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Effect of the duration of estradiol priming prior to progesterone administration on endometrial gene expression in anestrous mares

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ABSTRACT

Field data indicate that a longer period of estrus prior to ovulation correlates positively with fertility. To test the hypothesis that the duration of exposure to estrogens prior to progesterone dominance influences endometrial function, we used anestrous mares to simulate varying durations of estrus (3 groups of 5 mares): long (LE), short (SE), and no estrus (NE), as determined by the duration of estradiol priming prior to progesterone treatment: 7, 2 and 0 days for the LE, SE and NE, respectively. Endometrial biopsies were recovered 4 days after progesterone administration in all groups for real time quantitative reverse transcription PCR (RT-qPCR) and immunohistochemical analyses. A total of 17 genes believed to contribute to a "receptive endometrium" for embryo development and viability were evaluated by RT-qPCR. Of the genes evaluated, the expression of FGF-2 (fibroblast growth factor-2) decreased with increased length of preceding estrus, whereas P19 (uterocalin) expression was higher in the LE than in the SE or NE groups. In conclusion, a lower abundance of FGF-2 and higher abundance of uterocalin, a lipocalin protein known to play an important role in providing lipids to the embryo, could contribute to a more receptive endometrium in mares following a long estrus.

1. Introduction

The estrous cycle of the mare is divided into two distinct phases: a) the luteal phase or diestrus, during which progesterone produced by the corpus luteum is the dominant ovarian steroid hormone; and b) the follicular phase or estrus, which is characterized by basal progesterone but elevated estrogen concentrations [1]. The inter-ovulatory interval (IOI) spans the entire duration of the estrous cycle and averages 21 days in length, with a range of 16–28 days [2]. Whereas diestrus has a relatively constant duration (with a reported mean of 14.9 d), the duration of estrus, i.e. the period between the end of the luteal phase (luteolysis) and ovulation, can vary markedly between mares and cycles, with a reported range of 3–13 d [1]. One of the most commonly used indicators of the estrous phase in mares is the presence of endometrial edema, which is a result of relatively high circulating estrogen concentrations combined with low progesterone [3]. Indeed, the endometrial edema profile of mares during estrus correlates well with circulating 17-β estradiol concentrations [4].

Differences in the duration of estrus, as indicated by ultrasonographically visible endometrial edema, appear to influence fertility. Recent studies have shown a positive correlation between the length of estrus and likelihood of pregnancy in mares following estrus induced with PGF2α analogues [5,6] or after spontaneous luteolysis [7]. Moreover, since embryo recovery from embryo transfer donors and the likelihood of pregnancy in Thoroughbred mares both increased with the length of the preceding estrus, a longer duration of estrus seems to be beneficial for fertilization, presumably by improving either oocyte quality and/or the oviductal environment.

However, it is less clear whether the length of estrus also influences the uterine environment and, in particular, receptivity and ability of the endometrium to support embryonic growth and development. Nevertheless, the switch in ovarian steroid dominance (estradiol to progesterone) during the transition from estrus to diestrus has been reported to affect endometrial gene expression

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in a manner that should help support embryo survival [8]. Moreover, we recently reported a positive correlation between the duration of estrus prior to embryo transfer into a recipient mare and the subsequent likelihood of pregnancy [9], which suggests a positive effect of a long preceding estrus on the quality of the endometrial environment, in terms of its ability to successfully support the establishment of pregnancy.

The importance of the pre-ovulatory period of estrogen dominance for subsequent fertility is also assumed in the protocols designed for using acyclic mares as embryo transfer recipients, i.e. estradiol priming prior to progestagen administration [10]. In this respect, it was shown [11] that ovariectomized mares treated with exogenous steroid hormones showed a uterine secretion composition similar to normal cycling mares and, in particular, that pretreatment with estradiol induced the secretion of some unique proteins of non-serum origin (one of them being indistinguishable in electrophoretic properties from pig uteroferrin). Hinrichs and coworkers [12] reported that failure to administer estrogens prior to progesterone administration to ovariectomized mares destined to receive embryos, resulted in the absence of the same estrogen-induced proteins.

In short, while it is increasingly clear that estrogen priming is important to optimally prepare the uterus to receive an embryo, it is not clear how estrogen exposure prior to ovulation alters endometrial function for its role in supporting embryo survival and development. One approach is to select a panel of endometrial genes known or thought to be involved in the support and development of the conceptus during the mobile phase and/or known to be regulated by estrogens, and to study how they are influenced by varying durations of estrogen priming. Therefore, the objective of this study was to determine the effect of the duration of estradiol treatment prior to progesterone administration on endometrial expression of selected genes and proteins 4 days after the onset of progesterone treatment in seasonally anestrous mares. It was hypothesized that a difference in the length of exposure to estradiol prior to progesterone treatment would influence the expression of endometrial genes thought to play an important role in supporting embryo development. To test this hypothesis, a panel of 17 genes meeting the above criteria were selected from previous publications [8,13].

2. Material and methods

2.1. Animals

Fifteen mixed-bred mares, ranging from five to 15 years old and weighing 330–490 kg were used in this study. Mares were maintained on tifton hay (*Cynodon dactylon*) with water and trace-mineralized salt provided *ad-libitum*. The experiment was conducted during the month of July at a commercial horse farm in Uberlândia, Minas Gerais, Brazil (Southern hemisphere). All animal procedures were completed in accordance with, and the approval of, the Ethics Commission on Animal Use from the Federal University of Uberlândia (CEUA UFU 077/17).

Only seasonally anestrous mares were used; they were selected based on ovarian activity, i.e., mares presenting ovarian follicles <20 mm in diameter, in the absence of visible uterine edema or a CL on three ultrasound examinations performed at seven-day intervals.

2.2. Hormone treatments

The selected anestrous mares were randomly assigned to three different groups: (1) Long estrus (LE): Five mares were treated with a total of 8 mg of estradiol benzoate (EB; Estrogin, Biofarm, Jaboticabal, SP, Brazil) administered in increasing doses; Day −7 (0.5 mg), Day −6 (1 mg), Day −5 (1.5 mg), Day −4 (2 mg) and Day −3 (3 mg) followed, 3 days later, by1500 mg of long acting progesterone (LA P4; Sincrogest Injetável, Ourofino Saude Animal, Cravinhos, SP, Brazil) once on Day 0; (2) Short estrus (SE): Five mares were treated with 2.5 mg of EB once on Day −2, followed by 1500 mg of LA P4 once on Day 0; and (3) No estrus (NE): Five mares were treated with 1500 mg of LA P4 once without previous EB treatment (Fig. 1).

2.3. Blood collection and steroid hormone assay

Blood samples were obtained by jugular venipuncture into heparinized tubes every 24 h between Day −7 and Day 4 (Day 0 = Day of progesterone administration). Samples were centrifuged (900×*g* for 10 min) and plasma was harvested and stored at − 20 °C

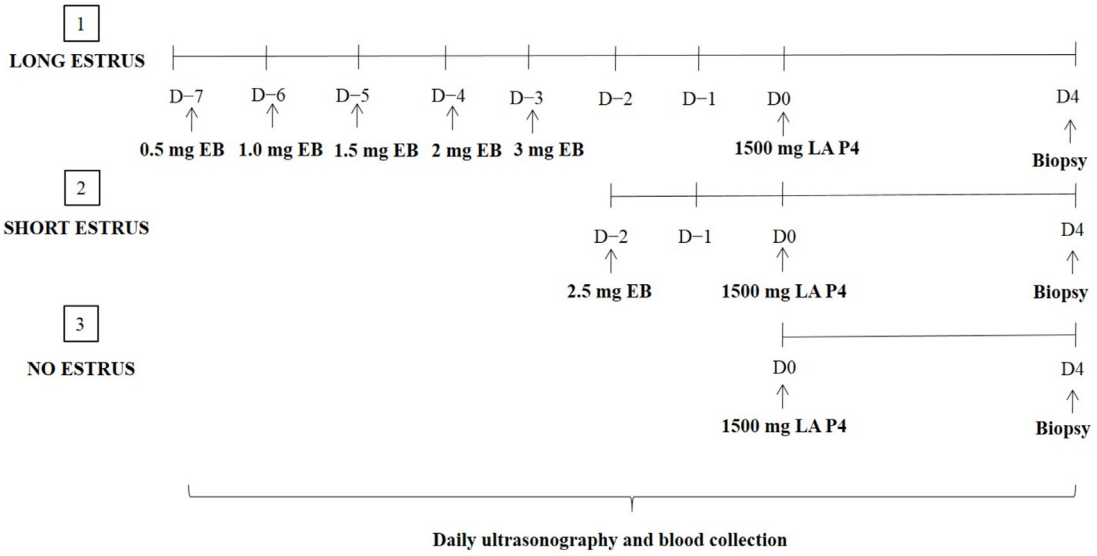

Fig. 1. Representative scheme for the estradiol benzoate (EB) followed by long-acting progesterone (LA P4) treatments in the long estrus and short estrus groups, as well as the LA P4 treatment in the no estrus group.

until used for hormone assay. The concentrations of plasma 17β-estradiol (E2) were determined daily from Day − 7 until Day 1 (1 day after progesterone treatment) whereas progesterone concentration was determined from Day 0 (just before progesterone treatment), to confirm the basal progesterone concentration expected in anestrous mares, to Day 4. Commercial radioimmunoassay kits were used to determine concentrations of 17β-estradiol and progesterone: RIA-4381 (DRG diagnostics GmbH, Germany), and IM 1188, (Beckman coulter, The Netherlands), respectively, as described previously in the horse [14], and validated in our laboratory [15]. The sensitivity of the assays, and the inter-assay and intra-assay coefficients of variation for 17β-estradiol and progesterone were 1.5 pg/mL, 11.5%, 3.6% and 0.1 ng/mL, 4.1% and 6.8%, respectively.

2.4. Transrectal palpation and ultrasound examinations

Transrectal palpation and B-mode ultrasonography were performed immediately before the start of hormone treatment and thereafter on a daily basis until four days after LA P4 administration. During the ultrasound examinations endometrial edema was evaluated and received a subjective score graded from 0 to 4 based on the prominence of the endometrial folds (0 = absence of uterine edema; 4 = intense edema and obvious endometrial folds).

2.5. Endometrial biopsy collection

Endometrial biopsies were recovered transcervically from the base of one of the uterine horns on Day 4 in all groups (Day 0 = Day of progesterone treatment), using an alligator jaw biopsy forceps (Botupharma, Botucatu, SP, Brazil). Biopsies were divided into two parts; one part was snap frozen in liquid nitrogen and stored at − 80C until being prepared for RT-qPCR, while the other part was fixed for 24h in 4% paraformaldehyde and then stored in 70% alcohol before being embedded in paraffin in preparation for immunohistochemistry (IHC).

2.6. Reverse transcription qPCR (RT-qPCR)

Endometrial RNA was extracted using a commercial kit (Invisorb® Spin Cell RNA Mini/Midi Kit) in accordance with the manufacturer's recommendation. Total RNA quantity and quality was determined by spectrometry (NanoDrop ND 1000; Isogen Life Sciences, De Meern, The Netherlands) and using an Agilent BioAnalyzer 2100 (Agilent, Palo Alto, CA) with an RNA 6000 Nano Chip, according to the manufacturers' instructions. Only samples showing a RIN higher than 8 were used for subsequent reverse transcription. Total RNA (1 μg) was treated with DNAse I for 30 min at 37C and 10 min at 65C (1 IU/μg; RNase-free DNase kit; Qiagen), and was followed by reverse transcription. The reaction was performed with 1 μg of total RNA in a final reaction volume of 20 μL, composed of 1x First Strand Buffer (Invitrogen, Breda, The Netherlands), 10 mM DTT (Invitrogen), 0.5 mM dNTPs (Promega, Leiden, The Netherlands), 1.8 U/ml Random primer (Invitrogen), 0.4 U/μl RNAsin (Promega) and 7.5 U/μL of Superscript III (Invitrogen). The reaction was incubated for 60 min at 50C and 5 min at 80C. Negative reverse transcription products were prepared from 0.5 μg of RNA using the same protocol, but omitting the superscript III.

All primer pairs were designed using Perl-Primer software (v1.1.14; Marshall 2004) based on the equine coding sequence, and were produced by Eurogentec (Seraing, Belgium). Primer details are listed in Table 1. Specificity of the PCR product for each target gene was assessed by DNA sequencing (ABI PRISM 310 Genetic analyzer, Applied Biosystem, Foster City, USA). For each gene, a standard curve was produced using a 10-fold serial dilution of a known quantity of the specific PCR product, to quantify expression. PCR was carried out in a 15 μL reaction mixture containing 1 μL of cDNA sample, 0.075 μL of primer (forward and reverse; Table 1), and 7.5 μL iQ SYBR® Green Supermix (Bio-Rad Laboratories) on an IQ5 real-time PCR detection system (Bio-Rad Laboratories, Veenendaal, The Netherlands). Cycle conditions were: denaturation for 3 min at 95C, followed by 40 cycles of amplification (15 s at 95C, 30 s at the primer specific annealing temperature and 30 s at 72C). This was followed by 1 min at 95C, 1 min at 55C and finished with a melting curve. Product specificity was evaluated by reading the melting

Table 1
Primer pairs used for the target and housekeeping genes for RT-qPCR on equine endometrium: gene name, annealing temperature (Ta), product size, sequences and accession number.

Name	Ta (℃)	Prod. Size (bp)	Forward	Reverse	Acc. nummer
GM2a	60	210	AAAGCCTGACTCTGGAACCT	GTCAAAGCGTCTAGTATATCACAG	XM_005599134.3
P19	64	104	TGAGCCGAAATTCATAGCGA	CCTCTTACCTTTCTGTGGAAGTG	NM_001082509.2
SLC36A2	60	210	GTTCAAAGATCCCGACACTG	CACACTGTCAAGAGGAATTTATGG	XM_005599277.3
HSPB7	63	184	AGCTTCTTGTCAACCTTCCC	GAATGTTCTGAATTCGGCCTC	XM_003364459.4
CRYAB	60	193	ATCTCTTCCCAACCTCTACTTCC	CACCTCAATCACATCTCCCA	XM_001501779.5
HSPB8	60	133	CACATTCTAAAGTTGCCAAACC	AATTGAGAGCCCAGAATAGGA	XM_001490413.4
IGF1	60	137	ACGCTCTTCAGTTCGTGTGT	CAGCCTCCTCAGATCACAGC	NM_001082498.2
SLC2A1	63	308	CACGGGCTTCGTGCCCATGT	GGGTCACGTCTGCCGTTCCG	NM_001163971
SLC2A3	58	233	CCAGGAGATGAAAGATGAGAG	ATAGTATTAACCACACCCGCA	XM_001498757.4 XM_023643092.1 XM_023643091.1 XM_023643090.1 XM_023643089.1
FGF7	62	254	TAAACGAGGCAAAGTCAAAGG	ACAAACATTTCTCCTCCACTG	NM_001163883.1
FGF9	60	254	ACGGCACCAGAAATTCACAC	AAATCAGCATGTTCCCATCCA	XM_005601094.3
FGF2	62	132	GAACCGTTATCTTGCTATGAAGG	CCAACTGGAGTATTTCCTTGAC	NM_001195221.1
ER-alpha	55	341	TCCATGATCAGGTCCACCTTCT	GGTGTCTGTCATCTTGTCCA	NM_001081772.1
ER-beta	60	194	TCAGCCTGTTCGACCAAGTG	CCTTGAAGTCGTTGCCAGGA	XM_001915519.2
PR	55	255	GTCAGTGGACAGATGCTGTA	CGCCTTGATGAGCTCTCTAA	XM_001498494.3
PGMRC1	58	280	TCAACGGCAAGGTGTTCGAC	GGCTCTTCCTCATCTGAGTA	XM_001914705.2
SPP1	54	268	AAACTGATCCTACTGATGACC	ATCTATGTCCTTGCTTTCCAC	XM_001496152.4
HPRTI	58	232	GAGATGTGATGAAGGAGATGG	CTTTCCAGTTAAAGTTGAGAGG	XM_001490189.2
PGK1	54	151	CTGTGGGTGTATTTGAATGG	GACTTTATCCTCCGTGTTCC	XM_001502668.3
SRP14	55	101	CTGAAGAAGTATGACGGTCG	CCATCAGTAGCTCTCAACAG	XM_001503583.2

curve with the iQ5 optical system software, and target gene concentrations were quantified by reference to their specific standard curve. Finally, relative gene expression was expressed as the ratio of target gene expression to the geometric mean for three reference genes (HPRT1, PGK1 and SRP14) validated to show stable expression using geNorm 3.5 [16].

2.7. Immunohistochemistry

Immunohistochemistry for estrogen receptor alpha (ERα) and for progesterone membrane receptor (PGMRC1) was performed on 5-mm sections cut from the paraffin blocks and mounted on SuperFrost Plus slides (ThermoFisher scientific, Waltham, MA, USA). The sections were deparaffinised and rehydrated by immersion in xylene (2 × 5 min) followed by decreasing concentrations of ethanol (EtOH; 100%, 96%, 70%: 2 × 3 min; Klinipath) and then rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked by immersing the sections in 1% H_2O_2 in methanol for 30 min. Antigen retrieval was performed by microwaving (at 840 W) for 30 min in preheated citrate-buffer (0.2% citrate, pH 6.0). After microwaving, the slides were cooled to room temperature over 30 min and then rinsed in PBS–0.05% Tween (PBST; 3 × 5 min). To block non-specific binding, sections were incubated with goat serum (diluted 1:10 in PBS) for 15 min at room temperature, then incubated with the specific primary antibody overnight at 4 C. The primary antibodies used were a mouse monoclonal anti-ERα (diluted 1:25 in PBS; ab C-311: sc787, Sigma Aldrich, Hertogenbosch, The Netherlands) and a rabbit polyclonal anti-PGRMC1 (progesterone receptor membrane component 1, diluted 1:100 in PBS; ab PGRMC1 10857, Sigma Aldrich, Hertogenbosch, The Netherlands). After rinsing in PBST (3 × 5 min), sections were incubated with the secondary biotinylated horse anti-mouse (for ERα) or horse anti-rabbit (for PGRMC) antibody (diluted 1:250 in PBS; BA-1000; Vector Laboratories, Peterborough, UK) for 30 min at room temperature. Sections were rinsed in PBS (3 × 5 min), then incubated with avidin-biotin-complex (ABC)–peroxidase (Vectastain ABC Kit; PK-4000; Vector Laboratories, Peterborough, UK) for 30 min at room temperature. After washing in PBS (3 × 5 min), the slides were incubated in freshly prepared 3,30-diaminobenzidine tetrahydrochloride solution (45 mL of 0.05 M Tris/HCl, pH 7.6, 5 mL diaminobenzidine and 5 mL H_2O_2) for 10 min. The slides were then washed for 5 min under running tap water, after which the nuclei were counterstained with haematoxylin (30 s) and slides were washed again for 10 min under running tap water. Finally, the sections were dehydrated in EtOH (70%, 96%, 100%; 2 × 3 min) followed by xylene (2 × 5 min) and mounted under a coverslip with Eukitt Mounting Medium (Electron Microscopy Systems). Sections were imaged using a digital camera (Color View II; Olympus) coupled to a microscope (Olympus BX42) using Cell B software (Olympus, Leiderdop, The Netherlands). For negative controls, the primary antibody was substituted with serum blocking solution or normal serum from the species in which the primary antibody was raised.

Sections for progesterone nuclear receptor (PR) and for P19 (uterocalin) were processed in The Institute for Pathology, Faculty of Veterinary Medicine, Leipzig University, Germany. In brief, the primary antibodies used were a mouse monoclonal anti-PR (diluted 1:100 in 1% bovine serum albumin (BSA; Boehringer-Ingelheim International Gmbh, Ingelheim am Rhein, Germany) in TBS; NCL-PGR-AB, Novocastra TM, Leica Biosystems Ltd, Newcastle, UK) and a rabbit polyclonal anti-uterocalin (diluted 1:150 in TBS; Courtesy of Prof. Dr. Twink Allen). After rinsing in TBS, sections were incubated with the secondary rat anti-mouse IgG antibody (for PR; diluted in 1% BSA in TBS; Code-No.: 415005100, Dianova, Hamburg, Germany) or secondary pig anti-rabbit IgG antibody (for uterocalin; diluted 1:100 in TBS, ZO196 DAKO) for 30 min at room temperature. Sections were rinsed in TBS (3 × 5 min) and then incubated with mouse-PAP (diluted 1:500 in 1% BSA in TBS) for PR and with rabbit-PAP (diluted 1:500 in TBS) for uterocalin (Dianova, Hamburg, Germany) for 30 min at room temperature. After washing in TBS (3 × 5 min), the slides were incubated in freshly prepared 3,3-diaminobenzidine tetrahydrochloride solution (200 mL of 0.1 M Imidazole/HCl, pH 7.1, and 70 μL H_2O_2) for 10 min. The slides were then washed (3x) for 5 min in TBS and for 5 min in Aquadest. After which the slides were counterstained with Papanicolaous solution (10 s; Merck B.V., Gernsheim, Germany) and washed again for 10 min under running tap water. Finally, the sections were dehydrated in Isopropanol (50%, 70%, 80%, 96%, 100%; 2 × 3 min) followed by xylene (3 × 3 min) and mounted under a coverslip. Sections were imaged with a digital camera (Color View II; Olympus) coupled to a microscope (Olympus BX42) using Cell B software (Olympus). As negative controls, the primary antibody was substituted by a nonrelated monoclonal antibody or normal serum from the species the primary antibody was raised in.

Stained slides were evaluated blindly by two different technicians and scored for intensity of staining in the nuclei and cytoplasm of stromal, glandular and luminal epithelial cells (0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining).

2.8. Statistical analyses

All data were analyzed using SPSS 20 for Windows (IBM Corp., New York, USA). Data for serum progesterone and 17-β estradiol concentrations were analyzed using a General Linear Model of Variance accounting for autocorrelation of sequential measurements within individuals, to identify any effects of Day, treatment group and Day by group interaction, after logarithmic transformation to obtain normally distributed data. If an effect of treatment group (LE, SE or NE) or an interaction of group and Day was apparent ($P < 0.05$), data were examined further using a post-hoc Tukey test to indicate differences in hormone concentration amongst groups within any given Day. The effect of treatment on the endometrial edema score was tested using the non-parametric Friedman test.

The RT-qPCR data were generated by the Relative Standard Curve Method [17]. Relative starting quantities were calculated from the Ct values from each sample, then the relative gene expression was obtained using the geometric mean of the housekeeping genes [16]. Finally, the data were subjected to logarithmic transformation to obtain normally distributed data sets. Data were analyzed by One Way ANOVA followed by a post-hoc Tukey test. Localization of the immunostaining for the proteins of interest (P19, PR, PGMRC1 and ERα) within the endometrium are reported descriptively; while the differences in the intensity of staining between groups were tested using a non-parametric Kruskal-Wallis test.

3. Results

The basal levels of 17-β estradiol (E2) (<1.5 pg/mL; range 0.7–1.4 pg/mL; on Day − 7) and progesterone (<1 ng/mL, range 0.04–0.5 ng/mL; on Day 0) in all mares at the onset of the study confirmed their status as seasonally anestrus (Fig. 2). As expected, the plasma concentration of E2 increased earlier (Day − 5; $P < 0.0001$) in the long estrus (LE) than in the other groups. E2 concentrations in the LE group increased gradually from Day − 5 to reach a peak on Day − 2 and then decreased gradually to reach baseline levels again on Day 1 (Fig. 2). The E2 concentrations in LE mares were significantly higher than those in SE mares for Days − 5

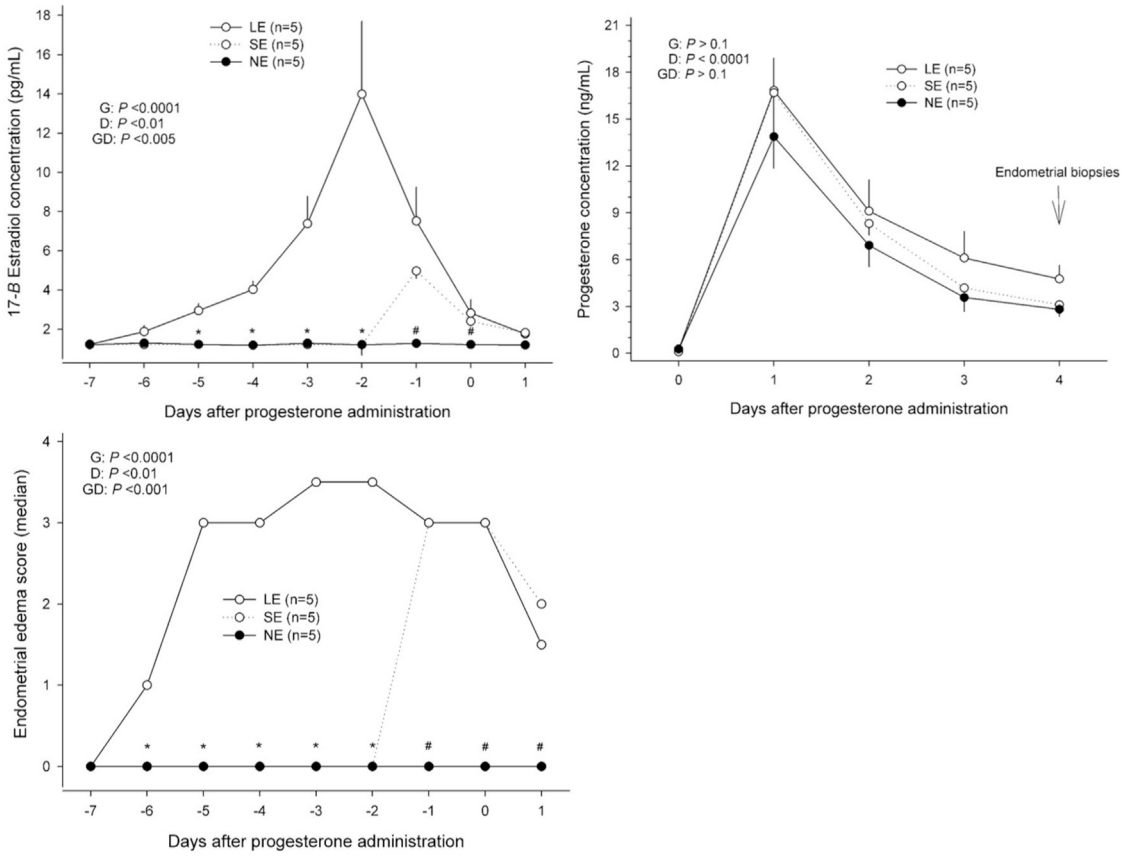

Fig. 2. Mean (±S.E.M) concentrations of 17-β estradiol (upper left panel) and progesterone (upper right panel) in mares treated with 1500 mg of long acting progesterone (LA P4) on Day 0 and varying doses of estradiol benzoate from Day − 7 to Day − 3 in mares in the long estrus group (LE; n − 5), a single dose on Day − 2 in mares in the short estrus group (SE; n = 5). Mares in the no estrus group (NE; n = 5) did not receive any estradiol treatment. The lower left panel represents the median scores for endometrial edema for the same groups of mares. The effect of Day (D), Group (G) and Group by Day interaction (GD) are shown. An asterisk (*) indicates a significant difference ($P < 0.05$) in the E2 concentration or median endometrial score within that Day, between mares from the LE and the other groups. The symbol # indicates a difference ($P < 0.05$) within a given Day between the NE group and the other groups.

to − 2, and in NE mares from Day − 5 to 0 (Fig. 2). The E2 concentration of mares in the NE group remained at baseline throughout the period studied. The differences in E2 concentrations between the NE and SE group were significant only for Days − 1 and 0 (Fig. 2). Likewise, the endometrial edema profile was different between groups ($P < 0.0001$), with a longer increase and higher peak of endometrial edema in mares from the LE compared to the NE and SE groups (Fig. 2). Mares from the NE group showed an absence of endometrial folding (score of 0) throughout the period of study.

The progesterone concentration profile after the single treatment of LA P4 was similar in all three groups ($P > 0.1$; Fig. 2), reaching a peak one day after the LA P4 treatment (Day 1), followed by a gradual decrease until Day 4, the day on which the endometrial biopsies were taken.

Of the 17 genes investigated (Table 2), three were differently expressed ($P < 0.05$) in the day 4 endometrium of mares with different preceding lengths of estradiol exposure: IGF-1, P19 and FGF-2, and expression of one further gene (FGF-7) approached significance ($P = 0.1$), (Fig. 3). The relative endometrial expression of IGF-1 mRNA was lower ($P = 0.007$) in mares from the SE group than mares from the LE and NE groups. The expression of FGF-2 mRNA in endometrium decreased with the length of estradiol treatment, being lowest ($P = 0.002$) in mares with a LE, followed by SE and highest in mares with no E2 treatment (Fig. 3). The expression of P19 mRNA was highest in the endometrium of mares from the LE ($P = 0.019$) compared with those from the SE and NE groups. Protein expression for P19 mirrored its mRNA abundance, with more intense and abundant staining in LE mares than in the other groups ($P < 0.05$); P19 immunostaining was mainly localized to the cytoplasm of glandular epithelial, stromal and luminal epithelial cells (Fig. 4a; Table 3).

The length of estrus did not affect ($P > 0.1$) the intensity of staining for ERα, PR or PGRMC1. ERα immune-staining was apparent throughout the whole endometrium with more intense staining in the nuclei than the cytoplasm, and in the glandular epithelium followed by the luminal epithelium and stroma (Fig. 4b; Table 3). PR immune-staining was weak and limited to some nuclei within the glandular epithelium and stroma (Fig. 4c). Finally, PGRMC1 immune-staining was strong, but limited to the cytoplasm of the glandular and luminal epithelium (Fig. 4d), no staining was observed in the stroma (Table 3).

4. Discussion

It has previously been shown that anestrous mares treated with appropriate doses of exogenous steroid hormones (estrogens and progesterone) show similar uterine morphology, and relative abundance of hormone receptor transcripts as cyclic mares [18], thereby establishing hormone treatment as a valid approach to studying the effect of the duration of estrogen stimulation on the endometrial gene expression profile. Day 8 embryos recovered

Table 2
Panel of genes selected for quantification of expression in endometrial tissue in mares with different durations of estrogenic stimulation (long, short and none: LE, SE and NE) prior to 4 days of progesterone dominance.

Gene symbol	Gene name	Function	P value
ERα	Estrogen receptor alpha	Hormone binding; regulated by estrogens	0.25
ERβ	Estrogen receptor beta	Hormone binding; regulated by estrogens	0.66
PR	Progesterone receptor	Hormone binding; regulated by estrogens	0.71
PGRMC1	Progesterone receptor membrane component 1	Hormone binding; regulated by estrogens	0.22
IGF-1	Insulin-like growth factor 1	Cell differentiation and growth	0.007
SPP1	Secreted phosphoprotein 1 (Osteopontin)	Best marker for "receptive endometrium" in women. Major component of the histotroph in pigs; regulated by estrogens	0.42
GM2a	Ganglioside activator	Lipid transport; oligosaccharide catabolism; regulated by estrogens	0.33
P19	Uterocalin	Lipid transport; regulated by progesterone	0.019
SLC2A1	Solute carrier 2A1	Glucose transport; regulated by progesterone	0.27
SLC2A3	Solute carrier 2A3	Glucose transport; regulated by progesterone	0.56
SLC36A2	Solute carrier 36A2	Amino-acid transport	0.84
FGF-2	Fibroblast growth factor 2	Growth factor activity; trophoblast adhesion; regulated by estrogens	0.002
FGF-7	Fibroblast growth factor 7	Growth factor activity; trophoblast adhesion; regulated by estrogens	0.10
FGF-9	Fibroblast growth factor 9	Growth factor activity; trophoblast adhesion; regulated by estrogens	0.91
CRYAB	Alpha-crystallin B chain	Protein tyrosine kinase signaling; anti-apoptosis; regulated by estrogens	0.85
HSPB7	Heat shock 27 kDa protein family, member 7	Response to stress; indicator of receptive endometrium in man; regulated by estrogens	0.74
HSPB8	Heat shock 22 kDa protein family, member 8	Response to stress; indicator of receptive endometrium inuman; regulated by estrogens	0.69

The existence of a difference in mRNA expression between the three groups (long, short, and no estrus) is indicate by the probability in the last column (P value). The mRNA expression for IGF-1, P19 and FGF-2 differed significantly between groups ($P < 0.05$). The genes were selected from previous publications (Klein et al., 2010; Gebhardt et al., 2012).

from donor mares have been reported to tolerate a wide degree of recipient mare negative uterine asynchrony, with no effect on the likelihood of pregnancy when embryos are transferred to recipient mares that ovulated as many as five days after the donor [19]. It is therefore common practice in commercial embryo transfer programs to transfer embryos recovered on day 8 after ovulation to cycling recipients on days 4–6 after ovulation [15] or to anestrous mares on days 4–5 after LA P4 treatment [20,21]. However, in cases

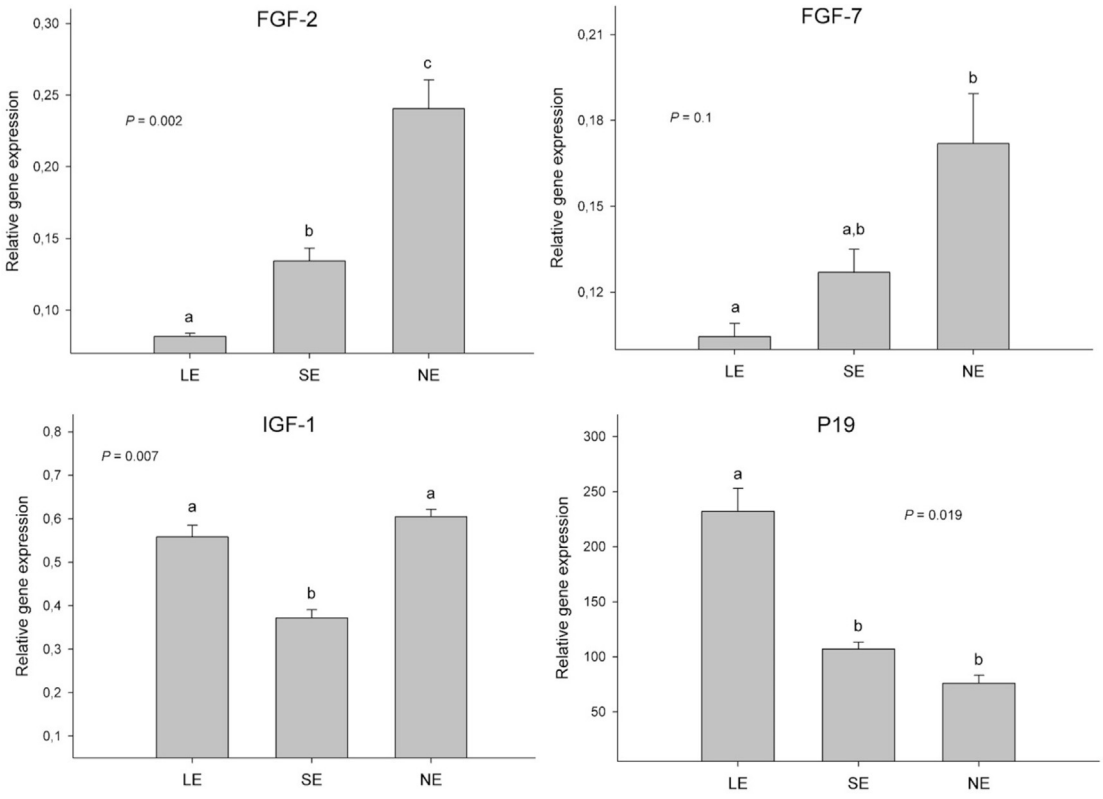

Fig. 3. Relative gene expression in the endometrium of mares 4 days after a single administration of 1500 mg of long acting progesterone immediately following a simulated long estrus (LE), short estrus (SE) or no estrus (NE), for fibroblast growth factor 2 (FGF-2; upper left panel), fibroblast growth factor 7 (FGF-7; upper right panel), Insulin-like growth factor 1 (IGF-1; lower left panel) and uterocalin (P19; lower right panel). Different letters (a, b, c) indicate a difference in RNA abundance amongst groups.

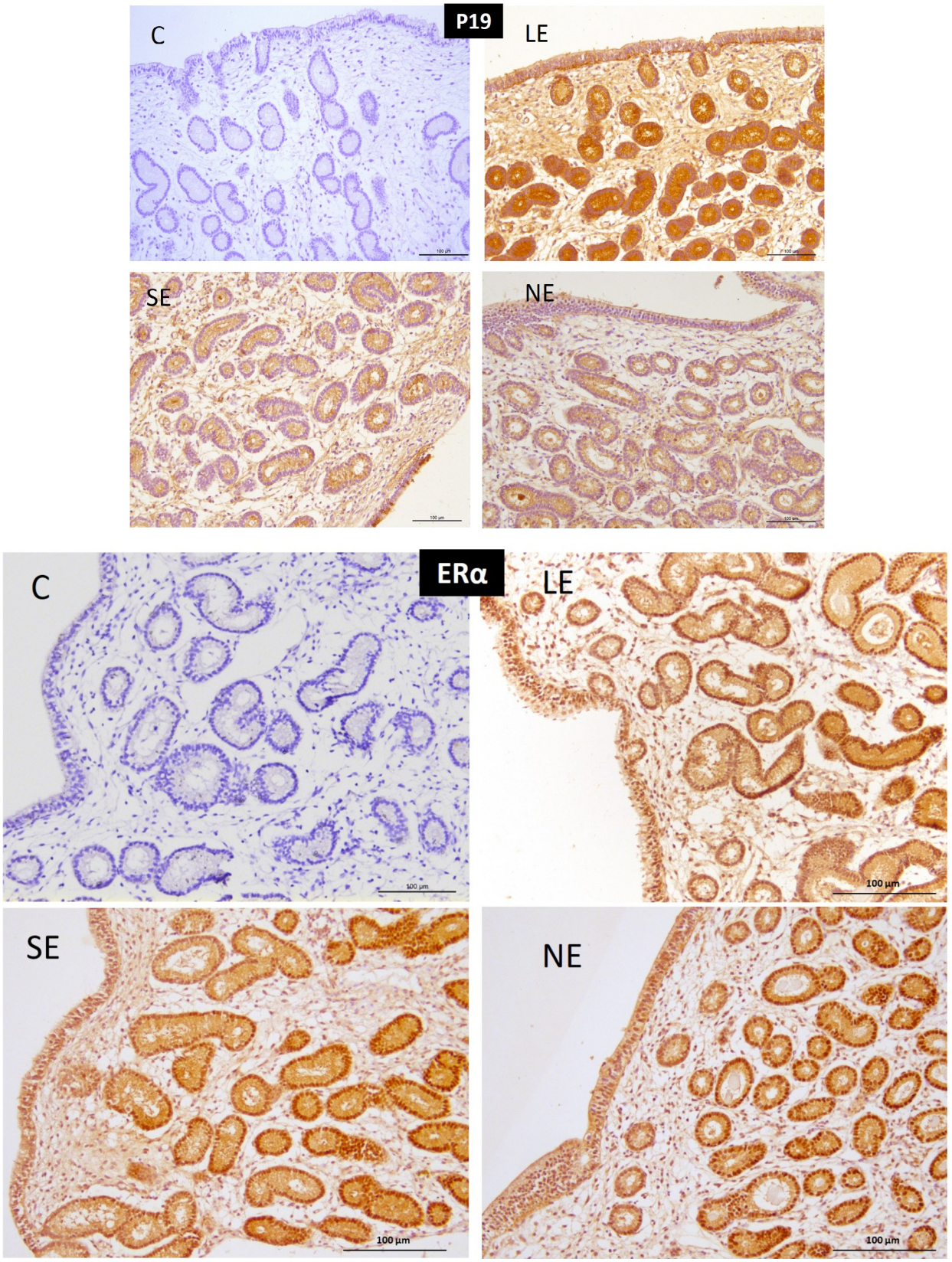

Fig. 4. Representative immunohistochemical images of endometrial samples from mares 4 days after a single administration of 1500 mg of long acting progesterone following a simulated long estrus (LE), short estrus (SE) or no estrus (NE), for P19, ERα, PR and PGRMC1. For each protein, a negative control slide (C) is shown.

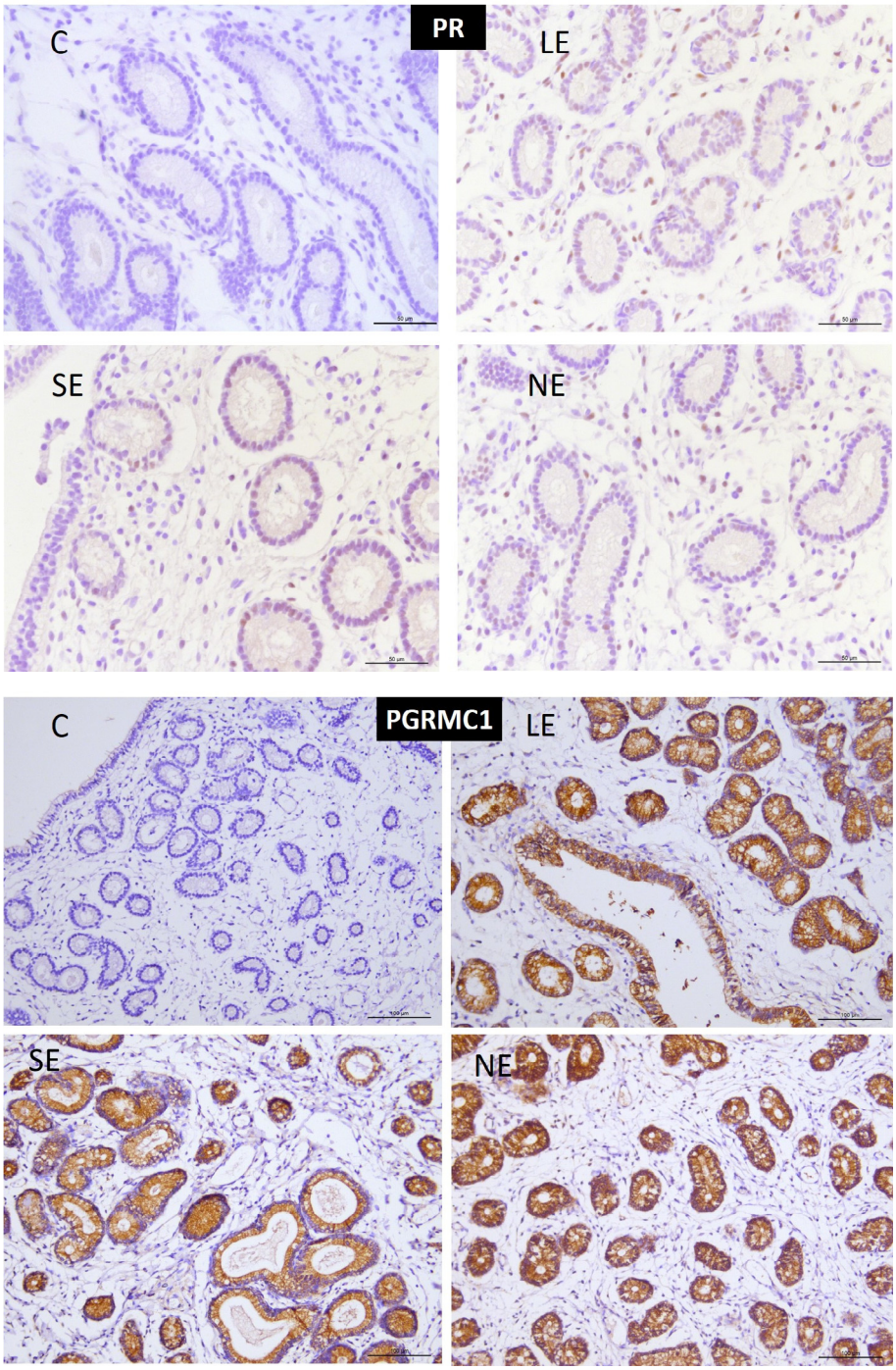

Fig. 4. (*continued*).

of asynchronous ET, the length of the preceding estrus appears to play a more important role in the outcome of ET than in synchronous recipients [9]. In this respect, the likelihood of pregnancy after ET of Day 8 embryos to asynchronous recipients (days 4–6 post-ovulation) with a short estrus (0–2 days) is significantly lower (55.9%) than that in either synchronous recipients (7–9 days post-ovulation) with a short estrous (≤3 days: 70.9%) or synchronous or asynchronous recipients with a long estrous (>3 days: 83.2% and 82.9%, respectively). In an attempt to improve our understanding of how the duration of estradiol stimulation prior to P4 dominance may affect uterine receptivity, we evaluated the endometrial expression of genes thought to be involved in supporting survival and early development of the conceptus and/or known to be regulated by estrogens, on Day 4 after LA P4 administration. Of the genes examined, only three were shown to be differentially expressed as a result of the duration of estrogen stimulation: P19, FGF-2 and IGF-1. It is important to emphasize that the genes examined in the present study were selected because they were previously reported to be differentially expressed either between different days of the estrous cycle [8], or between pregnant and

Table 3
Immune-staining scores for P19, PR, PGRMC1 and ERα expression in the endometrium of mares exposed to different durations of estrogen priming prior to four days of progesterone exposure.

PGRMC1	Stroma		Glandular epithelium		Luminal epithelium	
	Nuclei	Cytoplasm	Nuclei	Cytoplasm	Nuclei	Cytoplasm
NE	0	0	0	3^{a}	0	3^{a}
SE	0	0	0	3^{a}	0	3^{a}
LE	0	0	0	3^{a}	0	3^{a}
Negative C	0	0	0	0^{b}	0	0^{b}
P19	**Stroma**		**Glandular epithelium**		**Luminal epithelium**	
	Nuclei	Cytoplasm	Nuclei	Cytoplasm	Nuclei	Cytoplasm
NE	0	1^{a}	0	$1,5^{a}$	0	1^{a}
SE	0	1^{a}	0	2^{a}	0	$1,5^{a}$
LE	0	2^{b}	0	3^{b}	0	3^{b}
Negative C	0	0^{c}	0	0^{c}	0	0^{c}
ERα receptor	**Stroma**		**Glandular epithelium**		**Luminal epithelium**	
	Nuclei	Cytoplasm	Nuclei	Cytoplasm	Nuclei	Cytoplasm
NE	2^{a}	$0.5^{a,b}$	3^{a}	2^{a}	2^{a}	1^{a}
SE	2^{a}	1^{b}	3^{a}	2^{a}	2^{a}	1^{a}
LE	2^{a}	1^{b}	3^{a}	2^{a}	2^{a}	1^{a}
Negative C	0^{b}	0^{a}	0^{b}	0^{b}	0^{b}	0^{b}
PR	**Stroma**		**Glandular epithelium**		**Luminal epithelium**	
	Nuclei	Cytoplasm	Nuclei	Cytoplasm	Nuclei	Cytoplasm
NE	1^{a}	0	1^{a}	0	0	0
SE	1^{a}	0	1^{a}	0	0	0
LE	1^{a}	0	1^{a}	0	0	0
Negative C	0^{b}	0	0^{b}	0	0	0

LE: Long estrus; SE: short estrus; NE: No estrus; C: negative control. Within a column, different letters (a, b, c) indicate a significant difference ($P < 0.05$) in the staining score between groups (0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining).

non-pregnant mares on day 13.5 post-ovulation [13]. In this respect, absence of an effect on many of the genes might be due either to the absence of an embryo, due to the duration of progesterone exposure prior to endometrial harvesting, or simply to the fact that they are not influenced by different lengths of preceding estrogen exposure. However, the expression of both P19 and FGF-2 were markedly influenced by the length of previous estradiol exposure.

The protein expressed by the P19 gene, uterocalin, is a member of the lipocalin family, and was first characterized by Stewart and coworkers in 1995 [22], as an approximately 19 kDa molecule secreted in large amounts into the lumen of the uterus of both pregnant and non-pregnant mares under the influence of progesterone. Uterocalin is produced and secreted by endometrial glandular and luminal epithelial cells during the luteal phase of the estrous cycle and early pregnancy [23], and its secretion can be induced by exogenous progesterone [22]. Although P19 mRNA was found only in endometrial maternal cells, uterocalin was originally isolated from the embryonic capsule [23] and has also been identified in the trophoblast and yolk sac fluid [23]. Uterocalin is thought to be a transport protein of uterine origin that carries lipids and/or small lipophilic molecules into the embryo through the capsule [23]. In the present study, P19 expression was significantly increased in the LE group compared to SE and NE groups. Since the peak plasma progesterone concentration and general profile between treatment and D4 were similar in all groups, these results demonstrate that a longer exposure and/or higher peak concentration of estradiol prior to P4 administration stimulates enhanced endometrial P19 mRNA and protein expression. It therefore seems reasonable to speculate that a more physiological (longer) estrus primes the endometrium to produce larger amounts of uterocalin from earlier stages of diestrus, which could play an important role (e.g. as lipid transporter) in supporting the survival of the early embryo, possibly by effects on its metabolism and nutrition.

Fibroblast growth factors (FGFs) regulate a range of biological functions, including cellular proliferation, survival, migration, differentiation and angiogenesis [24]. The FGF family was originally identified via a protein capable of promoting fibroblast proliferation, and is now known to comprise 22 members [24]. Endometrial FGF-2, FGF-7 and FGF-9 have all been described to play roles in uterine receptivity and implantation in mares, and are known to be regulated by estrogens [8,13,25,26]. Expression of FGF-2 mRNA in the equine endometrium increases during estrus but is constant during diestrus and early pregnancy [25]. Endometrial FGF-2 mRNA expression then increases as pregnancy advances from day 21–28, suggesting a role during late preimplantation development [25]. In the present study, only FGF-2 was differently expressed between groups, although between group differences in FGF-7 expression approached significance. For both FGFs, mRNA abundance decreased as a function of duration of endometrial exposure to estradiol prior to LA P4 administration. A direct comparison (data not presented) between Day 7 pregnant mares and mares from the LE group showed similar expression of FGF-2, indicating a similarly reduced expression of this gene during normal early pregnancy. The physiological relevance of this finding still needs to be elucidated.

Morphological and functional changes of the endometrium during the estrous cycle to prepare the uterus for the establishment of pregnancy, are mainly induced by the ovarian hormones estradiol and P4 [8]. Estrogens exert their effects through their receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), and mediate cellular proliferation and secretory protein production during estrus. In addition, estrogen stimulates expression of both its own receptors and that of progesterone in the endometrium of estrous mares [8,27,28]. The role of P4 in maternal support of conceptus survival and development are classically thought to be mediated by the nuclear progesterone receptor (PR) [27,28]. However, P4 can also act via plasma membrane receptors (including progesterone receptor membrane component 1: PGRMC1), which is expressed in equine trophectoderm [29] during early pregnancy, and has been proposed to mediate cellular differentiation, modulation of apoptosis and steroidogenesis [30]. Intriguingly, expression of PR ERα and ERβ is down-regulated by P4 and is, therefore, low in mid to late diestrus and early pregnancy. Moreover, the suppression of PR expression in endometrial epithelial cells is known to be a pre-requisite for the expression of genes coding for secretory proteins [31]. By contrast, endometrial PGRMC1 is downregulated during estrus but increases during diestrus and pregnancy, indicating upregulation by P4 [32] which agrees with our results. Although there were no significant differences in ERα, ERβ, PR and PGRMC1 mRNA expression between treatments, given the importance of the steroid hormones and their receptors in endometrial preparation for pregnancy, and because mRNA expression does not always directly reflect translation to protein [33], we also evaluated protein expression. The length of preceding estrus exposure did not affect endometrial protein expression for any of the steroid receptors on Day 4 after LA P4 administration; this suggests that stimulation of P19 and inhibition FGF-2 expression must have been exerted through a steroid hormone receptor-independent mechanism.

Insulin-like growth factor 1 (IGF-1) is thought to play an important role as a regulator of embryonic and fetal development [13]. Both the conceptus and endometrium in early pregnant mares

have been reported to express IGF-1, and *in vitro* secretion was shown not to be estrogen dependent [34]. In cows, IGF-1 secreted into the uterine lumen was higher during progesterone dominance [35]. In the current study, IGF-1 mRNA expression was lower in the SE group than in either the LE or NE groups, such that there was no clear association with the length of the exposure to estradiol.

A limitation of the study was that the mares from the LE group were subjected to more transrectal examinations than the mares from the SE and NE groups. It is unknown, whether this difference may have influenced the results.

In conclusion, from a panel of 17 selected genes thought to be influenced by estrogens and/or involved in the formation of an endometrium "receptive" to embryo survival and development, only three were differentially expressed (P19, FGF-2 and IGF-1), and only two exhibited a clear association with the length of estrogen treatment prior to P4 (P19 and FGF-2). Although it is tempting to speculate that a longer estrus induces a more receptive endometrium via the enhanced synthesis of P19, it is possible that additional genes and/or pathways contribute to the apparent positive effects of estrogen exposure, while further studies on early pregnancy following varying durations of preceding estrogen priming are needed to validate the positive effect of a long estrus on embryo metabolism and viability.

Conflicts of interest

The authors declare no conflicts of interest.

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