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Administration of 2.5 mg of estradiol followed by 1,500 mg of progesterone to anovulatory mares promote similar uterine morphology, hormone concentrations and molecular dynamics to those observed in cyclic mares

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ABSTRACT

To test the hypothesis that the administration of 2.5 mg of estradiol benzoate (EB) followed by 1500 mg of long acting progesterone (LA P4) causes similar uterine changes and molecular dynamics in anovulatory mares to those observed in cyclic ones, we evaluated the changes of estrogen (ERα and ERβ) and progesterone receptors (PR) in anestrous, transitional and cyclic mares by RT-qPCR and immunohistochemistry. In addition, we evaluated uterine edema, tonus and estrogens and progesterone plasma profile. Endometrial biopsies were taken from anestrous and transitional mares immediately before EB injection, 48 h after EB administration and five days after LA P4 was given. In cyclic mares, biopsies were collected at estrus and at five days after ovulation. Similar estrogen peaks were achieved after the injection of the single EB dose between treated and cyclic groups, as well as maximum uterine edema. Uterine tone was increased to diestrus levels after administration of 1500 mg of LA P4. Changes in relative abundance of transcripts for PR, ERα and ERβ when progesterone stimulated endometrium was compared to estrogen stimulated endometrium were similar between cyclic and non-cyclic treated mares. However, apparent decreased PR in the endometrial glandular epithelium was not observed in non-cyclic mares five days after LA P4 administration as observed at five days after ovulation in cyclic mares. The protocol produced similar endometrial edema, uterine tonus and changes in relative abundance of PR, ERα and ERβ transcripts to those observed in cyclic mares during late estrus and early diestrus, as well as similar estradiol and estrogen conjugate plasma concentrations.

1. Introduction

Embryo transfer (ET) is a worldwide technology in the equine industry. The technique is a valuable tool to increase the number of foals from genetically valuable donor mares, for obtaining foals from subfertile mares and to obtain embryos from mares that are competing in equestrian modalities [1]. The selection of the recipient mare has a major effect on the embryo transfer success. However, the availability of recipient mares has become a limiting factor, especially during the beginning of the breeding season, as part of the recipients are in anestrous due to seasonal reproductive activity. In addition, differentiated feed management usually provided to donor and recipient mares can delay the onset of cyclicity in the recipient mares [2].

In order to increase the offer of recipient mares in ET programs, estrogen and/or progesterone (P4) treatments may be administered

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to non-cyclic recipient mares. Several studies reporting the pregnancy rates after estrogen and/or progestins injections have been described [3–7]. Because it has been demonstrated that estradiol (E2) increases the expression of P4 uterine receptors and that the equine embryo secretes estrogens during the early gestational phase [8], estrogen was administered prior to and together with P4 to ovariectomized mares and pregnancy rates ranging from 70 to 80% were obtained within the different evaluated hormonal protocols [4]. It was suggested that, regardless of E2 injection, the primary requirement for pregnancy establishment and maintenance in non-cyclic recipient mares is an adequate concentration of exogenous progestins [4].

In the past few years, various doses of E2 has been given to intact non-cyclic recipient mares and after observation of uterine edema, long acting progesterone (LA P4) [6,7] or altrenogest [2,9] has been administered three to eight days prior to embryo transfer. Recently, it was demonstrated that the administration of a single dose of 2.5 mg of estradiol benzoate to anestrous mares produces similar estrogen concentration to that found in cyclic mares [10]. Moreover, it is known that plasma P4 concentration after injection of 1500 mg of LA P4 at seven day intervals is compatible with P4 concentrations found in cyclic mares during the luteal phase (>4 ng/mL) [11].

Estrogen and P4 mediate many of their effects through nuclear receptors, which are transcription factors that regulate expression of target genes [12]. Estrogen can bind and activate two types of nuclear receptors (i.e., ERα and ERβ) encoded by different genes, *ESR1* and *ESR2* [13]. Estrogen receptor α (ERα) is the predominant estrogen receptor (ER) in the uterus [14]. It has been suggested that ERβ modulates the uterotrophic effects of ERα [14]. Estrogen acts via its receptors to mediate cellular proliferation and secretory protein production during estrus. In addition, it is widely accepted that estrogen increases the expression of its own receptor and P4 receptor (PR) in the endometrium during the estrous cycle in mares [15–17]. On the other hand, P4 is required for maternal support of conceptus survival and development during pregnancy [18]. The actions of P4 are mediated by PR. In ruminants, loss of PR in the endometrial luminal and glandular epithelium prior to the stages of uterine receptivity and implantation seems to be required for onset of differentiated functions in terms of secretory proteins during early pregnancy [18].

Considering that recipient mares are responsible for carrying a pregnancy to term, it is important to provide an appropriate uterine environment for the conceptus development in non-cyclic recipient mares. The protein immunostaining and changes in abundance of transcripts for ER and PR have not yet been described in non-cyclic mares treated with hormonal protocols to become embryo recipients. In previous studies [2,9], we could demonstrate that the administration of 2.5 mg of estradiol benzoate (EB) followed by progestin administration was able to establish and maintain pregnancy in either anestrous or transitional mares. Therefore, to test the hypothesis that the administration of a single dose of 2.5 mg of EB followed by 1500 mg of LA P4 in anestrous and transitional mares promote similar uterine edema, tone and molecular dynamics to those observed in non-treated cyclic mares, we described and compared protein immunolocalization and changes in relative abundance of transcripts for ERα, ERβ and PR, as well as the uterine characteristics and estrogen and P4 profile during the hormonal treatment.

2. Material and methods

2.1. Animals and experimental design

Twelve mares of different breeds, ranging from five to 15 years and weighing 350–450 kg were used in the study. Mares were selected based on histological evaluations of endometrial inflammation and fibrosis by biopsy [19], which only mares classified as categories I (normal endometrium) and IIA (mild inflammation and/or fibrosis) were included. Mares were maintained on coast-cross hay (*Cynodon dactylon*) with water and trace-mineralized salt *ad-libitum*. The experiment was conducted from June to December at the Equine Reproduction and Biotechnology Center – Lageado Farm, School of Veterinary Medicine and Animal Science, Botucatu – São Paulo, Brazil. Animal procedures were approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (CEUA-95/2012).

Mares were assigned to anestrous, transition or cyclic (control) groups. Anestrous mares were those presenting ovarian follicles <20 mm in diameter, absence of a CL and P4 concentration <1 ng/mL on three evaluations at seven day intervals. Transitional mares were those showing multiple follicles of 20–30 mm in diameter, absence of a CL and P4 concentration <1 ng/mL, considering that treatment was started when at least one of the follicles reached 30 mm. Cyclic group consisted of non-treated cyclic mares showing regular estrous cycles and ovulatory follicles. The same twelve mares were used throughout the experiment, i.e., anestrous mares were reevaluated during the transitional period and afterwards during the cyclic phase.

2.2. Hormonal treatment

Anestrous and transition groups were treated with a single dose of 2.5 mg of intramuscular EB (Estrogin®, Farmavet, SP, Brazil) and 48 h after its administration 1500 mg of long acting P4 in oil (LA P4; Sincrogest Injetável®, Ourofino, SP, Brazil) was given intramuscularly. Cyclic group did not receive EB and LA P4 treatments, although ovulation was induced with 1500 IU of hCG (Vetecor®, Hertape Calier, MG, Brazil) after detection of at least a 35 mm follicle and uterine edema, to synchronize evaluation days between natural and artificial cycles (Fig. 1).

2.3. Blood collection and steroid hormones assay

Blood samples were obtained via jugular venipuncture into heparinized tubes every 24 h, starting from three days before ovulation (D−3) until five days after (D5) in the cyclic group. In anestrous and transition groups, samples were obtained immediately before EB injection (D−2) until five days after LA P4 administration (D5). Samples were collected into heparinized tubes, centrifuged (900×g/10 min) and plasma was harvested and stored at − 20 °C until assayed (Fig. 1). The concentrations of plasma 17β-estradiol (E2), estrogen conjugate (EC) and progesterone (P4) were measured by validated radioimmunoassay (RIA) for E2 [20], EC [21], and using a commercial RIA kit according to manufacturer's recommendations (Immunotech Beckman Coulter – Brea, CA, USA) for P4. When using estrone-3-sulfate as the standard (100%) for EC assay, the antibody cross-reacted with estrone (200%), 17β-estradiol (100%), equilin (50%), estrone-3-glucuronide (38%), estradiol-3-sulfate (21%), estradiol-3-glucuronide (6.8%) and cross reaction was less than 0.5% with all the non-estrogenic steroids tested. The antiserum for P4 assay presented low cross-reactivity to other steroid hormones, as follows: 5α-pregnanedione (15.02%), 5β-pregnanedione (8.12%), 6β-hydroxiprogesterone (5.1%) corticosterone (4.07%), 11-desoxycorticosterone (2.56%), 16α-hydroxiprogesterone (1.82%) and 17α-hydroxiprogesterone (1.15%).

The extraction efficiency for the E2 assay was 88%. The sensitivity of the assays were 8 pg/mL for E2, 0.1 ng/mL for EC and 0.05 ng/mL for P4. The intra- and inter-assay coefficients of variation were 3.9% (n = 6) and 8.9% (n = 8) for E2, 3.0% (n = 6) and 7.3%

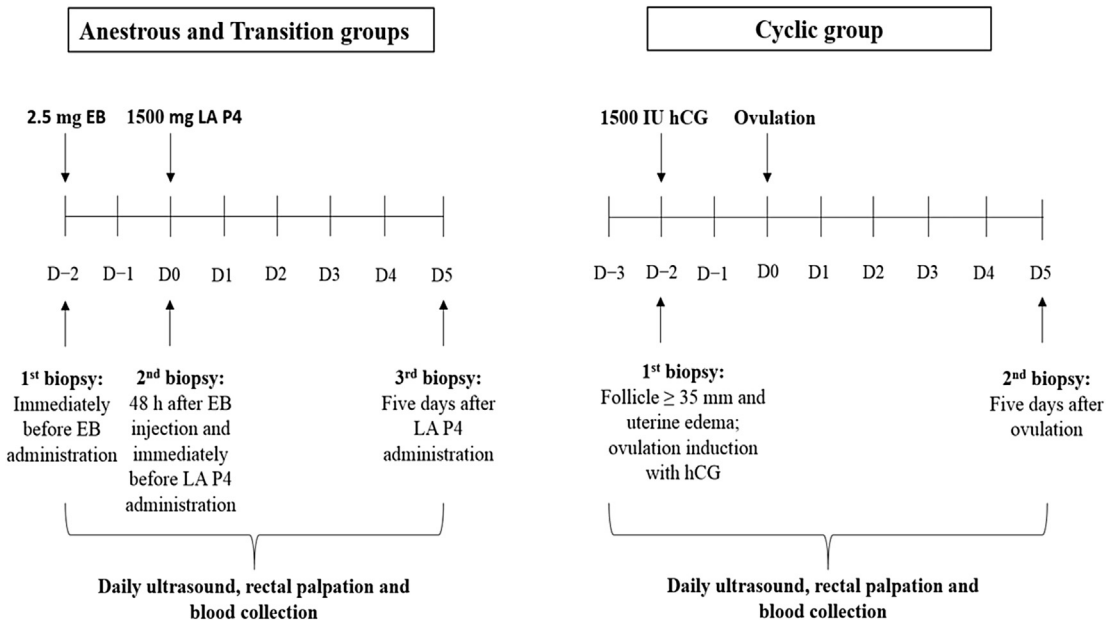

Fig. 1. Representative scheme of the days in which endometrial tissues were collected by biopsy and hormonal treatments were administered using 2.5 mg of estradiol benzoate (EB) followed by 1500 mg of long acting progesterone (LA P4) in anestrous and transition groups. In the cyclic group, ovulation was induced after detection of a ≥35 mm follicle and uterine edema, to synchronize evaluation days between natural and artificial cycles. Daily ultrasound, transrectal palpation and blood collections were performed in all groups.

(n = 4) for EC, and 12% (n = 6) and 12.6% (n = 3) for P4.

2.4. Transrectal palpation and ultrasound examinations

Transrectal palpation and B-mode ultrasonography (5 MHz linear transducer; Mindray® DP-3300Vet, Shenzhen, China) were performed daily prior to and during hormonal treatment in anestrous group (Fig. 1) After the end of treatment in anestrous group, ultrasound examinations were performed at two-day intervals until the transitional phase was characterized. Once in transition, mares were transrectally palpated and scanned by ultrasound as described for anestrous group. After treatment in transition group was completed, ultrasound evaluations were also performed at two-day intervals until the detection of the first ovulation of the season. In the cyclic group, ultrasonography was performed on a daily basis, starting from the second ovulation of the season (D0) until five days after the following ovulation (third ovulation of the season).

Transrectal palpation was performed immediately before and during hormonal treatments to evaluate uterine tone, scored as 0 to 3 (0 = lack of uterine tone; 1 = minimum uterine tone; 2 = intermediate uterine tone; 3 = maximum uterine tone) [22], and ultrasound examinations were performed to evaluate uterine edema, which also received a scoring system from 0 to 3 (0 = lack of uterine edema; 1 = minimum uterine edema; 2 = intermediate uterine edema; 3 = maximum uterine edema) [23]. Uterine tone and edema were also evaluated in the cyclic group, starting from the detection of a 35 mm follicle until five days after ovulation.

2.5. Tissue collection

Endometrial tissue samples were collected immediately before EB treatment, 48 h after EB administration and five days after LA P4 injection in anestrous and transition groups. In cyclic group, samples were collected during estrus, when a follicle of ≥35 mm and uterine edema (score 2 to 3) were observed, and during diestrus, on day five after ovulation (Fig. 1).

Endometrial samples were recovered transcervically using an alligator jaw biopsy forceps (Botupharma, Botucatu, SP, Brazil) from the base of one of the uterine horns. Biopsies were divided in two parts and one part was frozen in liquid nitrogen and stored at −80 °C for RT-qPCR, while the other part was fixed for 24 h in 10% formalin and stored at 70% alcohol for histology and immunohistochemistry (IHC).

2.6. Reverse transcription qPCR (RT-qPCR)

Total cellular RNA was extracted from endometrium samples using trizol (Trizol® Reagent, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendation. The extracted RNA was diluted in 40 μL of RNase-*free* water. The RNA (1 μL) was quantified via spectrophotometry (NanoDrop ND1000®) and samples with a 260/280 ratio of 1.9 or greater were used for analysis. The RNA samples (2 μg/reaction) were treated with RNase-free DNase I (Ambion®, Life Technologies, Carlsbad, CA, USA) for 30 min at 37 °C, followed by treatment with DNase Inactivation Reagent (room temperature for 2 min). Quantification of RNA was performed once more by spectrophotometry and treated samples were normalized to the same final concentration of 2400 ng using endonuclease free water. Synthesis of cDNA was performed with 2400 ng of total RNA per 40 μL of reaction using random hexamers and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Primers specific for estrogen receptor α (*ESR1*), estrogen receptor β (*ESR2*), progesterone (*PGR*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and beta-2-microglobulin (*B2M*) were selected based on primers previously validated for the mare's endometrium [24,25] (Table 1). Primers efficiencies ranged from 93.8% to 99.3%.

Each PCR reaction was performed in duplicate, using final volume of 20 μL: 2 μL of cDNA, 10 μL of GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), 1 μL of each primer (forward and reverse) and endonuclease free water q.s.p. A 'no template' control was included in duplicate on each plate to show that amplicon

Table 1
Primer sequences and gene accession numbers used for estrogen receptor α (*ESR1*), estrogen receptor β (*ESR2*), progesterone receptor (*PGR*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and beta-2-microglobulin (*B2M*) transcripts.

Gene	Accession number	Primer sequence (5′-3′)	Product size (bp)
ESR1[a]	NM_001081772.1	Forward:TCCATGGAGCACCCAGGAAAGC Reverse: CGGAGCCGAGATGACGTAGCC	125
ESR2[a]	NM_001309479.1	Forward: TCCTGAATGCTGTGACCGAC Reverse: GTGCCTGACGTGAGAAAGGA	116
PGR[a]	XM_014741094.1	Forward: CTTCCCCGACTGCGCGTACC Reverse: TTGTGTGGCTGGAAGTCGCCG	81
GAPDH[a]	NM_001163856.1	Forward: AGAAGGAGAAAGGCCCTCAG Reverse: GGAAACTGTGGAGGTCAGGA	87
ACTB[b]	NM_001081838	Forward: CGACATCCGTAAGGACCTGT Reverse: CAGGGCTGTGATCTCCTTCT	99
B2M[b]	NM_001082502	Forward: GTGTTCCGAAGGTTCAGGTT Reverse: ATTTCAATCTCAGGCGGATG	102

[a] Primers validated for the mare's endometrium according to Silva et al. (2014) [25].
[b] Primers validated for the mare's endometrium according to Klein et al. (2011) [24].

contamination was absent. Quantitative PCR was completed using 7500 fast Real Time PCR systems (Applied Byosystems®), with the following cycling conditions: 10 min at 95 °C (initial denaturation), 40 cycles at 95 °C for 15 s (denaturation) and 60 °C for 15s (annealing/extension), followed by a melting curve. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run. A standard curve was made using serial 10-fold dilution of a "pool" of equine endometrium cDNAs used as template in the RT-qPCR reactions.

Changes in gene expression were calculated by mean quantification cycle (Cq) and then normalized for the endogenous reference gene *B2M* to generate delta Cq values. *GAPDH* and *ACTB* were also tested as endogenous reference genes, since *GAPDH*, *ACTB* and *B2M* have been shown to be stably expressed across equine endometrium [24], however *B2M* was the most stably expressed gene in our experimental conditions as demonstrated by NormFinder software results (B2M = 0.372; BACT = 0.387; GAPDH = 0.458). Changes in relative abundance of transcripts were calculated by the relative expression ratio (*R*) method [26]. Data were analysed in SDS 7500 v.2.05 software (Applied Byosystems®).

2.7. Immunohistochemistry

Formalin fixed samples were dehydrated, embedded in paraffin and cut at 4 μm. Paraffin sections were deparaffinized and rehydrated in xylene and serial ethanol dilutions. Antigens retrieval were performed by heating tissue sections for 30 min in citrate buffer solution at pH 6. After heat treatment, all slides were left to cool down for 20min. Endogenous peroxidases were inhibited with 8% H_2O_2 in methanol for 20 min. Thereafter, incubation with 12% skimmed milk powder for 1 h at 27 °C was performed to minimize non-specific antibody binding. Sections were incubated with the primary antibodies ERα (1:100, mouse monoclonal SC-311, Santa Cruz Biotechnology), PR (1:100, mouse monoclonal PR-2C5, Invitrogen) and ERβ (1:100, mouse monoclonal PPG5/10, Abcam) for 18 h (overnight). The next day, section were washed with dilution buffer at pH 7.4 and treated with secondary antibody (*N*-Histofine Simple Stain® - Nichirei Biosciences Inc.) for 30 min at 27 °C. Sections were washed and developed with diaminobenzidine substrate (Liquid DAB Cromogen® - Dako) for 3 min in room temperature. Subsequently, sections were washed and counterstained with Mayer's Hematoxylin for 90 s, washed with tap water for 5 min, dehydrated and mounted for analysis. Negative controls were prepared using dilution buffer instead of primary antibody.

Slides were observed at 10×, 20×and 40×magnification under a light microscope. Staining intensity and distribution of ERα, ERβ and PR in luminal epithelium, glandular epithelium and stroma were described by three observers blinded to mare reproductive status and treatment.

2.8. Statistical analysis

The mean relative expression ratio for *ESR1*, *ESR2* and *PGR* were compared between treatments within anestrous and transition groups, as well as within treatments or cycle stages between groups, with the mixed model for repeated measures. The Tukey's test was used to adjust the P-values that resulted from multiple comparisons. The analysis was performed with PROC MIXED [27].

Descriptive analysis were produced (measures of central tendency and dispersion) for the hormone concentration results to characterize the study sample. All response variables were not normally distributed and therefore were log-transformed for analysis. Data were presented as geometric means with confidence intervals to show values in the original scale. A repeated measures model [28] was constructed with PROC MIXED [27] to compare the mean E2, EC, and P4 concentrations among collection days and treatments. The auto regressive covariance structure provided the best fit to the data and was used to model the correlation between repeated observations within the same mare. A *P*-value of 0.05 or less was considered statistically significant.

3. Results

3.1. Intervals between treatments

The mean interval between the last hormonal treatment (LA P4) in anestrous group to the onset of treatment in transition group was 64.6 ± 9.2 days (mean ± SEM). The mean interval between the last hormonal treatment (LA P4) in transition group to the first ovulation of the season in the cyclic group was 27.4 ± 2.9 days (mean ± SEM). Data from cyclic group were collected starting from the second ovulation of the season.

3.2. Steroid concentrations

Estradiol and EC peaks were observed 24 h after EB injection in treated groups and there were no differences ($P > 0.05$) on estrogens average concentrations between treatments (Fig. 2a and b). In the cyclic group, estradiol and estrogen conjugate peaks were observed on D−2 and there were no differences ($P > 0.05$) when compared to estrogens peaks of the treated groups. There were no differences on estrogens concentrations after LA P4 injection (from D1 onwards) between groups ($P > 0.05$; Fig. 2a and b).

Progesterone peaks were observed 24 h after injection of

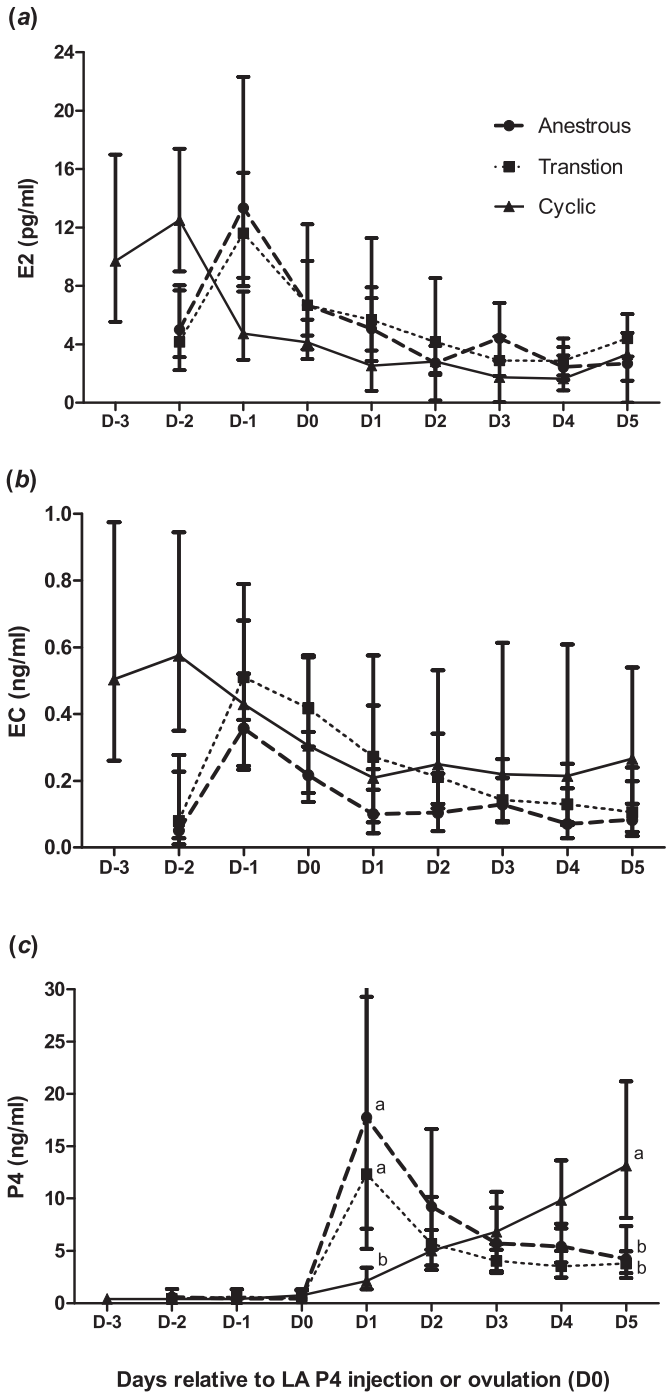

Fig. 2. Hormone concentrations of (a) estradiol (E2), (b) estrogen conjugate (EC) and (c) progesterone (P4) after injection of 2.5 mg of estradiol benzoate (EB) on day −2 (D−2) followed by 1500 mg of long acting progesterone (LA P4) on day 0 (D0) in anestrous and transition groups. Hormone concentrations for E2, EC and P4 from the Control group are also shown, starting three days before ovulation (D−3). Different letters between groups within correspondent days indicate statistical difference ($P < 0.05$).

1500 mg of LA P4 in non-cyclic treated groups. After peak, P4 concentrations showed a sharp decrease on D2, followed by a less pronounced decrease until D5 in treated groups. There were no statistical differences between treated groups after P4 administration (Fig. 2c; $P > 0.05$). Progesterone concentration in the cyclic group was low before ovulation and started to increase from D1, rising gradually until D5. Concentration of P4 was significantly lower ($P < 0.05$) in the cyclic group on D1 and significantly higher on D5 when compared to the treated groups ($P < 0.05$; Fig. 2c).

3.3. Uterine edema and tone

The scores given to uterine edema and tone in cyclic and non-cyclic treated mares are shown in Table 2. In the anestrous group, a lack of uterine edema was observed immediately before initiation of treatment (D −2). In this group, maximum edema was found 24 and 48 h after EB injection. Following LA P4 injection, a sharp reduction was observed on D1, remaining low until D5. Regarding to uterine tone, an increase was detected starting from D2 after LA P4 injection, reaching the intermediate level on D4.

In the transition group, a minimum to intermediate edema was observed before treatment initiation, reaching a maximum level 24–48 h after EB injection. After LA P4 administration, there was a gradual edema decrease, which was found in a minimum level on D5. An increase in uterine tone after LA P4 administration was observed sooner in the transition group as compared to anestrous group, reaching the intermediate level on D2.

Uterine edema in the cyclic group was found at a maximum level two days before ovulation, decreasing gradually to an intermediate to moderate level on ovulation day (D0). After ovulation, edema was observed at a minimum level, showing a slight increase on D5, where an intermediate to moderate edema was detected. An increase in uterine tone was observed starting from day one after ovulation, reaching an intermediate level on D2.

3.4. Changes in relative abundance of transcripts for PGR, ESR1 and ESR2

In anestrous group, there was an increase in relative abundance of *PGR* and *ESR1* transcripts (1.74 fold and 2.07 fold, respectively) after EB administration (Fig. 3a and b), however this increase was not different ($P > 0.05$) from the slight decrease in relative abundance of *PGR* and *ESR1* transcripts (−0.66 fold and −0.43 fold, respectively) after EB injection in transition group (Fig. 3a and b). In both anestrous and transition groups, there was a decrease in relative abundance of *ESR2* transcripts after EB administration (−0.89 fold and −1.89 fold, respectively; Fig. 3c), which was not significantly different between groups ($P > 0.05$). After LA P4 administration in anestrous group, a slight increase in relative abundance of *PGR* (0.34 fold), *ESR1* (2.67 fold) and a lack of change of *ESR2* (−0.004 fold) transcripts were observed, which were not different ($P > 0.05$) from the decrease in relative abundance of *PGR* (−0.42 fold), *ESR1* (−0.27 fold) and *ESR2* (−2.78 fold) transcripts after LA P4 injection in transition group (Fig. 3). In addition, within groups, there were no differences ($P > 0.05$) when changes in relative transcripts abundance were compared after EB and after LA P4 administration (Fig. 3).

When changes in transcripts abundance between five days after LA P4 injection and 48 h after EB were compared to changes between five days after ovulation and estrus, the decrease observed on *PGR* mRNA expression was not different ($P > 0.05$) between cyclic (−1.25 fold), anestrous (−1.28 fold) and transitional mares (−0.62 fold; Fig. 4a). There were no differences ($P > 0.05$) between anestrous and transition groups when changes in relative abundance of transcripts for *ESR1* (Fig. 4b) and *ESR2* (Fig. 4c) were compared between the moments after LA P4 was given and after EB administration. In addition, when changes in relative abundance five days after ovulation were compared to estrus, there were no differences between treated and cyclic group for *ESR1* and *ESR2* transcripts ($P > 0.05$; Fig. 4a and b).

Table 2
Median scores of uterine edema and tone in anestrous or transitional non-cyclic mares after estradiol benzoate (EB) treatment using a single dose of 2.5 mg on D−2, followed by the injection of 1500 mg of long acting progesterone 48 h after EB administration (D0). The median scores for cyclic mares at the corresponding days to treated mares are also shown.

Groups	Uterine edema								Uterine tone					
	Days relative to progesterone injection or ovulation (D0)													
	D−2	D−1	D0[a]	D1	D2	D3	D4	D5	D0[a]	D1	D2	D3	D4	D5
Anestrous	0	3	3	1	1	1	1	1	1	1	1.5	1.75	2	2
Transition	1.5	3	3	2	1.5	1.5	1.5	1	1	1.5	2	2	2	2
Cyclic	3	2	1.5	1	1	1	1	1.5	1	1.5	2	2	2	2

[a] Uterine edema and tone observed immediately before progesterone injection.

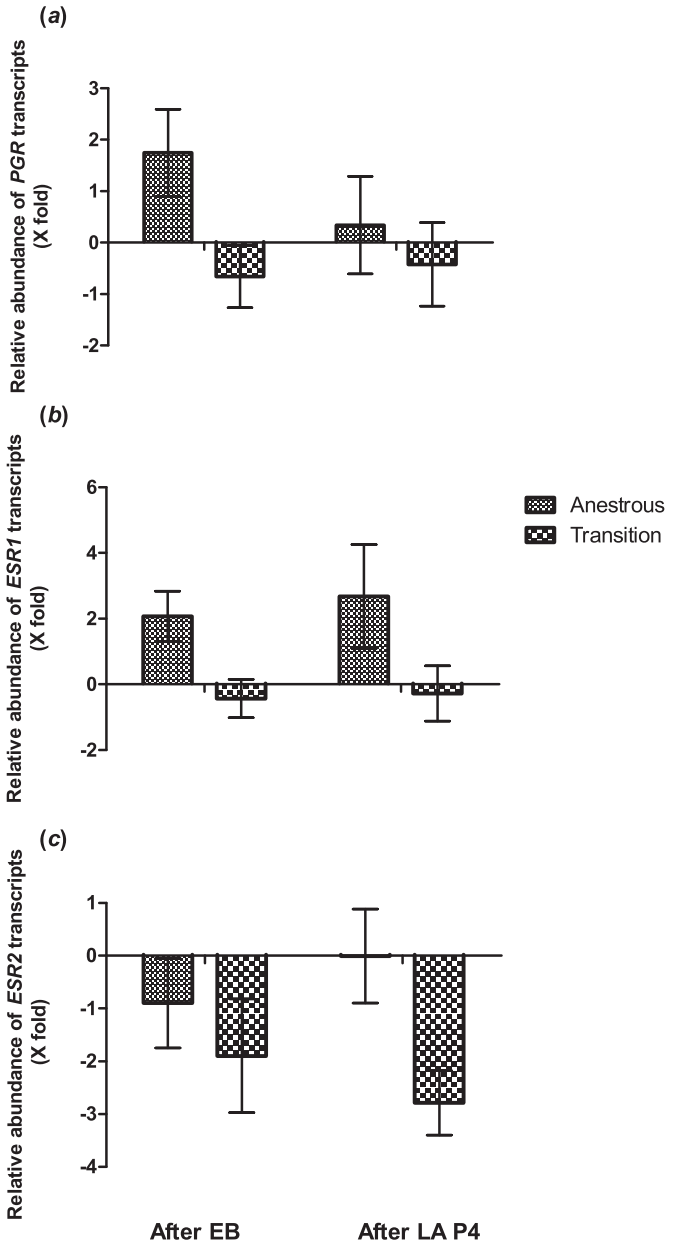

Fig. 3. Changes in relative abundance of transcripts for (a) progesterone receptor – *PGR*, (b) estrogen receptor α – *ESR1*, (c) and estrogen receptor β - *ESR2* at 48 h after estradiol benzoate treatment (After EB) and after long-acting progesterone treatment (after LA P4), in anestrous and transition groups. Data are expressed as mean ± SEM of the relative expression ratio of each transcript.

3.5. Endometrial immunostaining and distribution of PR, ERa and ERβ

In anestrous and transition groups, ERα and ERβ immunostaining were mostly localized to the nucleus of luminal epithelium, glandular epithelium and stroma before (Figs. 4a,d and 5a,d) and after treatment with EB (Fig. 5b,e and 6b,e). Following LA P4 injection, ERα and ERβ immunoreaction were also found in the glandular epithelium cytoplasm in both treated groups (Figs. 5c,f and 6c,f). Immunolabeling for PR in anestrous and transition groups were detected in the nuclei of the stroma, luminal epithelium and mostly in the glandular epithelium nuclei, prior to (Fig. 7a,d) and after EB treatment (Fig. 7b,e). After LA P4 treatment, PR immunostaining was localized to the nuclei of stroma and luminal epithelium, and in the nuclei and cytoplasm of glandular epithelium in both treated groups (Fig. 7c,f). In the cyclic group, immunolabeling for ERα, ERβ and PR were detected in the nuclei of stroma and luminal epithelium, as well as in the cytoplasm and nuclei of glandular epithelium at estrus and five days after ovulation (Figs. 5h and i, 6h,i and 7h,i).

In anestrous group, ERα and ERβ immunostaining five days after LA P4 administration (Figs. 5c and 6c) appeared to be more intense than prior to treatment initiation (Figs. 5a and 6a). In addition, apparently greater ERα, ERβ and PR immunolabeling, particularly in the glandular epithelium cytoplasm, were detected five days after LA P4 administration (Figs. 5c, 6c and 7c) as compared to 48 h after EB injection (Figs. 5b, 6b and 7b). In transition group, ERα cytoplasmic immunolabeling appeared to be stronger five days after LA P4 administration (Fig. 5f) than prior to treatments (Fig. 5d) and when compared to 48 h after EB injection (Fig. 5e). Subjectively, immunostaining for ERβ was more intense five days after P4 injection (Fig. 6f) as compared to 48 h after EB was given (Fig. 6e). For the cyclic group, cytoplasmic PR immunostaining appeared to be greater at estrus ($P < 0.05$; Fig. 7h) than five days after ovulation (Fig. 7i). There were no apparent differences observed for ERα and ERβ immunolabeling between cycle stages (Figs. 5h and i and 6h,i).

When the effects of treatment on receptors immunostaining were compared between treated groups (anestrous and transition), ERα and PR cytoplasmic immunolabeling at 48 h after EB administration appeared to be greater in transition group when compared to anestrous group (Figs. 5e,b and 7e,b). Moreover, when the effects of treatment on receptors immunostaining were subjectively compared between treated and cyclic groups, immunostaining for ERα and PR were more intense at estrus in cyclic group when compared to 48 h after EB administration in anestrous group (Figs. 5h,b and 7h,b). In addition, staining for ERα at estrus appeared to be greater than at 48 h after EB injection in transition group (Fig. 5h,e). There were no apparent differences on ERβ immunostaining between groups (Fig. 5). Immunolabeling before initiation of treatments were also compared between treated groups and subjectively a more intense staining for ERα was found before

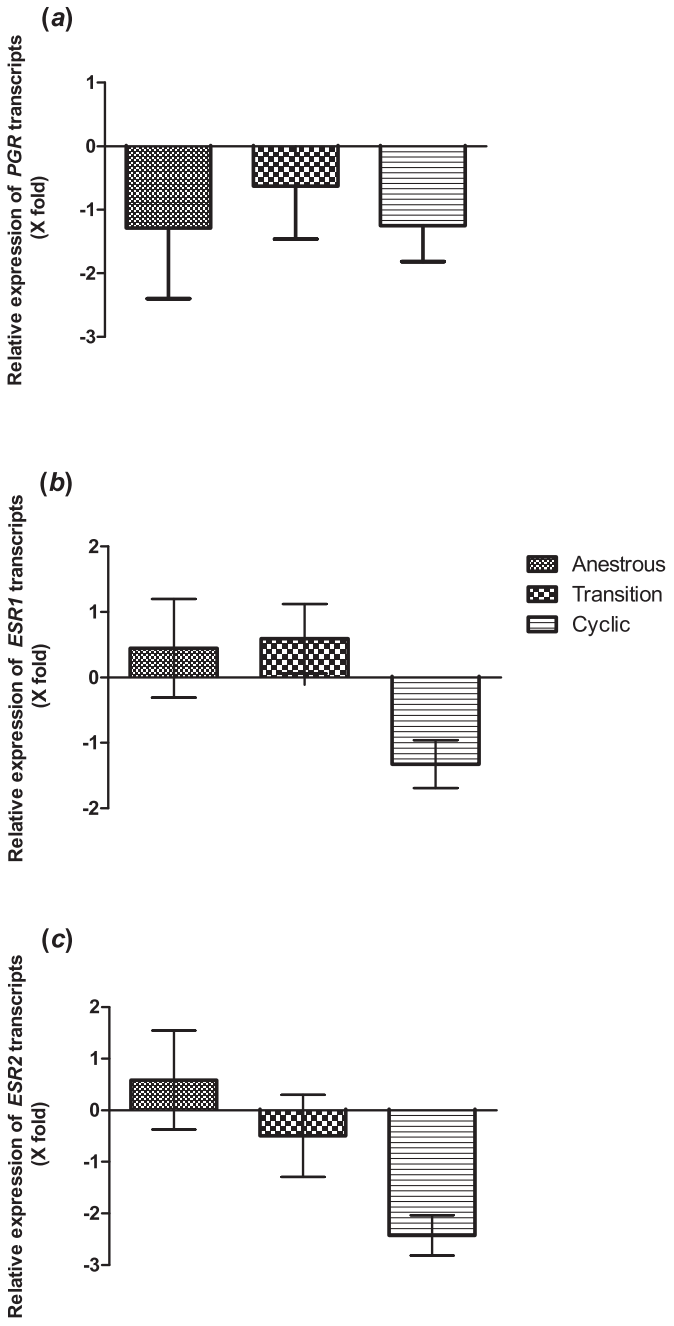

Fig. 4. Changes in relative abundance of transcripts for (a) progesterone receptor – *PGR*, (b) estrogen receptor α – *ESR1*, (c) and estrogen receptor β - *ESR2* when transcripts abundance after LA P4 treatment were compared to abundance after EB treatment (LA P4/EB) or abundance five days after ovulation was compared to estrus (Diestrus/Estrus), in anestrous, transition and cyclic groups. Data are expressed as mean ± SEM of the relative expression ratio of each transcript.

treatments in transition group as compared to anestrous group (Fig. 5d,a).

4. Discussion

Here we report, for the first time, protein immunostaining and changes in relative abundance of transcripts for ERα, ERβ and PR in non-cyclic mare's endometrium after the administration of 2.5 mg of EB followed by 1500 mg of LA P4 for preparing anovulatory mares to become embryo recipients. Moreover, we could demonstrate that the aforementioned protocol was effective at causing similar uterine edema, tone and plasma estradiol concentrations to those observed during the late estrus and early diestrus in cyclic mares.

Similar estradiol and estrogen conjugate peaks were achieved after the injection of the single dose between treated and cyclic groups. It is generally considered that measuring the active form of estrogen, the 17β-estradiol, provides the best information in terms of hormone signal delivered to the target cell. However, because circulating E2 concentrations are low in some animals, the accuracy of this method is sometimes limited. In contrast, EC concentrations are 100-fold higher than plasma E2 concentrations and are therefore easier to measure [21]. In the present study, only the peak values were above the E2 assay sensitivity. Because conjugated estrogens parallels plasma E2 and the same physiological information is obtained by estrogen conjugate (estrone sulfate) analysis [21], we also provided the EC profile in non-cyclic treated and cyclic mares.

The dose of 2.5 mg of EB was effective at causing high endometrial edema 24 h after injection in anestrous and transitional mares. High edema was also observed in the cyclic group, two days before ovulation, in agreement with previous reports on cyclic mares [29]. Moreover, a greater edema median score was detected prior to treatment initiation in transition group as compared to anestrous, probably due to the different follicular population size detected in anestrous (follicles <20 mm) and transitional mares (20–30 mm follicles). Considering that the larger the follicle the greater the ability to synthesize estrogen [30], the presence of a discrete to intermediate edema in transition group as compared to a lack of edema in the anestrous group could be explained. However, no difference on peripheral plasma E2 concentration was detected at this time between treated groups. A study was performed comparing estrogen and P4 concentrations between uterine fluid and peripheral blood of non-pregnant and pregnant pony mares, in which the authors indicated that changes occurring within the uterine environment are not discernible in the systemic circulation, especially during early pregnancy [31]. It is likely that local estrogens in the endometrium were not detected either by peripheral plasma concentrations during transitional phase in the present study.

After P4 administration, uterine edema and estrogen concentrations from both treated groups decreased to low levels, similarly to cyclic mares' concentrations after ovulation. However, a slight edema and estrogen rise was observed on day five after ovulation in the cyclic group. Ultrasonic uterine echotexture studies have revealed the presence of a slight increase in edema in early diestrus during the first half of the ovulatory season but not during the second half [32], which may reflect follicular growth that occurs in some mares in early diestrus or estrogen production by the developing corpus luteum [21]. In the present study, diestrus data were collected on the third estrous cycle of the breeding season, although we do not have accurate information on the follicular activity after ovulation.

Administration of 1500 mg of LA P4 in treated mares increased uterine tone to the intermediate level characteristic of diestrus in cyclic mares [29]. In addition, the dose given was effective in terms of reaching the minimum P4 concentrations required for pregnancy establishment and maintenance (>2.5 ng/mL) [4] until day five after its injection, although P4 profiles were different between cyclic and non-cyclic treated mares. Despite the gradual P4 decrease after peak in the treated groups, for embryo transfer purposes, performed on average on day four to six after P4

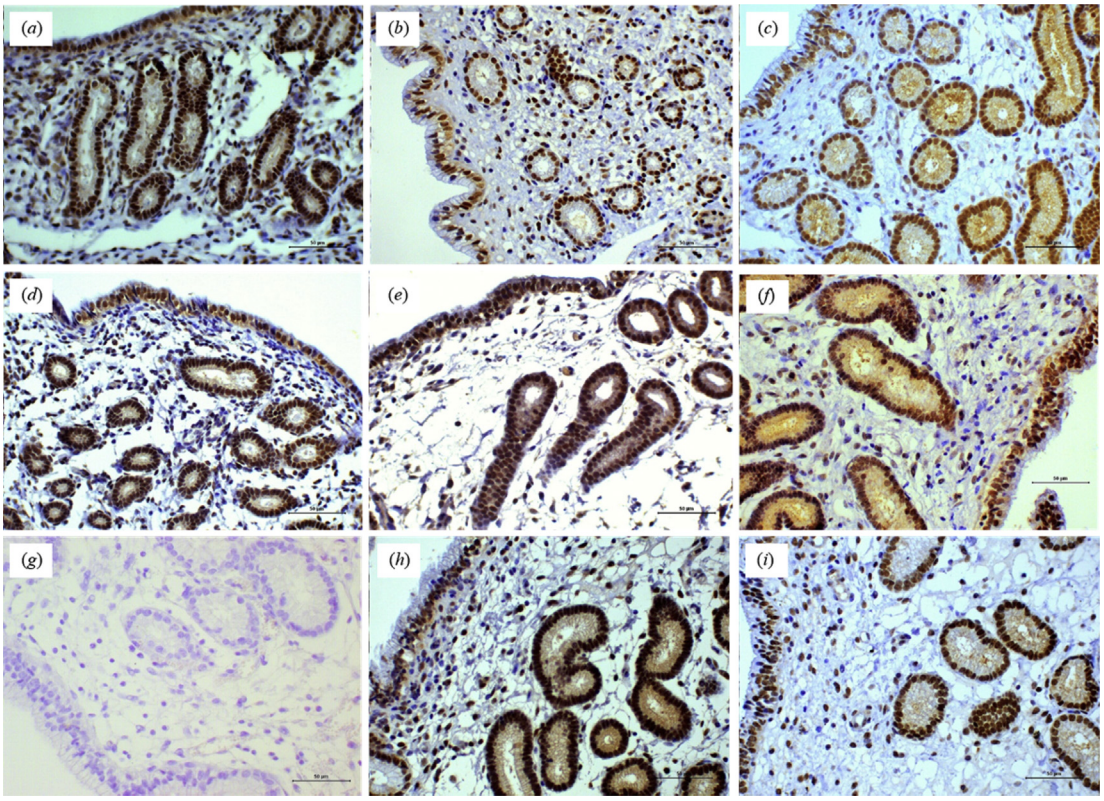

Fig. 5. Photomicrographs of the equine endometrium immunostained for estrogen receptor α (ERα). The figure panels show immunoexpression before treatments in anestrous (a) and transition (d) groups, at 48 h after estradiol benzoate treatment in anestrous (b) and transition (e) groups, and at five days after long acting progesterone administration in anestrous (c) and transition (f) groups. Figures (h) and (i) show immunohistochemical expression at estrus and five days after ovulation, respectively, in the cyclic group. Figure (g): negative control. Bars = 50 μm.

injection, another LA P4 dose is commonly given after transfer if an embryo is recovered, what ensures appropriate levels until pregnancy diagnosis.

It is well established that mRNA abundance of estrogen and P4 receptors in the mare's endometrium are stimulated by E2 and down-regulated by P4 [14,25,33]. Accordingly, *ESR1*, *ESR2* and *PGR* transcripts were decreased at early diestrus when compared to estrus in this study. When relative transcripts abundance five days after LA P4 injection were compared to transcripts abundance 48 h after EB administration within treated groups, the decrease found in *PGR* transcripts was similar to the decrease observed in the cyclic group when diestrus was compared to estrus. On the other hand, *ESR1* transcripts were increased in anestrous and transition groups, although such increase was not statistically different from the decrease found in the cyclic group.

As for *ESR1* transcripts, when *ESR2* transcripts at five days after LA P4 injection were compared to 48 h after EB administration in treated groups, there were no differences from the changes found in the cyclic group, when diestrus and estrus were compared. Moreover, changes in relative abundance of transcripts for *PGR*, *ESR1* and *ESR2* were similar after EB administration or LA P4 injection between anestrous and transition groups; although no statistical differences were detected, a variation in the relative abundance of transcripts was observed between groups after both hormone treatments. The similarity in transcripts changes observed in treated and cyclic groups suggest that the hormonal protocol was able to provide similar dynamics to that observed in cyclic mares.

As observed for mRNA abundance and as previously described [14,15], immunostaining for PR appeared to be more intense at estrus when compared to diestrus in cyclic mares. However, apparent differences for ERα and ERβ immunostaining were not observed. Increased levels of ERα during the pre-ovulatory phase in relation to late diestrus (8–13 days after ovulation) have been reported in mares [14,25] although no evidence for differential regulation of ERβ among cycle stages has been described [25,34]. It is possible that the lack of difference on ERα protein immunostaining between estrus and diestrus herein could be due to the early stage of diestrus (five days after ovulation) in which tissues were collected.

In addition, there were no apparent differences on receptor staining intensity after EB injection for ERα and PR in anestrous and transition groups. Interestingly, ERα and ERβ immunostaining in the glandular epithelium cytoplasm appeared to be more intense after LA P4 injection than prior to or after EB treatment, in both treated groups. Staining for ERα was observed in both the cell nuclei and cytoplasm of the glandular epithelium during estrus and diestrus in cyclic mares, with a pronounced staining observed in the nucleus, in agreement with recent reports [25]. Although ER and PR have been identified as nuclear receptors, it has been reported that they are continuously shuttled between the cytoplasm and cell nucleus [35,36]. In addition, predominant cytoplasmic ER has been found in early pregnant mares [37]. Furthermore, it is now widely accepted that both nuclear/cytoplasmic ERα and ERβ exist and, regardless to their location in the cell, the receptors have similar affinity for the steroid ligand [36]. Alternatively, cytoplasmic ER may function as membrane associated ER to mediate non-genomic actions of estrogen [36,38]. To identify the effects of E2 and P4 on ERα and PR expression, ovariectomized mice were treated with exogenous hormones [39]. Among other findings, the

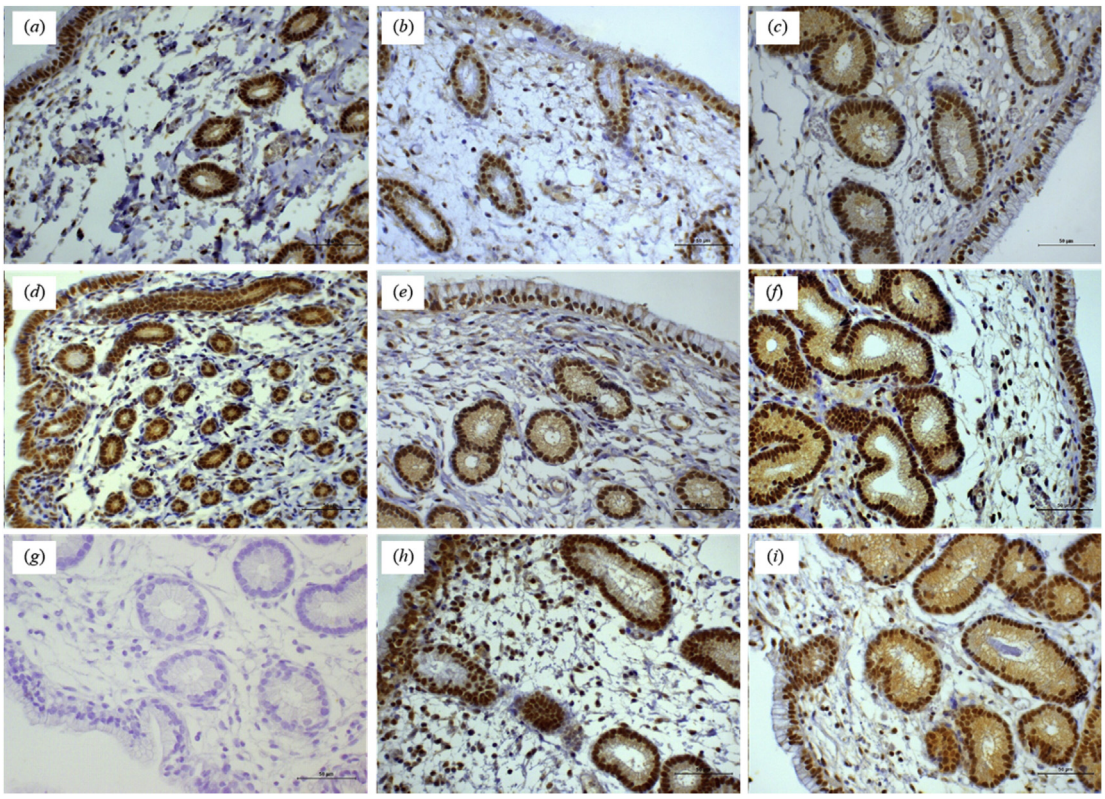

Fig. 6. Photomicrographs of the equine endometrium immunostained for estrogen receptor β (ERβ). The figure panels show immunoexpression before treatments in anestrous (a) and transition (d) groups, at 48 h after estradiol benzoate treatment in anestrous (b) and transition (e) groups, and at five days after long acting progesterone administration in anestrous (c) and transition (f) groups. Figures (h) and (i) show immunohistochemical expression at estrus and five days after ovulation, respectively, in the cyclic group. Figure (g): negative control. Bars = 50 μm.

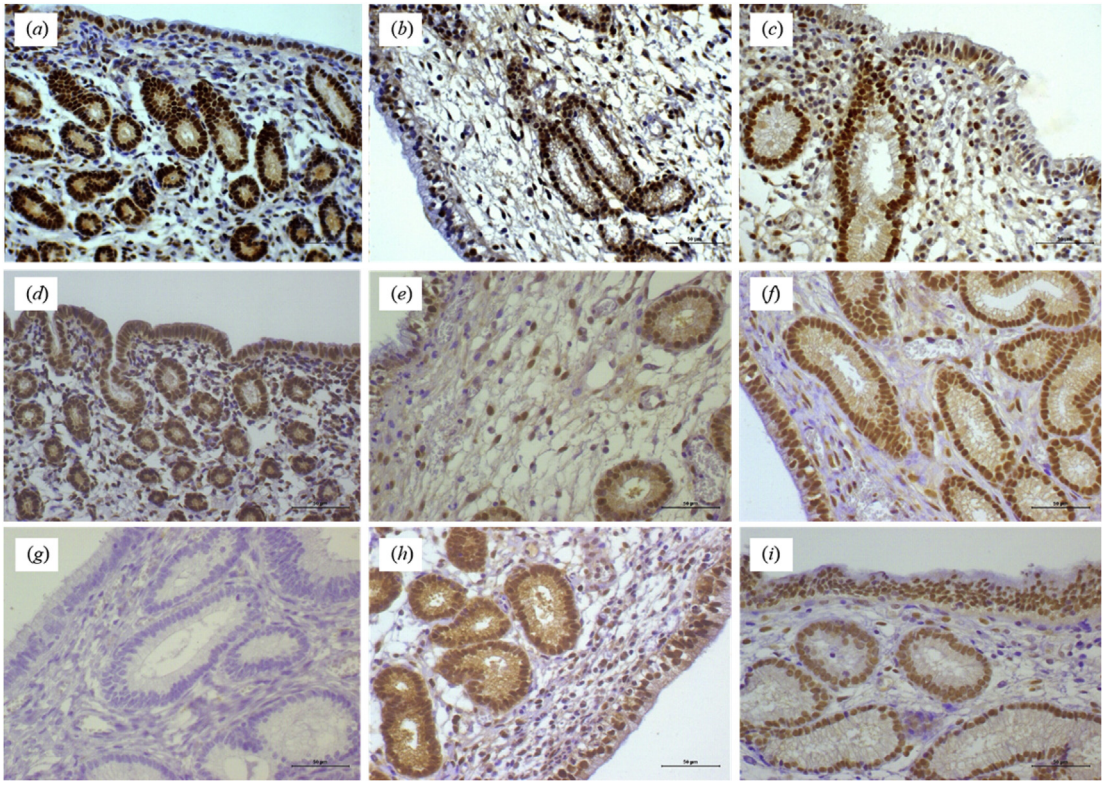

Fig. 7. Photomicrographs of the equine endometrium immunostained for progesterone receptor (PR). The figure panels show immunoexpression before treatments in anestrous (a) and transition (d) groups, at 48 h after estradiol benzoate treatment in anestrous (b) and transition (e) groups, and at five days after long acting progesterone administration in anestrous (c) and transition (f) groups. Figures (h) and (i) show immunohistochemical expression at estrus and five days after ovulation, respectively, in the cyclic group. Figure (g): negative control. Bars = 50 μm.

authors observed that ERα is dependent on P4 stimulation as it was found to be upregulated in the glandular epithelium after administration of estrogen and progesterone. It appears that glandular epithelium ERα and ERβ can also be upregulated by P4 in non-cyclic treated mares.

The surge in hormone levels and the differential expression pattern of the steroid hormone receptors play a critical and important role during estrous cycle and in pregnancy establishment and maintenance. Several studies in mares have determined that both PR and ER are evident during estrus when P4 concentrations are low and estrogen concentrations are increased [14,15,25]. Thereafter, PR and ER both decline in late diestrus, when circulating P4 levels are high. Spatially, the PR and ER are located in the nuclei of the luminal and glandular epithelia and stromal fibroblasts during estrus, whereas in late diestrus and early pregnancy these are reduced in the luminal and glandular epithelium but persist in the stroma [14,18].

In sheep the loss of PR in the glandular epithelia is a prerequisite for the expression of genes for secretory proteins [18]. Loss of PR in the luminal and glandular epithelium, not in the stroma, were found to be reduced or absent as early as day 20 of pregnancy also in the mare [37]. In the present experiment, PR expression in the cyclic group was apparently decreased in early to mid diestrus (day five after ovulation), particularly in the cytoplasm, but still showed pronounced labeling in the luminal and glandular epithelium nuclei. However, such decrease was not evidenced on day five after LA P4 administration in treated groups. Perhaps the different hormonal profile between non-cyclic progesterone treated groups and cyclic mares could have interfered on the PR expression. Moreover, successful pregnancies after embryo transfer are achieved using these LA P4 protocols. However, additional injections of LA P4 are given, especially after embryo transfer and pregnancy diagnosis, which may cause a further reduction on PR expression in luminal and glandular epithelium. Additional studies are needed to clarify these events in non-cyclic treated embryo recipient mares.

In conclusion, the dose of 2.5 mg of EB caused consistent endometrial edema and estrogens concentrations to those observed at late estrus in cyclic mares. Additionally, the administration of 1500 mg of LA P4 increased uterine tone to characteristic levels of diestrus found in cyclic mares and acceptable P4 concentrations to that observed during the early luteal phase. Changes in relative abundance of *PGR*, *ESR1* and *ESR2* transcripts were similar between cyclic and non-cyclic treated mares when P4 stimulated endometrium was compared to estrogen stimulated endometrium. However, decreased PR immunostaining in the glandular epithelium was not subjectively observed in the non-cyclic mares five days after LA P4 administration, as observed at five days after ovulation in the cyclic mares. Furthermore, the apparent increase in ERα and ERβ immunostaining in the glandular epithelium cytoplasm after LA P4 administration suggest that P4 may upregulate ER protein expression after the injection of 2.5 mg of EB in anestrous and transitional mares, as opposed to what is observed in cyclic mares.

Acknowledgments

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