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Short communication: Concentrations of the mammalian lignan enterolactone in preovulatory follicles and the correlation with intrafollicular estradiol in dairy cows fed extruded flaxseed

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ABSTRACT

Flaxseed is a rich source of lignans that can be metabolized to the mammalian lignan enterolactone (EL), which may elicit weak estrogenic or antiestrogenic effects. The objectives of this study were to examine the effects of feeding an extruded flaxseed supplement to dairy cows on concentrations of EL in plasma and preovulatory follicles and the association with intrafollicular estradiol (E_2). Twenty-four multiparous 256-d-pregnant Israeli Holstein cows were fed either a standard diet both pre- and postpartum (control; n = 12) or provided with an extruded flaxseed supplement (n = 12), at 7.9 and 9.2% of DM, pre- and postpartum, respectively. Follicular fluid (FF) aspirations were conducted at 84 ± 16 d in lactation as follows: 7 to 8 d following behavioral estrus, cows were injected with prostaglandin F_{2a} and 48 h later follicles >7 mm were aspirated. Follicles were regarded as preovulatory when the E_2-to-progesterone ratio was >1. Plasma EL concentrations were not different between treatment groups; however, concentrations of EL in FF of preovulatory follicles were 1.7 times higher in extruded flaxseed-supplemented cows than in control. Across-treatment analysis revealed a positive correlation between concentrations of EL in plasma and in FF. In addition, intrafollicular EL concentrations were positively correlated with E_2 concentrations (r = 0.50), and with the intrafollicular E_2-to-progesterone ratio. In conclusion, supplementing dairy cows with extruded flaxseed increased EL concentrations in preovulatory follicles. Intrafollicular EL was correlated with E_2 concentrations; therefore, the possible effects of EL from flaxseed on follicular steroidogenesis should be considered.

Key words: flaxseed, enterolactone, follicle, intrafollicular estradiol

Short Communication

Flaxseed (*Linum usitatissimum*) is the richest source of the plant lignan secoisolariciresinol diglycoside (**SDG**), a precursor of the mammalian lignans enterolactone (**EL**) and enterodiol (Thompson et al., 1991). Lignans are polyphenolic compounds with a wide range of biological activities, including antioxidant, antitumor, and weakly estrogenic and antiestrogenic properties; they also inhibit enzymes involved in the metabolism of sex hormones (Martin et al., 1996; Kitts et al., 1999). In ruminants, plant lignans are metabolized to mammalian lignans by both ruminal and fecal microbiota (Côrtes et al., 2008; Zhou et al., 2009). Gagnon et al. (2009) demonstrated that ruminal microbiota plays an important role in the metabolism of flaxseed lignans in dairy cattle. Lignan metabolites are present in biological fluids of cattle such as plasma, milk, and semen (Dehennin et al., 1982; Gagnon et al., 2009). In recent years, interest has been growing in the effects of dietary compounds in dairy cow feeds that can have estrogenic or antiestrogenic effects. The structural similarity of EL to estradiol ($\mathbf{E_2}$) enables these lignans to bind to estrogen receptors and elicit weak estrogenic or antiestrogenic effects (Carreau et al., 2008). In a study conducted in cattle, isoflavones (phyto-estrogens that are present in soybean) and their metabolites inhibited LH-stimulated progesterone ($\mathbf{P_4}$) secretion (Piotrowska et al., 2006). Flaxseed is used in dairy cows diets; however, the presence of EL in ovarian follicles and its possible effects on steroidogenesis are not documented. In the present work it was hypothesized that feeding dairy cows an extruded flaxseed supplement would increase plasma and follicular EL concentrations, and this could affect steroidogenesis in preovulatory follicles. Therefore, the objectives were (1) to examine the effects of feeding dairy cows with an extruded flaxseed supplement that contained 0.5% SDG on EL concentrations in plasma and follicular fluid (**FF**) obtained from preovulatory follicles, and (2) to examine the association between EL concentrations in plasma and FF and between intrafollicular EL and E_2 concentrations.

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The Volcani Center Animal Care Committee approved all the procedures involving animals. Twenty-four multiparous Israeli Holstein 256-d-pregnant dry cows at the Volcani Center experimental farm (Bet Dagan, Israel) participated in the study. Cows were blocked by previous lactation milk and fat yields, lactation number, BW, and BCS and were randomly allocated to treatment groups. Cows were group-housed in a shaded outdoor pen with adjacent outside yard, which was equipped with a real-time electronic individual feeding system. Each station was equipped with an individual identification system (S.A.E. Afikim, Kibbutz Afikim, Israel). The dietary treatments continued until 100 d in lactation and were as follows: (1) controls (n = 12) were fed standard diets both prepartum and postpartum according to NRC (1989) recommendations; (2) **EXF** (n = 12) were fed prepartum with a dry cow diet (NRC, 1989) supplemented with 1 kg/d per cow (7.9% DM) of an extruded flaxseed supplement (Valomega-160; Valorex, Combourtille, France). Postpartum, EXF cows received a lactating cow diet that included 9.2% (DM basis) of the same supplement. The pre- and postpartum diets contained 1.49 and 1.76 Mcal/kg of DM of NE_L and 13.3 and 17.0% CP, respectively. Diets from control and EXF groups were isonitrogenous and isoenergetic, and ether extract contents were similar in postpartum diets. The compositions and contents of the diets were described in Zachut et al. (2010a). The extruded supplement contained flaxseed and wheat bran at 700 and 300 g/kg, respectively, and contained 21.7% CP and 30.4% ether extracts. Cows were individually fed once a day at 1100 h and supplements were hand-mixed into the TMR. According to manufacturer's information, the EXF supplement contained 0.5% SDG of DM (Valomega-160; Valorex).

The reproductive health of the genital tract of all the participating cows was examined by 2 successive examinations, separated by a 10-d interval, by means of transrectal ultrasound examination with an Aquila 5-MHz linear array transducer (Pie Medical, Maastricht, the Netherlands), and only cows with a healthy genital tract were assigned to follicular aspiration. The follicular aspiration procedure was conducted at 84 ± 16 d in lactation. On d 7 and 8 after an observed behavioral estrus, the cows received a PGF_{2a} analog injection (2.5 mL of Estrumate, 625 μg of cloprostenol; Coopers Animal Health Ltd., Berkhamsted, UK); and 48 h later, FF from follicles >7 mm in diameter was aspirated as described. The cows were sedated with an i.m. injection of 1 mL of 2% Rompun (XYL-M2 Veterinary, xylazine base 20 mg/mL; VMD, Arendonk, Belgium) and were given a local anesthetic of 5 mL of 2% lidocaine HCl (2% esracain at 200 mg/10 mL; Rafa Laboratories, Jerusalem, Israel), injected epidurally between the last sacral and first caudal vertebrae. The ovaries were examined with an ultrasound scanner (Scanner 200; Pie Medical) and the diameter of the large follicles was measured. Follicles >7 mm were aspirated individually with an ultrasound scanner connected to a 7.5-MHz vaginal-sector transducer equipped with a needle guide and connected to an MP86 suction pump (Biometra, Goettingen, Germany) set at a flow rate of 25 to 30 mL/min. The needles used were 18 gauge and were changed between follicles and each follicle was collected to a separate tube. Blood samples were collected on the day of $PGF_{2\alpha}$ injection and on the day of follicular aspiration for determination of plasma P_4 concentrations to assess regression of the CL in response to $PGF_{2\alpha}$ injection. The blood samples were collected from the jugular vein into evacuated tubes containing lithium heparin (Becton Dickinson System, Cowley, UK). Blood samples and follicular fluid were centrifuged at 3,000 × *g* for 20 min at room temperature and stored at −25°C for subsequent analysis. The concentrations of P_4 in plasma as well as P_4 and E_2 in FF were determined by RIA (Diagnostic Products, Los Angeles, CA). Follicular fluid was diluted by 100 and 500 times for P_4 and E_2 determination, respectively. The minimal detectable concentrations of P_4 and E_2 were 0.2 and 20 ng/mL, respectively. The intra- and interassay coefficients of variation for the P_4 assay were 9.2 and 8.5% respectively. The intra- and interassay coefficients of variation for the E_2 assay were 4.1 and 3.6%, respectively. The follicles with an E_2-to-P_4 ratio >1 were regarded as E_2-active (Ireland and Roche, 1982).

The concentrations of EL in plasma and FF of preovulatory follicles were determined as described by Gagnon et al. (2009) using an enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI). The kit is a competitive assay that recognizes both enantiomeric forms of EL and uses a standard curve that covers the range from 15.6 to 2,000 pg/mL. Concentrations of EL in plasma and FF, as well as follicular P_4 and E_2 concentrations were analyzed using the General Linear Model procedure of SAS (version 9.2; SAS Institute, Cary, NC). The model included the effects of cow, treatment, parity, and DIM. Across-treatment correlations analysis was performed with the REG procedure of SAS; $P < 0.05$ was accepted as significant.

The concentrations of EL in plasma did not differ between EXF cows and controls (0.33 and 0.25 μmol/L, respectively; $P < 0.27$; SEM = 0.04; range 0.16–0.52 μmol/L). Follicular aspirations were conducted in all cows, and in 2 control cows and 1 EXF animal no dominant follicles were found. Among these 3 cows, in 2 of them plasma progesterone was <0.5 ng/mL at day of $PGF_{2\alpha}$ injection. The average plasma P_4 concentrations

at day of $PGF_{2\alpha}$ injection and on the day of follicular aspiration were 3.8 ± 1.5 and 0.3 ± 0.2 ng/mL, respectively, with no differences between treatment groups.

In total, 11 control and 15 EXF E_2-active follicles were collected from 9 and 11 control and EXF cows, respectively. In 4 cows, 2 E_2-active follicles were observed with a large difference in E_2-to-P_4 ratio between follicles. In these cows, the follicle with the higher E_2-to-P_4 ratio was considered as preovulatory. No differences in the FF concentrations of P_4 or E_2 were observed between control and EXF cows (85.9 and 74.3 ng/mL, respectively, for P_4; 1,920.7 and 2,079.9 ng/mL, respectively, for E_2). The concentrations of EL in FF were 1.7 times higher in the EXF as in the control group (0.19 and 0.11 μmol/L, respectively, SEM = 0.03, $P < 0.05$).

In the present study the EXF cows' average postpartum DM consumption was 27.1 ± 0.2 kg/d, which contained 9.2% of extruded flaxseed supplement. The EXF supplement contained 0.5% SDG of DM; therefore, on average, each cow on the EXF diet consumed 12.5 g/d of SDG. As SDG from the feed is metabolized into EL, mainly in the rumen (Gagnon et al., 2009), it is plausible that the higher concentrations of intrafollicular EL found in the EXF group resulted from their intake of SDG. Data from all cows was logarithmically transformed for correlation analysis, and across treatments analysis revealed a positive correlation (r = 0.61, $P < 0.05$) between the concentrations of EL in plasma and the follicular concentrations of EL in E_2-active follicles. Lignan metabolites have previously been shown to be present in biological fluids (i.e., plasma and milk) after cows were supplied with SDG from flaxseed (Gagnon et al., 2009). In dairy cows, the composition of FF reflects, to a certain extent, the composition of plasma, as FF fatty acid composition is comparable with the plasma profile (Zachut et al., 2010b, 2011) and follicular metabolite and ionic composition is correlated with serum concentrations (Leroy et al., 2004). The correlation between the concentrations of the mammalian lignan EL in plasma and in FF, observed in the present study, could imply that alterations in plasma EL concentrations caused by dietary treatments might affect ovarian follicles through modification of their inner environment.

Across-treatment analysis revealed a positive correlation (r = 0.50, $P < 0.04$; Figure 1) between concentrations of follicular EL and E_2 concentrations in FF. Flaxseed is also a source of α-linolenic acid (n-3), which could affect steroidogenesis in the follicles of EXF cows (Zachut et al., 2010b). However, no treatment-related differences in E_2 concentrations in FF were observed, and the effect of EL was observed regardless of treatments; therefore, we focused on the direct effect of EL on follicular steroidogenesis. The E_2-to-P_4 ratio is an indicator of the viability of the preovulatory follicle (Ireland and Roche, 1982), and the association between the follicular E_2-to-P_4 ratio and the follicular EL concentration that was observed in the present study indicated a positive correlation (r = 0.65, $P < 0.003$). The various reported effects of phyto-estrogens on follicular steroidogenesis are inconsistent. Thangavelu et al. (2008) did not observe changes in intrafollicular E_2 concentrations in cows that exhibited higher fecal EL concentrations when fed whole flaxseed at 750 g/d. In another study, EL was demonstrated to lower the activities of the steroidogenic enzymes aromatase and 17β-hydroxysteroid in vitro (Brooks and Thompson, 2005). Tiemann et al. (2007) incubated porcine granulosa cells with phyto-estrogens and found a reduction in P_4 synthesis and decreased 3β-hydroxysteroid gene, but they did not observe changes in E_2 synthesis. Enterolactone has been shown to have both estrogenic and antiestrogenic properties (Martin et al., 1996; Kitts et al., 1999); thus, the positive correlation between intrafollicular EL and E_2 observed in the present study may be the result of a positive feedback of EL on the granulosa cells of preovulatory follicles. However, these findings are inconsistent with other reports that indicated a negative effect of phyto-estrogens on follicular steroidogenesis. Therefore, the effects of EL on steroidogenesis warrants further investigation.

In conclusion, our study is the first to demonstrate the presence of EL in FF of preovulatory follicles and the positive correlation between plasma and intrafollicular EL concentrations. In addition, follicular EL concentrations were positively correlated with E_2 concentrations in preovulatory follicles. Further studies are

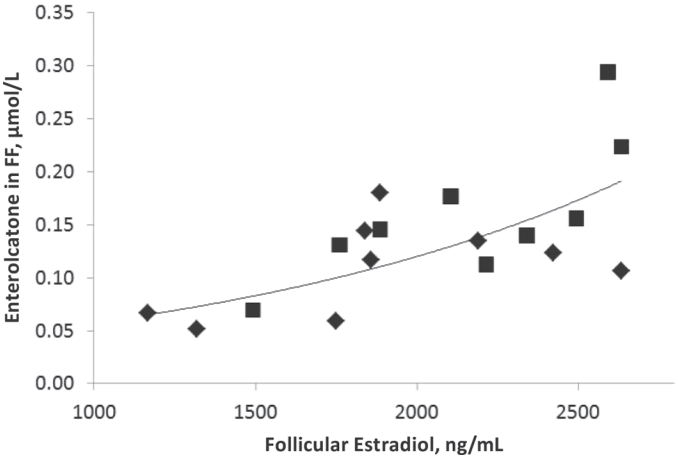

Figure 1. Correlation between intrafollicular enterolactone concentrations and estradiol concentrations in preovulatory follicles. Follicular fluid (FF) of preovulatory follicles from control cows (◆) and cows fed an extruded flaxseed supplement (■) that contained 0.5% secoisolariciresinol diglycoside.

required to elucidate the correlation between EL and steroidogenesis in preovulatory follicles and the possible effects on ovarian function.

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