Kafkas Univ Vet Fak Derg
22 (6): 837-846, 2016
DOI: 10.9775/kvfd.2016.15443

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

# Effects of Pyometra on Some Oxidative Stress Parameters, Inflammatory Mediators and Neutrophil Segmentation in Bitches [1]

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[1] *This research was supported by "Research Fund of Istanbul University with the grant number 17144*

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**Article Code: KVFD-2016-15443 Received: 09.03.2016 Accepted: 27.07.2016 Published Online: 08.08.2016**

## Abstract

Pyometra is characterized by uterine bacterial infection with the accumulation of pus in the uterus often initiates systemic inflammation in the body and may lead to multiple organ dysfunction syndrome (MODS). The main objectives of this study is to evaluate C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), superoxide dismutase (SOD), catalase (CAT), and thiobarbituric acid-reactive substances (TBARS) in bitches with pyometra and control bitches to explore the possible variety between the groups to detect the morbidity of the disease and contribution of these parameters in the condition of MODS. All bitches underwent ovariohysterectomy (OVH) and blood sampling was performed just before and after 15 days of the surgery. TNF-α, CRP, and IL-6 levels were significantly higher in pyometra group before the OVH ($P<0.05$, $P<0.001$, $P<0.001$, respectively), and CRP and IL-6 levels were significantly high after 15 days of OVH ($P<0.001$, $P<0.05$, respectively). Two-segmented neutrophils were significantly lower in pyometra group before ($P<0.05$) and after OVH ($P<0.01$). It is revealed that with this study, CRP and IL-6 could be used for inflammation detection when leucocyte number was normal in operated bitches with pyometra. Oxidative stress was not apparent in bitches with pyometra. Also, platelet (PLT) level and neutrophil segmentation index (NSI) might be good indicators to identify the chronic phase and the severity of the disease and risk of disseminated intravascular coagulation (DIC) in bitches with pyometra.

***Keywords:*** *Pyometra, C-reactive protein, Disseminated intravascular coagulation, Platelet, Neutrophil segmentation index, Dog*

# Köpeklerde Pyometranın Bazı Oksidatif Stres Parametreleri, İnflamatorik Mediyatörler ve Nötrofil Segmentasyonu Üzerine Etkileri

## Özet

Uterusun bakteriyel invazyonu sonucu irin toplanmasıyla karakterize olan pyometra sıklıkla sistemik bir enflamasyonu başlatabilir ve bu da hastayı çoklu organ yetmezliğine sürükleyebilir (MODS). Çalışmanın amaçlarından biri, pyometralı ve sağlıklı köpeklerde kanda C-reaktif protein (CRP), tümör nekrozis faktör-α (TNF-α), interlökin-6 (IL-6), süperoksit dizmutaz (SOD), katalaz (CAT) ve tiyobarbitürik asit reaktif ürünleri (TBARS) gibi parametrelere bakılarak gruplar arasındaki varyasyon tespitiyle morbidite tahmininin yapılması ve bu parametrelerle MODS arasındaki ilişkinin ortaya çıkarılmasıdır. Çalışmaya dahil edilen köpeklerden kan alınarak ovariohisterektomi operasyonu uygulandı, on beş gün sonra ikinci kez kan alınarak aynı parametreler tekrar değerlendirildi ve bu süreçte grup içi ve gruplar arasındaki değişime bakıldı. TNF-α, CRP ve IL-6 düzeyleri operasyon öncesi pyometralı grupta önemli oranda yüksek bulundu (sırasıyla, $P<0.05$, $P<0.001$, $P<0.001$), Ovariohisterektomi sonrasında 15. gün yapılan kan değerlendirmelerinde ise CRP ve IL-6'nın pyometralı grupta hala yüksek olduğu görüldü (sırasıyla, $P<0.001$, $P<0.05$). Çift segmentli nötrofillerin sayısı pyometralı köpeklerde operasyon öncesi ve sonrası gruplara ve zamana bağlı olarak önemli düzeyde düşük olduğu tespit edildi ($P<0.01$). Bu çalışmayla CRP ve IL-6 değerlerinin pyometralı köpeklerde ovariohisterektomi sonrası enflamasyonun devam edip etmediğinin bir göstergesi olarak kullanılabileceği sonucuna varıldı. Yine trombosit (PLT) düzeyi ve nötrofil segmentasyon indekslerinin (NSI) pyometranın kronik yapısının ve şiddetinin belirlenmesinde iyi birer gösterge olduğu kanaatine varılmıştır. Aynı zamanda, pyometralı köpeklerin kanamayı arttıran dissemine intravasküler koagülasyon (DIC) riski altında olabileceğinin ortaya konulduğu düşünülmektedir.

***Anahtar sözcükler:*** *Pyometra, C-reaktif protein, Dissemine intravaskuler koagülasyon, Trombosit, Nötrofil segmentasyon indeksi, Köpek*

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# INTRODUCTION

Pyometra is a frequent endometrial problem in bitches, prolonged effects of oestrogen and following progesterone dominance in the uterus lead to hormonally mediated differences in the endometrium [1]. Accumulated endometrial secretions, lack of uterine contractility, reduced leucocyte response and bacterial invasion from ascendant genitourinary system may lead to uterine infection and consequent pyometra in bitches [2,3]. High percentage of occurrence makes pyometra one of the most important diseases in intact bitches [4].

Systemic inflammatory response syndrome (SIRS) is a response to stimulus severe enough to cause the release of inflammatory mediators into the blood stream. Pyometra sometimes might turn into SIRS when it is not diagnosed properly or treated adequately. In under some conditions SIRS might lead to multiple organ dysfunction syndrome (MODS) and the development of MODS can increase the risk of mortality [5]. An imbalanced immune response to proinflammatory cytokines may cause SIRS turn into MODS. Proinflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumour necrosis factor-α (TNF-α) are considered to be associated with MODS. Some of the criteria in animals with SIRS are fever or hypothermia, tachycardia, hypocapnea or tachypnea, decreased or increased leucocyte number and increased percentage of band neutrophils. Unfortunately, SIRS cannot be only detected with these criteria and only 64% of sepsis condition can be diagnosed in dogs. In humans, TNF-α level increases in the presence of SIRS and MODS, IL-6 and C-reactive protein (CRP) levels increase in septic conditions [5].

Increased levels of reactive oxygen species (ROS)-superoxide radical ($\cdot O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH\cdot$)- in a cell may be a cause for DNA damage and it leads to inhibition of some proteins. In addition to this process decreased antioxidant levels called literally oxidative stress may be a cause of many diseases [6]. ROS are toxic substances and they are susceptible to causing damage as a starter of lipid peroxidation on cell structure and cell membrane [7]. Detoxification of ROS materials depends on the antioxidant defense system that includes some enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathion peroxidase (GPX) [8]. Malondialdehyde (MDA) is by-product of lipid peroxidation in the body and its assessment shows lipid peroxidation especially to determine the oxidative damage on the cellular membranes. MDA is measured as Thiobarbituric acid-reactive substances (TBARS) [8-10]. The published data for reference values of oxidative stress parameters in dogs and cats are very limited with many contradictory results.

Haematological abnormalities occur in bitches with pyometra. Because of the infection increased white blood cell (WBC) counts commonly with a regenerative left shift and decreased red blood cell (RBC) counts also present in bitches with pyometra. Causes of anaemia are might be different and one of them is thought to be neutrophil oxidative metabolism that destructs the cell membranes of erythrocytes leading to pyometra-associated anaemia [10].

The aim of this study are 1) to measure CBCs, serum SOD, CAT, TBARS and plasma concentrations of CRP, TNF-α and IL-6 in healthy old intact dioestrus bitches, 2) to determine whether these parameters are associated with severity of the pyometra and used as a diagnostic marker in pyometra bitches 3) to determine whether these oxidative stress parameters, inflammatory mediators and neutrophilic segmentation/or other haematological parameters are differentiated during the healing period of the OVH in healthy old and pyometra bitches.

# MATERIAL and METHODS

### *Animals*

The study was performed on 27 bitches with pyometra (pyometra group; PG) and 8 clinically healthy bitches in dioestrus (control group; CG). Mean body weight of PG was 20.18±2.39 kg and CG was 15.15±5.20 kg. All of the bitches were geriatric exception of one in each group. Bitches submitted to study were treated against internal and external parasites and vaccinated against common diseases by their owners. Health status of bitches was checked by laboratory and physical examinations that also include vaginoscopy and transabdominal ultrasonography (Easote Piemedical MyLab Five Vet, Netherlands). OVH was performed in both groups on the same day of the admission to hospital/diagnosis of pyometra.

### *Anamnesis Data*

Written informed consent was obtained from all dog owners after being explained the purpose of the study. A detailed case history of the animals (polyuria, polydipsia, appetite loss, vomiting, antiparasitic drug status, vaccination schedule, and any other diseases etc.) was taken and recorded with thorough clinical examination. All procedures were approved by the Istanbul University Ethic Committee (Proposal #1702011).

### *Haemotology and Biochemistry Analyses*

Blood samples were collected from the cephalic or jugular vein under aseptic precautions when the bitches come to hospital (before the anaesthesia of OVH) and transferred into two EDTA and one plain blood collection tubes (three tubes for each blood sample). The plain tubes were centrifuged after immediately blood collection at 4°C and 3.000 rpm for 15 min and sera-separated before analysis of biochemistry parameters. One of the EDTA tubes were then subjected to centrifugation at 4°C and 3.000 rpm for 10 min to segregate plasma and erythrocytes. Complete blood cell count (CBC) that includes measurement of RBC,

WBC, PLT, haemoglobin (HGB), haematocrit (HCT), medium cell volume (MCV), medium cell haemoglobin (MCH), medium cell haemoglobin concentration (MCHC) and some biochemical parameters (glucose, creatinine, urea, alanine and aspartate aminotransferase) were analysed at Istanbul University Veterinary Faculty's Teaching Hospital according to our routine laboratory methods. Remaining sera and plasma were transferred into eppendorf tubes and stored at -86°C for the analysis of TBARS, SOD, CAT, TNF-α, IL-6 and CRP.

Blood sampling were performed two times just before and two weeks after the OVH. Administration of mix of vitamins was not applied after surgeries not to cause any possibility of interaction with the antioxidant levels.

### *OVH, Tissue Collection and Histopathologic Examination*

OVHs were performed routinely under general isoflorane (Forane Liquid; Aesica Queenborough, UK) anaesthesia. Three transverse sections from different parts of each uterine horn were cut for the histopathological examination, and all uterine tissues were fixed in 10% histologic grade buffered formalin (Histologic Grade formaldehyde; Sigma-Aldrich) embedded in paraffin, and standard sectioning and staining procedures were used. Sections were cut at 5 to 7 µm, mounted on slides, and stained with haematoxylin and eosin. Slides were evaluated at magnification × 20.

### *Antioxidants and Inflammatory Markers*

The concentrations of all parameters were measured with commercially available canine sandwich enzyme-linked immunosorbent assay (ELISA) kits. TNF-α was measured using the canine TNF-α ELISA (CK-E90814, Hangzhou Eastbiopharm Co. Ltd., China), IL-6 was measured using the canine IL-6 ELISA (CK-E90948, Hangzhou Eastbiopharm Co. Ltd., China), CRP was measured using the HS-CRP canine ELISA (CK-E91348, Hangzhou Eastbiopharm Co. Ltd., China), TBARS was measured using the canine TBARS ELISA (CK-E91350, Hangzhou Eastbiopharm Co. Ltd., China), SOD was measured using the canine SOD ELISA (CK-E91351, Hangzhou Eastbiopharm Co. Ltd., China), and CAT was measured using the canine CAT ELISA (CK-E91349, Hangzhou Eastbiopharm Co. Ltd., China). Assay procedures were performed according to the manufacturer's instructions. Finally, microplates were read using a microtiter reader (µQuant, BioTek Instruments, USA). The detection range of CRP was 0.05-30 mg/L, IL-6 was 5-2.000 ng/L, TNF-α was 5-1.000 ng/L, SOD was 0.5-200 ng/mL, CAT was 1-300 ng/mL and TBARS was 0.5-100 nmol/mL. Intra- and inter-assay coefficient of variations (CV) for all parameters were <10% to <12%, respectively.

### *Blood Smear Preparation and Staining*

Slides were prepared for each blood sample (both pyometra and control) and a single smear were made per slide. For the smear preparation well mixture of whole blood sample anticaogulated with 2.0 mg EDTA per millilitre was used and smeared uniformly with the method by Chung et al.[11]. The slides were air-dried for 5 min before staining and May-Grünwald's-Giemsa's azur eosin methylene blue solution (contains methanol) stain (Merck KGaA, Darmstadt, Germany) was used for staining. Stained slides were rinsed with deionized water, air-dried and evaluated.

### *Counting of WBCs and Neutrophil Segmentation Index (NSI) in the Blood Smears*

The slides of 35-blood smear were analysed and WBCs enumerated with a conventional binocular Olympus light microscope system with 100× immersion objective (UPLSAPO-100XOPH, Olympus). A drop of immersion oil was placed directly at the feathered edge of the smears for observing and a total of hundred leukocytes were differentiated manually for each blood smear sample. Neutrophils were additionally classified in accordance to the number of lobes and NSI was calculated (number of neutrophil with 5 lobes or more × 100/number of neutrophil with 4 lobes). According to this formula values greater than 16.9 were considered abnormal and considered as a sensitive indicator for right shift [12].

### *Body Condition Score (BCS) and Type of Diet*

"Five-point" BCS was accepted for the study. Type of food fed to the bitch during the past year was asked to the owner and diet categories were determined with the method by Lund et al.[13].

### *Statistics*

Descriptive statistics (mean and standard error) and repeated ANOVA test was applied for haematological and biochemical analyses. Group (pyometra and control) was added to statistical model, as Between-Subject Effects while sampling time and Sampling Time×Group interaction were included in the model as within subject effects. Moreover, independent sample *t*-test was applied to compare for each sampling time. Additionally paired *t*-test was used to compare the values obtained at $0^{th}$ day and $15^{th}$ day. A value of $P<0.05$ was used to indicate the statistical significance.

## RESULTS

None of the bitches in PG had ruptured uterus noticed during surgery and none of them died during or within 15 days of the surgeries (mortality rate 0%). PG consists of six Terriers, four Cocker Spaniels, four Mongrels, four Golden Retrievers, three German Shepherds, two Pekignese, two Rottweillers, a Siberian Husky, and a Samoyed, whereas CG consists of four Terriers, a German Shepherds cross, a King Charles Spaniel, a Rottweiller, and a Pekignese. Both group of bitches were fed with mostly popular dry + homemade food as follows by popular dry food.

***Table 1.*** *Haematology and serum biochemistry variables measures in CG and PG before and 15 days after OVH*
***Tablo 1.*** *Kontrol ve pyometra grubu köpeklerde ovariohisterektomi öncesi ve 15 gün sonrası hematoloji ve serum biyokimyası değerleri*

| Variable | Control (n=8) Mean (±SE) | Pyometra (n=27) Mean (±SE) | T-test Significance | Group | T | G×T |
|---|---|---|---|---|---|---|
| RBC 0th day (×10⁶ µL) | 6.74±1.35 | 5.82±0.20 | * | * | NS | NS |
| RBC 15th day (×10⁶ µL) | 6.63±0.54 | 5.59±0.20 | * | | | |
| Significance | NS | NS | | | | |
| WBC 0th day (×10³ µL) | 14.03±1.70 | 38.30±5.25 | * | * | NS | NS |
| WBC 15th day (×10³ µL) | 14.11±1.51 | 19.50±2.34 | NS | | | |
| Significance | NS | ** | | | | |
| HCT 0th day (%) | 39.07±1.28 | 47.50±2.68 | ** | ** | NS | NS |
| HCT 15th day (%) | 37.93±1.42 | 46.88±8.94 | ** | | | |
| Significance | NS | NS | | | | |
| HGB 0th day (g/dL) | 15.54±0.10 | 12.79±0.40 | ** | ** | NS | NS |
| HGB 15th day (g/dL) | 15.20±1.10 | 12.23±0.50 | ** | | | |
| Significance | NS | NS | | | | |
| PLT 0th day (×10³ µL) | 496.75±79.60 | 314.30±35.00 | * | NS | NS | NS |
| PLT 15th day (×10³ µL) | 463.88±77.52 | 470.40±53.26 | NS | | | |
| Significance | NS | ** | | | | |
| MCV 0th day fl | 72.00±1.48 | 67.30±0.54 | *** | ** | NS | NS |
| MCV 15th day fl | 72.00±1.74 | 67.37±1.29 | NS | | | |
| Significance | NS | NS | | | | |
| MCH 0th day (pg) | 23.50±0.33 | 21.90±0.21 | *** | *** | NS | NS |
| MCH 15th day (pg) | 23.25±0.31 | 21.48±0.23 | *** | | | |
| Significance | NS | * | | | | |
| MCHC 0th day (%) | 32.13±0.44 | 32.63±0.24 | NS | NS | *** | NS |
| MCHC 15th day (%) | 31.50±0.42 | 31.37±0.26 | NS | | | |
| Significance | * | *** | | | | |
| Glucose 0th day (mg/dL) | 109.8±5.37 | 103.60±7.90 | NS | NS | NS | NS |
| Glucose 15th day (mg/dL) | 104.13±5.88 | 100.44±2.92 | NS | | | |
| Significance | NS | NS | | | | |
| Urea 0th day (mg/dL) | 30.8±3.4 | 42.22±8.8 | NS | NS | NS | NS |
| Urea 15th day (mg/dL) | 36.25±9.6 | 44.74±8.0 | NS | | | |
| Significance | NS | NS | | | | |
| Creatinine 0th day (mg/dL) | 0.9±0.05 | 1.10±0.13 | NS | NS | NS | NS |
| Creatinine 15th day (mg/dL) | 0.9±0.06 | 1.09±0.14 | NS | | | |
| Significance | NS | NS | | | | |
| AST 0th day (IU/L) | 26.13±1.73 | 47.80±7.61 | NS | NS | NS | NS |
| AST 15th day (IU/L) | 27.3±2.2 | 31.10±3.70 | NS | | | |
| Significance | NS | * | | | | |
| ALT 0th day (IU/L) | 40.5±8.94 | 28.89±4.30 | NS | NS | NS | NS |
| ALT 15th day (IU/L) | 38.3±7.73 | 33.52±6.32 | NS | | | |
| Significance | NS | NS | | | | |

***Abbreviations:*** ***CG****, control group;* ***PG,*** *pyometra group;* ***OVH,*** *ovariohysterectomie;* ***RBC,*** *red blood cell;* ***HCT,*** *hematocrit;* ***HGB,*** *hemoglobin;* ***PLT,*** *platelet;* ***MCV,*** *medium cell volume;* ***MCH,*** *medium cell hemoglobin;* ***MCHC,*** *medium cell hemoglobin concentration;* ***AST,*** *aspartate aminotransferase;* ***ALT,*** *alanine aminotransferase;* ***T****, Time;* ***G×T,*** *Group × Sampling Time interaction; Significance, comparison within the same group for each sampling time. *P<0.05, **P<0.01 ***P<0.001,* ***NS:*** *P>0.05*

***Table 2.*** *Antioxidant status and inflammatory mediators of bitches in CG and PG before and 15 days after OVH*
***Tablo 2.*** *Kontrol ve pyometra grubu köpeklerde ovariohisterektomi öncesi ve 15 gün sonrasında antioksidan ve inflamatorik mediyatörlerin durumu*

| Variable | Control (n=8) (Mean±SE) | Pyometra (n=27) (Mean±SE) | T-test Significance | Group | T | G×T |
|---|---|---|---|---|---|---|
| SOD 0th day (ng/mL) | 1.71±0.35 | 1.26±0.19 | NS | * | NS | NS |
| SOD 15th day (ng/mL) | 1.91±0.30 | 1.07±0.17 | * | | | |
| Significance | NS | NS | | | | |
| CAT 0th day (ng/mL) | 1.05±0.20 | 0.88±0.11 | NS | NS | NS | NS |
| CAT 15th day (ng/mL) | 1.56±0.27 | 1.03±0.15 | NS | | | |
| Significance | NS | NS | | | | |
| TBARS 0th day (nmol/mL) | 1.94±0.30 | 1.07±0.16 | * | * | * | NS |
| TBARS 15th day (nmol/mL) | 1.14±0.23 | 0.92±0.12 | NS | | | |
| Significance | NS | NS | | | | |
| CRP 0th day (mg/L) | 2.06±8.03 | 58.35±4.37 | *** | *** | * | * |
| CRP 15th day (mg/L) | 1.69±5.47 | 31.49±2.98 | *** | | | |
| Significance | NS | *** | | | | |
| IL-6 0th day (ng/L) | 10.19±9.20 | 63.73±5.01 | *** | *** | *** | *** |
| IL-6 15th day (ng/L) | 10.10±3.78 | 20.62±2.06 | * | | | |
| Significance | NS | *** | | | | |
| TNF-α 0th day (ng/L) | 4.89±3.84 | 14.03±2.09 | * | * | NS | NS |
| TNF-α 15th day (ng/L) | 4.44±1.80 | 6.13±0.98 | NS | | | |
| Significance | NS | ** | | | | |

***Abbreviations: : CG,*** *control group;* ***PG,*** *pyometra group;* ***OVH,*** *ovariohysterectomie;* ***SOD,*** *super oxide dismutase;* ***CAT,*** *catalase;* ***TBARS,*** *thiobarbituric acid reactive substances;* ***C-RP, C*** *reactive protein;* ***IL-6,*** *interleukin 6;* ***TNF-α,*** *tumour necrosis factor alpha.* ***T,*** *Time;* ***G×T,*** *Group × Sampling Time interaction; Significance, comparison within the same group for each sampling time. *P<0.05, **P<0.01, ***P<0.001,* ***NS:*** *P>0.05*

### *Clinical Signs, Haematology and Biochemical Parameters*

The most common clinical signs in PG were vaginal discharge (open-cervix; 81.48%), inappetance (51.85%), polyuria/polydipsia (48.15%), lethargy (33.33%), weight loss (25.92%), and vomiting (14.81%). Mean BCS of PG was 3.15±0.72 and CG was 3.25±0.46. Mean body temperature of PG was 38.58±0.14°C, and CG was 38.16±0.08°C. Heart rate was 100±18 beats $min^{-1}$ in PG, and 90±16 beats $min^{-1}$ was in CG. Respiratory rate was 30±12 breaths $min^{-1}$ in PG, and 20±6 breaths $min^{-1}$ was in CG.

The mean and the standard error values of CBC and biochemical parameters are presented in *Table 1*. At 0th day in PG, WBC count ranged from 9.0-105.0 ($\times 10^3$ µL), and in CG 9.4-16.6 ($\times 10^3$ µL). WBC counts decreased after OVH, but were still elevated in 16 bitches in PG. The mean and the standard error values of SOD, CAT, TBARS, TNF-α, CRP, and IL-6 are presented in *Table 2*.

### *Leukocyte Profile and NSI*

Leukocyte profile and NSIs of all bitches are presented in *Table 3* and *Table 4*, respectively. Although WBC counts were in normal range, NSI were high in two bitches with pyometra (#p22, #p23) before, and 15 days after OVH in #p22 *(Table 4, Table 5)*. PLT counts were high in accordance with NSIs in #p22 and #p23 bitches before, and 15 days after OVH in #p22. Both bitches had open cervix pyometra. The graphs in *Fig. 1* provide a good example of how three bitches with pyometra responded to uterine infection differently.

### *Histopathology*

Endometrial epitheliums were enlarged, columnar, and vacuolated in both groups. Papillary proliferations and hyperplasia were seen on the surface of the epithelium. In the CG, endometrial proliferation and glandular elongation were prominent. The stroma of the uterine tissues was oedematous and secretions were frequently present in the lumens of the glands.

In the PG, endometrium had moderate to severe cellular infiltration composed of many viable and degenerative neutrophils, fewer epitheloid macrophages, plasma cells and lymphocytes. Inflammatory cells, fibrin and karyorrhectic debris diffusely filled the lumen infiltrating the ulcerated endometrium. The surface epithelium was composed of columnar cells with foamy cytoplasm partially detached from the lamina propria. Varying sized cystic glands and papillary changes in the glandular epithelium were also found. Endometrial lymphatics were ectatic and blood vessels were congested.

***Table 3.*** *Leucocyte profile and segmented neutrophils' of bitches in CG and PG before and 15 days after OVH*
***Tablo 3****. Kontrol ve pyometra grubu köpeklerde ovariohisterektomi öncesi ve 15 gün sonrasında lökosit profili ve nötrofil segmentasyonu*

| Variable | Control (n=8) Mean (±SE) | Pyometra (n=27) Mean (±SE) | T-Test Significance | Group | T | G×T |
|---|---|---|---|---|---|---|
| Band neutrophil 0th day | 23.88±2.87 | 35.63±3.74 | NS | | | |
| Band neutrophil 15th day | 21.88±2.42 | 18.56±1.75 | NS | NS | * | NS |
| Significance | NS | NS | | | | |
| Two segmented neutrophil 0th day | 20.00±1.30 | 13.30±1.43 | * | | | |
| Two segmented neutrophil 15th day | 25.63±2.01 | 16.22±1.50 | ** | ** | * | NS |
| Significance | NS | NS | | | | |
| Three segmented neutrophil 0th day | 20.00±1.74 | 12.90±1.40 | * | | | |
| Three segmented neutrophil 15th day | 18.25±1.86 | 20.11±1.77 | NS | NS | NS | * |
| Significance | NS | NS | | | | |
| Four segmented neutrophil 0th day | 6.63±1.92 | 8.30±1.33 | NS | | | |
| Four segmented neutrophil 15th day | 7.00±0.80 | 10.63±1.15 | NS | NS | NS | NS |
| Significance | NS | NS | | | | |
| Five segmented neutrophil 0th day | 1.88±0.90 | 3.33±0.84 | NS | | | |
| Five segmented neutrophil 15th day | 1.25±0.62 | 3.96±1.33 | NS | NS | NS | NS |
| Significance | NS | * | | | | |
| Six segmented neutrophil Day 0 | 0.13±0.13 | 0.96±0.47 | NS | | | |
| Six segmented neutrophil 15th day | 0 | 1.00±0.30 | NS | NS | NS | NS |
| Significance | NS | ** | | | | |
| Seven segmented neutrophil 0th day | 0 | 0.19±0.9 | NS | | | |
| Seven segmented neutrophil 15th day | 0 | 0.44±0.21 | NS | NS | NS | NS |
| Significance | NS | * | | | | |
| Eight segmented neutrophil 0th day | 0 | 0.04±0.04 | NS | | | |
| Eight segmented neutrophil15th day | 0 | 0.11±0.06 | NS | NS | NS | NS |
| Significance | NS | NS | | | | |
| Nine segmented neutrophil 0th day | 0 | 0 | NS | | | |
| Nine segmented neutrophil 15th day | 0 | 0 | NS | NS | NS | NS |
| Significance | NS | NS | | | | |
| Lymphocyte 0th day | 17.25±2.99 | 11.37±1.46 | NS | | | |
| Lymphocyte 15th day | 17.13±1.46 | 16.00±1.47 | NS | NS | NS | NS |
| Significance | * | NS | | | | |
| Monocyte 0th day | 7.88±1.01 | 9.67±1.12 | NS | | | |
| Monocyte 15th day | 3.38±0.94 | 5.41±0.60 | NS | NS | ** | NS |
| Significance | * | NS | | | | |
| Eosinophil 0th day | 2.13±1.63 | 0.44±0.19 | NS | | | |
| Eosinophil 15th day | 5.50±1.13 | 3.89±0.85 | NS | NS | ** | NS |
| Significance | NS | NS | | | | |
| Basophil 0th day | 0 | 0.07±0.05 | NS | | | |
| Basophil 15th day | 0 | 0.33±0.13 | NS | NS | NS | NS |
| Significance | NS | NS | | | | |

***Abbreviations:*** ***CG,*** *control group;* ***PG,*** *pyometra group;* ***OVH,*** *ovariohysterectomie;* ***T,*** *Time;* ***G×T,*** *Group × Sampling Time interaction; Significance, comparison within the same group for each sampling time.*P<0.05, **P<0.01, ***P<0.001,* ***NS:*** *P>0.05*

***Table 4.*** *0th day and 15th day of NSIs' in both groups of bitches*
***Tablo 4.*** *Her iki gruptaki köpeklerde 0. ve 15. gün nötrofil segmentasyon indeksleri*

| Bitch no. | 0th Day NSI | 15th Day NSI | Bitch no. | 0th Day NSI | 15th Day NSI |
|---|---|---|---|---|---|
| P1 | 1.27 | 0 | P19 | 0 | 3.61 |
| P2 | 4.84 | 5.66 | P20 | 4.41 | 3.61 |
| P3 | 0 | 4.94 | P21 | 1.27 | 0 |
| P4 | 0 | 2.63 | P22 | **70.27** | **102.86** |
| P5 | 0 | 10.53 | P23 | **44.23** | 3.33 |
| P6 | 0 | 0 | P24 | 2.53 | 3.33 |
| P7 | 2.38 | 13.43 | P25 | 0 | 7.23 |
| P8 | 7.14 | 4.94 | P26 | 3.57 | 10.53 |
| P9 | 0 | 2.63 | P27 | 4.05 | 0 |
| P10 | 16 | 3.61 | C1 | 9.33 | 1.39 |
| P11 | 0 | 3.92 | C2 | 1.82 | 1.56 |
| P12 | 5.88 | 3.92 | C3 | 2.53 | 0 |
| P13 | 1.30 | 0 | C4 | 5.17 | 5.71 |
| P14 | 12.68 | 13.43 | C5 | 0 | 0 |
| P15 | 3.23 | 0 | C6 | 2.86 | 5.71 |
| P16 | 2.66 | 3.61 | C7 | 0 | 0 |
| P17 | 14.47 | 2.63 | C8 | 1.39 | 0 |
| P18 | 10.66 | 7.23 | | | |

***Abbreviations; P,*** *pyometra bitches;* ***C,*** *control bitches. Values bold indicate high segmentation index with significance*

***Table 5.*** *Haematology variables measures in #p22 and #p23 bitches before and 15 days after OVH*
***Tablo 5.*** *p22 ve p23 numaralı köpeklerde ovariohisterektomiden hemen önce ve 15 gün sonrasına ait hemogram değerleri*

| Variable | #p22 | #p23 | Reference Intervals |
|---|---|---|---|
| RBC 0th day (×10⁶ µL) | 6.28 | **5.24** | 5.6-8 |
| RBC 15th day (×10⁶ µL) | 7.50 | 5.60 | |
| HGB 0th day (g/dL) | **13.70** | **11.30** | 14-19 |
| HGB 15th day (g/dL) | **15.90** | **12** | |
| HCT 0th day (%) | 43 | **37** | 40-55 |
| HCT 0th day (%) | 52 | **35** | |
| WBC 0th day (×10³ µL) | 15.90 | 9 | 6-16.6 |
| WBC 15th day (×10³ µL) | 12.40 | 7 | |
| PLT 0th day (×10³ µL) | **571** | **490** | 150-400 |
| PLT 15th day (×10³ µL) | **516** | 400 | |
| MCV 0th day fl | 69 | 70 | 65-75 |
| MCV 15th day fl | 70 | 70 | |
| MCH 0th day (pg) | 22 | 22 | 22-26 |
| MCH 15th day (pg) | **21** | 23 | |
| MCHC 0th day (%) | **32** | **31** | 33-36 |
| MCHC 15th day (%) | **30** | **32** | |

***Abbreviation: OVH,*** *ovariohysterectomie. Bold values indicate abnormality*

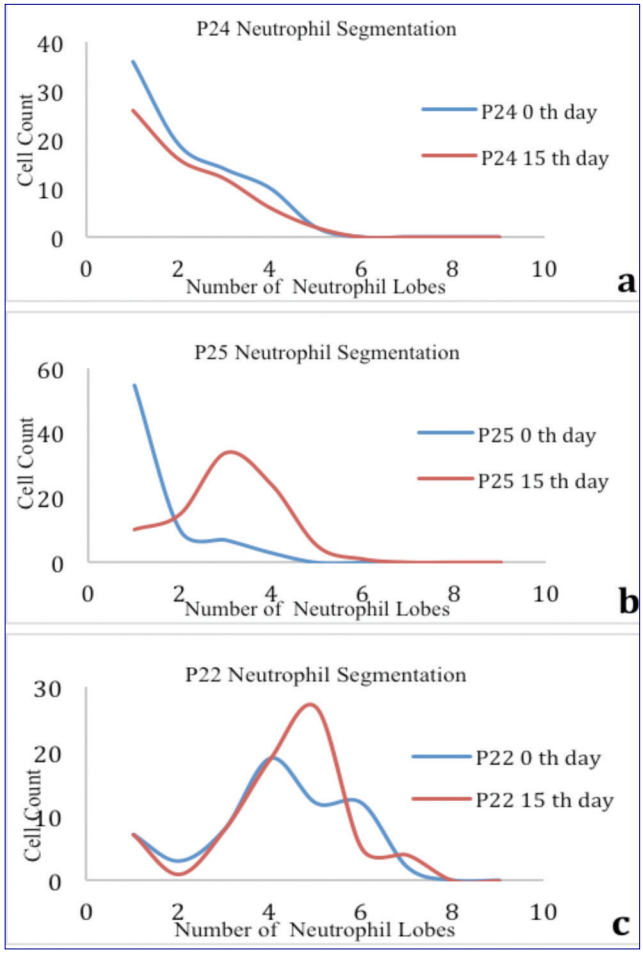


**Fig 1.** Neutrophilic segmentation profiles in #p24, #p25, #p22 bitches with pyometra. Blue line shows the first blood sampling (0th day), red line shows the second blood sampling (15th day). X-axis shows the segmentation of the neutrophils (0-9 lobes), and Y-axis shows the number of neutrophils. a. #p24 shows left shift at 0th and 15th days, b. #p25 shows left shift at 0th day, and moderate right shift at 15th day, c. #p22 shows intense right shift at both sampling days

**Şekil 1.** p24, p25 ve p22 no'lu pyometralı köpeklerde nötrofil segmentasyon profili. Mavi çizgi ilk kan örneklemesini (0. gün), kırmızı çizgi ikinci kan örneklemesini (15. gün) göstermektedir. X ekseni nötrofil segmentasyonunu (0-9 lob), Y ekseni nötrofil sayısını göstermektedir. a. #p24 0. ve 15. günlerde sola kayma, b. #p25 0. günde sola kayma ve 15. günde orta düzeyde sağa kayma, c. #p22 ise her iki günde de sağa kayma göstermektedir

## DISCUSSION

One of the main aims of our study was to determine a clinically more specific marker in veterinary medicine for the prognosis of pyometra by measuring biochemical parameters. The ideal parameter should estimate the morbidity and mortality of critically ill patients at high risk of development SIRS and consequently MODS. In the present study, comparison was made between the evaluation of CBCs, NSI, IL-6, TNF-α, CRP, SOD, CAT, and TBARS in bitches with or without pyometra before and after 15 days of OVH.

In our study mortality rate in PG was 0% within the 15

days of OVH. In a previous study, the mortality rate in PG was 4% [5]. In humans with pyometra accompanied by SIRS, the mortality rate was found 6-7% [14] that is much higher compared to dogs. Finding of 0% mortality in our study indicates that the risk of development of MODS through SIRS in PG is very low. Because the majority of bitches in our study had open-cervix pyometra this might lowered the endotoxemia and SIRS and consequently impacts on the mortality rate. Oxidative stress was not obviously occurred in PG in this study and also this might reflected on the mortality rate. Respiratory and heart rates and body temperatures were similar with the previous studies [5,15]. Breed distribution in PG was similar with the breeds with high risk, but three German shepherd bitches in PG that was determined to have a low risk of developing pyometra was in contrast by Egenvall et al.[4].

Although BW of bitches in PG presented higher than CG in our study, BCSs were higher in CG. Our result with higher BW in PG was in accordance with the previous study [14], but our opinion here BCSs is more predictive value about the adipose tissue in the body and this high BW of the bitches in PG only shows more medium and large breed of dogs involved in the PG. Inflammatory parameters such as CRP, IL-6 and TNF-α are known to systematically rise in obesity [16,17], but higher levels of those in PG were associated with the inflammation of the uterus, not with the obesity because of this group had normal BCS.

Of the variables, RBC, WBC, PLT, HCT, HGB, MCH and MCV showed significantly different between the PG and CG at 0$^{th}$ day in this study. Lower levels of RBC, MCH, and MCV in PG show microcytic anaemia and also high HCT levels shows dehydration of the bitches which points the chronic phase of the disease. Microcytic anaemia in PG may be occurred because of the blood loss into the uterus, iron deficiency, and toxic effects of the pyometra on the bone marrow or lipid peroxidation damage on erythrocytes membranes. In contrast with the other studies [5,18] none of the biochemical variables were found significantly different between the groups, before and 15 days after OVH. PLT was significantly lower in PG before the OVH, but increased after 15 days. This result of decreased PLT in pyometra bitches was in accordance with the previous studies [5,19] and it was noted that bitches with endometritis-pyometra were under risk of disseminated intravascular coagulation (DIC), which decreases the PLT, and other coagulation factors and increase the risk of haemorrhage [19]. In another study [20], it was revealed plasma coagulation factors were decreased in dogs with sepsis but PLT levels were not differed. Haemorrhagic shock after the day of the pyometra surgery was seen previously in a bitch [21] and diagnosed as DIC. Also some studies [22,23] have shown that *Escherichia coli* endotoxemia is responsible for DIC in bitches with pyometra. In contrast, in our study, PLT levels were higher in some bitches with open cervix-pyometra for example in two bitches (#p22, #p23) with high NSI that shows the chronic phase of the pyometra disease. PLTs are known to be having an important role in inflammatory processes and in a study [24] high PLT and plateletcrit levels were found to be significantly associated with advanced endometriosis that is a chronic inflammatory disease in women. In our opinion, in chronic phase of pyometra especially in open-cervix pyometra which is not performed for a long time because of the unconcern of the owner, PLT level and NSI get higher because of the chronic inflammation of the uterus and aging of neutrophils in circulation, but in closed-cervix pyometra endotoxemia occurs more rapidly causing DIC with thrombocytopenia and consequent haemorrhage.

Cytokines acts as mediators in immune system may give valuable information about the mechanism, the severity and the prognosis of the disease [25]. In our study we focused on IL-6 and TNF-α cytokines that are considered to induce synthesis of acute phase proteins such as CRP. Previously, IL-6 has been shown to have major importance in acute phase response and hepatic regeneration [25]. In our study, IL-6 values were significantly different before and 15 days after OVH in PG and higher than expected (3.24-16.6 ng/L) in CG. Neuman et al.[25] reported that IL-6 concentration in healthy bitches should be <1 pg/mL, this value is similar with the human studies [26,27] and with that study it was found that IL-6 concentrations were much higher in dogs with acute disease. Because pyometra is an acute disease, measurement of IL-6 concentrations in pyometra cases may give an opinion about the course of the disease. In that study, IL-6 level in bitches with pyometra similar with dogs with acute hepatitis and varied between 1-202 pg/mL. In a newer study [28] revealed that, IL-6 concentrations were ranged from 45-4656 pg/mL in the non-surviving dogs while the surviving dogs had a range of 0-405 pg/mL in an intensive care unit. IL-6 found to be a good prognostic factor in critically ill dogs ($P$<0.001) but its concentrations in bitches with pyometra (a part of the survivors) were not given to the reader [28]. Our results of IL-6 concentrations within the accordance with this study, and none of the bitches died during the study. In another study [5], IL-6 concentrations were not significantly differed between pyometra and control groups, although Dabrowski et al.[29] found out that assessment of serum IL-6 and IL-10 were good prognostic factors in the healing period after OVH in bitches with pyometra.

The TNF-α concentrations were significantly higher before and 15 days after OVH in PG. This result is in accordance with the Fransson et al.[5]. It is known that TNF-α has a very short half-life and difficult to detect after starting of an infection within 24 h [5]. In our opinion, high levels of TNF-α concentrations after OVH might due to continuing of systemic infection in some bitches with pyometra in our study. The CRP concentrations were significantly higher before and 15 days after OVH in PG. This result is in accordance with the other studies and it was

able to distinguish SIRS status with CRP concentration [5,18,30]. In our opinion, CRP can serve clinically as an important marker in pyometra for the determination of the severity and continuity of the inflammation in case of no other inflammation occurs in other parts of the body.

It is reported that TBARS and CAT levels increase but SOD levels decrease by age [8,31-34]. In our study, SOD and CAT values were higher in CG although not significantly difference was found between the groups before OVH. According to our results none of the groups did show oxidative stress before and after OVH and additionally CAT and TBARS values were elevated after 15 days of OVH exception of SOD value. In another study [35] in healthy bitches, OVH was related with oxidative stress after 30 days of the surgeries. Our results have shown accordance with the earlier results of that study. Also similar results of oxidative stress parameters in PG and CG may be explained with the majority of the bitches in our study had open-cervix pyometra. The food type were fed to the bitches was not constant in our study but very much similar between the both groups and the results of any parameter in this study was not affected by the diet.

A response characterized by leukocytosis with a left shift and neutrophilia was mostly apparent in PG before the surgery. Each bitch was responded in different ways to inflammation (pyometra) and stress (OVH) as observed by neutrophil segmentation profiles *(Fig. 1a-c)*. Some of the bitches in PG still had an infection 15 days after OVH as shown by a left shift *(Fig. 1a)*, ceasing of the infection *(Fig. 1b)*, and had leukocytosis or normal leucocyte number with a right shift (high NSI) before and after OVH *(Fig. 1c)*. This bitch *(Fig. 1c)* is believed to have a uterine infection for longer duration before seeking a veterinary care. Right shift-hypersegmentation of neutrophils is a nonspecific indicator of aging of these cells in circulation [36] and refers to chronicle of the disease like in these two bitches with pyometra (#p22, #p23). Band neutrophils were found higher but not significant before OVH in PG while in other studies significantly higher in pyometra bitches [5,19]. In our study, two segmented neutrophils were found significantly lower in PG before and after OVH. Lymphopenia, eosinophilia, and monocytosis were seen in PG before OVH and this result within the accordance with a similar study [37].

Different breeds of dogs that have different cytokines, hormones and humoral factors, and many different breeds were included in our study and in our opinion this heterogeneity might affected some of our results.

As a conclusion, CRP and IL-6 could be used for inflammation detection after OVH in pyometra. Oxidative stress was not apparent with the parameters of TBARS, CAT, and SOD in PG. Also, surgery itself did not generate oxidative stress in bitches of both groups as detected after 15 days of the OVHs. Because thrombocytosis is a marker of chronic inflammation [24], in future studies PLT and other factors relevant with PLT should be determined for the chronic phase of the pyometra and sepsis in bitches. Additionally, NSI can give very valuable information about the severity of the pyometra and blood smear should be prepared from especially critically ill cases to see how immune system respond to an infection by an individual.

## Acknowledgement

The authors thank Prof. Dr. Bülent Ekiz for his assistance with the statistical analyses.

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