

Expression of prostaglandin F2 alpha receptors (PTGFR) and C-reactive protein (CRP) in the canine uterus and cervix with opened- and closed-cervix pyometra

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Abstract

Pyometra is a common reproductive disorder in female dogs. This study investigated the protein expression of prostaglandin F2 alpha receptors (PTGFR) and C-reactive protein (CRP) in responses to inflammatory events of uterine and cervical tissues in dogs. Uterus and cervix samples were collected from six dogs which had closed-cervix pyometra (n = 3) and opened- cervix pyometra (n = 3). Healthy dogs in diestrus (n = 3) and anestrus (n=3) were used as controls. Tissues were histologically prepared and stained with hematoxylin and eosin. Immunohistochemistry (IHC) and western blot analysis for the PTGFR and CRP were used to verify the protein expressions. Leukocyte infiltration was markedly presented in some layers of the uterine and cervical tissues of closed- and opened-cervix pyometra. Closed-cervix pyometra had a distinct severity of muscular layers of the uterus compared with opened-cervix pyometra. Plasma cell infiltration was significantly increased in both uterine and cervical pyometra tissues when compared with the controls ($P < 0.0001$). Western blot analysis confirmed the expression of PTGFR and CRP in normal and pyometra tissues. In the uterus and cervix, the positive CRP reactivity was weakly expressed (T score = 1.00–2.37). The expression of CRP as well as PTGFR reactivity did not significantly differ among the groups ($P > 0.05$). Inflammatory responses, pathological changes, and PTGFR expression did not correlate with CRP accumulation in uterine and cervical tissue of closed- and opened-cervix pyometra.

Keywords: Pyometra, Prostaglandin F2 alpha receptor, C-reactive protein, Immunohistochemistry

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Introduction

Pyometra is a common reproductive disorder in female dogs and is classified as an endometritis with pus accumulation in the uterus. This condition is highly regulated by endogenous and/or exogenous progesterone. The progesterone levels gradually increase and are maintained at a high concentration during diestrus, subsequently increasing endometrial proliferation and glandular activity. The high progesterone level induces an increase in luminal secretion and suppresses myometrial contractions; it also decreases local immune responses (Prapaiwan *et al.*, 2017; Kempisty *et al.*, 2013). The disease is classified based on pathological findings on the histology of the reproductive tracts as cystic hyperplasia-pyometra complex (Dow, 1959). In addition, the closed-cervix and opened-cervix pyometra can also be identified by the presence of purulent vaginal discharge (Fieni *et al.*, 2014). Uterine and cervical pathological histology demonstrated a proliferative and secretive endometrial overreaction of estrogen and progesterone, leading to hyperplasia of the endometrium (Kempisty *et al.*, 2013). These hormones also induce cervical closure (Borresen, 1975; Smith, 2006). However, there is no difference in sex steroid receptor expressions between opened- and closed- cervix pyometra (Prapaiwan *et al.*, 2017). The high progesterone levels prompted infection by pathological bacteria such as *Escherichia coli* in approximately 75-91% of pyometra cases (Kempisty *et al.*, 2013; Agostinho *et al.*, 2014). These bacteria activate the inflammatory responses upon binding to the endometrium (Sirivaidyapong and Chotimanukul, 2010; Leitner *et al.*, 2003; Bigliardi *et al.*, 2004; Ishiguro *et al.*, 2007; Agostinho *et al.*, 2014; Yoon *et al.*, 2017).

The $\text{PGF}_{2\alpha}$ produced in the epithelial cells of the endometrium, plays a pivotal role in the control of the length of diestrus in dogs by the luteolytic mechanism and regulates myometrial contraction (Wallace *et al.*, 2009; Gogny *et al.*, 2010; Hagman *et al.*, 2006). This eicosanoid binds specifically to the transmembrane G protein-coupled PTGFR receptors (FP type) and activates the myometrium contraction of the female reproductive tract (Blesson *et al.*, 2012). Several clinical studies have reported that exogenous $\text{PGF}_{2\alpha}$ administration promoted uterine contractility and the emptying of exudates in the pyometra uterine. However, this treatment was unsuccessful in the case of Dow's type IV with endometrial and myometrium atrophy (Romagnoli, 2017; Dow, 1959). For inflammation of reproductive tissue, E- and F-series prostanoids are mostly synthesized, especially $\text{PGF}_{2\alpha}$. The $\text{PGF}_{2\alpha}$ signals are upregulated via chemokine (C-X-C motif) ligand 1 (CXCL1) to induce the production of matrix metalloproteinase and angiogenic factors that, in turn, promote the remodeling of tissue and eliminate the occupying bacteria (Mallison *et al.*, 1991; Cassese *et al.*, 2003; Girolamo *et al.*, 1997; Slocombe *et al.*, 2013; Wallace *et al.*, 2009). However, it is still unclear whether the PTGFR receptors are involved in the pathogenesis and types of pyometra in dogs.

Apart from the $\text{PGF}_{2\alpha}$ cascade, inflammatory responses such as infiltrations of leukocytes in blood or

tissue could also play an essential role in the pathogenesis of pyometra. Leukocytosis is generally an inflammatory response in the case of pyometra and is commonly used for clinical diagnosis. However, a normal leukogram could also be observed in opened-cervix pyometra because inflammation was minimized, with limited pus accumulation (Mojzisova *et al.*, 2000). This shortcoming could be tested by the activation of acute-phase protein (APP). This protein is produced by the liver and functions in the response to the inflammation by inducing the release of cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor alpha from the inflammatory site (Peisajovich *et al.*, 2008; Eckersall, 2008; Osman *et al.*, 2003). There are several kinds of APP, such as CRP, fibrinogen, haptoglobin, alpha 1-acid glycoprotein, ceruloplasmin, albumin, transferrin, and serum amyloid A (Cerón *et al.*, 2005; Murata *et al.*, 2004). Serum CRP is a sensitive criterion and has become a specific marker for systemic inflammation in dogs with no effect of breed, age, sex, estrous cycle and using of NSAIDs (Kuribayashi *et al.*, 2003; Schmidt and Eckersall, 2015). Comparing CRP with other APPs, the levels of CRP rapidly increased within 1 to 2 days in the response of inflammation, and it also rapidly decreased within 1 day after treatment (Dabrowski *et al.*, 2009; Matijatko *et al.*, 2007). Therefore, the levels of CRP have been used for the diagnosis and prognosis of several inflammatory diseases (Eckersall, 2010; Cerón *et al.*, 2005; Kjølgaard-Hansen, 2004). Healthy dogs have a low serum CRP (< 10 mg/l), but the CRP levels increase up to 10-100 times within 24-48 hours of inflammation (Kuribayashi *et al.*, 2003; Yamamoto *et al.*, 1994; Schmidt and Eckersall, 2015). However, information on the inflammatory responses, especially the relationship between the presence of CRP and inflammation responses in pyometra dogs, is limited. This study examined the expressions of prostaglandin F2 alpha receptor (PTGFR) and C-reactive protein (CRP) in reproductive tissues (cervix and uterus of dogs) with clinical open- and closed-cervix pyometra.

Materials and Methods

Experimental design: 18 dogs were submitted in this study which was ranged 5-10 years old (means 6.2 years old). Reproductive organs (cervix and uterus) of dogs with opened (n = 3) and closed (n = 3) cervix pyometra were included in the study. Diestrus (n = 6) and anestrus (n = 6) dogs without any reproductive disorders were used as controls. Uterine and cervical tissues were examined for histology, leukocyte infiltrations, and protein expression (CRP and PTGFR) by immunohistochemistry (IHC) and confirmed of protein expression by western blot analysis.

Formalin-paraffin tissue block process: All tissues were collected after a routine ovariohysterectomy at the Veterinary Teaching Hospital of Prince of Songkla University, Faculty of Veterinary Science, Prince of Songkla University, Thailand. After excision, the tissues were fixed in 10% (w/v) formaldehyde for 24-48 h and then embedded in paraffin. Sections with a thickness of 4 μm were cut and prepared on a silane-

coated slide for hematoxylin-eosin staining and immunohistochemistry.

Hematoxylin and eosin staining: After processing, the tissue was deparaffinized by xylene and then rehydrated in an ethanol series (100%, 95%, 95%, 80% (v/v)). Staining was performed by hematoxylin and eosin dye. The 95% (v/v) ethanol wash was used to remove excessive eosin, and dehydration was performed by a series of concentrated ethanol and xylene. The stained tissues were mounted under a cover slip using mounting solution, pathological examination and counting of inflammatory cells were performed under a bright-field microscope (BX5, Olympus, Shinjuku, Japan). The images were recorded and visualized using the cellSens software (Olympus, Shinjuku, Japan).

Immunohistochemistry (IHC): Deparaffinization, dehydration, and rehydration were performed as mentioned above. For immunohistochemistry (IHC), the PTGFR protein was detected by the Dako REAL™ EnVision™ detection system (modified from Tiptanavattana *et al.*, 2013). The antigen retrieval technique was activated by heating the samples in 0.01 M citrate buffer pH = 6.0 (VWR BDH Prolabo, Poole, UK) using a microwave at 560 W, 15 min, for PTGFR and 800 W, 15 min, for CRP. Endogenous peroxidase activity was blocked by 3% (v/v) hydrogen peroxide (VWR BDH Prolabo, Poole, UK) for 15 min. Non-specific blocking was performed with incubation of 2% (w/v) bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature and subsequently with primary antibodies diluted in sterile PBS at 4°C overnight. Rabbit polyclonal primary antibody was used at a dilution of 1:50 for PTGFR (Abcam®, AB188993, Cambridge, UK). For CRP detection, rabbit monoclonal primary antibody against CRP at 1:50 dilution (Abcam®, AB32412, Cambridge, UK) was used. Negative controls were performed similar to CRP and PTGFR, but the primary antibody was replaced with PBS. The protein targets were detected and visualized with a Dako REAL™ EnVision™ system (DakoCytomation, Glostrup, Denmark) according to the manufacturer's recommendations and counterstained with Mayer's hematoxylin. Protein expression was examined under a bright-field microscope (BX5, Olympus). The images were recorded and visualized using the cellSens software (Olympus) and Adobe Photoshop CS6 version 13.0.1 (Adobe Systems, San Jose, CA, USA).

Counting of inflammatory and immunolocalized cells: Following H&E staining, the immune cells including lymphocytes, macrophages, plasma cells, neutrophils, and eosinophils were classified according to previous reports (Veiga *et al.*, 2017; Kunkitti *et al.*, 2011; Bosschere, 2001). Classification and determination of leukocyte infiltration were performed under a microscope with 100x magnification. In total, means of five independent areas were evaluated by blinded 3

researchers. The data were collected and calculated as percentage of leukocyte infiltration.

Protein expression of uterine and cervical compartments was determined as described elsewhere (Prapaiwan *et al.*, 2017; Kunkitti *et al.*, 2011; Srisuwatanasagul *et al.*, 2010). Briefly, three compartments of the cervix, namely the surface epithelium (SE), the subepithelial layer of the stroma (STR), and the muscular layer (MYO), were examined. The uterus was classified into four compartments: surface epithelium (SE), glandular epithelium (GE), subepithelial layer of the stroma (STR), and myometrium (MYO). Immunostaining was examined microscopically at 40x magnification for at least five areas in each compartment (Prapaiwan *et al.*, 2017; Ribeiro *et al.*, 2012; Kunkitti *et al.*, 2011; Srisuwatanasagul *et al.*, 2010; Sukjumlong *et al.*, 2003; Kaeoket *et al.*, 2001). The proportions were estimated as follows (1–4): 1 for low proportion (< 30% positive cells), 2 for moderate proportion (30–60% positive cells), 3 for high proportion (> 60–90% positive cells), and 4 for almost all cells positive (more than 90%). Intensity (marked 0–3) was defined as follows: 0 for non-detected, 1 for weak-intensity, 2 for moderate intensity, 3 for strong intensity. The total scores were calculated as the summary of intensity and proportional scores of each compartment of the tissues for PTGFR and CRP (Srisuwatanasagul *et al.*, 2010; Srisuwatanasagul *et al.*, 2006; Sukjumlong *et al.*, 2005; Vermisch, 2000).

Western blot analysis: The uterus and cervix tissues were harvested as previously described, chopped using a surgical blade, and soaked in 500 µl of lysis buffer (containing 1 mM EDTA, 0.5% (v/v) Igepal (Nonidet P-40 detergent), 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and protease inhibitors). The soaked tissues were homogenized using a homogenizer and incubated at 4°C for 30 min. After centrifugation at 3,000 rpm for 5 min, 50 µl of the protein in the supernatant was incubated with 2x SDS-PAGE sample loading buffer containing 2% (v/v) β mercaptoethanol and heated at 95°C for 10 min. The protein samples were separated by 12.5% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in 5% skim milk in TBST (20 mM Tris, 137 mM NaCl and 0.1% Tween-20) and subsequently separated and incubated with rabbit anti-CRP and rabbit anti-PTGFR (Abcam) at 4°C overnight. After washing three times for 5 min each in TBST, the membrane was incubated by donkey anti-rabbit antibody conjugated-horseradish peroxidase (HRP) (DakoCytomation) for 1 h. After washing three times in TBST, the chemiluminescent bands on the membrane were revealed by Enhanced Chemiluminescence reagent (ECL; Perkin Elmer) and imaged using the ImageQuant LAS 500 (GE Healthcare Bio-Science AB, Sweden).

Statistical analysis: The pathological findings were described and compared among the four groups. The percentages of lymphocytes, macrophages, plasma cells, neutrophils, and eosinophils were presented as mean ± standard deviation (SD).

Immunohistochemical staining data were presented as total score (T). The T score was formulated by the proportion (P) plus the intensity (I) score. Two-way ANOVA was used to compare scores among different groups and for comparison of the means. The data were analyzed with GraphPad Prism (King's College London, UK). A P value < 0.05 was considered statistically significant.

Results

Histopathology of uterus and cervix: Microscopic findings of uterine and cervical tissues with pyometra were differentially presented from normal control dogs

(Fig. 1). Uterine tissues of the two types of pyometra were pathogenic, with different severities of the cystic endometrial hyperplasia and diffused suppurative hemorrhagic endometritis (Figs. 1 and 2). Fibrinopurulent hemorrhagic exudate was increasingly present on the surface epithelium of closed cervix pyometra compared with opened cervix pyometra. Leukocyte infiltration was also increased at the submucosa layer. Moreover, significant findings, presented in closed-cervix pyometra, were severe stromal hemorrhage and myometrium atrophy (Fig. 2). Leukocyte infiltration in the cervix was located at the stroma, germinal epithelium, and tunica muscularis in both closed- and opened-cervix pyometra (Fig. 3).

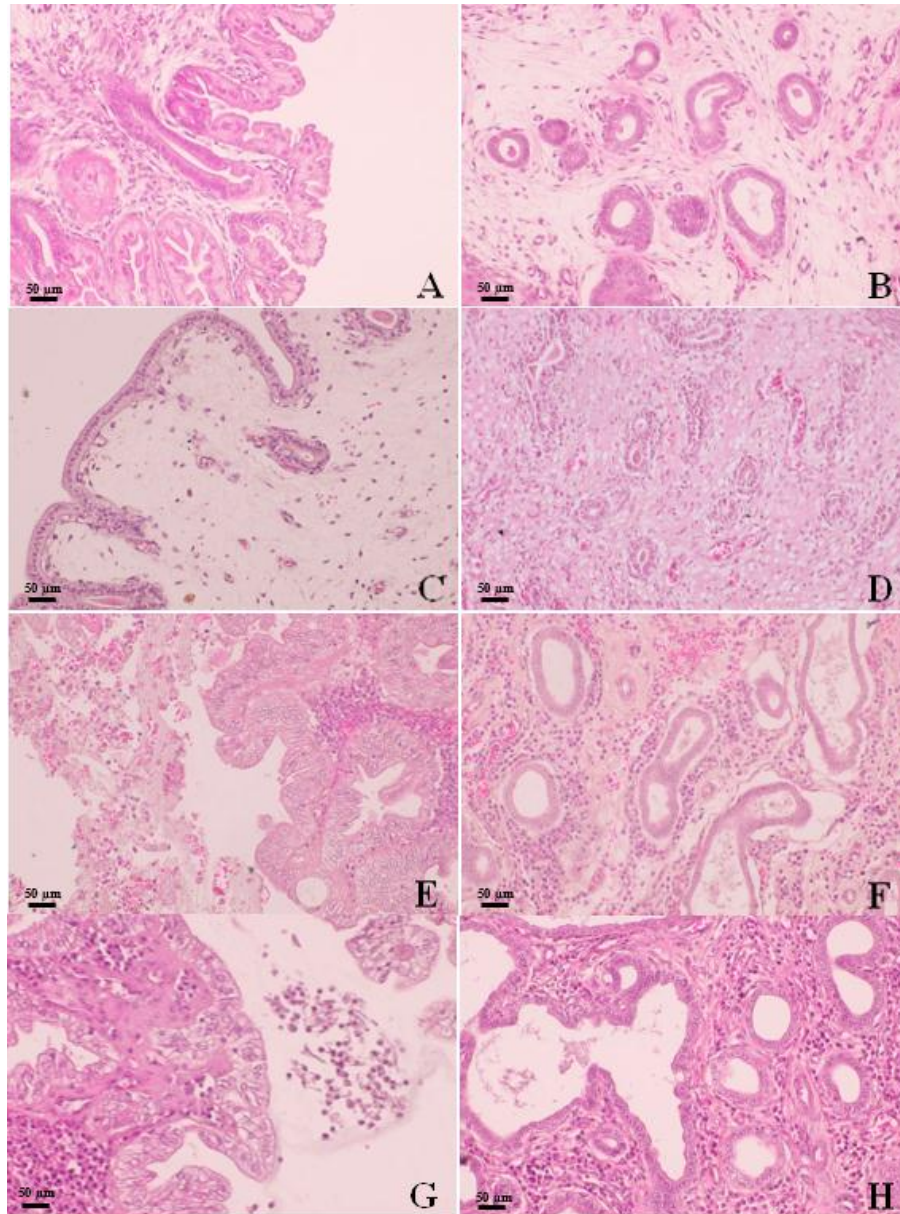


Figure 1 Histopathological findings on the uterus of closed-cervix (E, F) and opened-cervix (G, H) pyometra compared with normal diestrus (A, B) and anestrus (C, D). SE of diestrus in the normal group showed pseudostratified columnar epithelium (A), STR showed glandular proliferation and dilation (B), SE of the anestrus group was a simple cuboidal epithelium (C), STR showed irregular connective tissue with inactive endometrial glands (D), SE of closed-cervix pyometra showed hyperplasia of pseudostratified columnar epithelium with fibrino-hemopurulent exudate on ulcerative epithelium, and cystic-like epithelium was formed by epithelial erosion in the lumen (E), numerous inflammatory cells, red blood cells, and fibroblasts infiltrated the stromal tissue (F), hyperplasia of pseudostratified columnar epithelium with mild fibrinopurulent exudate was presented, along with cystic endometrium (G), glandular hyperplasia of endometrial gland with inflammatory cells in stroma with mild congestion (H).

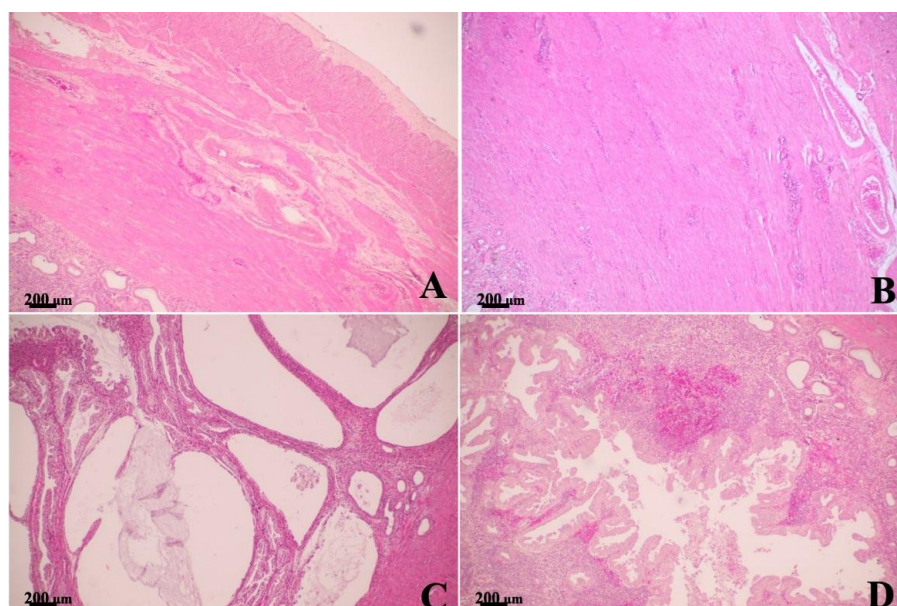


Figure 2 The MYO of the uterus in the closed-cervix pyometra group (A) was thinner compared with that in the open-cervix pyometra group (B), Cystic formation in the uterus of the closed-cervix pyometra group (C), Diffuse hemorrhagic endometritis in the uterus of the open-cervix pyometra group (D).

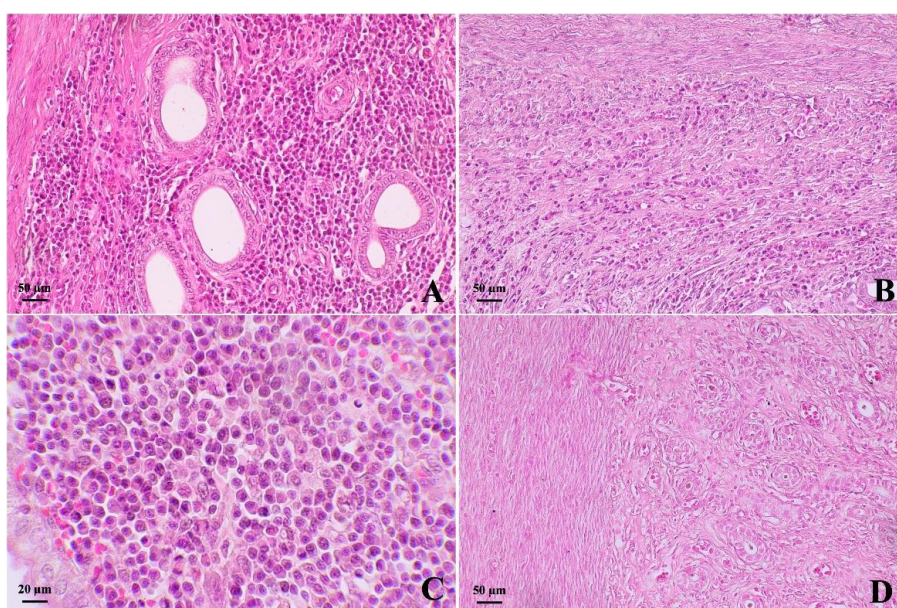


Figure 3 Leukocyte infiltration in uterine and cervical tissues. STR in the open cervix of the pyometra group with the accumulation of leukocytes (40x, A). GE in the open cervix of the pyometra group with mild accumulation of leukocytes (40x, B). Plasma infiltration in STR of uterine tissue (100x, C). Cervical tissue of normal anestrus (D).

Infiltration of inflammatory cells in uterus and cervix:

The cellular inflammatory response was characterized by leukocyte infiltration in the uterine and cervical tissues; these included lymphocytes, plasma cells, macrophages, neutrophils, and eosinophils. In normal reproductive tissue, both in diestrus and anestrus, all types of leukocytes were not significantly different ($P > 0.05$). However, lymphocyte infiltration was absent in the uterus and cervix of anestrus dogs.

For pyometra, most inflammatory cells in uterine and cervical tissues were plasma cells, especially in the uterine tissue of closed-cervix pyometra (Table 1). Plasma cell infiltration of opened-cervix and closed-cervix pyometra was significantly increased in both uterine and cervical tissues when compared with the

control groups ($P < 0.0001$). However, plasma cell infiltration in the cervix was lower than that in the uterus (Table 1). In the cervix, plasma cells, lymphocytes, and neutrophil populations of closed-cervix pyometra were significantly more abundant than in the opened-cervix pyometra ($P < 0.05$, Table 1).

PTGFR and CRP expressions: The reactivity (brown staining) of PTGFR in the uterus and cervix was presented at the cytosol of epithelial cells and glandular epithelial cells with nuclear staining. Smooth muscle cells were stained at the nuclear and perinuclear areas (Fig. 4, Table 2). The expression of PTGFR in the uterine STR of closed-cervix pyometra (T score = 3.6) was significantly lower when compared

with that of normal tissues ($P < 0.05$). We found no statistically different expressions of PTGFR in the uterine layers ($P > 0.05$). In each group, the T scores of uterine SE in normal diestrus and opened-cervix pyometra groups and the GE in normal anestrus and closed-cervix pyometra markedly expressed PTGFR were 7.0, 6.2, 6.76 and 6.07, respectively. The T scores of the PTGFR level in the MYO of the uterus ranged from 4.08-4.82 in all groups ($P > 0.05$). Regarding cervical tissue, this study revealed no statistically different expressions of PTGFR in all cervical layers and among the different groups ($P > 0.05$). However, the T scores of

expressions in all layers were high (from 4.77 to 6.62). The CRP expression was only observed in the cytoplasm and nucleus of macrophages at the STR of the uterus and cervix. The expression of CRP at the STR of the uterus was observed in the opened- and closed-cervix pyometra (Fig. 5). However, the T scores of CRP immunostaining were weakly expressed (ranging from 1.00-2.37) and did not statistically differ among the groups ($P > 0.05$). In all cases, western blot analysis confirmed the expression of PTGFR and CRP proteins in the uterus and cervix of normal and pyometra tissues (Fig. 6).

Table 1 Total counts of each type of leukocytes for uterine and cervical tissues (Mean \pm SD)

Type of leukocyte	Mean of total number of leukocytes (cells per $0.31 \times 10^3 \text{ mm}^2$ area)							
	Uterus				Cervix			
	Normal anestrus	Normal diestrus	Opened-cervix pyometra	Closed-cervix pyometra	Normal anestrus	Normal diestrus	Opened-cervix pyometra	Closed-cervix pyometra
Lymphocyte	0 ^a	0.2 \pm 0.1 ^a	12.2 \pm 3.4 ^a	13.7 \pm 5.8 ^a	0 ^A	0.3 \pm 0.2 ^A	1.9 \pm 0.2 ^{AB}	6.4 \pm 0.2 ^C
Plasma cell	0.2 \pm 0.1 ^a	0.7 \pm 0.7 ^a	60.7 \pm 3.3 ^b	108.4 \pm 14.9 ^c	0 ^A	0.1 \pm 0.1 ^A	10.8 \pm 2.3 ^B	17.9 \pm 3.1 ^C
Macrophage	0.1 \pm 0.1 ^a	0.6 \pm 0.6 ^a	1.1 \pm 0.4 ^a	5.5 \pm 2.2 ^a	0.1 \pm 0.1 ^A	0.6 \pm 0.5 ^{AB}	0.7 \pm 0.2 ^{AB}	3.6 \pm 1.2 ^{BC}
Neutrophil	0.3 \pm 0.2 ^a	1.4 \pm 0.9 ^a	1.8 \pm 0.0 ^a	9.9 \pm 5.5 ^a	0.3 \pm 0.3 ^A	0.6 \pm 0.4 ^A	1.4 \pm 0.4 ^{AB}	5.5 \pm 0.3 ^C
Eosinophil	0 ^a	0.1 \pm 0.1 ^a	1.8 \pm 0.7 ^a	1.8 \pm 0.7 ^a	0.1 \pm 0.1 ^A	0.1 \pm 0.1 ^A	0.1 \pm 0.1 ^A	0.3 \pm 0.1 ^A

Different superscripts within the same row (in each tissue) indicate significantly different values ($P < 0.05$).

Table 2 Means of T score of PTGFR and CRP expression on uterine and cervical tissues (Mean \pm SD)

Expression		Uterus				Cervix				
		Normal anestrus	Normal diestrus	Opened-cervix pyometra	Closed-cervix pyometra	Normal anestrus	Normal diestrus	Opened-cervix pyometra	Closed-cervix pyometra	
PTGFR	SE	5.51 ± 0.64	7.00 ± 0.00	6.20 ± 0.44	5.91 ± 0.56	SE	5.51 ± 0.66	6.42 ± 0.21	5.69 ± 0.48	6.62 ± 0.14
	STR	5.58 ± 0.14	5.55 ± 0.10	4.62 ± 0.38	3.60 ± 0.55*	STR	4.77 ± 0.74	5.65 ± 0.45	5.85 ± 0.43	5.16 ± 0.08
	GE	6.76 ± 0.08	6.78 ± 0.22	5.04 ± 0.53	6.07 ± 0.28					
	MYO	4.82 ± 0.26	4.58 ± 0.32	4.08 ± 0.47	4.11 ± 0.17	MYO	4.93 ± 0.54	5.26 ± 0.51	5.22 ± 0.53	5.28 ± 0.58
CRP	SE	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	SE	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	STR	1.20 ± 0.20	1.00 ± 0.00	2.37 ± 0.77	1.60 ± 0.60	STR	1.38 ± 0.38	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
	GE	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00					
	MYO	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	MYO	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00

* Different superscripts within the same row (in each tissue) indicate significantly different values ($P < 0.05$).

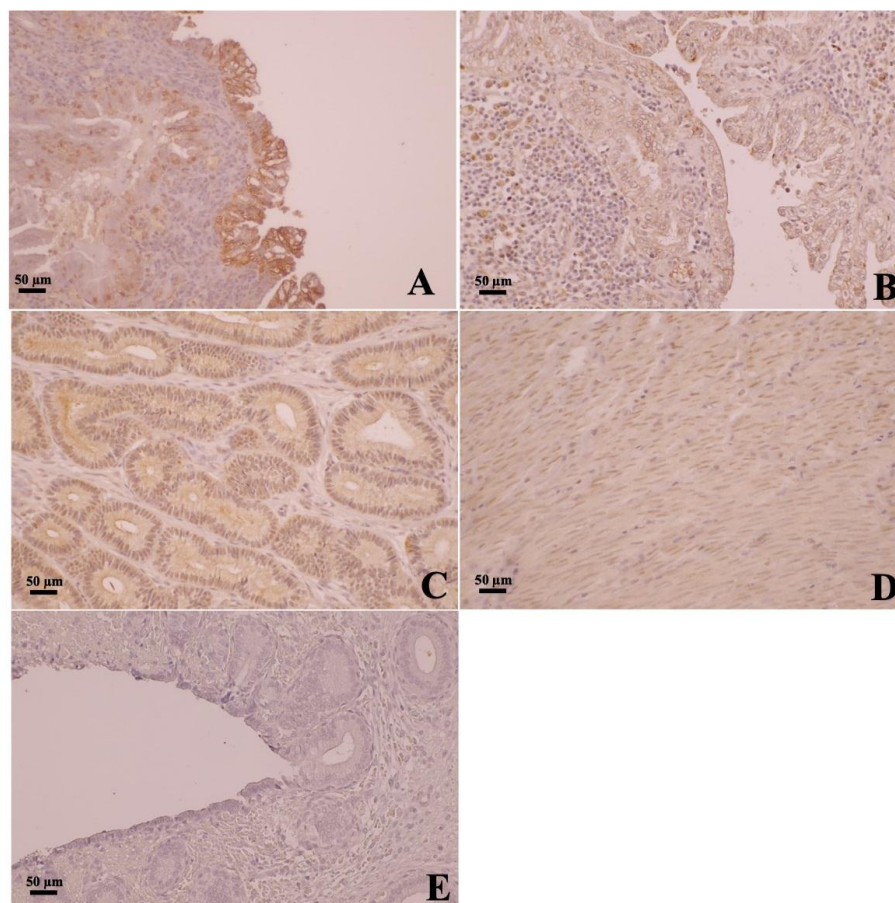


Figure 4 Reactivity of prostaglandin F-2 alpha receptor in SE (T score = 6.42 ± 0.21 , A), STR (T score = $5.55.00 \pm 0.10$, B), GE (T score = 4.08 ± 0.47 , C), and MYO (T score = 5.26 ± 0.51 , D). A and D; cervical tissue of normal diestrus. B; uterine tissue of normal diestrus. C; uterine tissue of open-cervix pyometra. E; negative control.

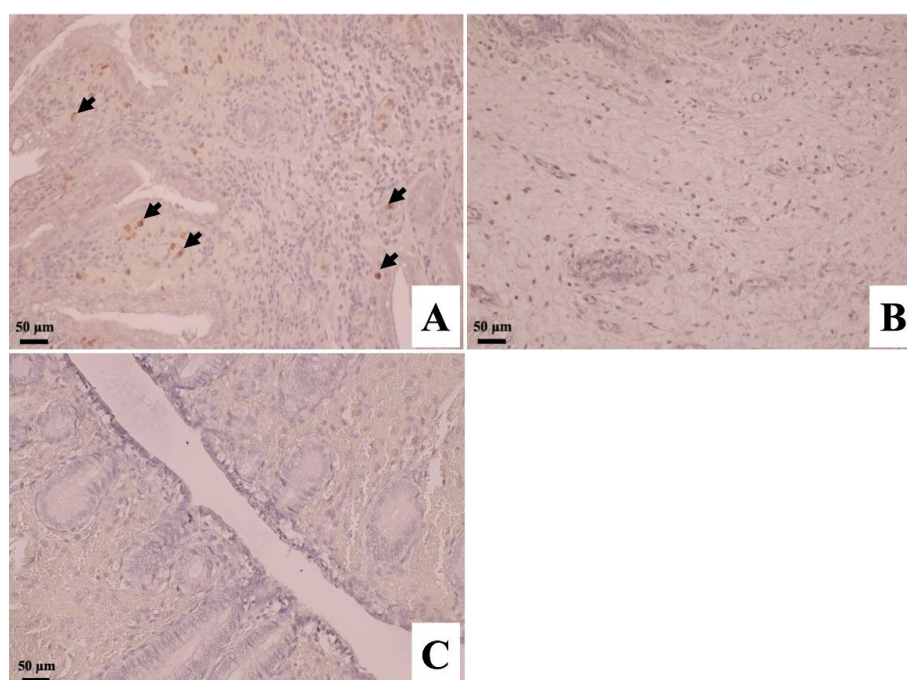


Figure 5 CRP reactivity by immunohistochemistry. C-reactive protein (CRP) expression (arrow) in leukocytes of uterine STR in open-cervix pyometra group (T score = 2.37 ± 0.77 , A). Normal diestrus group (T score = 1.00 ± 0.00 , B). Negative control of CRP receptor immunostaining (C).

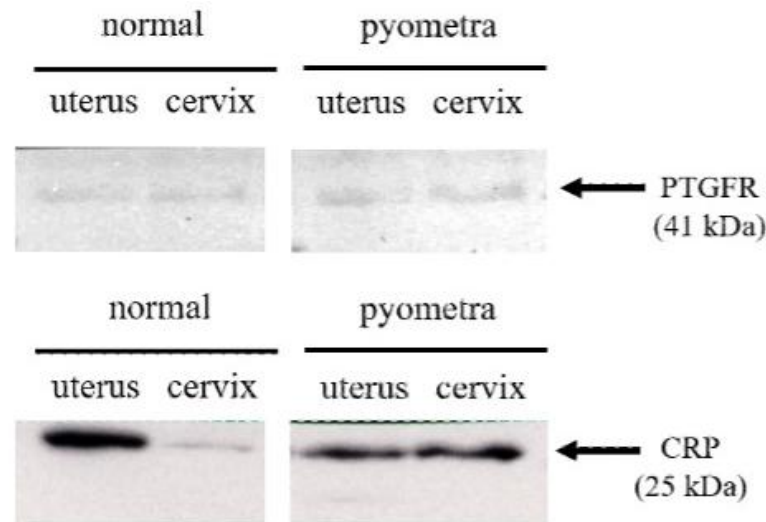


Figure 6 To confirm the protein expression by western blot analysis, this result shows that PTGFR and CRP were expressed in the uterus and cervix of normal and pyometra tissues.

Discussion

This study revealed a significant difference in the inflammatory status by pathological changes, inflammatory events, PTGFR, and CRP of opened-cervix and closed-cervix pyometra tissues. Inflammation of closed-cervix and opened-cervix pyometra was predominantly demonstrated by increased plasma cell infiltration at the stroma of uterine and cervical tissues. These findings reveal the chronic inflammatory stage of female reproductive tract pathogenesis, as described previously (Kempisty *et al.*, 2013, Hardy and Osborne, 1974; Fransson and Ragle, 2003, Mallison *et al.*, 1991; Slocombe *et al.*, 2013, Baithalu *et al.*, 2010). Plasma cell accumulation was stimulated by microbial infection or specific antigens. Thereafter, the inflammatory cascade induced and extended the life span of plasma cells in tissues by cytokines (i.e., TNF α , APRIL, BAFF, IL-6, and CXCL) (Mallison *et al.*, 1991, Cassese *et al.*, 2003 Girolamo *et al.*, 1997; Slocombe *et al.*, 2013). Our results indicate the relationship between mean plasma cells/area of closed-cervix pyometra in the uterus and cervix and the high severity of septic signs in dogs (Jitpean *et al.*, 2017). High levels of serum CRP occurred in both closed- and opened-cervix pyometra in dogs (Nakamura *et al.*, 2008; Sproston and Ashworth, 2018; Enginler *et al.*, 2014). Recently, CRP was introduced as a major inflammatory marker of acute-phase protein; it is more sensitive than the blood leukocyte profile in dogs, for example in terms of inflammatory condition, infectious disease, and malignant tumors (Nakamura *et al.*, 2008; Sproston and Ashworth, 2018; Pepys and Hirschfield, 2003; Galezowski *et al.*, 2010). Our study revealed the low reactivity (T score varied from 1.00–2.37) of CRP by immunostaining; there was no significant difference in both uterus and cervix among the groups, similar to previous studies on other tissues where no CRP was accumulated in the tissues (Baidoshvili *et al.*, 2002; Sproston and Ashworth *et al.*, 2018). Therefore, CRP can be used as an inflammatory markers for pyometra in

clinical fields for 24–72 hours after inflammation up to 1–2 weeks (Sproston and Ashworth *et al.*, 2018).

Generally, PGF 2α plays a role in luteolysis at the end of the diestrus period and regulates myometrial contraction (Wallace *et al.*, 2009; Gogny *et al.*, 2010; Hagman *et al.*, 2006), and there are reports about the competence of prostaglandin analogue for pyometra treatment in dogs (Fieni *et al.*, 2014; Thirumurugan and Rajasundaram, 2011; Gobello *et al.*, 2003). Our study showed no differences in the T scores of PTGFR between opened- and closed-cervix pyometra. These findings may reflect the successful administration of PGF 2α in opened- and closed-cervix pyometra, as reported elsewhere (Fieni, 2006). Exogenous PGF 2α induces uterine contraction, decreases uterine contents, and activates luteolysis (Nelson *et al.*, 1982). However, we found no differences in the expression of its receptors in the pyometra uterus (except stroma) and the cervix compared with normal anestrus and normal diestrus. Inflammatory responses of pyometra have been described regarding the presence of PGF 2α in the pyometrial fluid of cows, dogs, and ferrets, associated with uterine bacterial infection and inflammation (Heap and Poyser, 1975).

In the current study, the PTGFR demonstrated high T scores in the SE and GE of the pyometra. This may reflect the response of inflammatory cells, especially when the plasma cells and neutrophils promote tissue remodeling and inflammatory responses via chemokine (C-X-C motif) ligand 1 (CXCL1) of PGF 2α (Mallison *et al.*, 1991, Cassese *et al.*, 2003 Girolamo *et al.*, 1997; Slocombe *et al.*, 2013; Wallace *et al.*, 2009). Moreover, the vascular endothelial growth factor, via the CXCL1 cascade, plays a classic role as the first line of defense against invading pathogens and may also remodel vascular uterine tissue in the normal menstrual cycle in humans (Ardi *et al.*, 2007; Gargett *et al.*, 2001). However, our study did not find a correlation between the expression of PTGFR and CRP in uterine and cervical tissue and leukocyte infiltration. For MYO, PTGFR expression was not significantly different among the groups, although the CEH with

chronic endometritis has pathological changes, especially severe myometrium atrophy (Dow, 1959). Endometrium degeneration may affect the expression of PTGFR in the uterine STR compartment, with a low T score in closed-cervix pyometra.

In conclusion, we provide evidence of an inflammatory response in closed- and opened-cervix pyometra of dogs. The PTGFR expression did not affect most layers of the uterus and the cervix of pyometra in dogs, but pathological severity was pronounced. Inflammatory cells, in particular the plasma cells that predominantly infiltrated in tissues of closed-cervix pyometra, were not correlated with CRP accumulation in an inflammation of the uterus and the cervix. However, its response in peripheral blood should be further investigated.

Conflict of interest statement: The authors declare that there is no conflict of interest that could be perceived as

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