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Expression patterns of *TLR4* mRNA and protein in the equine endometrium during early pregnancy

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Abstract: Toll-like receptors (TLRs) are an important part of the innate immune system and play a role in activating the acquired immune system. TLR4 is the best characterised of the many TLRs; its signalling mechanisms are known to be mediated by many inflammatory pathways and by endogenous substances. The aim of the present study was to elucidate the expression profiles for *TLR4* mRNA and protein and determine the cell-specific localisation of TLR4 in the equine endometrium during the oestrous cycle and early pregnancy. Endometrial biopsy samples were obtained from mares during early pregnancy (on day 14, P14, $n = 4$) and, for comparison, on day 14 of the oestrous cycle (late dioestrus, LD, $n = 4$) and when luteolysis was completed (after luteolysis, AL, $n = 4$). Total RNA was extracted and transcribed into cDNA, and the relative expression of *TLR4* mRNA was quantified by qPCR. Immunohistochemistry was used to define the spatio-temporal localisation of TLR4 protein in the uterus. Expression of *TLR4* mRNA was reduced ($P < 0.05$) on P14 compared to LD. The expression of *TLR4* mRNA was similar for P14 and AL. TLR4 protein was localised mainly in the luminal and glandular epithelia, but was also present in endothelial cells of capillaries. Compared to P14, TLR4 protein was abundant in both luminal and glandular epithelia of uteri of mares in LD. These results suggest that downregulation of TLR4 in the endometrial epithelia in early pregnancy may play a role in the immune tolerance mechanisms that protect the conceptus (embryo/foetus and associated membranes).

Keywords: Toll-like receptor 4; early pregnancy; equine endometrium

The maternal immune system protects the developing embryo/foetus against harmful immune responses during pregnancy; this is known as immune tolerance. Compared to other factors with roles in early recognition of pregnancy in the equine endometrium, knowledge of the immunological events that occur during early pregnancy is quite limited (Noronha and Antczak 2010; Antczak 2012). Klein and Trodsson (2011) indicated that immune regulatory genes display decreased expression profiles during the early stage of equine pregnancy, concluding that this reflects the protection of the semi-allograft conceptus from the dam's immune system. When equine trophoblast cells were incubated with peripheral blood lymphocytes *in vitro*, the proliferation of lymphocytes was suppressed by more than 50% (Flaminio and Antczak 2005). These findings indicate unique immune regulation in the equine endometrium during early pregnancy.

The innate immune system plays a critical regulatory role during pregnancy, providing a suitable environment for the allogeneic conceptus (embryo/foetus and associated membranes) (Abrahams and Mor 2005; Koga and Mor 2007). Toll-like receptors (TLRs) are an important part of the innate immune system, and they are also involved in the activation of the acquired immune system (Akira 2003). Apart from their roles in pathogen recognition, TLRs interact with secreted or released endogenous mo-

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lecules, such as reactive oxygen species, heat shock proteins, proteins released from dying cells and so forth (Chen et al. 2011; Critchley and Fildes 2012). Therefore, activation of TLRs can lead to both physiological and pathological processes, such as pro- and anti-inflammatory responses (Kannaki et al. 2011). TLRs in the reproductive system are involved in ovulation, fertilisation, placental function, trophoblast invasion, parturition and protection of the reproductive tract from pathogens, and they also mediate communication among other components of the immune system (Holmlund et al. 2002; Koga and Mor 2007; Liu et al. 2008). TLRs are generally produced by different types of immune cells, but they are also expressed in some reproductive tissue cells, such as the granulosa, luteal, endometrial luminal and stromal cells, the trophoblast and the cervix (Gonzalez et al. 2007; Shimada et al. 2008; Luttgenau et al. 2016).

TLR4 is among the best characterised of the TLRs, and its signalling mechanism is mediated by many inflammatory pathways and by endogenous substances (Vaure and Liu 2014). During maternal recognition of equine pregnancy, it is critical to orchestrate the interaction between the conceptus and uterus. The conceptus must develop, prevent luteolysis and escape from the maternal immune system to maintain pregnancy. According to previous studies, day 14 is an important day in equine pregnancy for the above-mentioned events (Sharp et al. 1997; Atli et al. 2010; Klein and Troedsson 2011; Klohonatz et al. 2015). These previous reports led us to hypothesise that the oestrous cycle and early pregnancy in mares could be changed by expression of innate immune system components, especially TLRs. Therefore, the aim of the present study was to analyse the expression profiles of *TLR4* mRNA and protein and to determine the cell-specific localisation of TLR4 in the equine endometrium during the oestrous cycle and early pregnancy.

MATERIAL AND METHODS

Animals and experimental design. The experimental design for biopsy collection from cyclic and pregnant mares was previously described by Atli et al. (2010). Briefly, ovulation was induced with hCG (1500 IU, IV, Chorulon; Intervet, Inc., Netherlands) in mares with obvious oedema in the uterus and a > 35-mm follicle on the ovary. Mares in the pregnancy group were inseminated with 750 million motile spermatozoa freshly collected from a stallion. Ovulation was observed by serial ultrasonography examination every 12 hours with the day of ovulation denoted as day zero (d0). When ovulation did not occur within 48 hours after insemination, the insemination procedure was repeated. All experimental procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine at Selcuk University (No. 040/2007).

Measurement of progesterone concentrations in the plasma of cyclic mares was performed as previously described (Atli et al. 2010). Briefly, blood samples were collected every 12 hours starting at least two days before the day of biopsy for measurements of concentrations of progesterone in plasma. Progesterone was quantified using an ELISA (Progesterone II, Germany). Endometrial biopsies were obtained from mares on the day of late dioestrus (LD, day 13.5–14, n = 4, high concentrations of progesterone in plasma) and after luteolysis at the beginning of the oestrus phase (AL, day 17–18, n = 4, low concentrations of progesterone and increasing concentrations of oestrogens) of the oestrous cycle. LD was defined as high concentrations of progesterone in plasma and no oedema in the endometrium of mares. Low concentrations of progesterone in plasma and obvious oedema in the endometrium were considered characteristic of AL. Biopsies were taken from pregnant mares on day 14 (P14; n = 4; high concentrations of progesterone in plasma and high concentrations of oestrogens from the conceptus within the uterine lumen). Pregnancy was diagnosed by the presence of a conceptus/blastocyst using ultrasonography. A piece of endometrial tissue was collected and snap-frozen immediately in liquid nitrogen and stored at –70 °C until RNA isolation. Another piece of endometrial tissue was fixed in 4% PFA (paraformaldehyde) solution overnight at 4 °C and then dehydrated in a 25%, 50%, 75% and 100% methanol (MeOH) series. Dehydrated samples were stored at –20 °C in 100% MeOH until evaluation using immunohistochemistry.

RNA extraction, cDNA synthesis and qPCR. Total RNA was extracted from frozen endometrial tissue using TRIzol® (Invitrogen, USA) according to the manufacturer's protocol. RNA samples with distinct 18 and 28S bands on agarose gels and an optical density (260/280) of 2 ± 0.1 by NanoDrop

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were used in the downstream applications. Total RNA was cleaned of gDNA contamination by RNAse-free DNAse I (Fermentas Life Sciences, USA) treatment, and 2 µg of total RNA was reverse-transcribed into cDNA (RevertAid™ First Strand cDNA Synthesis Kit, Fermentas Life Sciences). Primers for *TLR4* (forward: 5-gacaataactttccggcttt-3; reverse: 5-gactgccttgaccttctctt-3; product size: 151 bp) and *GAPDH* (forward: 5-atcaccatcttccaggagcgaga-3; reverse: 5-gtcttcgggtggcagtgatgg-3, product size: 341 bp) were designed using the Primer3 program. qPCR was employed to determine the expression profiles of the genes of interest during the oestrous cycle and early pregnancy. qPCR samples consisted of 10 µl SYBR Green Master Mix (2X; Fermentas Life Sciences), 5 pmol of each primer, 1 µl cDNA, with ddH_2O added to a final volume of 20 µl.

Thermal cyclic conditions consisted of initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation, annealing and amplification (95 °C 30 s, 60 °C 1 min, 72 °C 30 s, respectively). Melting curve analysis was performed as follows: 95 °C for 1 min, followed by fluorescence measurements at 1° increments from 55 °C to 95 °C. In each run, a negative control with no cDNA template was included. To verify reaction specificity, amplification products were run on a 2% agarose gel. *GAPDH* was used as the reference gene for normalisation, as it was previously determined to be the most stable housekeeping gene in the present experimental setting (Kayis et al. 2011). To determine the dynamic range and amplification efficiencies of the real-time PCR assays for *TLR4* and *GAPDH*, amplifications were performed in duplicate using specific primers with two-fold serial dilution series of pooled cDNA collected from the endometrium. Real-time PCR amplification efficiency was 95–105% for *TLR4* and *GAPDH*.

Immunohistochemistry. Paraffin-embedded sections of endometrium from pregnant and cyclic mares (~ 5 micrometer) were deparaffinised in xylene, rehydrated through a descending ethanol series and boiled in 10 mM sodium citrate (pH 6.0) for 20 minutes. Tissues were washed with a solution containing 25 mM Tris-HCl, pH 7.5; 140 mM NaCl; 2.7 mM KCl; and 0.1% Tween-20 (TBSTw). After cooling to room temperature, parts of the sections were limited with a super PaP pen in a water repellent circle and then covered with 3% H_2O_2 in methanol to block endogenous peroxidase. After rinsing with TBSTw, non-specific binding sites were blocked for 1 hour in blocking solution (TBSTw containing 1% blocking reagent (Roche Diagnostics, USA), 5% normal goat serum, and 1% bovine serum albumin). The sections were incubated overnight at 4 °C in a humidified chamber with 1 : 100 rabbit anti-TLR4 (AA 780-830, antibodies-online.com, polyclonal) in blocking solution. After several washes with TBSTw, tissues were incubated for 1 hour with blocking solution containing 1 : 250 biotin-conjugated goat anti-rabbit antibody. After rinsing with TBSTw, avidin-biotin complex (Vectastain® Elite ABC Kit, PK6100, Vector Laboratories, Ltd., USA) was added, and the sections were incubated for 30 minutes. After serial rinsing with TBSTw (5 × 5 min), DAB chromogen substrate (DAKO K3466) was added to achieve specific staining. The reaction was stopped with distilled water, and haematoxylin (10%) was added for counterstaining. The sections were dehydrated with a graded ethanol series, rinsed with 100% xylene and mounted. Sections were incubated with rabbit IgG antibody as a negative control. Bright-field images were captured using a Nikon Eclipse E80i (Nikon Instruments, Inc., USA).

Statistical analysis. The real-time PCR data (threshold cycle; Ct) were calculated as fold-changes in expression in the pregnant and cyclic mares. During the fold-change calculation, mean Ct values from the means of the cyclic mares (LD) were used as reference points, and the Ct values of the pregnant and cyclic mares were used to calculate the fold-changes from the reference points according to the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). Normalised data were analysed using the Relative Expression Software Tool (REST2009; Pfaffl et al. (2002)), which calculates group-wise comparisons and statistical analyses of relative expression results for qPCR. Differences were considered significant at $P < 0.05$.

RESULTS

TLR4 mRNA expression in the equine endometrium

The real-time PCR amplification product of *TLR4* mRNA was detected in endometria from both cyclic and pregnant mares, and the expected 151 base pair bands were detected (data not shown). The relative expression of *TLR4* mRNA in endometrial samples collected from the pregnant and cyclic mares is

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shown in Figure 1. The expression of *TLR4* mRNA was less ($P < 0.05$) in endometria from mares on P14 compared to mares in LD ($P < 0.05$). The expression of *TLR4* mRNA was similar ($P > 0.05$) for mares on P14 and mares AL.

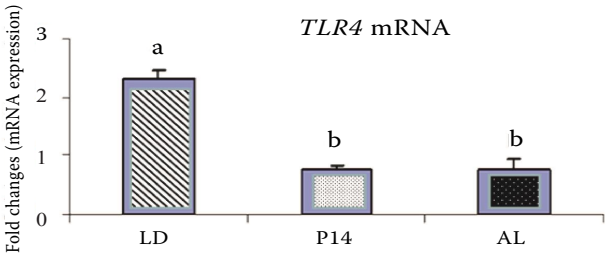

Figure 1. At P14 of pregnancy the expression of *TLR4* mRNA is significantly suppressed in equine endometrium, compared to LD. The expression profile of *TLR4* mRNA was similar between P14 and AL

AL = after luteolysis; LD = late dioestrus; P14 = pregnancy on day 14

[a,b] Statistically significant differences at $P < 0.05$

TLR4 expression in the equine endometrium

The abundance of TLR4 protein was greatest in the luminal and glandular epithelia of the endometrial samples and endothelial cells of capillaries. Compared to P14, the abundance of TLR4 was greater in both luminal and glandular epithelia on mares in LD. The negative control (NC) exhibited no background staining (Figure 2).

DISCUSSION

TLR4 is a pattern-recognition receptor that triggers immune responses to various infectious agents (Mogensen 2009). TLR4's primary ligand is bacterial lipopolysaccharide, and activation of TLR4 causes the release of various pro-inflammatory mediators and cytokines. Apart from the control of infections, secreted or released molecules, such as reactive oxygen species, heat shock proteins and products from both intracellular and extracellular degradation, as well as molecules produced by a conceptus, can activate the TLR4 system as endogenous ligands (Fan et al. 2003; Zhang and Schluesener 2006; Kannaki et al. 2011; Kaya et al.

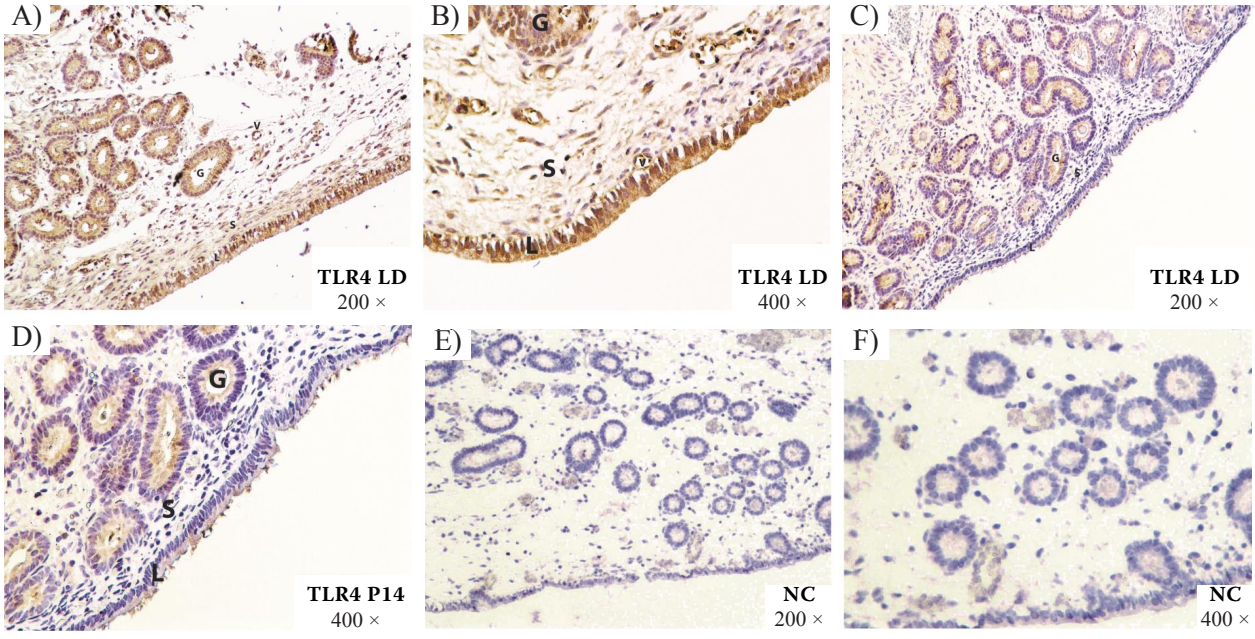

Figure 2. Representative images showing localisation of TLR4 protein, which is particularly apparent in both luminal and glandular epithelium of the endometrium, but also appeared to be present in capillary endothelial cells. When compared to P14 **(C, D)**, a prominent strong signal in both the luminal and glandular epithelium of the endometrium corresponding to TLR4 was detected in LD **(A, B)**. Negative control (NC) exhibited no background staining **(E, F)**

G = glandular epithelium; L = luminal epithelium; LD = late dioestrus; P14 = pregnancy on day 14; S = stroma; V = capillary endothelial cells

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2015; Luttgenau et al. 2016). Therefore, TLR4 secreted by non-immune cells also plays a role in tissue restructuring, reorganisation and regeneration (Zhang and Schluesener 2006). In addition to the protective effects of TLR4 against infection, changes in the expression of *TLR4* mRNA and protein in the equine endometrium during early pregnancy suggest that it may have roles in endometrial tissue.

This is the first study to describe *TLR4* mRNA and protein expression in equine endometrium at a critical stage in early pregnancy. The role of TLR4 in the immunological response to equine endometritis was previously elucidated (Marth et al. 2016; Siemieniuch et al. 2016). In mares, breeding-induced endometritis models indicate that endometrial profiles of *TLR4* mRNA increase significantly and very rapidly (at 3 h) after *E. coli* inoculation into the cyclic uterus. Activation of endometrial cells through TLR4 ultimately leads to an increase in cytokine production that is likely necessary for effective elimination of bacteria. According to those results, TLR4 plays a role in inflammatory pathways (Marth et al. 2016). Similarly, expression profiles for TLR4 have been described in the endometria of humans, sheep, mice, cattle and goats (Aflatoonian et al. 2007; Tirumurugaan et al. 2010; Silva et al. 2012; Ruiz-Gonzalez et al. 2015). Many studies have also examined TLR4 expression in relation to bacterial and viral infections (Sheldon and Roberts 2010; Chotimanukul and Sirivaidyapong 2011; Swangchan-Uthai et al. 2012; Ju et al. 2014; Luttgenau et al. 2016). However, studies that have aimed to determine the role of TLR4 in physiological reproductive processes are limited. The roles of TLR4 in the physiological processes (ovulation, fertilization, pregnancy, and parturition) of the reproductive system were reviewed by Kanaki et al. (2011). Moreover, *TLR4* mRNA and protein were detected in a limited number of ovine trophoblast cells during early pregnancy (Kaya et al. 2015). The present study revealed that expression of *TLR4* mRNA and protein in the equine endometrium is low during the critical peri-implantation stage of pregnancy. This is consistent with the idea that the presence of the conceptus in the uterus in early pregnancy suppresses the immune system (Klein and Troedsson 2011). The expression of TLR4 was examined throughout pregnancy in pigs, and there was high expression of TLR4 during the implantation stages of pregnancy (15–26 days). It was concluded that TLR4 is necessary for successful implantation (Ansari et al. 2015). The different expression profiles of TLR4 between mares and gilts/sows can be explained by the later onset of the implantation process in pregnant mares.

Immunolocalisation of the TLR4 protein was demonstrated in endometrial epithelial and stromal cells of mares with subclinical endometritis (Siemieniuch et al. 2016). Similar to those results, the results of the present study confirmed the presence of TLR4 protein in both uterine luminal and glandular epithelia and in endothelial cells of capillaries of mares during early pregnancy and during the oestrous cycle. The results of the present study also clearly demonstrated that the abundance of TLR4 protein was less in both luminal and glandular epithelia of mares on P14 than mares AL. The expression of *TLR4* mRNA in the human endometrium is significantly greater in the progesterone-induced secretory phase compared to the proliferative phase (Aflatoonian et al. 2007). In canine endometrium, the expression of TLR2 and TLR4 is significantly increased toward the end of dioestrus (Silva et al. 2012). Similarly, in the present study, expression of both *TLR4* mRNA and protein were greater in mares during dioestrus when the endometrium is under the influence of progesterone, compared to AL.

In conclusion, the differential regulation of the expression of TLR4 in luminal and glandular epithelia and endothelial cells of capillaries in the uteri of mares suggests that these cells play an immunological role at the maternal-conceptus interface during early pregnancy. Downregulation of expression of TLR4 in the endometrium of mares on P14 may play a role in an early immune tolerance mechanism to protect the equine conceptus, and this may be driven by factors from the conceptus such as oestrogens (Raeside et al. 2004) or interferon delta (Cochet et al. 2009).

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