Soybean-Derived Phytoestrogens Regulate Prostaglandin Secretion in Endometrium During Cattle Estrous Cycle and Early Pregnancy

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Phytoestrogens acting as endocrine disruptors may induce various pathologies in the female reproductive tract. The purpose of this study was to determine whether phytoestrogens present in the soybean and/or their metabolites are detectable in the plasma of cows fed a diet rich in soy and whether these phytoestrogens influence reproductive efficiency and prostaglandin (PG) synthesis during the estrous cycle and early pregnancy in the bovine endometrium. In *in vivo* Experiment 1, we found significant levels of daidzein and genistein in the fodder and their metabolites (equol and p-ethyl-phenol) in bovine serum and urine. The mean number of artificial inseminations (AIs) and pregnancy rates in two kinds of herds, control and experimental (cows fed with soybean 2.5 kg/day), were almost double in the soy-diet herd in comparison with the control animals. In *in vivo* Experiment 2, three out of five heifers fed soybean (2.5 kg/day) became pregnant whereas four out of five heifers in the control group became pregnant. The concentrations of a metabolite of $PGF_{2\alpha}$ (PGFM) were significantly higher in the blood plasma of heifers fed a diet rich in soybean than those in the control heifers throughout the first 21 days after ovulation and AI. The higher levels of PGFM were positively correlated with equol and p-ethyl phenol concentrations in the blood. In *in vitro* experiments, the influence of isoflavones on PG secretion in different stages of the estrous cycle was studied. Although all phytoestrogens augmented the output of both PGs throughout the estrous cycle, equol and p-ethyl-phenol preferentially stimulated $PGF_{2\alpha}$ output. The results obtained lead to the conclusion that soy-derived phytoestrogens and their metabolites disrupt reproductive efficiency and uterus function by modulating the ratio of $PGF_{2\alpha}$ to PGE_2, which leads to high, nonphysiological production of luteolytic $PGF_{2\alpha}$ in cattle during the estrous cycle and early pregnancy. Exp Biol Med 230:189–199, 2005

Key words: phytoestrogens; estrous cycle; cow; prostaglandins

The role of estrogenic substances derived from plants has been studied for almost half a century (reviewed in 1–3). Environmental estrogens are divided into two main groups: phytoestrogens and xenoestrogens (4). Xenoestrogens are man-made synthetic products, whereas phytoestrogens are derived from plants. There is some evidence that consumption of soy diets containing phytoestrogens has some positive effects on human and animal health. Phytoestrogens are thought to reduce the risk of mammary cancer (5, 6), act as antioxidants (7), and modify certain enzyme activities (8). Moreover, the consumption of soybeans by postmenopausal women has been associated with other health benefits, such as reducing the risk of cardiovascular disease (9, 10) and cancer (11), stopping the progression of atherosclerosis (12, 13), and having positive effects on hot flashes, vaginal symptoms, cognitive function or dementia (14), and bone preservation (15). On the other hand, these substances also have some hazardous effects, especially in animals fed with pasture rich in phytoestrogens (16, 17). Ingestion of clover pasture rich in plant estrogens was shown to cause infertility in cattle (18). There is increasing evidence that phytoestrogens can disrupt reproductive efficiency in various species, including humans (19, 20), rats (21), and cows (22).

Phytoestrogens may act like antagonists or agonists of endogenous estrogens (20, 23–25). Endogenous estrogens control the estrous cycle in ruminants influencing prosta-

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Table 1. Comparison of Feeding Systems in the Z and W Herds[a]

Characteristic	Herd Z (control cows)	Herd W (soybean-fed cows)
Number of animals	10	12
Mean lactation (liter milk/animal/year)	6800	9750
Filling fodder (kg/animal/day)	1.6 kg bruised sunflower grain, 1.6 kg wheat bran, 4.5 kg bruised rye	2.5 kg bruised soy, 1.5 kg bruised rape, 5 kg bruised grain

[a] For volume fodder, both herds received 0.6 kg dry beet pulp, 40 kg silage from maize, and 2 kg hay. Each animal of both herds received 200 g premix SANO 2050 lascan TOP vitamins and minerals/animal/day.

glandin (PG) synthesis (reviewed in 26). For example, the removal of 17-β-estradiol (E_2) on Day 8 of the cycle by destroying ovarian follicles with x irradiation in ewes resulted in prolongation of the estrous cycle and lack of luteolysis (27). On the other hand, administration of E_2 to heifers on Day 13 of the cycle initiated luteolysis by increasing the $PGF_{2\alpha}$ concentration (28). The role of PGs in the reproductive processes of many species is well established (29). In ruminants, $PGF_{2\alpha}$ is the major luteolytic agent (30), whereas PGE_2 has luteoprotective and anti-luteolytic properties (31, 32). Therefore, the development and maintenance of the corpus luteum (CL) as well as establishment of pregnancy may depend on the ratio of luteolytic $PGF_{2\alpha}$ to luteotropic PGE_2 (33). In view of the structural and functional similarities of phytoestrogens and endogenous estrogens, we suspect that these plant-derived substances modulate prostaglandin synthesis in the bovine endometrium.

The fodder commonly used for feeding dairy cattle contains phytoestrogens, such as genistein, daidzein, formonentin, and biochanin A (34). Lundh *et al.* (35) showed that, in cows and ewes, daidzein and genistein present in the fodder are immediately converted in the rumen to equol and p-ethyl-phenol, respectively. The concentration of daidzein and genistein decreases within 1 hr after feeding, whereas equol and p-ethyl-phenol are present in the blood of cows for many hours after feeding (35). Although metabolism of phytoestrogens from synthetically prepared fodder that is rich in phytoestrogens has been thoroughly investigated by Lundh et al. (34, 35), little is known about the effects of feeding cattle with fodder rich in phytoestrogens derived from natural soybean. Therefore, the present study was undertaken to identify which metabolites of phytoestrogens are present in the blood of cows fed a diet rich in soybean, and what phytoestrogen concentrations are needed to have an effect on the bovine reproductive tract. Furthermore, we examined whether phytoestrogens identified in soybean and their metabolites regulate PG output from the bovine endometrium *in vitro* and if they influence the $PGF_{2\alpha}$ to PGE_2 ratio during the estrous cycle in cattle.

Materials and Methods

Animals and Collection of Endometrial Tissues. The study consisted of *in vivo* experiments and an *in vitro* experiment. The *in vivo* experiment was performed on mature, normally cycling Holstein/Polish black and white (75% and 25%, respectively) cows (n = 22) and heifers (n = 10). All experimental procedures concerning live animals were approved by the Local Animal Care and Use Committee (agreement 4/2001/N).

For the *in vitro* experiment, bovine uteri were obtained at a local abattoir within 30 min after exsanguination and were transported, on ice, to the laboratory within 1 hr. Bovine uteri were classified into five stages of the estrous cycle (early luteal I, Days 1–4; early luteal II, Days 5–8; mid luteal, Days 8–12; late luteal, Days 12–15; and follicular, Days 18–21). The stages of the estrous cycle were estimated by macroscopic observation of the ovaries and uterus (36). The uterine horns were separated from each other and from the remaining tissue.

Endometrial Tissue Culture. Endometrial strips were washed three times in sterile saline containing 100 IU/ml penicillin and 100 μg/ml streptomycin. The tissue was then cut into small pieces (approximately 20–30 mg) with a scalpel and subsequently washed in sterile saline. The individual endometrial tissues were placed in culture glass tubes (12 × 75 mm) containing 3 ml of culture medium (Dulbecco's Modified Eagle's Medium and Ham's F-12 medium 1:1 [volume/volume {v/v}]; Sigma Chemical Company, St. Louis, MO; #D8900) supplemented with 0.1% BSA (Boehringer Mannheim GmbH, Mannheim, Germany; #735078), 100 IU/ml penicillin, and 100 μg/ml streptomycin. The tissues were incubated in a shaking water bath at 37°C as described previously (36). The media were continuously gassed with 5% CO_2 in air during incubation.

Experiments. *Experiment 1. Effects of soy-derived phytoestrogens on reproductive efficiency.* The experiment was carried out on two animal farms, with different feeding systems. On one farm in Zalesie, 12 cows were fed a standard diet (Herd Z), and on the other farm in Watkowice, 12 animals were fed a diet containing soybean (Herd W). All experimental cows had given birth to healthy offspring in September and October of the previous year (2002). After calving, the cows were fed during lactation with the scheme shown in Table 1.

In the next year, all cows of both herds were impregnated by artificial insemination (AI) with the semen of the same bull, called Macassar (no. 95113-4-5). The semen was purchased from the Station of Animal Insemination and Breeding in Bydgoszcz, Poland. The

onset of the estrus was confirmed by standing behavior and determined by farm workers as well as by the inseminating veterinarian *via* rectal examination. The estrus was taken as Day 0 of the estrous cycle. Only the cows with behavioral signs of estrus underwent artificial insemination after positive rectal examination. After inseminations, the cows were observed until early October 2003. During the observation, blood samples were taken from the jugular vein every 3 months and the condition of reproductive organs was examined rectally. At the end of the experiment (early October 2003), urine samples were also taken from the observed cows. The blood plasma was separated by centrifugation (2000 *g*, 10 min, 4°C) and stored at –20°C until determination of hormones was made. Concentrations of free and conjugated phytoestrogens in plasma and urine of cows of the two examined herds were determined by high-performance liquid chromatography (HPLC).

Experiment 2. Effect of soy-derived phytoestrogens on prostaglandin secretion during early pregnancy. The objectives of this experiment were to examine (i) the influence of a high soybean diet on the concentrations of P4, PGFM (a metabolite of $PGF_{2\alpha}$—13,14, keto $PGF_{2\alpha}$), and PGE_2 in the blood plasma of experimental heifers after artificial insemination and (ii) the possible influence of phytoestrogens present in the soybean on the reproductive efficiency of heifers.

The experiment was carried out on Herd Z from July to September 2004. We chose 10 normally cycling Holstein/Polish black and white (75% and 25%, respectively) heifers (18–20 months of age and 400–450 kg body weight). The animals in Herd Z were fed a standard diet (Table 1). Two weeks after weighing and choosing the animals for the experiment, the estrus was synchronized using implants of a progesterone analog (Crestar; Intervet, Boxmeer, Holland) as described previously (37). When Crestar was removed the animals (n = 10) were divided into two groups: control (n = 5) and soybean diet (n = 5). Control animals were continuously fed a standard diet (Table 1, Herd Z). Five other heifers were fed 2.5 kg/day of soybean instead of bruised sunflower grain and wheat bran until Day 21 of either the estrous cycle or pregnancy. The onset of estrus was confirmed by standing behavior as determined by workers on the farm as well as by the veterinarian *via* rectal examination. The estrus was taken as Day 0 of the estrous cycle. Seventy-two and 84 hr after Crestar removal, each heifer was inseminated twice with the semen of a bull called Math TV (no. 29960031953). The semen was a gift from Union Nord-Ouest Genetique (UNOG, Lisiex, Bosc-Berenger; St. Saens, France). Only the heifers with behavioral signs of estrus underwent AI after positive rectal examination. The blood samples were collected via puncture of the jugular vein on Days 0, 2, 5, 8, 12–18, and 21 of either pregnancy or estrous cycle. The blood plasma was separated by centrifugation (2000 *g*, 10 min, 4°C) and stored at –20°C until determination of hormones was made. Concentrations of free and conjugated phytoestrogens in plasma were determined by HPLC.

Experiment 3. Determination of effective dose of soy-derived phytoestrogen in vitro. Endometrial slices from early luteal I stage (Day 1–4 of the estrous cycle) were treated for 24 hr with various concentrations (0.1 n*M* to 1 μ*M*) of equol (#45405, Fluka Chemie GmbH, Seelze, Germany), p-ethyl-phenol (#821290, Merck & Co., Inc., Whitehouse Station, NJ), daidzein (#30405 Fluka Chemie), genistein (#345834, Calbiochem-Novabiochem GmbH, Darmstadt, Germany) or E_2 (#75262, 1 n*M*; Fluka Chemie), and tumor necrosis factor-α (TNFα, 0.6 n*M*; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) for a positive control. After 24 hr of incubation, the conditioned media were collected in tubes with 10 μl EDTA, 1% aspirin (#A2093; Sigma), solution (pH 7.3), and frozen at –20°C until measurement of $PGF_{2\alpha}$. The conditioned media were examined for the concentrations of $PGF_{2\alpha}$ by enzyme immunoassay (EIA).

Experiment 4. Effect of soy-derived phytoestrogens on prostaglandin output at different stages of the estrous cycle in vitro. Endometria were taken from cows at five stages of the estrous cycle: early-I luteal (n = 4), early-II luteal (n = 4), midluteal (n = 4), late luteal (n = 4), and follicular (n = 4). Endometrial slices were exposed to daidzein and genistein and their metabolites, equol and p-ethyl-phenol (10 n*M* each) or E_2 (1 n*M*), and TNFα (0.6 n*M*) for a positive control (38). The phytoestrogen concentration of 10 n*M* was chosen because a preliminary experiment showed that it was the most effective dose. After 24 hr of incubation, the conditioned media were collected in tubes with 10 μl EDTA, 1% aspirin (#A1093; Sigma), solution (pH 7.3), and frozen at –20°C until measurement of $PGF_{2\alpha}$ and PGE_2. The tissues were blotted with filter paper and weighed to obtain the concentration per gram tissue.

Analytical Methods. *Determination of phytoestrogens and their conjugates in soybean.* Phytoestrogens were extracted from soybean and identified by HPLC-UV-mass spectroscopy (MS). Pulverized soybean (ca. 0.15 g) was defatted with n-hexane by subsequent sonication and centrifugation (5 × 1 ml). Defatted soy was extracted with 1 ml of 80% methanol containing 0.3 *M* hydrochloric acid by a 30-sec sonication. The mixture was vortexed for 30 secs, again sonicated and vortexed, and centrifuged for 5 min (5000 *g* at 4°C). The supernatant was collected in a 5-ml volumetric flask. That step was repeated five times. The obtained extract was directly submitted to HPLC analysis. Standard compounds (daidzein, daidzin, genistein, genistin, glycitein) were dissolved in 80% methanol containing 0.3 *M* hydrochloric acid, and their concentration was confirmed by UV measurement. Concentration of the daidzin 6-OMalGlc, 6-OAcGlc conjugates, genistin 6-OMalGlc, 6-OAcGlc conjugates, and glycitin were calculated from the daidzin, genistin, and glycitein standard curves, respectively.

Chromatographic determinations were done on a Shimadzu HPLC LC-10 gradient system (Shimadzu, Kyoto,

Japan) consisting of a system controller, two pumps, UV detector set at 254 nm, MS detector (QP8000α), autosampler with 5-μl injection loop, and column oven. All chromatographic determinations were performed at 35°C, at a flow rate of 0.2 ml/min on a C18(2) Luna 3μ column, 150 × 2 mm (Phenomenex, Torrance, CA). The mobile phase was composed of a mixture of solvent A (water, with 0.05% formic acid [v/v]) and solvent B (acetonitrile). Gradients were as follows: 10%B, 28%B, 37%B, 60%B, 60%B, 10%B, 10%B at gradient times t_G = 0, 14, 44, 49, 54, 55, and 75 mins, respectively. Identities were confirmed by mass spectrometry. Mass spectrometer settings were curve desolvation line (CDL) temperature, 160°C; CDL voltage, –70 V; probe voltage, –2,5 kV; defragmentation voltage, –50 V; nitrogen as nebulizer gas flow at 2.8 ml/min. Three replications were done for each analysis.

Determination of phytoestrogens and their conjugates in bovine plasma and urine. Plasma and urine concentration of phytoestrogens and their metabolites were measured as described previously (39). Nonconjugated daidzein, genistein, or their metabolites were determined on HPLC after extraction from blood plasma. To 50 μl of plasma, 50 μl of 0.2 *M* sodium acetate buffer, pH 5, and 900 μl of methanol/acetic acid (100:5, v/v) were added. The mixture was vortexed for 30 secs, sonicated for 30 secs, again vortexed for 30 secs, and centrifuged for 5 mins at 4°C and 5000 *g*. The supernatant was diluted with 100 m*M* of lithium acetate (1:1, v/v), centrifuged for 2 mins at 4°C and 5000 *g*, and 20 μl was injected onto an HPLC column (TSKgel ODS-80TS, 5 μm, 150 × 4.6 mm; TOSOH, Tokyo, Japan). The flow of the mobile phase, composed of water/methanol/acetic acid (58:40:2, v/v/v) containing 50 m*M* of lithium acetate, was 0.9 ml/min. The eluate was monitored with an amperometric detector (ICA-3062; TOA, Tokyo, Japan) with the working potential set at +950 mV. When necessary, samples were diluted with the mobile phase before HPLC analysis.

Enzymatic hydrolysis of the conjugates of daidzein, genistein, and their metabolites and determination of concentrations of released free forms in plasma and urine. Cow plasma and urine samples (50 μl) were mixed with 50 μl of sulfatase type H-5 solution in 0.2 *M* acetate buffer, pH 5 (the preparation contained 500 U of β-glucuronidase per 25 U of sulfatase), and the mixture was incubated at 37°C in a shaking water bath for 1 hr. Daidzein, genistein, and their metabolites released during the incubation and their nonconjugated forms present in plasma before the hydrolysis were extracted with 900 μl of methanol/acetic acid (100:5, v/v) and determined as described above. The result was the total plasma concentration of daidzein, genistein, and their respective metabolites.

Blood biochemical analyses. Blood was collected from the jugular vein. Serum activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and asparagine aminotransferase (AST) were determined using kits (Pointe Scientific, Lincoln Park, MI). The levels of electrolytes (phosphorus [P^+], magnesium [Mg^{2+}], calcium [Ca^{2+}]) were determined with an electrolyte analyzer (EasyLyte; Medica, Bedford, MA) and ion-selective electrodes. Glucose content was measured by the oxidase method. Total protein content was measured with a kit (Alpha Diagnostics, San Antonio, TX). Cholesterol content was measured enzymatically with a kit (Pointe Scientific). Colorimetric and kinetic assays were done with a spectrophotometer (UV/VIS s 330; Marcel Euro, Marcel Sp. z.o.o, Warszawa, Poland).

Determination of Hormones. Progesterone concentrations in plasma samples were assayed using a direct EIA as described previously (37). The P4 standard curve was produced for P4 concentrations ranging from 0.39 pg/ml to 25 ng/ml. The intra- and interassay coefficients of variation averaged 6.6% and 8.4%, respectively.

The concentrations of PGFM in the plasma samples were determined with a direct EIA, as described previously (37). The anti-PGFM serum (WS4468-5) was donated by Dr. W.J. Silvia, University of Kentucky, Lexington, KY. The PGFM standard curve was produced for PGFM concentrations ranging from 32.5 pg/ml to 8000 pg/ml. The intra- and inter-assay coefficients of variation were, on average, 7.6% and 10.4%, respectively.

The concentrations of PGE_2 in the blood samples and in the culture medium were determined by a direct EIA test as described previously (37). The anti-PGE_2 serum was donated by Dr. S. Ito, Kansai Medical University in Osaka, Japan. Cross-reactivities of the anti-PGE_2 serum, determined by measuring the inhibition of binding of peroxidase-labeled PGE_2 to this antiserum, were as follows: PGE_2, 100%; PGE_1, 18%; PGJ_2, 14%; PGA_1, 10%; 15-keto PGE_2, 8.8%; PGB_2, 6.7%; PGA_2, 4.6%; PGD_2, 0.13%, and $PGF_{2\alpha}$, 2.8%. For blood plasma samples, the PGE_2 standard curve was produced for PGE_2 concentrations ranging from 0.07 ng/ml to 20 ng/ml. For medium samples, the standard curve was produced for PGE_2 concentrations ranging from 0.39 ng/ml to 100 ng/m. The intra- and interassay coefficients of variation were, on average, 6.9% and 9.7%, respectively.

The concentration of $PGF_{2\alpha}$ in the culture medium was determined with the direct EIA test as described previously (38). The $PGF_{2\alpha}$ standard curve ranged from 0.016 ng/ml to 4 ng/ml. The intra- and interassay coefficients of variation were, on average, 7.1% and 11.3%, respectively.

Statistical Analysis. Least squares means and SEM were determined in the preliminary *in vivo* observations. Differences in biochemical indexes in the blood were analyzed using Student's *t* test (GraphPad PRISM Version 4.00; GraphPad Software, San Diego, CA). The differences of mean number of cover and the percentage of the pregnancy were analyzed using an X2 test (GraphPad PRISM). Plasma concentrations of hormones in Experiment 2 were analyzed using ANOVA with repeated measures (GraphPad PRISM). Prostaglandins (PGFM, PGE_2) and P4 in the jugular plasma samples, collected during the estrous

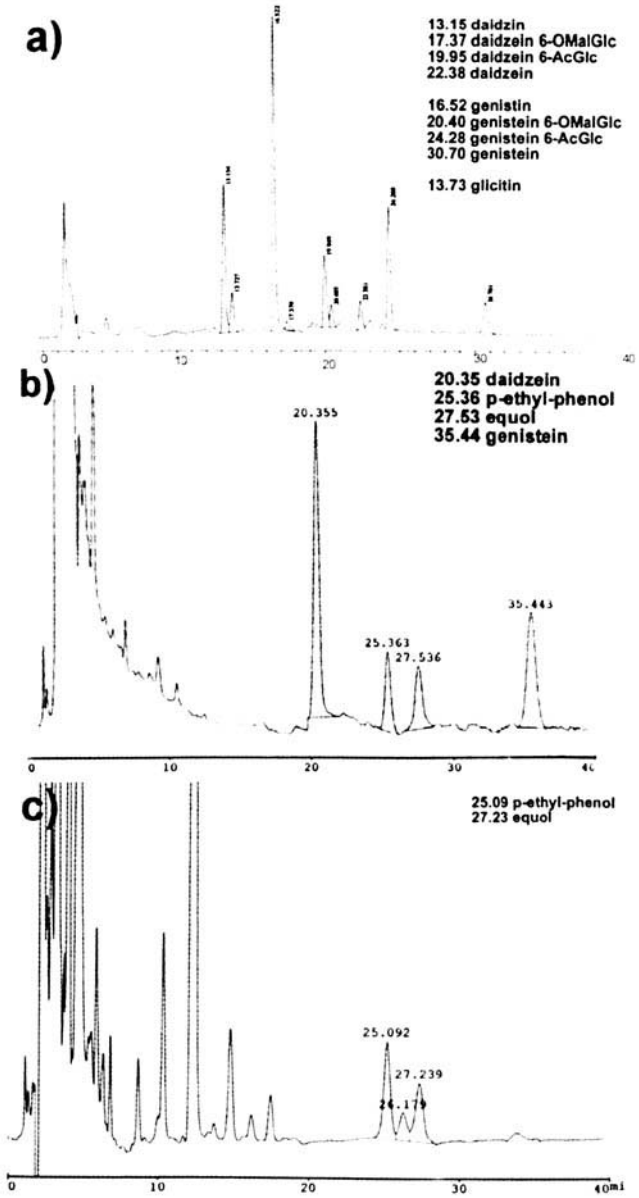

Figure 1. ECD-high–performance liquid chromatography (HPLC) chromatograms: phytoestrogens in the soybean–containing fodder (a), phytoestrogens standards used in HPLC analysis (b), phytoestrogens in the plasma of cows fed with silage with soybean (c).

cycle, were analyzed with a repeated measures design approach with treatments (soy-been fed *vs.* control heifers) and time of sample collection (days of the cycle) being fixed effects, with all interactions included. All analyses were done using repeated measures ANOVA tests followed by Bonferroni's multiple comparison test (GraphPad PRISM; $P < 0.05$ was considered significant). The obtained data from the *in vitro* experiments (the concentrations of PGE_2 and $PGF_{2\alpha}$ in the cultured media) were exposed as the mean ± SEM of values obtained in 3–4 separate experiments, each performed in triplicate. The statistical significance of differences between controls and treated groups was assessed by one-way ANOVA, followed by the Dunnett comparison test (GraphPad PRISM).

Results

Experiment 1. *Effects of soy-derived phytoestrogens on reproductive efficiency.* The total phytoestrogen content of fodder used to feed cows from Herd W was approximately 1900 μg/g (Fig. 1b). The phytoestrogens consisted mainly of daidzein and genistein glucosides. The same phytoestrogens were found in the fodder used to feed cows from Herd Z, but the total phytoestrogen content was smaller than 300 μg/g. The cows fed soybeans (Herd W) had significant levels of p-ethyl-phenol and equol in urine (365 ± 67 μ*M* and 160 ± 24 μ*M*, respectively) and in the plasma (1.6 ± 0.31 μ*M* and 1.2 ± 0.28 μ*M*, respectively; Fig. 1c). In contrast, no phytoestrogens were detected in urine or plasma of cows fed the standard diet (Herd Z). The phytoestrogen metabolites present in plasma and urine formed glucuronide and/or sulfate conjugates.

Blood analyses and other characteristics of Herds Z and W are compared in Table 2. One year after the beginning of observations, the pregnancy rate of cows fed the standard diet (100%) was significantly greater ($P < 0.01$) than that of cows fed the diet based on soybeans (60%). The mean number of cover for experimental cows (Herd W) differed significantly from the mean number of cover for control cows (4.0 ± 0.78 *vs.* 2.2 ± 0.33; $P < 0.01$). Moreover, plasma concentrations of total cholesterol, protein, and ALT activity were higher in the cows fed with soybeans (Herd W) compared with cows fed with standard diet ($P < 0.05$).

Experiment 2. *Effect of soy-derived phytoestrogens on prostaglandin secretion during early pregnancy.* In the serum of soybean-fed heifers, only the

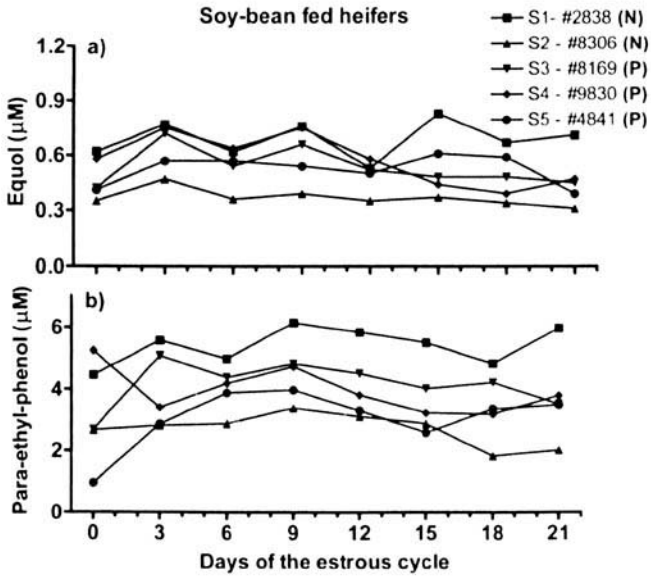

Figure 2. Concentration of equol (a) and para-ethyl-phenol (b) in the peripheral blood plasma of heifers fed with soybean (2.5 kg soybean/animal/day; $n = 5$) at 21 days after artificial insemination.

Table 2. Effect of Different Feeding Schemes, Standard Diet (Herd Z) and Diet with Soybean (Herd W), on the Mean Number of Cover, Percent of Pregnancy, Glucose, Cholesterol, Phosphorus (P^{-}), Magnesium (Mg^{2+}), Calcium (Ca^{2+}), Total Protein Concentrations, and Activity of Alanine Aminotransferase (ALT), Asparagine Aminotransferase (AST), Alkaline Phosphatase (ALP) in Serum[a]

Characteristic	Herd Z (control) x ± SEM (n = 10)	Herd W (soy diet) x ± SEM (n = 12)
Number of cover	2.2 ± 0.33	4.0 ± 0.78**
Percent of the pregnancy	100	60**
Glucose (m*M*)	3.139 ± 0.13	3.384 ± 0.11
Cholesterol (mg/L)	925.4 ± 76.3	1390 ± 153.7*
P^{-} (mg/L)	57.14 ± 3.9	57.62 ± 2.6
Mg^{2+} (m*M*)	10.19 ± 0.6	9.600 ± 0.3
Ca^{2+} (mg/L)	86.58 ± 2.2	89.74 ± 5.6
Total protein (g/L)	73.59 ± 1.42	94.13 ± 4.23***
ALT (U/L)	14.93 ± 1.89	25.50 ± 2.22**
AST (U/L)	72.21 ± 5.33	67.08 ± 4.32
ALP (U/L)	48.01 ± 3.69	51.86 ± 3.93

[a] Asterisks indicate significant differences between two herds among one row (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), as determined by X2 test (no. of cover and percentage of pregnancy) and by Student's t test.

conjugated forms of p-ethyl phenol and equol were found in high concentration (Fig. 2). Phytoestrogens and their metabolites in the blood of control animals were almost undetectable. Only p-ethyl-phenol was found in the blood of the control animals, and its concentration was low (0.45 ± 0.078 μ*M*; data not shown). The pregnancy rates of the heifers fed the high-soy diet (3/5) and the heifers fed the standard diet (4/5) were not significantly different as determined by the presence of CL in the ovary and a P4 concentration higher that 2 ng/ml at Day 21 after insemination (Fig. 3). The concentrations of PGFM in the blood of heifers fed soybeans were significantly higher throughout the experimental period than those in the control heifers ($P < 0.01$; Fig. 4). The higher levels of PGFM were positively correlated with equol and p-ethyl phenol concentrations in the blood ($P < 0.001$). The differences of PGE_2 concentrations between the two groups of heifers were not significantly different (Fig. 5; $P > 0.05$). PGE_2 concentrations in both examined groups of heifers increased from Day 8 through Day 12 of the experiment from 0.49 ± 0.085 ng/ml to 1.634 ± 0.35 ng/ml, remaining at this high level in pregnant animals and decreasing to 0.43 ± 0.04 ng/ml in nonpregnant heifers (data not shown).

Experiment 3. *Determination of the effective dose of the soy-derived phytoestrogens in vitro.* Both daidzein and genistein and their metabolites, equol and p-ethyl-phenol, stimulated $PGF_{2\alpha}$ secretion by bovine endometrium

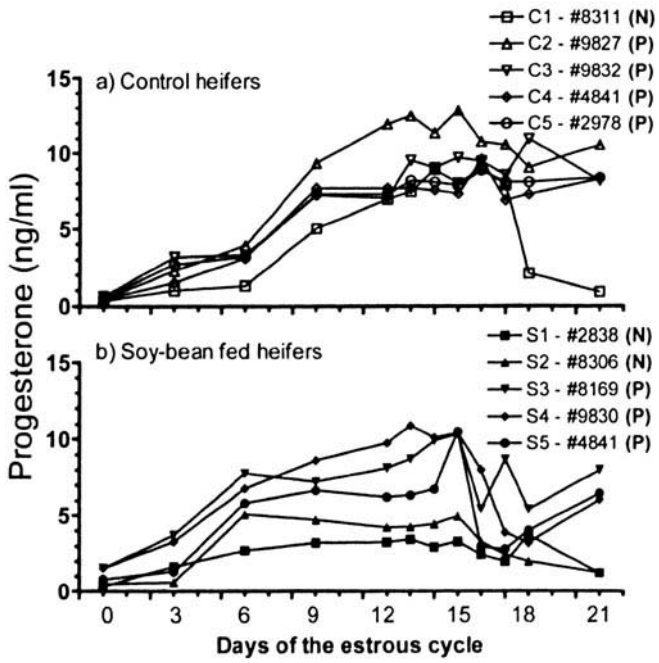

Figure 3. Concentration of progesterone in the peripheral blood plasma of heifers fed with standard diet (a) and 2.5 kg/day/animal of soybean (b).

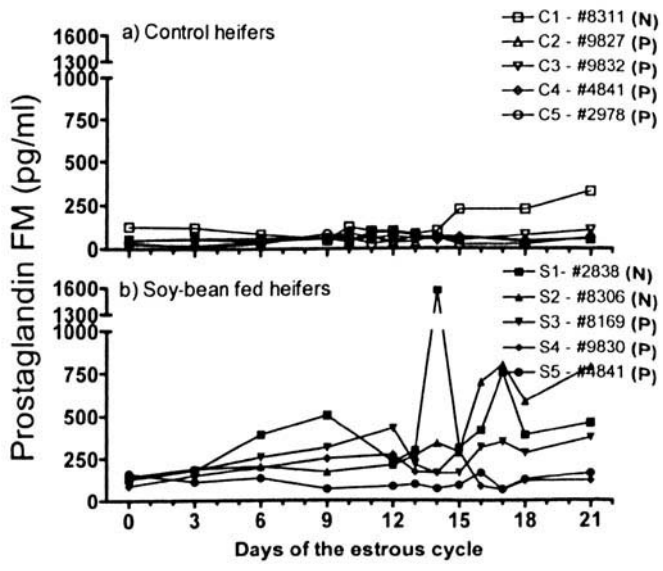

Figure 4. Concentration of a metabolite of prostaglandin $F_{2\alpha}$-13,14, keto $PGF_{2\alpha}$ (PGFM) in the blood plasma of heifers fed standard diet (a) and 2.5 kg/day/animal of soybean (b).

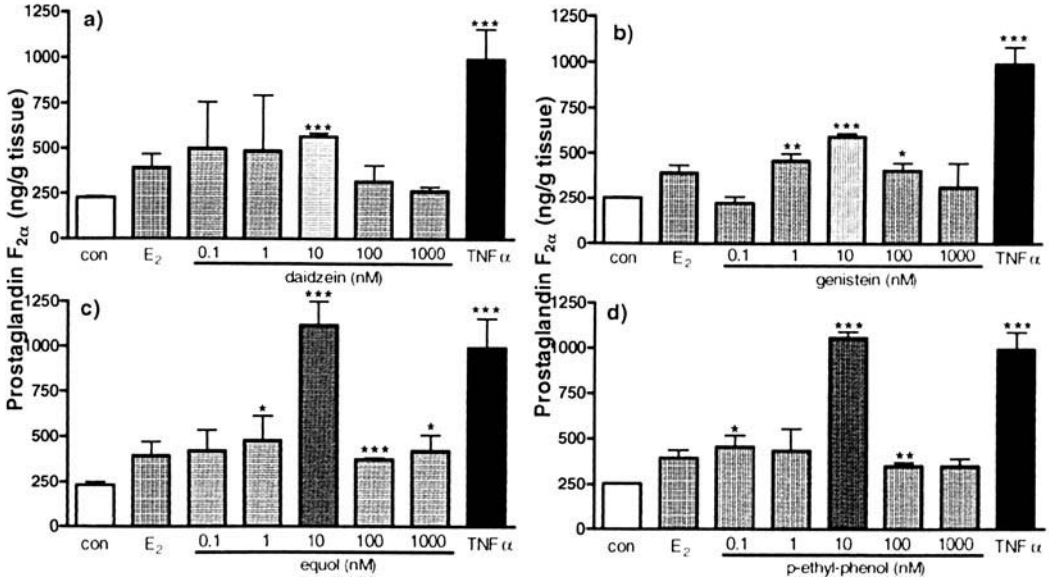

Figure 5. Effects of various concentrations (0.1–1000 n*M*) of phytoestrogens and their metabolites on $PGF_{2\alpha}$ production by bovine endometrial slices. Daidzein (a), genistein (b), equol (c), and p-ethyl-phenol (d), estradiol-17β (E_2; 1 n*M*) or tumor necrosis factor-α (TNFα; 0.06 n*M*) were added to the medium for 24 hr after 1 hr of preincubation. Asterisks indicate significant differences between control and treated groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), as determined by one-way ANOVA followed by the Dunnett comparison test.

during the early Stage I ($P < 0.01$; Fig. 5). The concentration of four compounds that gave the most significant increase ($P < 0.001$) was 10 n*M*. Therefore, a concentration of 10 n*M* was chosen for further studies.

Experiment 4. *Effect of soy-derived phytoestrogens on prostaglandin output at different stages of the estrous cycle in vitro.* Phytoestrogens and their metabolites stimulated the secretion of $PGF_{2\alpha}$ (Fig. 6) and PGE_2 (Fig. 7) in different stages of the estrous cycle in the bovine endometrium. $PGF_{2\alpha}$ output was strongly stimulated (approximately 6-fold), whereas PGE_2 output was stimulated by only about one third this amount (Table 3).

Discussion

We found large amounts of daidzein and genistein in the soybean commonly used for feeding dairy cattle. These phytoestrogens occur in plants as glycosides and are hydrolyzed in the rumen by microorganisms (40). Daidzein is metabolized in the rumen to equol, whereas genistein is metabolized to p-ethyl-phenol (35). High concentrations of both of these metabolites were found in plasma and urine. Cattle fed the high-soy diet (Herd W) had higher serum concentrations of cholesterol, protein, and ALT than cattle fed the diet without soybean (Herd Z). These high concentrations may be due to the high total fat and protein content in the soy diet. However, all the serum constituents that were examined were in their normal physiological ranges, showing that the examined animals were in proper condition without any metabolic disorders.

The high-soy diet decreased the pregnancy rate. This confirms the findings of many other studies that phytoestrogens can disturb reproductive processes on many different regulatory levels (reviewed in 41). Phytoestrogens can inhibit hypophyseal luteinizing hormone secretion (42). This causes a decrease of progesterone production, which in

Table 3. Effects of Equol and p-Ethyl-Phenol on the $PGF_{2\alpha}$ and PGE_2 Output in Endometrial Slices in Various Phases of the Estrous Cycle (Fold of Stimulation); Fold of Stimulation Was Defined on the Basis of Data from the Saline-Treated Slices (Control) in Each Phase of the Estrous Cycle; the Data are Shown as the Mean ± SEM from Four Experiments

	Equol		p-Ethyl-phenol	
Phase of the estrous cycle	$PGF_{2\alpha}$	PGE_2	$PGF_{2\alpha}$	PGE_2
Early luteal I	4.4 ± 0.51^a	1.7 ± 0.16^b	4.3 ± 0.5^a	1.8 ± 0.17^b
Early luteal II	7.3 ± 0.72^a	1.2 ± 0.12^b	5.9 ± 0.58^a	1.1 ± 0.11^b
Midluteal	9.5 ± 0.38^a	2.5 ± 0.13^b	7.9 ± 0.49^a	2.2 ± 0.12^b
Late luteal	4.3 ± 0.43^a	1.8 ± 0.17^b	3.1 ± 0.31^a	1.6 ± 0.16^b
Follicular	7.15 ± 0.73^a	1.6 ± 0.16^b	5.8 ± 0.68^a	1.3 ± 0.13^b

^{a,b} Different superscript letters indicate significant differences ($P < 0.05$), as determined by one-way ANOVA followed by the Dunnett comparison test.

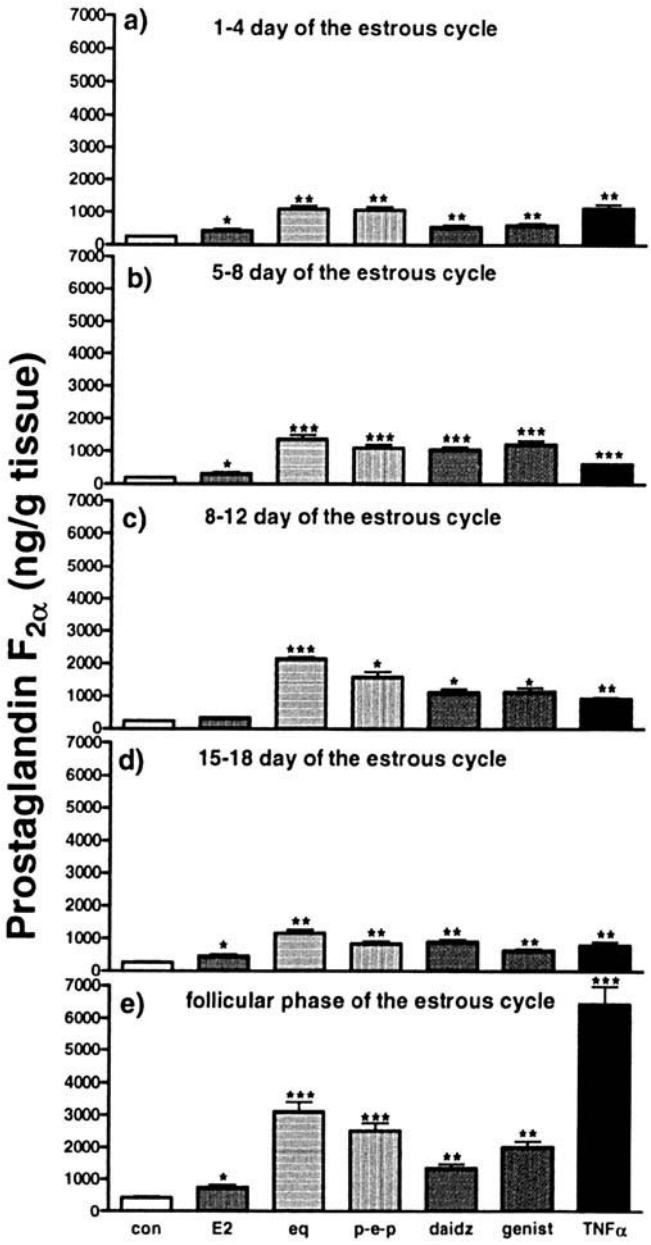

Figure 6. Effects of equol (eq), p-ethyl-phenol (p-e-p), daidzein (daidz), and genistein (genist) on $PGF_{2\alpha}$ output from the cultured bovine endometrium at (a) early luteal phase I (Days 1–4 of the estrous cycle), (b) early luteal phase II (Days 5–8 of the estrous cycle), (c) midluteal phase (Days 8–12 of the estrous cycle), (d) late luteal phase (Days 15–18 of the estrous cycle), and (e) follicular phase (Days 18–21 of the estrous cycle). Phytoestrogens and their metabolites (10 n*M*), E_2 (1 n*M*) and TNFα (0.06 n*M*) were added 24 hr before the end of culture. Asterisks indicate significant differences between control and treated groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), as determined by one-way ANOVA followed by the Dunnett comparison test.

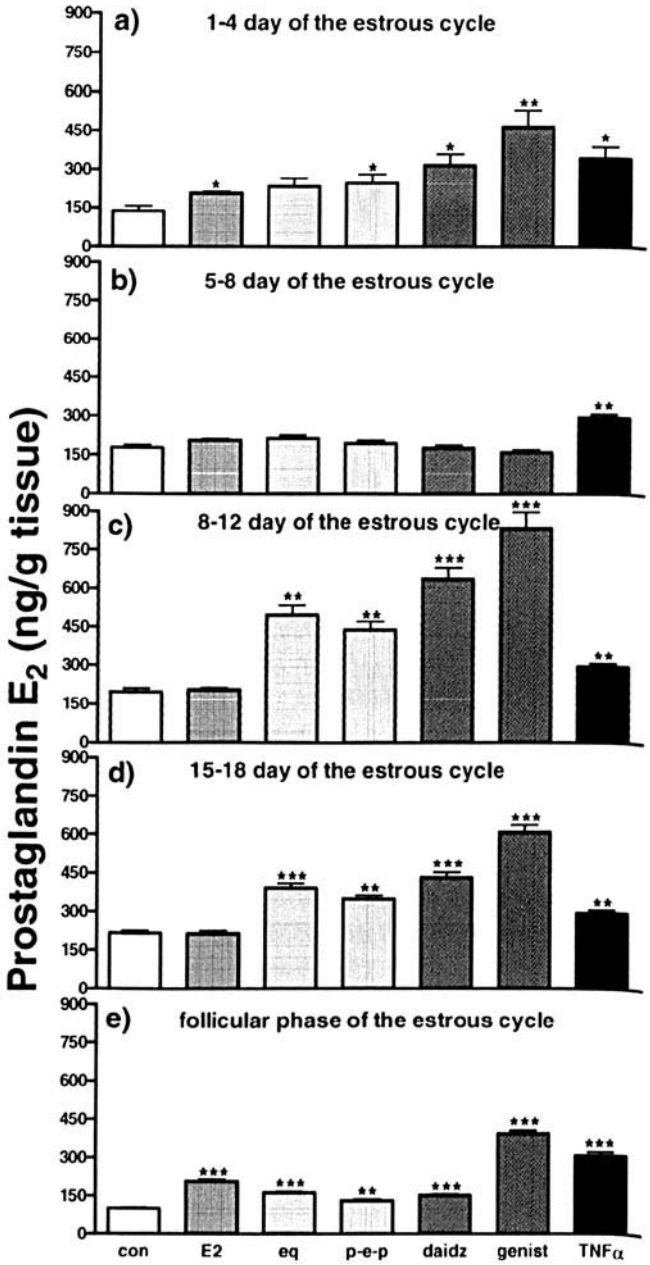

Figure 7. Effects of equol (eq), p-ethyl-phenol (p-e-p), daidzein (daidz), and genistein (genist) on PGE_2 output from the cultured bovine endometrium at (a) early luteal phase I (Days 1–4 of the estrous cycle), (b) early luteal phase II (Days 5–8 of the estrous cycle), (c) midluteal phase (Days 8–12 of the estrous cycle), (d) late luteal phase (Days 15–18 of the estrous cycle), and (e) follicular phase (Days 18–21 of the estrous cycle). Phytoestrogens and their metabolites (10 n*M*), E_2 (1 n*M*), and TNFα (0.06 n*M*) were added 24 hr before the end of culture. Asterisks indicate significant differences between control and treated groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$), as determined by one-way ANOVA followed by the Dunnett comparison test.

turn leads to high abortion rate (43). The decrease of pregnancy rate can also be attributed to phytoestrogen-dependent inhibition of endogenous estrogens production leading to disturbances in follicle development and lack of estrus (4, 20). Therefore, phytoestrogens may disturb estrus and ovulation through their effects on the central nervous system. Moreover, high concentrations of estrogenic substances on the day of insemination can be associated with early embryonic loss (44). High productivity of dairy cows may lead to a higher insemination rate (45). A high protein diet often reduces reproductive efficiency in cows (46). We hypothesize that phytoestrogens decrease fertility in cows

by modulating the production of prostaglandins. As shown in Experiments 1 and 2, the high-soy diet significantly decreased the rate of successful pregnancies as well as increased the mean insemination rate and PGFM concentration in the serum of soy-fed animals.

Phytoestrogens inhibit the binding of (H^3)-E_2 or (H^3)-organon to their respective receptors, but the relative affinities of (H^3)-E_2 and (H^3)-organon are lower than those of E_2 (20, 47, 48). The affinities of phytoestrogens for estrogen receptors are only 0.1% to 1% of those of circulating estrogens (E_2 or estrone) (1, 23, 49). Thus, the many biological effects attributed to phytoestrogens may be due to their relatively high concentrations. The p-ethyl-phenol and equol concentrations that we detected in plasma of cows fed soybean (1.6 ± 0.31 μ*M* and 1.2 ± 0.28 μ*M*, respectively) were more than a thousand times greater than the concentrations of endogenous E_2 (1–10 n*M*) (50). These high concentrations may compensate for the much weaker affinity of phytoestrogens for estrogen receptors (47). It has been previously shown that the concentrations of phytoestrogens in plasma of pregnant women consuming soybeans are over 1000 times higher than E_2 concentrations and 10,000–100,000 higher than E_2 concentrations during the menstrual cycle (1, 17, 24). The phytoestrogen concentrations used in the present *in vitro* study (10 n*M*) were lower than the concentrations of conjugated phytoestrogens found in the blood plasma (1.6 ± 0.31 μ*M* and 1.2 ± 0.28 μ*M*). About 5%–0% of all phytoestrogens in bovine plasma are in the free form (34, 40). The concentrations of free phytoestrogens and their metabolites were below the detection limit of our HPLC system. Thus, the concentrations of phytoestrogens and their metabolites that we used in the present *in vitro* study were based on the free, unconjugated daidzein (0.2–0.4 n*M*) and equol (4–8 n*M*) found by Lundh *et al.* (34, 35) in plasma of cows fed with moderately estrogenic silage. In our *in vitro* experiments, phytoestrogen metabolites (equol and p-ethyl-phenol) turned out to be much more potent disruptors than the original phytoestrogens themselves. The stronger effects of the metabolites may be due to their higher affinities for estrogen receptors than original phytoestrogens. This hypothesis is supported by findings of other authors (reviewed in 2, 47) who showed that phytoestrogen metabolites are about 100%–150% more active than environmental estrogens.

Phytoestrogens derived from soybean and their metabolites stimulated $PGF_{2\alpha}$ and PGE_2 production in the cultured bovine endometrium at different stages of the estrous cycle. However, the strongest effects of phytoestrogen metabolites (p-ethyl-phenol and equol) on $PGF_{2\alpha}$ secretion were observed in the phases of the estrous cycle (early II and midluteal; Table 3). Prostaglandin E_2 and $PGF_{2\alpha}$ are crucial for proper development and maintenance of the CL. The maintenance of CL and P4 production is regulated by several luteotropic factors, including PGE_2 (51). Stimulation of $PGF_{2\alpha}$ production disturbs the ratio PGE_2 to $PGF_{2\alpha}$. Proper $PGF_{2\alpha}$ to PGE_2 ratio is important for the maternal recognition of pregnancy, for maintaining the function of CL, and embryo implantation and development (30, 52). A strong stimulation of $PGF_{2\alpha}$ production by phytoestrogens or their metabolites can lead to a disturbance (*i.e.*, an increase) of this ratio that may interfere with early embryo development and implantation. During embryo development and implantation the $PGF_{2\alpha}$ to PGE_2 ratio should decrease. This relaxes the blood vessels and increases blood flow in the uterus, which prepares it for embryo implantation (31). The decreased $PGF_{2\alpha}$ to PGE_2 ratio also stimulates P4 synthesis (53). In our study, phytoestrogens and their metabolites greatly increased $PGF_{2\alpha}$ production and moderately but significantly increased PGE_2 production. These changes may interfere with embryo implantation in the uterus. Because $PGF_{2\alpha}$ has a direct and negative effect on bovine embryo development *in vitro* (54), the strong stimulation of $PGF_{2\alpha}$ production compared with PGE_2 production that we observed (6–7 times greater; Table 3) may be one of the reasons for the early embryo mortality or abortion.

Phytoestrogens and their metabolites also strongly stimulated $PGF_{2\alpha}$ output from the bovine endometrium at the late luteal and follicular phases of the estrous cycle. Stimulation of $PGF_{2\alpha}$ secretion by estrogenic-like substances during luteolysis (*i.e.*, during the late luteal phase and follicular phase) may accelerate the positive feedback loop between $PGF_{2\alpha}$ and other regulators of luteolysis, such as oxytocin (OT) (26, 30, 52, 55). Estradiol-17β increases OT-stimulated $PGF_{2\alpha}$ production in cultured bovine endometrial cells (32) as well as amplifies the stimulatory effect of OT on endometrial $PGF_{2\alpha}$ synthesis (56). Additionally, gonadal steroids upregulate OT gene expression in the hypothalamus and upregulate OT receptors in the uterus; thus, they can alter the frequency of the central OT pulse generator, leading to the pulsatile $PGF_{2\alpha}$ output from the endometrium during luteolysis in ruminants (30, 57). Therefore, if phytoestrogens and their metabolites act like endogenous estrogens, especially in nonpregnant animals, at the time of luteolysis and ovulation, they may amplify the mechanisms that return the cow to cyclicity after labor.

In view of the structural and functional similarity of phytoestrogens, their metabolites, and endogenous estrogens, we presume that plant-derived estrogens that are present in the plasma of cows fed soybean modulate PG synthesis in the bovine endometrium at the enzyme level. Thus, phytoestrogens and their metabolites, like endogenous estrogens, may influence the activity and expression of key enzymes taking part in the PG synthesis (26, 58): cyclooxygenase-2 (COX-2) (32, 59), PG synthases (60), and even PGE-9-keto-reductase, which converts PGE_2 to $PGF_{2\alpha}$ (61, 62). Our previous *in vitro* study (63) revealed that phytoestrogens and their metabolites affected the expression of COX-2 and PGE synthase in cultured bovine stromal and epithelial cells of the endometrium. Equol and p-ethyl-phenol strongly stimulated PGF synthase expression at the protein level in the epithelial cells (63), which are the

main source of luteolytic $PGF_{2\alpha}$ production in the bovine endometrium (32, 62). Thus, phytoestrogens may reduce the PGE_2 to $PGF_{2\alpha}$ ratio during the estrous cycle and pregnancy. Reduction of the PGE_2 to $PGF_{2\alpha}$ ratio would modulate the expression of key enzymes taking part in PG synthesis in the bovine endometrium.

The presented data provide a possible explanation of how soybean-derived phytoestrogens and their metabolites act as endocrine disruptors, leading to disruption of the reproductive processes and to temporal infertility of cows. Phytoestrogens and their active metabolites disrupt the ratio PGE_2 to $PGF_{2\alpha}$, which leads to the nonphysiological production of luteolytic agent in cattle during the estrous cycle and pregnancy. Therefore, phytoestrogen-dependent inhibition of the PGE_2 to $PGF_{2\alpha}$ ratio might be the reason for the early embryo mortality that occurs with cows fed a high-soy diet.

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