Phytoestrogens reduce the effect of polychlorinated biphenyls on the contractility of bovine myometrium *in vitro*

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ABSTRACT: Proper regulation of uterine contractility, both spontaneous and oxytocin (OT)-amplified, is essential for successful fertilization and maintenance of gestation. Previously, we showed that polychlorinated biphenyls (PCBs), regarded as environmental pollutants, contributed to the increase in contractions of cyclic bovine uterus. In this paper, we hypothesized that phytoestrogens, plant-derived components, counteract the disruptive effects of PCBs. Therefore, we examined the *in vitro* effect (24–72 h) of coumestrol, daidzein, and genistein (10^{-6}, 10^{-5}M each) on spontaneous and OT-stimulated (10^{-7}M) myometrial strips contractions. Moreover, the effect (48 h) of coumestrol (10^{-6}M) or daidzein (10^{-5}M), jointly with PCBs (77, 100 ng/ml; 126, 153, 10 ng/ml), on myometrial contractions was registered. The myometrium was the most relaxed ($P < 0.05$) when treated with coumestrol, while the force of myometrial spontaneous contractions increased the most ($P < 0.05$) when treated with daidzein. Daidzein (10^{-5}M) also effectively increased the OT-stimulated force of contractions ($P < 0.01$). All phytoestrogens used affected the frequency and amplitude of myometrial contractions, though less markedly. Furthermore, coumestrol or daidzein reduced ($P < 0.05$) the spontaneous and OT-stimulated force of myometrial contractions, evoked by PCBs. Data suggest the effect of PCBs on myometrial contractility can be markedly reduced by some phytoestrogens.

Keywords: phytoestrogens; PCBs; oxytocin; myometrium; contractions; cattle

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants with various industrial applications, which were widely used in industry (Safe, 1992; Borja et al., 2005). However, their lipophilic properties and resistance to degradation (Borja et al., 2005) still tend to accumulate PCBs in the environment (Safe, 1992; Franek and Hruska, 2005) and in the tissues of living bodies (Clarkson, 1995). Exposure to PCBs is associated with early abortion in monkeys (Arnold et al., 1990) and premature parturition in human (Taylor et al., 1989). Moreover, PCBs increase the frequency of contractions of pregnant rat uteri (Tsai et al., 1996; Bae et al., 1999) and the force of contractions of the uteri in cyclic cows (Wrobel et al., 2005). Meanwhile, correct contractility of the uterine and oviduct muscle facilitates transport of sperm and oocyte, thereby enabling fertilization. The force of myometrial contractions is increased by oxytocin (OT) and prostaglandin (PG) $F_{2\alpha}$ (Huszar and Roberts, 1982; McKillen et al., 1999). The ability of OT to stimulate contractions of the myometrium rises around ovulation, because of an estradiol-dependent increase in the number of OT receptors (Johnson, 1992; Holda et al., 1996).

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PCBs can affect cells via estradiol (Andric et al., 2000; McLachlan, 2001) or aryl hydrocarbon receptors (Hahn, 1998; Andric et al., 2000), or via glucocorticoid receptors as found recently (Mlynarczuk and Kotwica, 2006). Also phytoestrogens, plant-derived compounds that resemble estradiol or synthetic estrogens (Kurzer and Xu, 1997; Moon et al., 2006), may activate estrogen receptors in the nucleus (Anderson et al., 1999). Although they have rather weak estrogenic activity (Setchell and Cassidy, 1999), when used in high concentrations, they may cause biological effects within the tissues of the reproductive tract (Slomczynska, 2004). Moreover, their effect is also dependent on the presence or absence of endogenous estrogens (Kurzer and Xu, 1997). Hence, the effects evoked by phytoestrogens can vary with different days of the estrous cycle (Slomczynska, 2004). Therefore, the aims of the experiments were (1) to study the influence of phytoestrogens on myometrial contractions before ovulation and (2) to determine whether phytoestrogens interfere with the effect of PCBs on basal and OT-stimulated contractility of myometrial strips.

MATERIAL AND METHODS

Tissue collection and preparation

Uterine horns, ipsilateral to the ovary with regressed *corpus luteum*, of healthy, no pregnant cows on days 19–21 (n = 14) were collected from a commercial slaughterhouse within 15–20 min of slaughter. The physiological status of the tissue was estimated by examining the ovarian morphology as described previously (Ireland et al., 1980; Fields and Fields, 1996). Uterine horns were immediately placed in ice-cold saline and transported to the laboratory within 1 hour. Longitudinal strips of the myometrial layers, 3–4 mm wide and 6–7 mm long, were dissected.

Strips incubation

Strips, as described previously (Wrobel et al., 2005), were individually immersed in 4 ml of aerated (95% air and 5% CO_2) physiological salt solution (PSS; 116mM NaCl, 4.6mM KCL, 1.16mM $NaH_2PO_4 \times H_2O$, 1.16mM $MgSO_4 \times 7\ H_2O$, 21.9mM $NaHCO_3$, 1.8mM $CaCl_2 \times 2H_2O$, 11.6mM dextrose, 0.03mM CaNaEDTA, pH 7.4; Bae et al., 2001) and incubated (4°C) without (control) and with phytoestrogens or PCBs, administered separately or jointly. Every of strips were used for one period of time incubation only. After every 24 h one set of strips was studied and the medium and treatments were changed in others. All materials used in these studies were purchased from Sigma Chemical Co., Poznan, Poland, unless otherwise stated.

Experiment 1

Myometrial strips (n = 5 cows, 21 strips per cow) were incubated (24, 48 and 72 h, *i.e.* 7 strips per one time period) with coumestrol, daidzein, or genistein (10^{-6}, 10^{-5}M each), and their were compared to one control strip in each time-group. Thus, the force, frequency, and amplitude of contractions of the set of 7 myometrial strips were registered after every 24 h, before and after OT (10^{-7}M) treatment. All doses of phytoestrogens used were based on our previous study, when we tested three concentrations of these phytoestrogens on endometrium cell culture in cow (Wrobel and Kotwica, 2005a).

Experiment 2

Based on the data from Experiment 1, myometrial strips were incubated for 48 h with a lower dose of coumestrol and a higher dose of daidzein. Strips of myometrium (8 per cow) were incubated for 48 h, in the first group (n = 5 cows) with coumestrol (10^{-6}M) or with either PCB 77 (100 ng/ml; AccuStandard, New Haven, USA, 99+% purity) or PCB 126 or 153 (10 ng/ml both), separately and jointly. In the second group (n = 4 cows), the experimental design was the same, but coumestrol was replaced by daidzein (10^{-5}M). Next, the force of myometrial contractions, before and after OT (10^{-7}M) treatment, was registered. All doses of PCBs used were based on our previous studies, when we tested their action on myometrium contractility in cow (Wrobel et al., 2005).

Measurement of myometrial contractility

After incubation, the uterine strips were fixed in a special chamber of HSE Schuler Organbath apparatus (March-Hugstetten, Germany). They were

suspended in organ bath containing Krebs-Ringers' solution (KRS; 120.3mM NaCl, 5.9mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM NaH_2PO_4, 15.5mM $NaHCO_3$, 11.5mM glucose, pH 7.4; Kotwica et al., 2003) for isometric force measurement as described previously (Wrobel et al., 2005). Briefly, one edge of a strip was connected with silk line and attached to an isometric contraction transducer (Type 372). The second one was fastened to the bottom of the tissue chamber. The KRS was maintained at 38°C and oxygenated (95% O_2 and 5% CO_2). All preparations were allowed to equilibrate for at least 90 min. Thereafter, spontaneous contractility was measured for 20 min. Subsequently, OT (10^{-7}M) was administered to the medium and myometrial contractions were measured for the next 20 min.

Data analysis

The mean value of the force of contraction was expressed as mN and was calculated from all measurements collected every 4 s before OT treatment. Fast Fourier transform analysis was used (OriginPro7.5, OriginLab Corporation) to determine the frequency and amplitude of contractions. The frequency of contractions expressed in Hz was converted to number of peaks per 100 s by means of OriginPro 7.5 program. Data obtained before and after OT treatment were analyzed using one-way ANOVA and Tukey's post test (GraphPad PRISM 2.00 software).

RESULTS

Effect of coumestrol, daidzein, and genistein on spontaneous and OT-stimulated force, frequency, and amplitude of myometrial strips contractions (Experiment 1)

Both the force and amplitude of myometrial contractions in control strips after 72 h (data not shown) were significantly lower compared to the tissue contractility after 24 and 48 hours. Therefore, data from this time were eliminated from further analysis. The response of myometrial contractions to phytoestrogens depends on the type of phytoestrogen, its doses, and time of treatment (Figure 1). Daidzein clearly increased ($P < 0.05$) the force of spontaneous and OT-stimulated contractions, whereas both doses of coumestrol decreased ($P < 0.05$) the force of spontaneous and OT-stimulated contractions of myometrial strips after 48 h (Figure 1 and 2). Genistein slightly decreased the force of spontaneous myometrial contractions compared to control after 24 hours. However, this effect was much more evident in OT-stimulated myometrium, treated with 10^{-5}M of genistein for 48 hours. Oxytocin increased ($P < 0.01$) the frequency of the control strips' contractions and those incubated with all phytoestrogens, compared to the frequency of spontaneous contractions (Figure 3). However, coumestrol (10^{-5}M) increased ($P < 0.01$) the frequency of spontaneous myometrial contractions after 24 hours. Moreover, daidzein (10^{-6}M) only increased ($P < 0.05$) the con-

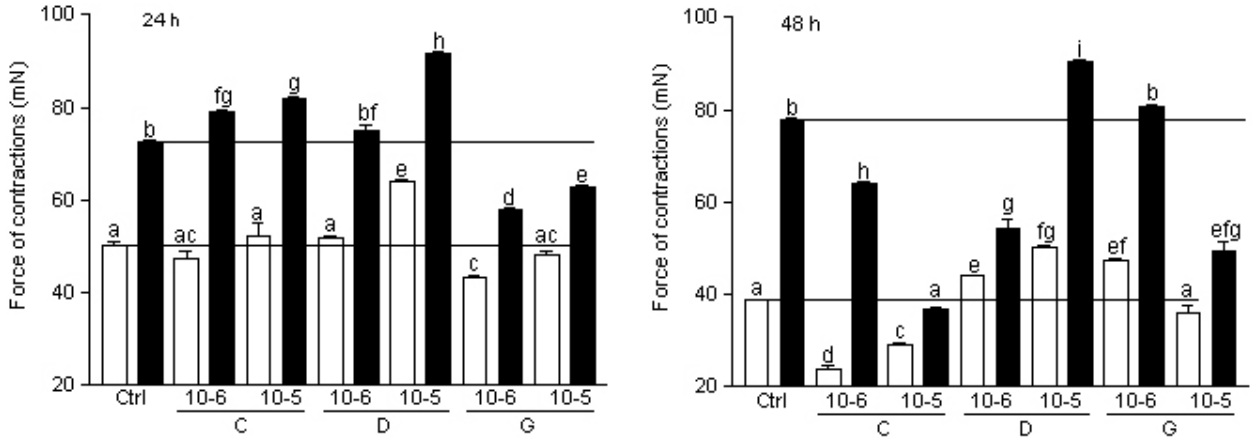

Figure 1. Effect of coumestrol (C), daidzein (D), and genistein (G; 10^{-5} and 10^{-6}M each) on spontaneous (basal) and OT-stimulated (10^{-7}M) mean (± SEM) force of contractions of myometrial strips from days 19–21 of the estrous cycle ($n = 5$), after 24 and 48 h of incubation

$^{a-i}P < 0.05$; ▭ basal; ▬ OT-stimulated

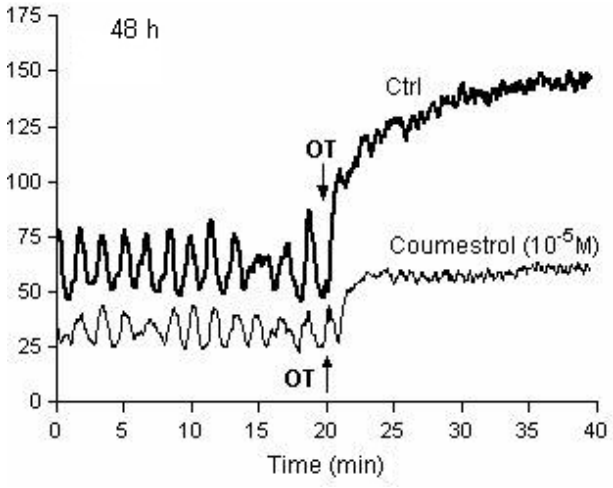

Figure 2. Individual chart of contractions of myometrial strips (control and coumestrol 10^{-5}M) after 48 h of incubation from one cow on days 19–21 of the estrous cycle, before and after oxytocin (OT; 10^{-7}M) challenge (arrow)

tractions of OT-stimulated myometrial strips after 48 h (Figure 3). In addition, genistein and daidzein (10^{-5}M), after 24 and 48 h, respectively, increased ($P < 0.05$) the amplitude of spontaneous myometrial strips' contractions (Figure 4), while none of the phytoestrogens influenced the amplitude of myometrial contractions compared to control ($P > 0.05$) in OT-stimulated strips. There was a tendency ($P > 0.07$) to reduce the amplitude of contractions of control strips and those incubated with phytoestrogens, after OT stimulation. However, only genistein and daidzein decreased ($P < 0.01$) the amplitude of OT-stimulated contractions (Figure 4), after 24 and 48 h, respectively.

Effect of coumestrol and daidzein, separately and jointly with PCBs, on spontaneous and OT-stimulated force of contractions of myometrial strips (Experiment 2)

All three PCB congeners increased ($P < 0.05$) the spontaneous and OT-stimulated force of myometrial contractions (Figure 5 and 6), whereas coumestrol alone decreased ($P < 0.05$) the force of contractions and inhibited completely ($P < 0.001$) basal and OT-stimulated force of spontaneous myometrial contractions evoked by all PCBs used (Figure 5). Similarly, to the all three PCB congeners used separately, daidzein increased ($P < 0.05$) the force of spontaneous and OT-stimulated myometrial contractions compared to control (Figure 6). Spontaneous force of myometrial contractions treated jointly with daidzein and PCB 77 or PCB 153 was lower compared to strips treated separately with these compounds ($P < 0.05$). Spontaneous force of contractions of strips treated jointly with daidzein and PCB 126 was also lower compared to those treated with PCB 126, while it was higher compared to daidzein given alone ($P < 0.05$). Daidzein and PCB congeners used increased OT-stimulated force of myometrial contractions ($P < 0.05$), but their effect was cumulative (Figure 6) only when daidzein was treated with PCB 153 jointly.

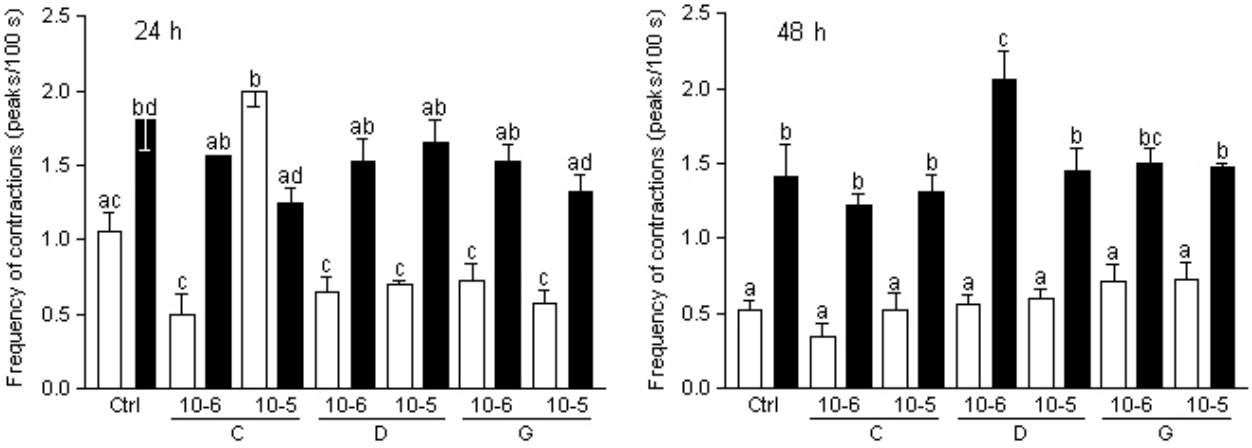

Figure 3. Effect of coumestrol (C), daidzein (D), and genistein (G; 10^{-5} and 10^{-6}M each) on spontaneous (basal) and OT-stimulated (10^{-7}M) mean (± SEM) frequency of contractions of myometrial strips from days 19–21 of the estrous cycle ($n = 5$), after 24 and 48 h of incubation

$^{a–d}P < 0.05$; ▭ basal; ▬ OT-stimulated

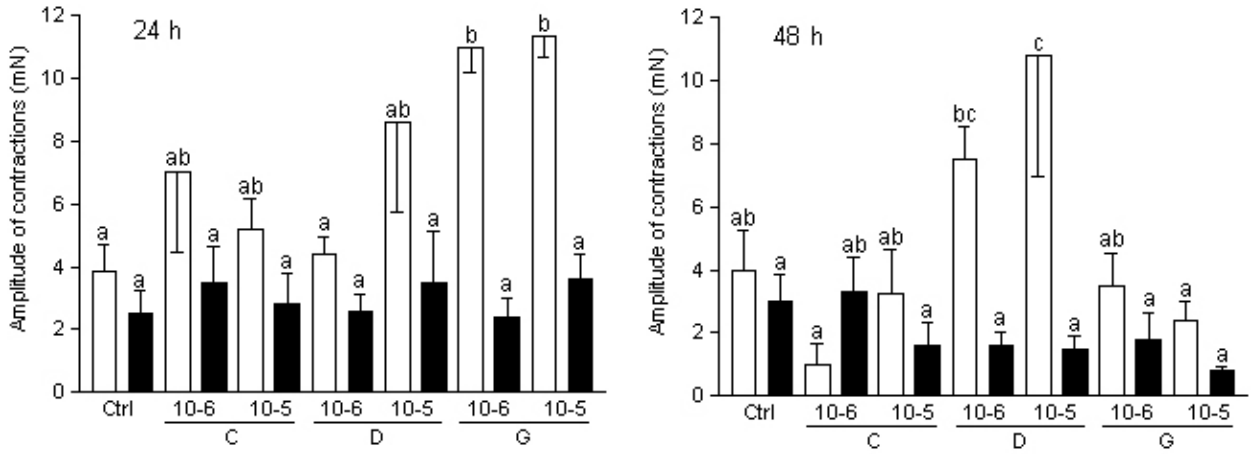

Figure 4. Effect of coumestrol (C), daidzein (D), and genistein (G; 10^{-5} and 10^{-6}M each) on spontaneous (basal) and OT-stimulated (10^{-7}M) mean (± SEM) amplitude of contractions of myometrial strips from days 19–21 of the estrous cycle (n = 5), after 24 and 48 h of incubation

$^{a–c}P < 0.05$; ▭ basal; ▬ OT-stimulated

DISCUSSION

It was found that PCBs, acting also via estrogen receptor (Andric et al., 2000; McLachlan, 2001), can stimulate myometrial contractions in rats (Tsai et al., 1996) and cattle (Wrobel et al., 2005). These studies revealed that two of three phytoestrogens (coumestrol and genistein) used decreased the force of spontaneous or OT-stimulated myometrial contractions. Moreover, in most cases, phytoestrogens did not affect the frequency and amplitude of contractions; hence, these parameters were not registered in the next experiments. There is a lack of data connecting the effects of phytoestrogens with myometrial contractions in bovine. However, our observations are partly in agreement with data obtained in porcine (Lee et al., 2004) and rabbits (Figtree et al., 2000) when genistein induced relaxation in arteries *in vitro*. Moreover, we found that phytoestrogens can restore the proper ratio of

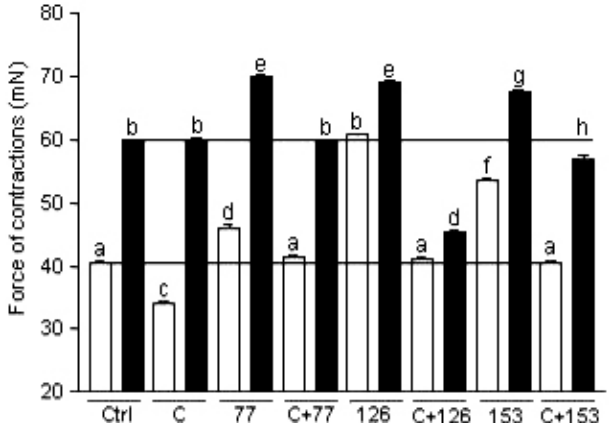

Figure 5. Effect of coumestrol (C; 10^{-6}M) compared with each of the PCBs (77 at the dose 100 ng/ml, and 126 or 153, both at the dose of 10 ng/ml) on spontaneous (basal) and OT-stimulated (10^{-7}M) mean (± SEM) force of contractions of myometrial strips from days 19–21 of the estrous cycle (n = 5), after 48 h of incubation

$^{a–h}P < 0.05$; ▭ basal; ▬ OT-stimulated

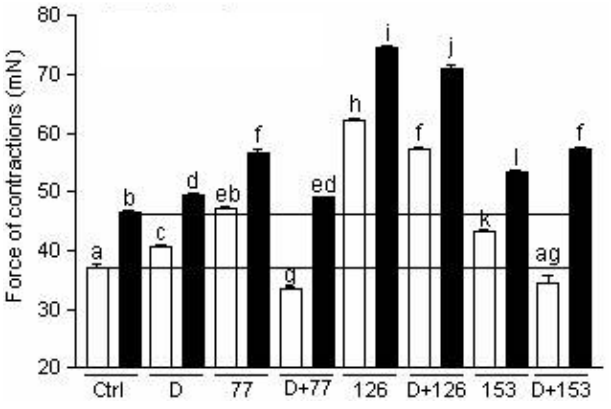

Figure 6. Effect of daidzein (D; 10^{-5}M), compared with each of the PCBs (77 at the dose 100 ng/ml, and 126 or 153, both at the dose of 10 ng/ml) on spontaneous (basal) and OT-stimulated (10^{-7}M) mean (± SEM) force of contractions of myometrial strips from days 19–21 of the estrous cycle (n = 4), after 48 h of incubation

$^{a–l}P < 0.05$; ▭ basal; ▬ OT-stimulated

PGE_2 and $PGF_{2\alpha}$ secretion from bovine endometrial cells, which was disrupted by PCBs (Wrobel and Kotwica, 2005a). Therefore, we presumed that phytoestrogens might also counteract the effect of PCBs on the myometrium.

Smooth muscle contractility is partly regulated by estradiol (Inoue, 1981; Johnson, 1992) and stimulated by prostaglandin $F_{2\alpha}$ (Huszar and Roberts, 1982; Yousufzai and Abdel-Latif, 1998). Estradiol increased intracellular calcium concentrations in rats (Boyle et al., 1987) and PCBs evoked a similar effect in cattle myometrial cells (Wrobel and Kotwica, 2005b). Genistein, but not daidzein, was found to be an inhibitor of calcium influx in isolated uterine (Kusaka and Sperelakis, 1995) or aortic (Petrescu et al., 2001) smooth muscle cells in rats (Ratz et al., 1999) or arteries (Li et al., 2004) in rabbits. Therefore, perhaps only daidzein stimulated the force of myometrial contractions in our studies. However, the mechanism of phytoestrogens action on uterine contractility seems to be more complicated. Because $PGF_{2\alpha}$ stimulated myometrial contractions in cattle (Ocal et al., 2004), we assume that phytoestrogens may influence myometrial contractility indirectly, via inhibition of $PGF_{2\alpha}$ secretion. This suggestion can be supported by data that PCBs stimulate secretion of $PGF_{2\alpha}$ (Wrobel and Kotwica, 2005a) and release of arachidonic acid, a substrate to PGs production (Bae et al., 1999), in cattle endometrial and rat myometrial cells, respectively. In contrast, phytoestrogens reduced the effect of PCBs (Wrobel and Kotwica, 2005a). Therefore, phytoestrogens may also reduce the stimulating effect of PCBs on uterine contractility in this manner. For this purpose, we have chosen two phytoestrogens, having an opposite effect; that is, coumestrol decreases and daidzein increases the force of myometrial contractions (see Exp. 1). However, incubation of strips together with either one of the phytoestrogens and PCBs was followed by reduction in the spontaneous force of contractions, compared to strips incubated with each of the PCBs separately. Moreover, coumestrol decreased this force to the control level. This evident effect by coumestrol may be a result of decreased muscle glycogen concentrations and inhibited insulin binding to muscle cell membrane in rats (Nogowski et al., 1992).

Estradiol increased OT mRNA and OT receptor concentration in myometrial cells (Picherit et al., 2000). Because the ability of OT to stimulate contractions of the myometrium is estradiol-dependent (Johnson, 1992; Holda et al., 1996), we tested the effect of phytoestrogens and compared it with the effect of PCBs. The most effective increase in OT-stimulated force of myometrial contractions was caused by the incubation of tissues with the highest dose of daidzein, but genistein and coumestrol reduced this parameter. This is partly in agreement with data that daidzein, but not genistein, sensitized ovariectomized rat uterine smooth muscle to contractile agents OT and $PGF_{2\alpha}$ (Picherit et al., 2000). It was also shown that daidzein inhibited estradiol binding to its receptor in the uterus, but on other hand, both daidzein and genistein inhibited the ability of OT to stimulate myometrial contractions (Picherit et al., 2000). This suggests that phytoestrogens might exhibit both estrogen-like and anti-estrogenic effects (Picherit et al., 2000). Supposedly, their metabolite(s) can affect the uterus in a different way. Nevertheless, coumestrol or daidzein, jointly with PCBs, decreased OT-stimulated force of contractions, while the effect of daidzein was less evident. Moreover, coumestrol could restore OT-stimulated contractions to normal levels.

In conclusion, these results suggest that phytoestrogens themselves are involved in the regulation of uterine contractility. Furthermore, they could partly reduce or reverse the effect of PCBs on myometrial contractions, supposedly via influence on prostaglandins secretion and synthesis as suggested earlier (Wrobel and Kotwica, 2005a). Thus, it is possible that a diet containing flavonoids can be a part of a feeding strategy to prevent disorders in the reproduction process of domestic animals.

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