blocking of mitotic cycle. Another possible mechanism may be a desensitization of receptor by a high concentration of ligand, if AST functions via a kind of receptor. On the other hand, many reports showed that *in vivo* administrations of high dose of AST (up to 700–920 mg/kg bodyweight in rats [2] and up to 400 mg/kg bodyweight in rabbit [38]) were without no adverse effects. (In this study, one mg/kg bodyweight of AST was fed to cows.) And, effective concentrations on P4 production were one-hundredth of the inhibitory concentration in this study. So, a risk of excess symptom in practical use is expected to be relatively low. In any case, it is important to clarify the effective dose of AST on P4 production *in vivo* in future.

Murata *et al.* [25] reported that AST (extracted from *Paracoccus carotinifaciens*) decreased the level of TBARS in a murine model. However, a similar effect of AST was not observed in this study (Fig. 3). Takimoto *et al.* [41] also showed no effect of AST from *Phaffia rhodozyma* on TBARS of organs in broiler chick. Increases in TBARS by the addition of LH are considered to be a by-product generated from the process of P4 synthesis using molecular oxygen [47]. When the increase in the rate of P4 production activity by AST was the same as that by LH, no significant differences were observed in TBARS per cell between the two treatments. And, the value of TBARS per cell in a AST treatment was greater than in control. These data indicated that AST stimulates the production of P4, but does not suppress the generation of lipid peroxides accompanying P4 production. A previous study demonstrated that Se decreased lipid peroxide levels in luteal cells [15]. The stimulating mechanism of P4 production may differ between two antioxidants. The mechanisms underlying AST-induced increase in the production of P4 in luteal cells are now being investigated in more detail.

Ruminants have the ability to easily absorb carotenes, but not xanthophylls, such as AST [8]. In addition, AST derived from *Haematococcus* is in an esterified form whose absorption rate in digestive tracts is said to be different from that of the non-esterified type in fish or humans [36]. In this study, we demonstrated that *Haematococcus* AST (SS-type) supplied as a dietary supplement was transferred to the blood plasma of cows. Although its concentration was very low in the plasma, it was still sufficient to expect positive effects on P4 production observed *in vitro*. These results suggested the possibility that AST functions as a CL-stimulating substance in ruminants. *Haematococcus* AST has been reported to contain approximately 94% of the monoester type, 2% of the diester type and 4% of the free type [10]. The absorption efficiency of esterified AST is said to be dependent on the specificity of esterases. In humans, the absorption efficiency of esterified AST was one-fifth to one-fourth compared with that of free-type AST [6]. In the present study, only 0.025% of total AST fed to cows for one day was detected in their blood plasma. Therefore, it currently remained unclear whether esterified AST derived from *Haematococcus* is absorbed by cows because it contains 4% of free-type AST.

It is interesting that AST-induced increases in the production of P4 differed between its optical isomers. Every AST added to the cell culture in this experiment was the non-ester type. So, the differences observed in the production of P4 in Fig. 4 were attributed to optical isomerism. Differences between R/S isomers in the distribution among organs or the absorption rate from the digestive tract have recently been reported. However, differences in their biochemical functions between two isomers have not yet been examined in detail. Cardounel *et al.* [4] employed electron paramagnetic resonance and showed that the polyene chain alone of AST was responsible for the scavenging of superoxide. Because the polyene chain does not play a role in optical isomerism, differences in the production of P4 between the optical isomers of AST (R/S) in the present study may not be explained by

superoxide scavenging ability derived from the polyene structure. Although the function of the six-membered carbocyclic structure generating optical isomerism of AST has not yet been clarified, the permeability into a cell may also explain differences between isomers. If AST functions in a cell, SS-type AST may permeate into the cell more easily than RR-type AST. The suppression of P4 production at the high concentration of AST was observed. Its adverse effect was stronger in pure optical isomers (SS and RR) than in racemic type (mixture of RR, SS and RS). The RS-type in racemic AST may rescue the suppression effects by RR- and SS-type AST at the high concentration.

There are two representative natural materials of AST for food supplements of domestic animals, namely, SS-type AST from *Haematococcus* or *Euphausia*, and RR-type AST from *Phaffia* yeast. The results of this study showed that SS-AST had stronger effects on the production of P4 in luteal cells than RR-AST. Therefore, the feeding of SS-AST (derived from *Haematococcus* or *Euphausia*) to ruminants may be advantageous. On the other hands, AST (racemic type) synthesized by the ordinary chemical method contains 50% of meso-AST (the RS-type) (remaining ASTs contain 25% of the RR-type and 25% of the SS-type). It is already available in the fish-raising industry. Moreover, differences in the structure of the polyene chain of AST generate geometrical isomers (E/Z). Although the all-E-isomer predominates in nature, the 9Z, 13Z and 15Z isomers are also present. Differences in the bioavailabilities and functions of these geometrical isomers have already been reported. The antioxidant potency of 9Z-AST was previously found to be higher than that of the all-E-AST *in vitro* [20]. It was reported that chemically synthesized AST included 74% all-E, 9% 9Z and 17% 13Z isomer [31]. Experiments using pure meso-AST or the pure geometrical isomers (E or Z type) of AST need to be conducted in the future.

In the present study, P4 production of luteal cells was enhanced by AST at rather low concentrations (0.1 to 10 nM). And, such levels of AST were observed in the plasma of cows fed SS-type AST. Although few studies have investigated the relationship between animal reproduction and AST *in vivo* until now, our results suggest the potential of AST to improve the function of the CL in cows. As mentioned above, previous paper reported a positive correlation between postpartum plasma P4 concentrations and conception rates [39]. Pre- and postpartum supplementation of AST might contribute to the increase of conception rate.

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