

Prostaglandin synthesis enzymes' gene transcription in bitches with endometritis

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Contents

Endometritis is a major cause of infertility in many domestic species. However, until now the pathogenesis of the endometritis in the bitch is unclear. The aim of this study was to evaluate the gene transcription pattern of prostaglandin (PG) synthesis enzymes (cyclooxygenase [COX2], PTGES-1 and PGFS) in the endometrium of bitches with or without endometritis. Thirty mixed breed bitches in dioestrus, aged between 1 and 5 years, and weighing between 10 and 30 kg were used. After ovariohysterectomy (OVX), uterine biopsy samples were collected from the middle part of both horns. Then, endometrial epithelium was collected using the cytobrush method and mRNA analysis was performed by real-time RT-PCR. Data were analysed with Kruskal–Wallis ANOVA using the SAS[®] software. Uterine condition was identified by endometrial biopsies (normal endometria [*n* = 11; NE], acute endometritis [*n* = 10; AE] and chronic endometritis [*n* = 9; CE]). The COX2, PTGES-1 and PGFS/AKR1C3 mRNA expression in bitches with and without endometritis was similar. Except for PGFS/AKR1C3, gene transcription of COX2 and PTGES-1 was significantly increased in AE compared with CE. In addition, COX2 gene transcription was significantly increased in AE compared with NE. In contrast, no differences were found for COX2, PTGES-1 and PGFS/AKR1C3 mRNA expression in the samples of NE compared with CE.

1 | INTRODUCTION

Canine breeding had a remarkable growth in the last decade. In this way, breeders and veterinarians have increased the interest to improve the reproductive efficiency of stud animals. Mistimed breeding is considered by most authors as the major cause of conception failure in the bitch (Johnston, Root Kustritz, & Olson, 2000; Zoldag, Kecskemethy, & Nagy, 1993). However, when proper time of ovulation is identified, the bitch has no anatomic abnormalities and the genital tract is clinically healthy, the cause of infertility may be difficult to identify (Johnston et al., 2000).

In the last few years, studies have shown that endometrial lesions in bitches affect uterine physiology (Mir et al., 2013; Schlafer, 2012). Endometrial alterations might induce implantation failure or defective embryonic or foetal development in bitch (Freshman, 1991). Moreover, uterine lesions such as endometritis, endometriosis, uterine fibrosis, atrophy or hypoplasia and degeneration of the endometrial

glands are known to play an important role in fertility in other species like women, cows and mares (LeBlanc & Causey, 2009; Schlafer, 2007; de Ziegler, Borghese, & Chapron, 2010).

Endometritis is a major cause of infertility in many domestic species. However, until now the pathogenesis of the endometritis in the bitch is unclear (Mir et al., 2013). The female with endometritis may have normal oestrous cycles, ovulation, fertilized eggs and early embryonic development, but fail in the maintenance of pregnancy due to an abnormal uterine environment that prevents embryonic implantation (Feldman & Nelson, 2000).

Endometrial biopsy is a valuable tool for identifying endometritis. Lesions within the endometrial epithelium and stroma are classified histologically as inflammatory (acute or chronic). Acute endometritis is characterized with mainly neutrophils and chronic endometritis is characterized with mainly lymphocytes. In chronic reactions, lymphocytes are often accompanied by scattered eosinophils (Fontaine et al., 2009; LeBlanc & Causey, 2009). Several cytokines and prostaglandins (PG) play

an important role in preparing the endometrium for implantation and mediating proinflammatory events (Gabler et al., 2009; Kauma, 2000). On the other hand, Silva, Leita, Ferreira-Dias, Lopes da Costa, and Mateus (2009), Silva et al. (2010) observed that pyometra-associated *E. coli* endotoxin release stimulates the upregulation of COX2, PGFS and mPGES-1 gene transcription in the endometrium (Silva et al., 2009, 2010). In addition, endometrial PGE₂ and PGF_{2 α} contents were significantly higher in pyometra than in normal dioestrus endometrial samples, which may further regulate the local inflammatory response (Silva et al., 2009, 2010). To our knowledge, there are not reports about the expression of PG synthesis enzymes in bitches with endometritis.

The aim of this study was to evaluate the gene transcription pattern of PG synthesis enzymes (cyclooxygenase [COX2], PGES-1 and PGFS/AKR1C3) in the endometrium of bitches with or without endometritis.

2 | MATERIALS AND METHODS

Thirty mixed breed bitches in dioestrus, mean age 2.1 ± 0.26 years (1–5 years) and weighing between 10 and 30 kg were used. OVX was performed between fifteen and thirty days after the end of the oestrus. Bitches were included in a programme for breeding control at a Municipal Pet Public shelter in the city of La Plata. The bitches were undergoing a thorough clinical and reproductive examination. Dioestrus was determined based on the history provided by the owner and confirmed in each bitch based on vaginal cytology and serum progesterone (P₄) concentration. Stage of the oestrous cycle was determined according to the percentage and type of cells present (Feldman & Nelson, 2000). P₄ was measured serum by chemiluminescence immunoassay (Elecys[®], Progesterone II; Roche, Mannheim, Germany). The intra-assay CVs for high-pool (4.87 ng/ml) and low-pool (0.3 ng/ml) P₄ were 4.5% and 2%, respectively. Samples of vaginal cytology and serum P₄ concentration were taken after clinical and reproductive examination, and before OVX.

The experiment was carried out in accordance with international recommendations specified in the guidelines for the care and use of laboratory animals and with the recommendations of the National Academy Science concerning the use of dogs as laboratory animals (National Research Council, 2002), and the approval of the IACUC of FCV UNLP (40-4-14 B). For OVX anaesthesia, bitches were pre-medicated with acepromazine (0.1 mg/kg sc, no more than 1 mg; Acedan, Laboratorio Holliday-Scott, Argentina) and tramadol (1 mg/kg im; Algen 20 Laboratorio Richmond, Argentina), anesthetized with propofol (4 mg/kg; Propovet, Laboratorio Richmond, Argentina) and maintained with isoflurane (Isoflurano USP, Laboratorio Baxter, Argentina, Slatter, 2003). After OVX, uterine biopsy samples were collected from the middle part of both horns, fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections were then cut at 2–4 μ m, deparaffinized and stained with haematoxylin and eosin, and were examined with light microscope at 10 \times and 40 \times magnifications (Olympus, Tokyo, Japan; Schlafer & Miller, 2007). All biopsies were

assessed by two investigators blinded to the bitches' identification and categorized based on a score ad hoc using previous reports (Galabova, Egerbacher, Aurich, Leitner, & Walter, 2003; Schlafer, 2012). Normal endometrium was defined by the absence of inflammatory cells or the presence of at least three neutrophils in the superficial endometrial epithelium per 40 \times field (Galabova et al., 2003). Acute endometritis was defined as the presence of at least four neutrophils in the superficial endometrial epithelium per 40 \times field and the presence of hyperaemia, vascular congestion or stromal oedema (Schlafer, 2012). Chronic endometritis was defined as the presence of lymphocytes, plasma cells and macrophage in the superficial endometrial epithelium and interstitial fibrosis (Schlafer, 2012). For the investigation of the mRNA expression in the endometrium of healthy bitches or bitches with endometritis, endometrial epithelium samples were collected ex vivo using a cytobrush technique (Gabler et al., 2009). Cells were collected by rotating the cytobrush (Medibrush[®] Plus, Medical Engineering Corporation, Argentina) in a clockwise direction while in contact with the uterine wall. Two samples were used to isolate RNA. Therefore, the cytobrushes were placed into two separate reaction tubes and stored at -80°C . Endometrial cells remaining in the cytobrush were resuspended in saline solution and centrifuged at 3000 g for 10 min (Serrano, Romero, Gómez, & Bravo, 2006). The supernatant was then discarded and the cell pellet resuspended and homogenized in 500 μ l of TRIzol[®] Reagent (Invitrogen, Carlsbad, California, United States). The extraction of RNA was performed according to the manufacturer's instructions of TRIzol[®] (Serrano et al., 2006; Yue et al., 2008). Concentration and purity of RNA were determined spectrophotometrically at 260 and 280 nm, and RNA quality was assessed by visualization of 28S and 18 rRNA bands after electrophoresis through a 1.5% gel agarose with ethidium bromide staining. Complementary DNA (cDNA) synthesis was obtained by reverse transcription of 500 ng of total RNA primed with 1 μ l of oligo (dT)15 primer (500 ng/ μ l) (Invitrogen[®]) and 1 μ l of random hexamers (500 ng/ μ l) (Invitrogen[®]). This mixture was heated at 70 $^{\circ}\text{C}$ for 5 min and cooled on ice for RNA denaturation. Subsequently, 1 μ l of dNTPs (10 mM), 4 μ l 5 \times transcriptase reaction buffer (Invitrogen[®]), 1 μ l RNasin (40 U/ μ l) (Invitrogen[®]) and 1 μ l of M-MLV reverse transcriptase enzyme (200 U/ μ l) (Invitrogen[®]) were added. The reactions were carried out for 1 hr at 37 $^{\circ}\text{C}$, 15 min at 42 $^{\circ}\text{C}$ and for a further 5 min at 94 $^{\circ}\text{C}$. cDNA samples were stored at -20°C until real-time PCR amplification (Silva et al., 2010).

Quantitative analysis of the mRNA expression of the selected factors in endometrial epithelial cells was performed using real-time PCR. The primer pairs as indicated in Table 1 were used to amplify specific canine transcripts. All primers were synthesized by Invitrogen[®] (Life Technologies, USA). Ribosomal protein L27 gene was chosen as the housekeeping gene (Silva et al., 2010). Real-time PCRs were performed using HOT FIREPol[®] EvaGreen[®] qPCR Mix Plus (Solis Biodyne, Estonia), following the protocol proposed by the manufacturer. Each reaction requires: 4 μ l of mix, specific primers, cDNA and DNase/RNase-free water to a final volume of 20 μ l. The reactions were conducted in the MiniOpticon Real-Time PCR detection system (Bio-Rad). Cycling parameters were as follows: 10 min of pre-incubation at 95 $^{\circ}\text{C}$, followed by 42 cycles of 15 s at 95 $^{\circ}\text{C}$, 20 s at

TABLE 1 Primer sequences for mRNA of target genes

Target gene	Sequence (5'-3')	GenBank accession number	References
RPL27	FW ACAATCACCTCATGCCACACA RV CTTGACCTTGGCCTCTCGTC	NM_001003102	Silva et al. (2010)
COX2	FW GTATGAGCACAGGATTTGACCCAGTA RV AATTCGGTGTGAGCAGTTTT	NM_001003354	Silva et al. (2010)
PTGES-1	FW CAGAGCCCACCCGGAATGA RV GGAAGAAGACGAGGAAGTGCAT	NM_001122854	Silva et al. (2010)
PGFS/AKR1C3	FW GGCCAAGAGCTTCAACGAGA RV AGGCTGCTCAGAGTCTCCATG	NM_001012344	Silva et al. (2010)

60°C and 20 s at 72°C. Melting curves were acquired to ensure that a single product was amplified in the reaction. After analysing the melting curves, the PCR products were run through a 2.5% agarose gel to confirm expected product size. The data of relative mRNA quantification were analysed with CFX MANAGER Software (Bio-Rad). Relative changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

2.1 | Statistical analysis

Data were analysed with Kruskal–Wallis ANOVA using the SAS® software (SAS, 2003). Serum concentrations of P₄ were analysed by PROC MIXED from SAS® 9.1 (SAS, 2003).

3 | RESULTS

Uterine condition was identified by endometrial biopsies (normal [$n = 11$; NE], acute endometritis [$n = 10$; AE] and chronic endometritis [$n = 9$; CE]). Mean serum P₄ concentrations in bitches with and without endometritis were similar (12.35 ± 3.25 vs. 11.02 ± 2.51 , respectively; $p > .75$). Mean age in bitches with and without endometritis was similar (2.18 ± 0.45 vs. 2.05 ± 0.34 years, respectively; $p > .82$). The COX2, PTGES-1 and PGFS/AKR1C3 mRNA expression in bitches with and without endometritis was similar ($p > .21$, $p > .40$ and $p > .88$, respectively). Except for PGFS/AKR1C3, gene transcription of COX2 and PTGES-1 was significantly increased in AE compared with CE ($p > .35$, $p < .02$ and $p < .10$, respectively). The COX2 mRNA expression was significantly increased in AE compared with NE ($p < .02$; Figures 1 and 2). However, no differences were found for PGFS/AKR1C3 and PTGES-1 mRNA expression in AE compared with NE ($p > .56$, $p < .20$; respectively). In addition, no differences were found for COX2, PTGES-1 and PGFS/AKR1C3 mRNA expression in the samples of NE compared with CE ($p > .87$, $p > .90$, $p > .74$; respectively).

4 | DISCUSSION

Our results show a high per cent of endometritis (63.3%). These findings agree with the results obtained by Gifford, Scarlett, and Schlafer

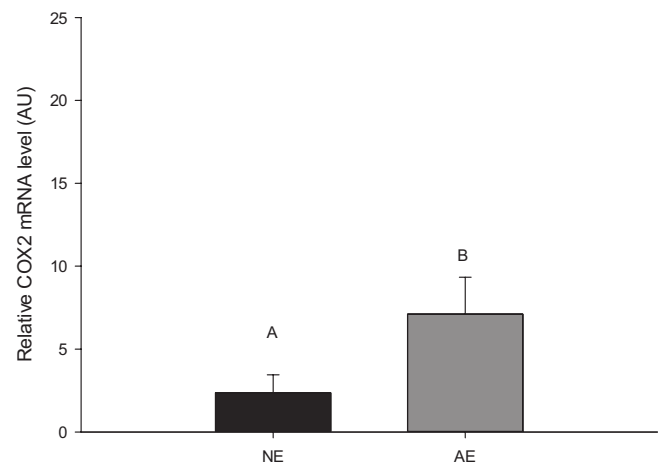


FIGURE 1 Relative mRNA level (Arbitrary Units, AU) evaluated by real-time PCR in normal endometria (NE, $n = 11$) and acute endometritis (AE, $n = 10$) of bitches for the genes COX2. Data are given as mean \pm SEM. Columns with different superscripts differ significantly at $p < .05$

(2014) who found that endometritis was the most prevalent uterine lesion (42.6%) and only found a normal endometrium in a 27.8% of samples. In agreement with previous findings, our result show that endometritis is a common disease in the bitch (Gifford et al., 2014; Mir et al., 2013).

Acute endometritis was found in higher per cent (33.3%) in our work compared with previous reports (Gifford et al., 2014; Mir et al., 2013). In our study, all samples were taken in early and middle dioestrus. The time of sampling could explain the high percentage of acute endometritis found in our study. Our results agree with the results obtained by Dow (1958) who studied cystic endometrial hyperplasia (CEH) with acute and chronic inflammation. Dow found that acute inflammation developed in dogs in the earlier luteal phase (0–40 days post-oestrus) and that more chronic inflammation developed later in the luteal period (50–90 days post-oestrus; Dow, 1958).

Our results show that COX2 and PTGES-1 gene transcriptions were significantly increased in AE compared with CE. In addition, COX2 gene transcriptions were significantly increased in AE compared with NE. These findings agree with the results obtained by Silva et al. (2009, 2010) who found COX2 and PTGES-1 gene transcriptions were

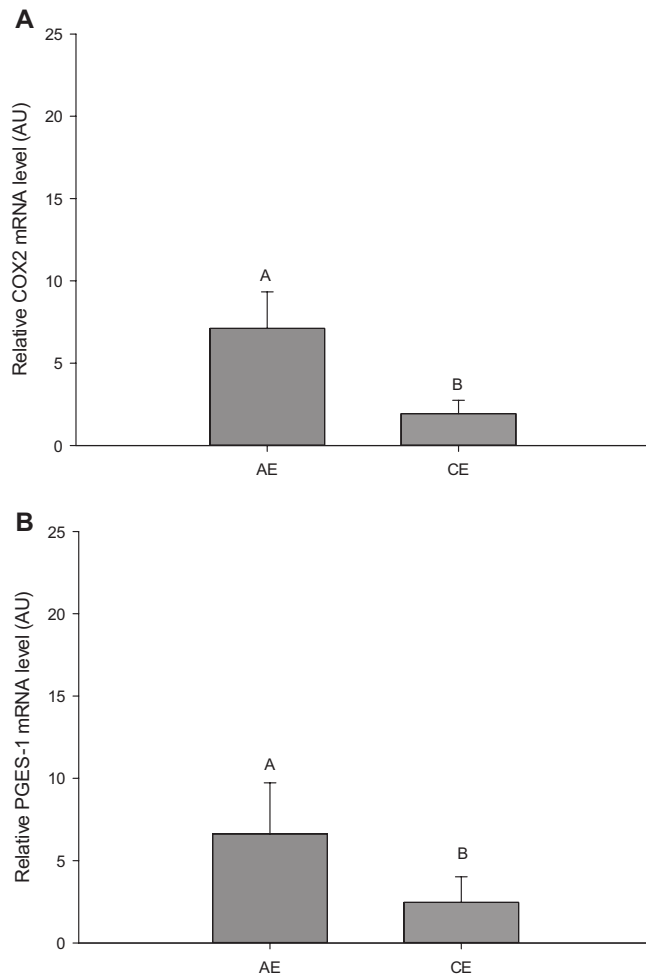


FIGURE 2 Relative mRNA level (Arbitrary Units, AU) evaluated by real-time PCR in acute endometritis (AE, $n = 10$) and chronic endometritis (CE, $n = 9$) of bitches for the genes COX2 (a) and PTGES-1 (b). Data are given as mean \pm SEM. Columns with different superscripts differ significantly

significantly increased in uteri with pyometra compared with normal uteri. These authors found that pyometra-isolated *E. coli* induces the upregulation of Toll-like receptors genes in canine dioestrus endometrium. This upregulation, which is probably the result of stimulation by LPS and lipoprotein *E. coli* constituents, leads to the endometrial upregulation of PG synthesis genes. This, in turn, results in a higher endometrial concentration of PG which may further regulate the local inflammatory response (Silva et al., 2009, 2010).

On the contrary to our results, Gabler et al. did not find differences for mPGES-1 and mPGES-2 mRNA expression in the samples of healthy cows compared with cows with subclinical or clinical endometritis. However, these authors found that cPGES, L-PGDS, IL-1-RN and IL-1 α were differently expressed in cow with subclinical/clinical endometritis compared with cows with a healthy endometrium, therefore suggesting that a dysregulated cytokine and/or prostaglandin profile in the uterus could be induced by subclinical endometritis or clinical endometritis (Gabler et al., 2009).

Silva et al. (2009, 2010) observed a higher PGF2 gene transcription in pyometra than in normal dioestrus endometria. In our study, the PGFS/AKR1C3 mRNA expression in bitches with and without endometritis was similar. However, PGFS/AKR1C3 mRNA expression was numerically higher in the endometrial epithelium of bitches with endometritis compared with healthy bitches. Future studies involving a higher number of samples included could be elucidating if PGFS/AKR1C3 mRNA expression is involved in the endometritis in bitches.

The results obtained in this study reflect the role of the PG in the generation of the inflammatory response. It is known that their biosynthesis is significantly increased in inflamed tissue and they contribute to the development of the cardinal signs of acute inflammation. PG production is generally very low in uninfamed tissues, but increases immediately in acute inflammation prior to the recruitment of leucocytes and the infiltration of immune cells. PG production depends on the activity of COXs. In addition, COX-2 is the more important source of prostanoid formation in inflammation and in proliferative diseases (Ricciotti & FitzGerald, 2011).

The real-time PCR technique is very accurate and sensitive, allows a high throughput and can be performed on very small samples; therefore, this method could be used for quantification of PG synthesis enzymes in bitches with endometritis. In conclusion, the results suggest that endometritis could induce PG synthesis enzymes profile in the uterus. This study is valuable to advance in the current knowledge on the causes of infertility in bitches and will be useful to design new courses of an effective treatment.

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CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

García Mitacek MC helped to design, conduct the experiment and process the samples, analysed data and drafted the manuscript. Stornelli MC and Praderio R helped to conduct the experiment and process the samples. De la Sota RL and Stornelli MA helped to design and conduct the experiment and critically revised the manuscript.

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