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Endometrial mRNA expression of key inflammatory genes and progesterone receptor in uterine tissue of bitches with endometritis

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ABSTRACT

Our study aimed to evaluate the gene transcription of Cyclooxygenase 1 (COX1), cytokines (Interleukin 6 [IL6], Interleukin 8 [IL8], and Leukemia Inhibitory Factor [LIF]), and Progesterone Receptor (PR) in clinically healthy bitches with a normal endometrium and with endometritis, without the presence of cystic endometrial hyperplasia. Forty-eight mixed breed bitches in dioestrus were used. Uterine biopsies were collected for diagnosis of a normal endometrium (n=15), and endometritis (n=30). Three samples were excluded from the study (two bitches with cystic endometrial hyperplasia and one bitch with atrophy). Samples were collected by cytobrush to quantify mRNA by qPCR. Data were analyzed using a generalized mixed model. COX1, IL6, and IL8 mRNA expression in bitches with endometritis was significantly higher than in a normal endometrium (P=0.0002, P=0.002, and P=0.003, respectively). In contrast, LIF and PR mRNA expression in bitches with endometritis was significantly lower than with a normal endometrium (P=0.006 and P=0.001, respectively). IL6 (AUC 0.67) was the most favorable biomolecular marker for predicting endometritis in bitches. There was an evident change in the mRNA expression of COX1, cytokines, and PR in clinically healthy bitches with a normal endometrium and endometritis without cystic endometrial hyperplasia. These results suggest that biomolecular markers such as COX1, IL6, IL8, LIF, and PR could help diagnose endometritis in a bitch.

Key words: bitch; endometritis; cyclooxygenase 1; cytokines; progesterone receptor

Introduction

Endometritis is an inflammation of the endometrial layer of the uterine tissue, characterized by infiltration of inflammatory cells, vascular congestion, and stromal edema, which can occur without clinical signs of illness (FONTAINE et al., 2009; MIR et al., 2013; GIFFORD et al., 2014; PRADERIO et al., 2019). In contrast, bitches with cystic endometrial hyperplasia-pyometra show

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several clinical signs, such as abdominal distension due to an enlarged fluid-filled uterus, polyuria, polydipsia, inappetence, emesis, and lethargy. If the cervix is open, vaginal discharge can also be observed (MIR et al., 2013; DE BOSSCHERE el at., 2001; CHRISTENSEN et al., 2012; HAAS et al., 2016; HAGMAN, 2017). Bitches with cystic endometrial hyperplasia present histological evidence of an increase in the size and number of glands, and a flattening of the gland epithelium (PRADERIO et al., 2019; FOSTER, 2012). Endometritis and cystic endometrial hyperplasia-pyometra may also present together or independently (SCHLAFER and GIFFORD, 2008). Thus, it is commonly accepted that endometritis and cystic endometrial hyperplasia can be different uterine conditions and are distinct from a normal endometrium, which is characterized by an intact endometrial epithelium and scarce inflammatory cells (GIFFORD et al., 2014; PRADERIO et al., 2019; DOW, 1959).

Cyclooxygenase 1 (COX1) is expressed constitutively in many tissues. The biosynthesis of prostaglandins is increased in inflamed tissue, and their production depends on COX1 and COX2 (RICCIOTTI and FITZGERALD, 2011; VOORWALD et al., 2015). Also, proinflammatory cytokines, such as interleukin 6 (IL6) and interleukin 8 (IL8), are produced during inflammation and have several activities, such as polymorphonuclear recruitment and infiltration in tissue inflammation (GHASEMI et al., 2012). On the other hand, leukemia inhibitory factor (LIF) is a cytokine whose expression increases during implantation in most mammals, and is considered essential to establishing a pregnancy (VOGIAGIS and SALAMONSEN, 1999; SCHÄFER-SOMI et al., 2009). During dioestrus in the non-pregnant bitch, the corpus luteum persists, so progesterone concentrations remain elevated to similar levels to those in pregnant bitches. Hence, progesterone stimulation in the uterus during dioestrus is considered one of the main causes of the cystic endometrial hyperplasia-pyometra complex (DE BOSSCHERE el at., 2001; FELDMAN and NELSON, 2000). Studies have shown that serum progesterone concentrations are not significantly different between bitches with cystic endometrial hyperplasia-pyometra and normal bitches (DE BOSSCHERE el at., 2001). However, PRADERIO et al. found significant differences in serum progesterone concentrations between bitches with endometritis and those with a normal endometrium (PRADERIO et al., 2019). In this sense, bitches with endometritis could be showing abnormalities in endometrial receptors for progesterone.

During the estrous cycle and pregnancy, endocrine and paracrine signals in the uterus require specific sex steroid receptor regulation, such as progesterone receptors (PR). Progesterone action on the endometrium is also known to induce some uterus diseases. Therefore, an understanding of changes in progesterone and PR, and their relationship with inflammatory and proinflammatory biomolecular markers related to genes that could be involved in the pathogenesis of endometritis, is essential. Differences in the expression of inflammatory-related biomolecular markers have been reported in bitches with pyometra. These suggest that inflammation in uterine tissue is associated with the activation of specific immune cells that are involved in the pathogenesis of pyometra in a bitch (SILVA et al., 2009; BUKOWSKA et al., 2014; KARLSSON et al., 2015). Furthermore, other biomolecular markers, different from proinflammatory-related genes, have differed in expression in bitches with pyometra compared with normal uteri (BUKOWSKA et al., 2014). Some researchers have shown differences in the expression of prostaglandins synthesis enzymes and cytokines between normal uteri compared with uterine tissue from cows and mares with clinical and subclinical endometritis (GHASEMI et al., 2012; GABLER et al., 2009; WAGENER et al., 2017). Recently, we reported a preliminary study in which we observed that the COX2, PGE synthase 1 (PTGES-1), and PGF synthase (PGFS/AKR1C3) expression did not differ between bitches with and without endometritis. However, García Mitacek *et al.* (2017) found that COX2 and PTGES-1 expression differed in the different endometritis subtypes and from a normal endometrium (GARCÍA MITACEK et al., 2017).

We hypothesized that the pattern of endometrial mRNA expression of COX1, cytokines (IL6, IL8,

and LIF), and PR is altered in uterine tissue of clinically healthy bitches with endometritis without the presence of cystic endometrial hyperplasia, compared with a normal endometrium. Therefore, this study aimed to evaluate the gene transcription of COX1, cytokines (IL6, IL8, and LIF) and PR in clinically healthy bitches with a normal endometrium and bitches with endometritis without the presence of cystic endometrial hyperplasia.

Materials and methods

Animal and collection of tissue. Forty-eight mixed breed, privately-owned, intact and clinically healthy bitches (*Canis lupus familiaris*) in dioestrus, with a mean age of 2.1±0.21 yr (range 1–5 yr) and weighing between 10 and 30 kg, were used in this study. The females were incorporated into a program for breeding control in La Plata, Argentina. Ovariohysterectomy was carried out during diestrus. A clinical and reproductive examination was performed. Dioestrus was determined on the basis of the history provided by the owner and confirmed by vaginal cytology and serum progesterone. Vaginal cytology samples were collected using a cotton-tipped swab. The swab was rolled over a clean microscopic slide, and smears were air-dried and stained with Tincion 15 (Biopur S.R.L., Rosario, Argentina). The estrus cycle stage was defined according to the percentage and type of cells present in the vaginal cytology (FELDMAN and NELSON, 2000). Blood samples were taken before ovariohysterectomy, centrifuged, and stored at -20°C until progesterone was measured by chemiluminescence immunoassay (Elecsys®, Progesterone II; Roche, Mannheim, Germany). The intra-assay CVs for high-pool and low-pool progesterone (15.48 and 0.95 nmol/L) were 4.5% and 2%, respectively. The study was performed following the international recommendations specified in the guidelines for the care and use of laboratory animal (ICLAS, 2012) and the National Academy of Science recommendations concerning the use of dogs as laboratory animals (CIOMS, 2012), as well as the approval of the Institutional Animal Care and Use Committee of Faculty of Veterinary Sciences, National University of La Plata. Before ovariohysterectomy, the bitches were pre-medicated with acepromazine (0.1 mg/kg S/C, no more than 1 mg total dose; Acedan, Laboratorio Holliday-Scott, Argentina) and tramadol (1 mg/kg I/M; Algen 20, Laboratorio Richmond, Argentina). Anesthesia was induced with propofol (4 mg/kg; Propovet, Laboratorio Richmond, Argentina) and maintained with isoflurane (Isoflurane USP, Laboratorio Baxter, Argentina (GARCÍA MITACEK et al., 2017; SLATTER, 2003). Uterine biopsy samples were obtained from the middle part of both uterine horns. Uterine biopsy samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Sections were then cut at 2–4 μm, deparaffinized, stained with H&E, and observed with a light microscope at 10X and 40X magnifications (Olympus, Tokyo, Japan). Later, samples were classified according to previous studies (PRADERIO et al., 2019; SLATTER, 2003). Only bitches with a normal endometrium and bitches with endometritis were included. Therefore, three samples were excluded from the study (two bitches with cystic endometrial hyperplasia and one bitch with atrophy).

RNA extraction and RT-qPCR. The cytobrush technique was performed to obtain cells from the endometrial epithelium to isolate RNA. In the uterine lumen, we introduced and rotated a cytobrush (Medibrush® Plus, Medical Engineering Corporation, Bs. As., Argentina). We obtained cells from the middle part of both horns. Then, the samples were placed into a reaction tube and stored at −80°C (GABLER et al., 2009; GARCÍA MITACEK et al., 2017). Cells from both horns were suspended separately in saline solution and centrifuged at 3000 X g for 10 min. The cell pellet was resuspended and homogenized in 500 μL of TRIzol® Reagent (Invitrogen, Carlsbad, California, United States (GABLER et al., 2009; GARCÍA MITACEK et al., 2017; SERRANO et al., 2006). Total RNA was extracted using TRIzol® (Life Technologies, Carlsbad, CA, United States), according to the manufacturer›s instructions. RNA quality was assessed by visualization of 28S and 18S rRNA bands after electrophoresis through a 1.5% gel agarose with ethidium bromide staining. Total RNA amounts were then determined using a NanoDrop One® (Thermo Fisher Scientific Inc., Wilmington,

DE, United States (GARCÍA MITACEK et al., 2017). The synthesis of the complementary DNA (cDNA) was obtained by reverse transcription of 500 ng of total RNA primed with 1 µL of oligo (dT) 15 primer (500 ng/µL) (Invitrogen, California, United States) and 1 µL of random hexamers (500 ng/µL; Invitrogen, California, United States). This mixture was heated at 70°C for 5 min and cooled on ice for RNA denaturation. Subsequently, 1 µL of dNTPs (10 mm), 4 µL 5× transcriptase reaction buffer (Invitrogen, California, United States), 1 µL RNaseOUT™ Ribonuclease Inhibitor (40 U/µL; Invitrogen, California, United States) and 1 µL of M-MLV reverse transcriptase enzyme (200 U/µL; Invitrogen, California, United States) were added. Reactions were performed for 1 hr at 37°C, 15 min at 42°C, and for a further 5 min at 94°C. cDNA samples were stored at −20°C until real-time PCR amplification (SILVA et al., 2009; GARCÍA MITACEK et al., 2017). Quantitative analysis of the mRNA expression of the selected factors was carried out using real-time PCR. The primer pairs are described in Table 1 and were synthesized by Invitrogen® (Life Technologies, California, United States). Ribosomal protein L27 (RPL27) and glyceraldehyde-3phosphate-dehydrogenase (GAPDH) were amplified as reference genes (SILVA et al., 2009; RAI et al., 2008; SILVA et al., 2010). Real-time PCRs were performed using HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis Biodyne, Tartu, Estonia), according to the manufacturer′s instructions. Each reaction required 4 µl of the mix, specific primers (Primer Forward [250 nM], and Primer Reverse [250 nM]), 1 µl of the cDNA and DNase/RNase-free water to a final volume of 20 µL. Reactions were carried out in the MiniOpticon Real-Time PCR detection system (Bio-Rad, California, United States). Cycling parameters were 10 min of pre-incubation at 95°C, followed by 42 cycles of 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C. Melting curves were acquired to ensure that a single product was amplified in the reaction. PCR products were run through a 2.5% agarose gel to confirm the expected product size. The relative mRNA quantification data were analyzed with CFX Manager Software (Bio-Rad, California, United States (GARCÍA MITACEK et al., 2017). Each sample was run in duplicate, and amplification with sterile nuclease-free water, instead of cDNA, was systematically carried out as a negative control. The target gene's relative expression was performed following the normalization of the level of mRNA expression for the geometric mean of 2 reference genes (RPL27 and GAPDH). The relative fold changes of target genes in the bitches with endometritis were calculated using a normal endometrium as a calibrator (JOHNSON et al., 2015; LIMA et al., 2015; SINGH et al., 2018). Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method; each sample was performed in duplicate, and the means of each sample were calculated. Then, the geometric mean was calculated using the Ct data for 2 reference genes (RPL27 and GAPDH) for each sample and the ΔΔCt was calculated (Ct sample - Ct reference). Then again, the $2^{-\Delta\Delta Ct}$ was calculated for each sample. The means and standard errors for each group (normal and endometritis) were calculated, thus providing a relative expression for each biomolecular marker expressed in arbitrary units (LIVAK and SCHMITTGEN, 2001).

Statistical analyses. RT-qPCR data are shown as least squares means ± standard errors. The differences between endometritis and a normal endometrium were analyzed with a generalized linear mixed model, Poisson distribution, and log link functions (PROC GLIMMIX, SAS® 9.4; SAS Institute Inc., Cary, NC, USA (YUAN, 2006; LIMA et al., 2015). The correlation coefficient of the genes (PG synthesis enzymes, cytokines, and PR) in bitches with a normal endometrium and endometritis was performed using Spearman›s rho correlation (PROC CORR; SAS® 9.4). Receiver operating characteristics (ROC) analysis was performed to test the potential biomolecular markers› predictive power for endometritis. The area under the curve (AUC) values were evaluated for endometritis vs. a normal endometrium using univariate ROC analyses. Progesterone serum concentrations were analyzed with a generalized linear mixed model (PROC GLIMMIX, SAS® 9.4).

Table 1. Primer sequences for mRNA of target genes

Target gene	Sequence (5´-3')	GeneBank accession number	References
RPL27	FW ACAATCACCTCATGCCCACA RV CTTGACCTTGGCCTCTCGTC	NM_001003102	(SILVA et al., 2010)
GAPDH	FW TATTGTCGCCATCAATGACC RV TACTCAGCACCAGCATCACC	NM_01003142	(RAI et al., 2008)
COX1	FW CACTCGTGTTCTGCCCTCTGT RV GCGTCTGGCAACTGCTTCTT	NM_001003023	(SILVA et al., 2010)
IL6	FW GGCTACTGCTTTCCCTACCC RV TTTTCTGCCAGTGCCTCTTT	NM_001003301	(RAI et al., 2008)
IL8	FW ACTTCCAAGCTGGCTGTTGC RV GGCCACTGTCAATCACTCTC	U10308	(SCHÄFER-SOMI et al., 2008)
LIF	FW GACAGACTTCCCACCATTCC RV GGGATTGAGGACCTTCTGGT	AF479881	(SCHÄFER-SOMI et al., 2008)
PR	FW CAGGTGTACCAGCCGTACCT RV ATTTCGAAAACCTGGCAATG	AF177470	(SCHÄFER-SOMI et al., 2008)

RPL27: ribosomal protein L27, GAPDH: glyceraldehyde-3phosphate-dehydrogenase, COX1: cyclooxygenase 1, IL6: interleukin 6, IL8: interleukin 8, LIF: leukemia inhibitory factor, PR: progesterone receptor

Results

All uterine samples were macroscopically normal. None of the samples showed lesions or luminal content compatible with pyometra. Dioestrus was confirmed on the basis of progesterone concentration. The mean serum progesterone concentration was 29.06±4.67 nmol/L. Serum progesterone concentrations were higher in bitches with a normal endometrium than in bitches with endometritis (42.29±10.81 vs. 22.26±3.81 nmol/L; P<0.0001). Only 33.3% of the bitches had a normal endometrium (n=15); the remaining 66.7% of the bitches had endometritis (n=30). A normal endometrium was characterized by fewer than 3 neutrophils, or the absence of inflammatory cells in the endometrium per 40X field. Endometritis was characterized by infiltration of inflammatory cells, hyperemia, vascular congestion or stromal edema, and interstitial fibrosis in the endometrium (Fig. 1).

COX1 expression was 3.1-fold greater (P=0.0002) in endometritis than in a normal endometrium. Also, IL6 and IL8 gene transcription was 2.5- and 2.1-fold greater (P=0.002 and P=0.003, respectively) in the endometrial tissue of bitches with endometritis compared to those with a normal endometrium. In contrast, LIF and PR were less expressed in endometritis compared to a normal endometrium (2.8 and 2.4-fold, P=0.006 and P<0.001; respectively; Fig. 2-4).

Furthermore, we observed a positive and significant correlation of PR with COX1 (0.9, P=0.03) and LIF (1.0, P=0.0001) in bitches with a normal endometrium.

In bitches with endometritis, we observed a positive and significant correlation between IL8 with IL6 (0.519, P=0.02). We also observed a positive and significant correlation between LIF with IL8 (0.584, P=0.005).

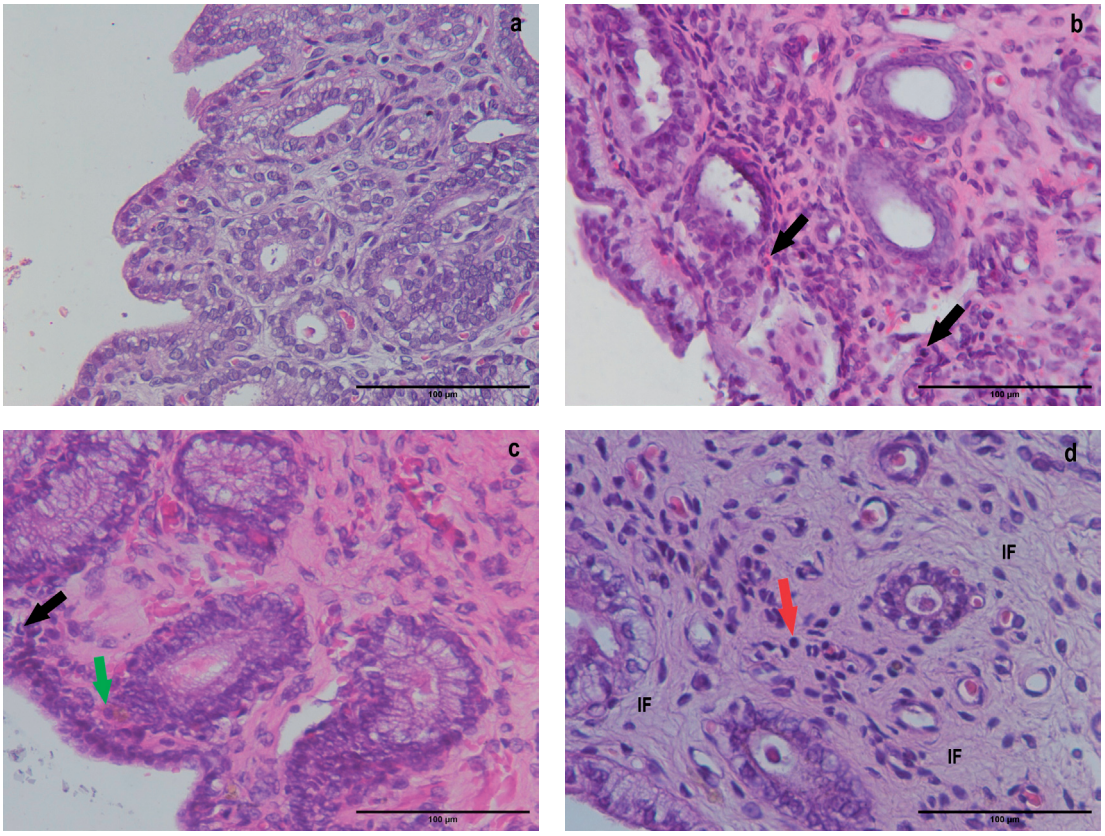

Fig. 1. Photomicrograph of endometrial tissue in a biopsy from a clinically healthy bitch with a normal endometrium (NE [33.3%]; a) and endometritis (E [66.7%]; b, c, d)
Black arrows indicate polymorphonuclear neutrophilic cells, green arrows indicate macrophages, red arrows indicate lymphocytes. IF: interstitial fibrosis (X400, H&E)

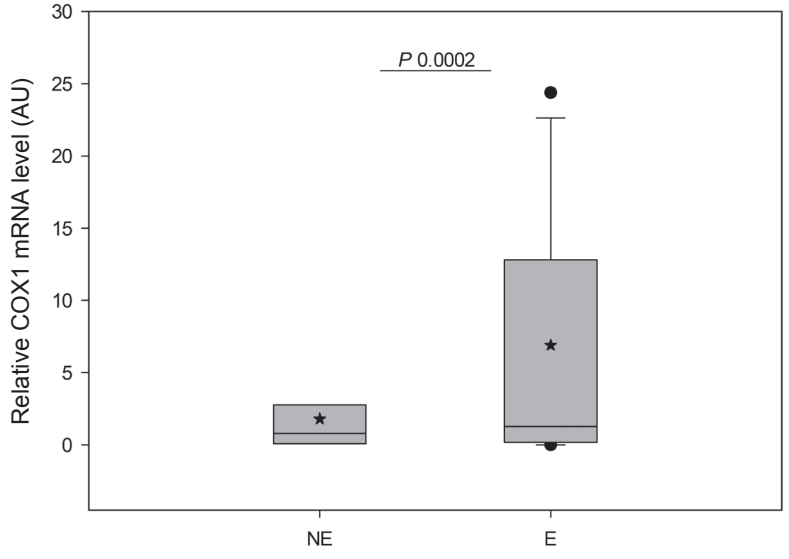

Fig. 2. Relative mRNA expression in arbitrary units (AU) of COX1 in a normal endometrium (NE) and endometritis (E)

Significant differences (P) between groups are indicated as a horizontal line. Median values are shown as stars and outliers as circles

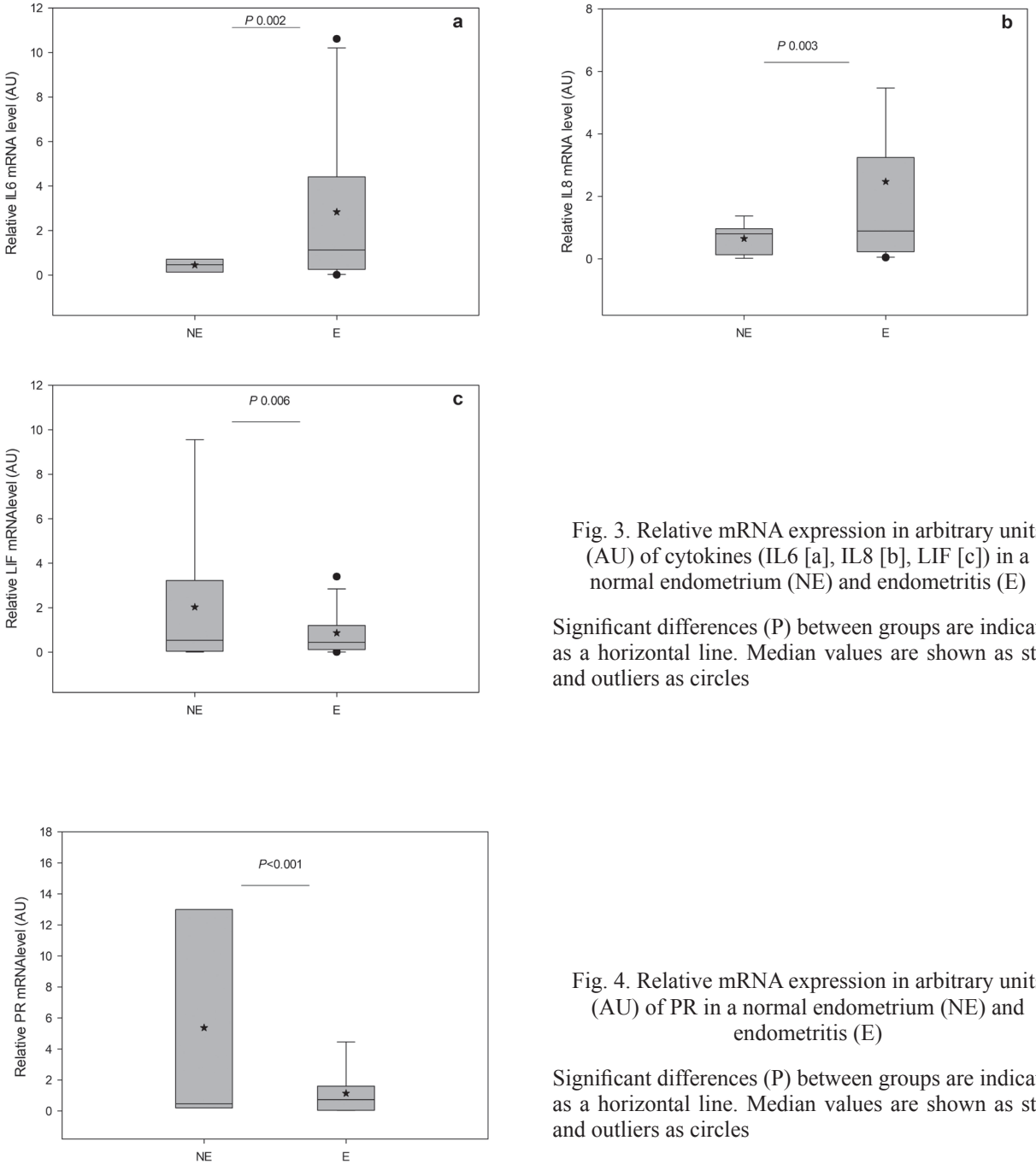

Fig. 3. Relative mRNA expression in arbitrary units (AU) of cytokines (IL6 [a], IL8 [b], LIF [c]) in a normal endometrium (NE) and endometritis (E)

Significant differences (P) between groups are indicated as a horizontal line. Median values are shown as stars and outliers as circles

Fig. 4. Relative mRNA expression in arbitrary units (AU) of PR in a normal endometrium (NE) and endometritis (E)

Significant differences (P) between groups are indicated as a horizontal line. Median values are shown as stars and outliers as circles

In ROC analysis, IL6 (AUC, 0.67) mRNA proved most favorable for suspecting endometritis among the 5 potential biomolecular markers. The AUC values of the 5 candidate transcripts ranged from 0.50 to 0.67 (Table 2).

Table 2. Area under the curve (AUC) values from Receiver operating characteristics (ROC) analysis to test the potential biomolecular markers' predictive power for endometritis vs. normal endometria

Gene	AUC	95% CI
COX1	0.63	0.40-0.85
IL6	0.67	0.48-0.86
IL8	0.60	0.40-0.81
LIF	0.50	0.28-0.73
PR	0.58	0.19-0.96

Discussion

In agreement with our hypothesis, there was an evident change in the mRNA expression pattern of COX1, cytokines, and PR in endometritis tissue compared to a normal endometrium. We showed that COX1, IL6, and IL8 mRNA expression in bitches with endometritis was higher, and LIF and PR mRNA were lower than in a normal endometrium. We also showed that IL6 (AUC 0.67) was the biomolecular marker most favorable for suspecting endometritis.

Several researchers have also identified a high prevalence of endometritis in dogs. CHRISTENSEN et al. (2012) found 50% endometritis, 35% cystic endometrial hyperplasia, and 30% fibrosis in bitches without clinical signs consistent with any underlying disease (CHRISTENSEN et al., 2012). In our work, 20% of the uterine biopsies showed endometritis without cystic endometrial hyperplasia and pyometra. FONTAINE et al. (2009) and GIFFORD et al. (2014) found a high prevalence of endometritis without the presence of cystic endometrial hyperplasia in sexually intact and infertile bitches (38% and 42.6% prevalence, respectively). In a previous study, our group identified a high percentage of bitches with endometritis (54%; PRADERIO et al., 2019). These findings agree with the results of the current study since we found a 66.7% prevalence of endometritis in clinically healthy bitches. However, it is essential to consider that we do not know if the bitches included in this study were fertile or infertile. Although several studies investigate the prevalence of endometritis without cystic endometrial hyperplasia in clinically healthy bitches, none have studied the expression of inflammatory genes by qPCR.

In our work, we observed that the COX1 gene transcriptions were increased in endometritis compared to a normal endometrium. Similarly, VOORWALD et al. (2015) detected COX1 overexpression in bitches with open pyometra. However, SILVA et al. (2009; 2010) did not find significant differences in COX1 gene transcription between pyometra and normal uteri. GABLER et al. (2009) suggested that a dysregulated cytokine and/or prostaglandin profile in the uterine tissue could be induced both by subclinical endometritis and clinical endometritis. In agreement with previous reports in cows, our findings in bitches suggest the same dysregulation in bitches with endometritis without clinical signs and without the presence of cystic endometrial hyperplasia.

In our study, IL6 and IL8 gene transcriptions were increased in endometritis compared to a normal endometrium. Similar results were obtained by other authors who reported that the expression of IL6 and IL8 was higher in the uterus of bitches with pyometra than healthy bitches (VOORWALD et al., 2015; BUKOWSKA et al., 2014; SINGH et al., 2018). KARLSSON et al. (2015) found that the level of IL8 was significantly higher in bitches with pyometra compared to that of healthy dogs. GHASEMI et al. (2012) also reported considerably increased IL8 expression in postpartum cows with subclinical endometritis compared to those without inflammation.

The inflammatory reaction in the endometrium in both diseases could support the similarity between our findings and the data reported relating to pyometra. In the same way, there were similarities between our results and data reported on subclinical endometritis in cows.

SCHÄFER-SOMI et al. (2009) observed that LIF expression was increased from pre-implantation to the placentation stage in dogs. Strikingly, in our work, we found the gene transcription of LIF significantly decreased in bitches with endometritis compared to those with normal endometria. This fact suggests endometritis could impact implantation and result in infertility.

PRADERIO et al. (2019), found serum progesterone concentrations to be lower in bitches with endometritis than those with normal endometria. We obtained similar results in the current work. DERUSSI et al. (2012) reported that the oviduct's PR expression did not differ between pregnant and non-pregnant bitches. On the other hand, they also reported that mRNA PR expression in uterine tissue during the early stages of pregnancy and during the luteal phase was higher than at any other time (DERUSSI et al., 2012). In agreement with PRADERIO et al. (2019), we found that the expression of PR was significantly decreased in bitches with endometritis compared with those with a normal endometrium. This finding supports the hypothesis that progesterone levels in endometritis could play a role in infertility pathophysiology. VERMEIRSCH et al. (2000) reported a different PR staining score in the uterus according to the estrous cycle stage, and between early and late metestrus. In our study, samples were taken throughout the diestrus without considering the stage. DE BOSSCHERE et al. (2002) reported an increase in PR-score immunolocalization in bitches with cystic endometrial hyperplasia-mucometra endometritis-pyometra compared with normal uterine levels. However, these differences were not statistically significant, while bitches with cystic endometrial hyperplasia-mucometra or endometritis-pyometra, treated with exogenous progesterone, presented reduced PR-scores in uterine cells, compared with non-treated cystic endometrial hyperplasia-mucometra or endometritis-pyometra groups (DE BOSSCHERE et al., 2002). This could be due to progesterone's well-known action on pyometra physiopathology (DE BOSSCHERE et al., 2002; PRAPAIWAN et al., 2017). In this way, a different PR expression and immunolocalization could be expected in other uterine pathologies. Whereas previous studies used immunohistochemistry and qPCR to identify and differentiate molecular biomarkers to study endometritis in bitches, only qPCR was used in our study. This may be a limitation of our study because no comparisons between qPCR and immunohistochemistry results can be made, which would be interesting for further work.

Our work found a significant and positive correlation between PR with LIF in a normal endometrium. However, we did not find this correlation in bitches with endometritis. Several studies have confirmed that progesterone and LIF are essential to pregnancy in bitches (SCHÄFER-SOMI et al., 2009; FELDMAN and NELSON, 2000). Therefore, this dysregulated gene expression in the uterine tissue could affect the uterine environment for the development of pregnancy.

Knowledge about the mechanisms regulating the uterine microenvironment and the factors that prevent implantation is still limited in relation to bitches. Knowing the modifications in expression of PG synthesis enzymes and cytokines allows us to understand the etiopathogenesis of some diestrual uterine disorders in bitches, such as endometritis without cystic endometrial hyperplasia. Also, the lower expression of PR and LIF in bitches with endometritis but without cystic endometrial hyperplasia compared to those with a normal endometrium could explain infertility or subfertility in bitches with endometritis, as the source of progesterone and LIF are essential to the establishment and maintenance of pregnancy in a bitch.

Previous investigations of endometritis in dairy cows have also used the cytobrush to obtain endometrial epithelial cells in order to study biomolecular markers (GABLER et al., 2009; WAGENER et al., 2017). Consequently, using these previous studies as a model, we chose to obtain RNA from cells recovered by the cytobrush technique to analyze the endometrial layer, and not include deeper layers of the uterine tissue. Endometrial samples taken by biopsy or cytobrush could be obtained from bitches with a presumptive diagnosis of endometritis. However, these procedures involve a surgical procedure. Lavage through transcervical catheterization by vaginal endoscopy could also be a useful and non-invasive method to obtain endometrial cells in order to obtain RNA and perform RT-qPCR (FONTAINE et al., 2009; SERRANO et al., 2006). These techniques could be used in the future to diagnose clinically healthy bitches with endometritis without performing surgery. However, future studies are

needed to elucidate this point and to know if the cytobrush, biopsy, and endometrial cells obtained by transcervical catheterization by vaginal endoscopy produce the same results.

In conclusion, this study's results suggest that the alterations in the pattern of expression of some genes could be related to uterine disease, and could be valuable tools to use as diagnostic methods. Furthermore, these biomolecular markers (COX1, IL6, IL8, LIF, and PR) could help diagnose endometritis in clinically healthy bitches using endometrial cells obtained by lavage through transcervical catheterization by vaginal endoscopy. More research is needed to understand the role of inflammatory gene transcription in reducing fertility in bitches with different endometritis types without cystic endometrial hyperplasia.

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SAŽETAK

Cilj je istraživanja bio procijeniti transkripciju gena ciklooksigenaze 1 (COX1), citokine (interleukin 6 [IL6], interleukin 8 [IL8] i faktor inhibicije leukemije [LIF]) i receptor progesterona (PR) u klinički zdravih kuja s urednim endometrijem i u onih s endometritisom bez cistične endometrijske hiperplazije. U istraživanju je sudjelovalo ukupno 48 kuja mješanki u diestrusu. Biopsijom maternice prikupljeni su uzorci za dijagnostiku (zdrav endometrij [n=15] i endometrij s endometritisom [n=30]). Tri su uzorka isključena iz istraživanja (dvije kuje s cističnom endometrijskom hiperplazijom i jedna s atrofijom). Uzorci su prikupljeni citočetkicama kako bi se qPCR-om kvantificirala mRNA. Podaci su analizirani pomoću generaliziranog mješovitog modela. Ekspresija mRNA COX1, IL6 i IL8 u kuja s endometritisom bila je znakovito veća nego u onih sa zdravim endometrijem (za COX1 P=0,0002, za IL6 P=0,002, a za IL8 P=0,003). Za razliku od toga, ekspresija mRNA LIF-a i PR-a u kuja s endometritisom bila je znakovito manja nego u kuja sa zdravim endometrijem (za LIF P=0,006, a za PR P=0,001). IL6 (AUC 0,67) pokazao se najboljim biomolekularnim markerom za predviđanje endometritisa u kuja. Zapažena je očita promjena u ekspresiji mRNA COX1, citokina i PR-a u klinički zdravih kuja s urednim endometrijem i endometritisom bez cistične endometrijske hiperplazije. Rezultati istraživanja upućuju na to da bi biomolekularni markeri kao što su COX1, IL6, IL8, LIF i PR mogli pomoći u dijagnostici endometritisa u kuja.

Ključne riječi: kuja; endometritis; ciklooksigenaza 1; citokini; receptor progesterona