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⁺, 10⁻⁷ M), 17β estradiol (E₂, 10⁻⁹ M), or P⁺ + E₂ 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, E₂ and P⁺ + E₂ increased PGE₂ and PGF₂α secretion as well as specific prostaglandin-endoperoxide synthase-2 (PTGS2), PGE₂ synthases (PGES), and PGF₂α synthases (PGFS) expression in the epithelial cells (P < 0.05). Additionally, E₂ and P⁺ + E₂ increased PTGS2 expression in stromal cells after 24 h (P < 0.05). In stromal cells, P⁺ + E₂ increased PGE₂ production as well as PGES expression after 24 h (P < 0.05). Both E₂ and P⁺ + E₂ increased PGF₂α 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.

Introduction

Ovarian steroids regulate female cyclicity. Progesterone (P⁺) and 17β estradiol (E₂) are synthesized from cholesterol through a series of complex pathways involving different enzymatic conversions. The sources of E₂ are mainly granulosa cells of the ovarian follicle (Ryan & Short 1965) and the placenta (Pashen & Allen 1979). The E₂ regulates sexual behavior, enhances uterine motility, and promotes the secretory activity of the entire reproductive tract. By contrast, P⁺ 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.
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 PGE\(_{2\alpha}\) 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 (Atlí 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 \(\mu\)g/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 antymycotic 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 \(5 \times 10^5\) viable cells/ml for epithelial cells and...
2×10⁵ 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 immunofluorescent 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 P₄ (10⁻⁷ M), E₂ (10⁻⁹ M), or P₄ + E₂ (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 P₄ (10⁻⁷ M), E₂ (10⁻⁹ M), or P₄ + E₂ (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 Fenzol (A&A Biotechnology, Gdansk, Poland) was added and then the cells were collected and kept frozen until RNA isolation.
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 P4 (10^-7 M), E2 (10^-9 M), or P4 + E2 (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 (A260/280) 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. 2012b). 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 mM 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)E2 and PGF2α secretion by equine epithelial cells after 12, 24, 48, and 72 h of incubation

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Prostaglandin (PG)E2 (ng/μg DNA)</th>
<th>Prostaglandin (PG)F2α (ng/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0.430 ± 0.011</td>
<td>0.832 ± 0.03</td>
</tr>
<tr>
<td>24</td>
<td>2.54 ± 0.33</td>
<td>3.14 ± 0.32</td>
</tr>
<tr>
<td>48</td>
<td>3.647 ± 0.23</td>
<td>3.96 ± 0.32</td>
</tr>
<tr>
<td>72</td>
<td>4.32 ± 0.53</td>
<td>4.94 ± 0.072</td>
</tr>
</tbody>
</table>

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 Newman–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 PGE2 and PGF2α secretion by epithelial and stromal cells after 12, 24, 48, and 72 h of incubation.

At 24, 48, and 72 h, OT increased PGE2 and PGF2α 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 PGE2 and PGF2α secretion compared with the control group (P<0.001 and P<0.05; Figs 4A and 5A).

After 12 h, E2 increased PGE2 secretion in epithelial cells (P<0.001; Fig. 2A), while P4, E2, and P4 + E2 increased PGE2 secretion after 24 and 48 h (P<0.01; Fig. 2B and C). Only P4 + E2 increased PGE2 secretion after 72 h (P<0.001; Fig. 2D).

Table 2  The basal in vitro prostaglandin (PG)E2 and PGF2α secretion by equine stromal cells after 12, 24, 48, and 72 h of incubation

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Prostaglandin (PG)E2 (ng/μg DNA)</th>
<th>Prostaglandin (PG)F2α (ng/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1.04 ± 0.107</td>
<td>0.74 ± 0.2</td>
</tr>
<tr>
<td>24</td>
<td>2.12 ± 0.16</td>
<td>1.94 ± 0.43</td>
</tr>
<tr>
<td>48</td>
<td>4.12 ± 0.96</td>
<td>3.05 ± 0.23</td>
</tr>
<tr>
<td>72</td>
<td>5.01 ± 0.16</td>
<td>4.82 ± 0.22</td>
</tr>
</tbody>
</table>
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).

**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 $P<0.05$.

**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...
The results were considered significantly different when one-way ANOVA followed by the Newman–Keuls comparison test. Prostaglandin (PG)F2α for (A) 12 h, (B) 24 h, (C) 48 h, and (D) 72 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.

Figure 3
Effect of P4 (10−7 M), 17βE2 (10−8 M), and P4+E2 (10−7 M/10−8 M) on prostaglandin (PG)F2α 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.

Ovarian steroids on PG production in the mare
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 E2 and P4 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 P4 or E2. P4 treatment did not affect PGF2α production, but while E2 was stimulatory during late diestrus, it had little or no effect during early diestrus (Vernon et al. 1981). In the second experiment, ovariec-tomized pony mares were injected over 3 weeks with P4, E2, or for 2 weeks with E2 and then for 1 week with P4. On day 21 of treatment, endometrial explants were incubated in vitro with ovarian steroids for 2 h (Vernon et al. 1981). Tissue exposure to P4 in vitro failed to alter PGF2α production. However, exposure of the tissue to E2 in vitro resulted in a dose-dependent stimulation of PGF2α production, with the greatest response to E2 in tissues from mares treated with P4 in vivo (Vernon et al. 1981). These authors proposed a hypothesis that P4 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 P4 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 E2 or P4 on uterine luminal PGF2α concentration in the ovariec-tomized mare. The pony mares received only E2, P4, or E2 followed by P4 (Zavy et al. 1984). P4-treated mares had a higher uterine luminal
PGF\textsubscript{2a} 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\textsubscript{2} (7 days) followed by P\textsubscript{4} (50 days). The findings showed that after ovarian steroid treatment, uterine luminal PGF\textsubscript{2a} 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\textsubscript{2} and P\textsubscript{4} increased PGF\textsubscript{2a} 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\textsubscript{2} imitated the follicular phase, P\textsubscript{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 \textit{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\textsubscript{2} seems to be a strong stimulator of PG production by epithelial cells, increasing production of PGE\textsubscript{2} as well as PGF\textsubscript{2a} 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\textsubscript{2a} in response to P\textsubscript{4} after 48 and 72 h as well as PGE\textsubscript{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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4}
\caption{Effect of P\textsubscript{4} (10\textsuperscript{\textminus7} M), 17\textbeta E\textsubscript{2} (10\textsuperscript{\textminus8} M), and P\textsubscript{4}+E\textsubscript{2} (10\textsuperscript{\textminus7} M/10\textsuperscript{\textminus9} M) on prostaglandin (PG)E\textsubscript{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\textsuperscript{\textminus7} M) was used as a positive control. All values are expressed as n-fold change from control. Asterisks indicate significant differences (*P \textless 0.05, **P \textless 0.01, and ***P \textless 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 \textless 0.05.}
\label{fig:figure4}
\end{figure}
The results were considered significantly different when one-way ANOVA followed by the Newman–Keuls comparison test. 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.

**Figure 5**
Effect \( P_4 \) \((10^{-7} \text{M})\), \( 17\beta \text{E}_2 \) \((10^{-9} \text{M})\), and \( P_4 + \text{E}_2 \) \((10^{-7} \text{M}/10^{-9} \text{M})\) on prostaglandin (PG)\( F_{2\alpha} \) secretion by stromal cells during incubation for (A) 12 h, (B) 24 h, (C) 48 h, and (D) 72 h. Oxytocin (OT; \( 10^{-7} \text{M} \)) 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.

Ovarian steroids did not affect PG production by stromal cells after 12 h, but after 24 and 48 h, \( P_4 + \text{E}_2 \) 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, \( \text{E}_2 \) seems to act as a short-term and \( P_4 \) as a long-term stimulator. In general, as mentioned earlier, in our study, \( \text{E}_2 \) affected PG\( F_{2\alpha} \) secretion from epithelial and stromal cells, but \( P_4 \) increased PG\( F_{2\alpha} \) 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 PG\( F_{2\alpha} \) 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 PTG\( S_2 \), PGES, and PGFS expression in epithelial cells and PTG\( S_2 \) 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.
The influence of P4 on PG production by epithelial cells is significant. Differences from our methodology. However, decreased PGF2 secretion of both PGF2 and PGE2 secretion by stromal cells. In that study, the synthetic phospholipid (PG)E2 synthases (PGES) (C and D), or prostaglandin F2 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.

Effect of P4 (10⁻⁷ M), 17β E2 (10⁻⁹ M), and P4+E2 (10⁻⁷ M/10⁻⁹ M) on prostaglandin-endoperoxide synthase-2 (PTGS2) (A and B), prostaglandin (PG)E2 synthases (PGES) (C and D), or prostaglandin F2 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.

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 PGF2α secretion but P4+P2 together increased PGE2 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 P4 or E2 (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, and angiogenesis. It was confirmed that endometrial PGF2α is a primary initiating factor for luteolysis (McCracken et al. 1999) in most non-primate species. The concentration of PGF2α 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 PGF2α decreased P4 secretion by equine steroidogenic cells in the mid luteal phase of the estrous cycle (Lukasik et al. 2010). Luteal regression involves secretion of endometrial PGF2α in

(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, E2 decreased PGE2 and PGF2α production, in contrast to P4, which increased secretion of both PGF2α and PGE2 after 72 h (Asselin et al. 1996). In the same study, in stromal cells, E2 similarly decreased PGF2α 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 P4 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.
Figure 7
Effect of P4 (10–7 M), 17β E2 (10–8 M), and P4+E2 (10–7 M/10–9 M) on prostaglandin-endoperoxide synthase-2 (PTGS2) (A and B), prostaglandin (PG)E2 synthases (PGES) (C and D), or prostaglandin F2α 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 E2 is a determinant of luteolysis initiation in cattle (Araujo et al. 2011a). The temporal relationship between the raise in E2 and spontaneous luteolysis that has been reported in cattle (Ginther et al. 2011a) was not detected in the mare (Ginther et al. 2011b). Thus, the mechanisms controlling endometrial PGF2α secretion in the mare are not yet well understood. Bearing in mind our findings, the association of P4+E2 may influence PGF2α secretion at the time of luteolysis. However, this suggestion requires further in vivo studies.

The luteotropic action of PGE2 produced by epithelial cells in response to P4 must also be taken into consideration. The antiluteolytic action of PGE2 in vivo was confirmed by Vanderwall et al. (1994) in the mare. Nonpregnant mares were continuously infused with PGE2 on days 10–16 postestrus, using osmotic minipumps surgically placed into the uterine lumen on day 10. This intrauterine administration of PGE2 maintained prolonged CL function. Moreover, upregulation of endometrial PGES mRNA transcription and PGE2 in the mid luteal phase of the estrous cycle was established (Szóstek et al. 2012b). Additionally, Lukasik et al. (2010) showed that PGE2 upregulated P4 secretion by equine luteal steroidogenic cells in vitro. Furthermore, the stimulatory effect of PGE2 on P4 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. embryonic 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 embryonic survival. It was stated that PGE2 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). PGE2 acts in an autocrine–paracrine manner on the PGE2 receptor (EP) to trigger intracellular signal transduction cascades and transcription of pro-angiogenic factors such as vascular endothelial growth factors (VEGFs),
Ovarian steroids on PG production in the mare

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2002). Kaczmarek 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 PGE2 and PGF2α secretion by epithelial cells. Similarly, P4 + E2 augmented PGF2α 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 PGE2 and PGF2α 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.

![Figure 8](http://joe.endocrinology-journals.org/C209)

**Effect of progesterone (P4, 10^{-7} M), 17βE2 (10^{-9} M), and P4+E2 (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.

<table>
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<tr>
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<th>Stromal cells</th>
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<td>Control</td>
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<td>100</td>
</tr>
<tr>
<td>P4 (10^{-7} M)</td>
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<td><em>150</em>*</td>
</tr>
<tr>
<td>E2 (10^{-9} M)</td>
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<td><em>100</em>*</td>
</tr>
<tr>
<td>P4/E2 (10^{-7}M/10^{-9} M)</td>
<td><em>200</em>*</td>
<td><em>200</em>*</td>
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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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