


# SOD, CAT, TBARS, and TNF- $\alpha$ Concentrations in Uterine Tissues of Bitches with Pyometra and Dioestrus Bitches

## Pyometralı ve Sağlıklı Diöstrustaki Köpeklerde uterus SOD, CAT, TBARS ve TNF- $\alpha$ Konsantrasyonları

Tuğba Seval Fatma TOYDEMİR KARABULUT 

Department of Gynaecology and Obstetrics, İstanbul University Faculty of Veterinary Medicine, İstanbul, Turkey

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ORCID ID of the author: T.S.F.T.K. 0000-0001-6416-8337

### Abstract

Decreased antioxidant levels may be a cause of many diseases. Pus accumulation in the uterus literally called pyometra and generally occurs in median or late life span of bitches. The objective of the study was to evaluate the concentrations of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), oxidative stress parameter-thiobarbituric acid-reactive substances (TBARS) and inflammatory mediator, tumour necrosis factor alpha (TNF- $\alpha$ ) in uterine tissues of bitches with and without pyometra. For this purpose, bitches with pyometra (n=27), and healthy bitches in dioestrus (n=8) included in the study and both of the groups went under ovariohysterectomy ope-

ration. SOD and CAT enzymes were found significantly higher in uterine tissues of dioestrus group (p<0.01, p<0.05, respectively). TNF- $\alpha$  did not differ in uterine tissues between the groups (p>0.05). Our data showing that decreased concentrations of antioxidant enzymes in the uterus occurs during the disease of pyometra in bitches. Due to antioxidants cannot deactivate free radicals occurred during the inflammatory process probably one of the main cause for uterine tissue damage in pyometra.

**Keywords:** Pyometra, oxidative stress, uterus, dioestrus, TNF- $\alpha$ , dog

### Öz

Azalmış antioksidan seviyeleri pek çok hastalığın sebebi olabilmektedir. Pyometra orta yaşlı ve yaşlı kısırlaştırılmamış dişi köpeklerde görülen uterusun irinle dolmasıyla karakterize bir hastalıktır. Çalışmamızın amacı oksidatif stres parametrelerinden süperoksit dizmutaz (SOD), katalaz (CAT), tiyobarbitürik asid maddeleri (TBARS) ve enflamatorik mediyatörlerden tümör nekroz faktörünün (TNF- $\alpha$ ) pyometralı köpek uterusunda ve sağlıklı köpek uterusunda bakıp karşılaştırarak gruplar arasındaki olabilecek varyasyonu tespit etmektir. Bu amaçla, pyometralı köpekler (n=27) ve diöstrustaki sağlıklı köpekler (n=8) çalışmaya dahil edildi ve her iki gruptaki köpek-

lere de ovariohisterektomi operasyonu uygulandı. Uterusta SOD ve CAT seviyeleri kontrol grubunda önemli oranda yüksek bulundu (sırasıyla, p<0,01, p<0,05). TNF-alfa konsantrasyonları gruplar arasında farklılık göstermemiştir. Elde ettiğimiz sonuçlar göstermektedir ki pyometra esnasında köpeklerde uterustaki antioksidan aktivitesi düşmektedir. Pyometra sürecinde antioksidan seviyesinin düşmesi serbest radikallerin artışına sebep olmaktadır ve bu da hastalık sürecindeki uterustaki doku yıkımının başlıca sebeplerindedir.

**Anahtar kelimeler:** Pyometra, oksidatif stres, uterus, diöstrus, TNF-alfa, köpek

### Introduction

Free radicals are a reactive and unstable ion species, which become stable by acquiring electrons from wherever they can,

which causes some adverse reactions resulting in cellular impairment. There are two types of free radicals: reactive oxygen species (ROS) and reactive nitrogen species (NOS) (Agarwall et al., 2005). Superoxide radical ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ )

**Address for Correspondence:** Tuğba Seval Fatma Toydemir Karabulut • **E-mail:** sevaltoydemir@yahoo.com

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and hydroxyl radical ( $\text{OH}^\cdot$ ) are referred as ROS and have become more recognizable in the last few decades. ROS are responsible for DNA damage, and thus are a cause of underlying diseases (Ercan et al., 2012, Todorova et al., 2005). More than 100 diseases including female reproductive disorders have been associated with ROS (Agarwall et al., 2005). Overabundance of ROS is termed "oxidative stress", and this stress is possibly an initiator of certain pregnancy-related disorders such as early pregnancy loss and embryopathies, in both humans and animals (Al-Gubory et al., 2010). Oxidative stress and antioxidants during pregnancy have been analysed in many studies on mammalian species (Gupta et al., 2007; Santos et al., 2004). It is clear that there is a link between oxidative stress and disorders of the female reproductive system, which is important in both human and animal reproduction (Al-Gubory et al., 2010).

The causes of infertility in bitches are difficult to identify and generally remain an unsolved problem in pet clinics. Infertility in bitches is presumed mainly caused by infectious agents (*Brucella canis*, *Campylobacter spp.*, *Escherichia coli*, etc.), however, there are other causes including uterine lesions, hormonal imbalances, systemic diseases (Mir et al., 2013) as well as oxidative stress (Agarwall et al., 2005, Burton and Jauniaux, 2011). Diseases related to oxidative stress and inflammation has been reported in numerous previous studies in dogs. When oxidative stress occurs, there may be no clinical signs, however, more energy is used for scavenging free radicals rather than for reproduction (Mohebbi-Fani et al., 2016) and this may lead to infertility in dogs.

Reactive oxygen species can be deactivated by antioxidants, which are 1) vitamins (A, C, and E) and 2) antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; and glutathione peroxidase, GPX). Antioxidants protect against peroxidation and DNA impairment (Al-Gubory et al., 2010, Todorova et al., 2005). If the balance between antioxidants and ROS fails, ROS is overproduced and binds to unsaturated fatty acids on cell membranes. This reaction is called "lipid peroxidation" and can be measured as thiobarbituric acid-reactive substances (TBARS) to show the degree of lipid peroxidation in an organism.

Superoxide dismutase is the first part of the defence mechanism during oxidative stress conditions and its main function is catalysing detoxification of superoxide radicals ( $\cdot\text{O}_2^-$ ) (Nakano et al., 1996, Todorova et al., 2005). Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is generated at the end of this reaction and together with GPX, CAT degrades  $\text{H}_2\text{O}_2$  into water and oxygen. CAT is a more active antioxidant enzyme under high concentrations of  $\text{H}_2\text{O}_2$  (Szczybal and Dabrowski, 2009).

Pyometra is sometimes a life-threatening disease, arising as a consequence of the systemic inflammatory response syndrome (SIRS). SIRS can be detected by determining the concentrations of cytokines in dogs, like in humans (Karlsson et al., 2012). Cytokines are mainly protein constructed elements that

are released into the inter cellular space by leucocytes. SOD also neutralizes superoxide radicals formed by tumour necrosis factor alpha (TNF- $\alpha$ ) cytokine (Agarwall et al., 2005).

In our study, oxidative stress biomarkers were chosen according to the results of some previous studies. In those studies, Cu SOD, Mn SOD, CAT and GPX (Sugino et al., 2000), malondialdehyde (MDA) and TBARS (Jozwik et al., 1999) were revealed to be present in the female reproductive tract.

The present study compared the level of oxidative stress by using TBARS, SOD and CAT, and the inflammatory mediator TNF- $\alpha$  during the dioestrus period in healthy bitches and bitches with pyometra. The hypothesis of the study was that, when the uterus gets infected, oxidative stress probably occurs in the uterine tissues, affecting the reproductive performance of bitches and also increasing the risk of pyometra disease.

## Materials and Methods

### Dogs

The studies were performed on 27 dogs with pyometra, with a mean body weight of  $20.18 \pm 12.41$  kg (pyometra group; PG), and 8 clinically healthy dogs in dioestrus with a mean body weight of  $15.15 \pm 14.72$  kg (dioestrus group; DG). There was only one young dog in each group while all others were geriatric in both groups. Dogs in both groups were free of common infectious diseases. Bitches were considered to be in dioestrus according to their vaginal cytology results (appearance of intermediate and parabasal cells, neutrophils, and large numbers of bacteria in vaginal smears) in DG. Five dogs in PG had closed-cervix pyometra while twenty-two had open-cervix pyometra. Physical examinations were performed on each dog to evaluate the general condition in both groups. Results of vaginal examination, vaginal cytology and ultrasonography were used to differentiate pyometra from other uterine pathologies such as mucometra and hydrometra. For this purpose, transabdominal ultrasonography was performed to visualise the uterus (Easote Piemedical MyLab Five Vet, Netherlands) and also vaginoscopy was used to see the cervix uteri and vagina. Visualisation of pus during vaginal examination and/or a hypoechoic fluid-filled uterus on ultrasonography was considered as pyometra.

### Tissue collection

Both groups of bitches underwent ovariohysterectomy (OVH). OVHs were performed under general anaesthesia, induced with an intravenous 4-8 mg/kg propofol solution (Propofol 1% Fresenius; Fresenius Kabi, DE) and continued with 2-4% isoflurane (Forane Liquid; Aesica Queenborough, UK). Excised uterine tissues were promptly rinsed and cleaned of blood and pus with cold saline to minimize the effect of blood on free radicals. Three parts of the uterus at different levels were taken for homogenization and stored in Eppendorf tubes at  $-86^\circ\text{C}$  until homogenization.

### Homogenization of uterine tissues and ELISA assays

Uterine tissues were first weighed and then rinsed with phosphate-buffered saline (PBS, pH 7.4) and homogenized (MIC-CRA-D1, ART Prozess&Labortechnik GmbH&Co. KG., Germany) in 5-10 ml cold PBS (proportion: 1/10). Uterine homogenates were centrifuged at 4°C for 30 min at 3000×g and then supernatants were analysed for TBARS, SOD, CAT and TNF-α. These parameters were measured with commercially available canine sandwich ELISA kits (TNF-α; canine TNF-α, CK-E90814; SOD; canine SOD ELISA, CK-E91351; CAT; canine CAT ELISA, CK-E91349; TBARS; canine TBARS ELISA, CK-E91350; Hangzhou Eastbio-pharm Co. Ltd., China) by the quantitative sandwich enzyme immunoassay technique (μQuant, Bio-Tech Instruments, High Point, NC, USA) according to the manufacturer's instructions. The detection range of TNF-α was 5-1000 ng/L, SOD was 0.5-200 ng/mL, CAT was 1-300 ng/mL, TBARS was 0.5-100 nmol/mL and intra- and inter-assay coefficients of variations for all parameters were <10% and <2%, respectively.

### Statistical analysis

Groups (pyometra and dioestrus) were added to the statistical model as between-subject effects and an independent sample t-test was applied to compare each sampling time for uterine analyses. A value of  $p < 0.05$  was used to indicate statistical significance.

### Results

#### Uterine concentrations of SOD, CAT, TBARS and TNF-α

The mean concentrations in the groups are shown in Table 1. SOD, CAT and TNF-α were higher in uterine tissues of DG compared with PG. Of these variables, SOD and CAT levels were significantly different between the two groups ( $p < 0.01$ ,  $p < 0.05$ , respectively). Concentrations of SOD, CAT, TBARS and TNF-α did not differ between the open- and closed-cervix pyometra bitches.

### Discussion

Reactive oxygen species production is correlated with inflammatory processes. The consumption of oxygen by activated macrophages and neutrophils occurs during inflammation. An overabundance of ROS is seen if ROS production cannot be controlled by antioxidative mechanisms and this is one of the causes of tissue damage (Agarwal et al., 2005).

In this study, comparisons were made between SOD, CAT, TBARS and TNF-α concentrations in uterine tissues of bitches in the dioestrus period and bitches with pyometra. The main aim of this study was to determine the effect of ROS occurring during pyometra on levels of antioxidant enzymes SOD and CAT and also on the lipid peroxidation product TBARS and their relationships with a cytokine, TNF-α.

In our study, SOD concentrations were higher in DG, which comprises animals under the influence of progesterone. In a previous study, changes in superoxide anion and SOD level were detected during the oestrus cycle of *Rattus norvegicus*. Low levels of superoxide radical and high levels of SOD were observed during the dioestrus period (Laloraya et al., 1990). In another study, the expression of SOD was investigated in corpora lutea collected from women. The Cu-Zn SOD expression in the corpora lutea paralleled levels of progesterone and these levels rose from the early to the mid luteal phase and decreased during regression of the corpus luteum. Enhanced expression of Cu-Zn SOD in luteal tissue from pregnant patients was found and it was correlated with high levels of hCG (Sugino et al., 2000). In addition to this, it was revealed that estrogen lowers mammalian uterine SOD levels (Jain et al., 1999). Our results are in accordance with these studies. Additionally, when compared with DG, SOD levels were significantly lower in PG ( $p < 0.01$ ). This is likely due to the increased levels of ROS during inflammation and infection occurring in the uterus such as in the case of pyometra.

Our findings showed that pyometra disease significantly decreases the activity of the antioxidant enzymes, SOD and CAT ( $p < 0.01$ ,  $p < 0.05$ , respectively), however, unfortunately, the level of antioxidant activity in the uterus at the beginning of the infection in these dogs is not known. TBARS concentrations were increased as expected in PG but the difference between the groups was not significant ( $p > 0.05$ ). At the initiation of uterine infection, antioxidant enzymes probably increase because of cytokines released from the leucocytes. In another study in bitches, SOD and CAT levels were found to be similar between the uterine tissues of bitches with pyometra and healthy ones (Szczubial and Dabrowski, 2009). This result might have been obtained in that study due to the fact that those bitches were recognized promptly at the initiation of pyometra. The duration of infection is thought to affect the activity of SOD and CAT concentrations in uterine tissue.

**Table 1.** Uterine concentrations of SOD, CAT, TBARS and TNF-α

Variable	Dioestrus group (Mean±SE) (n=8)	Pyometra group (Mean±SE) (n=27)	t-test significance
SOD (ng/mL)	67.93±18.78	31.11±2.32	**
CAT (ng/mL)	67.69±18.52	39.40±3.80	*
TBARS (nmol/mL)	89.49±21.32	97.90±10.14	NS
TNF-α (ng/L)	371.45±64.56	342.99±32.85	NS

SOD: super oxide dismutase; CAT: catalase; TBARS: thiobarbituric acid reactive substances; TNF-α: tumour necrosis factor alpha

\* $p < 0.05$ ; \*\* $p < 0.001$ ; NS:  $p > 0.05$

In another study, in women with endometriosis, high TNF- $\alpha$  concentrations were detected in the peritoneal fluid (Bedaiwy and Falcone, 2003). Cytokines influence the redox status of the ectopic endometrium in patients with endometriosis. Also, the antioxidant MnSOD neutralizes superoxide anions generated by cytokine TNF- $\alpha$  (Agarwal et al., 2005). In our study, TNF- $\alpha$  concentrations did not differ in uterine tissues between DG and PG ( $p>0.05$ ) and no relationship was found between concentrations of TNF- $\alpha$  and pyometra occurrence.

In conclusion, our data showed that pyometra disease negatively changes the oxidative status of the uterus. Additionally, significantly decreased concentrations of SOD and CAT found in infected uteri compared with dioestrus uterine tissue show the importance of these antioxidant enzymes in the uterus. Also, it was assumed that one of the main causes of uterine damage in pyometra is the generation of free radicals during the inflammatory process, which cannot be deactivated by decreased levels of antioxidants.

**Ethics Committee Approval:** Ethics committee approval was received for this study from the ethics committee of İstanbul University (1702011).

**Informed Consent:** Written informed consent was obtained from patients' parents who participated in this study.

**Peer-review:** Externally peer-reviewed.

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