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# Expression of enzymes and receptors of leukotriene pathway genes in equine endometrium during the estrous cycle and early pregnancy

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ABSTRACT

The aims of the present study were to elucidate the expression profiles of leukotriene (LT) pathway mRNA transcription and to determine the possible interaction of LT and prostaglandin (PTG) pathways genes in equine endometrium during the estrous cycle and early pregnancy. Endometrial biopsies were obtained from mares on the day of ovulation (d0), at late diestrous (LD, n = 4), and after luteolysis in the estrus phase (AL, n = 4) of the cycle. Biopsies were also taken on Days 14 (P14; n = 4), 18 (P18, n = 4), and 22 (P22, n = 4) during early pregnancy that were comparable days to cyclic sampling days. A mixed model was fitted on the normalized relative mRNA levels, quantified by qPCR in duplicate, and least significant difference test was employed to detect significantly different group(s). In addition, to determine the degree of contribution of each gene to separation of treatment groups, the multivariate projection method partial least square regression discriminant analysis was used. The expression of 5-lipoxygenase mRNA was greater on d0 and LD, declined at AL, and was suppressed by early pregnancy. Leukotriene $A_4$ hydrolase mRNA expression increased at LD and during early pregnancy, but was significantly greater at LD compared with P14. The expression of LT $C_4$ synthase mRNA was only induced at LD. Cysteinyl leukotriene receptors (CysLT1 and CysLT2) mRNA expressions were decreased by both cyclic changes and early pregnancy, whereas 5-lipoxygenase-activating protein and B leukotriene receptor mRNA expressions were not affected by early pregnancy or stages of the estrous cycle. Partial least square discriminant analysis suggests that LT and PTG pathway enzymes and receptors appear to behave similarly in terms of mRNA expression. In conclusion, the expression profiles of LT pathway genes are demonstrated in equine endometrium for the first time by the present study, and the present data suggest that LT pathway mRNA transcriptions are tightly regulated during early pregnancy in mares.


## 1. Introduction

Arachidonic acid is the main substrate for the enzymes that produce prostaglandins (PTGs) and leukotrienes (LTs). Arachidonic acid is converted into prostaglandin $H_2$ ($PTGH_2$) by cyclooxygenase (prostaglandin H synthase, PTGS) enzymes, and arachidonate 5-lipoxygenase (5-LO) transforms arachidonic acid into leukotriene $A_4$ ($LTA_4$). 5-Lipoxygenase-activating protein (FLAP) is a membrane anchor protein necessary for the activation of 5-LO. $LTA_4$ is used for the production of leukotriene $B_4$ ($LTB_4$) by $LTA_4$

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hydrolase ($LTA_4H$) or cysteinyl $LTC_4$ by $LTC_4$ synthase ($LTC_4S$). The actions of LTs are exerted through G-coupled receptors: B leukotriene receptor for $LTB_4$ and cysteinyl leukotriene receptors (CysLT1/CysLT2) for $LTC_4$ [1].

Both PTGS and 5-LO enzymes share arachidonic acid, and these two pathways are proposed to affect the functions of each other [2]. Inhibition of 5-LO could lead to increased production of PTGs because more substrate would be available for PTGS enzymes; however, the opposite also seems to be correct as PTG production was affected negatively in 5-LO knockout mice [3]. Similarly, inhibition of PTGS activity could also cause an increased production of LTs. However, different PTGS inhibitors may or may not inhibit the activity of 5-LO, but this inhibition of PTGS does not necessarily result in increased consumption of arachidonic acid by 5-LO [2]. These results, although controversial, indicate that these two pathways are not completely independent from each other and they have converging functions [4].

LTs belong to a family of lipid mediators and are involved in the innate immune and inflammatory response. Along with their functions related to the innate immune response, there is evidence that LTs may be associated with luteal regulation. LTs could enroll in the occurrence of luteolysis in ewe and cow [5,6]. In cows and mares, natural luteolysis involves stimulation of $PGF_{2\alpha}$ secretion in a pulsatile manner from the endometrium, which is then transferred to the corpus luteum (CL) [7–9]. Similar to the ruminant and the porcine, the equine endometrium is regulated for the luteolytic or anti-luteolytic pathway, and any failure in anti-luteolytic mechanism during maternal recognition of pregnancy causes embryonic losses [7]. Infusion of 5-LO inhibitors inside the uterus causes an extension of the CL lifespan by delaying luteolysis in cow [10] and ewe [11,12]. These data suggest that intrauterine regulation of LTs production may regulate the maintenance of the CL in ruminants. Korzekwa et al. [13] have clearly demonstrated the regulation of LTs production by analyzing mRNA expression of the associated enzymes and receptors in bovine luteal tissue during the estrous cycle. The data suggested that LTs are generated in the CL and are regulated according to the stages of the estrous cycle.

Our earlier study [14] evaluated the expression profile of genes involved in PTG action in equine endometrium and reported that both the estrous cycle and early pregnancy regulate the luteolytic process through differential expression of genes in PTG cascade. In the light of this evidence, the objective of this study was to demonstrate the expression of LT pathway genes in equine endometrium. We also aimed to evaluate the expression patterns of LTs and PTGs pathway genes during the estrous cycle and early pregnancy in equine endometrium. Therefore, this study may contribute to better understanding of the arachidonic acid catabolism in equine endometrium during the estrous cycle and early pregnancy.

## 2. Materials and methods

### 2.1. Materials

TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit), RNAse-free DNAse I, qPCR Master Mix (2×) for real-time PCR (Maxima SYBR), and dNTP set and Taq DNA polymerase (Fermentas Life Sciences, Glen Burnie, MD, USA) were obtained. Specific oligonucleotide primers for LT pathway genes were synthesized by Metabion International AG (Martinsried, Germany).

### 2.2. Animals and experimental procedures

All animal procedures and the experimental design of this study are explained in detail by Atli et al. [14,15]. Briefly, 10 mares and 1 stallion (aged 5–16 years and weighing 400–450 kg) were used. Fertility examination and uterine health were evaluated (biopsy scores were I or IIA according to the classification of Kenney and Doig [16]). Mares were placed individually in the equestrian center in the Faculty of Veterinary Medicine, Selcuk University, Turkey, provided haylage and water *ad libitum*, and were fed with grain pellets twice a day. The Ethics Committee of Faculty of Veterinary Medicine, Selcuk University, approved all the experimental procedures.

Mares with obvious edema in uterus and >35 mm follicle on the ovary was induced with hCG (1500 IU, iv) for ovulation. When a mare is scheduled for pregnant endometrium sampling, it was inseminated with 750 million motile spermatozoa collected freshly from the stallion. Ultrasonography was employed every 12 hours until the detection of ovulation, and the day of ovulation was accepted as day zero (d0). In pregnant group, if the ovulation had not occurred within 48 hours after insemination, the insemination procedure was repeated.

Endometrial biopsies were obtained from mares on the day of ovulation (d0, n = 4), at late diestrus (LD, Days 13.5–14, n = 4, high plasma progesterone), and after luteolysis in the beginning of the estrus phase (AL, Days 17–18, n = 4, low progesterone; <1 ng/mL) of the cycle. Biopsies were collected in pregnant mares on Days 14 (P14; n = 4) and 18 (P18, n = 4) that were comparable days to cyclic sampling days. In a 21-day cycle, Day 14 corresponds to LD and Day 18 to after luteolysis (AL). Biopsies were also obtained on Day 22 of pregnancy (P22; n = 4). The endometrial tissue samples were snap-frozen immediately in liquid nitrogen and stored at −70 °C until RNA isolation. In an estrous cycle or pregnancy, only one biopsy sampling was performed. Following biopsy, mares were rested for recovery for one cycle period. Within a cyclic or pregnant group, biopsy samples for any particular day were taken from four different mares. Mares were used for multiple time points (three to five times) during the experimental period; therefore, a given mare was used for different days of the cycle and pregnancy groups. Twelve-hour blood sampling for plasma progesterone measurement was started at least 2 days before the day of biopsy.

### 2.3. RNA extraction, cDNA synthesis, and qPCR

RNA isolation protocol and quality measurements from equine endometrium were performed according to Kurar et al. [17]. Primers were either obtained from published primer sequences (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) [18] or derived from equine sequences by