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—Original Article—

Relationships Between Uterine Blood Flow, Peripheral Sex Steroids, Expression of Endometrial Estrogen Receptors and Nitric Oxide Synthases During the Estrous Cycle in Mares

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Abstract. The objective of this study was to investigate the relationships between uterine perfusion and estrogen, progesterone and the uterine nitric oxide synthase (NOS) system in five trotter mares during the estrous cycle. Color Doppler sonography for measurement of uterine blood flow and collection of blood for determination of plasma estrogen and progesterone concentrations were performed on days 0 (= ovulation), 1, 5, 11 and 15 and daily during estrus (days –1 to –4) of one estrous cycle; endometrial biopsy collection for mRNA expression analysis of NOS and estrogen receptors was performed on days 0, 1, 5, 11, 15 and –3. Blood flow in each uterine artery was assessed by calculating the mean time-averaged maximum velocity (TAMV) and the pulsatility index (PI). Plasma concentrations of estrogen and progesterone were determined using specific enzyme immunoassays. The mRNA expressions of endothelial NOS (eNOS), inducible NOS (iNOS) as well as estrogen receptors α (ERα) and β (ERβ) were quantified using reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The TAMV and PI had a biphasic pattern during the estrous cycle ($P<0.05$), with maximum and minimum, respectively, values on days 5 and –4. Estrogen receptor mRNA concentrations increased significantly during days 15 (ERα) and –3 (ERβ). Transcript expression of eNOS, but not iNOS, had a biphasic pattern during the cycle ($P<0.05$) with maximum levels on days 5 and –3 and correlated positively with TAMV ($r=0.81$, $P=0.05$). We infer that the uterine NOS system, especially eNOS, plays an important role in the regulation of uterine blood flow during the estrous cycle in mares.

Key words: Endometrial messenger RNA expression, Estrous cycle, Nitric oxide synthases, Steroid hormones, Uterine perfusion

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Characteristic uterine blood flow patterns have been reported during the estrous cycle in mares based on color Doppler sonography [1, 2]. Uterine perfusion had a wave-shaped profile throughout the cycle, showing maximal blood flow during estrus and the early luteal phase [1-4]. Variations in uterine blood flow in cycling mares have been attributed to changes in circulating estrogen concentrations. However, due to a weak correlation between the resistance to uterine blood flow and plasma estrogen concentrations, other factors may also regulate uterine blood flow [2].

Changes in uterine blood flow during the estrous cycle in many species are mediated mainly by estrogens [5]; although flow was enhanced by high plasma estradiol concentrations [6, 7] and decreased during the luteal phase [8, 9], the mechanisms involved have not been elucidated. Estrogens appear to act via two pathways: the genomic and non-genomic pathways [10]. Briefly, in the classical genomic pathway, estrogens bind to their nuclear receptors, subsequently regulating the transcription of target genes. For example, administration of estradiol increased estrogen receptors within 48 h in cyclic ewes [11]. Furthermore, a non-genomic pathway with a more rapid response that does not involve the synthesis of new proteins has been proposed [12]. In that regard, uterine blood flow increased 30 min after intra-arterial estradiol administration in oophorectomized ewes [13]. The rapid vasodilatory effect of estrogen was attributed to its direct action on vascular smooth muscle cells or to endothelial cell activation [12] with subsequent release of nitric oxide (NO) [14].

Nitric oxide, a volatile gas, is a potent vasodilator [15] and the main mediator for estrogen-induced stimulation of uterine blood flow. However, it may also have estrogen-independent actions [16, 17]. Endothelial nitric oxide synthases (eNOS) and inducible nitric oxide synthases (iNOS), which are responsible for the production of NO, have been detected in the endometrium in humans, sheep, rodents [18, 19], cows [20] and horses [21-23]. Since there were changes in the expression of eNOS during the estrous cycle in mares, it was speculated that the NOS system was involved in the regulation of their endometrial cyclicity [21, 22].

The objective of the present study was to investigate the involvement of steroid hormones and nitric oxide synthases in the regulation of uterine blood flow in cycling mares.

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Materials and Methods

Mares and housing

Five clinically healthy trotter mares, with a mean age of 12.2 years (range, 8 to 17), were investigated. Two mares were multiparous, and the other three were nulliparous. They were kept in box stalls with straw for bedding, fed hay and grain, with *ad libitum* access to water, and given daily access to a paddock.

Study design

The mares were examined throughout one estrous cycle. Color Doppler assessments of blood flow in uterine arteries were conducted on days 0 (=day of ovulation; in mares with double ovulations=day of first ovulation), 1, 5, 11 and 15 and daily during estrus on days –4, –3, –2 and –1. Blood samples were taken after each examination. Endometrial biopsies were collected on days 0, 1, 5, 11 and 15 and on the second day of estrus (day –3).

Mare management prior to and during the study

Every day during the study period, the mares were teased using a stallion, and the uterus and ovaries were examined using B-mode sonography. Estrus was defined as the days on which mares showed behavioral sings of estrus and an endometrial edema was detectable. During estrus, ovarian follicular development was monitored for detection of ovulation. The day of ovulation (day 0) was defined as the day of disappearance of a preovulatory follicle.

To ensure that endometritis was not present, a uterine swab (Accu Cul Shure®, Accu-Med, NY, USA) was collected transcervically from all mares on the day of ovulation. The uterine swabs were streaked onto blood, Gassner and Edwards agar and incubated at 37 C for 24 h for bacteriological evaluation. Samples were incubated an additional 24 h when no growth was detected after the first 24 h. In addition, the swabs were rolled onto a glass slide, which was stained (Dade® Diff-Quick-Schnellfärbelösung), and the cytology was examined microscopically.

Blood flow measurements

Both the left and right uterine arteries were examined transrectally, as previously described [1]. Doppler measurements were conducted using a Toshiba SSH 140A ultrasound device (Toshiba, Tokyo, Japan) equipped with a 7.0-MHz microconvex transducer (PVF-738-F). Blood flow waveforms were displayed on-line and recorded on video tapes. In retrospect, Doppler calculations were completed (off-line) using two similar consecutive waveforms, with a maximum ratio between diastolic and systolic frequency shift. Blood flow was characterized by the time-averaged maximum velocity (TAMV) and the pulsatility index (PI), which indicates the resistance to blood flow in the uterine vascular bed. Based on the envelope of the Doppler shift spectrum, TAMV was calculated from the time-averaged maximum frequency shift over the cardiac cycle (TAMF) using the following equation: $TAMV = (TAMF \times c) / (2F \times \cos\alpha)$, with c=ultrasound propagation speed, F=transmitted wave frequency and α=angle between the ultrasound beam and the blood flow direction. The PI was calculated as the ratio of the difference between the peak systolic frequency shift (PSF) and the minimum diastolic frequency shift (MDF) to TAMF: $PI = (PSF–MDF)/TAMF$. The average of the TAMV and PI values, respectively, of two consecutive pulse waves were used in the statistical analyses.

Measurement or steroid hormones

Blood samples were obtained by venipuncture of the jugular vein, with collection of the samples in an evacuated blood tube (Venoject® Vacuum System, Terumo Europe N.V., Leuven, Belgium) after each sonographic examination. Plasma was separated by centrifugation (2,000 × *g* for 20 min) and frozen at –20 C. Steroid hormone concentrations were determined by enzyme immunoassays, as previously described [24, 25]. Unless otherwise stated, all reagents for analysis of steroid hormone concentrations were purchased from Sigma (Munich, Germany) as indicated by their corresponding catalogue number.

In brief, the plasma progesterone concentration (P_4) was measured directly in 5 μl plasma (antibody, clone 2H4 (P1922); antigen, progesterone-7α-BSA; enzyme, progesterone-7β-horseradish peroxidase (P3659)). Total estrogen (E) analysis (rabbit anti-horse antibody, Code 2/3 Whst.; antigen, estradiol-17β-hemisuccinate-BSA (E0756); enzyme, estradiol-17β-hemisuccinate-horseradish peroxidase (E8883)) was performed after hydrolysis of unconjugated estrogens (acetate buffer: pH 4.8; 0.4% glucuronidase aryl sulfatase from helix pomatia) and ether (30% butyl-methyl ether and 70% petrol ether) extraction of conjugated estrogens. The intra- and interassay coefficients of variation were <14% for estrogens and <16% for progesterone.

Endometrial biopsy and mRNA expression analysis

Endometrial biopsy samples were collected transcervically using Kevorkian biopsy forceps (Hauptner, Solingen, Germany). The tissue samples (10 × 5 × 5 mm) were immediately transferred into liquid nitrogen (–196 C) and stored at –80 C until expression analysis.

Total RNA was isolated from endometrial biopsy specimens according to Chomczynski and Sacchi [26] using TriPure® Isolation Reagent (Roche Diagnostics, Mannheim, Germany). Contamination by genomic DNA was minimized by an additional DNase digestion according to the manufacturer's protocol (RQ1, Promega, Madison, WI, USA). Total RNA was purified using NucleoSpin® RNA II (Macherey-Nagel, Düren, Germany) and quantified spectroscopically (260 nm) using a Biophotometer (Eppendorf, Hamburg, Germany).

A two-step quantitative real-time RT-PCR was undertaken, as previously described [21]. In brief, total RNA was reverse transcribed to cDNA, as previously described [27]. Specific equine transcripts were amplified using the following commercial primers (MWG, Ebersberg, Munich, and Amersham Pharmacia Biotech, Freiburg, Germany): 18S rRNA (5′- TCAAGAACGAAAGTCGGAGG -3′, forward; 5′-GGACATCTAAGGGCATCACA-3′, reverse [488 bp]), eNOS synthase (5′-GAAGCACCTGGAGAATGAGC -3′, forward; 5′- TCTGGCTGGTAGCGGAAG -3′, reverse [149 bp]), iNOS synthase (5′-GCCAAGGTCTGAGCTACCTG-3′, forward; 5′-GAGTGCCTGGCTGAGTGAG-3′, reverse [200 bp]), estrogen receptor-α (5′-AGGGAAGCTCCTATTTGCTCC-3′, forward; 5′-CGGTGGATGTGGTCCTTCTCT -3′,

reverse [234 bp]) and estrogen receptor-β (5′-GCTTCGTGGAGCTCAGCCTG-3′, forward; 5′- AGGATCATGGCCTTGACACAGA -3′, reverse [262 bp]). The quantity and integrity of endometrial cDNA were checked by denaturing gel electrophoresis, as well as PCR specific for the housekeeping gene 18S rRNA. Quantitative real-time PCR reactions using the LightCycler® DNA Master SYBR Green I protocol (Roche Diagnostics, Mannheim, Germany) were carried out as previously described [28]. Absolute quantification was performed as described by Ulbrich *et al*. [28] using serial dilutions of corresponding amplicons, followed by an 18S rRNA normalization. The authenticity of each amplicon was confirmed through melting-curve analysis, as well as by sequencing of the resulting PCR products (TOPLAP, Munich, Germany).

Statistical analysis

The TAMV and PI values of the left and right uterine arteries were compared using the Pearson correlation coefficient and paired Student's *t*-test. To assess changes, the mean values of each variable from all examinations were defined as 100%, whereas values for each examination day were expressed in relation to the mean. The effect of day on relative values of uterine TAMV and PI, plasma concentrations of estrogen and progesterone and the mRNA expressions of ERα, ERβ, eNOS and iNOS were tested using one-way ANOVA for repeated measures. Relationships between relative blood flow values and plasma steroid hormonal concentrations of all examinations days and between blood flow values and mRNA expressions in the endometrium on days 0, 1, 5, 11, 15 and –3 were examined using Pearson correlation coefficients (r). Correlation coefficients were calculated using the means of the respective days of examination to analyze cycle-associated effects. Statistical analyses were conduced with the Statistical Analysis System (SAS Institute, Cary, NC, USA). Values are presented as means ± SEM. For all analyses, $P \leq 0.05$ was considered significant, whereas $0.05 < P \leq 0.10$ was considered to indicate a trend.

Results

Clinical findings

No abnormalities of the reproductive tract were detected in any mare during the study. There was no indication of endometrial pathology based on bacteriological or cytological examination of uterine swab specimens. In two of the mares, a second ovulation occurred 1 day after the first ovulation. In all mares, the ovulations occurred on the left ovary. The length of the cycle was (mean ± SEM) 24.2 ± 3.0 days, and estrus lasted 4.8 ± 0.2 days.

Uterine blood flow

The blood flow values of the left and right uterine arteries and of the uterine arteries ipsilateral and contralateral to the preovulatory follicle and corpus luteum, respectively, did not differ in terms of TAMV (P=0.88) and PI (P=0.85), but they were correlated (TAMV, r=0.46, P=0.002; PI, r=0.50, P=0.001). Therefore, the mean values were used for further analysis. For both end points, there was an effect of day of cycle (TAMV, P=0.03; PI, P=0.04), and both TAMV and PI had a biphasic pattern (Fig. 1 and Table 1). The relative TAMV values were low on days 0, 1, 11 and –3 to –1 and higher ($P \leq 0.05$) on days 5 and –4. The TAMV values on day 15 did not differ significantly (P>0.05) from any other day. The relative PI values started high on days 0 and 1, decreased ($P \leq 0.05$) on day 5, showed a transient increase ($P \leq 0.05$) on day 11 and decreased ($P \leq 0.05$) again on day 15. A second increase ($P \leq 0.05$) occurred between days –4 and –3. Values from day –3 onwards were not significantly different (P>0.05) from any of the other examination days.

Steroid hormone concentrations

There was an effect of day for the plasma concentrations of P_4 (P=0.004) and E (P=0.05). The relative P_4 concentrations were low during estrus and high during diestrus ($P \leq 0.05$, Fig. 1). The relative E concentrations were low during diestrus and increased ($P \leq 0.05$) towards estrus (Fig. 1). The absolute values of the plasma concentrations of E and P_4 are shown in Table 1.

Endometrial mRNA expression

There were effects of day for the endometrial mRNA expressions of ERα and ERβ (P=0.03 and P=0.05, respectively). From days 0 to 11, the relative mRNA concentration of ERα remained (P>0.05) at a low to moderate level (Fig. 1). By day 15, the value had increased relative to days 0, 1 and 5 ($P \leq 0.05$) and tended to be higher than on day 11 (P=0.08). Between days 15 and –3, the relative mRNA expression of ERα did not change (P>0.05). The relative mRNA expression of ERβ decreased ($P \leq 0.05$) between days 0 and 5 and then increased ($P \leq 0.05$) until day –3. There was a positive correlation between the relative expressions of ERα and ERβ mRNA (r=0.89; $P \leq 0.05$). The absolute values of the mRNA expressions of ERα and ERβ are shown in Table 1.

There was an effect of day for the endometrial mRNA expression of eNOS (P=0.04). The pattern was biphasic (highest on day 5 and –3; Fig. 1). In contrast, there was no effect of day (P=0.26) for the expression of iNOS mRNA (Fig. 1). Note that the absolute data for NOS were previously published [21].

Relationships between uterine blood flow, steroid hormone concentrations and endometrial mRNA expressions of estrogen receptors and nitric oxide synthases

There was no correlation between the relative changes in TAMV and plasma concentrations of E and P_4 (E: r=–0.06, P=0.91; P_4: r= 0.08, P=0.87) nor between the TAMV and mRNA expressions of ERα and ERβ (ERα, r=0.43, P=0.40; ERβ, r=0.53, P=0.27). However, TAMV was correlated with the expression of eNOS mRNA (r=0.81, P=0.05). There was no significant correlation between iNOS and TAMV (r=0.72, P=0.10), but iNOS mRNA expression correlated positively with mRNA expression of ERβ (r=0.81, P= 0.05) and the plasma concentrations of E (r=0.85, P=0.03).

Discussion

This is apparently the first study reporting an association between uterine blood flow and the expression of the endometrial NOS system in mares. There was a significant correlation (P=0.05) between TAMV and the expression of eNOS mRNA, both showing a similar biphasic pattern throughout the estrous cycle. This pat-

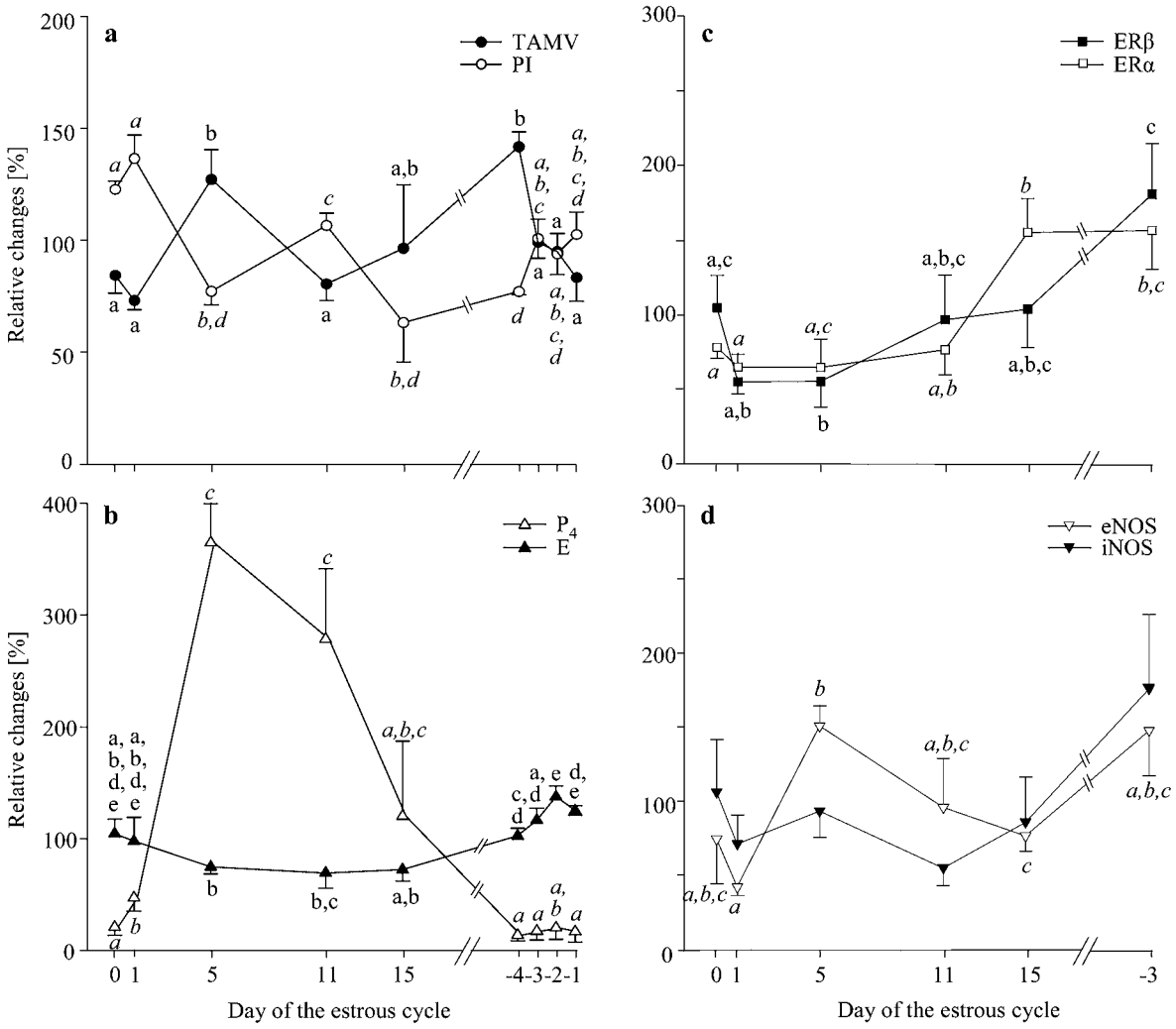

Fig. 1. Relative changes of (a) the time-averaged maximum velocity (TAMV) and Ppulsatility index (PI) of both uterine arteries, (b) plasma concentrations of total estrogens (E) and progesterone (P_4), (c) endometrial mRNA expressions of estrogen receptor α (ERα) and estrogen receptor β (ERβ) and (d) endothelial nitric oxide synthases (eNOS) and inducible nitric oxide synthases (iNOS) of five mares during the estrous cycle (day 0, ovulation). Values are presented as means ± SEM. Means of all values of the estrous cycle=100%. a–e) Within endpoint variables, values with different letters differ (P≤0.05).

tern was consistent with that previously reported for uterine blood flow [1–4] and similar to that reported for the immunohistochemical activity of eNOS protein in the endometrium of cyclic mares [22]. It has been suggested that eNOS plays a predominant role in regulating the uterine vasculature of mares [21, 22], perhaps due to induction of vasodilation rather than induction of vessel growth [22]. In sheep, eNOS may be responsible for NO-mediated uterine vasodilation [19, 29, 30]; exogenous estradiol-17 beta (E_2) increases the eNOS mRNA expression [19, 30] and protein expression [29] in endothelial cells of the uterine artery. Furthermore, iNOS may also regulate uterine vasodilation, perhaps by an endothelium-independent pathway, as it is not expressed in the uterine endothelium [19]. In the present study, although the iNOS mRNA expression pattern was similar to that of eNOS, there was no significant effect of day of cycle.

In contrast to other species [5, 6, 31], there was no significant correlation between uterine perfusion and the plasma estradiol concentrations during the estrous cycle. In an earlier study utilizing daily assessments in cycling mares, there was no correlation between uterine perfusion and peripheral estrogen concentrations during diestrus and only a weak correlation during estrus [2]. Furthermore, the plasma progesterone concentration and uterine perfusion in this study were not significantly correlated, in agreement with previous findings [2]. Therefore, we concluded that factors other than or in addition to steroid hormones regulated uterine blood flow during the estrous cycle in mares.

Since the effect of a hormone depends not only on its peripheral concentration, but also on its tissue receptors, the mRNA expressions of estrogen receptors α and β in endometrial tissue were characterized. The expressions of both receptors changed signifi-

Table 1. Absolute mean values ± SEM for the time-averaged maximum velocity (TAMV) and pulsatility index (PI) of both uterine arteries, plasma concentrations of estrogen (E) and progesterone (P_4) and mRNA expressions of estrogen receptor α (ERα) and estrogen receptor β (ERβ) during one estrous cycle of five mares

Days of estrous cycle	TAMV (cm/sec)	PI	E (pmol/ml)	P_4 (pmol/ml)	ERα (fg/ng)*	ERβ (ag/ng)*
0	10.8 ± 1.1	3.1 ± 0.5	2.9 ± 0.6	2.5 ± 0.6	2.5 ± 0.5	3.6 ± 1.2
1	9.3 ± 0.3	3.4 ± 0.5	3.0 ± 1.1	5.9 ± 0.9	2.0 ± 0.3	1.9 ± 0.3
5	15.9 ± 0.9	1.9 ± 0.2	2.0 ± 0.2	48.4 ± 4.2	2.2 ± 0.9	2.2 ± 1.1
11	10.4 ± 1.3	2.7 ± 0.4	1.8 ± 0.3	40.5 ± 10.3	2.3 ± 0.5	3.3 ± 1.3
15	16.3 ± 2.9	1.7 ± 0.3	1.9 ± 0.3	19.5 ± 13.3	4.8 ± 0.9	3.7 ± 1.0
–4	18.3 ± 1.6	1.9 ± 0.3	2.9 ± 0.5	1.6 ± 0.5	–	–
–3	13.0 ± 1.8	2.6 ± 0.6	3.2 ± 0.5	2.2 ± 1.0	4.6 ± 0.5	6.0 ± 1.3
–2	12.4 ± 1.8	2.3 ± 0.4	3.8 ± 0.5	2.7 ± 1.5	–	–
–1	10.9 ± 2.0	2.7 ± 0.6	3.4 ± 0.4	2.4 ± 1.4	–	–

* fg and ag, respectively, specific mRNA / ng total RNA.

cantly during the cycle, with highest values on day 15 (ERα) and day –3 (ERα and ERβ). However, in a previous study [32], the ERα mRNA levels in 25 mares were similar during estrus and on days 5, 10 or 15 of the cycle, whereas in other studies [33, 34], the levels were highest around estrus and low during mid- to late-diestrus. Clearly, additional studies are needed to resolve these apparent discrepancies.

The pattern of ERα mRNA expression was similar to that of the plasma estrogen concentration with the exception of that on day 15, when the ERα mRNA expression was high while the estrogen concentration was low. Since this high expression of ERα mRNA occurred concurrent with low PI values on day 15, we inferred that these two events may be associated.

Based on the present findings, we inferred that uterine blood flow during the estrous cycle was regulated by a steroid hormone-mediated stimulation of NOS. In that regard, we observed two increasing phases of uterine blood flow: the first increasing phase (days 0 to 5) was accompanied by increasing concentrations of progesterone and increasing NOS mRNA expression; the second increasing phase (days 11 to estrus) was accompanied by increasing concentrations of estradiol and increasing NOS mRNA expression. Both estrogens and progesterone can cause systemic and uterine vascular relaxation and increased blood flow, which are mediated primarily by NO [16, 30, 35, 36], as reported in sheep [16, 19, 30, 37], pigs [36] and rats [12, 35]. Also, we assume that both hormones can cause NO-mediated vasorelaxation in the uterine vascular bed during the estrus cycle in the mare; progesterone seems to stimulate uterine blood flow during early diestrus, and estradiol seems to stimulate it towards estrus. The estrogen-induced production of NO may occur both by immediately increasing eNOS activity through non-genomic pathways via membrane-bound estrogen receptors or in the longer term by upregulating eNOS protein through genomic pathways via binding to nuclear ER [38]. This hypothesis was further supported by a recent study [29] describing that exogenous E_2, P_4 or both increased eNOS protein in the uterine artery of ovariectomized ewes. However, the specific mechanism by which progesterone caused eNOS activation has not been clarified.

We conclude that the uterine NOS system plays a major role in regulation of uterine perfusion during the estrous cycle in mares. In particular, eNOS may have an important role in increasing uterine blood flow during estrus and the early luteal phase in mares.

Acknowledgments

Absolute data for NOS were previously published [21].

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