Kafkas Univ Vet Fak Derg
17 (2): 319-323, 2011
DOI:10.9775/kvfd.2010.3644

RESEARCH ARTICLE

POMC, iNOS, PGES, IL-4, IL-5 and IL-10 Gene Expression in Peripheral Blood Mononuclear Cells of Cyclic and Pregnant Mares [1]

Ercan KURAR * Mehmet Osman ATLI ** & ***
Aydın GÜZELOĞLU ** Ahmet SEMACAN **

[1] *This study was supported by TÜBİTAK-TOVAG (105 O 652)*
* Selcuk University, Faculty of Veterinary Medicine, Department of Genetics, TR-42031 Konya - TÜRKİYE
** Selcuk University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, TR-42031 Konya - TÜRKİYE
*** Dicle University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynaecology, TR-21280 Diyarbakır - TÜRKİYE

Makale Kodu (Article Code): KVFD-2010-3644

Summary

Although a number of different clinical and laboratory methods are used, there is still need for a practical, reliable and economical diagnosis method for early pregnancy in mares. Objective was to provide a preliminary background for understanding immunological modifications at molecular level during early pregnancy in mare and thereby to allow development of a practical pregnancy test. Blood samples were collected from 10 pregnant and 4 cyclic mares on days of ovulation (d0), 4 and 8. Total RNA samples were isolated from peripheral blood mononuclear cells (PBMC) and cDNA synthesis was performed. Pregnancy dependent gene expression profiles of proopiomelanocortin (POMC), nitric oxide synthase (iNOS), prostaglandin E synthase (PGES), interleukin-4 (IL-4), IL-5 and IL-10 were evaluated at mRNA level using semiquantitative Reverse Transcriptase-Polymerase Chain Reaction. Data were analyzed by General Linear Model and possible differences between all mean factors were determined by Tukey's analysis. No gene expressions were observed for PGES, IL-5 and iNOS in PMBCs. Expressions of POMC, IL-4 and IL-10 were not significant on d0, 4 and 8, which suggested that pregnancy or cyclic status had no effect on expression of these genes. Only, IL-10 mRNA expression was lower in pregnant mares than cyclic mares ($P<0.05$).

Keywords: *Horse, Pregnancy, PBMC, Gene expression, RT-PCR*

Siklik ve Gebe Kısrak Periferal Kan Mononükleer Hücrelerinde POMC, iNOS, PGES, IL-4, IL-5 ve IL-10 Gen Ekspresyonu

Özet

Kısrakların erken gebelik tanısında farklı klinik yöntemler ve laboratuvar analizleri kullanılmaktadır. Ancak pratik, güvenilir ve ekonomik bir gebelik tanı yöntemine halen ihtiyaç bulunmaktadır. Bu çalışmada, gebelikle birlikte immun sistemde meydana gelen degişimlerin moleküler düzeyde belirlenmesi, gebelik immunolojisinin anlaşılması ve sonuçta pratik bir gebelik testi geliştirilmesi için bilimsel alt yapının hazırlanması amaçlanmıştır. Kan örnekleri, 10 baş gebe ve 4 baş gebe olmayan (siklik) kısraktan ovulasyon gününden başlayarak (0. gün), 4. ve 8. günlerde alındı. Periferal kan mononükleer hücrelerinden (PKMH) total RNA izolasyonu ve cDNA sentezi gerçekleştirildi. Proopiomelanocortin (POMC), nitrik oksit sentaz (iNOS), prostaglandin E sentaz (PGES), interlökin-4 (IL-4), IL-5 ve IL-10 gen ekspresyonlarında gebeliğe bağlı muhtemel değişiklikler mRNA seviyesinde Reverz Transkriptaz-Polimeraz Zincir Reaksiyonu (PZR) ile semi-kantitatif olarak araştırıldı. İstatistiksel analizlerde General Linear Model ve modeldeki bütün etkenler için ortalama değerler (means) arasındaki farklılık olasılıkları Tukey analizi kullanılarak belirlendi. PKMH'de PGES, IL-5 ve iNOS gen ekspresyonları tespit edilemedi. İstatistiksel analizler sonucunda POMC, IL-4 ve IL-10 gen ekspresyonlarının 0., 4. ve 8. günler arasında mRNA düzeyinde herhangi bir farklılık göstermediği belirlendi. Gebeliğin veya siklik olmanın bu genlerin mRNA ekspresyonu üzerine uyarma veya baskılama şeklinde bir etkisi olmadığı tespit edildi. Yalnızca, IL-10 mRNA konsantrasyonunun gebe hayvanlarda siklik hayvanlara göre düşük olduğu belirlendi ($P<0.05$).

Anahtar sözcükler: *At, Gebelik, PKMH, Gen ekspresyonu, RT-PZR*

İletişim (Correspondence)
+90 332 2233597
aguzeloglu@selcuk.edu.tr

INTRODUCTION

Majority of horses (90%) are known as seasonal polyestric and exhibit their sexual activities between spring and fall in Turkey with limited number of estrus (about 3-6). In this period, number of ovulatory cycles is limited [1]. Normal reproductive cycle is critically important in horse breeding. Planning of this limited mating period and diagnosis of pregnancy as early as possible, especially in the first week after mating, is clinically important in thoroughbred horse breeding.

A number of different clinical method are used for early diagnosis of pregnancy in mares such as observation of estrus symptoms, stallion checking, rectal palpation, ultrasonography as well as lab methods including evaluation of hormone (eCG, oestron sulfate, progesteron) and the early pregnancy factors [2,3]. There is still however need for a practical, reliable and economical pregnancy diagnosis method in mares.

In mammalians, embryo has to be recognized by the mother for maintaining the pregnancy. This procedure, which may be species specific, is generally maintained by expression of some signals from embryo. Therefore, mother can hormonally and immunologically prepare for pregnancy. The communication between the embryo and the mother inhibits development of immunological reactions against embryo/fetus, which is genetically different than mother. In this immunological insensitivity, direct and indirect factors involve through local-stimulation of embryo and some factors that are produced in the oviduct-uterus by impact of embryo. It is well known that there is a reduction in number of blood lymphocytes [4] and in symptoms of autoimmune diseases after fertilization [5].

Pregnancy requires a tight regulation between the immune and endocrine systems by which a molecular level communication is formed between different cytokines, growth factors and hormones [6]. Equine early embryo secretes an immunoregulatory prostanoid, prostaglandin E_2 (PGE_2) and events such as proliferation of T lymphocytes and termination of T helper-1 (Th-1) cytokines including IL-2, IFN-γ and TNF-α are under control of PGE_2. PGE_2 also stimulates Th-2 cytokines such as IL-4, IL-5 and IL-10 [7-9]. Also, Dixit and Parvizi [10] reported that nitric oxide (NO) and adrenocorticothropic hormon (ACTH), that are known as immunosuppressive agents, are expressed by bovine lymphocytes during the pregnancy.

The objective of this study was to evaluate expression of genes including POMC, iNOS, PGES, IL-4, IL-5 and IL-10 that have effect in immune system of peripheral blood mononuclear cells (PBMC). Results are expected to allow evaluating immunological aspect of early mare pregnancy and providing scientific basis for development of a novel and practical pregnancy diagnosis method.

MATERIAL and METHODS

Animal Material and Experimental Procedure

Animal material included reproductively sound 10 Arabian mares and one stallion aging from 5 to 16. Animals were obtained from TIGEM Anatolian Agricultural Station Horse Breeding Center, Eskisehir, TURKEY and TIGEM Karacabey Horse Breeding Center, Bursa, TURKEY. All experimental procedures were approved by the Ethics Committee of Faculty of Veterinary Medicine at Selcuk University (# 2007/0034 on 07.13.2007). Experimental procedure was previously reported elsewhere [11]. Briefly, all animals were evaluated for infectious diseases and reproductive performance before the study. In the beginning of the season, mares were synchronized for estrous and ovulation and were evaluated for criteria including follicular development and endometrial edema. Mares were inseminated after daily inspection of ovaries for determination of preovulatory follicle (≥35 mm). Four mares remained uninseminated and were used as negative control (cyclic). Blood samples were collected from 10 pregnant and 4 cyclic mares on days of ovulation (d0), 4 and 8. Embryonic vesicle was detected on day 9-11 and pregnancy was verified on day 14-15 by ultrasonographic examination. All animals were housed at the Equestrian Center of Selcuk University.

Peripheral Blood Mononuclear Cell (PBMC) Isolation and Total RNA Extraction

PBMCs were isolated as previously described [12]. Briefly, 10 ml blood sample was centrifuged at 300 g for 20 min at 4°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°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.[13]. 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™ FirstStandart cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions.

Polymerase Chain Reaction

Relative mRNA expression levels of genes were analyzed by semiquatitative RT-PCR. Polymerase Chain Reactions (PCR) were performed on a Bio-RAD MyCycler thermal cycler in 15 µl reaction volume including 1x Mg^{++} free PCR buffer, 0.125 mM dNTP, 1.5 mM $MgCl^{++}$, 0.375 units of *Taq* polymerase (Fermentas), 5 pMol each primer *(Table 1)* and 2 µl cDNA sample as template. A touchdown-PCR profile [14] was used with two steps. The first step was an

initial denaturation at 95°C for 4 min, followed by 16 cycles of denaturation at 94°C for 30 sec, annealing beginning at 60°C and ending at 52°C for 30 sec and extension at 72°C for 1 min. The annealing temperature was decreased 0.5°C per cycle until it reached 52°C. At the second step, 25 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min was applied.

The resulting PCR products were separated by electrophoresis on 2-3% agarose gels. After ethidium bromide staining, band sizes and densities were determined using GenoMini Image Capture and Analysis System (VWR, Leuven, Germany).

Statistical Analyses

Data were analyzed using General Linear Model (GLM) where status of animal (pregnant or cyclic), days (0, 4 and 8) and interaction (pregnant or cyclic * days) were included. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control and covariate for normalization of the expression data in the model. Possible differences between means for all factors were determined by Tukey's analysis. Differences of $P \leq 0.05$ were accepted as statistically important.

RESULTS

Expressions of POMC, IL-4, IL-10 and GAPDH genes were determined in PBMC *(Fig. 1)*. PGES, IL-5 and iNOS gene expressions were not determined at mRNA levels *(Fig. 1)*. However, expressions of these genes were determined in total mRNA samples from equine endometrium *(Fig. 2)*.

Expression profiles of POMC, IL-4 and IL-10 during the early pregnancy and estrus cycle were illustrated in *Fig. 3*. In order to determine the real differences, the beginning of the exponential phases (quantity of PCR product is doubled at each cycle), cycle numbers were determined as 33, 35, 30 and 25, and semiquantitative evaluation of RT-PCR analyses for POMC, IL-4, IL-10 and GAPDH were performed at indicated number of cycles.

POMC expressions were slightly higher in pregnant mares on days 0 and 8 but lower in day 4. POMC and IL-4 expressions were not statistically significant between pregnant and cyclic mares. However, mRNA concentration of IL-10 was significantly lower in pregnant mares during early pregnancy on the time points evaluated.

Table 1. *Primers used in PCR analyses*
Tablo 1. *PZR analizlerinde kullanılan primerler*

Gene	Primer Sequence		PCR Product (bp)	Gene Bank Accession Code
	Forward	Reverse		
PGES	ggaacgacatggagaccatctac	gaagggatgcccaatcccctag	311	AY057096
iNOS	gccaaggtctgagctacctg	gagtgcctggctgagtgag	200	AJ493657
IL-5	gatgggaacctgatgattcctact	tccccttggacagtttgattct	107	U91947
IL-4	atccagggatgcaaatacga	ttgaggttcctgtccagtcc	239	AF305617
IL-10	ttacctggaggaggtgatgc	taccacagggttttccaagg	399	U38200
POMC	ttctgagctccgagaagagcca	aaacgagccgagtatctgccgctg	203	*
GAPDH	atcaccatcttccaggagcgaga	gtcttctgggtggcagtgatgg	341	**

** Primers were designed based on the published DNA sequence [15] and the resulting PCR product was verified by DNA sequencing, ** Obtained from Boerboom et al.[16]*

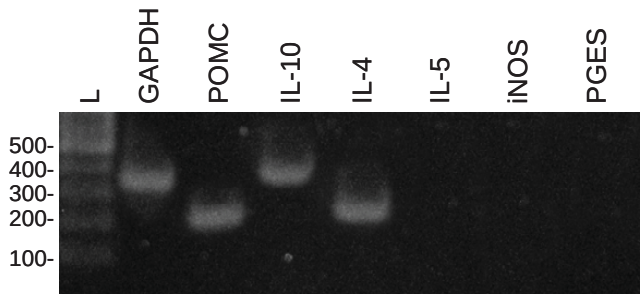

Fig 1. RT-PCR analysis of the genes used in the study (L: 100-bp DNA size standard)

Şekil 1. Çalışmada kullanılan genlerin RT-PZR analizi (L: 100-bç DNA standartını ifade etmektedir)

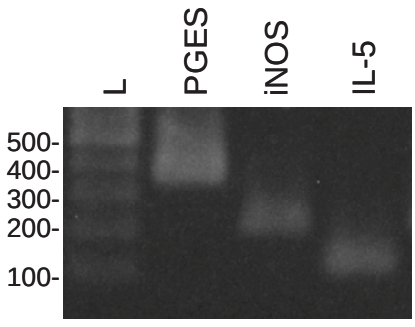

Fig 2. PCR analysis of IL-5, iNOS and PGES genes in cDNA samples of mare endometrium (L: 100-bp DNA size standard)

Şekil 2. IL-5, iNOS and PGES genlerinin kısrak endometrium cDNA örneklerinde PZR analizi (L: 100-bç DNA standartını ifade etmektedir)

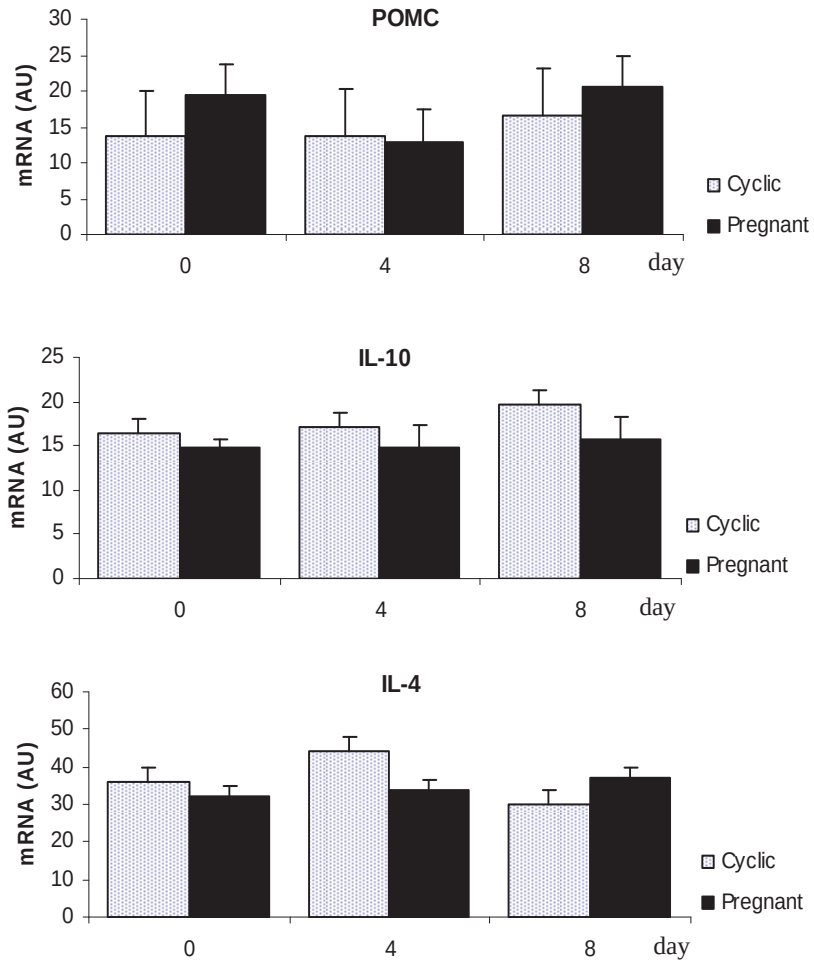

Fig 3. Expression of POMC, IL-10 and IL-4 genes at mRNA level

Şekil 3. POMC, IL-10 ve IL-4 genlerinin mRNA seviyesinde ekspresyonu

DISCUSSION

For sustaining the pregnancy, a tight regulation is established between the immune and endocrine systems at the beginning of pregnancy. For this purpose, a molecular level communication is formed between different cytokines, growth factors and hormones [6]. Fertilized ovum reaches into uterus from oviduct about 144-156 h after ovulation and secretes an immunoregulatory prostanoid, PGE_2. Some critical immunological modulations, for instance proliferation of T lymphocytes and termination of T helper-1 (Th-1) cytokines including IL-2, IFN-γ and TNF-α, are under control of PGE_2. PGE_2 is also responsible for stimulation of Th-2 cytokines such as IL-4, IL-5 and IL-10 [7-9]. It has been reported that such Th-1 down regulation by PGE_2 is critically important for prevention of transplant rejections. These findings suggested a possible immune modulatory role of PGE_2 during pregnancy [17]. Furthermore, some tumor tissues produce PGE_2 to suppress immune reactions against tumor tissues [18].

These findings demonstrated that PGES can be a potential candidate gene. In this study, PBMC expression of PGES that produce PGE_2 was used to evaluate the effect of early pregnancy. No gene expression was determined for PGES and IL-5 genes in total RNA samples isolated from PBMC *(Fig. 1)*. However, expressions of these genes were determined in cDNA samples isolated from equine endometrium [11], which suggests that primers used for these genes are informative and suitable for equine gene expression studies *(Fig. 2)*. These findings demonstrated that PGES and IL-5 were not expressed in mare PMBCs during the early pregnancy and early in estrous cycle.

It is well known that immune tolerance against human embryo can be explained by transition from cellular to humoral immune response. During the pregnancy, immune system prefers humoral response against intracellular pathogens instead of cellular response that is normally applied. Over expression of maternal Th-1 cytokines that are part of cellular response negatively affects embryo development [19]. Therefore, shift from production of Th-1 cytokines to Th-2 cytokines were determined in early human pregnancy [20,21]. Expression of IL-4 and IL-10, which are Th-2 cytokines, were determined in PBMCs *(Fig. 1)*. Statistical analyses illustrated that expression of IL-4 was not different on days 0, 4 and 8. However, IL-10 expression on days 0 and 4 were significantly lower ($P<0.05$) in pregnant mares than cyclic mares *(Fig. 3)*. This finding partially may indicate the alteration from cellular to humoral response in early pregnancy.

Beside its immunosuppressive role, ACTH has an effect to stimulate glucocorticoids. On day 7 after ovulation that is the period accepted as reception of the embryo by mother, blood lymphocytes produce ACTH at gradually increasing levels throughout pregnancy [10,22]. In this time period, bovine embryo initiates expression of paternal MHC molecules. Therefore, ACTH affects mother's immune system in the favor of embryo during the pregnancy particularly at the beginning of implantation. Local production of this hormone allows immune tolerance against the embryo and thereby plays role in immunological acquiescence of embryo. In this study, primers derived from polycistronic POMC locus were used to evaluate ACTH expression. Although POMC expression was determined in PBMCs of both pregnant and cyclic mares, no differences were observed between on days 0, 4 and 8 *(Fig. 3)*.

Nitric oxide (NO) is similarly expressed by bovine lymphocytes throughout the pregnancy to suppress immune system [10]. Nitric oxide exhibits its immunosuppressive role by transition from Th-1 lymphocytes to Th-2 lymphocytes (from cellular response to humoral response) [23]. However, in this study, nitric oxide synthase (iNOS) gene was used to test NO expression; but, iNOS mRNA was not determined in PBMCs.

As a result PGES, IL-5 and iNOS expressions were not observed in PBMCs during first 8 days of the cycle after the ovulation. IL-10, POMC and IL-4 expressions were determined in PBMC, but POMC and IL-4 expressions were not different between pregnant and cyclic mares. These findings demonstrated that these genes are not useful for development of an alternative method for diagnosis of early pregnancy in mares. There is need to evaluate expression of other candidate genes that may have an effect on immunogenetical inertness of the dam in the early pregnancy.

Aknowledegements

The authors would like to thank the staff at the Equestrian Center of Selcuk University for handling and care of the animals used in this study. The animal material was kindly provided by TIGEM Anatolian Agricultural Station Horse Breeding Center, Eskisehir, TURKEY and TIGEM Karacabey Horse Breeding Center, Bursa, TURKEY.

REFERENCES

1. Hyland JH: Reproductive endocrinology: It's role in fertility and infertility in the horse. *Br Vet J,* 14, 1-16, 1990.

2. Kılıçarslan MR, Soylu MK, Şenünver A, Kırşan İ, Carioğlu B: Kısraklarda erken gebelik tanısında ultrasonografik tekniklerin kullanımı. *Kafkas Univ Vet Fak Derg,* 2, 147-150, 1996.

3. Foristall KM, Roser JF, Liu IKM, Lasley B, Munro CJ, Carneiro GF: Development of a tandem hormone assay for the detection of pregnancy in the miniature mare. *44th Annual Convention of the American Association of Equine Practitioners.* Baltilmore, Maryland, USA. 1998.

4. Jiang SP, Vacchio MS: Multiple mechanisms of peripheral T cell tolerance to the fetal "allograft". *J Immunol,* 160, 3086-3090, 1998.

5. Wilder RL: Hormones, pregnancy and autoimmune diseases. *Ann NY Acad Sci,* 84, 45-50, 1998.

6. Weetman P: The immunology of pregnancy. *Thyroid,* 9, 643-646, 1999.

7. Trebble TM, Wootton SA, Miles EA, Mullee M, Arden NK, Ballinger AB, Stroud MA, Burdge GC, Calder PC: Prostaglandin E2 production and T cell function after fish-oil supplementation: Response to antioxidant cosupplementation. *Am J Clin Nutr,* 78, 376-382, 2003.

8. Meyer F, Ramanujam KS, Gobert AP, James SP, Wilson WT: Cutting edge: Cyclooxygenase-2 activation suppresses Th1 polarization in response to *Helicobacter pylori. J Immunol,* 171, 3913-3197, 2003.

9. Walker W, Rotondo D: Prostaglandin E2 is a potent regulator of interleukin-12- and interleukin-18-induced natural killer cell interferon-gamma synthesis. *Immunology,* 111, 298-305, 2004.

10. Dixit VD, Parvizi N: Pregnancy stimulates secretion of adrenocorticotropin and nitric oxide from peripheral bovine lymphocytes. *Biol Reprod,* 64, 242-248, 2001.

11. Atli MO, Kurar E, Kayis SA, Aslan A, Semacan A, Celik, S, Guzeloglu A: Evaluation of genes involved in prostaglandin action in equine endometrium during estrous cycle and early pregnancy. *Anim Reprod Sci,* 122, 124-132, 2010.

12. Yankey SJ, Hicks BA, Carnahan KG, Assiri AM, Sinor SJ, Kodali K, Stellflug JN, Ott TL: Expression of the antiviral protein Mx in peripheral blood mononuclear cells of pregnant and bred, non-pregnant ewes. *J Endocrinol,* 170, R7-11, 2001.

13. Kurar E, Atlı MO, Güzeloğlu A, Özşensoy Y, Semacan A: Comparison of five different RNA isolation methods from equine endometrium for gene transcription analysis. *Kafkas Univ Vet Fak Derg,* 16 (5): 851-855, 2010.

14. Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS: Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res,* 19 (14): 4008, 1991.

15. Soverchia L, Mosconi G. Ruggeri B, Ballarini P, Catone G, Degl'Innocenti S, Nabissi M, Polzonetti-Magni AM: Proopiomelanocortin gene expression and beta-endorphin localization in the pituitary, testis, and epididymis of stallion. *Mol Reprod Dev,* 73, 1-8, 2006.

16. Boerboom D, Brown KA, Vaillancourt D, Poitras P, Goff AK, Watanabe K, Dore M, Sirois J: Expression of key prostaglandin synthases in equine endometrium during late diestrus and early pregnancy. *Biol Reprod,* 70, 391-399, 2004.

17. Morichika T, Takahashi HK, Iwagaki H, Yagi T, Saito S, Kubo S, Yoshino T, Akagi T, Mori S, Nishibori M, Tanaka N: Effect of prostaglandin E2 on intercellular adhesion molecule-1 and B7 expression in mixed lymphocyte reaction. *Transplantation,* 75, 2100-2105, 2003.

18. Smyth GP, Stapleton PP, Barden CB, Mestre JR, Freeman TA, Duff MD, Maddali S, Yan Z, Daly JM: Renal cell carcinoma induces prostaglandin E2 and T-helper type 2 cytokine production in peripheral blood mononuclear cells. *Ann Surg Oncol,* 10, 455-462, 2003.

19. Hill JA, Choi BC: Immunodystrophism: Evidence for a novel alloimmune hypothesis for recurrent pregnancy loss involving Th1-type immunity to trophoblast. *Semin Reprod Med,* 18, 401-405, 2000.

20. Wegmann TG, Lin H, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: Is successful pregnancy a TH2 phenomenon? *Immunol Today,* 14, 353-356, 1993.

21. Reinhard G, Noll A, Schlebusch H, Mallmann P, Ruecker AV: Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochem Biophys Res Commun,* 245, 933-938,1998.

22. Dixit VD, Parvizi N: Lymphocytic secretion of adrenocorticotropic hormone throughout pregnancy in cows and its possible physiological role. *Reprod Dom Anim,* 3, 171, 2000.

23. Roozendaal R, Vellenga E, Postma DS, De Monchy JG, Kauffman HF: Nitric oxide selectively decreases interferon-gamma expression by activated human T lymphocytes via a cGMP-independent mechanism. *Immunology,* 98, 393-399, 1999.