Polish Journal of Veterinary Sciences Vol. 28, No. 3 (2025), 429–439

DOI 10.24425/pjvs.2025.156068

Original article

Differential mRNA expression of luteal TLR2, TLR4, TLR7 and interleukin-8 across canine reproductive states

C. Peker[1], E.H. Uçar[1], M. Köse[2], M. Polat[3], M.O. Atlı[4]

[1] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Aydın Adnan Menderes University, Işıklı, 09020, Efeler, Aydın, Turkey
[2] Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Dicle University, Fetih, 21200, Sur, Diyarbakır, Turkey
[3] Graduate School of Health Sciences, Dicle University, Kıtılbıl, 21280, Sur, Diyarbakır, Turkey
[4] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Harran University, Asya, 63200, Eyyübiye, Şanlıurfa, Turkey

Correspondence to: C. Peker, e-mail: cevdet.peker@adu.edu.tr, tel.: +90-554-8495988

Abstract

Toll-like receptors (TLRs) are essential components of both innate and adaptive immunity, influencing various physiological and pathological processes. It is also known that immune system components are present in the corpus luteum (CL) and regulate its functions. This study aimed to, for the first time, examine the mRNA expression levels of *TLR2*, *TLR4*, *TLR7*, and interleukin-8 (*IL-8*) in canine luteal tissue samples collected during early diestrus (EDI) (n=5), early pregnancy (EPR) (n=5), and pyometra (PYO) (n=5), a pathological condition in which the CL remains active. The luteal tissue samples were obtained via ovariohysterectomy and analyzed using real-time quantitative polymerase chain reaction (RT-qPCR). It was found that the mRNA expression levels of *TLR2*, *TLR7*, and *IL-8* were highest in the PYO group ($p<0.05$). However, no significant difference was observed in *TLR4* mRNA expression between groups ($p>0.05$). This study reveals distinct expression profiles of TLRs and IL-8 in canine luteal tissue under different physiological and pathological states. In conclusion, pyometra may induce changes in the mRNA expression patterns of TLRs and *IL-8* in canine luteal tissues. Further research involving protein-level analyses with larger sample sizes is necessary to better understand the roles of these molecules in the regulation of luteal function.

Keywords: bitch, corpus luteum, diestrus, pregnancy, pyometra, toll-like receptor

Introduction

The reproductive physiology of bitches differs significantly from that of other domestic animals (Papa and Kowalewski 2020). Notable differences include variations in the luteal phase and its regulatory mechanisms. The corpus luteum (CL), like in all animal species, is a temporary endocrine gland that develops from the remaining cells of the follicle after ovulation, and progesterone (P4) is the primary steroid hormone it secretes (Arosh et al. 2004, Stocco et al. 2007). In bitches, regardless of pregnancy status, the CL is the sole source of P4 (Kowalewski 2014), and P4 primarily regulates the sexual cycle and supports pregnancy establishment and maintenance (Arosh et al. 2004, Stocco et al. 2007). The P4 production pattern in the luteal phase appears similar in cyclic diestrus and pregnant bitches until just before parturition, although pregnant bitches generally show higher overall P4 levels (Kowalewski et al. 2006). The luteal phase in bitches can be divided into two phases. The first is the early luteal phase, characterized by a steady rise in P4 levels, which lasts 2-4 weeks. During this period, the CL reaches its peak P4 secretion capacity and is resistant to luteolytic factors. The second stage is the mature CL phase, during which P4 levels begin to decline, and the CL is quite sensitive to luteolytic treatments (Kowalewski et al. 2013).

In the bitch, the CL is active not only in physiological processes such as diestrus and pregnancy but also in conditions like pyometra and other uterine pathologies, including cystic endometrial hyperplasia and pseudoplacental endometrial hyperplasia. Pyometra is an inflammatory disease characterized by the accumulation of purulent discharge in the uterus of unspayed bitches. It is the most common and severe reproductive disorder in bitches and can lead to septicemia or toxemia, resulting in the death of affected animals (Johnston et al. 2001, Kida et al. 2006, Smith 2006, Schlafer and Gifford 2008). The disease often occurs 4 weeks to 4 months after the end of estrus (Smith 2006). Although the pathogenesis is not fully understood, pyometra is accepted as a disease of diestrus, and the prolonged and repeated effect of P4 results in cystic hyperplasia of the endometrial glands (Santana and Santos 2021). Elevated levels of P4 also cause suppression of cellular immunity (Sugiura et al. 2004). Based on available information, it is assumed that the CL plays an important role in the physiological processes involving the sexual cycle and pregnancy in bitches and is directly related to pyometra infection.

Luteal tissue is composed of a heterogeneous mixture of steroidogenic, endothelial, and immune cells. Previous studies have demonstrated that immune cells and their effector cytokines play a crucial role in regulating CL functions (Shirasuna et al. 2012, Shirasuna et al. 2015, Gadsby et al. 2017). It is well established that the immune system functions in two ways: innate and acquired immunity. The acquired immune system is activated through the inflammatory response initiated by the innate immune system (Kundakci and Pirat 2012). Toll-like receptors (TLRs) are a family of highly conserved pattern recognition receptors (PRRs) in mammals that effectively recognize and cooperate with pathogen-specific molecular patterns (PAMPs), such as peptidoglycan, lipopolysaccharide (LPS), and nucleic acids, to trigger the innate immune response (Bazzocchi et al. 2005, Kannaki et al. 2011). Recognition of PAMPs triggers the production of various inflammatory mediators, which are responsible for the development of a comprehensive immune response (Kempisty et al. 2013). Thus, as an essential component of innate immunity, TLRs are also involved in activating adaptive immunity (Akira 2003). Toll-like receptors are divided into two groups based on their cell location and ligands. The first group (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) is located on the cell surface and specifically recognizes compounds derived from various pathogens. The second group (TLR3, TLR7, and TLR9) is located within intracellular organelles and recognizes nucleic acids or nucleotide derivatives (Kannaki et al. 2011, Kundakci and Pirat 2012). Toll-like receptor-2 activates the immune system by recognizing ligands such as lipoteichoic acid, lipopeptides/lipoproteins, and peptidoglycan from Gram-positive and Gram-negative bacteria (Zähringer et al. 2008). Toll-like receptor-4 is the first and one of the best-characterized members of the TLR family. Many inflammatory pathways and endogenous substances mediate the transduction mechanism of TLR4 (Akashi et al. 2000). The primary ligand of TLR4 is LPS, the endotoxin of Gram-negative bacteria. Toll-like receptor-4 detects LPS in conjunction with accessory molecules (CD14, MD2, and lipopolysaccharide-binding protein) and induces the release of various proinflammatory factors that ultimately initiate the inflammatory process (Jungi et al. 2011). Toll-like receptor-7, an intracellular receptor, is activated by viral or bacterial nucleic acids (Aflatoonian and Fazeli 2008, Kawai and Akira 2010). It also induces the production of type I interferon, which is crucial for host immunity (Crozat and Beutler 2004). Recently, studies have investigated the expression of these receptors in different animal species and various genital tissues, especially in relation to the estrous cycle, pregnancy, and genital diseases (Silva et al. 2010, 2012, Chotimanukul and Sirivaidyapong 2011, 2012, Atli et al. 2012, 2017, 2018, Patra et al. 2014, Lüttgenau et al. 2016, Gadsby et al. 2017, Kaya et al. 2017, Yoo et al. 2019). Moreover, when a specific PAMP binds to a TLR, it triggers a sig-

naling cascade that produces proinflammatory cytokines (Patra et al. 2014). Various cell types produce cytokines in response to inflammation. Interleukin-8 (IL-8) is a chemotactic cytokine produced by various cells in response to inflammatory stimuli and plays a crucial role in directing neutrophils to sites of inflammation (Koch et al. 1992). In humans and rabbits, IL-8 has been identified in the ovarian theca, granulosa, luteal granulosa, and vascular endothelial cells (Arici et al. 1996). Interleukin-8 is secreted in the CL and leads to neutrophil activation. In bovine ovaries, its expression is higher during the early luteal phase than in other cycle phases (Jiemtaweeboon et al. 2011).

The similarity between the luteal process and hormonal mechanisms in pregnant and non-pregnant bitches, the presence of complex mechanisms affecting the lifespan and function of the CL, and the occurrence of pyometra during the luteal phase increase interest in luteal tissue in bitches. Therefore, there is a need to investigate the effects of TLRs and cytokines on various reproductive tissues and processes, especially in the CL. This study aims to examine the levels of mRNA expressions of *TLR2* and *TLR4* on the cell surface, *TLR7* in intracellular organelles, and *IL-8* in the luteal tissue of bitches with early diestrus, early pregnancy, and pyometra, which are in physiological and pathological processes under P4 dominance. The findings are expected to improve understanding of the role of innate immune mechanisms in luteal physiology and contribute to the development of pyometra.

Materials and Methods

Ethical statements

This study was approved by the Animal Research Local Ethics Committee of Aydin Adnan Menderes University (64583101/2023/97). Written informed consent was obtained from all animal owners before participation.

Animals and tissue samples

The current study was conducted using archived luteal tissue samples from 15 bitches at different reproductive states, including both physiological and pathological conditions, as reported in our recently published study (Ucar et al. 2024). No new animals or tissue samples were collected for this research. The bitches were medium to large in size, aged 3 to 10 years, and weighed between 17 and 44 kg. All animals were privately owned and housed in home environments with similar husbandry and housing conditions.

Experimental design and clinical examinations

The bitches included in the study were divided into three groups: Early pregnancy (EPR), early diestrus (EDI), and pyometra (PYO). To assign bitches to these groups, a detailed anamnesis was initially obtained, followed by a thorough clinical examination to assess their overall health. Next, gynecological examinations were performed, including vaginal cytology, ultrasonography, and serum P4 measurement. Ultrasonographic examinations were conducted using an ultrasound device (Esaote MyLab30Vet, Genoa, Italy) equipped with an 8 MHz microconvex probe. Complete blood count and serum biochemical analyses were also conducted in bitches diagnosed with pyometra. After completing these diagnostic procedures, groups were defined based on the following criteria:

Early dioestrus group (EDI, n=5) included bitches that: (I) had their proestrus bleeding monitored by their owners, (II) were regularly examined during estrus, ovulation, and dioestrus stages through vaginal cytology and ultrasonography, (III) were between 25 and 30 days post-ovulation, and (IV) were confirmed to be non-pregnant.

Early pregnancy group (EPR, n=5) included bitches that: (I) had their proestrus bleeding monitored by their owners and a known mating date, (II) were confirmed pregnant through ultrasonography, with gestational age estimated using fetometric measurements, and (III) were between days 25 and 30 of gestation.

Pyometra group (PYO, n=5) included bitches that: (I) were presented at least four weeks after their last observed proestrus bleeding, (II) showed clinical signs of pyometra such as vaginal discharge, anorexia, polyuria, polydipsia, vomiting, and lethargy, and (III) were diagnosed with pyometra based on clinical, cytological, and ultrasonographic examinations. The diagnosis of pyometra was confirmed by ultrasonographic appearance of an enlarged uterus with convoluted and tubular structures filled with anechoic or hypoechoic fluid, usually containing purulent material.

Although the study included various dog breeds, all were medium to large-sized breeds (Golden Retriever, Labrador Retriever, Anatolian Shepherd Dog, Rottweiler, Beagle, Pointer, and Wolfdog), and breed diversity was comparable across the groups. Nulliparous and multiparous dogs were mixed within the groups. The age ranges of individuals in the EDI, EPR, and PYO groups were 3-5, 3-5, and 3-10 years, respectively, while their body weights ranged from 18-44, 19-38, and 17-25 kg, respectively. No significant differences were found among the groups regarding age or body weight, and the distribution of these variables was homogeneous (Ucar et al. 2024).

Clinical evaluations showed that all bitches in the EDI and EPR groups were healthy. Four dogs in the PYO group had the open-cervix form of pyometra. Dogs in the PYO group exhibited clinical signs consistent with pyometra, accompanied by systemic illness indicators, including leukocytosis (mean: 36.70 x 10^3/μL) and an average body temperature of 39.18°C. Additional individual-level clinical data are detailed in our previous publication for compliance with ethical publishing guidelines (Ucar et al. 2024).

Progesterone measurements

As part of the previous study (Ucar et al. 2024), serum P4 levels were measured in all bitches to support the clinical findings regarding the functionality of the luteal structures. For the measurement process, venous blood samples were collected from all bitches and centrifuged at 3000 rpm for 15 minutes. Progesterone levels were then determined in the obtained serum samples using Vet Chroma (Anivet Inc., Chuncheon, South Korea). The mean P4 levels were determined to be 25.35 ng/mL in the EDI group, 21.95 ng/mL in the EPR group, and 10.61 ng/mL in the PYO group, confirming the presence of luteal activity. Progesterone levels in bitches with pyometra were found to be significantly lower compared to those in the other groups ($p<0.05$).

Ovariohysterectomy and sample collection

Ovariohysterectomy procedures were carefully planned in the EDI and EPR groups, whereas in the PYO group, they were performed immediately after the diagnosis of pyometra. The anesthesia protocol involved induction with 4-6 mg/kg of propofol (Propofol-PF 1%, 200 mg/20 mL; Polifarma, Turkey) and maintenance with 2-3% isoflurane (Isoflurane USP 100%; ADEKA, Turkey). All surgeries were performed through a ventral midline approach along the linea alba. After preparing the surgical site with proper aseptic and antiseptic techniques, a midline incision was made from the umbilical region toward the pelvis. Under sterile conditions and following standard surgical procedures, the abdominal cavity was entered, and the uterus and ovaries were identified and ligated using absorbable suture material. The tissues were then carefully excised with hemostasis and removed. Closure of the abdominal wall and skin was performed using absorbable suture material. The same experienced surgeon conducted all surgical procedures. Throughout the perioperative period, fluid deficits and needs were calculated and corrected by administering appropriate volumes of balanced electrolyte solutions.

Following ovariohysterectomy, the luteal structures on the removed ovaries were examined and confirmed based on their macroscopic characteristics, including solid consistency, good vascularization, and a soft-elastic texture. All ovaries looked normal to the naked eye, with no signs of disease. One CL sample was taken from each dog's ovary. The sampling did not differentiate between the left and right ovary; instead, the CL with the best appearance was chosen. The selected CL was carefully separated from the surrounding tissue and cut into small pieces, which were then placed into 2 mL sterile cryo tubes (Isolab, Germany). These tubes were stored in liquid nitrogen until real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Each individual's CL tissue was treated as an independent biological replicate; no samples were pooled or combined.

RNA isolation, RT reaction, and quantitative PCR

Approximately 50 mg of luteal tissue was placed into an RNase- and DNase-free homogenization tube containing TRIzol™ reagent (Invitrogen, USA), kept in an ice-water bath, and mechanically disrupted using a homogenizer (Silent Crusher M, Heidolph, Germany). Total RNA was then extracted following the manufacturer's protocol. The purity of RNA was assessed by agarose gel (1%) electrophoresis, and the optical density at 260/280 nm (NanoDrop ND-2000, Thermo Scientific, Wilmington, DE, USA) was 2±0.1 for all RNA samples. Two micrograms of total RNA were treated with DNase I (Fermentas Life Sciences, USA) to remove any genomic DNA contamination and then reverse-transcribed into first-strand complementary DNA (cDNA) using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA) according to the manufacturer's instructions.

Primers for *TLR2*, *TLR4*, *TLR7*, *IL-8*, and housekeeping genes [Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Beta-actin (ACTB)] were designed using Primer3 based on sequences from the NCBI gene database or from published primer sequences. Details of all the primers used are provided in Table 1. To assess the dynamic range and amplification efficiencies of the real-time PCR assays for each gene, amplifications were performed with specific primers in duplicate with a two-fold serial dilution series (1/2, 1/4, 1/8, 1/16) of pooled cDNA collected from CLs. The real-time PCR amplification efficiencies ranged between 95% and 105%.

All real-time PCR reactions were set up as follows: 5 μL SYBR Green Master Mix (2X), 0.1 μL of each primer, 1.0 μL cDNA, and ddH_2O to reach a final volume of 10 μL. Thermal cycling conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, and

extension at 72 °C for 30 seconds. All reactions were among the EDI, EPR, and PYO groups were evaluated

Table 1. Primer sequences, amplicon sizes, and GenBank accession numbers for target and reference genes analyzed in luteum tissue.

Primer	Forward/ Reverse	Primer Sequence	Product size (bp)	GeneBank accession number
Target genes				
TLR2	Frw	CACTTCAATCCCCCGTTCAA	66	NM_001005264.3
	Rev	AATAATCCACTTGCCGGGAATA		
TLR4	Frw	CCTCTTGTCATTGGATACACTAG	105	NM_001002950.3
	Rev	TGCTGTTGTCCTTGTTGTTCCTTGA		
TLR7	Frw	CTGCTCTGCTCTCTTCAACCA	140	NM_001048124.2
	Rev	ATCTGGCTCCAAGGAGTTTGG		
IL-8	Frw	CTGGGACAAGAGCCAGAAAG	102	NM_001003200.1
	Rev	GCTGCAGAAAGGACAAAAGC		
Housekeeping genes				
GAPDH	Frw	GCCAAGAGGGTCATCATCTC	109	NM_001003142.2
	Rev	AGGAGGCATTGCTGACAATC		
ACTB	Frw	AAGCCAACCGTGAGAAGATG	175	NM_001195845.3
	Rev	AAGGCGTACCCCTCGTAGAT		

TLR – Toll-Like Receptor, IL-8 – Interleukin 8, GAPDH – Glyceraldehyde-3-Phosphate dehydrogenase, ACTB – Beta-actin.

performed on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Melting curve analysis was performed as follows: 95°C for 1 min, then fluorescence was measured at every 1°C increment between 60°C and 95°C. The specificity of each primer set was confirmed by melt-curve analysis, which showed a single sharp peak for all targets. To verify RT-qPCR reaction specificity, amplification products were also examined after separation on a 2% agarose gel. In each run, a negative control without cDNA template was included. The RT-qPCR processes were carried out in triplicate. To identify the most suitable reference genes for the study, a panel of housekeeping genes was evaluated using the geNorm (Pfaffl et al. 2004) and BestKeeper (Vandesompele et al. 2002) software programs. GAPDH and ACTB were identified as the most suitable reference genes for this experimental model. Additionally, the use of GAPDH as a housekeeping gene has been previously validated by our group (Atli et al. 2012). The geometric mean of GAPDH and ACTB was used as a normalization factor to calculate fold changes in the real-time PCR data. All RT-qPCR experiments were designed, validated, and reported in accordance with the MIQE guidelines (Bustin et al. 2009).

Statistical analysis

Statistical analysis of the study was performed using SPSS version 25.0 (IBM SPSS Statistics, Chicago, IL, USA). Changes in mRNA transcript levels based on quantification cycle (Cq) values obtained from real-time PCR. For relative quantification, the mean Cq values of the EDI group served as the reference, and fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method, as described by Livak and Schmittgen (2001). The normality of the data distribution was assessed using the Shapiro-Wilk test. In the EPR group, TLR2 ($p=0.043$) and TLR7 ($p=0.039$) data did not show a normal distribution, whereas TLR4 and IL-8 data were normally distributed ($p>0.05$). Homogeneity of variances was assessed using Levene's test. Variances were homogeneous for TLR4, TLR7, and IL-8 ($p>0.05$), while TLR2 showed a significant variance heterogeneity among groups ($p=0.017$). Based on these assumptions, TLR2 and TLR7 data were analyzed using the non-parametric Kruskal-Wallis H test. When significant differences were detected, pairwise post hoc comparisons were performed using Dunn's test with Bonferroni correction. For TLR4 and IL-8, one-way analysis of variance (ANOVA) was applied. When statistically significant differences were found, Tukey's Honestly Significant Difference (HSD) test was used for multiple comparisons between groups. All results were reported as mean ± standard deviation (SD), and a p-value of <0.05 was considered statistically significant. The exact p-values obtained from the post hoc comparisons were presented in the Results section. Graphs were generated using GraphPad Prism version 9.5.0 (GraphPad Software, San Diego, CA, USA).

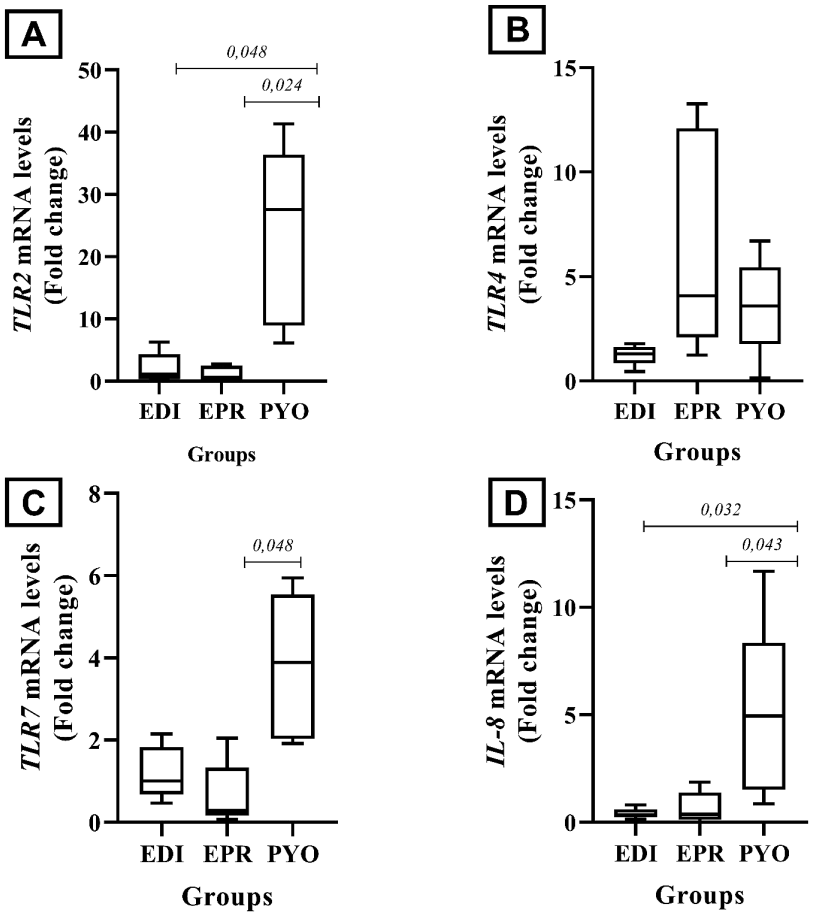

Fig. 1. Box-plot distribution of relative mRNA expression levels of *TLR2* (A), *TLR4* (B), *TLR7* (C), and *IL-8* (D) in luteal tissues of bitches in early diestrus (EDI), early pregnancy (EPR), and pyometra (PYO) groups. Statistical analysis was conducted using the Kruskal-Wallis test followed by Dunn's post hoc test with Bonferroni correction for TLR2 and TLR7 due to violations of normality and/or homogeneity assumptions. For TLR4 and IL-8, one-way ANOVA was applied, followed by Tukey's Honestly Significant Difference (HSD) test for multiple comparisons. P-values are reported for significant pairwise comparisons ($p<0.05$). Box plots display the median (horizontal line), interquartile range (boxes), and minimum and maximum values (whiskers).

Results

The mean±SD values of mRNA expression levels for *TLR2*, *TLR4*, *TLR7*, and *IL-8* were calculated for each experimental group. In the EDI, EPR, and PYO groups, *TLR2* expression was 2.09±2.48, 1.20±1.18, and 23.67±14.45; *TLR4* expression was 1.25±0.49, 6.49±5.28, and 3.61±2.34; *TLR7* expression was 1.21±0.65, 0.65±0.80, and 3.80±1.78; and *IL-8* expression was 0.41±0.25, 0.67±0.74, and 4.94±4.17, respectively.

The distribution of mRNA expression levels of *TLR2*, *TLR4*, *TLR7*, and *IL-8* in the luteal tissues of the study groups is summarized in the box plots shown in Figs. 1A, 1 B, 1C, and 1D, respectively. *TLR2* mRNA expression was significantly higher in the PYO group compared to EDI (p=0.048) and EPR (p=0.024). No significant difference was observed between the EPR and EDI groups (p=1.00). There was no statistical difference in *TLR4* mRNA expression between groups (p=0.083). *TLR7* mRNA expression was significantly higher in the PYO group compared to the EPR (p=0.048) and was similar to the level observed in the EDI (p=0.095). No significant difference was found between the EPR and EDI groups regarding *TLR7* expression (p=0.452). *IL-8* mRNA expression, similar to the expression patterns observed for the other TLRs analyzed, was significantly higher in the PYO group compared to the EDI (p=0.032) and EPR (p=0.043) groups. There was no difference in IL-8 mRNA expression between the EDI and EPR groups (p=0.984).

Discussion

In this study, the mRNA expression levels of *TLR2*, *TLR4*, *TLR7*, and *IL-8* were determined for the first time in the luteal tissue of bitches with two physiologic (early diestrus and early pregnancy) and a pathologic (pyometra) reproductive condition. The presence of

luteal tissue was confirmed in our previous study, based on anamnesis, clinical examination findings, serum P4 levels, and the macroscopic appearance of the ovaries (Ucar et al. 2024).

The common characteristics of our study groups included the presence of active luteal tissue in their ovaries and being under the influence of P4. In our previous publication, we confirmed the presence of active luteal structures in the ovaries of all subjects by determining the mRNA expression of StAR (Ucar et al. 2024). StAR is crucial for converting cholesterol to P4 and contributes significantly to P4 production (Stocco 2001). Hoffmann et al. (1996) reported that the StAR mRNA profile in bitches increases from early to mid-luteal phase and decreases toward regression. In our previous study, we also measured the plasma P4 concentrations of the bitches in the groups and found that they showed a similar pattern to the StAR mRNA profile (Ucar et al. 2024). Notably, P4 levels were lower in bitches with pyometra than in bitches in early pregnancy and early diestrus. The low P4 and StAR mRNA expression observed in our prior study in bitches with pyometra suggest that they are in the late stages of diestrus (Ucar et al. 2024). This aligns with the disease's pathogenesis (Smith 2006) and the anamnesis of the patient owners. In the same study (Ucar et al. 2024), similar StAR mRNA expression was observed in bitches in late diestrus and bitches with pyometra, which can also support this phenomenon. Additionally, one study documented that mRNA expression of 3-beta-hydroxysteroid dehydrogenase, responsible for converting pregnenolone to P4 during P4 biosynthesis in canine luteal tissue, was highest in early diestrus and gradually decreased in late diestrus. This finding was similar to the P4 profile (Kowalewski et al. 2013). It may also provide further evidence for why P4 levels were lower in the PYO group than in the EDI and EPR groups, indicating that our dogs with pyometra are in the late luteal stage.

The primary approach of our study was to evaluate the physiological conditions (EDI and EPR groups) in the presence of luteal structures, then include pathological conditions (PYO group). In our previous study conducted on sheep, we emphasized that certain TLR expressions in CL have alterations related to pregnancy and may contribute to its development and maintenance (Atli et al. 2018). Therefore, we hypothesized that the mRNA profiles of *TLR2* and *TLR4* might differ between the EDI and EPR. However, despite the slight increase in TLR4 expression observed in early pregnant bitches, similar *TLR2* and *TLR4* mRNA expression profiles were noted in both the EDI and EPR groups. This similarity could be because these groups have the same time frame after ovulation and exhibit similar P4 secretion patterns, which is a characteristic feature of the luteal phase in bitches. A study documenting the similarity of *TLR2* and *TLR4* expression in the luteal phase of cattle during the early luteal stage and pregnancy (Gadsby et al. 2017) supports our current findings. Similarly, Nitsche et al. (2010) support our results from a systemic perspective by reporting similar expression levels of these TLRs in human maternal neutrophils between pregnant and non-pregnant individuals.

In our research, we also examined pyometra, a late luteal phase disorder in bitches. Luteal expression of *TLR2* and *TLR4* was significantly upregulated following intravenous LPS administration in cattle, indicating a possible direct effect of LPS on luteal tissue (Lüttgenau et al. 2016). Elevated LPS levels in the follicles of cows with postpartum metritis support this perspective (Magata et al. 2015). Based on this, we hypothesized that the strong inflammatory response caused by pyometra could be activated in the uterus and involve the luteal tissue, potentially leading to increased expression of *TLR2* and *TLR4*. We found that the luteal *TLR2* mRNA profile of the PYO group was significantly higher than that of the bitches in the EPR and EDI groups. However, luteal *TLR4* mRNA levels appeared to be higher in the EPR group and lower in the EDI group on the graph; no statistically significant difference was observed between the groups. The high *TLR2* mRNA expression could be caused by the stimulation of the innate immune defense mechanism against bacterial infections in bitches with pyometra. However, the similarity in *TLR4* mRNA expression might be due to its suppression as a protective response against excessive inflammatory reactions, as has been proposed for endometrial *TLR2* expression (Chotimanukul and Sirivaidyapong 2011). This suggests that the excessive inflammatory response of the luteal tissue could be prevented, helping to maintain homeostasis. Additionally, the comparable *TLR4* mRNA expression may be linked to a unique expression pattern specific to canine luteal tissue, or the slight increase in *TLR4* expression seen in early pregnant bitches might indicate a role in regulating the immune system during pregnancy. The effects of TLR2 and TLR4 in canine pyometra have also been examined in various studies on the endometrium (Silva et al. 2010, 2012, Chotimanukul and Sirivaidyapong 2011, 2012). During the diestrus period, increased expression of *TLR2* and *TLR4* was observed in the endometrium of bitches with pyometra caused by Escherichia coli, and this was associated with strong leukocyte infiltration (Silva et al. 2010). The same researchers reported that the endometrial *TLR2* gene was upregulated in bitches with pyometra (Silva et al. 2012). Chotimanukul and Sirivaidyapong (2011) also showed high *TLR4* expression in the endometrium of

infected bitches compared to healthy ones. They also noted an increase in glandular epithelium and stroma during healthy diestrus, which they suggested could be related to the high P4 levels. Progesterone has also been reported to induce *TLR4* expression in the endometrium (Yoo et al. 2019). Additionally, human endometrial *TLR4* mRNA expression has been shown to be higher during the secretory phase compared to the proliferative phase (Aflatoonian et al. 2007). Another reason for the similar *TLR4* expression between the groups in our study could be that, as we found in our previous publication (Ucar et al. 2024), serum P4 levels were higher in the EDI and EPR groups compared to the PYO group. The high P4 in these groups may have increased luteal *TLR4* mRNA expression, similar to what is seen in the endometrium, resulting in comparable findings to the PYO group.

It may also be noteworthy to analyze the expression of *TLR2* and *TLR4* mRNA in relation to the phase of the luteal period in each group. In one of our previous studies, we observed that the mRNA profiles of *TLR2* and *TLR4* in cattle during the luteal phase increase significantly as luteal regression progresses (Atli et al. 2018). Gadsby et al. (2017) found that *TLR2* and *TLR4* mRNA profiles increase during the regression phase of bovine luteal tissue. Another study also documented that the mRNA expression of *TLR2* and *TLR4* rises in the CL of cattle until the mid-luteal phase (Lüttgenau et al. 2016). During luteal tissue regression, there are changes in the concentrations of various molecules, including reactive oxygen species, endothelium-derived proteins, and lipophilic molecules, which can serve as endogenous ligands to induce TLR2 and TLR4 signaling (Piccini and Midwood 2010, Skarzynski et al. 2010, Pate et al. 2012). Furthermore, luteolysis is defined as a proinflammatory event, and during luteal regression, there is an increase in proinflammatory gene expression (Atli et al. 2012) and immune cell infiltration (Penny 2000). We observed that the expression of *TLR2* and *TLR4* was upregulated in the CL of sheep at various time points along with PGF2α-induced luteolysis (Atli et al. 2018). A similar pattern was observed in the endometrium of dogs (Silva et al. 2012). It was found that mRNA transcription and protein expression of TLR2 and TLR4 were lower in the early diestrus phase compared to the late diestrus phase. This has been associated with a reduced immune response to conception and increased susceptibility to pyometra (Silva et al. 2012). In the present study, we found that pregnant and healthy diestrus bitches were in the early phase of the luteal period, while pyometra bitches were in the late phase. The high *TLR2* mRNA expression in the PYO group could also be related to the fact that these animals were in the late stage of the luteal period. As noted in studies (Atli et al. 2017, Gadsby et al. 2017), the increase in TLR2 may suggest its possible role in the immune-mediated regression process.

Toll-like receptor-7 mRNA expression was demonstrated only in the endometrium of bitches throughout the estrous cycle (Silva et al. 2012). Gadsby et al. (2017) reported no change in *TLR7* mRNA profile in the luteal tissue of cows during the estrous cycle or pregnancy, except for a slight increase toward the end of diestrus. Our previous study also documented that ovine luteal tissue exhibits similar *TLR7* mRNA profiles at various time points during the estrous cycle and pregnancy (Atli et al. 2018). The results of the current study are consistent with those of previous studies and indicate that luteal *TLR7* expression is consistent in bitches across physiological states, including early diestrus and pregnancy, without a distinct profile. Additionally, similar *TLR7* mRNA expression in peripheral blood leukocytes of early pregnant and non-pregnant sheep, as reported in another study, further supports our findings (Kaya et al. 2017). However, we observed that luteal *TLR7* mRNA expression was significantly higher in bitches with pyometra than in pregnant bitches. This may be because, as with TLR2, the upregulation of luteal *TLR7* is stimulated by ligands derived from pyometra infection, and the suppression of luteal immune activity, including TLR7, is only partially downregulated in pregnant bitches to maintain pregnancy.

Interleukin-8 is known to be highly expressed in the CL, particularly during the early luteal phase, indicating its role in inflammation and immune responses within this reproductive structure (Jiemtaweboon et al. 2011). The expression of *IL-8* is crucial for neutrophil recruitment and activation, as well as for enhancing inflammatory responses (Polec et al. 2009). Toll-like receptor-2-mediated response to ligands in monocytes and granulocytes stimulates the release of IL-8, a proinflammatory cytokine. In our study, *IL-8* mRNA exhibited a similar expression pattern to that of TLR2. It is highest in the PYO group, indicating a strong inflammatory response to bacterial infection and neutrophil migration. The similar expression pattern in the EDI and EPR groups is likely due to the comparable hormonal mechanisms in pregnant and non-pregnant bitches.

Pyometra is more than just a localized infection of the uterus; it is a serious clinical condition often accompanied by a systemic inflammatory response that requires urgent intervention (Hagman, 2018). This systemic immune activation can affect the expression levels of not only target genes but also reference genes. In fact, several studies have shown that commonly used housekeeping genes such as GAPDH and ACTB can

vary in expression under inflammatory or septic conditions (Piehler et al. 2010, Bednarz-Misa et al. 2020). However, in our study, GAPDH and ACTB showed stable Cq values across the groups, with no significant differences in their expression. Moreover, these two genes have been tested as reference genes in various RT-qPCR-based gene expression studies on canine reproductive tissues and have been validated as suitable reference genes in most cases due to their stable expression profiles (Du et al. 2016, Nowak et al. 2020). These findings support the reliability of the experiment and the transcriptional stability of our data. On the other hand, the upregulation of TLRs in tissues may indicate not only a local immune response but also systemic immune activation. TLRs are known to respond to both PAMPs and damage-associated molecular patterns (DAMPs) during systemic infection (Takeuchi and Akira, 2010). Therefore, the observed increase in TLR expression in conditions accompanied by systemic inflammation, such as pyometra, may reflect immune activation at both local and systemic levels. In this context, future studies incorporating systemic inflammatory markers, such as interleukin-6 and tumor necrosis factor-alpha, would help provide a more comprehensive understanding of the systemic effects on both target and reference genes. Such an approach would improve the biological interpretation and relevance of RT-qPCR data.

Although our study is the first to examine the mRNA expression of selected TLRs and *IL-8* in the luteal tissues of bitches under different reproductive conditions, it has certain limitations. This work provides a basic initial molecular characterization based on the analysis of mRNA expression levels of the selected genes. One limitation of our study is the absence of protein-level validation, such as Western blot or immunohistochemical analyses, to support the transcriptional results. Since post-transcriptional and post-translational mechanisms can influence transcript abundance, it may not always correlate with protein expression levels (Wang, 2008). Therefore, future studies should include protein level validation to confirm the functional importance of the transcriptional changes observed in luteal tissues under different reproductive states. Another limiting factor is the modest sample size, which may have constrained the study's statistical power. In this context, ethical considerations, particularly regarding the collection of luteal tissues from pregnant bitches, can be considered a determining factor. However, to minimize the impact of the sample size, our study employed comprehensive clinical assessments to ensure accurate classification of subjects, and aimed to reduce biological variability by selecting uniform individuals. This method was designed to improve data quality. Future larger-scale studies are recommended to validate these initial findings.

Conclusions

In conclusion, this study is the first to investigate and reveal differential expression patterns of *TLR2*, *TLR4*, *TLR7*, and *IL-8* mRNA in the canine luteal tissue across early diestrus, early pregnancy, and pyometra. The expression of TLR mRNA in the canine luteal tissue may vary depending on the state of the uterus (pregnancy or pyometra), luteal stage, and serum P4 level. Additional comprehensive studies that include both mRNA and protein analyses of TLRs and cytokines across various reproductive states, including different phases of the sexual cycle and pregnancy, are needed to clarify the influence of the immune system on the complex mechanisms of the luteal phase.

Acknowledgements

The present study was supported by Aydın Adnan Menderes University Scientific Research Projects (VTF-22021). It was carried out with the assistance of facilities from Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, and Dicle University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, and Harran University, Department of Reproduction and Artificial Insemination.

References

Aflatoonian R, Fazeli A (**2008**) Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression. J Reprod Immunol 77: 7-13.

Aflatoonian R, Tuckerman E, Elliott SL, Bruce C, Aflatoonian A, Li TC, Fazeli A (**2007**) Menstrual cycle-dependent changes of Toll-like receptors in endometrium. Hum Reprod 22: 586-593.

Akashi S, Shimazu R, Ogata H, Nagai Y, Takeda K, Kimoto M, Miyake K (**2000**) Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J Immunol 164: 3471-3475.

Akira S (**2003**) Toll-like receptor signaling. J Biol Chem 278: 38105-38108.

Arici A, Tazuke SI, Attar E, Kliman HJ, Olive DL (**1996**) Interleukin-8 concentration in peritoneal fluid of patients with endometriosis and modulation of interleukin-8 expression in human mesothelial cells. Mol Hum Reprod 2: 40-45.

Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA (**2004**) Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. Endocrinology 145: 2551-2560.

Atli MO, Akbalık ME, Kose M, Alak I, Atli Z, Hitit M (**2022**) Expression pattern and cellular localization of two critical non-nuclear progesterone receptors in the ovine corpus luteum during the estrous cycle and early pregnancy. Anim Reprod Sci 243: 107026.

Atli MO, Bender RW, Mehta V, Bastos MR, Luo W, Vezina CM, Wiltbank MC (**2012**) Patterns of gene expression in the bovine corpus luteum following repeated intrauterine infusions of low doses of prostaglandin F2alpha. Biol Reprod 86: 130.

Atli MO, Kose M, Hitit M, Kaya MS, Bozkaya F (**2018**) Expression patterns of Toll-like receptors in the ovine corpus luteum during the early pregnancy and prostaglandin F2α-induced luteolysis. Theriogenology 111: 25-33.

Atli MO, Köse M, Kaya MS, Aydilek N, Güzeloğlu A, Wiltbank MC (**2017**) Toll-like receptor 2 and 4 expression in the bovine corpus luteum during the different stages of the estrous cycle. Anim Reprod 14 : 1270-1277.

Bazzocchi C, Mortarino M, Comazzi S, Bandi C, Franceschi A, Genchi C (**2005**) Expression and function of Toll-like receptor 2 in canine blood phagocytes. Vet Immunol Immunopathol 104: 15-19.

Bednarz-Misa I, Neubauer K, Zacharska E, Kapturkiewicz B, Krzystek-Korpacka M (**2020**) Whole blood ACTB, B2M and GAPDH expression reflects activity of inflammatory bowel disease, advancement of colorectal cancer, and correlates with circulating inflammatory and angiogenic factors: Relevance for real-time quantitative PCR. Adv Clin Exp Med 29: 547-556.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (**2009**) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622.

Chotimanukul S, Sirivaidyapong S (**2011**) Differential expression of Toll-like receptor 4 (TLR4) in healthy and infected canine endometrium. Theriogenology 76: 1152-1161.

Chotimanukul S, Sirivaidyapong S (**2012**) The localization of Toll-like Receptor 2 (TLR 2) in the endometrium and the cervix of dogs at different stages of the oestrous cycle and with pyometra. Reprod Domest Anim 47: 351-355.

Crozat K, Beutler B (**2004**) TLR7: A new sensor of viral infection. Proc Natl Acad Sci U S A 101: 6835-6836.

Du M, Wang X, Yue YW, Zhou PY, Yao W, Li X, Ding XB, Liu XF, Guo H, Ma WZ (**2016**) Selection of reference genes in canine uterine tissues. Genet Mol Res 15: 15028138.

Gadsby JE, Tyson Nipper AM, Faircloth HA, D'Annibale-Tolhurst M, Chang J, Farin PW, Sheldon IM, Poole DH (**2017**) Toll-like receptor and related cytokine mRNA expression in bovine corpora lutea during the oestrous cycle and pregnancy. Reprod Domest Anim 52: 495-504.

Hagman R (**2018**) Pyometra in small animals. Vet Clin North Am Small Anim Pract 48: 639-661.

Hoffmann B, Riesenbeck A, Klein R (**1996**) Reproductive endocrinology of bitches. Anim Reprod Sci 42: 275-288.

Jiemtaweeboon S, Shirasuna K, Nitta A, Kobayashi A, Schuberth HJ, Shimizu T, Miyamoto A (**2011**) Evidence that polymorphonuclear neutrophils infiltrate into the developing corpus luteum and promote angiogenesis with interleukin-8 in the cow. Reprod Biol Endocrinol 9: 79.

Johnston SD, Kustritz MR, Olson PNS (**2001**) Disorders of the canine uterus and uterine tubes (oviducts). In: Johnston SD, Kustritz MR, Olson PNS (Eds). Canine and Feline Theriogenology. Philadelphia, WB Saunders pp 463-471.

Jungi TW, Farhat K, Burgener IA, Werling D (**2011**) Toll-like receptors in domestic animals. Cell Tissue Res 343: 107-120.

Kannaki TR, Shanmugam M, Verma PC (**2011**) Toll-like receptors and their role in animal reproduction. Anim Reprod Sci 125: 1-12.

Kawai T, Akira S (**2010**) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11: 373-384.

Kaya MS, Kose M, Guzeloglu A, Kıyma Z, Atli MO (**2017**) Early pregnancy-related changes in toll-like receptors expression in ovine trophoblasts and peripheral blood leukocytes. Theriogenology 93: 40-45.

Kempisty B, Bukowska D, Wozna M, Piotrowska H, Jackowska M, Zuraw A, Ciesiolka S, Antosik P, Maryniak H, Ociepa E, Porowski SZ, Brussow KP, Jaskowski JM, Nowicki M (**2013**) Endometritis and pyometra in bitches: a review. Vet Med-Czech 58: 289-297.

Kida K, Baba E, Torii R, Kawate N, Hatoya S, Wijewardana V, Sugiura K, Sawada T, Tamada H, Inaba T (**2006**) Lactoferrin expression in the canine uterus during the estrous cycle and with pyometra. Theriogenology 66: 1325-1333.

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM (**1992**) Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 258: 1798-1801.

Kowalewski MP (**2014**) Luteal regression vs. prepartum luteolysis: regulatory mechanisms governing canine corpus luteum function. Reprod Biol 14: 89-102.

Kowalewski MP, Fox B, Gram A, Boos A, Reichler I (**2013**) Prostaglandin E2 functions as a luteotrophic factor in the dog. Reproduction 145: 213-226.

Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G, Hoffmann B (**2006**) Characterization of the canine 3beta-hydroxysteroid dehydrogenase and its expression in the corpus luteum during diestrus. J Steroid Biochem Mol Biol 101: 254-262.

Kundakci A, Pirat A (**2012**) Toll-Like Receptors. J Turk Soc Intens Care 10: 63-73.

Livak KJ, Schmittgen TD (**2001**) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408.

Lüttgenau J, Herzog K, Strüve K, Latter S, Boos A, Bruckmaier RM, Bollwein H, Kowalewski MP (**2016**) LPS-mediated effects and spatio-temporal expression of TLR2 and TLR4 in the bovine corpus luteum. Reproduction 151: 391-399.

Magata F, Ishida Y, Miyamoto A, Furuoka H, Inokuma H, Shimizu T (**2015**) Comparison of bacterial endotoxin lipopolysaccharide concentrations in the blood, ovarian follicular fluid and uterine fluid: a clinical case of bovine metritis. J Vet Med Sci 77: 81-84.

Nitsche JF, Jiang SW, Brost BC (**2010**) Toll-like receptor-2 and Toll-like receptor-4 expression on maternal neutrophils during pregnancy. Am J Reprod Immunol 64: 427-434.

Nowak M, Aslan S, Kowalewski MP (**2020**) Determination of novel reference genes for improving gene expression data normalization in selected canine reproductive tissues – a multistudy analysis. BMC Vet Res 16: 440.

Papa PC, Kowalewski MP (**2020**) Factors affecting the fate of the canine corpus luteum: Potential contributors to pregnancy and non-pregnancy. Theriogenology 150: 339-346.

Pate JL, Johnson-Larson CJ, Ottobre JS (**2012**) Life or death decisions in the corpus luteum. Reprod Domest Anim 47: 297-303.

Patra MK, Kumar H, Nandi S, Loyi T, Islam R (**2014**) Upregulation of TLR-4 and proinflammatory cytokine transcripts as diagnostic indicator of endometritis in buffaloes. J Appl Anim Res 42: 256-262.

Penny LA (**2000**) Monocyte chemoattractant protein 1 in luteolysis. Rev Reprod 5: 63-66.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (**2004**) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 26: 509-515.

Piccinini AM, Midwood KS (**2010**) DAMPening inflammation by modulating TLR signalling. Mediators Inflamm 2010: 672395.

Piehler AP, Grimholt RM, Ovstebø R, Berg JP (**2010**) Gene expression results in lipopolysaccharide-stimulated monocytes depend significantly on the choice of reference genes. BMC Immunol 11: 21.

Polec A, Tanbo T, Fedorcsak P (**2009**) Cellular interaction regulates interleukin-8 secretion by granulosa-lutein cells and monocytes/macrophages. Am J Reprod Immunol 61: 85-94.

Santana CH, Santos RL (**2021**) Canine pyometra-an update and revision of diagnostic terminology. Braz J Vet Pathol 14: 1-8.

Schlafer DH, Gifford AT (**2008**) Cystic endometrial hyperplasia, pseudo-placentational endometrial hyperplasia, and other cystic conditions of the canine and feline uterus. Theriogenology 70: 349-358.

Shirasuna K, Jiemtaweeboon S, Raddatz S, Nitta A, Schuberth HJ, Bollwein H, Miyamoto A (**2012**) Rapid accumulation of polymorphonuclear neutrophils in the corpus luteum during prostaglandin F(2α)-induced luteolysis in the cow. PLoS One 7: e29054.

Shirasuna K, Matsumoto H, Matsuyama S, Kimura K, Bollwein H, Miyamoto A (**2015**) Possible role of interferon tau on the bovine corpus luteum and neutrophils during the early pregnancy. Reproduction 150 : 217-225.

Silva E, Henriques S, Brito S, Ferreira-Dias G, Lopes-da--Costa L, Mateus L (**2012**) Oestrous cycle-related changes in production of Toll-like receptors and prostaglandins in the canine endometrium. J Reprod Immunol 96: 45-57.

Silva E, Leitao S, Henriques S, Kowalewski MP, Hoffmann B, Ferreira-Dias G, Lopes da Costa L, Mateus L (**2010**) Gene transcription of TLR2, TLR4, LPS ligands and prostaglandin synthesis enzymes are up-regulated in canine uteri with cystic endometrial hyperplasia-pyometra complex. J Reprod Immunol 84: 66-74.

Skarzynski DJ, Okuda K (**2010**) Inter- and intra-cellular mechanisms of prostaglandin F2alpha action during corpus luteum regression in cattle. Soc Reprod Fertil Suppl 67: 305-324.

Smith FO (**2006**) Canine pyometra. Theriogenology 66: 610-612.

Stocco C, Telleria C, Gibori G (**2007**) The molecular control of corpus luteum formation, function, and regression. Endocr Rev 28: 117-149.

Stocco DM (**2001**) StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 63: 193-213.

Sugiura K, Nishikawa M, Ishiguro K, Tajima T, Inaba M, Torii R, Hatoya S, Wijewardana V, Kumagai D, Tamada H, Sawada T, Ikehara S, Inaba T (**2004**) Effect of ovarian hormones on periodical changes in immune resistance associated with estrous cycle in the beagle bitch. Immunobiology 209: 619-627.

Takeuchi O, Akira S (**2010**) Pattern recognition receptors and inflammation. Cell 140: 805-820.

Ucar EH, Cetin H, Atli MO (**2018**) Effect of multiple low-dose PGF2α injections on the mature corpus luteum in non-pregnant bitches. Theriogenology 113: 34-43.

Ucar EH, Peker C, Hitit M, Kose M, Tatar M, Bozkaya F, Atli MO (**2024**) Altered luteal expression patterns of genomic and non-genomic progesterone receptors in bitches at different reproductive states. Theriogenology 218: 153-162.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (**2002**) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.

Wang D (**2008**) Discrepancy between mRNA and protein abundance: insight from information retrieval process in computers. Comput Biol Chem 32: 462-468.

Yoo I, Han J, Lee S, Jung W, Kim JH, Kim YW, Kim HJ, Hong M, Ka H (**2019**) Analysis of stage-specific expression of the toll-like receptor family in the porcine endometrium throughout the estrous cycle and pregnancy. Theriogenology 125: 173-183.

Zähringer U, Lindner B, Inamura S, Heine H, Alexander C (**2008**) TLR2–promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. Immunobiology 213: 205-224.