The Effect of Soybean-Derived Phytoestrogens on Concentrations of Plasma Isoflavones, 15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$ and Progesterone in Dairy Cows

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Abstract

The objective of the study was to determine the effect of soybean-derived phytoestrogens and their metabolites on the activity of sex hormones during the oestrous cycle in multiparous lactating dairy cows. The experiment was carried out on 4 multiparous lactating Holstein cows in the form of replicated Latin square in double reversal design. The experiment in the total length of 168 days was divided into 4 periods of 42 days, each consisting of a 21-day preliminary period and a 21-day collecting period. Cows were divided into 2 groups of 2 cows. The control group (C) was fed a diet based on extruded rapeseed cake while the experimental group (S) was fed a diet containing extruded full-fat soya. The intake of total isoflavones was 3297 mg/d in S and 58.0 mg/d in C ($P < 0.001$). The concentrations of individual isoflavones, it is daidzein, genistein and equol in plasma were significantly higher in the experimental group S (49.3, 78.7 and 218.8 ng/ml, respectively) than in the control group C (13.5, 42.9 and 18.3 ng/ml, respectively, $P < 0.001$). Plasma concentration of progesterone throughout the oestrous cycle was not influenced by the diet used ($P > 0.05$). Plasma concentration of prostaglandine PGFM throughout the oestrous cycle in the experimental group (S) tended to be higher ($P = 0.095$) than in the control group (C). No differences in the length of the oestrous cycle between the cows fed different diets were observed.

Isoflavones, prostaglandins, bovine, oestrous cycle

Soybeans or soybean products are used in diets of high yielding dairy cows, especially during early lactation, as an excelent source of energy and high-quality protein (Chouinard et al. 1997). Nevertheless, soybeans are known as the major dietary source of phytoestrogens (Bingham et al. 1998). Phytoestrogens may induce various pathologies in the female reproductive tract, such as disorders in the ovarian and uterine function (Rosselli et al. 2000) resulting in disruption of the reproductive processes, decreased fertility or even temporal infertility in cows (Woclawek-Potocka et al. 2005b). Recently, several studies have been conducted to evaluate the effect of soybean phytoestrogens on the bovine reproductive tract, oestrous cycle and hormonal profile of fertile cows (e. g. Woclawek-Potocka et al. 2005ab, 2006, 2008; Piotrowska et al. 2006).

In ruminants, endogenous estrogens are known to control the oestrous cycle by influencing prostaglandin synthesis (Goff 2004). Furthermore, in bovines, prostaglandine $PGF_{2\alpha}$ is the major luteolytic agent (McCracken et al. 1999), whereas prostaglandine PGE_2 has luteoprotective and antiluteolytic properties (Kennedy 1983; Asselin et al. 1996). Optimal $PGF_{2\alpha}$ to PGE_2 ratio is essential for endometrial receptivity, maintenance of corpus luteum (CL), and progesterone (P_4) secretion (Milvae et al. 1996). Although it has been recently shown that soy-bean derived phytoestrogens regulate both $PGF_{2\alpha}$ and PGE_2 secretion (Woclawek-Potocka et al. 2005b), in the subsequent study of Woclawek-Potocka et al. (2005a) it has been demonstrated that phytoestrogens preferentially

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stimulated $PGF_{2\alpha}$ synthesis in the epithelial cells of bovine endometrium. Based on the stronger phytoestrogen-dependent stimulation of $PGF_{2\alpha}$ compared to PGE_2 production in epithelial cells, it appears that phytoestrogens and their metabolites mainly modulate the $PGF_{2\alpha}$ to PGE_2 ratio that cause premature luteolysis.

The objective of the study was to determine the effect of soybean-derived phytoestrogens and their metabolites on the activity of sex hormones during the oestrous cycle in multiparous lactating dairy cows.

Materials and Methods

Animals and diets

The experiment was carried out on 4 high-yielding lactating Holstein cows (lactation 3–4, week 16–46 of lactation) with similar milk production (27.3 kg ± 1.7) that were divided into 2 groups according to milk yield. The control group of animals was fed a diet based on extruded rapeseed cake (C); the experimental group was fed a diet based on extruded full-fat soya (S). The experiment was carried out in the form of replicated Latin square in double reversal design (Tempelman 2004). The trial in the total length of 168 days was divided into 4 periods of 42 days. Each period consisted of a preliminary period (21 d) and a collecting period (21 d). Before the beginning of the experiment, the oestrus was synchronized by application of $PGF_{2\alpha}$ (Oestrophan, Bioveta a. s., CR). The day of oestrus was considered to be day 0 of the collecting period. The length of oestrous cycle was determined on the basis of signs of the next oestrus and by the veterinarian via rectal examination. The animals were under regular veterinary rectal examination during the whole experiment.

Sampling and analysis

Cows were fed individually twice daily (6.30 and 16.30 h) the diet based on maize silage, meadow hay and supplemental mixture (Table 1). Samples of feed and feed refusals were taken twice a week in the collecting periods and used for the determination of the content of basal nutrients and phytoestrogens (daidzein, genistein). Dry matter (DM) was determined by drying at 103 ºC for 4 h. The content of PDIN, PDIE (digestible protein in the intestine when rumen fermentable N supply or energy supply are limited) and NEL (net energy of lactation) was calculated according to Sommer (1994). The contents of daidzein and genisten in feed and feed refusals were analysed using HPLC-DAD as given in details below.

Blood samples were taken into heparinised tubes from vena jugularis after morning milking (8:00 h) during the collecting period three times a week. The blood plasma was separated by centrifuge (1500 × *g* for 15 min, 4 °C) and stored at -20 °C until analyses. For determination of prostaglandine PGFM (15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$) in blood plasma, prostaglandine stabiliser was added to each tube with blood and mixed thoroughly prior centrifugation. The content of phytoestrogens and their metabolites was analysed using HPLC-MS/MS. The level of P_4 in blood plasma was measured by imuno-chemiluminescence method in automatic analyser IMMULITE (DPC).

Table 1. Composition of diet in g/kg of dry matter

Component	C[1]	S[1]
Maize silage	465	484
Meadow hay	79	82
Supplemental mixture	456	434
Total	1000	1000
Composition of supplemental mixture		
Sugarbeet chippings	145	153
Barley	265	293
Oat	269	297
Rapeseed oil	12	–
Extruded rapeseed cake	258	–
Protex (extruded full-fat soya)	–	206
Premix (sum)[2]	51	51
Total	1000	1000

[1] C – control group fed a diet containing extruded rapeseed cake, S – experimental group fed a diet containing extruded full-fat soya

[2] Premix contains (g/kg in supplemental mixture): sodium chloride (NaCl) 6; dicalciumphosphate (DCP) 18; limestone ($CaCO_3$) 16; sodium bicarbonate ($NaHCO_3$) 1; monosodiumphosphate (MSP) 2; magnesiumphosphate (MgP) 2; microelements and vitamin mixture 6

The PGFM concentration in blood plasma was determined with an EIA using HRP-labeled PGFM and anti-PGFM serum (Skarzynski et al. 2003).

Determination of phytoestrogens and their metabolites

Contents of target compounds were determined after their releasing from bonded forms. Hydrolysis of glycosides was carried out by acidic hydrolysis from feed samples and by enzymatic hydrolysis from biotic samples. HPLC/DAD method was used for determination of phytoestrogens in plant materials and LC-MS/MS method was employed for determination of estrogenic compounds in biotic samples.

High purity standards of daidzein (≥ 98%) and genistein (≥ 95%) were purchased from

Sigma-Aldrich (Germany), eqoul (≥ 99%) and internal standard 4-hydroxybenzofenon (4-HBPE) (≥ 99%) were purchased from Fluka (Germany).

Feed samples

Homogenised samples of feed were hydrolysed with 6 mol/l hydrochloric acid and ethanol under the reverse condenser at the boiling point of ethanol. After hydrolysis the extract was clean up by SPE procedure on Oasis HLB, Waters (UK) cartridges.

The analytical column used for experiments was LichroCART LiChrospher 100 RP8 (250 × 4 mm, 5 μm) with analytical precolumns LichnoCART LiChrospher 100 RP8 (4 × 4 mm, 5 μm) (Merck, Germany). Mobile phase methanol and 1% acetic acid water solution (v/v) with gradient elution at a flow-rate 1 ml/min was used. The absorption maxima using for detection of total daidzein and genistein was 260 nm.

The HPLC analysis was carried out on an HP 1200 liquid chromatograph coupled with a diode array detector (DAD) (Hewlett Packard, USA).

The limit of detection for total isoflavones obtained under the described method was 3.5 mg/kg for daidzein and 2.8 mg/kg for genistein. The repeatability expressed as relative standard deviation (n = 6) was 4.1% and 4.9%, respectively.

Plasma samples

Target analytes were hydrolysed from possible conjugates by enzymatic hydrolysis with Helix pomatia enzyme β-glucuronidase/sulfatase in sodium acetate buffer at 37 °C. After hydrolysis the analytes were extracted by ethylacetate.

The analytical column used for experiments was Discovery C18, (150 × 3 mm, 5 μm) with analytical precolumns Discovery C18 Guard column (20 × 4 mm, 5 μm) (Supelco, Germany). Mobile phase methanol and 1% acetic acid water solution (v/v) with gradient elution at a flow-rate 0.7 ml/min was used. For MS/MS detection APCI at positive ionization mode was used with monitoring of transitions (m/z) 255.3 → 199.3 for daidzein, 271.4 → 215.3 for genistein, 243.1 → 123.1 for equol and 199.2 → 121.2 for 4-HBPE. Analytes were quantified by the method of internal standard.

Liquid chromatograph HP 1100, (Hewlett Packard, USA) coupled with mass spectrometry detector - ion trap, Finnigan LCQ Deca, (Finnigan, USA) operated in selected reaction monitoring (SRM) mode was used for analysis.

The limit of detection obtained under the described method was 2.5 ng/ml for daidzein and equol, and 5 ng/ml for genistein. The repeatability expressed as relative standard deviation (n = 6) was 5.0% for daidzein, 6.8% for genistein and 3.5% for equol in plasma samples.

Statistical analyses

Data obtained in the experiment were analysed using GLM procedure of the SAS/STAT, Version 8 according to the following model: $Y_{ijk} = \mu + T_i + C_j + D_k + (T \times C)_{ij} + \varepsilon_{ijk}$, where μ= general mean, T_i = treatment effect (i = 2), C_j = cow effect (j = 4), D_k = day (replication) effect (k = 8), $(T \times C)_{ij}$ = interaction effect, ε_{ijk} = residual error. Results are expressed as means with standard error of the mean (SE)

Results

The nutrient intake is presented in Table 2. The intake of DM, PDIN, PDIE and NEL did not differ significantly between groups ($P > 0.05$). Average concentration of total isoflavones (aglycones and glycones) daidzein and genistein determined in extruded full-fat soya was 150.9 mg/kg and 222.6 mg/kg, respectively, resulting in the average total isoflavones intake of 3297 mg/d in S. The concentration of total daidzein and genistein in extruded rapeseed cake was under the limit of detection of used analytical method. Low intake 58.0 mg/d of total isoflavones in C was found.

Concentrations of isoflavones and studied hormones in plasma are given in Table 3. The concentrations of daidzein, genistein and equol in plasma were significantly higher in S than in C ($P < 0.001$). Plasma concentration of prostaglandine PGFM throughout the experiment in S tended to be higher ($P = 0.095$) than that in C. The concentration of P_4 in the plasma in S did not differ significantly throughout the experiment in comparison to C ($P > 0.05$).

As shown in Fig. 1 higher plasma concentrations of daidzein were found in S ($P < 0.001$) compared to C. Similar tendencies between S and C were observed in concentration of genistein (Fig. 2). High plasma concentrations of equol were found in S ($P < 0.001$) compared to C with almost undetectable levels of equol (Fig. 3). The p-ethyl phenol in the plasma was not detected. The plasma concentrations of PGFM in S varied with the days of oestrous cycle

Table 2. The nutrient intake

Intake	Unit	C[1]	S[1]	SE[2]	*P*[3]
DM[4]	kg/d	18.9	18.9	0.28	0.883
PDIN[5]	kg/d	1.5	1.6	0.13	0.759
PDIE[5]	kg/d	1.6	1.7	0.09	0.811
NEL[6]	MJ/d	116	117	1.92	0.329
Daidzein	mg/d	31.6	1318.9	48.55	< 0.001
Genistein	mg/d	26.4	1978.1	69.91	< 0.001

[1] C – control group fed a diet containing extruded rapeseed cake, S – experimental group fed a diet containing extruded full-fat soya
[2] SE – standard error of the mean
[3] *P* – level of significance
[4] DM – dry matter
[5] PDIN, PDIE - digestible protein in the intestine when rumen fermentable supply of N or energy are limiting, respectively
[6] NEL - net energy of lactation

Table 3. Plasma concentration of isoflavones, prostaglandine PGFM and progesterone (P_4)

Component	Unit	C[1]	S[1]	SE[2]	*P*[3]
Daidzein	ng/ml	13.5	49.3	7.58	< 0.001
Genistein	ng/ml	42.9	78.7	10.75	< 0.001
Equol	ng/ml	18.3	218.8	11.13	< 0.001
PGFM	ng/ml	0.042	0.126	0.04	0.095
P_4	nmol/ml	6.834	8.45	0.77	0.504

[1] C – control group fed a diet containing extruded rapeseed cake, S – experimental group fed a diet containing extruded full-fat soya
[2] SE – standard error of the mean
[3] *P* – level of significance

with the highest concentration on Day 15 (0.349 ± 0.259 ng/ml, Fig. 4). The plasma concentration of PGFM in C varied only little throughout the experiment. As shown in Fig. 5 the plasma P_4 concentration increased in both of the examined groups of cows from Day 0 to 15 of the oestrous cycle, with the highest concentration on Day 15 being 9.883 ± 2.810 mmol/l in C and 11.930 ± 2.200 in S, respectively. Starting on Day 15 to 21 of the oestrous cycle the concentration of plasma P_4 declined in both groups. No differences in the length of the oestrous cycle between cows fed C and S diet (17.0 ± 0.87 days of the oestrous cycle and 17.3 ± 1.15 days, respectively) was found.

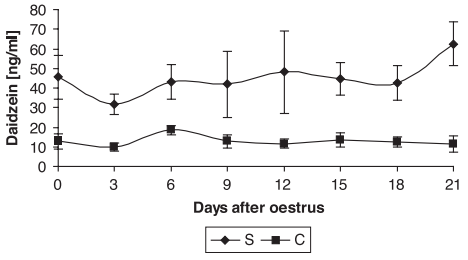

Fig. 1. Concentration of daidzein in the peripheral blood plasma of high-yielding lactating Holstein cows fed extruded full-fat soya diet (S) and extruded rapeseed-cake diet (C) during the oestrous cycle

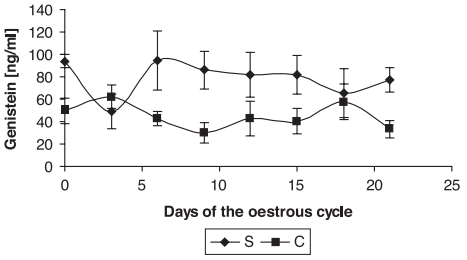

Fig. 2. Concentration of genistein in the peripheral blood plasma of high-yielding lactating Holstein cows fed extruded full-fat soya diet (S) and extruded rapeseed-cake diet (C) during the oestrous cycle

Discussion

Extruded full-fat soya used in this experiment contained 150.9 mg/kg of daidzein and 222.6 mg/kg of genistein. Thus, average total isoflavones intake in S was 3297 mg/d. Although the concentration of isoflavones in extruded rapeseed cake was under the limit of

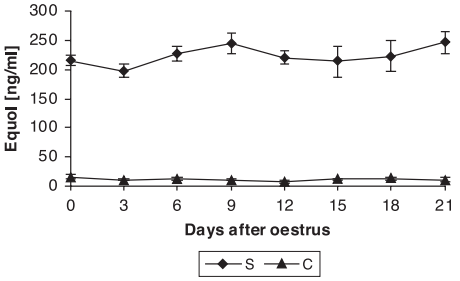

Fig. 3. Concentration of equol in the peripheral blood plasma of high-yielding lactating Holstein cows fed extruded full-fat soya diet (S) and extruded rapeseed-cake diet (C) during the oestrous cycle

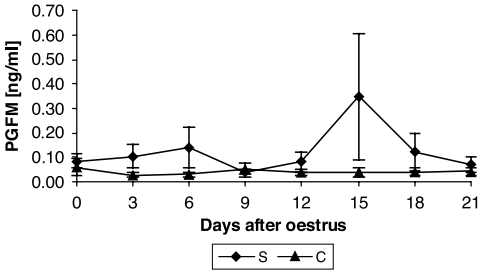

Fig. 4. Concentration of prostaglandin PGFM in the peripheral blood plasma of high-yielding lactating Holstein cows fed extruded full-fat soya diet (S) and extruded rapeseed-cake diet (C) during the oestrous cycle

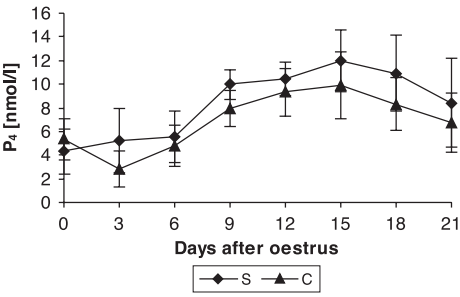

Fig. 5. Concentration of progesterone (P_4) in the peripheral blood plasma of high-yielding lactating Holstein cows fed extruded full-fat soya diet (S) and extruded rapeseed-cake diet (C) during the oestrous cycle

detection, low intake of total isoflavones (58.0 mg/d) in C diet was found. Low concentrations of isoflavones in control diets were also found in studies of Piotrowska et al. (2006) or Woclawek-Potocka et al. (2005b).

In the present experiment, cows fed S diet had significantly higher concentrations of daidzein, genistein and equol in plasma in comparison to C ($P < 0.001$), and p-ethyl-phenol was not detected. On the other hand, Woclawek-Potocka et al. (2005b) or Piotrowska et al. (2006) found significant concentrations of p-ethyl-phenol and equol in the plasma of cows fed soybeans but they detected neither daidzein nor genistein. In contrast to our findings, in the above mentioned studies no phytoestrogens or their metabolites in plasma of cows fed the control diet were detected. This discrepancy can be caused by differences in dynamics of isoflavones and their metabolites in the plasma after administration of soybeans. As described in the study of Woclawek-Potocka et al. (2008), daidzein concentration in plasma of cycling heifers increased after single dose of soybeans during 0.5 h ($P < 0.05$) and then remained constant until 2 h post feeding. Three hours after soybean feeding the daidzein concentration started to decrease to same levels as at the beginning of the experiment. Genistein concentration in blood plasma increased during 0.5 h after soybean feeding ($P < 0.05$) and then remained constant. Similarly, the equol concentration in the blood plasma was increasing up to 4 h after soybean administration ($P < 0.05$) and then remained at an approximately constant level until the end of the experiment. On the other hand, the p-ethyl phenol concentration in the blood plasma remained constant during first 3 h of the experiment ($P > 0.05$) and started to increase ($P < 0.05$), beginning at 4 h after feeding. Blood samples for determination of isoflavones and their metabolites in our experiment were taken approximately 1 h after feeding, thus our findings are in accordance with the above-mentioned study.

Endogenous estrogens (E2) are known to modulate the oestrous cycle in ruminants by influencing the synthesis of prostaglandins (Goff 2004), as demonstrated e.g. in the study of Zhang et al. (1991). E2 has been proved to stimulate cell viability and proliferation in the female reproductive tract of many species (Reynolds and Redmer 1998). Due to the fact that phytoestrogens are structurally similar to E2 (Middleton and Kandaswami

1986) and act as agonists or antagonists of endogenous E2 (Rosselli et al. 2000, Dubey et al. 2000), it can be assumed that they influence the same reproductive processes as regulated by E2. As already mentioned, in bovines, $PGF_{2\alpha}$ is the major luteolytic agent (McCracken et al. 1999) whereas PGE_2 exerts luteoprotective properties (Asselin et al. 1996). Thus, the development and maintenance of the CL whether during the oestrous cycle or for establishment of pregnancy depends on the optimum $PGF_{2\alpha}$: PGE_2 ratio (Milvae et al. 1996; Arosh et al. 2004). In the endometrium, the $PGF_{2\alpha}$ is synthetised mainly by the epithelial cells and PGE_2 by the stromal cells (Skarzynski et al. 2000; Asselin et al. 1996). In their recent *in vivo* and *in vitro* studies, Woclawek-Potocka et al. (2005a,b) have shown that soy-bean derived phytoestrogens regulate both PGE_2 and $PGF_{2\alpha}$ secretion in endometrium of cycling and early pregnant heifers or cows with preferential increases in $PGF_{2\alpha}$ secretion (Woclawek-Potocka 2005a,c). Furthermore, equol and p-ethyl-phenol, via the preferential decrease of the viability of stromal cells (Woclawek-Potocka 2005c), can negatively influence the production of PGE_2 and may cause additional disturbances in prostaglandins synthesis and disrupt the physiologic ratio between PGE_2 and $PGF_{2\alpha}$.

Due to the fact that $PGF_{2\alpha}$ is rapidly metabolised in the organism, a metabolite of 15-keto-13,14-dihydroprostaglandin $F_{2\alpha}$ (PGFM) was determined in blood plasma of cows in our experiment as described by Skarzynski et al. (2003). Plasma concentration of PGFM throughout the experiment in S tended to be higher ($P = 0.095$) than in C. This is in disagreement with Woclawek-Potocka et al. (2005b) who found significantly higher ($P < 0.01$) plasma concentrations of PGFM in soybean-fed heifers than in control animals. This discrepancy can be caused by a higher variability in PGFM concentrations determined in their study with the highest level reaching up to 1.6 ng/ml while the highest concentration of PGFM in our experiment with multiparous cows was 0.349 ng/ml. The plasma concentrations of PGFM in C varied only little throughout the experiment and were similar to those determined by Woclawek-Potocka et al. (2005b).

The concentration of P_4 in the plasma in S did not differ significantly throughout the experiment in comparison to C ($P > 0.05$). This is in agreement with Piotrowska et al. (2006) or Woclawek-Potocka et al. (2005b). Similarly to our findings, in both of the mentioned studies the P_4 concentrations increased from Day 0 to 12-15 and then started to decline. In the present study, no significant difference was detected between P_4 concentrations in S compared to C in dependence on days of oestrous cycle. On the other hand, Piotrowska et al. (2006) found that the P_4 concentration in soybean-fed animals on Day 15 and 18 of oestrous cycle was significantly lower than in standard diet-fed heifers.

In our experiment, the length of the oestrous cycle was not affected by the treatment ($P > 0.05$). This is in agreement with Piotrowska et al. (2006). On the other hand, the average length of the oestrous cycle in our experiment was shorter than that reported in the above-mentioned study. This shortage was probably caused by regular per rectum examinations that were used to examine the ovaries.

The length of the oestrous cycle in cattle is commonly 18-24 days with 21 days considered the average (Rioux and Rajotte 2004). Nevertheless, the intensity and duration of oestrous behaviours during the oestrous cycle is highly variable among individuals. Social interactions, housing and management factors, the physical environment impinging on the animal, nutritional status age and physiological state, genetic factors and presence of the bull can influence oestrus in cattle (Orihuela 2000). The oestrous cycle length in the cows in this experiment was probably influenced by the frequent interventions of a veterinarian (three times a week in the collecting period). Frequent rectal examinations could cause congestion of the endometrium and consequent shortening of the oestrous cycle in the cows.

In conclusion, the effect of soybean-derived phytoestrogens and their metabolites on the activity of sex hormones during the oestrous cycle was studied on multiparous (lactation

3–4) lactating dairy cows. Inclusion of extruded full-fat soya to their diet resulted in a significant increase in daidzein, genistein and, in particular, equol concentrations in blood plasma. Although these compounds tended to increase the plasma concentration of PGFM throughout the oestrous cycle ($P = 0.095$), plasma concentration of P_4 throughout the cycle was not influenced by the treatment. Further, no differences in the length of the oestrous cycle were observed. These data suggest that if cows are continuously exposed to a diet that include soya, phytoestrogen active metabolites may act chronically and locally on the reproductive tract.

Vliv sojových fytoestrogenů na koncentraci isoflavonů, 15-keto-13,14-dihydroprostaglandinu $F_{2\alpha}$ a progesteronu v plazmě dojnic

Cílem studie bylo stanovení vlivu sojových fytoestrogenů a jejich metabolitů na aktivitu pohlavních hormonů během estrálního cyklu u multiparních laktujících dojnic. Pokus byl proveden na čtyřech multiparních laktujících holštýnských dojnicích formou opakovaného latinského čtverce s „double reversal design“. Pokus v délce 168 dnů byl rozdělen do 4 period, každá v délce 42 dnů, složených z 21 dnů přípravného období a 21 dnů odběrového období. Dojnice byly rozděleny do 2 skupin. Kontrolní skupina (C) byla krmena krmnou dávkou obsahující extrudované řepkové pokrutiny zatímco pokusná skupina (S) byla krmena krmnou dávkou s obsahem extrudované plnotučné sóji. Celkový příjem izoflavonů byl 3297 mg/d u S a 58,0 mg/d u C ($P < 0,001$). Koncentrace jednotlivých izoflavonů, tj. daidzeinu, genisteinu a equolu v plazmě u S (tj. 49,3, 78,7 a 218,8 ng/ml v tomto pořadí) byla průkazně vyšší než u C (13,5, 42,9 a 18,3 ng/ml v tomto pořadí, $P < 0,001$). Koncentrace progesteronu v plazmě během estrálního cyklu nebyla pokusným zásahem ovlivněna ($P > 0,05$). Koncentrace prostaglandinu PGFM v plazmě během estrálního cyklu u S měla tendenci k vyšším hodnotám ($P = 0,095$) než u C. Rozdíly v délce estrálního cyklu mezi dojnicemi krmenými C a S dietou nebyly zjištěny.

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