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—Full Paper—

Fecal and Urinary Lignans, Intrafollicular Estradiol, and Endometrial Receptors in Lactating Dairy Cows Fed Diets Supplemented with Hydrogenated Animal Fat, Flaxseed or Sunflower Seed

Govindarajan THANGAVELU[1)], Marcos Germán COLAZO[2)], Masahito OBA[1)], Michael Kane DYCK[1)], Erasmus Kojo OKINE[1)] and Divakar Justus AMBROSE[1,2)]

[1)]Dairy Research and Technology Centre, Department of Agricultural Food and Nutritional Science, University of Alberta and [2)]Alberta Agriculture and Rural Development, Edmonton, Alberta T6H 5T6, Canada

Abstract. We hypothesized that the inclusion of flaxseed in the diets of lactating dairy cows will increase urinary and fecal concentrations of the lignans, secoisolariciresinol diglycoside (SDG), enterolactone and enterodiol, reduce intrafollicular concentrations of IGF-I and estradiol, and subsequently reduce estradiol and oxytocin receptor expression in the endometrium. To test this hypothesis, 27 cycling, lactating Holstein cows were assigned to 1 of 3 diets supplemented with saturated fatty acids (SAT), flax (FLX), or sunflower (SUN) seed. Rations were formulated to provide 750 g supplemental fat/cow/d in all dietary groups. Ovulation (Day 0) was synchronized, and 5 d later, follicles > 8 mm were ablated by an ultrasound-guided procedure in all cows. Samples of blood (Days 0 to 14), follicular fluid (Day 5 and 15), endometrium (Day 15), as well as urine and feces were collected in a subset of the animals. The fecal concentrations of SDG and enterodiol were higher ($P<0.05$) in cows fed FLX than in those fed SAT or SUN. Enterodiol increased ($P<0.05$) in urine samples of cows fed FLX, compared to those of cows fed SUN. However, follicular estradiol concentrations on Day 5 and 15 and endometrial concentrations of estradiol and oxytocin receptors on Day 15 did not differ among the dietary groups. Mean plasma progesterone concentrations were higher ($P<0.05$) in cows fed FLX and SUN than in those fed SAT. In summary, a diet supplemented with flaxseed increased the concentrations of SDG and enterodiol in feces, as hypothesized, but did not alter intrafollicular concentrations of IGF-I or estradiol, or endometrial populations of oxytocin or estrogen receptors in lactating dairy cows.

Key words: Dairy cow, Endometrial receptors, Estradiol, Flaxseed, Lignan

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Early embryonic loss is a major contributor to reproductive inefficiency in dairy cattle, and upto 80% of embryonic losses occur between 8 and 16 days of gestation [1]. One potential approach to reduce embryonic mortality in cattle is through nutritional management [2]. In recent studies, the dietary inclusion of flaxseed (rich in ∝-linolenic acid; ALA) increased conception rate in dairy cattle [3, 4], likely through a reduction of embryonic losses [4, 5]. More recently, we have shown [6] that early embryonic development is accelerated in cows fed unsaturated fatty acids compared to those fed a saturated fat source.

Diminished or delayed $PGF_{2\alpha}$ production during the critical window of pregnancy recognition can potentially enhance embryo survival. Although a direct role for ALA in $PGF_{2\alpha}$ suppression in bovine endometrial cells *in vitro* has been reported [7], this has not been unequivocally demonstrated in bovine *in vivo* models [8, 9].

The estradiol-active dominant follicle plays a major role in the luteolytic process in ruminants. It is suggested that an increase in the intrafollicular concentrations of estradiol elevates uterine $PGF_{2\alpha}$ production by stimulating the activity of enzymes controlling $PGF_{2\alpha}$ synthesis and inducting the formation of endometrial oxytocin receptors (OTR; [10]), which are essential for the pulsatile release of $PGF_{2\alpha}$ leading to luteolysis.

Flaxseed is the richest known dietary source of the plant lignan, secoisolariciresinol diglycoside (SDG), a precursor for the mammalian lignans, enterolactone and enterodiol [11], which are diphenolic compounds, formed by intestinal bacteria in humans and rodents [12]. An increase in urinary lignans was negatively correlated to plasma IGF-I concentrations in rats [13]. These mammalian lignans have also exerted antiestrogenic properties in both *in vivo* [14] and *in vitro* [15] non ruminant studies. Diets containing lignan precursors (e.g. SDG) increased the production of steroid hormone binding proteins from the liver, contributing to a decrease in the concentrations of circulating estradiol and testosterone [16]. However, it is not known if diets containing flaxseed can contribute to the production of mammalian lignans and to the reduction of intrafollicular estradiol concentrations in cattle.

Therefore, the primary objectives were to compare SDG, enterolactone and enterodiol concentrations in urine and feces, intrafollicular concentrations of IGF-I and estradiol, and that of estradiol receptor-α (ERα) and OTR in the endometrium, in cows fed diets supplemented with hydrogenated animal fat high in saturated fatty acids (SAT), flaxseed (FLX) or sunflower seed (SUN). We also determined intrafollicular and plasma concentrations of progesterone and plasma IGF-I.

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Correspondence: D Ambrose (e-mail: divakar.ambrose@gov.ab.ca)

Materials and Methods

Animals and diets

The study was conducted at the Dairy Research Unit of the University of Alberta, Edmonton (53° 34' N, 113° 31' W), between September 2005 and February 2006, with all animal experimental procedures approved by the University of Alberta Animal Policy and Welfare Committee (Protocol # 2005-33c). A total of 27 cyclic, lactating Holstein cows were randomly but equally assigned to 1 of the 3 dietary groups. The experimental design is shown in Fig. 1. At initiation of experimental diets, cows averaged 53 ± 4.1 d postpartum and 2.2 ± 0.25 lactations, respectively. Total mixed rations (TMR) were formulated to meet or exceed the requirements of a 650 kg lactating cow, as per NRC guidelines [17]. Ingredients and diet composition are presented in Table 1. Rations were formulated to provide 750 g of supplemental fat/cow/d from hydrogenated animal fat (Energy Booster 100; United Nutrients Corporation Inc., Plattsville, ON, Canada) high in saturated fatty acids (SAT), whole flaxseed (FLX) or whole sunflower seed (SUN), both high in unsaturated fatty acids. Cows were housed in tie-stalls and had unrestricted access to water. Cows were fed once daily at 0930 h, allowed 1 h of daily exercise (between 1030 and 1130 h), and milked twice daily between 0400 and 0600 h, and between 1530 and 1730 h. Diets were delivered individually by a Data Ranger (American Calan, Northwood, NH, USA) and orts were weighed daily, prior to commencement of feeding.

Body weight and body condition score (BCS; 1=emaciated, 5= obese; [18]) were recorded before the initiation of the experimental diets.

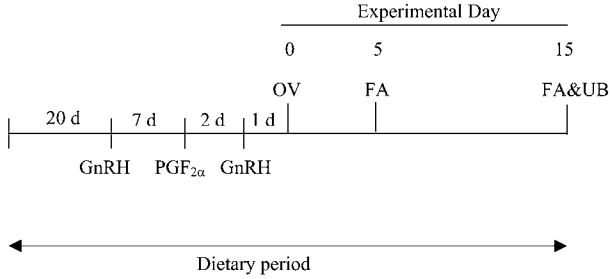
Synchronization of ovulation

Twenty days after the initiation of diets, ovulation was synchronized in all cows by the administration of 100 μg GnRH (gonadorelin acetate; Fertiline, Vetoquinol Canada, Lavaltrie, QC, Canada) followed by 25 mg of $PGF_{2\alpha}$ (dinoprost tromethamine, Lutalyse; Pfizer Animal Health, Kirkville, QC, Canada) 7 d later, and a second GnRH given 2 d after $PGF_{2\alpha}$ [19]. Transrectal ultrasonography (Aloka-500V scanner attached to a 7.5 MHz linear transducer; Aloka, Tokyo, Japan) was performed daily in all cows from the day of $PGF_{2\alpha}$ administration until ovulation (Day 0).

Sample collection and analyses

Feed, feces and urine: Samples of TMR were taken every two weeks and processed as previously described [6] to determine dry matter intake (DMI).

Fecal and urine samples were obtained from 6 and 3 animals, respectively, in each dietary group, to determine SDG, enterolactone and enterodiol concentrations concentrations. Samples were collected at the end of the experimental period, when cows had received the diets for about 45 d. Fecal samples were collected transrectally in 50 ml plastic containers (Fisher Scientific, Ottawa, ON, Canada) and urine samples were collected into 500 ml wide-mouth polypropylene square bottles (Nalgene Nunc International, Rochester, NY, USA) initially, and then transferred to 50 ml plastic containers. Samples were stored at –20 C until the determination of lignan concentrations.



GnRH = gonadotropin releasing hormone
$PGF_{2\alpha}$ = prostaglandin $F_{2\alpha}$
OV = ovulation
FA = follicular ablation
UB = uterine biopsy

Fig. 1. Experimental design. Twenty-seven lactating Holstein cows were assigned randomly, but equally, to 1 of 3 diets containing saturated fatty acids (SAT, high in palmitic acids), whole flaxseed (FLX, high in α-linolenic acid), or sunflower seed (SUN, high in linoleic acid). Twenty days after diet initiation, ovulation (Day=0) was synchronized in all animals with GnRH, PGF and GnRH (Ovsynch). Ultrasound-guided follicle ablation (FA) was performed on Days 5 and 15.

Fecal and urine samples were sent to the Laboratory Analytical Facilities (Department of Nutritional Sciences, University of Toronto, ON, Canada) for quantification of lignans. The concentrations of SDG, enterolactone and enterodiol were determined, in thawed urine samples, as previously described by Liu *et al*. [20]. The fecal lignans were also determined by the same methodology, except that thawed samples were extracted twice using 70% methanol at 60 C for 2 h.

Follicular fluid

Follicular fluid samples were collected from the largest (dominant) follicle by transvaginal follicular aspiration [21] in all 27 cows on Day 5, and in a subset of 14 cows on Day 15 (one cow was in estrus 3 d before the scheduled date of follicle aspiration; hence excluded). Samples were immediately centrifuged at 1,500 × *g* for 20 min at 4 C to remove cellular debris and subsequently stored at –20 C until estradiol, progesterone, and IGF-I concentrations were determined.

Concentrations of progesterone and estradiol were determined as previously described [22]. Briefly, samples were thawed at 37 C for 10 min and centrifuged at 3,000 × *g*, for 20 min at 4 C. The centrifuged sample was diluted to 1:500 with zero calibrator for estradiol and 1:100 for progesterone. Estradiol and progesterone concentrations in follicular fluid were analyzed using the Coat-a-Count kit (Diagnostic Products Corporation, Los Angeles, CA, USA). All samples were analyzed in one assay; the intra-assay coefficient of variation was 9.8 and 7.6% for estradiol and progesterone, respectively. The sensitivity of the assays were 8 pg/ml and 0.01 ng/ml for estradiol and progesterone respectively.

The estradiol:progesterone ratio was determined in the follicular fluid samples collected on Day 15; follicles were classified as estradiol-active when the ratio was > 1 [23].

Concentrations of total IGF-I were also determined in follicular fluid samples (Days 5 and 15) by a homologous double antibody radioimmunoassay according to the method of Novak *et al*. [24]. The antibody, rabbit anti-human-IGF-I serum (product name AFP4892898; gift from Dr. AF Parlow through the NIDDK's National Hormone and Pituitary Program, CA). The inter- and intra-assay coefficients of variation were 17.7 and 5.6% and the sensitivity of the assay was 16.4 ng/ml.

Endometrial tissue

Transcervical uterine biopsy was performed [24] on Day 15 to collect endometrial samples in 15 animals. Briefly, after emptying the rectum, the vulva and perianal area was washed and disinfected with povidone iodine (Prepodyne solution; West Penetone, Anjou, QC, Canada). Epidural anaesthesia was induced by administering 3 to 5 ml of 2% lidocaine hydrochloride (Bimeda-MTC Animal health, Cambridge, ON, Canada). A 25 cm long uterine biopsy forceps (Kevorkian-Younge; Fine Surgicals, Hempstead, NY, USA) was modified for use in cattle by lengthening it to 58 cm (CMS, Saskatoon, SK, Canada). The biopsy forceps was introduced into the uterus transcervically taking a rectovaginal approach, and diverted into the uterine horn ipsilateral to the side of the dominant follicle. An endometrial sample was collected from a location about 5 cm beyond the external bifurcation of uterus. The samples were placed in cryovials (Fisher Scientific) and immediately stored in liquid nitrogen at –196 C.

The abundance of ERα and OTR populations in the endometrium was determined by western blot as described by Guzeloglu *et al*. [26] and Wu *et al.* [27], respectively, with modifications. Briefly, the collected endometrial tissues were homogenized for 30 sec in 0.5 ml of homogenizing buffer [50 mM Tris, pH 8.0, 300 mM NaCl, 1 mM Na_3VO_4, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM ethylene glycol-bis (ß-aminoethyl ether)-N N N'N'-tetraacetic acid: EGTA, 0.5 mM phenylmethylsulfonyl fluoride, 10% v/v glycerol, 1% v/v Nonidet P-40 and 10 μg/ml each of aprotinin, leupeptin and pepstatin] followed by centrifugation at 11,000 rpm (Beckman J2-21, rotor JA020.1; Beckman Coulter, Fullerton, CA, USA) for 15 min at 4 C. The protein concentrations were determined in supernatants of the homogenized samples by using BCA protein assay reagent kit (Pierce, Rock ford, IL, USA). Volumes of homogenizing extract from each cow corresponding to 200 μg of protein were loaded onto 10% separating gel and 4% stacking gel, subjected to SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes overnight.

Membranes were blocked for 3 h in 2% (w/v) of Amersham ECL advanced blocking agent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) in Tris-buffered saline containing 0.1% Tween-20 (TBST) which were then incubated with mouse monoclonal ERα antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA; No. Sc-787) or anti-rat OTR (1:500; Alpha Diagnostics, San Antonio, TX, USA; No. OTR 11A) overnight for binding of the corresponding proteins. Secondary antibodies were goat anti-mouse IgG2a-HRP (1:4,000 Santacruz biotechnology, No.Sc-2061) or goat anti-rabbit IgG (H+L) peroxidase purified (1:4,000, Alpha diagnostics; No. 20320) for ERα and OTR receptors, respectively. Protein bands were detected using Amersham ECL advance western blotting kit (GE Healthcare Bio-Sciences) and assessed for relative protein quantities based on band optical density (OD) as determined with a Typhoon Trio+ scanner (GE Healthcare Bio-Sciences), using Image Quant TL software version 2005 (GE Healthcare Bio-Sciences).

Blood

Blood samples (5 cows/dietary group) were collected in the afternoon, by coccygeal venipuncture daily from Days 0 to 8 and on alternate days thereafter, up to Days 14, into 10 ml Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing sodium heparin. The blood samples were immediately placed on ice and centrifuged, within 1 h, at 4 C for 20 min at 1500 × g (Rotanta 460 R; Hettich Zentrifugan, Tuttlingen, Germany), plasma separated and stored at –20 C until progesterone and IGF-I concentrations were determined.

Plasma concentrations of progesterone were determined in all samples using a commercially available solid-phase radioimmunoassay kit (Coat-a-Count; Diagnostic Products, Los Angeles, CA, USA) [28]. Inter- and intra-assay coefficients of variation were 5.4 and 9.9% respectively.

Concentrations of total IGF-I were determined only in Day 5 plasma samples, by a homologous double antibody radioimmunoassay as previously described by Novak *et al.* [24]. The inter- and intra-assay coefficients of variation were 17.7 and 5.6% and the sensitivity of the assay was 16.4 ng/ml.

Statistical analyses

Throughout this paper, data are reported as means ± standard error (SEM). Differences were considered significant when probability (P) values were ≤0.05, and a tendency toward significance was indicated if the P was < 0.15 but > 0.05. One way non-parametric analysis of variance (ANOVA) using Kruskal-Wallis method by Statistix (Analytical software, Tallahassee, FL, USA) was used to determine the effects of diet on estradiol and progesterone concentrations in follicular fluid, plasma IGF-I, and the concentration of SDG, enterolactone and enterodiol in fecal and urine samples. Pearson correlation was used to determine the relationship of IGF-I concentration between Day 5 plasma and follicular fluid. Plasma progesterone concentration was analyzed using the MIXED procedure of SAS [29] for repeated measures with the following model:

$$Y_{ijk}=\mu + D_i + \beta j + (\alpha\beta)ij + e_{ijk}$$

Where μ is the population mean, D_i is a population parameter corresponding to treatment i, β_j is the fixed effect of time j, $(\alpha\beta)_{ij}$ is the effect of treatment by time interaction, and e_{ijk} is the residual error. The covariance structure of the repeated measurements for each variable was modeled separately according to the lowest value of fit statistics for Akaike's Information Criterion, Akaike's Information Criterion Corrected, and Bayesian Information Criterion, and an appropriate structure fitted [30]. Treatment (diet) effects on endometrial receptors were determined using the General Linear Models procedure.

Table 1. Ingredients and composition of the experimental diets[1]

Item	SAT	FLX	SUN
Ingredients (% of dry matter)			
Alfalfa hay	12	12	12
Barley silage	27.5	27.5	27.5
Concentrate mix[2]	45.5	45.5	45.5
Flaxseed	0	10	0
Sunflower seed	0	0	10.5
Energy booster[3]	3.75	0	0
Soybean meal	6.25	2.5	4.5
Beet pulp	5	2.5	0
Nutrient composition (% of DM)			
Organic matter	92.4	92.2	92.1
CP	18.1	18.1	18.1
NDF	32.5	32.6	32.9
ADF	19.7	19.5	18.9
Lipid	6.52	6.50	6.51

[1]Reprinted from Thangavelu *et al*.[6]. Copyright with permission from Elsevier; License # 1873860013487. [2]Concentrate mix contained rolled barley grain 62.15, corn grain ground 15.1, canola meal (solvent extracted) 2.2, corn gluten meal 12.7, dairy premix 2.7, magnesium oxide 0.2, limestone 1.8, sodium bicarbonate 1.2, molasses 1.4 and biophosphorus (Ca 21%, P 17%) 0.5%. [3]Energy Booster: saturated fatty acids 85; unsaturated fatty acids 15% (C16:0.47; C18:0.36; C18:1.14; and C18:2.1%).

Table 2. Means (± SEM) of the lignans, secoisolariciresinol diglucoside (SDG), enterolactone and enterodiol in frozen-thawed fecal (n=6/dietary group) and urine samples (n=3/dietary group) [on wet basis] of lactating Holstein cows fed saturated fatty acids (SAT), whole flax (FLX) or sunflower (SUN) seeds

	Fecal (μg/g)			Urine (μg/ml)		
	SAT	FLX	SUN	SAT	FLX	SUN
SDG	0.2 ± 0.1^{b}	34.7 ± 13.7^{a}	0.3 ± 0.2^{b}	0.4 ± 0.3	0.1 ± 0.04	0.0 ± 0.0
Enterolactone[1]	10.0 ± 2.5	15.2 ± 2.8	8.6 ± 4.2	65.9 ± 3.9	91.1 ± 28.8	51.1 ± 10.8
Enterodiol	0.5 ± 0.2^{b}	20.4 ± 2.5^{a}	0.4 ± 0.1^{b}	0.6 ± 0.3^{ab}	8.3 ± 0.3^{a}	0.2 ± 0.0^{b}

[ab] Means with different superscripts within rows and samples were significantly different ($P<0.01$). [1]Cows fed FLX tended ($P<0.15$) to have higher fecal enterolactone concentration than cows fed SUN.

Results

Diet composition, DMI, body weight and BCS

Ingredients and composition of the experimental diets are shown in Table 1. DMI, body weight and BCS did not differ among dietary groups. Average DMI and body weight of cows fed SAT, FLX or SUN were 16.8 ± 0.5 and 608 ± 23.4, 17.0 ± 0.5 and 630 ± 28.2 or 17.4 ± 0.5 and 604 ± 41.4 kg, respectively. Overall BCS of cows fed SAT, FLX or SUN was 2.7 ± 0.1, 2.6 ± 0.04, 2.6 ± 0.1 respectively.

Lignans

The concentrations of SDG, enterolactone and enterodiol in urine and fecal samples are shown in Table 2.

There was an increase in the concentration of SDG in fecal samples of cows fed FLX ($P<0.05$) relative to those fed SAT or SUN, but not in urine samples. The mean enterodiol concentration was also higher ($P<0.05$) in fecal samples of cows fed FLX than in those of cows fed SAT or SUN. Although the concentrations of enterolactone in urine samples did not differ among dietary groups, fecal enterolactone tended to be higher in cows fed FLX than in cows fed SUN.

There was no correlation among lignan concentrations (in urine or feces) and concentrations of estradiol or IGF-I in follicular fluid, or IGF-I in plasma (data not shown).

Follicular fluid estradiol and progesterone

Follicular fluid estradiol and progesterone concentrations on Day 5 were not different among the 27 cows fed SAT, FLX or SUN. When all 14 cows from which follicular fluid was aspirated on Day 15 were considered, estradiol and progesterone concentrations did not differ among diets. Five of the 14 cows (SAT=1; FLX=2; SUN=2) did not have an estradiol-active follicle on Day 15. Therefore, we reanalyzed the estradiol and progesterone data considering only the 9 cows that had an estradiol-active follicle. Even though there was no statistical difference in follicular fluid estradiol concentrations, progesterone concentrations tended to be higher in cows fed SAT on Day 15. Estradiol and progesterone concentrations in follicular fluid on Days 5 and 15 by dietary groups are presented in Table 3.

Table 3. Median (Day 5) and mean (± SEM; Day 15) of follicular estradiol and progesterone concentrations (ng/ml; Day 0=ovulation) of lactating Holstein cows given diets supplemented with saturated (SAT) fatty acids, flaxseed (FLX) or sunflower seed (SUN), high in unsaturated fatty acids

	SAT	FLX	SUN	P value
Estradiol				
Day 5 (n=27) [1]	129.0	150.0	246.0	0.29
Day 15 (n=14) [2]	313 ± 194.3	161 ± 79.5	302 ± 114.5	0.65
Day 15 (n=9) [3]	398 ± 246.6	252 ± 103.5	486 ± 32.7	0.45
Progesterone				
Day 5 (n=27) [1]	60.0	60.0	70.0	0.42
Day 15 (n=14) [2]	84 ± 5.7	91.2 ± 20.4	83.6 ± 21.7	0.94
Day 15 (n=9) [3]	80 ± 5.8	60 ± 11.5	57 ± 3.3	0.12

[1] Follicular fluid samples from all 27 cows were used on Day 5. [2] Follicular fluid samples were collected only from 14 cows on Day 15. [3] Only samples from cows with estradiol-active follicles on Day 15.

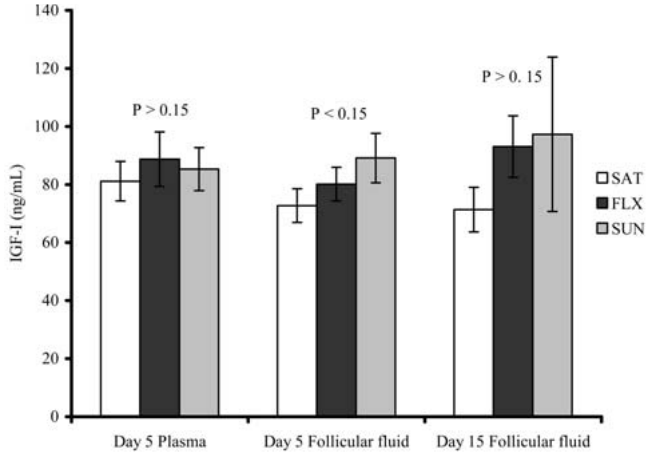


Fig. 2. Mean (± SEM) concentrations (ng/ml) of IGF-I on plasma (Day 5) and follicular fluid (Days 5 and 15) of cows fed saturated fatty acids (SAT), whole flax (FLX) or sunflower (SUN) seeds. Ultrasound-guided follicle ablation was performed in all animals on Days 5 and 15. Concentrations of IGF-1 were not different among Day 5 plasma (P=0.85) or among Day 15 follicular fluid (P=0.38) samples. Day 5 follicular fluid IGF-I concentrations tended (P=0.14) to be higher in cows fed SUN.

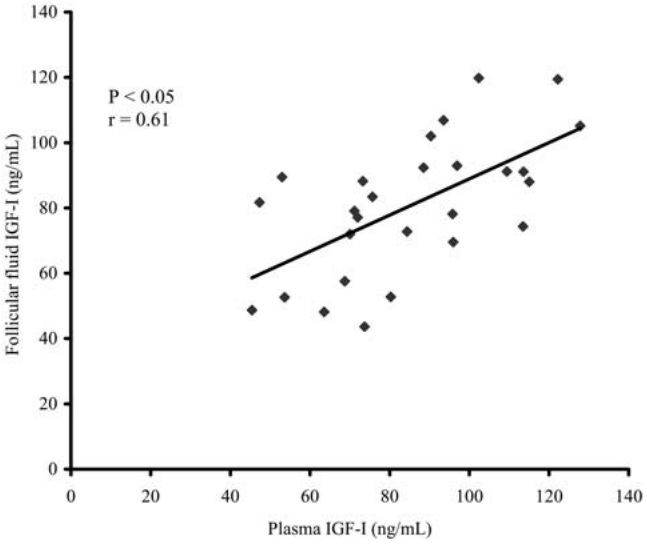


Fig. 3. Positive correlation between plasma and follicular fluid IGF-I concentrations (ng/ml) on Day 5 in cows (n=27) fed SAT (saturated fatty acid), whole flax (FLX) or sunflower (SUN) seed.

IGF- I

Overall means ± SEM for plasma and follicular fluid IGF-I concentrations are presented in Fig. 2. Diets had no effect on Day 5 plasma or Day 15 follicular fluid IGF-I concentrations. However, follicular fluid IGF-I concentration on Day 5 tended to be higher in cows fed SUN than in those fed SAT. Although there was no correlation among follicular fluid concentrations of IGF-I on Day 5 and that of estradiol (r= –0.07) or progesterone (r= –0.14), the concentrations of IGF-I in plasma and follicular fluid on Day 5 were positively correlated (r=0.61; P< 0.05) (Fig. 3).

Estradiol- and oxytocin-receptors

Each endometrial biopsy sample weighed between 250 and 300 mg. We only analyzed samples from cows that had an estradiol-active follicle due to its role in the luteolytic process (n=9; 3 cows/dietary group). Diet had no influence on the abundance of endometrial ERα and OTR. Overall means (± SEM) for endometrial ERα and OTR, expressed in arbitrary units, were 28.5 ± 5.5, 21.9 ± 5.5 and 32.7 ± 5.5 and 0.8 ± 0.08, 0.9 ± 0.08 and 0.6 ± 0.1 for cows fed SAT, FLX or SUN, respectively.

Plasma progesterone

One cow from the SAT group came into estrus on Day 12; therefore, she was excluded from the analysis. Overall mean (± SEM) plasma progesterone concentrations of cows fed SAT, FLX or SUN were 2.1 ± 0.2, 2.5 ± 0.1 and 2.7 ± 0.1, respectively (P<0.05). Progesterone concentration was also affected by day (P<0.05). Even though progesterone concentrations tended to increase faster in cows fed SUN, the interaction between day and dietary group was not significant (Fig. 4).

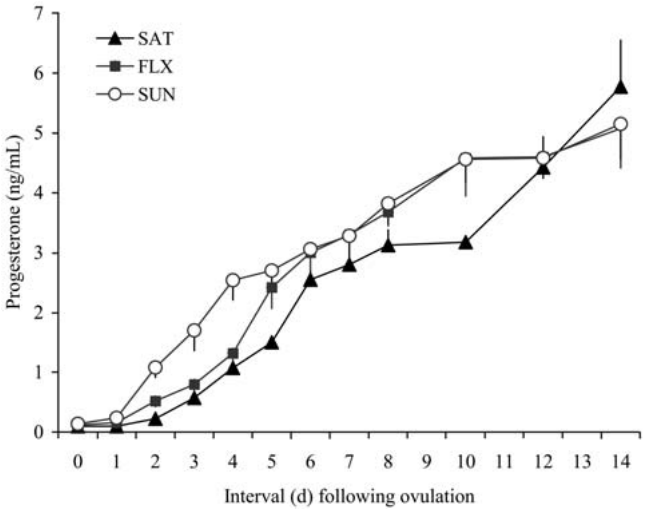


Fig. 4. Mean plasma (± SEM) progesterone concentrations (ng/ml) in lactating Holstein cows (n=14) fed saturated fatty acids (SAT), whole flax (FLX) or sunflower (SUN) seeds from Day 0 to Day 14. Ovarian status of all animals was synchronized with an Ovsynch program and day of ovulation (Day 0) was confirmed by ultrasonography. Cows fed SAT had lower ($P<0.05$) progesterone concentration than that in cows fed SUN from Day 2 to Day 5 and only on Day 5 when compared to cows fed FLX. Cows fed FLX had lower progesterone concentration when compared to cows fed SUN from Day 2 to Day 4 ($P<0.05$). In addition, cows fed SAT tended to have lower plasma progesterone concentration from Day 6 to 8 than cows fed SUN or FLX, respectively.

Discussion

To our knowledge, this is the first study to demonstrate that the inclusion of flaxseed in the rations of lactating dairy cows can increase the concentrations of mammalian lignans in feces and urine.

Flaxseed is the richest known source of the mammalian precursor SDG with levels of up to 1800 times higher than that in sunflowerseed [31]. In rodents, diets supplemented with either flaxseed or SDG (of flaxseed-origin) caused irregularity and lengthening of the estrous cycle similar to those observed with tamoxifen known to have antiestrogen activity [14]. More recently enterolactone and enterodiol reduced estrogen production in estrogen-receptor-positive human breast cancer cells *in vitro* [15]. Based on these [14, 15] and other [13, 16] reports we hypothesized that a diet enriched with flaxseed will increase the production of lignans (SDG, enterolactone and enterodiol), decrease IGF-I and estradiol concentrations at the ovarian level, and subsequently reduce ERα and OTR populations in the uterus of lactating dairy cows.

Despite an increase in the concentration of SDG and enterodiol in feces, and the latter in the urine of cows fed FLX, the concentrations of estradiol in follicular fluid were not significantly reduced. In contrast, a previous study [9] reported that lactating dairy cows fed non-enzymatically-treated whole linseed (i.e., flaxseed) had higher peak plasma estradiol concentrations during estrus, than that of cows fed a diet with no added fat. In that study, cows fed flaxseed had both an increase in the number and diameter of follicles, which may have contributed to the increased circulating estradiol.

In rodents and humans, SDG is converted into enterodiol, which inturn is converted into enterolactone by intestinal bacteria via demethylation and dehydroxylation [16]. The digestive system of ruminants is complex and remarkably different from that of monogastric animals; therefore, the lignan production pathway may be entirely different. Another possible explanation for the failure of the FLX diet to decrease intrafollicular estradiol concentrations in the present study is that the quantity of enterodiol and enterolactone produced or absorbed in these cattle was insufficient to exert antiestrogenic properties. In humans, consumption of only 25 g of SDG per day resulted in plasma concentrations of up to 500 ng/ml enterodiol and enterolactone [32]. An analytical procedure sensitive enough to quantify the potentially low concentrations of lignan in bovine plasma was not readily available; thus, plasma lignan concentrations could not be determined in the present study. Therefore it is not clear if plasma enterodiol and enterolactone concentrations were elevated to levels comparable to that observed in human studies.

Insulin-like growth factor-I is present in circulation and follicular fluid of various species and has the ability to regulate steroidogenesis [33]. Estradiol production of bovine granulosa cells, cultured *in vitro*, was enhanced by IGF-I but the IGF-I-enhanced aromatase activity was also influenced by gonadotropins and follicle size [34]. Ginther *et al*. [35] reported that the administration of IGF-I directly into ovarian follicles of dairy heifers increased follicular fluid estradiol concentrations. In rats fed diets supplemented with either flaxseed (5%) or SDG (1.5 mg/d) plasma IGF-I concentrations were reduced compared to rats fed a control diet, which did not include flaxseed [13]. In the present study, a diet supplemented with flaxseed did not affect IGF concentration in either follicular fluid or plasma. In agreement with our findings, another study [36] also reported that plasma IGF-I concentrations in beef steers fed 5% ground flaxseed were not different from that of steers fed a control diet. Similarly, in lactating dairy cows fed a protected form of whole flaxseed, plasma IGF-I concentrations did not differ from that of cows fed a diet with no added fat [9].

The liver is the major target organ for lignans in rats [37]. High producing dairy cows have increased liver metabolism due to the high blood flow to the liver [38]. Considering that lactating dairy cows were used in the present study, rapid clearance of lignans may have occurred due to increased liver metabolism, thus reducing the potential for any anti-IGF-I / anti-estrogenic activity.

As mentioned earlier, flaxseed is a rich source of ALA and *in vitro* studies have reported a direct role for ALA in $PGF_{2\alpha}$ suppression [7]. However, this has not been clearly demonstrated in studies [8, 9] where the 13, 14, dihydro-15 keto $PGF_{2\alpha}$ (PGFM) concentration was measured *in vivo*, after oxytocin administration. It is well established that the administration of estradiol in cyclic cows will stimulate $PGF_{2\alpha}$ secretion [39] and ERα is upregulated during luteolysis in sheep [40]. There is also an increase in OTR populations around the time of luteolyis [41, 42] that plays a key role in amplifying endometrial $PGF_{2\alpha}$ release in ruminants [10]. Although an increase in estradiol and that of endometrial expres-

sions of ERα and OTR will contribute to an increase in $PGF_{2\alpha}$, most studies have measured PGFM as an indicator for luteolysis. However, our approach was different in that we determined the contributors to $PGF_{2\alpha}$-increase, i.e., estradiol, and endometrial ERα and OTR. We did not detect any significant differences in either endometrial ERα or OTR among the diets and this may be explained by the lack of difference in intrafollicular estradiol concentrations among the diets. It is also possible that endometrial biopsies were performed at a stage when OTR concentrations were low, as Mann and Lamming [42] reported that OTR concentrations were lowest on Day 15 of the estrous cycle, and continued to increase reaching peak concentrations after the completion of luteolysis.

Flax and sunflower seed based diets significantly increased mean plasma progesterone concentrations in the present study, but this does not support earlier findings [4, 9]. Whereas one study [9] reported that in cows fed either a protected form of whole flaxseed or whole soybean (high in linoleic acid) plasma progesterone concentrations in the early luteal phase were significantly lower than in cows fed a control diet with no added fat, another study [4] reported that progesterone concentrations did not differ among cows fed diets supplemented with either flax or sunflower seed. The present results, however, partially support our recent finding [6] that cows fed flaxseed had higher concentrations of progesterone than those fed saturated fatty acids on Days 7 and 8 of the estrous cycle. The reasons for the inconsistencies among studies are unclear. In a recent study [43] isoflavones, phytoestrogens of soy bean origin, and their metabolites inhibited LH-stimulated progesterone secretion in cattle. The effects of lignans and other classes of phytoestrogens on steroidogenesis in cattle remains to be investigated.

Results from the present study indicated a positive correlation between intrafollicular and circulating IGF-I, which is consistent with an earlier report [44]. Even though there is an intra-organ IGF-I system present in the ovary, the positive correlation between IGF-I in plasma and follicular fluid indicates that at least a part of the IGF-I in follicular fluid is of hepatic origin.

In conclusion, the dietary inclusion of flaxseed in dairy cows increased concentrations of SDG in feces, and that of enterodiol in urine and feces, without reducing intrafollicular concentrations of IGF-I or estradiol, or that of endometrial ERα and OTR.

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