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RESEARCH ARTICLE

Expression of *Toll-like receptors* (TLRs) in the equine peripheral blood mononuclear cells (PBMCs) during the early pregnancy and estrous cycle

Ercan Kurar[1], Mehmet Osman Atli[2], Aydin Guzeloglu[1*], Seyit Ali Kayis[3], Ahmet Semacan[4]

Özet

Kurar E, Atlı MO, Güzeloğlu A, Kayış SA, Semacan A. Atların periferal kan mononükler hücrelerinde (PKMH) erken gebelik ve östrus siklusu sürecinde *Toll-like reseptörlerinin* (*TLRs*) ekspresyonu. **Eurasian J Vet Sci, 2012, 28, 1, 15-20**

Amaç: Günümüzde kısraklarda birçok gebelik teşhis metodu olmasına rağmen, mümkün olabilecek en erken zamanda gebeliğinin teşhis edilmesi için pratik, güvenilir ve ekonomik bir yönteme halen ihtiyaç bulunmaktadır. Allojenik fötusa karşı immun tolerans erken gebelik esnasında kritik bir öneme sahiptir. *Toll-like reseptörlerinin* (*TLRs*) doğal immun sistemin önemli bir parçası olduğu bilinmektedir. Sunulan çalışmada, kısraklarda erken gebelikte periferal kan mononükler hücrelerinde (PKMH) *TLRs* ekspresyonunun incelenmesi amaçlandı.

Gereç ve Yöntem: Kan numuneleri aynı üç kısraktan östrus siklusunun ve gebeliğin 0 ve 8. günlerinde toplandı. Total RNA örnekleri PKMH'lerinden izole edildi ve cDNA sentezlendi. mRNA düzeyindeki *TLRs* ekspresyonları iki defa real-time PZR kullanılarak kuantifiye edildi. İstatistiksel analiz için Relative Expression Software Tool (REST2009) kullanıldı.

Bulgular: RT-PZR ile PKMH'lerde *TLRs* (1-10) ekspresyonları tespit edildi. Bununla beraber, real-time PZR ölçümleri siklik veya gebelik durumunun *TLRs* ekspresyonu üzerine etkisini göstermekte başarısız kaldı.

Öneri: Bu sonuçlara bakıldığında, atlarda gebeliğin erken döneminde PKMH'lerinde *TLR* genlerinin ekspresyonlarının gebeliğin erken anlaşılması için iyi bir markör olmadığı söylenebilir.

Abstract

Kurar E, Atli MO, Guzeloglu A, Kayis SA, Semacan A. Expression of *Toll-like receptors* (*TLRs*) in the equine peripheral blood mononuclear cells (PBMCs) during the early pregnancy and estrous cycle. **Eurasian J Vet Sci, 2012, 28, 1, 15-20**

Aim: Practical, reliable and economical pregnancy diagnosis method for as early as possible time of pregnancy in mares is still requirement. Immune tolerance against allogenic fetus is critically important during the early pregnancy. Toll-like Receptors (*TLRs*) are known to be an important part of innate immune system. Aim was to evaluate expression of TLRs in peripheral blood mononuclear cells (PBMCs) during the early pregnancy in mare.

Material and Methods: Blood samples were collected on days of ovulation (d0) and 8 during estrous and pregnancy from three same mares. Total RNA samples were isolated from PBMCs and cDNA synthesis was performed. Expressions of *TLRs* at mRNA levels were quantified by using real-time RT-PCR in duplicates. Relative Expression Software Tool (REST2009) was used for statistical analysis.

Results: *TLRs* (1 to 10) were found to be expressed in PMBCs by RT-PCR. However, real-time PCR measurements failed to show effects of pregnant or cyclic status on expression of *TLRs.*

Conclusion: According to these results, it may suggest that determination of *TLRs* gene expression in equine PBMCs during early pregnancy is not a good marker in understanding early equine pregnancy.

[1]Department of Genetics, [4]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, [3]Department of Animal Science, Faculty of Agriculture, Selcuk University, 42075, Konya, [2]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Dicle University, 21280, Diyarbakir, Turkey
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*aguzeloglu@selcuk.edu.tr

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► Introduction

In horse breeding industry, diagnosis of pregnancy as early as possible, especially in the first week after breeding, is important for embryo transfer studies and re-schedule of non-pregnant animals in the restricted breeding season (Hyland et al 1990, Allen and Antczak 2000). Observation of estrus symptoms, stallion checking, rectal palpation, ultrasonography as well as laboratuaric diagnostic methods including measurements of oestrone sulfate, progesteron, eCG and the early pregnancy factors are still applied methods for early diagnosis of pregnancy in mares (Aslan et al 1997, Foristall et al 1998, Kılıçarslan et al 1999, Metacalf et al 2000).

In order for establishing and maintaining the pregnancy, dam has to make hormonal and immunological preparations (Antczak and Allen 1989, Hyland et al 1990, Weetman et al 1999). Activation of immunological reactions against embryo/fetus, which is genetically different than mother, are regulated by the communication between the embryo and the dam (Weetman et al 1999). It is well known that there is a reduction in number of blood lymphocytes and in symptoms of autoimmune diseases after fertilization (Wilder 1998, Jiang and Vacchio 1998). Increase in expression level of interferon-tau stimulated genes is reported in non-reproductive organs including peripheral mononuclear blood cells in bovine (Gifford et al 2007, Gibbs et al 2010). Moreover, it has been reported that nitric oxide (NO) and adrenocorticotropic hormone (ACTH) that are known as immunosuppressive agents are expressed by bovine lymphocytes during the pregnancy (Dixit and Parvizi 2000, Dixit and Parvizi 2001). Among immunological factors, Toll-like Receptors (TLRs) are known to be an important part of innate immune system (Akira 2003). Apart from their roles in inflammations, regulatory roles of TLRs in physiological processes such as pregnancy have been demonstrated by numerous studies (Abrahams and Mor 2005, Kannaki et al 2011). Some functions of TLRs in reproductive system involve ovulation, fertilization, placental function, trophoblast invasion, parturition, protection of reproductive tract from pathogens and providing a communication among the other members of immune system components (Holmlund et al 2002, Aflatoonian et al 2007, Gonzales et al 2007, Koga and Mor 2008). In our previous studies (Atli et al 2010a, Atli et al 2010b), it was emphasized that both embryonic factors and ovarian steroids hormones regulate expression of *TLRs* genes at mRNA levels in equine endometrium.

Objective of this study was to evaluate expression of TLRs in equine peripheral blood mononuclear cells (PBMC) with the perspective of evaluating immunological aspect of early mare pregnancy and providing scientific basis for development of a novel and practical pregnancy diagnosis methods.

► Materials and Methods

- *Animal materials*

All experimental procedures were approved by the Ethics Committee of Faculty of Veterinary Medicine at Selcuk University (#2007/34). Three reproductively sound three mares and a stallion were used as animal materials of the present study. Fertility examination, housing and feeding of animals, insemination procedure, and detection of pregnancy and definition of estrous cycle in mares were described earlier (Atli et al 2010c). Moreover, all animals were evaluated for infectious diseases. Blood samples were collected on days of ovulation (d0) and 8 days after ovulation (d8) in both estrous cycle and pregnancy.

- *Peripheral blood mononuclear cell (PBMC) isolation and total RNA extraction*

PBMCs were isolated as previously described (Kurar et al 2011). Briefly, 10 mL blood sample was centrifuged at 300 g for 20 min at 4 ^{0}C. The buffy coat was harvested and resuspended in 1:5 V:V 0,87% Tris-NH_4Cl lysis buffer. Samples were kept at 37 ^{0}C for 10 min and then centrifuged at 300 g for 10 min. The PBMC pellet was washed with 10 mL PBS buffer and used for total RNA extraction procedures. RNA isolation, quality control, genomic DNA removal by DNAse-I and cDNA synthesis procedures were conducted as described by Kurar et al (2010). Briefly, total RNA isolation was performed by using Trizol Reagent (Invitrogen, USA). Two µg RNA samples were first cleaned for possible genomic DNA contamination by DNAse-I and then subjected to reverse transcriptase reaction for first strand complementary DNA (cDNA) synthesis using RevertAid™ First Standart cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions.

- *Detection of TLRs expression in PBMC*

Primers for *TLR1-10* genes were derived from equine sequences or conserved mammalian sequences by using Primer3 from NCBI (http://www.ncbi.nlm.nih.gov/) database. The primer pair sequences and product sizes are shown on Table 1. PCR reactions included 1x Mg^{++} free PCR buffer, 0.125 mM dNTP, 1,5 mM $MgCl^{++}$, 0.35 U of *Taq* polymerase, 5 pMol each primer (Table 1) and 2 µL cDNA as template. A touchdown-PCR profile was used with two steps. The first step was an initial denaturation at 95 ^{0}C for 4 min, followed by 16 cycles of denaturation at 94 ^{0}C for 30 sec, annealing beginning at 60 ^{0}C and ending at 52 ^{0}C for 30 sec and extension at 72 ^{0}C for 1 min. The annealing temperature was decreased 0.5 ^{0}C per cycle until it reached 52 ^{0}C. At the second step, 25 cycles of 94 ^{0}C for 30 sec, 52 ^{0}C for 30 sec, and 72 ^{0}C for 1 min was applied. PCR amplification products were separated by electrophoresis on 2% agarose gels and were visualized after ethidium bromide staining. Amplified PCR products were confirmed by sequencing (Iontek,

Table 1. TLRs primers used for PCR and Real-time PCR.

Locus	Primer sequence	Product size (bp)
TLR1	5'-tcttgccaccctactgtgaa-3'	159
	5'-atgagcaattggcagcacac-3'	
TLR2	5'-tggccagaaaagatgaaata-3'	194
	5'-aagaaggaggcatctggtag-3'	
TLR3	5'-tgtctttgacgatcaggtgt-3'	245
	5'-aaccatgatactgaggtgga-3'	
TLR4	5'-agactgggtgaggaatgaat-3'	151
	5'-gacaataactttccggcttt-3'	
TLR5	5'-gactgccttgaccttctctt-3'	164
	5'-tagttgaagctgagcaggag-3'	
TLR6	5'-caaagcagggaacaatccat-3'	206
	5'-ccacaatggtgacaatcagc-3'	
TLR7	5'-tgctggttcaaaaacagaga-3'	145
	5'-ggcatttgaggaaagaaaga-3'	
TLR8	5'-agctaacgacgtgtgtttca-3'	183
	5'-aagtttgggatgtggaaaga-3'	
TLR9	5'-attacctggccttcttcaattg-3'	100
	5'-ctgccattgctcagagccttc-3'	
TLR10	5'-actggcaacatgtcacacct-3'	151
	5'-agatgggcaagctaccttct-3'	
GAPDH	5'-atcaccatcttccaggagcgaga-3'	341
	5'-gtcttcgggtggcagtgatgg-3'	

Istanbul, Turkey) and using restriction endonuclease digestion.

- *Quantification of expression levels by real-time PCR*

Real-time PCR was used to evaluate the expression profiles of *TLR1-10* on days 0 and 8 of the early pregnancy and estrous cycle in the equine PBMCs. The reaction was set up as follows: 10 µL SYBR Green Master Mix (2x), 5 pMol of each primer (Table 1), 1µL cDNA and ddH_2O up to 20 µL of final volume. Thermal cyclic conditions were initial denaturation at 95 ^{0}C for 10 min followed by 40 cycles of denaturation, annealing and amplification (95 ^{0}C 30 sec, 60 ^{0}C 1 min, 72 ^{0}C 30 sec) on a Mx3005P™ 3005 Real-Time PCR System (Agilent Technologies Inc., Santa Clara CA, USA). Melting curve analysis was performed as follows: 95 ^{0}C 1 min, then fluorescence measurement was done at every 1-degree increments between 55 ^{0}C and 95 ^{0}C. In each run, a negative control with no cDNA template was included. From the RNA extraction to the real-time PCR, whole procedure was performed twice as technical replicate. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was selected as a housekeeping gene in order to normalize real-time PCR data.

- *Statistical analysis*

Primer efficiencies were calculated according to Schefe et al (2006) by using five data points of log transformed fluorescence graph of the exponential phase of the PCR kinetic curve. Then, each PCR reaction was evaluated in order to determine whether it would be included or excluded in further analysis.

The data collected was threshold of cycle (Ct) values from day 0 and 8 of the cyclic and pregnant animals. Mean Ct values of technical replicates were obtained. Before statistical analysis, normalization were performed according to $2^{-\Delta Ct'}$ method described by Livak and Schmittgen (2000). Normalized data were analyzed by using Relative Expression Software Tool (REST2009; Pfaffl et al 2002). Groups were considered to be statistically significantly different when $P < 0.05$.

► Results

cDNA samples were used by PCR to amplify *TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10.* Resulting PCR products were separated by electrophoresis on 2% agarose gels and visualized after ethidium bromide staining (Figure 1). The steady-state concentrations of mRNA for *TLR1-10* in the PBMCs taken on day 0 and 8 of the estrous cycle are shown in Figure 2. The concentrations of mRNA for *TLR1-10* did not show any increase on day 8 compared to day 0 in the estrous cycle. The steady-state concentrations of mRNA for *TLR1-10* in the PBMCs taken on day 0 and 8 of pregnancy are shown in Figure 3. Similar to estrous cycle days (0, 8) profiles, early pregnancy did not affect expression of *TLR1-10* in equine PBMCs. When compared to day 8 of the estrous cycle, *TLRs* expression did not show significant changes on day 8 of pregnancy in equine PBMCs (Figure 4).

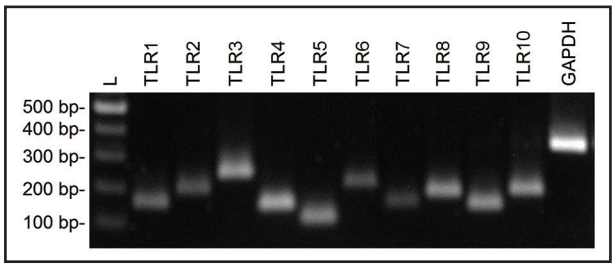

Figure 1. Agarose gel electrophoresis of TLRs and GAPDH PCR amplification products along with 100 bp-DNA size standard (L).

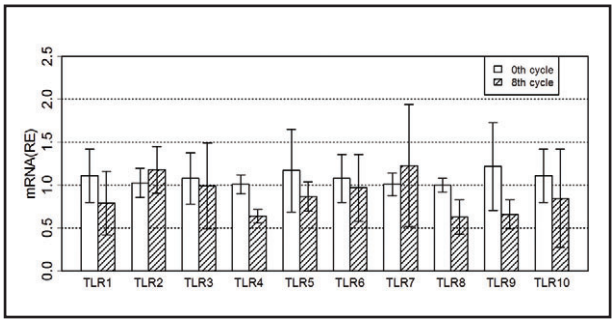

Figure 2. Relative expression (RE) of TLR1-10 mRNAs on day 0 (d0), on day 8 of the estrous cycle in equine PBMCs. Data were expressed as fold change (± SEM).

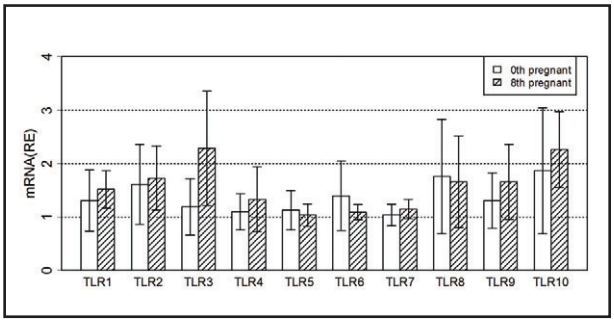

Figure 3. Relative expression (RE) of TLR1-10 mRNAs on day 0 (d0) and on day 8 of pregnancy in equine PBMCs. Data were expressed as fold change (± SEM).

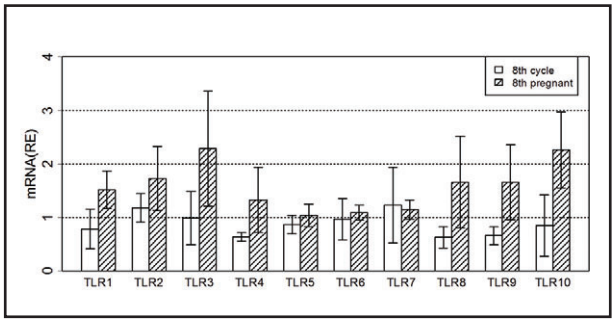

Figure 4. Relative expression (RE) of TLR1-10 mRNAs on day 8 of the estrous cycle and on day 8 of pregnancy in equine PBMCs. Data were expressed as fold change (± SEM).

► Discussion

In the present study, expressions of *TLRs* at mRNA levels were determined in equine PBMCs by PCR and also their expression profiles during the early pregnancy (0 and 8 days) and estrous cycle (0 and 8 days) were shown by real-time PCR. Collecting samples from the same animal in both the estrous cycle and pregnancy results in evaluation of expression of TLRs without any compromising effects of disease states that may lead to inappropriate conclusions. Day of ovulation (d0) was used as a control point for both the estrous and pregnancy days (day 8). With this model, any effect of animal variation on TLRs expression has been eliminated. Expressions of *TLR* genes compared within groups (d0 vs. 8) and between groups (day 8 of the estrous cycle and day 8 of the pregnancy) were not changed significantly.

TLRs employ their functions mainly by recognition of structurally conserved molecules of microbial organisms including bacteria, viruses and fungi. TLRs also interacts with some hosts' endogenous molecules (Koga and Mor 2002, Zarember and Godowski 2002, Akira 2003). It is well known that immune response is regulated in order to protect the embryo in the uterus (Antzcak and Allen 1989). It has been reported that most mammalian species express 10-13 different types of TLRs in different tissues (Young et al 2004, Alfatoolian et al 2007, Tirumurugaan et al 2010, Atli et al 2010b, Kannaki et al 2011). In this study, we found expressions of all analyzed *TLRs (1 to 10)* in equine PBMCs. Similarly, expressions of all analyzed *TLRs (1 to 10)* were also detected in the equine endometrium during the estrous cycle and early pregnancy (Atli et al 2010a, Atli et al 2010b). While expression of *TLRs* are regulated in the equine endometrium during the estrous cycle and early pregnancy, findings of this study indicated that neither the estrous cycle nor early pregnancy status affected mRNA levels of *TLR* genes in equine PBMCs 8 days after ovulation.

Embryonic factors secreted from conceptus plays important roles in maternal recognition of pregnancy (Mann and Lamming 2001, Spencer and Bazer 2002). For incidence, interferon-tau induces expression interferon-tau stimulated genes including *Mx1, Mx2* and *ISG15* (Roberts et al 1997, Ott et al 1998, Hicks et al 2003). It has been emphasized that measurements of those genes expression in bovine PBMCs by qPCR is a good indicator for determination of early bovine pregnancy (Gifford et al 2007, Green et al 2010). However, equine peripheral blood mononuclear cells did not show any significant changes for Mx1, Mx2 (Hicks et al 2003). In our previous study (Kurar et al 2011), we had examined expressions of *PGES, IL-5, iNOS, POMC, IL-4* and *IL-10* on d0, 4 and 8 of pregnancy. Gene expressions for *PGES, IL-5* and *iNOS* were not observed in PMBCs. Meanwhile, expressions of POMC, *IL-4* and *IL-10* were not showed any significant changes on d0, 4 and 8 of pregnancy and the estrous cycle in equine *PBMCs*. Consistent with these results, we could also not find any significant changes for *TLRs* in equine PBMCs.

► Conclusions

The results indicate that at this early stage of pregnancy, embryonic factors do not affect *TLRs* expression levels at PBMC level. Therefore, it may suggest that determination of *TLRs* gene expression in equine PBMCs during early pregnancy is not a good marker in understanding early equine pregnancy.

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