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Endometritis in the bitch: Immunohistochemical localization of cyclooxygenase 2

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Abstract

Background: In several mammals, subfertility or infertility associated with endometritis was reported. Although there have been studies about endometritis in bitches, the pathophysiological mechanisms are not completely known.

Aim: This study aimed to evaluate the immunohistochemical expression of Cyclooxygenase 2 (COX2) in clinically healthy bitches with normal uterine tissue and bitches with endometritis.

Methods: Forty-eight mixed breed bitches in diestrus were used. Uterine biopsies were collected for diagnosis [normal endometrium ($n = 15$; NE), cystic endometrial hyperplasia ($n = 1$), atrophy ($n = 2$), acute endometritis ($n = 9$; AE), subacute endometritis ($n = 7$; SE), and chronic endometritis ($n = 14$; CE)]. Immunostaining and quantification of positively stained cells was performed on full-thickness uterine biopsies. Data were analyzed by the GLIMMIX procedure of SAS.

Results: COX2 immunostaining was scattered and restricted to cells in the stroma in bitches with NE. However, in bitches with endometritis, strong staining was observed in the luminal epithelium, glandular epithelium, and stromal cells. Staining was also observed in inflammatory cells localized in the stroma as well as inside of the glands. The percentage of COX2 positive stromal cells in bitches with AE, SE, and CE was significantly higher compared with NE ($p < 0.005$). In addition, the percentage of COX2 positive stromal cells in bitches with SE, and CE was significantly lower compared with AE ($p < 0.003$).

Conclusion: COX2 could be involved in the pathophysiological mechanisms producing endometritis without the presence of cystic endometrial hyperplasia in bitches. However, further researches on this topic are required.

Keywords: Bitch, COX2, Endometritis, Immunohistochemical.

Introduction

Diseases of the canine uterus were studied by several researchers. The most prevalent uterine lesions in the bitch are endometritis, cystic endometrial hyperplasia, and cystic endometrial hyperplasia-pyometra complex (De Bosschere *et al.*, 2001; Silva *et al.*, 2010; Bukowska *et al.*, 2014; Gifford *et al.*, 2014; Hagman, 2017; Praderio *et al.*, 2019). It was reported that clinically healthy bitches (CHB) with normal vaginal cytology could suffer endometritis, showing in the histopathological study inflammation of the endometrial tissue (Fontaine *et al.*, 2009; Mir *et al.*, 2013). Some researchers think that cystic endometrial hyperplasia and endometritis occurred together, but in some cases, cystic endometrial hyperplasia occurred without endometritis (Dow, 1959; Gifford *et al.*, 2014). Moreover, bitches with cystic endometrial hyperplasia-pyometra complex show several clinical signs (Haas *et al.*, 2016). In the last years, it has been observed that several mammals with subfertility or infertility as the only clinical sign suffer endometritis. Although endometritis had been reported in CHB, the pathophysiological mechanisms

it is not completely known (Fontaine *et al.*, 2009; Mir *et al.*, 2013; Gifford *et al.*, 2014). Cyclooxygenase 2 (COX2) is a prostaglandin synthesis enzyme, and it is induced during the inflammation. Thus, the increase in prostaglandin production because the over-expression of COX2 could be impairing the implantation of fertilized oocytes because an abnormality in the endometrium (Ota *et al.*, 2001). It has been reported that COX2 expression may be abnormal in several reproductive diseases, such as pyometra and endometriosis (Ota *et al.*, 2001; Silva *et al.*, 2010). In this sense, Silva *et al.* (2010) reported that COX2 immunostaining was observed in luminal epithelium, glandular epithelium, and stromal cells in bitches with pyometra. In addition, staining was also observed in inflammatory cells localized in the stroma as well as in glandular lumen. Besides, in bitches with normal endometria COX2 immunostaining was observed in the cells of the stroma and small capillaries (Silva *et al.*, 2010). Therefore, it is probably that normal uterus and uterus with endometritis without cystic endometrial hyperplasia in CHB show different immunostaining patterns.

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The aim was to evaluate the immunohistochemical expression of COX2 in the endometrium of CHB with a normal uterus and in CHB with endometritis without the presence of cystic endometrial hyperplasia.

Materials and Methods

Forty-eight mixed breed, privately owned, intact, CHB, in diestrus, aged between 1–5 years and weighing between 10 and 30 kg were used in this study. Bitches were included in a program for breeding control at a municipal pet public shelter in the city of La Plata. Ovariohysterectomy was performed between 15 and 50 days after the end of the estrus. All bitches enrolled in the study were undergoing a thorough clinical and reproductive examination and were clinically healthy. Diestrus was determined based on the history provided by the owner and confirmed in each bitch based on ovarian structures, serum progesterone (P_4), and vaginal cytology (Feldman and Nelson, 2000). Blood samples were centrifuged and stored at -20°C until P_4 was measured by chemiluminescence immunoassay (Elecsys[®], Progesterone II; Roche, Mannheim, Germany). The intra-assay CVs for high-pool and low-pool P_4 (4.87 and 0.3 ng/ml) were 4.5% and 2%, respectively. Vaginal cytology was stained with Tinción 15[®] (Biopur, Rosario) and was examined at 10 \times and 40 \times magnification. The experiment had the approval of the IACUC of FCV UNLP (40-4-14 B). After ovariohysterectomy (OVX), uterine biopsy samples were collected from the middle part of both horns. The samples were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Then, sections were cut at 2–4 μm , deparaffinized and stained with H&E, and were observed with a light microscope at 10 \times and 40 \times magnifications (Olympus, Tokyo, Japan; Schlafer and Miller, 2007).

After biopsies were classified, samples from normal uterine tissue and uterus with endometritis without the presence of cystic endometrial hyperplasia were selected to perform the immunohistochemical study (Fig. 1).

Immunostaining and quantification of positively stained cells were performed on full-thickness uterine biopsies. Sections were cut from paraffin embedded tissues

and mounted on slides. Tissues were deparaffinized, rehydrated, and washed under running tap water (5 minutes). Immunohistochemical detection of COX2 was performed by an immunoperoxidase method using COX2 polyclonal primary antibody (1:200, aa 584-598; Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions (Kowalewski *et al.*, 2006; Santelices Iglesias *et al.*, 2018). For negative control, the primary antibody was replaced with PBS. For positive control, was used the macula densa of sections of dog kidney (Fig. 2). Revealing was achieved using a chromogenic solution (3, 3'-diaminobenzidine tetrahydrochloride; Dako). For counterstaining the hematoxylin was used (Khan *et al.*, 2001; Santelices Iglesias *et al.*, 2018). One immunostained section per uterine tissue was evaluated at a 40 \times ; a minimum of three arbitrarily chosen views with approximately 100 cells was evaluated (Kowalewski *et al.*, 2006). Data were analyzed with GLIMMIX procedure of SAS 9.4 with Poisson distribution and log link functions (SAS Institute Inc., Cary, NC). Three orthogonal contrasts were designed to compare differences between means. Data are shown as least squares means \pm standard errors. Statistical significance was set at $p < 0.05$.

Ethical approval

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, and the approval of the IACUC of the Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata (40-4-14 B).

Results and Discussion

The uterine biopsies samples were classified in normal endometrium (NE; $n = 15$), acute endometritis (AE; $n = 9$), subacute endometritis (SE; $n = 7$), chronic endometritis (CE; $n = 14$; Fig. 1), cystic endometrial hyperplasia ($n = 1$), and atrophy [$n = 2$ (Table 1)]. Normal endometrium was characterized by the presence of less than three neutrophils or the absence of inflammatory cells in endometrium per 40 \times field (Galabova *et al.*, 2003).

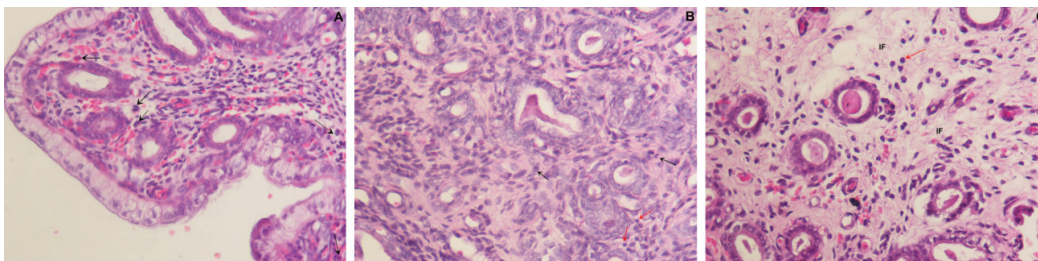


Fig. 1. Photomicrograph of endometrial tissue in a biopsy from acute endometritis (AE; A), subacute endometritis (SE; B), and chronic endometritis (CE, C). Black arrows indicate polymorphonuclear neutrophils, red arrow indicate lymphocytes. IF: interstitial fibrosis (40 \times , H&E).

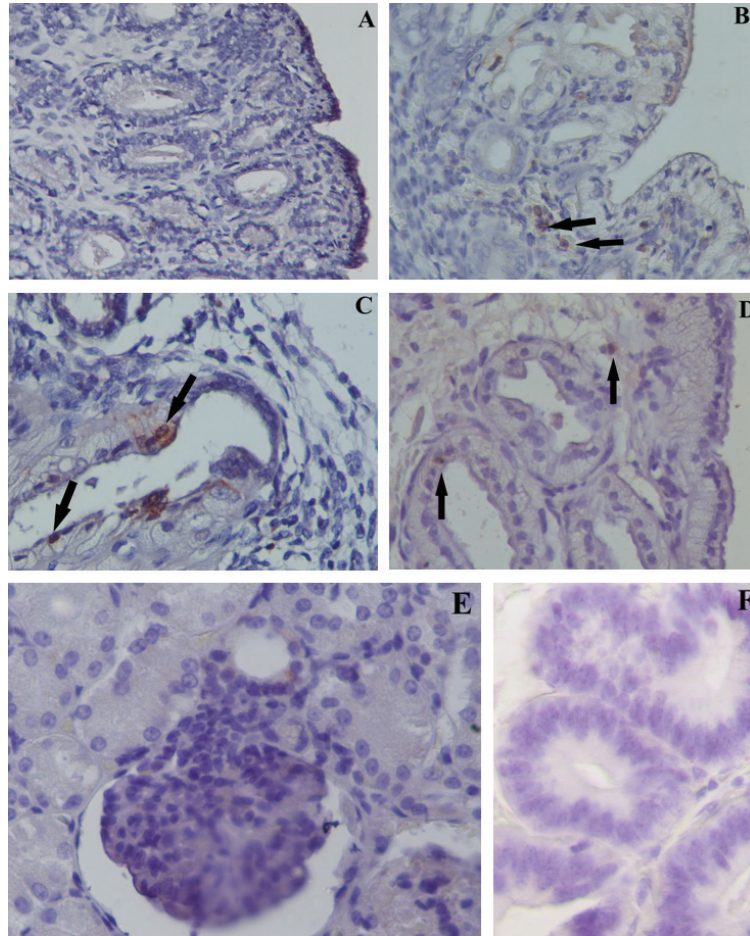


Fig. 2. Expression of COX2 by immunohistochemistry in NE (A, 40×), AE (B, 60×), SE (C, 60×), and CE (D, 60×). Black arrow indicates stained inflammatory cells. Positive control: Expression of COX2 in the macula densa of a section of canine kidney (E, 60×). Negative control: (F, 60×).

Acute endometritis was characterized by the presence of neutrophils in endometrium per 40× field and the presence of hyperemia, vascular congestion, or stromal edema. Subacute endometritis was characterized by the presence of edema, polymorphonuclear, and mononuclear cells. Chronic endometritis was characterized by the presence of lymphocytes, plasma cells, and macrophage in endometrium and interstitial fibrosis (Schlafer, 2012; Gifford *et al.*, 2014; García Mitacek *et al.*, 2017; Praderio *et al.*, 2019). Endometrial hyperplasia was defined by an increase in the size and number of glands with no change in the stroma except for edema, presence of hypertrophic and hyperplastic glandular cells with clear vacuolated cytoplasm. When the glands become cystic, and the epithelium of the glands becomes flattened, the biopsy was defined as cystic endometrial hyperplasia (Foster, 2012). Atrophy was defined by lining mucosa covering a thin layer of condensed stroma in the depths of which are the inactive glandular remnants (Schlafer and Foster, 2016; Praderio *et al.*, 2019). Mean serum P₄

concentrations in bitches with and without endometritis were similar (11.26 ± 2.43 vs. 19.83 ± 5.34 , respectively; $p > 0.09$). Vaginal cytology samples showed between 70% and 80% intermediate cells, 10%–15% parabasal cells, 5%–10% superficial cells per field and neutrophils presence at 40× magnification. COX2 immunostaining was scattered and restricted to cells in the stroma in bitches with NE. However, in bitches with endometritis, strong staining was observed in luminal epithelium, glandular epithelium, and stromal cells (Fig. 2). Staining was also observed in inflammatory cells localized in the stroma as well as inside of the glands. The percentage of COX2 positive stromal cells in bitches with AE, SE, and CE were significantly higher compared with NE ($p < 0.005$). In addition, the percentage of COX2 positive stromal cells in bitches with SE and CE was significantly lower compared with AE ($p < 0.003$). However, no differences were found in the percentage of COX2 positive stromal cells in bitches with SE compared with CE ($p > 0.33$; Fig. 3).

Table 1. Data of the age, breed, parity and days post-estrus at the time of OVX in bitches include in the study.

Bitch	Age (years)	Breed	Number of Parity	Days post-estrus at the time of ovariectomy	Uterine biopsies samples
1	2	Mixed	0	50	NE
2	2	Mixed	1	45	NE
3	2	Mixed	0	50	NE
4	1	Mixed	0	15	NE
5	1	Mixed	0	50	NE
6	4	Mixed	0	35	NE
7	2	Mixed	0	50	NE
8	1	Mixed	0	30	NE
9	4	Mixed	1	43	NE
10	5	Mixed	0	50	NE
11	1	Mixed	0	15	NE
12	2	Mixed	0	30	NE
13	1	Mixed	0	15	NE
14	2	Mixed	0	15	NE
15	2	Mixed	0	50	NE
16	3	Mixed	0	15	AE
17	5	Mixed	0	30	AE
18	5	Mixed	0	50	AE
19	3	Mixed	0	43	AE
20	1	Mixed	0	45	AE
21	1	Mixed	0	48	AE
22	1	Mixed	0	33	AE
23	5	Mixed	0	15	AE
24	1	Mixed	0	50	AE
25	1	Mixed	0	50	SE
26	1	Mixed	0	45	SE
27	3	Mixed	0	15	SE
28	1	Mixed	0	20	SE
29	1	Mixed	0	43	SE
30	3	Mixed	2	19	SE
31	1	Mixed	0	50	SE
32	2	Mixed	0	50	CE
33	4	Mixed	0	50	CE
34	1	Mixed	0	50	CE
35	1	Mixed	0	30	CE
36	2	Mixed	1	17	CE
37	2	Mixed	2	32	CE
38	1	Mixed	0	32	CE
39	2	Mixed	0	50	CE
40	2	Mixed	0	22	CE
41	2	Mixed	0	43	CE
42	3	Mixed	1	20	CE

continued

Bitch	Age (years)	Breed	Number of Parity	Days post-estrus at the time of ovariohysterectomy	Uterine biopsies samples
43	5	Mixed	0	50	CE
44	1	Mixed	0	15	CE
45	2	Mixed	0	30	CE
46	3	Mixed	0	50	Cystic endometrial hyperplasia
47	1	Mixed	0	50	Atrophy
48	1	Mixed	0	33	Atrophy

NE: normal endometrium; AE: acute endometritis; SE: subacute endometritis; CE: chronic endometritis.

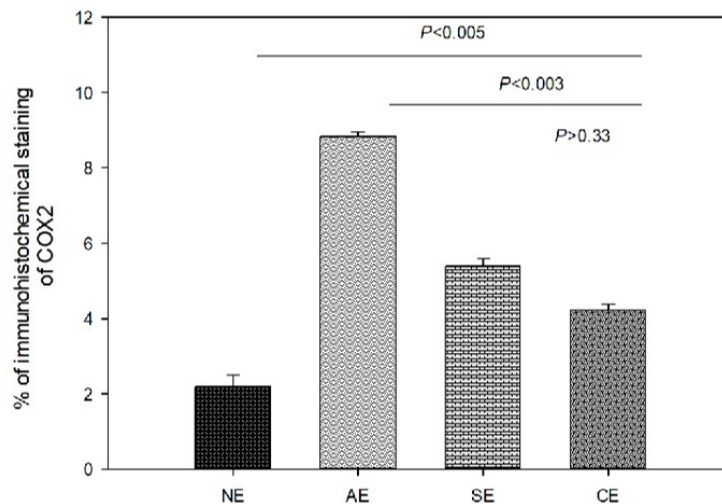


Fig. 3. Percentage of immunohistochemical staining of COX2 in stromal cells in bitches with NE, AE, SE, and CE.

Immunohistochemical expression of COX2 in the endometrial epithelium and stromal cells was also observed in cases of pyometra in the bitch. In addition, the COX2 expression was higher at the glandular and luminal epithelium as well as in the inflammatory cells in bitches with pyometra compared with control group (Silva *et al.*, 2010). Similarly, in our work, we observed the expression of COX2 in the luminal epithelium, glandular epithelium, and stromal cells in bitches with endometritis without the presence of cystic endometrial hyperplasia. In woman, Chishima *et al.* (2002) observed that COX2 staining was denser in the ectopic endometriosis implants when compared with eutopic endometrium (Chishima *et al.*, 2002). In addition, Ota *et al.* (2001) found that the expression of COX2 in the glandular epithelium was higher from endometriosis samples compared with control group. In contrast with our results, in woman no differences in the expression of COX2 in stromal cells were found in endometriosis compared with control group;

however, the expression tended to be slightly higher (Ota *et al.*, 2001).

It has been reported that COX2 in normal uterine tissue is concerned in cell proliferation and regeneration, promotion of angiogenesis, and immunity (Chishima *et al.*, 2002). On the other hand, several reproductive disorders showed a high expression of COX2. This fact has been correlated with pathological abnormalities, such as infertility (Ota *et al.*, 2001; Chishima *et al.*, 2002). Therefore, is considered that high expression of COX2 with abnormal prostaglandin generation contributes to the pathophysiology of uterine diseases (Chishima *et al.*, 2002).

In conclusion, our results showed that the percentage of COX2 positive stromal cells in bitches with endometritis was significantly higher compared with NE. This fact could be involved in the pathophysiological mechanisms in CHB showing endometritis without cystic endometrial hyperplasia. However, further research studies into the involvement of COX2 in bitches are required.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Authors contribution

García Mitacek MC helped to design, conduct the experiment, and process the samples, analyzed 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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