Influence of polychlorinated biphenyls (PCBs) and phytoestrogens on prostaglandin $F_{2\alpha}$ and E_2 secretion from bovine endometrial cells at a postovulatory stage of the estrous cycle

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ABSTRACT: Polychlorinated biphenyls (PCBs) and phytoestrogens were found to affect contractions of bovine uterus. Prostaglandins (PG) $F_{2\alpha}$ and E_2 are also involved in the uterine contractility. Hence the aim of these studies was to investigate the effect of PCBs and some phytoestrogens on PG secretion from endometrial cells obtained on days 1–5 of the oestrous cycle. Cells were incubated in aerated atmosphere at 38°C for 24 h, separately with the mixture of PCBs – Aroclor 1248 (10 ng/ml), with individual congeners -77, -126 or -153 (each at the dose 100 g/ml), coumestrol, daidzein or genistein (each at the dose 10^{-6}M) or jointly each PCB with each of the phytoestrogens. Using the TOX1-kit neither Aroclor 1248 (Ar 1248) nor individual congeners were found to affect the viability of cells compared to the control ($P > 0.05$). All used PCBs markedly increased the metabolite of $PGF_{2\alpha}$ (PGFM) concentrations ($P < 0.05$) but not PGE_2 ($P > 0.05$). Hence the ratio of $PGF_{2\alpha}$ to PGE_2 was also increased by PCBs. However, when these cells were incubated with each of the phytoestrogens, there was a decrease in both $PGF_{2\alpha}$ and PGE_2 secretion compared to the control ($P < 0.05$) but without altering the $PGF_{2\alpha}$: PGE_2 ratio. Moreover, phytoestrogens could clearly reduce the concentrations of PGFM elicited by PCBs, and they reduced PGE_2 secretion compared to that evoked by PCB-126 and –153 only. Thus phytoestrogens can restore the proper ratio of $PGF_{2\alpha}$: PGE_2 secreted by the bovine endometrium.

Keywords: PCBs; phytoestrogens; endometrium; $PGF_{2\alpha}$; PGE_2; cattle

The postovulatory stage of the oestrous cycle is a critical time for fertilization and further fate of the ovum. Prostaglandins (PG), synthesized in the endometrium from arachidonic acid (AA) as a precursor (Asselin et al., 1996), are involved in the process of ovulation, luteolysis and implantation (Goff, 2004). Prostaglandin $F_{2\alpha}$ is the main luteolysin in most mammals including bovines (Inskeep and Murdoch, 1980; Silvia et al., 1991; Poyser, 1995). In contrast, PGE_2 may have a luteoprotective effect and it is involved in the implantation of blastocyst (Asselin et al., 1996). Estradiol (E_2) was found to be important for the timing of luteolysis because its removal prolonged the duration of oestrous cycle (Zhang et al., 1991; Goff, 2004) while the administration of E_2 in mid-cycle increased the plasma metabolite of prostaglandin $F_{2\alpha}$-13,14,-dihydro-15-keto-PGF2α (PGFM) concentrations and initiated luteolysis (Thatcher et al., 1986). The time and duration of E_2 treatment is very critical for the final effect. The administration of E_2 during the mid-luteal phase in ewes caused premature luteolysis whereas daily injections of E_2 during the early stage of the cycle prolonged the lifespan of CL, and this is thought to be due to the influence on endometrial oxytocin (OT)R (Goff, 2004). The blood level of E_2 in cows is much lower compared to the other domestic animals (Hirako et al., 2005), and this suggests that the sensitivity of target cells to oestrogens is higher in bovines

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than in the other species. Therefore endocrine disrupting chemicals may cause abnormalities in the reproductive function of cattle, even at concentrations too low to induce such effects in the other species (Hirako et al., 2005).

PCBs that have widely been used in the industry are now persistent environmental contaminants and resistant to degradation (Safe, 1992, 1994; Franek and Hruska, 2005). They can increase concentrations of PGFM in pregnant guinea-pig (Lundkvist and Kindahl, 1989) or to release AA from neutrophils (Tithof et al., 1996) and from the myometrium (Bae et al., 1999) in rat, and they can also mimic the oestrogen effect upon cells (Kristan et al., 2005). A few studies demonstrated that isoflavonoids, plant phenolic compounds found in soybeans and other legumes, could inhibit (Whitten et al., 1994; Ruh et al., 1995) or mimic the effect of E_2 (Miksicek, 1993; Benassayag et al., 2002). Therefore possible health risks or benefits of these compounds (Baker, 1998; Humfrey, 1998; Setchell, 2001) are worthy of further studies.

We have found that PCBs affect the force and frequency of contractions of myometrial strips from cows (Wrobel et al., 2005). This effect is assumed to be elicited by PCB influence on endometrial secretion of PGs and that phytoestrogens can modify this process. Therefore in these studies the effect of PCBs and selected phytoestrogens on endometrial secretion of $PGF_{2\alpha}$ and PGE_2 was investigated.

MATERIAL AND METHODS

Tissue collection

Uterine horns ipsilateral to the ovary with corpus luteum (CL) were collected from non-pregnant cows from a commercial slaughterhouse on day 1–5 and 19–21 of the oestrous cycle (Ireland et al., 1980) and transported in ice-cold saline to the laboratory within one hour. All materials used in these studies were purchased from Sigma-Aldrich Co., Poland, unless otherwise stated.

Isolation of endometrial cells

Endometrial cells were isolated as described by Skarzynski et al. (2000) with some modifications. Briefly, after flushing the lumen of the isolated uterine horn with saline, it was filled with the mixture (25–30 ml) of collagenase IA (5 mg/ml) and dispase (0.2 g/100 ml, Gibco) diluted with M-199 and supplemented with 0.1% BSA. Then both ends of the uterine horn were sutured with silk and it was placed for 1 h in a water bath (38°C) gently shaken. Thereafter, the medium was collected and centrifuged 3-times (1000 × g for 10 min at 4°C). After each centrifugation the supernatant was removed and cells were suspended in 10 ml of M-199 with 0.1% BSA. Moreover, suspended cells were filtered: through a steel sieve (180 µm) before the first centrifugation, and through three layers of nylon filters (270, 180, 105 µm) after the last centrifugation. Then, cells were suspended in DMEM/F-12 HAM supplemented with 5% calf serum (FCS). Thereafter, the uterine horn was cut off and the remaining cells were collected with blunt spatula. These cells were suspended for 30 min at 38°C in the mixture of collagenase and dispase and centrifuged as above. Viability of cells was estimated by exclusion of 0.04% trypan blue dye and cells with viability only above 85% were used in further studies. Homogeneity of obtained cells was determined by means of Burker chamber soon after the cells were obtained from the lumen of the uterine horn (n = 10). Mean (±SEM) amount of epithelial cells was 86.1 ± 4%, 8.5 ± 1% non-epithelial cells, and 5.4 ± 1% of dead cells in the whole population of cells.

Cell culture

Cells (2×10^5/ml) suspended in DMEM/Ham's-F12 and supplemented with 5% FCS and 20 µg/ml gentamycin were seeded into 48-well plates (Nunc GmbH&Co.KG, Wiesbaden, Germany) and cultured (Heraus BB-6060, Hanau, Germany) at 38°C in 95% air and 5% CO_2 atmosphere. Cells attached to the bottom of the well after 72h were washed with M-199 + 0.1% BSA and incubated in DMEM/Ham's-F12 medium containing 0.1% BSA supplemented with ascorbic acid (20 µg/ml), sodium selenite (5 ng/ml, ICN), transferrin (5 µg /ml), and 20 µg/ml of gentamycin.

Preliminary studies

Doses of PCBs we used were the same as in previous studies (Wrobel et al., 2005) when the contractility of bovine myometrial strips was measured.

In order to avoid the criticism that these doses of PCBs could affect the viability of cells, this parameter was measured. For this purpose endometrial cells from three cows on day 1–5 of the oestrous cycle were incubated for 48 h with Ar 1248 (10 and 100 ng/ml; LGC Promochem, Teddington, UK) and with individual congeners of PCBs (-77, -126, -153; 100 ng/ml; AccuStandard, New Haven, USA). The cell viability was estimated by means of TOX1 test (*in vitro* toxicology assay kit, MTT based), which is correlated with the activity of mitochondrial dehydrogenases. Cells were also incubated with Actinomycin D (500 ng/ml), which served as a negative control.

Further, the time of incubation was established. The effect of Ar 1248 (10 and 100 ng/ml) after 24 and 48 h on the secretion of $PGF_{2\alpha}$ from endometrial cells (day 1–5 of the oestrous cycle; $n = 5$) was studied. Arachidonic acid (AA; 20 µg/ml) was used as a positive control. Each treatment in this cell culture and in the other individual cell cultures was done in triplicates. After incubation, the medium was collected into tubes containing 10 µl of 30 µM EDTA, 1% acetylosalicylic acid, and stored (–20° C) until determination of PGFM and PGE_2 concentrations. Protein concentrations in cells were measured by Bradford (1976) method and hormone concentrations were expressed per milligram of protein. Since the response of cells was more evident after 24 h, this time was selected in further studies.

Moreover, the effect of individual congeners of PCB (-77, -126, -153; 100 ng/ml) on the secretion of $PGF_{2\alpha}$ from endometrial cells from days before (day 19–21; $n = 6$) and after ovulation (day 1–5; $n = 6$) was investigated.

Experimental design

Cells from day 1–5 ($n = 7$) of the oestrous cycle were incubated for 24 h with either Ar 1248 (10 ng/ml) or individual congeners of PCB (-77, -126, -153; 100 ng/ml) separately or jointly with each of the phytoestrogens (coumestrol, daidzein and genistein) at the dose of 10^{-6}M.

Hormone assays

The concentration of PGFM in the medium, which reliably reflects $PGF_{2\alpha}$ secretion from endometrial cells (Skarzynski et al., 1999), was determined by EIA using horseradish peroxidase-labelled PGFM as a tracer (1 : 40 000; final dilution) and anti-PGFM serum (1 : 80 000) described earlier (Homanics and Silvia, 1988) was donated by Dr. W.J. Silvia (University of Kentucky, Lexington, USA). The standard curve ranged from 62.5 to 32 000 pg/ml. The intra- and inter-assay coefficients of variation were 6.7% and 11.7%, respectively.

PGE_2 concentrations were determined also by EIA using horseradish peroxidase-labelled PGE_2 (1 : 30 000; final dilution) and anti-PGE_2 serum (diluted 1 : 35 000; kindly donated by Dr. W.W. Thatcher, University of Florida, Gainesville, USA). The standard curve ranged from 78 do 20 000 pg PGE_2/ml. The intra- and inter-assay coefficients of variation were 7.5% and 12.1%, respectively.

Data analysis

Due to some variations of acquired data concentrations of PGs were expressed as percent, compared to the control accepted as 100%, and analysed by one-way ANOVA followed by Tukey's test (GraphPad PRISM, GraphPad Software, Inc., San Diego, CA).

RESULTS

Preliminary studies showed that: (a) Neither Ar 1248 nor any of PCB congeners affected the viability of cells compared to the control ($P > 0.05$) whereas actinomycin D reduced ($P < 0.001$) the viability of cells by about 90% compared to the control (Figure 1). (b) Effect of Ar 1248 on the secretion of $PGF_{2\alpha}$ from endometrial cells was the most evident after 24 h of incubation compared to the control and to the data after 48 h (Figure 2), therefore in further studies cells were incubated for 24 hours. (c) All three PCBs congeners in the used doses also stimulated $PGF_{2\alpha}$ secretion from cells more markedly ($P < 0.05$) after 24 h (data not given). Since there was no clear difference in $PGF_{2\alpha}$ secretion from cells collected before and after ovulation, those from days 1–5 of the cycle were used in further studies. (d) All phytoestrogens, except genistein at the dose of 10^{-5}M, decreased the secretion of $PGF_{2\alpha}$ (Figure 3a) and all phytoestrogens decreased the secretion of PGE_2 (Figure 3b) compared to the control ($P < 0.05$).

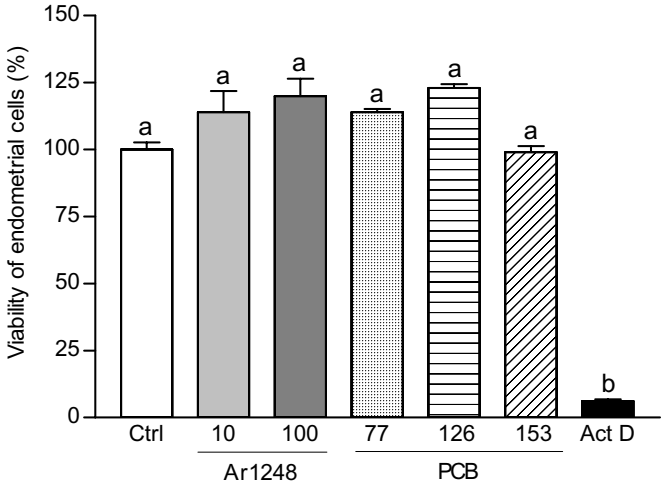

Figure 1. Mean (± SEM) viability of endometrial cells (2×10^5/ml) from days 1–5 of the estrous cycle ($n = 2$) after incubation (48 h) with Ar 1248 (10 ng/ml grey bar or 100 ng/ml dark bar) and PCB congeners: 77, 126, or 153 (100 ng/ml). Actinomycin (Act)D (500 ng/ml) was used as a negative control

[a, b]($P < 0.001$)

Therefore phytoestrogens at the dose of 10^{-6}M were used in further studies.

The concentration of PGFM in the medium after 24 h cell incubation with Ar 1248 (10 ng/ml) or with each of the three PCB congeners (100 ng/ml) was higher ($P < 0.05$) compared to the control (Figure 4a–d) whereas we found less PGFM in the medium above cells incubated with phytoestrogens (10^{-6}M) only, compared to the control ($P < 0.05$). However, when cells were incubated with Ar 1248 jointly with each of the three phytoestrogens (Figure 4a); with PCB-77 and daidzein or with genistein (Figure 4b); with PCB-126 and daidzein (Figure 4c); with PCB-153 and daidzein or with genistein (Figure 4d), the concentrations of PGFM in the medium decreased ($P < 0.05$) compared to cells incubated with PCBs only. Moreover, phytoestrogens reduces the PGFM concentration, elicited by PCBs, to the control values. There was a non-significant difference ($P > 0.05$) in the secretion of PGE_2 from cells incubated with Ar 1248 and with each of the PCB congeners compared to the control (Figure 5a–d). However, when these cells were incubated with each of the phytoestrogens, there was a decrease in PGE_2 secretion compared to the control ($P < 0.05$). Furthermore, cells incubated with Ar 1248 and coumestrol or daidzein (Figure 5a) and with each of the PCB congeners and coumestrol (Figure 5b–d) secreted less PGE_2 compared to the control ($P < 0.05$). Coumestrol also decreased ($P < 0.05$) the secretion of PGE_2 from cells incubated with PCB 126 and 153.

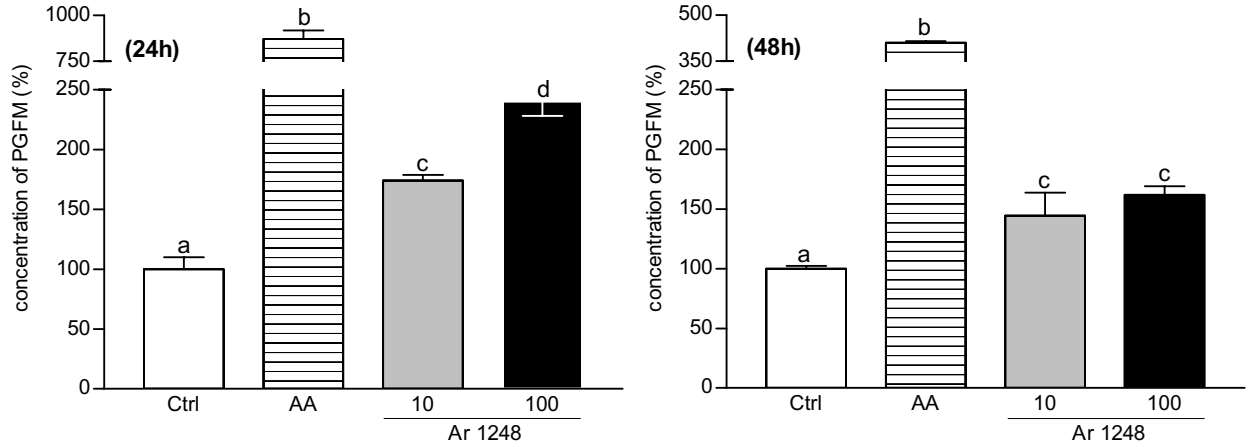

Figure 2. Effect of Ar 1248 (10 and 100 ng/ml) on PGFM concentrations (mean ± SEM) in the medium. Endometrial cells from day 1–5 of the estrous cycle ($n = 6$) were incubated with PCBs for 24 and 48 hours. Arachidonic acid (AA; 20 µg/ml) was used as a positive control

[a–d]($P < 0.05$)

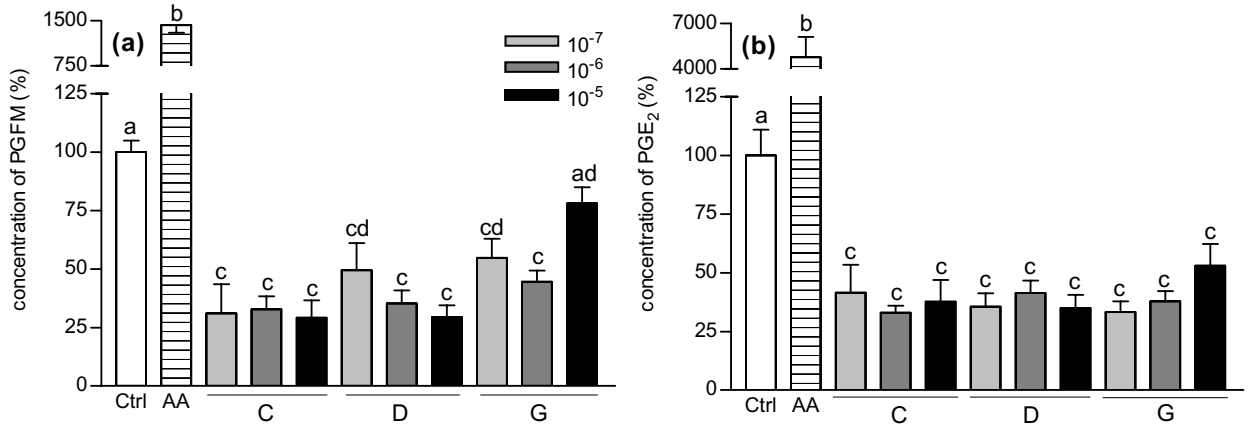

Figure 3. Mean (± SEM) concentrations of PGFM (a) and PGE_2 (b) in the medium after 24 h of incubation of endometrial cells (2×10^5/ml) from day 1–5 of the estrous cycle (n = 6) with phytoestrogens (coumestrol C; daidzein D, genistein G at doses 10^{-7}, 10^{-6}, 10^{-5} M). Arachidonic acid (AA; 20 µg/ml) was used as a positive control

$^{a–d}(P < 0.05)$

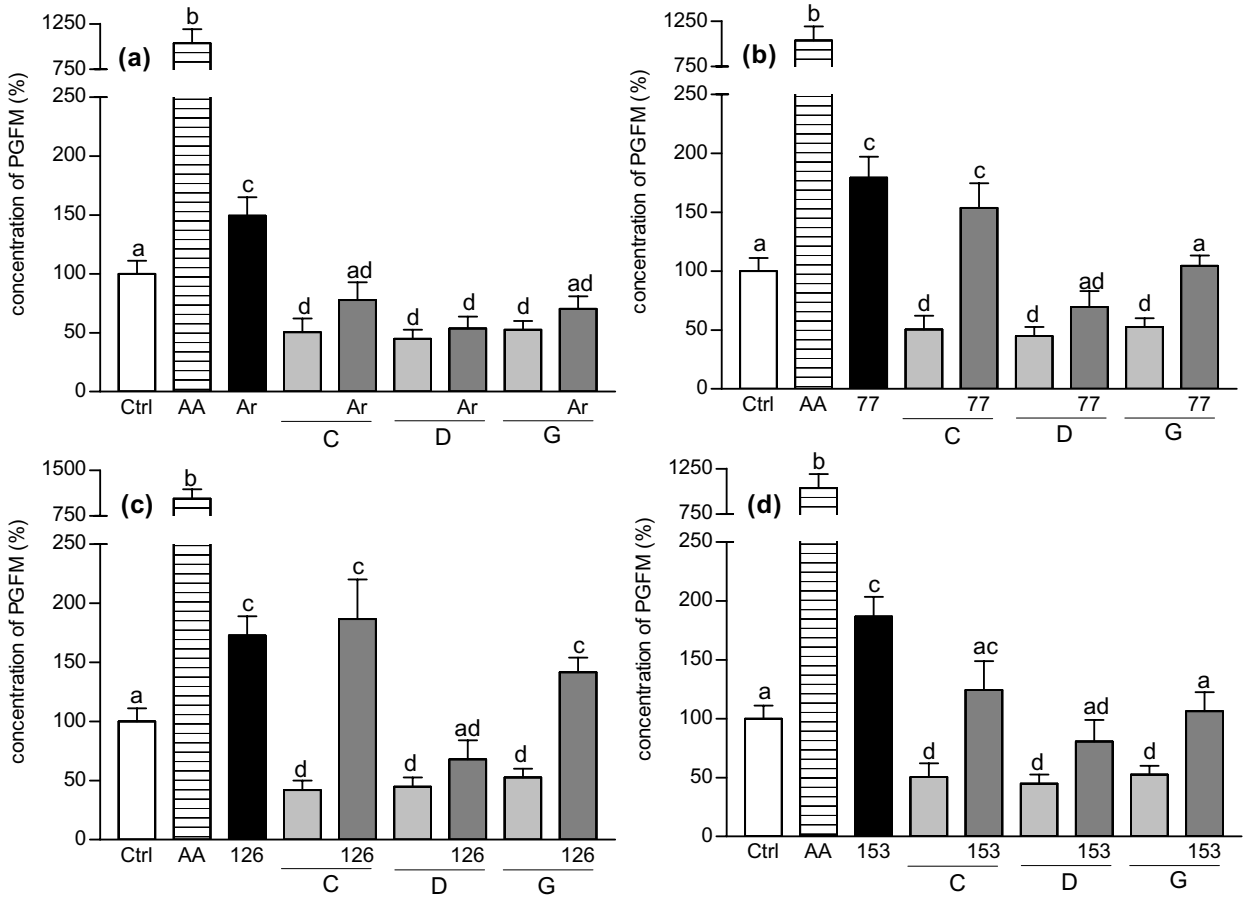

Figure 4. Effect of Ar 1248 (a) at a dose 10 ng/ml and PCB congeners: 77 (b), 126 (c), 153 (d), each at a dose 100 ng/ml, with or without phytoestrogens (10^{-6}M each): coumestrol (C), daidzein (D), genistein (G) on PGFM concentration (mean ± SEM) in the medium. Endometrial cells from day 1–5 of the estrous cycle (n = 7 cows) were incubated with PCBs for 24 hours. Arachidonic acid (AA; 20 µg/ml) was used as a positive control

$^{a–d}(P < 0.05)$

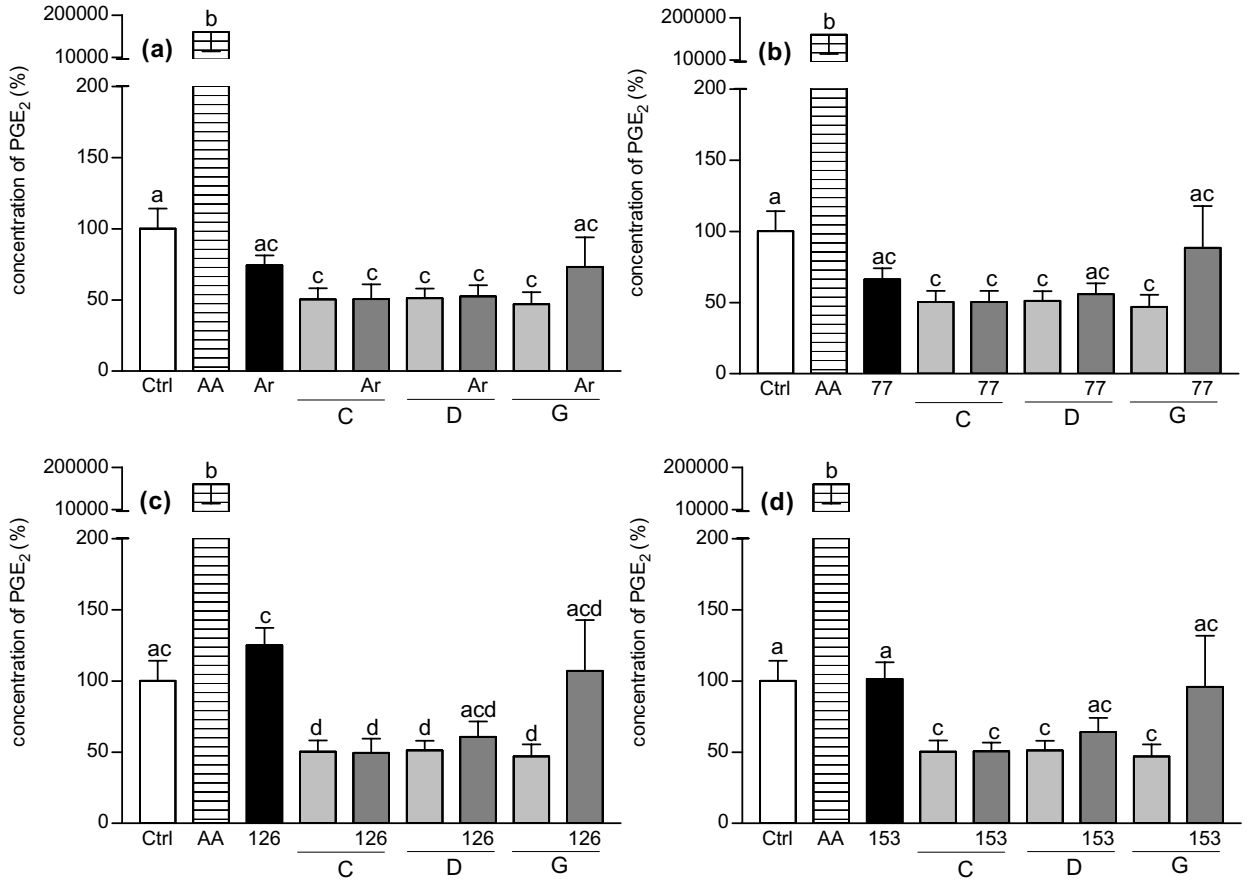

Figure 5. Effect of Ar 1248 (a) at a dose 10 ng/ml and PCB congeners: 77 (b), 126 (c), 153 (d), each at a dose 100 ng/ml, with or without phytoestrogens (10^{-6}M each): coumestrol (C), daidzein (D), genistein (G) on PGE_2 concentration (mean ± SEM) in the medium. Endometrial cells from day 1–5 of the estrous cycle (n = 7 cows) were incubated with PCBs for 24 hours. Arachidonic acid (AA; 20 µg/ml) was used as a positive control

$^{a–d}(P < 0.05)$

DISCUSSION

Since neither Ar 1248 nor individual PCB congeners affected the viability of endometrial cells compared to the control, we assume that the data on PGs secretion were acquired from live and responsive cells. This was also confirmed by the increase in PGFM and PGE_2 concentrations in the medium compared to the control when the cells were supplemented with AA – used as a positive control.

The doses of PCBs we used were applied in animals and in humans (De Saeger et al., 2005), and in our previous studies the same doses were used to measure the effect of PCBs on the contractions of bovine myometrium (Wrobel et al., 2005). The doses of phytoestrogens we used were similar to those used by Miksicek (1993), Ruh et al. (1995), Stromeier et al. (2005) and were also similar to those found in urine and blood plasma of cows fed soybean (Woclawek-Potocka et al., 2005). Besides that, all three phytoestrogens were often tested in a number of laboratories (Blomquist et al., 2005; Kristan et al., 2005; Schmidt et al., 2005), mainly due to a large amount of daidzein and genistein present in soybean, commonly used for the feeding of dairy cattle. These compounds are partly hydrolyzed in the rumen to equol and 6'-hydroxy-O-DMA (Kurzer and Xu, 1997; Janning et al., 2000) and in this form they have higher affinity to estrogen receptor (ER) and therefore they are more active than the native phytoestrogens (Benassayag et al., 2002). Nevertheless, about 21% of daidzein and 9% genistein were still measured in urine 24 h after feeding (Kurzer and Xu, 1997). Moreover, we

used coumestrol that is present in clover and other corps. Its metabolism has not been described in animals and humans yet but it is believed that it still has a biological activity after rumen passage (Kurzer and Xu, 1997; Wu et al., 2004) and it can affect the function of the uterus (Lapcik et al., 2003). Thus we assume that phytoestrogens used in the present studies are less active than their metabolites, and therefore they can be a more sensitive tool to investigate their influence on the effects elicited by PCBs.

PCBs increased the secretion of $PGF_{2\alpha}$ from the bovine endometrium obtained both before and after ovulation. Therefore it can be assumed that PCBs accumulated in the body can influence luteolysis by an increase in $PGF_{2\alpha}$ secretion or they can impair uterine contractility. Moreover, it was shown that PCBs had a negative effect on the bovine embryo development *in vitro* (Scenna et al., 2004) and they could increase the myometrial contractions (Bae et al., 1999; Wrobel et al., 2005). On the other hand, phytoestrogens decreased PGFM concentrations in the current study and concomitantly they can inhibit a specific binding of 17 β-estradiol to ER in the rat and human uterus and in the human mammary gland in a dose-dependent manner (Benassayag et al., 2002). We did not observe a similar dose-dependent effect of phytoestrogens, nevertheless the present data suggest they could overcome the PCB effect on $PGF_{2\alpha}$ secretion from endometrial cells. Interestingly, phytoestrogens at the dose of 10^{-9} or 10^{-8}M increased PG secretions from endometrial cells obtained after ovulation (Woclawek-Potocka et al., 2005). Similarly, genistein was able to stimulate the growth of MCF-7 cells at nanomolar concentrations and to inhibit it at micromolar doses (Whitten et al., 1994). Therefore it can be assumed that the dose of phytoestrogens is critical for the final effect they evoke. Our data indicate that the dose of 10^{-5}M–10^{-7}M is able to reduce the influence of PCBs on endometrial PG secretion. It was seen most markedly in daidzein, which reduced the effect of PCBs on $PGF_{2\alpha}$ secretion. Genistein did not diminish the action of PCB-126 while coumestrol reduced the effect of PCB mixture – Ar 1248 only. On the other hand, all phytoestrogens decreased the secretion of $PGF_{2\alpha}$ from cells incubated with PCB-153 to the control level. Moreover, the PGFM concentration decreased to the control values when cells were incubated jointly with coumestrol and Ar 1248, daidzein with PCB-77 or PCB-126, and genistein together with Ar 1248 or PCB-77. Thus each of the phytoestrogens can inhibit the effect of PCBs on $PGF_{2\alpha}$ secretion at different intensity.

In contrast, PCBs have a very minute effect or no effect on PGE_2 secretion while all three phytoestrogens could clearly decrease the secretion of this prostaglandin. Merely coumestrol reduced the influence of PCB-126 and PCB-153 on PGE_2 secretion. On the other hand, coumestrol jointly with each PCB congener and daidzein with Ar 1248 can decrease PGE_2 secretion below the control values. The ratio of $PGF_{2\alpha}$: PGE_2 is crucial for the establishment of pregnancy and its maternal recognition (Bazer, 1992; McCracken et al., 1999). Thus PCBs, via the influence on PG secretion, can impair the process of fertilization and embryo implantation. However, phytoestrogens may have a direct effect on endometrial PG secretion but they can also reduce the action of PCBs on PG release.

The potential beneficial role of nutritional phytoestrogens in animal and human health was shown (Setchell and Cassidy, 1999), but the mechanism of their effect in target cells is not yet completely understood. Clinical and experimental studies demonstrated that phytoestrogens could notably modulate the impact of oestrogens on the target cells (Benassayag et al., 2002). Some PCB congeners have an affinity to ER (Rollerova and Urbancikova, 2000) and the long-term effect of PCBs can also affect the gene expression for estradiol receptor (Tsai et al., 1997). PCBs may mimic the effects of E_2 on PGs synthesis in this way. Therefore it is possible that phytoestrogens compete with PCBs for ER binding sites and they diminish the effect exerted by PCBs in this way.

In conclusion, our results indicate that PCBs and phytoestrogens are involved in the regulation of PGs secretion. They disrupt the ratio of $PGF_{2\alpha}$: PGE_2 secreted by the endometrium, but phytoestrogens can partly reduce the effect elicited by PCBs. Thus the diet containing plant flavonoids can be an important part of feeding strategy to prevent disorders in reproduction of domestic animals.

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