Evaluation of tissue trauma and healing on the basis of tumour necrosis factor-alpha, interleukin-6 and C-reactive protein in peripheral blood during and after pyometra in bitches

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Summary

In this study, inflammation in the blood of bitches with pyometra (PG) was compared before and 15 days after ovariectomy (OVH). The results were compared with those for control dioestrus bitches (CG) to reveal the evidence of inflammation in blood after a routine surgery. Inflammation was tracked by tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) and C-reactive protein (CRP), using immunocytochemistry (ICC) and immunofluorescence capture of blood cells in cell blocks. ICC is performed mainly during routine cytological examinations, whereas the use of cell blocks in blood examination is uncommon. Insofar as we know, this is the first study using cell block techniques on canine blood samples. Three commercially available antibodies against TNF- α , IL-6 and CRP, forming two panels, were evaluated. A standard streptavidin-biotin complex technique was used for ICC. TNF- α and IL-6 labelling was scored for colour and intensity, and CRP for immunofluorescence capture. TNF- α and IL-6 colour and intensity scores differed significantly between the PG and CG groups, and were higher in PG before OVH ($P < 0.01$, $P < 0.01$, $P < 0.001$, $P < 0.01$, respectively). IL-6 intensity was significantly greater in PG 15 days after OVH ($P < 0.05$). CRP capture in PG was strong before OVH and high in both groups 15 days later. Low-dose anti-inflammatory agents or an anti-cytokine therapy may be useful in pyometra treatment in the future because these treatments may offer protection from systemic inflammatory response syndrome before and after OVH.

Keywords: female dog, inflammation, anti-cytokine therapy, SIRS

Pyometra is the accumulation of pus in the uterus. Bacterial colonization of the uterus can lead to systemic inflammation that threatens life (7). In an intact bitch, repeated cycles of oestrogen and progesterone stimulation can result in endometrial pathology (8). Cystic endometrial hyperplasia can support bacterial growth, and a secondary colonization by *Escherichia coli* provides the primary opportunistic pathogen (11). *E. coli* originating from faeces can enter by the vaginal route as an ascending infection. Canines are especially susceptible in the luteal phase of their estrous cycle. Hematogenous or lymphatic sources are also possible (8).

Complications of *E. coli*-associated pyometra can include serious-to-fatal sepsis and endotoxemia. The

endotoxin of *E. coli* is a very potent inflammatory stimulant; it is a lipopolysaccharide (LPS) that activates blood platelets and may cause hypercoagulation (disseminated intravascular coagulation, or DIC) (13). The host immune response includes leukocyte release of pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-8 and tumour necrosis factor-alpha (TNF- α) (4, 13, 16). Cytokines, especially IL-6, trigger production of acute-phase proteins (APP) (6, 11), such as C-reactive protein (CRP), which is synthesized in the liver and may be useful as an inflammatory indicator in blood (5). CRP binds to the surface of dead cells and promotes phagocytosis (9). In an intensive care unit (ICU), inflammation is tracked mainly by detection of TNF- α , IL-6 and CRP in circulation (25). TNF- α has

a very short half-life in circulation, and is elevated in blood during the acute phase of inflammation (3, 18). Interleukin-6 is a prognostic marker in dogs, with high blood levels indicating poor prognosis (25). CRP has been found to be a good indicator for an ongoing systemic inflammatory response syndrome (SIRS) during pyometra (7).

Cytokines induce a normal immune response. However, dysregulated cytokine signalling associated with LPS and/or sepsis can result in autoimmunity (6), cytokine storm, SIRS, septic shock, multiple organ dysfunction syndrome (MODS) and death (1, 4, 13, 27). The duration of SIRS also determines the risk of developing MODS (14).

The aims of this study were 1) to evaluate TNF- α , IL-6 and CRP in bitches with pyometra before and during the recovery period following ovariohysterectomy (OVH) and to compare the results with those from healthy dioestrus bitches that underwent elective OVH; 2) to determine whether OVH would increase blood inflammatory markers in pyometra-affected and healthy bitches; and 3) to evaluate the „cell blocks” method for efficacy in blood preservation, which, in turn, facilitates determining TNF- α , IL-6 and CRP.

Material and methods

Dogs. Dogs enrolled in this study were patients of the Faculty of Veterinary Medicine at Istanbul University. This randomized trial included two groups of bitches: one group with pyometra (PG; $n = 21$) and a control group of healthy dioestrus bitches (CG; $n = 5$). The mean ages of the dogs at the start of the study were 7.9 ± 0.57 years for PG and 5.7 ± 1.36 years for CG. According to a five-point body condition scoring system, the mean body condition scores were 3.05 ± 0.16 for PG and 3.40 ± 0.24 for CG. Vaginoscopy and ultrasonography (Easote Pie Medical MyLab Five Vet, Netherlands) were used to assess the uterus of study subjects. Fluid in the uterus presents as anechoic or hypoechoic ultrasonography, which may reflect pyometra, hydrometra, or mucometra. Clinical signs, vaginal cytology, clinical chemistry and patient history can support a differential diagnosis. OVHs were performed on the day of arrival. Seventeen (PG) bitches had pyometra with an open cervix, and four had pyometra with a closed cervix (#P5, #P9, #P13, #P16). Bitches in CG had no known diseases and had applied only for OVH. A broad-spectrum antibiotic therapy (ceftriaxone or enrofloxacin) was given to all bitches in PG and CG after OVHs. There was no specific grouping for antibiotic selection in the study. Ceftriaxone was administered to 7 bitches in the PG and 1 bitch in CG, whereas enrofloxacin was given to 14 animals in PG and to 4 in CG. Owner permission for study participation was obtained before blood samples were collected. The study was approved by the Istanbul University Ethics Committee (#1702011).

Sampling procedures. Blood was taken aseptically from a vena cephalica antebrachii or a vena saphena parva; 1 mL of blood was collected into polystyrene tubes that contained

1 mL of 7.5% buffered formaline (Histologic Grade Formaldehyde; Sigma-Aldrich). The tubes were capped tightly and gently shaken by hand for mixture.

Blood was also collected into EDTA tubes to determine the complete blood cell count (CBC). Specimens were analyzed using a ProCyte Dx Hematology Analyzer (Idexx, USA) at our hospital laboratory. The bitches in both groups were sampled twice, on admission to the hospital (before OVH) and 15 days later.

Cell blocks. Cell blocks were prepared according to Kung et al. (15). Blood samples were fixed and incubated at room temperature for 4-6 hours, and then cell pellets were embedded in 2% bacteriological agar in Eppendorf tubes and left at room temperature for cooling. Embedded cell blocks were wrapped in filter paper and processed routinely as tissue specimens. Sections of 3-4 μm were cut from cell blocks with a rotary microtome. One section of each specimen was stained with haematoxylin and eosin (H&E) to observe cell morphology, while three sections were placed on positive-charged adhesion microscope slides (Citoglass, Citotest Labware Manufacturing Co. Ltd.) and reserved for immunocytochemistry (ICC). Blood smears were also prepared to compare cell morphology between H&E slides and cell blocks. For this purpose, slides were prepared and air-dried for 5 min before staining. Smears were stained with May-Grünwald-Giemsa azur methylene blue solution stain (MGG; Merck KGaA, Darmstadt, Germany) and rinsed with deionized water and air dried before evaluation.

Immunocytochemical detection of TNF- α and IL-6. Slides of cell blocks were deparaffinized, followed by antigen retrieval and the endogen peroxidase and protein blocking procedures. The slides were incubated with antibody to TNF- α (diluted 1 : 150; 1.5 h, room temperature, R&D Systems, Cat. no: MAB1507) and IL-6 (diluted 1 : 200; 1.5 h, room temperature, R&D Systems, Cat. no: AF1609) and then treated with a commercial secondary antibody kit (Invitrogen, Histostain-Plus IHC Kit, HRP, broad spectrum, Cat. No: 85-9043, Paisley, UK). These steps were followed by marking with 3,3'-Diaminobenzidine (DAB) chromogen (Invitrogen, DAB-Plus Substrate Kit, Cat. No: 00-2020, Paisley, UK). Lastly, the sections were counterstained with Mayer's haematoxylin. Negative control sections were incubated with antibody diluent instead of the primary antibody. Antigen retrieval was performed using citrate buffer solution.

Immunofluorescence capture of CRP. Direct immunofluorescence labelling of CRP from cell blocks was performed according to Mera et al. (20). The slide sections were dried overnight at 37°C. The slides were de-waxed, rehydrated, washed in Tris-saline buffer (pH 7.8), and trypsinised for 40 min at 37°C in a solution of 0.1% calcium chloride and 0.05% trypsin. The slides were washed again with Tris-saline buffer (pH 7.8) and incubated in a moist chamber for 30 min with commercially available fluorescein conjugates of rabbit anti-canine C-reactive protein Ab (diluted 1 : 1000; 20 min, room temperature, Assaypro; RPE-Canine C-Reactive Protein Ab, Cat. no: 11922-05051, USA). The slides were washed three times with phosphate

buffered saline (PBS; pH 7.4, 0.1 M, Sigma-Aldrich, Germany) before being mounted (Dako, fluorescent mounting medium, USA).

Evaluation of TNF- α , IL-6 and CRP. The slides were examined using an Olympus BX50 light microscope and the Olympus DP2-BSW software program. Scoring for evaluation of TNF- α and IL-6 was based on ICC staining colour and intensity. The results were graded from 0 to +++ (0 – no staining; + – weak staining; ++ – moderate staining; +++ – intense staining). Five areas on each slide were selected randomly for this purpose, and the percentage of cells with various staining intensities was determined (2). The slides were examined under a 100 $\times$ objective with immersion oil.

CRP expression was determined using an Olympus microscope equipped with FITC exciter and barrier filters, with incident illumination from an Olympus U-LH100HG 100 W light source. The staining of the slides was scored according to fluorescence intensity as 0 – no capture, + – weak capture, ++ – moderate capture or +++ – intense capture. Evaluation was done under a 100 $\times$ objectives with immersion oil.

The ICC sections were evaluated by two observers blinded to the clinical status of the bitches.

Statistical analyses. Descriptive statistics and repeated ANOVA tests were applied to CBC and ICC results for TNF- α and IL-6. An independent sample t-test was applied to compare each sampling time. Group, sampling time, and sampling time $\times$ group interaction were included in the models. A paired t-test was used to compare values between days 0 and 15. For interpretation of ICC results, the scores of 0 to +++ were numbered as 1 to 4. A value of $P < 0.05$ was determined as an indicator of statistical significance.

Results and discussion

Prior to OVH, the mean body temperature was $38.66 \pm 0.16^\circ\text{C}$ in PG and $38.2 \pm 0.08^\circ\text{C}$ in CG ($P > 0.05$). At surgery, none of the bitches were diagnosed with peritonitis. Fifteen days after surgery, all bitches in both groups had good appetite, with no surgery site inflammation or other health problems.

The mean and standard error values for CBC comparisons are presented in Table 1. In PG, WBCs were lower and PLT levels were higher 15 days after OVH ($P < 0.01$). MCHC was lower in both groups after 15 days ($P < 0.05$ in CG; $P < 0.001$ in PG).

Tab. 1. CBC results in CG and PG before OVH and 15 days after OVH

Variable	Control (n = 5) Mean $\pm$ SE	Pyometra (n = 21) Mean $\pm$ SE	Repeated ANOVA		
			Group	T	G $\times$ T
RBC 0 th day ($\times 10^6 \mu\text{L}$)	6.24 $\pm$ 0.68 ^{a, A}	5.80 $\pm$ 0.21 ^{a, A}	NS	NS	NS
RBC 15 th day ($\times 10^6 \mu\text{L}$)	6.00 $\pm$ 0.74 ^{a, A}	5.54 $\pm$ 0.24 ^{a, A}			
WBC 0 th day ($\times 10^3 \mu\text{L}$)	16.09 $\pm$ 2.20 ^{a, A}	38.92 $\pm$ 6.20 ^{a, A}	NS	NS	NS
WBC 15 th day ($\times 10^3 \mu\text{L}$)	13.52 $\pm$ 1.85 ^{a, A}	18.46 $\pm$ 2.61 ^{a, C}			
HCT 0 th day (%)	45.2 $\pm$ 3.85 ^{a, A}	38.95 $\pm$ 1.38 ^{a, A}	NS	NS	NS
HCT 15 th day (%)	44 $\pm$ 4.60 ^{a, A}	37.86 $\pm$ 1.58 ^{a, A}			
HGB 0 th day (g/dL)	14.70 $\pm$ 1.46 ^{a, A}	12.87 $\pm$ 0.42 ^{a, A}	NS	NS	NS
HGB 15 th day (g/dL)	14.08 $\pm$ 1.60 ^{a, A}	12.36 $\pm$ 0.55 ^{a, A}			
PLT 0 th day ($\times 10^3 \mu\text{L}$)	524 $\pm$ 97.57 ^{a, A}	285.05 $\pm$ 35.95 ^{b, A}	NS	NS	NS
PLT 15 th day ($\times 10^3 \mu\text{L}$)	488.8 $\pm$ 107.74 ^{a, A}	450.05 $\pm$ 63.76 ^{a, C}			
MCV 0 th day fl	72.8 $\pm$ 2.35 ^{a, A}	67.29 $\pm$ 0.67 ^{b, A}	*	NS	NS
MCV 15 th day fl	73 $\pm$ 2.77 ^{a, A}	67.43 $\pm$ 1.60 ^{a, A}			
MCH 0 th day (pg)	23.8 $\pm$ 0.37 ^{a, A}	22.05 $\pm$ 0.25 ^{c, C}	**	NS	NS
MCH 15 th day (pg)	23.4 $\pm$ 0.4 ^{a, A}	21.67 $\pm$ 0.25 ^{c, C}			
MCHC 0 th day (%)	32.40 $\pm$ 0.51 ^{a, B}	32.86 $\pm$ 0.28 ^{a, A}	NS	**	NS
MCHC 15 th day (%)	31.60 $\pm$ 0.51 ^{a, B}	31.67 $\pm$ 0.29 ^{a, C}			

Explanations: T – sampling time; G $\times$ T – group $\times$ sampling time interaction; a, b, c – means with different superscript letters differ significantly in the same row; A, B, C – means with different superscript letters differ significantly in the same column; ^{a, A} – $P < 0.05$; ^{b, B} – $P < 0.01$; ^{c, C} – $P < 0.001$; NS – $P > 0.05$

Fig. 1. Appearance of blood cells: (A) Blood smear. Leukocytes (black arrow). MGG. (B) "Cell blocks" of blood. Leukocytes (black arrow). H&E

Cell morphology on blood smears vs. cell blocks. Erythrocytes and leukocytes appeared larger and clearer than cells from cell block sections (Fig. 1A). In the case of specimens from cell blocks, erythrocytes were visible, but their shapes, borders and cytoplasm were less obvious, especially with larger numbers of cells in examination areas. Platelets were not apparent on slides from cell blocks (Fig. 1B). Clumps of bacteria were observed on cell block slides from some bitches with pyometra before OVH.

Comparison of TNF- α , IL-6 and CRP labelling between PG and CG. The means and standard errors for TNF- α and IL-6 are presented in Table 2. TNF- α colour and intensity scores were higher in PG at day 0 ($P < 0.01$). IL-6 colour and intensity scores were higher in PG at day 0 ($P < 0.001$, $P < 0.01$), and IL-6 intensity remained significantly greater, compared with CG, at day 15 ($P < 0.05$). Strong or moderate labelling of TNF- α and IL-6 was seen in all bitches before OVH in PG (Fig. 2A, B and Fig. 3A, B, respectively). Although most of the samples were positive for TNF- α and IL-6 labelling after 15 days, negative

Tab. 2. TNF- α and IL-6 labelling in cell blocks: colour and intensity scores before OVH and 15 days after OVH

Variable	Control (n = 5) Mean $\pm$ SE	Pyometra (n = 21) Mean $\pm$ SE	Repeated ANOVA		
			Group	T	G $\times$ T
TNF- α colour 0 th day	1.6 $\pm$ 0.24 ^{a, A}	2.76 $\pm$ 0.17 ^{c, A}	*	**	*
TNF- α colour 15 th day	2.4 $\pm$ 0.40 ^{a, A}	2.86 $\pm$ 0.16 ^{a, A}			
TNF- α intensity 0 th day	2.0 $\pm$ 0.55 ^{a, A}	3.38 $\pm$ 0.18 ^{b, A}	*	NS	NS
TNF- α intensity 15 th day	2.4 $\pm$ 0.51 ^{a, A}	3.10 $\pm$ 0.18 ^{a, A}			
IL-6 colour 0 th day	1.4 $\pm$ 0.24 ^{a, A}	2.71 $\pm$ 0.14 ^{c, A}	**	NS	NS
IL-6 colour 15 th day	1.8 $\pm$ 0.37 ^{a, A}	2.57 $\pm$ 0.16 ^{a, A}			
IL-6 intensity 0 th day	1.4 $\pm$ 0.24 ^{a, A}	2.71 $\pm$ 0.17 ^{b, A}	**	NS	NS
IL-6 intensity 15 th day	1.6 $\pm$ 0.24 ^{a, A}	2.62 $\pm$ 0.18 ^{b, A}			

Explanations: TNF- α – tumournecrosis factor alpha; IL-6 – interleukine-6; T – sampling time; G $\times$ T – group $\times$ sampling time interaction; a, b, c – means with different superscript letters differ significantly in the same row; A, B, C – means with different superscript letters differ significantly in the same column; ^{a, A} – $P < 0.05$; ^{b, B} – $P < 0.01$; ^{c, C} – $P < 0.001$; NS – $P > 0.05$

TNF- α and IL-6 labelling was seen in some PG bitches (Fig. 2C and Fig. 3C, respectively). Positive labelling of TNF- α and IL-6 antigen was seen in leukocytes before OVH in some PG bitches that showed a strong diffuse labelling of these antigens in serum at day 15 (Fig. 4A, B).

CRP capturing was present with different intensities (++, +++) before OVH in all PG samples. Only three bitches in CG showed no CRP capturing (–), while in

Fig. 2. (A) Strong labelling of TNF- α antigen in blood (+++, +++; #P14, before OVH). ICC. (B) Moderate labelling of TNF- α antigen in blood (+, ++; #P3, before OVH). ICC. (C) Negative labelling of TNF- α antigen in blood (–, –; #P1, 15 days after OVH). ICC. All slides from cell blocks

Fig. 3. (A) Strong labelling of IL-6 antigen in blood (+++, +++; #P14, before OVH). ICC. (B) Moderate but diffuse labelling of IL-6 antigen in blood (+, ++; #C3, 15 days after OVH). ICC. (C) Negative labelling of IL-6 antigen in blood (–, –; #P1, 15 days after OVH). ICC. All slides from cell blocks

two others the intensity of CRP capturing was weak (+). All bitches in both groups showed strong CRP capturing at day 15 (++++) (Fig. 5).

In this study, CBC results, TNF- α and IL-6 antigen expressions and CRP capture were compared before

and 15 days after OVH in bitches with pyometra and bitches in normal dioestrus. WBCs in PG were greater before OVH than they were 15 days later ($P < 0.01$). The change could have also been a physiological response to lower stress during the second examination.

Fig. 4. (A) Positive labelling of TNF- α antigen inside leukocytes (++, ++; #P19, before OVH). ICC. (B) Strong and diffuse labelling of TNF- α antigen (+++, +++; #P19, 15 days after OVH). ICC. All slides from cell blocks

Fig. 5. Fluorescence images of red blood cells capturing CRP: low intensity (left side), high intensity (right side). (Top images show few cells; bottom images show diffuse cells with serum capturing CRP). All slides from cell blocks

PLT levels in PG were lower before OVH, returning to normal levels 15 days after OVH. A low level of PLT has been described in bitches with pyometra (24) and is seen in other acute infections. The presence of DIC may increase the risk of haemorrhage (23). CBC results in our study were in accordance with previous studies (8, 23, 24).

In another study by Maciel et al. (18), there was no difference in TNF- α levels between dioestrus and pyometra bitches before OVH. In our study, on the other hand, TNF- α and IL-6 labelling as well as CRP capture in cell blocks were higher in PG compared with CG prior to OVH, and they were still higher in most PG bitches 15 days afterwards. Only one bitch in PG responded well to antibiotic therapy and OVH, and she had a negative labelling of TNF- α and IL-6 after 15 days. There was no difference between the effects of the antibiotics used (ceftriaxone and enrofloxacin) on TNF- α and IL-6 labelling or on CRP capture in our study. Fifteen days after OVH in PG, eighteen bitches still showed strong positive labelling (++, +++) for TNF- α and thirteen bitches for IL-6.

Expressions of TNF- α and IL-6 increased in CG after OVH ($P > 0.05$). After 15 days, all bitches in both groups showed a higher intensity of CRP capture.

Routinely, TNF- α , IL-6, and CRP in serum are detected by enzyme-linked immunosorbent assay (ELISA). In our study, immunocytochemistry (ICC) and immunofluorescence tests were tried on cell blocks. Cell block techniques are used infrequently to preserve blood cells. Cell blocks have gained importance in routine cytopathology, and there are many methods of preparation (10, 21). In our study, agar embedding was chosen. As described by Jain et al. (10), heat-related artefacts were observed in cell morphology. Thus, it is not advisable to use cell block techniques if morphology of cells at the histological level is important; also, atypia are difficult to recognize. However, repetitive use of the same blood sample with this method made it possible to identify cytokine expression in peripheral blood.

Reproductive infections and trauma can cause sepsis in dogs (22), and pyometra can be associated with DIC, SIRS, septic shock and MODS. The safest and most effective treatment for pyometra is OVH (12), although surgery is also associated with various degrees of inflammation. Thus, corticosteroids, anti-inflammatory agents, anti-endotoxine antibodies and anti-cytokine therapies have been evaluated in humans and some animal species (17, 19, 26, 28). According to our results, it would be beneficial to develop a cytokine- or toxin-targeting drug to support treatment of canine pyometra.

In conclusion, it is thought that TNF- α , IL-6, and CRP evaluations in blood are very useful for scientists and clinicians to track inflammation in circulation.

TNF- α and IL-6 antigen expressions, as well as CRP capture, were observed before and 15 days after OVH, mostly in PG. CRP capture was also noted in some CG bitches.

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