Ovarian steroids affect prostaglandin production in equine endometrial cells *in vitro*

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Abstract

This study aimed to evaluate the influence of ovarian steroids on equine endometrial epithelial and stromal cells, specifically i) prostaglandin (PG) production in a time-dependent manner, ii) specific PG synthases mRNA transcription and protein expression, and iii) cell proliferation. After passage I, cells were exposed to vehicle, oxytocin (OT, positive control, 10^{-7} M), progesterone (P_4, 10^{-7} M), 17β estradiol (E_2, 10^{-9} M), or $P_4 + E_2$ for 12, 24, 48, or 72 h. Following treatment, PG concentration was determined using the direct enzyme immunoassay (EIA) method. Alterations in *PG* synthases mRNA transcriptions, *PG* synthases protein expression, and cell proliferation in response to the treatments were determined after 24 h using real-time PCR, western blot, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide respectively. After 24 h, E_2 and $P_4 + E_2$ increased PGE_2 and $PGF_{2\alpha}$ secretion as well as specific prostaglandin-endoperoxide synthase-2 (PTGS2), PGE_2 synthases (PGES), and $PGF_{2\alpha}$ synthases (PGFS) expression in the epithelial cells ($P < 0.05$). Additionally, E_2 and $P_4 + E_2$ increased PTGS2 expression in stromal cells after 24 h ($P < 0.05$). In stromal cells, $P_4 + E_2$ increased PGE_2 production as well as PGES expression after 24 h ($P < 0.05$). Both E_2 and $P_4 + E_2$ increased $PGF_{2\alpha}$ production by stromal cells after 24 h ($P < 0.05$). Ovarian steroids affected proliferation of stromal and epithelial cells during the 24-h incubation period ($P < 0.05$). We provide evidence that ovarian steroids affect PG production in equine endometrial cells, upregulating PTGS2, PGES, and PGFS expression. Ovarian steroid-stimulated PG production could be an important mechanism occurring in the equine endometrium that is involved in the regulation of the estrous cycle and early pregnancy.

Key Words
- ► prostaglandin
- ► ovarian steroids
- ► endometrium
- ► mare

Introduction

Ovarian steroids regulate female cyclicity. Progesterone (P_4) and 17β estradiol (E_2) are synthesized from cholesterol through a series of complex pathways involving different enzymatic conversions. The sources of E_2 are mainly granulosa cells of the ovarian follicle (Ryan & Short 1965) and the placenta (Pashen & Allen 1979). The E_2 regulates sexual behavior, enhances uterine motility, and promotes the secretory activity of the entire reproductive tract. By contrast, P_4 is produced by steroidogenic cells of the corpus luteum (CL; Short 1967) and the placenta (Pashen & Allen 1979). This ovarian steroid affects endometrial secretion, inhibits gonadotropin-releasing hormone secretion and reproductive behavior, and promotes pregnancy maintenance.

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Additionally, ovarian steroids are involved in the regulation of endometrial prostaglandin (PG) production in many species (Okuda *et al.* 2002, Goff 2004, Siemieniuch *et al.* 2010). Nonetheless, the influence of ovarian steroids on PG production by homogeneous equine endometrial epithelial and stromal cells remains unclear. The *in vitro* model using cells isolated from equine endometrium recently developed by our team seems to be a good model for studying the physiological regulators of equine endometrial secretory function (Szóstek *et al.* 2012a).

In numerous species, PGs play critical roles in many reproductive events such as luteolysis, fertilization, and implantation (Weems *et al.* 2006). It was shown *in vivo* that, in the mare, $PGF_{2\alpha}$ has a luteolytic action (Douglas & Ginther 1976, Zavy *et al.* 1978), in contrast to PGE_2, which is a luteotropic agent (Vanderwall *et al.* 1994). Moreover, the highest endometrial PGE_2 synthases (*PGES*) and $PGF_{2\alpha}$ synthases (*PGFS*) mRNAs transcription was described in mid and late luteal phases of the estrous cycle respectively (Szóstek *et al.* 2012b).

Regarding the PG synthesis cascade, the prostaglandin-endoperoxide synthase (PTGS, COX) enzyme converts arachidonic acid (AA) into PGH_2 (Simmons *et al.* 2004, Weems *et al.* 2006). There are two isoforms of PTGS named PTGS1 and PTGS2. The PTGS1 isoform has been considered to be constitutively expressed while PTGS2 is the inducible isoform (Simmons *et al.* 2004). PGFS and PGES are downstream enzymes and catalyze the conversion of PGH_2 into $PGF_{2\alpha}$ and PGE_2 respectively. The expression patterns of enzymes responsible for specific PG production during the estrous cycle and early pregnancy in the mare have been recently described (Atli *et al.* 2010, Szóstek *et al.* 2012b). However, the putative role of ovarian steroids on PG production and the pathways triggered, as well as their effects on equine endometrial cell proliferation, are subjects of our interest. Thus, this study evaluated the influence of ovarian steroids on equine endometrial epithelial and stromal cells, specifically i) PG production in a time-dependent manner, ii) specific PG synthases expression, and iii) cell proliferation.

Materials and methods

Animals and endometrial tissue collection

Uteri ($n=30$) from cyclic mares at the early luteal phase of their estrous cycle were collected *postmortem* from April until the end of July at a local abattoir. The mares were healthy as stated by official governmental veterinary inspection. The phases of the estrous cycle were identified based on P_4 and E_2 analysis in blood serum and macroscopic observation of the ovaries (Roberto da Costa *et al.* 2007). The early luteal phase is characterized by the presence of corpus hemorrhagica with a plasma concentration of $P_4 > 1$ ng/ml. At the mid luteal phase, the well-developed CL is associated with follicles 15–20 mm in diameter and $P_4 > 6$ ng/ml. At the late luteal phase, a regressing CL is present, together with follicles 30–35 mm in diameter and a concentration of P_4 from 1 to 2.5 ng/ml. The follicular phase is characterized by absence of an active CL and presence of follicles with various sizes, but always > 35 mm diameter, with a concentration of $P_4 < 1$ ng/ml (Roberto da Costa *et al.* 2007). Moreover, the phases were differentiated by plasma E_2, which is present at a basal concentration in the luteal phase (around 2–10 pg/ml), but reaches values above 20 pg/ml in the follicular phase (Ginther 1992).

The entire uterus was collected within 5 min of an animal's death, placed in sterile, incomplete (Ca^{2+}- and Mg^{2+}-free) Hank's balanced salt solution supplemented with gentamicin (20 µg/ml; Sigma–Aldrich, #G1272) and 0.1% (w/v) BSA (Sigma–Aldrich, #A9056), kept on ice and transported in 40 min to the laboratory. A small piece of endometrium from each uterus was placed in buffered 4% (v/v) paraformaldehyde for histological analysis (Roberto da Costa *et al.* 2007), for further classification according to the scoring system developed by Kenney (1978). Only cells derived from the endometria that were classified as I according to the Kenney (1978) classification were used in this study. The animal treatment and tissue collection procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (agreements no. 51/2011).

Epithelial and stromal cells isolation and culture

The epithelial and stromal cells were isolated following the methodology described previously (Szóstek *et al.* 2012a). The cells were cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The culture medium was DMEM/Nutrient F-12 Ham (DMEM/Ham's F-12; Sigma–Aldrich, D8900) supplemented with 10% (v/v) fetal calf serum (FCS) (Sigma–Aldrich, #C6278) and 1% (v/v) antibiotic and antimycotic solution (Sigma–Aldrich, #A5955); it was changed every 2–3 days. After reaching 90–95% confluency (day 5 or 7 of the incubation of stromal or epithelial cells respectively), the cells were trypsinized as described previously (Szóstek *et al.* 2012a). Next, depending on the experiment, the cells were seeded at a density of 5×10^5 viable cells/ml for epithelial cells and

2×10^5 viable cells/ml for stromal cells in 24- or 96-well plates. The viability of both cell types was more than 87%. Cell culture homogeneity was evaluated using immuno-fluorescent staining for epithelial and stromal cell specific markers (cytokeratin and vimentin respectively) as described previously (Szóstek *et al.* 2012a).

Experimental procedures

Experiment 1: effect of ovarian steroids on time-dependent PG production by endometrial cells

Epithelial cells (Fig. 1A; $n = 5$ for each treatment time) and stromal cells (Fig. 1B; $n = 5$ for each treatment time) derived from passage I were placed in a 24-well plate in culture medium DMEM/Ham's F-12 supplemented with 10% (v/v) FCS and 1% (v/v) antibiotic and antimycotic solution. When the cells reached 95, 90, 80, or 70% confluency, the medium was replaced with fresh DMEM without phenol red (Sigma–Aldrich, D#2960) supplemented with 0.1% (w/v) BSA and 1% (v/v) antibiotics and antimycotic solution. The most effective dose of ovarian steroids was established in a preliminary study (data not shown). The epithelial and stromal cells were incubated with either vehicle or with P_4 (10^{-7} M), E_2 (10^{-9} M), or $P_4 + E_2$ (10^{-7} M/10^{-9} M) added to the culture medium for 12, 24, 48, and 72 h (95, 90, 80, or 70% confluence respectively). Oxytocin (OT; 10^{-7} M) was used as a positive control as described recently (Szóstek *et al.* 2012a,b). In the above-mentioned study, we showed that epithelial as well as stromal cells are capable of producing PG in response to OT

(Szóstek *et al.* 2012a,b). Positive control demonstrated that the condition of the experiment can give a positive outcome even if the results are not positive. In this case, endometrial cells have active molecular mechanism to produce PG.

The conditioned media were collected into tubes with 5 µl EDTA, 1% (v/v) acetylsalicylic acid solution (Sigma–Aldrich, #A2093), and frozen at $-20\,°C$ until later PG measurement. For single-step DNA isolation, 250 µl of TRI Reagent (Sigma–Aldrich, #T9424) was added to each well. Cells were then collected from four wells for each treatment. DNA was isolated according to the TRI Reagent manufacturer's procedure. DNA content was used to standardize the results.

Experiment 2: effect of ovarian steroids on PG expression in epithelial and stromal cell cultures

Experiment 2.1: effect of ovarian steroids on PG synthases mRNAs transcription in epithelial and stromal cell cultures Stromal ($n = 5$) and epithelial ($n = 5$) cells derived from passage I were placed in 24-well plates. When the cells reached 90% confluency, the medium was replaced with fresh DMEM without phenol red supplemented with 0.1% (w/v) BSA and 1% (v/v) antibiotics and antimycotic. Epithelial and stromal cells were incubated with either vehicle or with P_4 (10^{-7} M), E_2 (10^{-9} M), or $P_4 + E_2$ (10^{-7} M/10^{-9} M) added to the culture medium for 24 h. After that, the culture medium was removed and cells were washed twice with PBS. To each well, 250 µl Fenozol (A&A Biotechnology, Gdansk, Poland) was added and then the cells were collected and kept frozen until RNA isolation.
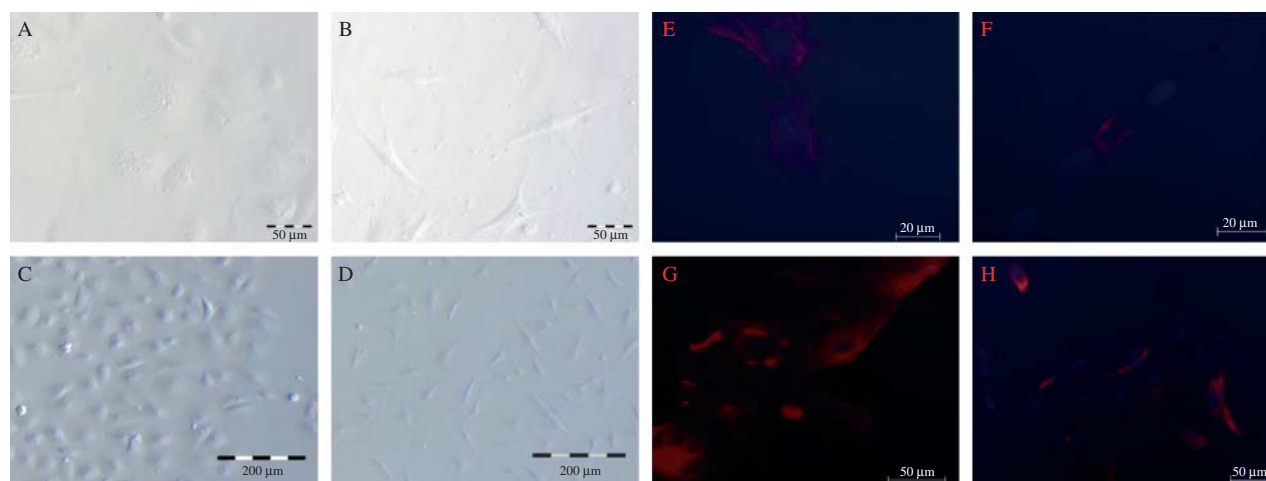

Figure 1
Representative morphology of cultured equine endometrium: (A and C) epithelial cells, (B and D) stromal cells. Epithelial cell identification by immunofluorescent staining for cytokeratin (E and G). Stromal cell identification by immunofluorescent staining for vimentin (F and H). The scale bar = 200 µm (magnification: ×10), 50 µm (magnification: ×40), and 20 µm (magnification: ×100).

Experiment 2.2: effect of ovarian steroids on PG synthases protein expression in epithelial and stromal cell cultures Stromal ($n=5$) and epithelial ($n=5$) cells derived from passage I were placed in six-well plates. When the cells reached 90% confluency, the medium was replaced with fresh DMEM without phenol red supplemented with 0.1% (w/v) BSA and 1% (v/v) antibiotics and antimycotic. Epithelial and stromal cells were incubated with either vehicle or with P_4 (10^{-7} M), E_2 (10^{-9} M), or $P_4 + E_2$ (10^{-7} M/10^{-9} M) added to the culture medium for 24 h. After that, the culture medium was removed and cells were washed twice with PBS. To each well, 250 μl radioimmunoprecipitation assay buffer (RIPA) with a protease inhibitor cocktail was added and then the cells were collected and kept frozen until RNA isolation.

Experiment 3: effect of ovarian steroids on epithelial and stromal cell proliferation

Stromal ($n=5$) and epithelial ($n=5$) cells derived from passage I were placed in 96-well plates. When the cells reached 50% confluency, the medium was replaced with fresh DMEM without phenol red supplemented with 0.1% (w/v) BSA and 1% (v/v) antibiotics and antimycotic solution. Then, endometrial cells were incubated under the same conditions as in Experiments 1 and 2. After 24 h, cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method using a TOX-1 Kit (Sigma–Aldrich, #7H258), according to the manufacturer's instructions.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from cultured endometrial cells in Experiment 2 using the Total RNA Prep Plus Kit (A&A Biotechnology) according to the manufacturer's instructions. RNA samples were stored at −80 °C. Before use, RNA concentration and quality were determined spectrophotometrically and with agarose gel electrophoresis. The absorbance ratio at 260 and 280 nm ($A_{260/280}$) was ∼2. Then, 1 μg RNA was reverse transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, # 205311) according to the manufacturer's instructions. The cDNA was stored at −20 °C until real-time PCR was carried out.

Real-time PCR

Real-time PCR was performed with an ABI Prism 7300 sequence detection system using SYBR Green PCR Master Mix (Applied Biosystems, # 4309155). The sequences for equine *PTGS2*, *PGES*, and *PGFS* primers were published recently (Szóstek *et al.* 2012*b*). After a preliminary study, *ACTB* was chosen as the best housekeeping gene. The sequence for *ACTB* was described by Cappelli *et al.* (2008). All primers were synthesized by GenoMed (Warszawa, Poland). Total reaction volume was 20 μl and contained 1 μl cDNA (1 ng), 2 μl forward and reverse primers each (250 nM), and 10 μl SYBR Green PCR Master Mix. Real-time PCR was carried out as follows: initial denaturation (10 min at 95 °C), followed by 40 cycles of denaturation (15 s at 95 °C) and annealing (1 min at 60 °C). After each PCR, melting curves were obtained by stepwise increases in temperature from 60 to 95 °C to ensure single-product amplification. The product specificity was also confirmed by electrophoresis on 2% (w/v) agarose gel. The data were analyzed using the method described by Zhao & Fernald (2005).

Western blot

For immunoblotting, protein fractions were obtained from total cell protein. Endometrial cells were collected from plates on ice in RIPA buffer containing 150 mM NaCl, 50 nM Tris base, pH 7.2, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, and 5 mM EDTA, in the presence of a protease inhibitor cocktail (#11697498001; Roche). Lysates were sonicated and centrifuged at 12 000 *g* for 15 min at 4 °C. The protein samples were stored at −70 °C for further analysis. The protein concentration was determined using Bicinchoninic Acid Protein Assay Kit (#23225; Thermo Scientific, Rockford, IL, USA). Equal amounts (50 μg) of membrane fraction were dissolved in SDS gel-loading buffer (50 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, and 2% β-mercaptoethanol), heated to 95 °C for 5 min and separated on a 10 or 15% SDS–PAGE. Separated proteins were electroblotted using a semi-dry transfer method onto PVDF membranes (Immobilon-P Transfer Membrane; # IPVH00010; Millipore, Billerica, MA, USA) in transfer buffer (0.3 mM Tris buffer, pH 10.4, 10% methanol; 25 mM Tris buffer, pH 10.4, 10% methanol; and 25 mM Tris buffer, pH 9.4, 10% methanol, 40 mM glycine). After blocking in 5% non-fat dry milk in TBS-T buffer (Tris-buffered saline, containing 0.1% Tween-20) for 1.5 h at room temperature, the membranes were incubated overnight with rabbit polyclonal anti-PTGS2, PGES, and PGFS (dilution for three antibodies 1:500 respectively; #SAB4502491, Sigma; #T6079, Sigma; and #ab84327, Abcam) or mouse monoclonal anti-β-actin antibody (concentration 0.05 μg/ml; #A2228; Sigma) at 4 °C. Subsequently, the proteins were detected by incubating the membrane with secondary polyclonal anti-rabbit alkaline phosphatase-conjugated antibody (dilution 1:30 000;

Table 1 The basal *in vitro* prostaglandin (PG)E$_2$ and PGF$_{2\alpha}$ secretion by equine epithelial cells after 12, 24, 48, and 72 h of incubation

	Prostaglandin (PG)E$_2$ (ng/µg DNA)	Prostaglandin (PG)F$_{2\alpha}$ (ng/µg DNA)
12 h	0.430 ± 0.011	0.832 ± 0.03
24 h	2.54 ± 0.33	3.14 ± 0.32
48 h	3.647 ± 0.23	3.96 ± 0.32
72 h	4.32 ± 0.53	4.94 ± 0.072

for PTGS2, PGES, and PGFS; #A3812; Sigma) or secondary polyclonal anti-mouse alkaline phosphatase-conjugated antibodies (dilution 1:30 000; for ACTB; #3562, Sigma) for 1.5 h at room temperature. After washing again in TBS-T buffer, immune complexes were visualized using the alkaline phosphatase visualization procedure. For densitometric analyses, the blots were scanned and the specific bands were quantified using Kodak 1D Image Analysis Software (Eastman Kodak). β-actin was used as an internal control for protein loading.

PG and ovarian steroid determinations

The concentration of PGE$_2$ in the conditioned medium was determined using a PGE$_2$ Enzyme Immunoassay (EIA) Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. The concentration of PGF$_{2\alpha}$ was determined using the direct EIA method as described previously by Uenoyama *et al.* (1997) with modification. The standard curve for PGE$_2$ ranged from 16.5 to 1000 pg/ml. The intra- and inter-assay coefficients of variation (CV) were 2.0 and 3.2% respectively. The standard curve for PGF$_{2\alpha}$ ranged from 0.19 to 50 ng/ml and CV were 4.5 and 3.6% respectively.

The concentration of P$_4$ in blood plasma was determined using EIA as described previously (Skarzynski *et al.* 2009). The standard curve for P$_4$ ranged from 0.0925 to 25 ng/ml and intra- and inter-assay CV were 2.5 and 4.3% respectively. The antibodies (anti-P$_4$, code SO/91/4; kindly donated by Dr S Okrasa, Warmia-Mazury University, Olsztyn, Poland) were characterized previously (Ciereszko *et al.* 2001).

The concentrations of E$_2$ in blood plasma were assayed by RIA after extraction with diethyl ether (extraction efficiency: 90%) as described previously (Fitko *et al.* 1996). The antibody (anti-E$_2$, code BS/88/754; gift from Dr B Szafranska, Warmia-Mazury University, Olsztyn, Poland) was characterized previously (Szafranska *et al.* 2002). The intra- and inter-assay CV averaged 5.5 and

6.5% respectively. The E$_2$ standard curve ranged from 0.5 to 80 pg/ml, and the effective dose for 50% inhibition (ED50) of the assay was 1.98 pg/ml. The intra- and inter-assay CV averaged 5.3 and 6.5% respectively.

Statistical analysis

The data are shown as the mean ± S.E.M. of values obtained in separate experiments, each performed in quadruplicate. The statistical analysis of data from Experiment 1 and Experiment 3 was performed using parametric one-way ANOVA followed by Newmann–Keuls comparison test (GraphPad Software, version 5, San Diego, CA, USA). The statistical analysis of Experiment 2 data was performed using nonparametric one-way ANOVA Kruskal–Wallis followed by Dunn's test. The results were considered significantly different when $P < 0.05$.

Results

**Experiment 1: effect of ovarian steroids on time-dependent PG production by endometrial cells** Figure 1 shows typical morphology of cultured epithelial and stromal cells. Tables 1 and 2 show the basal *in vitro* PGE$_2$ and PGF$_{2\alpha}$ secretion by epithelial and stromal cells after 12, 24, 48, and 72 h of incubation.

At 24, 48, and 72 h, OT increased PGE$_2$ and PGF$_{2\alpha}$ secretion in both epithelial and stromal cells ($P < 0.01$; Figs 2, 3, 4 and 5B, C and D). After 12 h, in stromal cells, OT increased both PGE$_2$ and PGF$_{2\alpha}$ secretion compared with the control group ($P < 0.001$ and $P < 0.05$; Figs 4A and 5A).

After 12 h, E$_2$ increased PGE$_2$ secretion in epithelial cells ($P < 0.001$; Fig. 2A), while P$_4$, E$_2$, and P$_4$+E$_2$ increased PGE$_2$ secretion after 24 and 48 h ($P < 0.01$; Fig. 2B and C). Only P$_4$+E$_2$ increased PGE$_2$ secretion after 72 h ($P < 0.001$; Fig. 2D).

Table 2 The basal *in vitro* prostaglandin (PG)E$_2$ and PGF$_{2\alpha}$ secretion by equine stromal cells after 12, 24, 48, and 72 h of incubation

	Prostaglandin (PG)E$_2$ (ng/µg DNA)	Prostaglandin (PG)F$_{2\alpha}$ (ng/µg DNA)
12 h	1.04 ± 0.107	0.74 ± 0.2
24 h	2.12 ± 0.16	1.94 ± 0.43
48 h	4.12 ± 0.96	3.05 ± 0.23
72 h	5.01 ± 0.16	4.82 ± 0.22
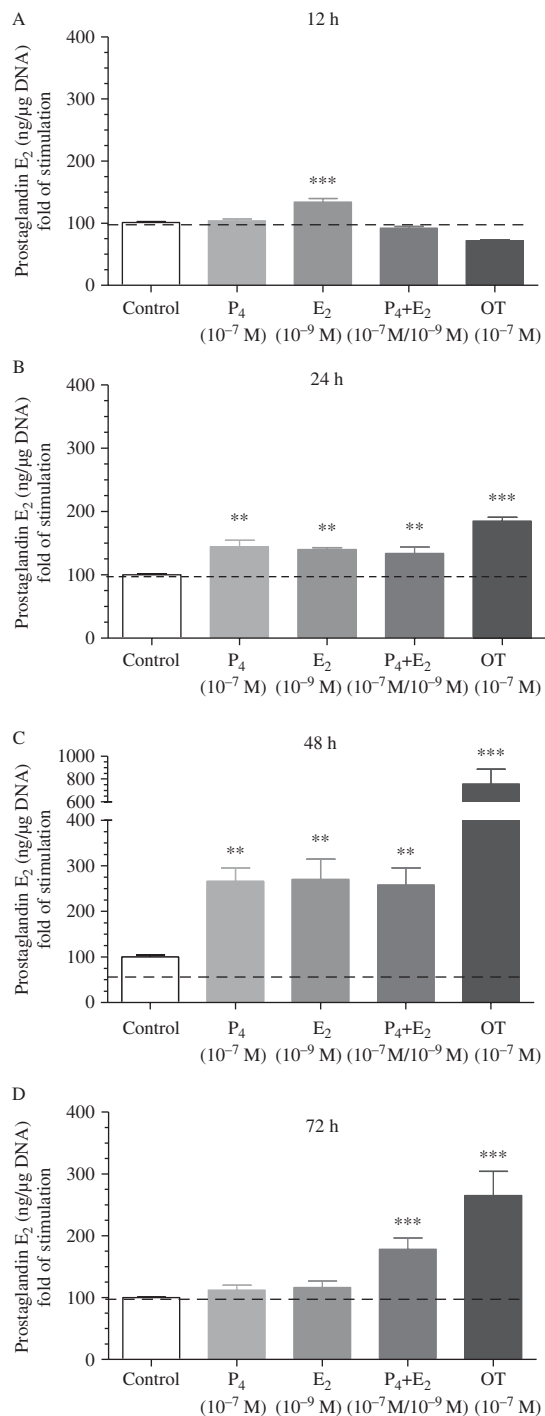
Figure 2

Effect of P_4 (10^{-7} M), 17β E_2 (10^{-9} M), and P_4+E_2 (10^{-7} M/10^{-9} M) on prostaglandin (PG)E_2 secretion by equine epithelial cells during incubation for (A) 12 h, (B) 24 h, (C) 48 h, and (D) 78 h. Oxytocin (OT; 10^{-7} M) was used as a positive control. All values are expressed as n-fold change from control. Asterisks indicate significant differences (**$P<0.01$ and ***$P<0.001$) from the respective control, as determined by parametric one-way ANOVA followed by the Newman–Keuls comparison test. The results were considered significantly different when $P<0.05$.

Secretion of $PGF_{2\alpha}$ was increased by E_2 and P_4+E_2 after 12, 48, and 72 h in epithelial cells ($P<0.05$; Fig. 3A, C, and D). After 24 h, all three treatments (P_4, E_2, and P_4+E_2) increased $PGF_{2\alpha}$ secretion ($P<0.01$, $P<0.05$, and $P<0.01$; Fig. 3B).

In stromal cells, P_4+E_2 increased PGE_2 secretion after 24 and 48 h ($P<0.001$ and $P<0.01$; Fig. 4B and C). After 72 h, P_4, E_2, and P_4+E_2 increased PGE_2 secretion ($P<0.05$, $P<0.05$, and $P<0.001$; Fig. 4D). After 24, 48, and 72 h, P_4, E_2, and P_4+E_2 increased $PGF_{2\alpha}$ secretion in stromal cells ($P<0.05$; Fig. 5B, C, and D).

Experiment 2: effect of ovarian steroids on PG expression in epithelial and stromal cell cultures

Experiment 2.1: effect of ovarian steroids on PG synthases mRNA transcriptions in epithelial and stromal cell cultures In epithelial cells, E_2 and P_4+E_2 upregulated *PTGS2* mRNA transcription compared with the control group ($P<0.05$; Fig. 6A). In the stromal cells, all ovarian steroids upregulated *PTGS2* mRNA transcription compared with the control group ($P<0.05$; Fig. 6B). Regarding *PGES* and *PGFS* mRNA transcriptions, P_4, E_2, and P_4+E_2 upregulated mRNA transcription of both enzymes in epithelial cells compared with the control group ($P<0.05$; Fig. 6C and E). In stromal cells, P_4 and the combination of P_4+E_2 upregulated *PGES* mRNA transcription compared with the control group ($P<0.01$; Fig. 6D). However, both E_2 and P_4+E_2 upregulated *PGFS* mRNA transcriptions in the stromal cells compared with the control group ($P<0.05$; Fig. 6F).

Experiment 2.2: effect of ovarian steroids on PG synthases protein expression in epithelial and stromal cell cultures In epithelial and stromal cells, E_2 and P_4+E_2 upregulated PTGS2 protein expression compared with the control group ($P<0.05$; Fig. 7A and B). In epithelial cells, all ovarian steroids upregulated PGFS and PGES protein expression compared with the control group ($P<0.05$; Fig. 7C and E).

Experiment 3: effect of ovarian steroids on epithelial and stromal cell proliferation

In epithelial cells, all ovarian steroid treatments increased cell proliferation during 24-h incubation ($P<0.05$; Fig. 8A). In stromal cells, E_2 and P_4+E_2 but not P_4 increased cell proliferation during 24-h incubation ($P<0.05$; Fig. 8B).

Discussion

This study demonstrates the response of homogeneous epithelial and stromal cells to P_4, E_2, or P_4+E_2. The second aim of the study was to verify whether the endometrial cell
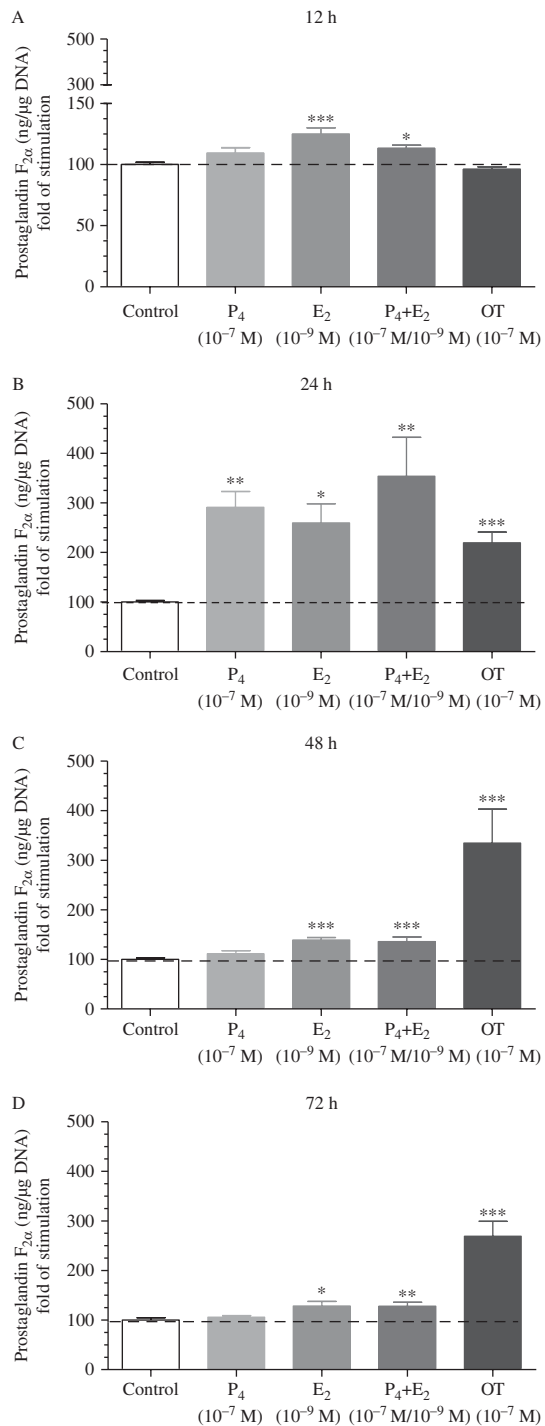
Figure 3

Effect of P_4 (10^{-7} M), 17β E_2 (10^{-9} M), and $P_4 + E_2$ (10^{-7} M/10^{-9} M) on prostaglandin (PG)$F_{2\alpha}$ secretion by equine epithelial cells during incubation for (A) 12 h, (B) 24 h, (C) 48 h, and (D) 78 h. Oxytocin (OT; 10^{-7} M) was used as a positive control. All values are expressed as *n*-fold change from control. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) from the respective control, as determined by parametric one-way ANOVA followed by the Newman–Keuls comparison test. The results were considered significantly different when $P < 0.05$.

capacity for producing PG is time dependent in response to ovarian steroids. In addition, our study examined regulation of PG synthases expression and cell proliferation by ovarian steroids.

Modulation of PG production by ovarian steroids in other domestic animal endometria is well described (Asselin *et al*. 1996, Skarzynski *et al*. 1999, Goff 2004, Blitek *et al*. 2007, Siemieniuch *et al*. 2010). However, data concerning the influence of E_2 and P_4 on PG production are still insufficient in the mare. It is notable that, in contrast to other animal species, *in vitro* experiments conducted into mare reproductive physiology are sparse. Only a few *in vitro* and *in vivo* studies concerning this issue are known but they have been carried out using different methodologies, times, and placement of ovarian steroid applications, dosage, or cell/tissue composition (Vernon *et al*. 1981, Zavy *et al*. 1984, Ababneh & Troedsson 2013, Galvão *et al*. 2013). Almost three decades ago, Vernon *et al*. (1981) carried out one of the first endometrial explant stimulations with ovarian steroids *in vitro*. In that study, the endometrial explants were obtained using two methods (Vernon *et al*. 1981). In the first experiment, explants were obtained from cyclic pony mares on days 4, 8, 12, 16, and 20 of the estrous cycle and then incubated for 2 h with P_4 or E_2. P_4 treatment did not affect $PGF_{2\alpha}$ production, but while E_2 was stimulatory during late diestrus, it had little or no effect during early diestrus (Vernon *et al*. 1981). In the second experiment, ovariectomized pony mares were injected over 3 weeks with P_4, E_2, or for 2 weeks with E_2 and then for 1 week with P_4. On day 21 of treatment, endometrial explants were incubated *in vitro* with ovarian steroids for 2 h (Vernon *et al*. 1981). Tissue exposure to P_4 *in vitro* failed to alter $PGF_{2\alpha}$ production. However, exposure of the tissue to E_2 *in vitro* resulted in a dose-dependent stimulation of $PGF_{2\alpha}$ production, with the greatest response to E_2 in tissues from mares treated with P_4 *in vivo* (Vernon *et al*. 1981). These authors proposed a hypothesis that P_4 is responsible for the production of the PG synthase system and/or the sequestration of PG precursors. The elaboration of this enzyme system or the recruitment of precursors may be slow and necessitate continuous, long-term P_4 exposure. Estrogen, on the other hand, may trigger or intensify PG synthase system in a response that is rapid and requires only short-term conditioning.

Subsequently, Zavy *et al*. (1984) demonstrated the influence of exogenous E_2 or P_4 on uterine luminal $PGF_{2\alpha}$ concentration in the ovariectomized mare. The pony mares received only E_2, P_4, or E_2 followed by P_4 (Zavy *et al*. 1984). P_4-treated mares had a higher uterine luminal
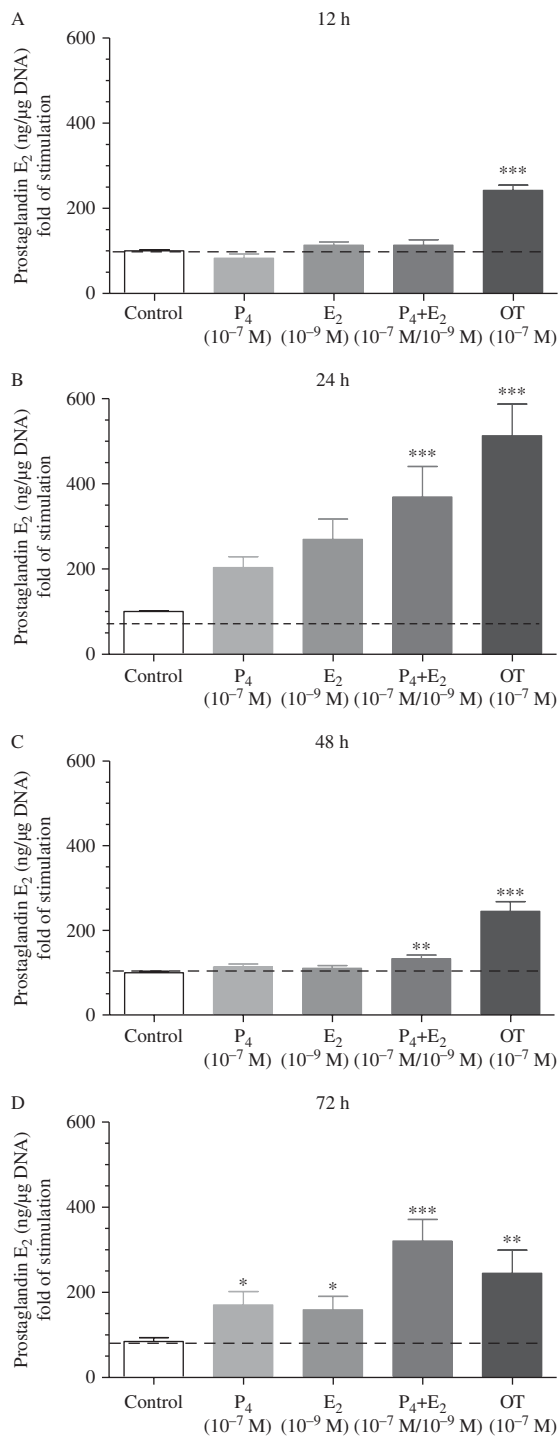
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Figure 4

Effect of P_4 (10^{-7} M), 17β E_2 (10^{-9} M), and $P_4 + E_2$ (10^{-7} M/10^{-9} M) on prostaglandin (PG)E_2 secretion by equine stromal cells during incubation for (A) 12 h, (B) 24 h, (C) 48 h, and (D) 78 h. Oxytocin (OT; 10^{-7} M) was used as a positive control. All values are expressed as n-fold change from control. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) from the respective control, as determined by parametric one-way ANOVA followed by the Newman–Keuls comparison test. The results were considered significantly different when $P < 0.05$.

$PGF_{2\alpha}$ concentration compared with all other groups, while no differences were detected among the other treatments (Zavy et al. 1984). Additionally, in the second experiment, uterine fluid was collected from mares before and after treatment with E_2 (7 days) followed by P_4 (50 days). The findings showed that after ovarian steroid treatment, uterine luminal $PGF_{2\alpha}$ concentration increased (Zavy et al. 1984). The data obtained from that study are not unequivocal and may reflect the particular manner of experimental design (Vernon et al. 1981, Zavy et al. 1984).

There is a lack of basic research to determine time-dependent PG production by homogeneous equine endometrial cells in response to ovarian steroids. At the cellular level, in our previous work, we have shown for the first time the effect of ovarian steroids on equine co-cultured stromal and epithelial cells in particular phases of the estrous cycle (Galvão et al. 2013). We showed previously that E_2 and $P_4 + E_2$ increased $PGF_{2\alpha}$ secretion by equine co-cultured endometrial cells from the mid luteal phase of the estrous cycle (Galvão et al. 2013). That study was the starting point for developing the methodology to verify the direct effect of ovarian steroids on homogeneous endometrial stromal and epithelial cells. Using the present culture method, we obtained a cell model minimal prior endogenous steroid influence. Results of treatment with E_2 imitated the follicular phase, P_4 mimicked the mid luteal phase and ovarian steroids in combination resembled the late luteal phase of the estrous cycle. The early luteal phase of the estrous cycle was selected based on a study in the cow (Fortier et al. 1988) showing that the estrous phase has a vast effect on uterine epithelial cell morphology and developmental patterns during primary in vitro culture. It has been confirmed that epithelial cell viability and attachment was the highest for epithelial cells obtained from days 4 to 6 compared to other days of the estrous cycle (Fortier et al. 1988).

Our findings showed clearly that the epithelial and stromal cells respond to ovarian steroids in a time-dependent manner. The E_2 seems to be a strong stimulator of PG production by epithelial cells, increasing production of PGE_2 as well as $PGF_{2\alpha}$ after only 12 h. All treatments stimulated PG production by the epithelial cells after 24 h. But the capacity of epithelial cells to produce $PGF_{2\alpha}$ in response to P_4 after 48 and 72 h as well as PGE_2 after 72 h was not maintained. Although epithelial cells comprise only a minor proportion of the endometrial tissue, they play an important role in domestic animal reproduction by secreting products into the uterine lumen that, e.g. interact with sperm and ovum, take part in implantation, cell proliferation, and angiogenesis. This may explain the
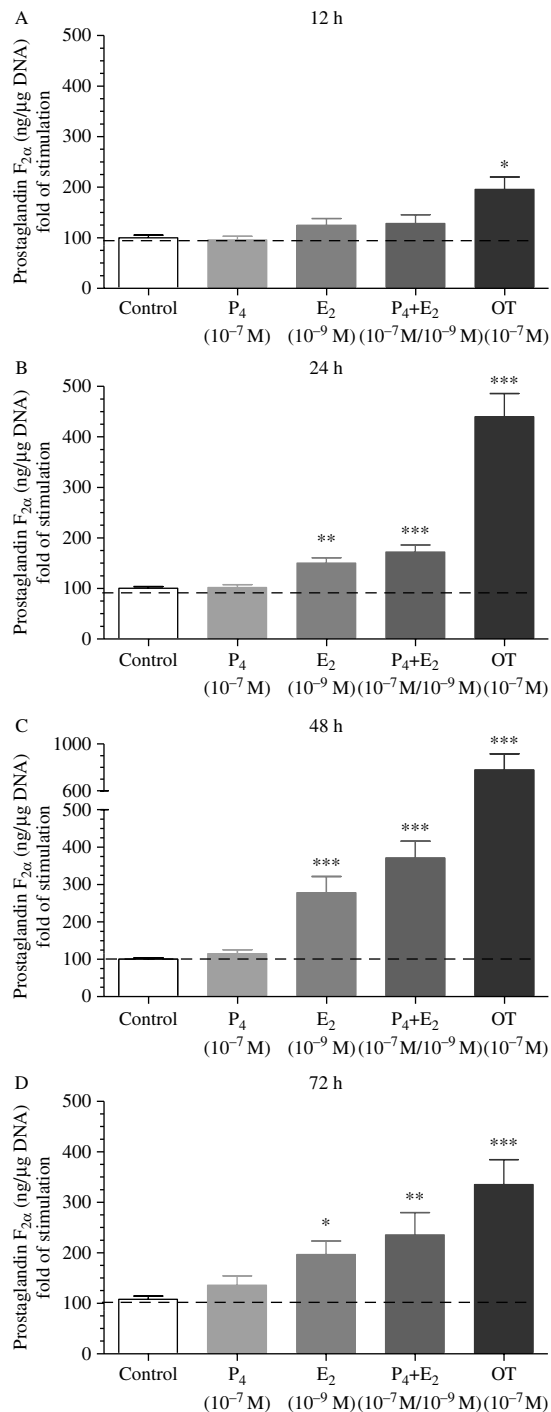
Figure 5

Effect P_4 (10^{-7} M), 17β E_2 (10^{-9} M), and P_4+E_2 (10^{-7} M/10^{-9} M) on prostaglandin (PG)$F_{2\alpha}$ secretion by stromal cells during incubation for (A) 12 h, (B) 24 h, (C) 48 h, and (D) 78 h. Oxytocin (OT; 10^{-7} M) was used as a positive control. All values are expressed as n-fold change from control. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$, and ***$P<0.001$) from the respective control, as determined by parametric one-way ANOVA followed by the Newman–Keuls comparison test. The results were considered significantly different when $P<0.05$.

high epithelial cell reactivity to treatment with ovarian steroids. In turn, the decrease in PG production by epithelial cells in response to ovarian steroids after 72 h may be connected with the physiology of epithelial cells that are much more sensitive to culture conditions than stromal cells (Szóstek *et al.* 2012a).

The time-dependent effect of ovarian steroids on PG production was more pronounced in stromal cells. Ovarian steroids did not affect PG production by stromal cells after 12 h, but after 24 and 48 h, P_4+E_2 stimulated PGE_2 production. After 72 h, the capacity of stromal cells to produce PGE_2 in response to all the ovarian steroid treatments was augmented.

Our findings are not mutually exclusive with results obtained by Vernon *et al.* (1981). As mentioned earlier, E_2 seems to act as a short-term and P_4 as a long-term stimulator. In general, as mentioned earlier, in our study, E_2 affected $PGF_{2\alpha}$ secretion from epithelial and stromal cells, but P_4 increased $PGF_{2\alpha}$ secretion only from epithelial cells. The endometrium is a tissue consisting of many types of cells. The response of endometrial tissue or co-culture is a result of the response by stromal as well as epithelial cells, which may differ from their individual responses. The difference between our study and that of Galvão *et al.* (2013) could be explained in this manner. Similarly, the P_4-stimulated $PGF_{2\alpha}$ increase in the uterine lumen reported in the study by Zavy *et al.* (1984) can be explained by the response of epithelial cells, which mostly secrete the uterine lumen compounds.

Until now, the influence of ovarian steroids on the AA metabolic pathway was not studied. Our study showed that PG production in response to ovarian steroids may be explained by upregulation of *PG* synthases expression in the endometrial cells. Generally, ovarian steroids upregulated PTGS2, PGES, and PGFS expression in epithelial cells and PTGS2 expression in stromal cells. The differences between *PG* synthases mRNA transcription and *PG* synthases may result from the combination of different posttranscriptional regulation processes. It was described that production and maintenance of cellular protein requires a vast series of linked processes, from transcription, processing, and degradation of mRNAs to translocation, localization, modification, and programmed destruction of the proteins themselves (Vogel & Marcotte 2012).

There are many studies in numerous other animal species concerning the influence of ovarian steroids on PG production (Salamonsen *et al.* 1991, Asselin *et al.* 1996, Blitek *et al.* 2007, Siemieniuch *et al.* 2010). The methodologies differ depending on the authors
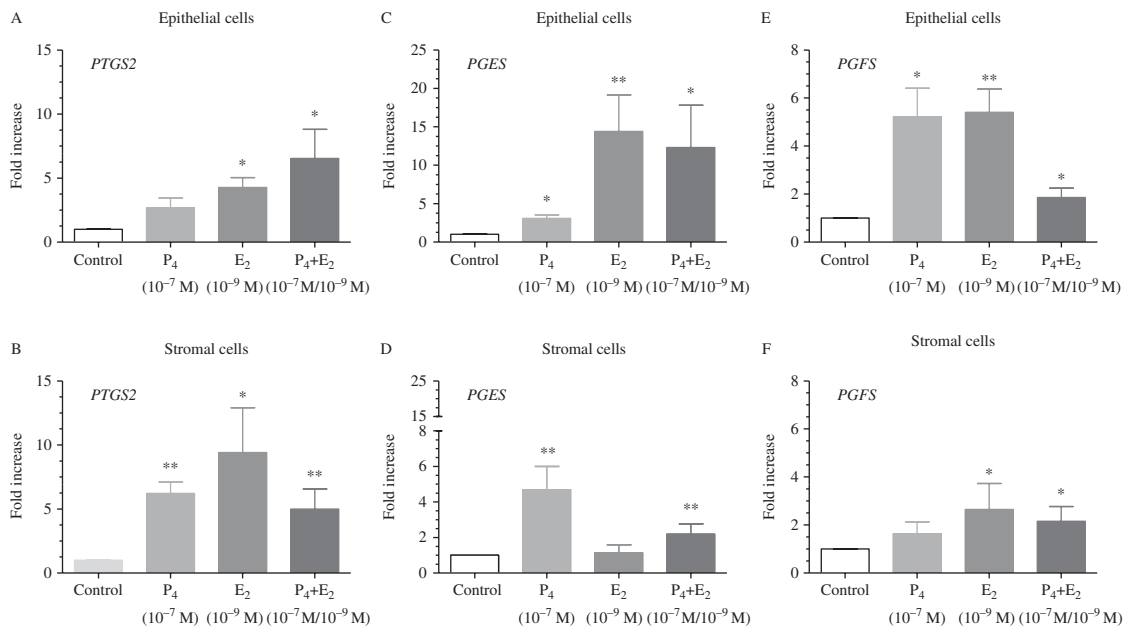
Figure 6

Effect of P_4 (10^{-7} M), 17β E_2 (10^{-9} M), and $P_4 + E_2$ (10^{-7} M/10^{-9} M) on prostaglandin-endoperoxide synthase-2 (*PTGS2*) (A and B), prostaglandin (PG)E_2 synthases (*PGES*) (C and D), or prostaglandin $F_{2\alpha}$ synthases (*PGFS*) (E and F) mRNA transcription in equine epithelial cells (A, C, and E) and stromal cells (B, D, and F) after 24-h incubation. Results are normalized against β-actin (*ACTB*). Data are presented as *n*-fold increase relative to control. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) from the respective control, as determined by nonparametric one-way ANOVA Kruskal–Wallis followed by Dunn's test. The results were considered significantly different when $P < 0.05$.

(Salamonsen *et al.* 1991, Asselin *et al.* 1996, Blitek *et al.* 2007). Asselin *et al.* (1996) showed an influence of ovarian steroids on PG production by endometrial epithelial and stromal cells obtained at days 2–5 of the estrous cycle (Asselin *et al.* 1996). In epithelial cells, E_2 decreased PGE_2 and $PGF_{2\alpha}$ production, in contrast to P_4, which increased secretion of both $PGF_{2\alpha}$ and PGE_2 after 72 h (Asselin *et al.* 1996). In the same study, in stromal cells, E_2 similarly decreased $PGF_{2\alpha}$ production after 72 h. The treatment and culture schemes proposed by Asselin *et al.* (1996) show significant differences from our methodology. However, the influence of P_4 on PG production by epithelial cells is in agreement with our findings. In the study by Asselin *et al.* (1996), endometrial cells were cultured in monolayers or on filter inserts to allow basal–apical polarization.

Nevertheless, data different from those obtained in our study were reported in the pig by Blitek *et al.* (2007). Those authors showed that ovarian steroids had no influence on $PGF_{2\alpha}$ secretion but $P_4 + E_2$ together increased PGE_2 secretion by stromal cells. In that study, the endometrial cells were obtained from days 12 to 13 of the estrous cycle and stimulated for 48 h in a medium with steroid-free calf serum alone or supplemented with P_4 or E_2 (Blitek *et al.* 2007). However, simple comparisons between

their findings and this study cannot be made because of differences in methodology. Additionally, the discrepancies in ovarian influence of steroids on PG production between the horse and other domestic animals may derive from different mechanisms consistent with anatomical and physiological differences.

PGs and ovarian steroids are fundamental components of estrous cycle regulation and take part in processes occurring in early pregnancy, e.g. embryo migration and development, implantation, or tissue growth and remodeling in the endometrium. We assumed that ovarian steroids through their influence on PG production modulate their action and indirectly affect luteolysis or CL maintenance, cell proliferation, and angiogenesis. It was confirmed that endometrial $PGF_{2\alpha}$ is a primary initiating factor for luteolysis (McCracken *et al.* 1999) in most non-primate species. The concentration of $PGF_{2\alpha}$ released from the uterus into the uterine vein is increased on day 14 of diestrus in the mare (Douglas & Ginther 1976). However, it is worthwhile to point out that our previous findings showed that $PGF_{2\alpha}$ decreased P_4 secretion by equine steroidogenic cells in the mid luteal phase of the estrous cycle (Lukasik *et al.* 2010). Luteal regression involves secretion of endometrial $PGF_{2\alpha}$ in
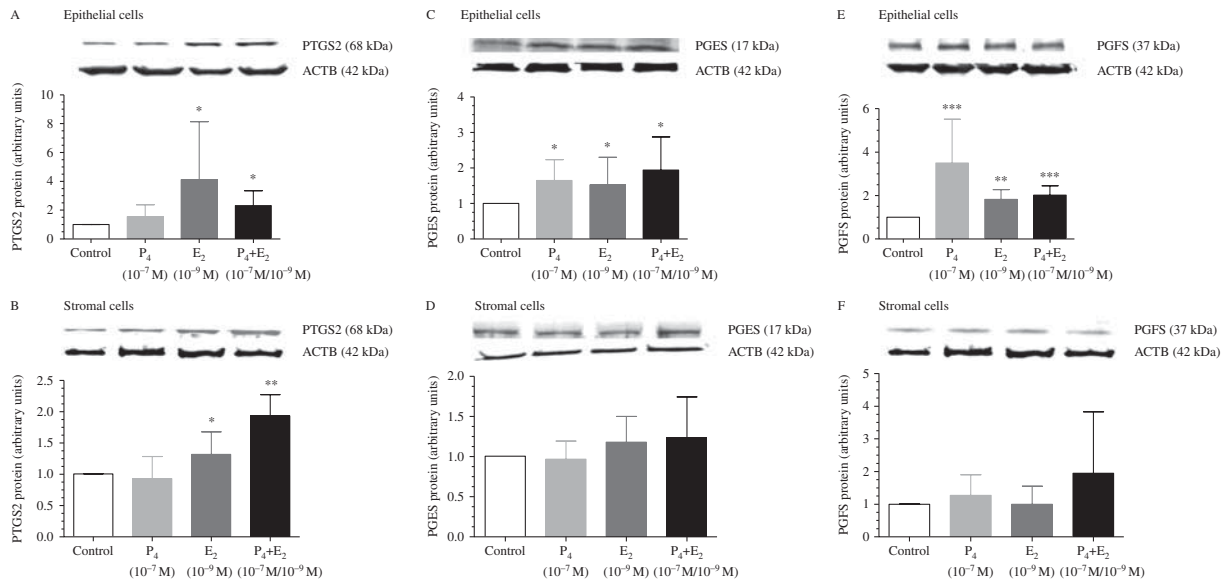
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Figure 7

Effect of P_4 (10^{-7} M), 17β E_2 (10^{-9} M), and $P_4 + E_2$ (10^{-7} M/10^{-9} M) on prostaglandin-endoperoxide synthase-2 (PTGS2) (A and B), prostaglandin (PG)E_2 synthases (PGES) (C and D), or prostaglandin $F_{2\alpha}$ synthases (PGFS) (E and F) protein expression in equine epithelial cells (A, C, and E) and stromal cells (B, D, and F) after 24-h incubation. Results are normalized against β-actin (ACTB). Data are presented as n-fold increase relative to control. Asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) from the respective control, as determined by nonparametric one-way ANOVA Kruskal–Wallis followed by Dunn's test. The results were considered significantly different when $P < 0.05$.

several sequential pulses during the luteolytic period (Ginther *et al.* 2009). It is important to note that E_2 is a determinant of luteolysis initiation in cattle (Araujo *et al.* 2009) but this effect was not confirmed in the mare (Ginther *et al.* 2011*a*). The temporal relationship between the raise in E_2 and spontaneous luteolysis that has been reported in cattle (Ginther *et al.* 2011*a*) was not detected in the mare (Ginther *et al.* 2011*b*). Thus, the mechanisms controlling endometrial $PGF_{2\alpha}$ secretion in the mare are not yet well understood. Bearing in mind our findings, the association of $P_4 + E_2$ may influence $PGF_{2\alpha}$ secretion at the time of luteolysis. However, this suggestion requires further *in vivo* studies.

The luteotropic action of PGE_2 produced by epithelial cells in response to P_4 must also be taken into consideration. The antiluteolytic action of PGE_2 *in vivo* was confirmed by Vanderwall *et al.* (1994) in the mare. Nonpregnant mares were continuously infused with PGE_2, on days 10–16 postestrus, using osmotic minipumps surgically placed into the uterine lumen on day 10. This intrauterine administration of PGE_2 maintained prolonged CL function. Moreover, upregulation of endometrial *PGES* mRNA transcription and PGE_2 in the mid luteal phase of the estrous cycle was established (Szóstek *et al.* 2012*b*). Additionally, Lukasik *et al.* (2010) showed

that PGE_2 upregulated P_4 secretion by equine luteal steroidogenic cells *in vitro*. Furthermore, the stimulatory effect of PGE_2 on P_4 production by steroidogenic cells was also confirmed in the cow *in vitro* (Kotwica *et al.* 2003).

Besides the endocrine role of PG in regulation of the estrous cycle, these molecules also play a role in auto/paracrine regulation of endometrial events, e.g. embryo implantation, cell proliferation, and angiogenesis. The uterus is an organ that exhibits continuous cyclic and dynamic changes that are necessary for its proper function. These changes are regulated by complex paracrine mechanisms occurring through the action of substances produced by the endometrial cells and by ovarian steroids produced. Endometrial vasculature changes are absolutely necessary to supply the nutrient and oxygen demands for endometrial gland function and embryo survival. It was stated that PGE_2 plays a role in promoting angiogenesis and inhibiting apoptosis (Tsujii & DuBois 1995, Tsujii *et al.* 1998). Both types of PG influence cell proliferation in human epithelial endometrial cells (Jabbour & Boddy 2003, Milne & Jabbour 2003). PGE_2 acts in an autocrine–paracrine manner on the PGE_2 receptor (EP) to trigger intracellular signal transduction cascades and transcription of pro-angiogenic factors such as vascular endothelial growth factors (VEGFs),
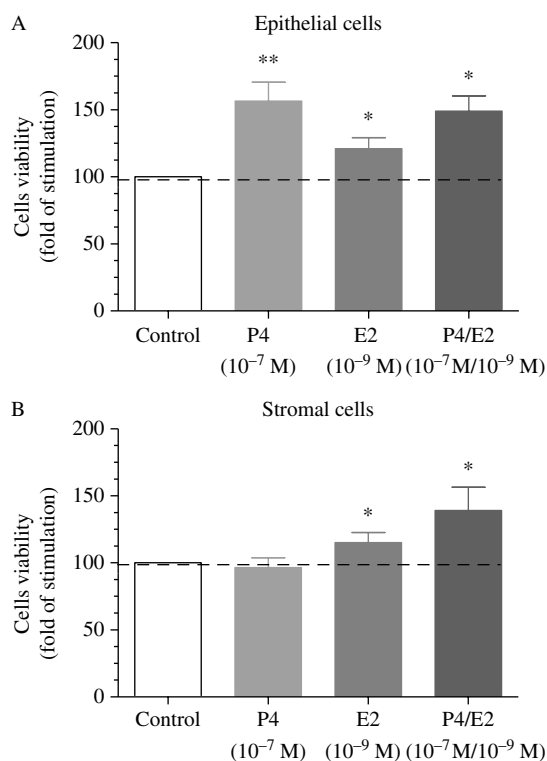
Figure 8
Effect of progesterone (P_4, 10^{-7} M), 17β E_2 (10^{-9} M), and $P_4 + E_2$ (10^{-7} M/10^{-9} M) on proliferation of equine (A) epithelial cells and (B) stromal cells after 24-h incubation. All values are expressed as *n*-fold change from control. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$) from the respective control, as determined by parametric one-way ANOVA followed by the Newman–Keuls comparison test. The results were considered significantly different when $P < 0.05$.

angiopoietin-1, and angiopoietin-2 (Tsujii *et al*. 1998, Sales *et al*. 2002). Then, these factors stimulate angiogenesis by acting on endothelial cells to cause vessel sprouting and tubular structure formation (Tsujii *et al*. 1998, Sales *et al*. 2002). Kaczmarek *et al*. (2008) reported that PGE_2 affects VEGF in porcine endometrial stromal cells. Therefore, ovarian steroids positively affected production of PGE_2, which is a potent angiogenic factor that could be indirectly involved in the regulation of vasculature events in the equine endometrium. Moreover, our previous data showed an increase in bovine aortic endothelial cell proliferation in response to incubation with culture media conditioned with follicular and mid luteal phase endometrial cells in the presence of ovarian steroids (Galvão *et al*. 2013). Additionally, the latter study determined that ovarian steroids increased nitric oxide production by endometrial cells. It can be assumed that ovarian steroids mediated PG production, which may affect cell proliferation and angiogenesis. However, this inference needs to be further tested.

In summary, the current study provides knowledge on the *in vitro* interactions between ovarian steroids and PG in the equine endometrium. The data showed that ovarian steroids affect PG secretion by equine endometrial epithelial and stromal cells in a time-dependent manner. Generally, ovarian steroids augmented PGE_2 and $PGF_{2\alpha}$ secretion by epithelial cells. Similarly, $P_4 + E_2$ augmented $PGF_{2\alpha}$ secretion by stromal cells. The upregulation of PTGS2, PGES, and PGFS expression in epithelial cells and PTGS2 expression in stromal cells seems to be the main cause of the endometrial response to ovarian steroids. The overall findings lead to the conclusion that PGE_2 and $PGF_{2\alpha}$ play important roles in local responses in the equine endometrium and as a result may be involved in many processes, e.g. regulation of the estrous cycle, cell proliferation, and angiogenesis. The results described here may contribute to a better understanding of endometrial physiology and form a basis for further research.

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