

DOI 10.24425/pjvs.2019.129963

*Original article*

# Uterine non-enzymatic antioxidant defence mechanisms (glutathione, vitamin C, copper and zinc) in diagnosis of canine pyometra

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## Abstract

The aim of the present study was to determine the concentrations of glutathione (GSH), vitamin C, copper (Cu) and zinc (Zn) in the uterine tissues in diagnosis of canine pyometra. Fourteen samples of uterine tissues from female dogs with pyometra and twelve samples of healthy uteruses (control) were used. The concentrations of GSH and vitamin C were determined in the uterine tissue homogenates using spectrophotometric methods. The concentrations of Cu and Zn were measured using atomic absorption spectrometer. The results obtained showed the significantly lower ( $p < 0.05$ ) concentration of GSH and the trend towards lower concentration of vitamin C in the pyometra samples compared to the control. The concentrations of Cu and Zn were similar in the uterine tissues from female dogs with pyometra and those from healthy female dogs. The lower GSH and vitamin C concentrations in the uterine tissues of female dogs with pyometra indicate that the non-enzymatic antioxidant mechanisms are impaired in the uterus of dogs with pyometra. These findings suggest that the imbalance of oxidative-antioxidative can play an important role in pathogenesis of canine pyometra.

**Key words:** oxidative stress, non-enzymatic antioxidants, uterus, pyometra, female dogs

## Introduction

Oxygen toxicity is an inevitable consequence of aerobic life and is associated with the production of reactive oxygen species (ROS) (Halliwell 2006, Kumar and Pandey 2015). At low concentrations of ROS are necessary for many processes in the organism, including maturation of cellular structures, cellular signalling, proliferation, differentiation, programmed cell death,

and phagocytosis (Pham-Huy et al. 2008, Ray et al. 2012). However, an uncontrolled production ROS leads to oxidative stress, which is a threat to the organism causing oxidative damage of enzymes, lipids, proteins, DNA and cell membranes, and results in tissue damage and disturbances in metabolism and physiological processes (Halliwell 2006, Durackova 2010).

The human and animal organism has enzymatic and non-enzymatic antioxidative mechanisms to counteract

oxidative stress by neutralizing or scavenging ROS or by breaking the chain reactions (Biswas 2016). The enzymatic antioxidative mechanisms include a variety of enzymes, the most important of which are superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT). In turn, the non-enzymatic antioxidative mechanisms, include a multitude of water-soluble and fat-soluble components, like vitamins C and E, glutathione and beta-carotene. The key non-enzymatic, water-soluble antioxidants include glutathione and vitamin C (Nordberg and Arner 2001, Halliwell 2006)

Glutathione (GSH) is a tripeptide synthesized in the cells from glutamate, cysteine and glycine. GSH has important antioxidative functions and is involved in xenobiotics detoxication (Meister and Anderson 1983). Antioxidative effects of GSH include ROS scavenging and participation in catalytic activities of such antioxidative enzymes as glutathione peroxidase (GSH-Px), glutathione transferase (GSH-Tr) and glutathione reductase (GSH-S-R) (Dringen 2000, Nordberg and Arnen 2001). GSH is the main source of thiol groups (-SH) in mammalian cells and is responsible for proper redox potential of thiol groups in the cell (Cotgreave and Gerdes 1998, Nordberg and Arnen 2001, Halliwell 2006).

Vitamin C (ascorbic acid) is considered as the most important antioxidant in extracellular fluids. Together with other water-soluble antioxidants, such as uric acid, bilirubin, albumins and thiol groups of proteins, it protects plasma lipids against oxidative damage. Moreover, vitamin C is involved in cellular antioxidative defence. At low levels and in the presence of transition metals, vitamin C can also act as a pro-oxidant (Frei *et al.* 1989, Naidu 2003).

Copper (Cu) and zinc (Zn) are cofactors of Cu-Zn-superoxide dismutase (Cu-Zn-SOD). Cu-Zn-SOD, the main antioxidative enzyme, catalyzes the dismutation of superoxide, which is constantly formed during aerobic metabolism (Klotz *et al.* 2003). Cu is also necessary for enzymatic action of ceruloplasmin, which has an antioxidant effect (Kankofer 2000). Cu and Zn may stimulate the synthesis of the metallothioneins – proteins having antioxidant properties (Klotz *et al.* 2003). Moreover, Cu and Zn stimulate protective cellular stress-signaling pathways, such as the antiapoptotic phosphoinositide-3-kinase/Akt cascade and may stabilize proteins, thereby rendering them less prone to oxidation (Klotz *et al.* 2003). Zn is also necessary to maintain the proper structure and function of cell membranes (Bray and Bettger 1990).

Oxidative stress and antioxidants are linked with a number of diseases in human and animals, including

disorders of the female reproductive system (Agarwal *et al.* 2005, Al-Gubory *et al.* 2010). Oxidative stress can occur if antioxidative defence mechanisms fail or if there is excess ROS formation (Sharma and Agarwal 2004). Numerous endogenous and exogenous conditions can induce oxidative stress (Halliwell 2006, Burton and Jauniaux 2011). One of the most important is inflammation (Mittal *et al.* 2014, Hussain *et al.* 2016, Biswas 2016). Inflammation and oxidative stress are closely related and tightly linked pathophysiological processes. One of them may appear before and after the other, but when one of them appears the other one is most likely to appear; and then both of them take part in the pathogenesis of many diseases (Biswas 2016).

Pyometra is a common inflammatory disease of uterus in female dogs (Smith 2006, Jitpean *et al.* 2012). It affects mainly older, nulliparous female dogs during the luteal phase of the oestrus cycle. Although the etiology of pyometra is still not completely understood it commonly accepted that hormonal and bacterial components play role in development this disease. It is thought that disease develops as a result of bacterial infection (mainly with *E. coli*) of the uterus, pathologically altered by progesterone influence (De Bosschere *et al.* 2001, Hagman 2017). Previous studies have shown the decreased activity of antioxidative enzymes in the uterine tissues from female dogs with pyometra (Szczubiał and Dąbrowski 2009, Toydemir Karabulut 2018); however, according to our knowledge, the concentrations of non-enzymatic antioxidants have not been examined yet.

The aim of the study was to evaluate the concentrations of the non-enzymatic antioxidants (GSH, vitamin C, Cu and Zn) in the uterine tissues from healthy female dogs and from female dogs with pyometra.

## Materials and Methods

The study was performed in accordance with animal protection regulations (Animal Experimentation Act dated 15 January 2015).

### Animals and design study

The study was performed on 26 female dogs of different breeds and mixed-breed undergoing ovariohysterectomy at the Department of Animal Reproduction, Faculty of Veterinary Medicine in Lublin. Fourteen dogs, aged 6 to 12 years, weighting 12 to 24 kg, between 4 and 7 weeks after oestrus, underwent ovariohysterectomy because of pyometra. To unify the group of dogs with pyometra only dogs with open cervix pyometra were selected for the study. Preliminary diagnosis of pyometra was made on the basis of medical history,

physical examination, routine haematological (Scil Vet ABC Plus+ Hematology Analyzer, Horiba ABX, Warsaw, Poland) and biochemical blood (Mindray BS-130 Chemistry Analyzer, Shenzhen Mindray Bio-medical Electronics Co, Ltd, ShenZen, China) analyses, and ultrasonography (Honda HS 2000, Honda Electronics CO, Ltd, Japan). In most cases of dogs with pyometra clinical examination revealed pyrexia, polydipsia, polyuria, anorexia, and apathy. In all dogs selected for study purulent discharge from the vagina appeared. Findings on abdominal ultrasound in all affected dogs indicated pyometra (enlarged uterus filled with fluid and thickened wall of uterus). The definitive diagnosis of pyometra was based on postoperative routine histopathological examination of uterus and positive bacterial culture from the uterine content. In all cases of pyometra *Escherichia coli* was identified in pus from uterus. The control group was twelve clinically healthy female dogs, aged 3 to 8 years, weighting 10 to 20 kg, between 4 and 8 weeks after oestrus. They underwent elective ovariohysterectomy on the owners' request. These dogs were classified as healthy after complete physical examination, haematology, biochemistry, ultrasonography, and postoperative histopathological examination of uterus.

To confirm the same phase of oestrus cycle (dioestrus) in both group of dogs vaginal cytology and serum progesterone concentration were performed using commercial ELISA kit (MyBioSource, Ins., San Diego, USA).

According to information obtained from the owners, both groups of dogs were fed similar commercial dog food for adult animals, without additional supplementation of vitamins and minerals.

All female dogs involved in the study were submitted to ovariohysterectomy according to standard surgical procedures.

### Tissue collection

In both group of animals immediately after ovariohysterectomy, uterine tissue samples of (2 cm<sup>2</sup>) were taken from the middle region of each uterine horn. Luminal exudate was excluded from endometrial samples. The samples of uterine tissue were homogenized in the Ultra Turrax T25 apparatus (Ikawerk, Janke and Kunkel Inc., Staufen, Germany). The homogenates were frozen at -76°C and kept deeply frozen until used for the determination of GSH, vitamin C, Cu, and Zn.

### GSH determination

The concentration of GSH in the homogenates of uterine tissues was determined using the ready kit (Glutathione Assay Kit, Cayman Chemical Company,

Ann Arbor, USA). The method is based on the reaction of SH groups of glutathione with DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] and the synthesis of yellow TNB (5-thio-2-nitrobenzoate anion). The amount of TNB correlates positively with the concentration of GSH in the sample. The absorbance was measured at 405 nm and recalculated into GSH content using the standard curves prepared with different dilutions of GSH. The results were expressed as μmol/g protein.

### Vitamin C determination

The procedure was performed as outlined by Omaye et al. (1979). Briefly, 1 mL of homogenate was mixed with 1 mL cold 5% HPO<sub>3</sub> and centrifuged for 20 min at 3500 x g at 4°C; 600 μl of supernatant were mixed with 300 μL of citrate acetate buffer (22 g of trisodium citrate dihydrate/100mL of distilled H<sub>2</sub>O, pH adjusted to 4.15 with glacial acetic acid) containing p-chloro mercuribenzoate (200 mg/100 mL of distilled H<sub>2</sub>O) and 300 μL of 2,6 dichlorophenylindofenol (100 mg/L of distilled H<sub>2</sub>O). Exactly after 30 s, the absorbance was measured at 520 nm and a few crystals of ascorbic acid were added to get rid of the colour. Then, the absorbance was re-measured. Controls were prepared accordingly but instead of supernatant, an equal volume of 5% HPO<sub>3</sub> was added. Two measurements of sample, one before addition of ascorbic acid, and one after, were subtracted. Two measurements of control, one before addition of ascorbic acid, and one after, were subtracted. The sample results were subtracted from the control results. The level of vitamin C was calculated from the standard curves prepared with ascorbic acid solution in 5% HPO<sub>3</sub> at concentrations of 0-100 μmol/L. The results were expressed as μmol/g protein.

### Protein determination

The protein content in the homogenates of uterine tissues was determined using the Lowry's et al. (1951) method.

### Cu and Zn determination

The concentrations of Cu and Zn in the homogenates of uterine tissues were determined using atomic absorption spectrometer (Agilent Vapor Generation Accessory VGA 77, Agilent Technology, USA). The results were expressed as μg/g tissue.

### Statistical analysis

The results were presented as means ± standard deviation (SD) and analysed using the computer

Table 1. The results of hematological and biochemical blood analysis in dogs with pyometra and healthy controls.

Parameter	Pyometra group	Control	Student's <i>t</i> -test significance
	n= 14	n=12	
RBC (x10 <sup>6</sup> /μL)	5.55 ±3.18	6.20 ±1.19	NS
Hb (g/dL)	12.60 ±3.54	15.00 ±2.62	NS
HCT (%)	41.65 ±5.92	43.71 ±3.56	NS
WBC (x10 <sup>9</sup> /L)	23.13 ±7.56	9.92 ±1.23	*
PLT (x10 <sup>9</sup> /L)	265.83 ±111.56	311.45 ±76.85	NS
ALT (IU/L)	56.83 ±8.73	23.21 ±3.65	*
AST (IU/L)	43.67 ±3.85	31.79 ±1.90	*
Urea (mg/dL)	59.20 ±6.23	23.11 ±3.44	*
Creatinine (mg/dL)	1.64 ±0.86	0.99 ±0.11	*
Glucose (mg/dL)	73.11 ±6.55	76.34 ±2.10	NS
Progesterone (ng/mL)	10.13 ±4.46	9.38 ±2.92	NS

\* p<0.05; NS – p>0.05

Table 2. The concentration of glutathione (GSH), vitamin C, copper (Cu) and zinc (Zn) in uterine tissues from dogs with pyometra and healthy controls.

Parameter	Pyometra group	Control	Student's <i>t</i> -test significance
	n=14	n=12	
GSH (μmol/g protein)	1.01 ±0.2	1.32 ±0.11	*
Vitamin C (μmol/g protein)	0.72 ±0.44	0.89 ±0.50	NS
Cu (μg/g tissue)	5.00 ±1.02	4.51 ±0.82	NS
Zn (μg/g tissue)	49.25 ±5.48	48.20 ±4.52	NS

\* p<0.05; NS – p>0.05

program STATISTICA version 10.0 (Statsoft USA). The Student's *t*-test was used to compare the results between the pyometra group and control. Differences were considered significant at p<0.05.

## Results

Table 1 shows the results of haematological and biochemical blood analysis in female dogs with pyometra and healthy female dogs. There were no statistically significant differences in the red blood cells (RBC) and platelets counts (PLT), haemoglobin content (Hb), haematocrit (HCT) as well as concentrations of glucose and progesterone. In female dogs with pyometra there were significantly higher (p<0.05) total count of white blood cells (WBC), activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), urea and creatinine concentrations, as compared to healthy dogs.

The results of determinations of GSH, vitamin C, Cu and Zn are presented in Table 2. The mean concentration of GSH was significantly lower (p<0.05) in the uterine tissues from dogs with pyometra than in healthy dogs. Similarly, the mean concentration of vitamin C

was markedly lower in the pyometra samples than in the samples from healthy uteruses, but a significant difference was not found. There were no significant differences in the mean concentrations of Cu and Zn between the groups.

## Discussion

The regulation of ROS generation is essential for good health of humans and animals. The antioxidative defence system, consisting of enzymatic and non-enzymatic mechanisms, is responsible for maintaining appropriate levels of ROS (Halliwell 2006). The uterus, like other organs, contains defence mechanisms controlling the ROS (Santos et al. 2016). The presented study related to the non-enzymatic antioxidant mechanisms of uterus of female dogs with pyometra. In our study the concentrations of GSH, vitamin C, Cu and Zn in the uterine tissues from female dogs with pyometra were determined. The results obtained indicate that the concentration of GSH was significantly decreased and the concentration of vitamin C tended to decrease in the uterine tissues from dogs with pyometra compared to the uterine tissues from healthy dogs.

These findings indicate that an antioxidative potential of uterus of female dogs with pyometra is reduced.

Previous studies have shown the decreased uterine activity of antioxidant enzymes in female dogs with pyometra (Szcubiał and Dąbrowski 2009, Toydemir Karabulut 2018). However, the concentrations of non-enzymatic antioxidants have not been previously determined in the uterine tissues of female dogs with pyometra. Our findings correspond with the results of studies in mares and queens with pyometra. El-Bahr and El-Deeb (2016) have found the significantly lower concentration of GSH in the serum of mares with pyometra compared to healthy mares. Similarly, Vilhena et al. (2018) have shown the significantly lower levels of various indicators of non-enzymatic antioxidant status in the serum of queens with pyometra than in control. The decreased GSH values have been observed in the serum or the tissues of various organs of dogs with various inflammatory processes compared to healthy controls (Center et al. 2002, Viviano et al. 2009). Studies in humans and farm animals have shown the reduced concentrations of GSH in the course of various reproductive disorders, which pathogenesis is associated with oxidative stress (Kankofer 2001, Sharma and Agarwal 2004, Agarwal et al. 2005, Lykkesfeldt and Svendsen 2007).

GSH is one of the most important agents of the antioxidative defence system in the cell. Its antioxidative action involves scavenging of ROS, repairing of oxidative DNA damages and protection against ROS-induced apoptosis (Dringen 2000). GSH is also a substrate for the antioxidative enzymes such as glutathione peroxidase, glutathione transferase and glutathione reductase (Nordberg and Arnen 2001). The important role GSH is maintaining the thiol groups in reduced form (Cotgreave and Gerdes 1998). It has been found that the lower level of GSH causes higher ROS production, which results in imbalanced immune response, inflammation and susceptibility to infection (Ghezzi 2011). The decreased GSH concentration in the uterus of female dogs with pyometra could be the cause of reduced activity of GSH-Px that has been found in the previous studies (Szcubiał and Dąbrowski 2009, Toydemir Karabulut 2018).

Like in the case of glutathione, the available literature does not provide data on the concentration of vitamin C in the uterine tissues of female dogs with pyometra. The plasma concentration of vitamin C has been significantly decreased in humans and animals with various inflammatory and / or infectious diseases (Banham et al. 1999, Karadeniz et al. 2008, Carr et al. 2017).

Vitamin C has numerous physiological roles in the organism through acting as an electron donor

(Figuroa-Mendez and Rivas-Arancibia 2015). Related to this oxidation-reduction potential, two major functions of vitamin C are as antioxidant and as enzyme cofactor (Levine et al. 2011). Vitamin C is the primary water-soluble, non-enzymatic antioxidant in plasma and tissues (Du et al. 2012). Vitamin C also participates in recycling of other important antioxidants, such as vitamin E and glutathione (Chan 1993, Levine et al. 2011). Moreover, vitamin C stimulates production and function of leucocytes, such as chemotaxis and phagocytosis as well as is involved in the regulation of gene expression (Levine et al. 2011, Matsui 2012).

The decreased GSH and vitamin C concentrations in female dogs with pyometra suggest that an excessive ROS production occurred in dogs with pyometra, leading to an intense consumption of antioxidant components. However, it is hard to say whether the reduction of antioxidant capacity of the uterus of female dogs occurred before the development of pyometra or was its consequence. It is known that concentrations of antioxidants increase to counteract the increase in ROS production. However, when ROS production exceeds antioxidant capacity, the concentrations of antioxidants is reduced as a result of their consumption (Halliwell and Whiteman 2004, Valko et al. 2007). Unfortunately, in our study the concentrations of GSH and vitamin C before the onset of pyometra and duration of the disease were unknown.

If ROS are not removed by endogenous antioxidants, the oxidative stress could occur, which is involved in tissue damages (Halliwell 2006). The increased production of ROS in the uterine tissues may be associated with infection and inflammatory reaction, that are elements of pathogenesis of canine pyometra (Smith 2006, Jitpean et al. 2012). Key component of inflammation is the infiltration of inflammatory cells, like neutrophils, monocytes, and lymphocytes, to the site of stimulus. At the site of inflammation the activated phagocytic cells, such as neutrophils and macrophages, produce large amounts of ROS to kill the invading agents and some of those ROS diffuse out of the phagocytic cells and induce localized oxidative stress and tissue injury (Mittal et al. 2014, Biswas 2016, Hussain et al. 2016). The production of ROS by immune cells must be controlled by antioxidative mechanisms. An excessive amounts of ROS are harmful for the immune cells, because they can attack cellular components and lead to cell damage or death. Thus, antioxidants play a key role in maintaining the proper functions of immune cells and in protecting them from oxidative stress (De la Fuente and Victor 2000). GSH and vitamin C are important cellular antioxidants, useful for immune cells (Eylar et al. 1993, Ghezzi 2011). The activity of arachidonic acid metabolizing

enzymes, such as cyclooxygenase and lipoxygenase may be another source of ROS during inflammation (De La Fuente and Victor 2000).

On the other hand, it is known that during inflammation ROS are involved in the activation the transcription factors, such as nuclear factor- $\kappa$ B and activator protein-1 (Tabas and Glass 2013), which in turn leads to up-regulation of number of inflammatory genes, including those encoding the pro-inflammatory cytokines and the matrix metalloproteinases – major tissue-destructive factors (De La Fuente and Victor 2000). Antioxidants, such as GSH and vitamin C inhibit the activation of the nuclear factor- $\kappa$ B produced by oxidative stress and thereby inhibit production of proinflammatory cytokines (De La Fuente and Victor 2000). Therefore, these antioxidants have an anti-inflammatory action (Grimble 1998). Taking into account the above data and the results obtained we may hypothesized that an uncontrolled, excessive production of ROS occurs in uterus of female dogs with pyometra and may affect the course of the disease.

Cu and Zn are two of the most abundant trace elements of body and are involved in the metabolism of oxygen and the biochemistry of redox reactions (Klotz et al. 2003). The decreased serum concentrations of Zn (Pasa et al. 2003, Chaudhuri et al. 2008) and Cu (Chadhuri et al. 2008) have been observed in various infectious diseases in dogs. It is thought that the decrease in serum Zn and Cu levels during inflammatory process results from increased synthesis of metallothioneins in liver and other tissues (Pasa et al. 2003). On the contrary, some authors have found the increased levels of Cu during inflammatory processes in dogs (Pasa et al. 2003, Fieten et al. 2012, Cedeno et al. 2016). In the literature there is no data available on the concentration of Cu and Zn in the uterine tissues from dogs with pyometra. In our study the concentrations of these trace elements did not differ significantly between the samples from dogs with pyometra and healthy ones.

In conclusion, the significantly lower concentration of GSH and the trend towards lower concentration of vitamin C in the uterine tissues of female dogs with pyometra indicate that the non-enzymatic antioxidant mechanisms are impaired in the uterus of dogs with pyometra. These findings suggest that the imbalance of oxidative-antioxidative can play an important role in pathogenesis of canine pyometra.

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