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Original Article

Direct effects of linoleic and linolenic acids on bovine uterine function using *in vivo* and *in vitro* studies

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Abstract. The aim of the present study was to evaluate the effects of continuous administration of linoleic acid or linolenic acid into the intra-uterine horn, ipsilateral to the corpus luteum, on the duration of the estrous cycle and plasma progesterone (P4) concentration. The effects of linoleic and linolenic acids on bovine uterine and luteal functions were also studied using a tissue culture system. Intra-uterine administration of linoleic or linolenic acid (5 mg/10 ml of each per day) in cows, between days 12 and 21, resulted in a prolonged estrous cycle compared to the average duration of the last one to three estrous cycles before administration in each group ($P < 0.05$). Moreover, plasma P4 concentration in cows treated with linoleic or linolenic acid was high between days 19 and 21 (linoleic acid), or on day 20 (linolenic acid), compared to that of the control cows (saline administration; $P < 0.05$ or lower). Both linoleic (500 µg/ml) and linolenic (5 and 500 µg/ml) acids stimulated prostaglandin (PG) E2 but inhibited PGF2α production by cultured endometrial tissue ($P < 0.01$), while P4 production by cultured luteal tissue was not affected. These findings suggest that both linoleic and linolenic acids support luteal P4 production by regulating endometrial PG production and, subsequently, prolonging the duration of the estrous cycle in cows.

Key words: Cow, Estrous cycle, Linoleic acid, Linolenic acid, Uterus

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Improving the productivity of livestock to ensure a stable supply of livestock products is important for the dairy industry. The decline in conception rates due to failure of insemination and early embryonic mortality remain challenging issues in cows. Adequate and appropriate feedings are essential for bovine reproductive activities. Diets are supplemented with fat primarily to increase the energy density of the diet to enhance reproduction in cows. Dietary supplementation of fatty acids (FAs), including those of the omega-3 (linolenic acid) and omega-6 (linoleic acid) families during the postpartum period, has the potential to increase pregnancy rates in dairy cows, and alter ovarian function by improving the energy status and increasing precursors for the synthesis of reproductive hormones, such as prostaglandins (PGs) [1, 2].

Postpartum supplementation of the diet with omega-3 FAs improves fertility by influencing follicular growth and ovulation in dairy cows [3–6]. Cows fed rolled flaxseeds, which are rich in omega-3 FAs, had a larger mean ovulatory follicle diameter than those fed rolled sunflower seeds, which are rich in omega-6 FAs [7]. Similarly, cows fed flaxseeds had fewer small follicles than those fed soybeans, which are rich in omega-6 FAs [8]. Moreover, cows that were fed diets enriched in linolenic acid, during the prepartum period, had a shorter interval from calving to first ovulation compared to those fed a diet enriched in oleic acid (omega-9 unsaturated FA) [9]. These findings suggest that omega-3 FAs have a greater effect on follicular development than other FAs, such as omega-6 or omega-9 FAs. In contrast, dietary supplementation of linoleic acid or linolenic acid resulted in a decrease in progesterone (P4) concentration in cows, compared to cows fed a control diet [10]. In contrast, increased concentrations of P4 have been reported in cows supplemented with conjugated linoleic acid [11], which is related to greater circulating amounts of insulin-like growth factor-I. In contrast, in another study on the supplementation of cows with omega-3 FAs resulted in similar plasma concentrations of P4 [12]. Thus, the effects of dietary supplementation of FAs on P4 production in cows have differed among research reports.

Fish oil contains substantial amounts of the long-chain omega-3 FAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). A diet enriched with omega-3 FAs can potentially reduce uterine PGF2α production [13, 14]. Other studies have shown significant inhibitory effects of fish oil on plasma 13, 14-dihydro-15-keto-prostaglandin F2α (PGFM) concentration [15, 16], which improved embryonic survival in cattle [17] and reduced pregnancy losses in lactating dairy cattle [18]. Plasma PGFM concentration was increased on the first day postpartum when cows were fed a linoleic acid supplement [19]. These findings suggest that omega-3 linolenic acid decreases plasma PGFM levels in cows, while omega-6 linoleic acid increases PGFM. *In vitro*, both linoleic and linolenic acids inhibit the secretion of eicosanoids in several cell types [20, 21], and omega-3 FAs reduce PGF2α secretion from bovine endometrial cells [22]. The addition of linoleic and linolenic acids at different volume ratios affected the mRNA abundance of PG synthesis-related genes in primary bovine endometrial cells [23]. In contrast, FA treatment had no effect on PGF2α or PGE2 release from the endometrium of both pregnant and non-pregnant cows [24], suggesting that the effects of linoleic and linolenic acids on *in vitro* PG production are not necessarily consistent with the results of *in vivo* experiments.

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Thus, although there have been various reports on the effects of adding linoleic and linolenic acids to the diet of cows on reproductive functions, no consensus has been reached. In this study, we examined the effects of linoleic and linolenic acids on the length of the estrous cycle and P4 concentration by administering them directly into the uterus. The effects of linoleic and linolenic acids on endometrial PG production and luteal P4 production were also examined using an *in vitro* tissue culture system.

Materials and Methods

All animal experiments were performed in accordance with guidelines approved by the Animal Ethics Committee of the Institute of Livestock and Grassland Science (#1911C005) and by the Tohoku Agricultural Research Center (#30-19), NARO.

Intra-uterine administration procedure

Japanese Black cows with normal estrous cycles were used in the experiment (12 animals in total). The days after parturition and parity of each treatment group were as follows: control (56.3 ± 2.8, 5.5 ± 1.8), linoleic acid (62.8 ± 3.8, 5.8 ± 1.1), and linolenic acid (56.5 ± 6.1, 5.8 ± 1.3). There was no statistical difference in the days after parturition or parity between the groups. On day 12 of the estrous cycle (day of estrus: day 0), 10 ml of saline containing 0.5% ethanol (control, n = 4), linoleic acid (5 mg/10 ml [99.5% saline, 0.5% ethanol]; 125-05821, FUJIFILM Wako Pure Chemical Corp., Osaka, Japan, n = 4), or α-linolenic acid (5 mg/10 ml [99.5% saline, 0.5% ethanol]; 128-05833, FUJIFILM Wako, n = 4) were injected into the tip of the uterine horn, ipsilateral to the corpus luteum (CL), using a deep insertion embryo transfer catheter (YT gun; Yamanetech Co., Ltd., Nagano, Japan). The same treatment was applied once a day until day 21 of the estrous cycle. All administrations were performed by the same operator to avoid variation in the results among operators. The status of the ovary and uterus following treatment was confirmed daily using rectal palpation and an Arietta 60 ultrasonographic imaging system (Hitachi-Aloka Medical Co., Ltd., Tokyo, Japan) until day 12 of the next estrous cycle. To determine the effect of the duration of the estrous cycle in the treatment groups, it was compared to the average duration of one–three estrous cycles before intra-uterine administration in each treatment group. Blood samples were drawn from the jugular vein into heparinized tubes every day from the day the treatment started (day 12 of the estrous cycle) until day 12 of the next estrous cycle. The tubes were then placed immediately in an ice bath and centrifuged (1200 × *g* at 4°C for 60 min) within one hour of blood collection. Plasma was stored at –30°C until further use.

Endometrial and luteal tissue culture

Bovine uteri and ovaries were obtained from Japanese Black cows at the institute ranch within 10–30 min of exsanguination. Uterine and luteal samples were collected from cows on days 10–12 of the estrous cycle (n = 4 animals). The uterine horn ipsilateral to the CL was obtained and immediately cut open to observe the endometrium. Endometrial tissues from the bovine uterus were separated using a modification of the previously described procedures [25]. The uterine lumen was washed three times with 30–50 ml of sterile physiological saline supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (P0781; Sigma-Aldrich Co., LLC, St. Louis, MO, USA), and 0.1% bovine serum albumin (BSA; 735078, Roche Diagnostics K.K., Tokyo, Japan). The uterine horn was cut transversely with scissors into several segments, which were slit to expose the endometrial surface. Caruncular endometrial strips were dissected from the myometrial layer with a scalpel and washed once in 50 ml of sterile saline containing penicillin and streptomycin. The endometrial strips were cut into small pieces (2 mm^3). Luteal tissues were obtained using a modified procedure as described previously [26]. The CL was washed three times with 30–50 ml of sterile physiological saline supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1% BSA. The CL was sliced into approximately 0.2 mm-thick layers with a tissue slicer (#KN-822; Natsume Seisakusyo Co., Ltd., Tokyo, Japan). The luteal strips were then cut into small square pieces (3 mm^2) using a sterile surgical blade.

Endometrial or luteal tissues (approximately 20–30 mg) were pre-incubated in Dulbecco's modified Eagle's medium (DMEM; D1152, Sigma-Aldrich) supplemented with 0.1% BSA. After pre-incubation for 4 h, the tissues were placed in culture medium (DMEM/Ham's F-12; 1:1 (v/v); D8900, Sigma-Aldrich) supplemented with 10% (v/v) calf serum (C6278, Sigma-Aldrich), 20 IU/ml penicillin, 20 µg/ml streptomycin, and 0.05 µg/ml amphotericin B (516104, EMD Millipore Corp., Billerica, MA, USA), and cultured at 37.5°C in a humidified atmosphere of 5% CO_2 in air. Cultured tissues were further incubated in medium with 0.005% ethanol (control), linoleic acid (5–500 µg/ml [99.995% medium, 0.005% ethanol]; 125-05821, FUJIFILM Wako) or α-linolenic acid (5–500 µg/ml [99.995% medium, 0.005% ethanol]; 128-05833, FUJIFILM Wako). After incubation for 18 h, the supernatant of the culture media was collected and stored at –30°C until use.

Enzyme immunoassay (EIA)

The P4 concentration in the plasma was determined using EIA, as described previously [27]. The standard curve ranged from 0.02 to 10 ng/ml. The ED50 of the assay was 0.19 ng/ml. The average intra- and inter-assay coefficients of variation were 3.7% and 13.2%, respectively.

The concentrations of PGE2, PGF2α, and P4 in the culture media were examined using commercial EIA kits (#500141, #516011, and #582601, respectively; Cayman Chemical Co., Ann Arbor, MI, USA). To determine the response of cultured endometrial and luteal tissues, the concentrations of PGE2 and PGF2α in the culture media, after stimulation with recombinant bovine tumor necrosis factor (TNF)-α (50 ng/ml; 2279-BT, R&D Systems Inc., Minneapolis, MN, USA), were examined. Both PGE2 (endometrium; 1624.6 ± 249%, CL; 383.5 ± 10%) and PGF2α (endometrium; 472.8 ± 43%, CL: 246.1 ± 42%) concentrations were significantly increased by the addition of TNF.

Statistical analyses

All experimental data are presented as the mean ± SEM. The statistical significance of the differences in the duration of the estrous cycle, before and during intra-uterine administration, was analyzed using a Student's *t*-test. Plasma P4 concentration and CL diameter between control and treated groups were analyzed using two-way repeated measures ANOVA with Fisher's PLSD test using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). The statistical significance of the differences in PG and P4 concentrations between the control and treated groups was analyzed using one-way ANOVA with Dunnett's multiple comparison post-hoc test using the KaleidaGraph 3.6 (Synergy Software, Reading, PN, USA) software package. Statistical significance was set at $P < 0.05$.

Results

During a preliminary experiment, 10 ml of saline containing 0.5% ethanol was injected into the tip of the uterine horn using a deep

insertion embryo transfer catheter. The dynamics of the injected saline were confirmed using an ultrasonographic imaging system immediately after injection, and 1 and 2 h later. Saline was diffusely absorbed into the deep uterine horn 2 h after administration. A significant prolongation of the estrous cycle was observed in cows treated with linoleic acid ($P < 0.01$) or linolenic acid ($P < 0.05$), compared to the average duration of the last one to three estrous cycles in individual cows (before the experiment); whereas saline treatment in control cows did not affect the duration of the estrous cycle (Fig. 1). The days of luteolysis, estimated using plasma P4 concentration (< 1.0 ng/ml), were 20.0 ± 0.0 (control), 24.5 ± 1.3 (linoleic acid) and 25.0 ± 3.0 (linolenic acid), respectively.

In contrast, P4 concentration in the blood of linoleic acid-treated cows was higher ($P < 0.05$) than that of the control cows during day 19–21 of the estrous cycle. P4 concentration in the blood of linolenic acid-treated cows tended to be higher than that of control cows during the same period and a significant difference was observed on day 20 of the estrous cycle (Fig. 2A). The diameter of CL in linoleic acid- or linolenic acid-treated cows was larger than that of control cows on days 19, 20, and 21 of the estrous cycle (Fig. 2B; $P < 0.05$). Furthermore, when plasma P4 concentration was examined from the next post-ovulation period to day 12, there was no significant difference between control cows and cows treated with linoleic or linolenic acid (Fig. 2C).

The production of PGE2 in cultured bovine endometrial tissue increased significantly after the addition of linoleic acid (500 µg/ml) or linolenic acid (5 and 500 µg/ml), compared to that in the control (Fig. 3; $P < 0.01$). PGF2α production decreased significantly after the addition of linoleic acid (500 µg/ml) or linolenic acid (5, 50, and 500 µg/ml), compared to the control (Fig. 4; $P < 0.01$). However, when the effects of linoleic and linolenic acids on P4 production in cultured bovine luteal tissues were examined, no significant changes were observed at any concentration (5, 50, and 500 µg/ml) when compared to the control (Fig. 5).

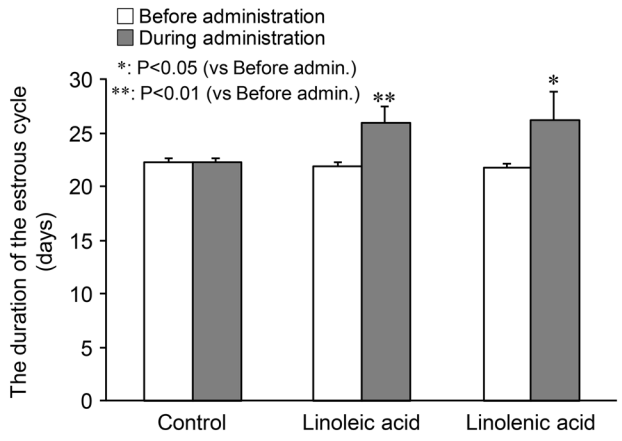

Fig. 1. Effects of continuous intra-uterine administration of linoleic or linolenic acid on the duration of estrous cycles in Japanese Black cows. Ten milliliters of saline containing 0.5% ethanol (control: n = 4), linoleic acid (5 mg/10 ml saline, n = 4), and linolenic acid (5 mg/10 ml saline, n = 4) were administrated to the intra-uterine horn, ipsilateral to the corpus luteum, from days 12 to 21 of the estrous cycle. This was done using a deep insertion embryo transfer catheter. Data are presented as the mean ± SEM. Prior to administration, data are presented as the mean ± SEM of one to three estrous cycles before intra-uterine administration in each treatment group (white columns). Asterisks show significant differences (* $P < 0.05$, ** $P < 0.01$).

Discussion

The results of the present study suggest that intra-uterine administration of linoleic or linolenic acid can prolong the length of the estrous cycle, which is thought to be due to the maintenance of the CL. To the best of our knowledge, this is the first report of linoleic or linolenic acid administered directly into the uterus to study their effects on reproductive function in cows. Furthermore, both linoleic and linolenic acids inhibited PGF2α but stimulated PGE2 production by endometrial tissue. However, they did not significantly affect P4 synthesis in luteal tissue under the culture conditions used in this study. PGE2 enhances luteal function by promoting P4 synthesis in the CL, while an endometrial PGF2α pulse has a regressive effect on the CL [28]. Therefore, it can be inferred that the maintenance of blood P4 levels observed in the *in vivo* experiments was not due to

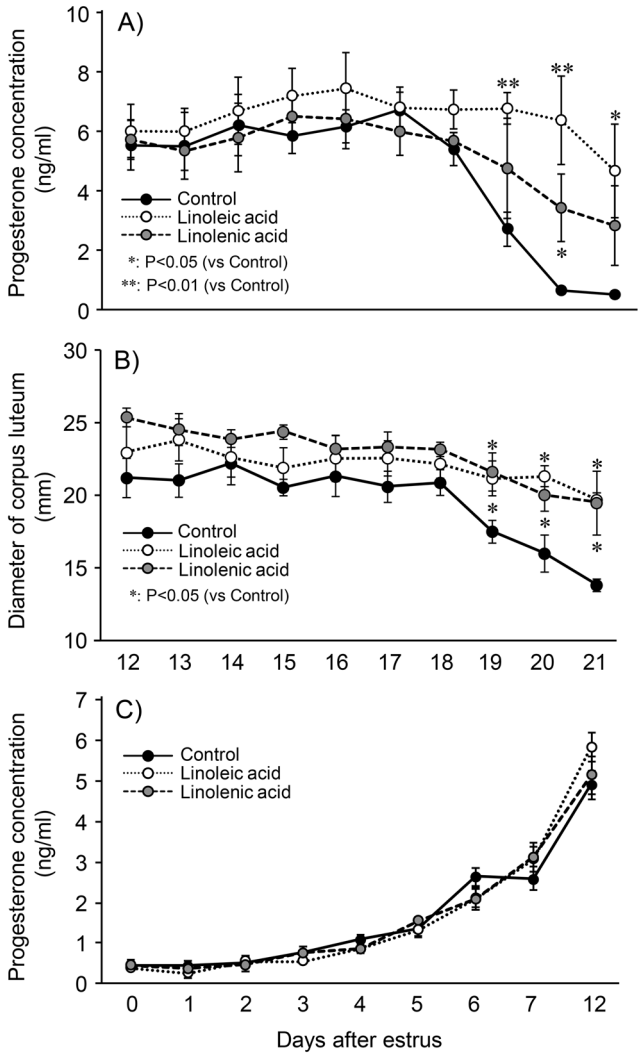

Fig. 2. Effects of continuous administration of linoleic or linolenic acid on (A) progesterone (P4) production and (B) corpus luteum diameter of Japanese Black cows. Ten milliliters of saline containing 0.5% ethanol (control: n = 4), linoleic acid (5 mg/10 ml saline, n = 4), and linolenic acid (5 mg/10 ml saline, n = 4) were administrated to the intra-uterine horn, ipsilateral to the corpus luteum, from day 12 to 21 of the estrous cycle. The effect on P4 production in the next estrous cycle (from day 0 to 12 of the estrous cycle) was investigated (C). Data are presented as the mean ± SEM. Asterisks show significant differences (* $P < 0.05$, ** $P < 0.01$).

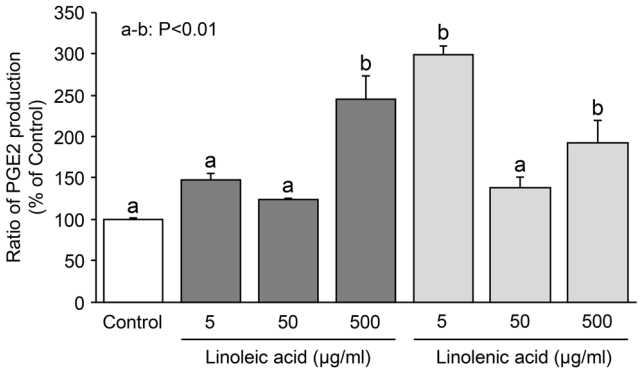

Fig. 3. Effects of linoleic and linolenic acids on prostaglandin E2 (PGE2) secretion by cultured bovine endometrial tissue. Data are the mean ± SEM of four cows per stage, and are expressed as relative ratios to the control (100%). In panels a and b, bars without common letters differ significantly (P < 0.01).

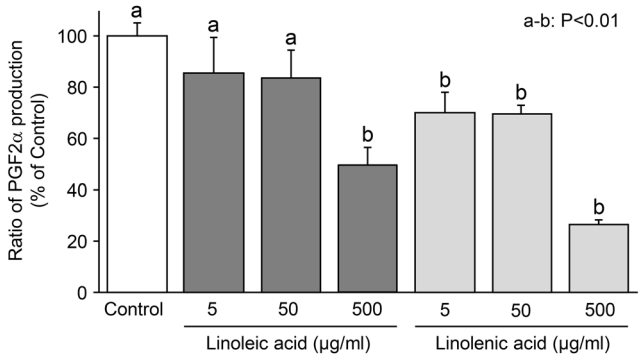

Fig. 4. Effects of linoleic and linolenic acids on prostaglandin F2α (PGF2α) secretion by cultured bovine endometrial tissue. Data are the mean ± SEM of four cows per stage, and are expressed as relative ratios to the control (100%). In panels a and b, bars without common letters differ significantly (P < 0.01).

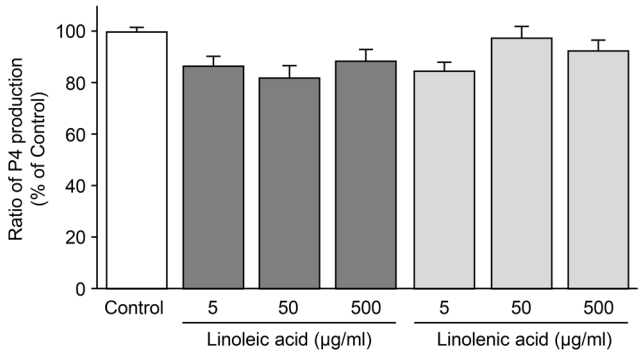

Fig. 5. Effects of linoleic and linolenic acids on progesterone (P4) secretion by cultured bovine luteal tissue. Data are the mean ± SEM of four cows per stage, and are expressed as relative ratios to the control (100%).

the direct effects of linoleic or linolenic acid on the CL, but rather because of the maintenance of the CL via PG production by the uterus.

A number of studies have demonstrated that dietary supplementation with omega-3 and omega-6 FAs influences fertility in dairy cows in a variety of ways. For example, cows fed omega-3 FAs showed increased follicle growth [10, 29] and the mean diameter of the ovulatory follicle [5–7]. In addition, the size of the dominant follicle increased in cows fed diets rich in linoleic and linolenic acids, compared to those fed saturated FAs [4]. Cows receiving supplementary omega-3 FAs had less plasma P4 and smaller CLs than cows receiving the control supplements. On the one hand, reduced plasma P4 in omega-3-supplemented cows may lead to a suboptimal uterine environment for embryo development and, hence, reduced fertility compared to cows receiving the control [30]. On the other hand, cows fed diets enriched with omega-3 or omega-6 FAs displayed higher plasma P4 concentrations than cows not fed a fat supplement [6]. A higher concentration of P4 two days after the injection of PGF2α in cows fed menhaden fish meal suggested delayed luteal regression [31]. Thus, oral administration of linoleic or linolenic acid in cows is unlikely to have a clear effect on reproductive function. In this study, the intra-uterine administration of linoleic or linolenic acid resulted in increased plasma P4 levels and maintained CL diameters compared to the administration of saline (control) at the time of luteolysis (days 19–21). Moreover, these FAs did not cause significant changes in plasma P4 concentrations during the next estrous cycle, suggesting that the effects of intra-uterine administration of these FAs are transient. Cholesterol and P4 concentrations in the blood during the luteal phase were higher when beef cows were fed calcium salts of palm oil, which contain saturated FA (palmitic acid), compared to those fed a control diet with no fat supplement [32]. Hence, one might speculate that FAs act on luteal P4 production in cows. However, no significant effects of linoleic and linolenic acids on P4 production in cultured bovine luteal tissues were observed in this study. A similar observation was reported in an *in vivo* study in which the P4 concentration of dairy cows fed a diet containing menhaden fish meal or calcium salts of fish oil did not change compared to cows fed a control diet and those fed a similar diet during the luteal phase [12, 33]. Therefore, the regulatory mechanisms of FAs on luteal P4 production and subsequent plasma P4 levels in cows may be intricately adjusted, but this needs to be clarified in detail.

Previous studies have shown significant inhibitory effects of fish oil, including omega-3 FA, on plasma PGFM concentration in cows [15, 16]. Circulating PGF2α is quickly metabolized to PGFM, which is relatively stable in the blood in the lungs [34], and PGFM concentration in serum is correlated with PGF2α activity in cows [16]. High concentrations of EPA and DHA in the caruncular tissue of cows fed omega-3 FA-enriched diets may reduce the PGF2α concentration [16]. Moreover, a decreased level of PGE2 was observed in cows fed omega-3 FA, compared to cows fed an omega-6 FA-rich diet or calcium salts of palm oil [35]. The plasma concentration of PGFM increased on the first day postpartum when cows were fed supplemental linoleic acid [19]. Thus, we investigated whether the intra-uterine administration of linolenic or linoleic acid affects plasma PGFM concentration in cows. However, intra-uterine administration of both linoleic and linolenic acids did not affect plasma PGFM concentration throughout the estrous cycle in this study (data not shown). Although we could not find an appropriate explanation for this phenomenon, different PGE2/PGF2α ratios in plasma or systemic/local effects of FAs might account for the discrepant results between oral and intra-uterine administration.

Omega-3 FAs have a cascade that leads to EPA and DHA synthesis, while omega-6 FAs have a cascade that leads to arachidonic acid synthesis [36]. Despite these different cascades, the effects of both linoleic and linolenic acids on endometrial PGF2α and PGE2

production were similar in this study. Previous studies demonstrated that a higher pregnancy rate and, simultaneously, lower pregnancy losses were observed in dairy cows fed a diet high in omega-3 FAs compared to those fed omega-6 or saturated FAs [5, 7, 37, 38]. Detailed differences in the effects of omega-3 and omega-6 FAs on the bovine endometrium, such as the effects of their concentrations or the duration of their action, will require further study.

Our *in vitro* data suggested that both linoleic and linolenic acids inhibit PGF2α synthesis in cultured endometrial tissues. PGF2α production from cultured luteal tissues was reportedly inhibited by linoleic and linolenic acids [39], and linolenic acid was shown to reduce ovarian and endometrial synthesis of PGF2α by reducing the activation of PG synthase and decreasing the availability of its precursor arachidonic acid [17]. Peroxisome proliferator-activated receptor alpha (PPARα), PPARD, and PPARγ are a family of nuclear receptors that are activated by binding natural ligands, such as FAs [40]. PPARD is thought to be involved in the pregnancy recognition process of cattle and may mediate some of the proposed beneficial effects of omega-3 FA supplementation on PG synthesis in the CL and endometrium [13, 39, 41]. The findings of these studies support the hypothesis that unsaturated FAs, including linoleic and linolenic acids, might not only stimulate PPARD expression, but also inhibit PGF2α synthesis in the bovine endometrium and CL.

Collectively, the present data suggest that the administration of unsaturated FAs (linoleic and linolenic acids), which are sources of various PGs, modulate the reproductive function in cows. In particular, it is suggested that these unsaturated FAs not only regulate endometrial PG production, but also indirectly support luteal P4 production.

Conflict of interests: The authors declare no conflicts of interest.

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