International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(4): 994-998

Received: 23-05-2018
Accepted: 25-06-2018

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Effect of ovariohysterectomy on oxidative stress markers in pyometra affected bitches

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Abstract
Canine pyometra has been studied extensively in the past few decades to find the causes and prognostic markers associated with this infective disease of intact bitches, which is known to cause a systemic inflammatory responses leading to multiple systemic dysfunctions. Besides the well described clinical symptoms and systemic effects of this diseases, limited research on antioxidative status and lipid peroxidation profile of this condition was done. To find the changes in antioxidative status and lipid peroxidation profile of pyometra affected bitches serum sample was collected from ten pyometra affected bitches during pre and post operative periods of surgery and compared against the control group (n=10). In the present study the antioxidative status of the pyometra affected bitches was altered due to decrease in superoxide dismutase and catalase enzymes level. After ovariohysterectomy levels of these enzymes were increased. This suggests that pyometra induces oxidative stress in the bitches and removal of causative pathogens alleviates this oxidative stress.

Keywords: Pyometra, oxidative stress, antioxidants enzymes, TBARS

Introduction
Pyometra either open or closed type in dogs is a life threatening disorder. It is most commonly seen in the middle aged bitches in diestrual phase of estrus cycle when uterus is being exposed to high levels of progesterone. It is hormone related disease with bacterial infection leading to endometritis and toxemia (Hardy and Osborne 1974) [11].

It has been reported that endometrial cell generates enormous reactive oxygen species due to its active metabolism. These are necessary for normal tissue function. When there is over production of free radicals tissue homeostasis becomes deranged due to loss of delicate balance by the antioxidant mechanism. (Celso Santos *et al.*, 2016).

Positive role of ROS in hormone signaling, angiogenesis, apoptosis, cell proliferation, prostaglandin secretion and progesterone mediated physiological events like decidualization are well documented (Suguino *et al.*, 2001, Agarwal 2005, Rizx *et.al*., 2013) [24, 1]. The activity of oxidatative stress enzymes in the endometrium suggest the existence of changes in antioxidant profile throughout estrus cycle. There are evidences of enormous and uncontrollable increased generation of free radicals which further deteriorates the progression of the disease.

Information concerning antioxidant enzymes in canine uterus and level of proinflammatory cytokines is scanty. Physiological knowledge gained indicates that there exist a relationship between a hormonal, biochemical haematological, antioxidative and inflammatory status. With this hypothesis the current study has been designed with the following objectives

With this hypothesis the current study has been designed to study the effects of ovariohysterectomy on oxidative stress markers in pyometra affected bitches and also to study the peroxidation product in pyometra affected bitches.

Materials and Methods
A clinical diagnosis of pyometra was made in ten bitches presented on the basis of medical history, clinical examination and haematological studies and the condition was confirmed with the help of ultra-sonographic studies. All the bitches were subjected to ovariohysterectomy for management of the condition and formed the experimental group. Ten apparently healthy bitches aged between 2 - 13 years were taken as a control blood samples were collected from pyometra affected bitches during both pre and post-operative periods.

Blood samples were collected on the day of surgery, 7th day and 14th day post operatively. Blood samples were collected from all the bitches by either cephalic or saphenous vein puncture. From each bitch, 2ml of blood was collected in EDTA vial and one sample of 2 ml in a clot activator serum vial. The 2 ml of blood was centrifuged at 1500 rpm for 20 minutes and serum samples were separated out and was stored at -20 degree Celsius for Antioxidants enzymatic estimation. The plasma was separated by centrifugation of EDTA blood at 3000 rpm for 15 minutes and was stored at -20 degree Celsius till further analysis for thiobarbituric acid reactive substance estimation The levels of Glutathione Peroxidase 1 (GPX1), Superoxide Dismutase (SOD), Canine Catalase (CAT) and TBARS were estimated using ELISA kits supplied by Sincere Biotech, Beijing101300, China.

Statiscal Analysis

The data were subjected to one- way analysis of variance (ANOVA) and post hoc test. Turkey's and Duncan's tests were performed to analyse the between group and within group variations. SPSS software version 20 for windows was used to perform all the statistical tests

Results

The mean and standard error for glutathione peroxidase, superoxide dismutase and catalase, TBARS were provided in Table 1.The mean and standard error for glutathione peroxidase (ng/ml) of control, preoperative, day of surgery, 7th day post-operative and 14th day post-operative were 6.63±0.30, 3.75±0.56, 4.44±0.90, 5.18±0.49 and 5.02±0.92 respectively.

Table 1: Results for evaluation of oxidative stress in pyometra affected bitches

Parameters	Control	Experimental groups				F value
		Preoperative Period	Day of surgery	7th day postoperative	14th day postoperative	
GSH-X(ng/ml)	6.63 ± 0.30^{b}	3.75 ± 0.56^{a}	4.44 ± 0.90^{a}	5.18 ± 0.49^{ab}	5.02 ± 0.92^{ab}	2.45^{NS}
SOD(ng/ml)	6.80 ± 0.24^{b}	3.42 ± 0.26^{a}	3.88 ± 0.46^{a}	3.80 ± 0.37^{a}	3.79 ± 0.69^{a}	9.88^{**}
Catalase(ng/ml)	6.41 ± 0.14^{ab}	5.57 ± 0.33^{a}	6.35 ± 0.37^{ab}	8.00 ± 0.52^{c}	6.90 ± 0.40^{b}	5.68^{**}
TBARS(ng/ml)	23.30 ± 1.41^{a}	25.15 ± 2.46^{a}	26.19 ± 2.72^{a}	26.19 ± 2.30^{a}	27.03 ± 1.70^{a}	0.43^{NS}

Glutathione peroxidase of experimental groups did not differ significantly when compared to control group. The mean and standard error for superoxide dismutase (ng/ml) of control, preoperative, day of surgery, 7th day post-operative and 14th day post-operative were 6.80±0.24, 3.42±0.26, 3.88±0.46, 3.80±0.37 and 3.79±0.69 respectively. There was a statistical significant difference between control and experimental groups ($P<0.01$).Among the experimental groups there was no significant difference observed.

The mean and standard error for catalase (ng/ml) of control, preoperative, day of surgery, 7th day post-operative and 14th day post-operative were 6.41±0.14, 5.57±0.33, 6.35±0.37, 8.00±0.52 and 6.90±0.40 respectively. There existed significant difference among control and experimental groups at the level of $P<0.01$, the catalase concentration during the day of surgery did not differ when compared to control group but after surgery there was an increase in the catalase concentration.

TBARS (ng/ml) concentration of control and experimental groups did not differ significantly. The mean and standard error for thiobarbituric acid reactive substance of control, preoperative, day of surgery, 7th day post-operative and 14th day post-operative were 23.30±1.41, 25.15±2.46, 26.19±2.72, 26.19±2.30 and 27.03±1.70 respectively.

Discussion

One of the key mechanisms responsible for tissue damage is oxidative stress caused by overproduction of reactive oxygen species. (ROS). The term ROS refers to radical and non-radical oxygen species formed by the partial reduction of oxygen, such as superoxide anion (O_2-), hydrogen peroxides (H_2O_2) or the hydroxyl radical (Ray *et al.*. 2012) [20]

Hydroxyl radical is the most reactive form of oxygen and shows high toxicity at the site of its formation. It rapidly reacts with most of the biological particles (lipids, proteins, nucleic acids, carbohydrates) present at the site and the adjacent phases of synthesis. Due to extremely high speed of the reaction, destructive effects of the hydroxyl radical once formed are difficult to avoid. Therefore, it is essential to prevent its formation by removing superoxide anion radical and hydrogen peroxide by antioxidant enzyme. (Halliwell and Whiteman, 2004) [10]

The main event related to ROS production is an inflammatory process (Barnes *et al.* 1997 and Babior *et al.*. 2000) [4]. In pyometra the inflammation develops in the uterine mucosa. At the inflammation site, complement fragments, opsonized bacteria, immunoglobulins and chemotactic peptides induce rapid consumption of oxygen in the phagocytic cells, called as "respiratory burst" and with the help of reactive oxygen species they destroy the absorbed microorganisms (Yu *et al.*, 1995). However, the lack of proper control of ROS production by antioxidative mechanisms result in their overproduction and cause damage to phagocytic cells as well as adjacent tissue cells.

Halliwell and Whiteman (2004) [10] demonstrated in vitro studies that ROS released by activated neutrophils and macrophages may be toxic for various somatic cells such as erythrocytes, epithelial cells, fibroblast or platelets.

The uterus, alike other organs, contains defense mechanism controlling the reactive oxygen species, the changes in the distribution pattern of antioxidant enzymes would reflect in the changes associated with morphologic events and metabolic activities within the tissue. (Rizk *et al.*, 2013) [21].

Glutathione Peroxidase (GSH-Px)

Glutatathione peroxidase is a tetramer who's each subunit contains one selenium atom bound with cysteine (Nordberg *et al*., 2001). Its main function is to remove hydrogen peroxide (Ursini and Bindoli *et al*., 1987) [28]. GSH-Px catalyzes the hydrogen peroxide glutathione reaction, which result in the oxidation of glutathione, it reduces organic peroxides, mainly lipid peroxides, to alcohols and in cases of lipid peroxides it discontinues the process of lipid peroxidation. The preoperative concentration of glutathione peroxidase is significantly lower than the control group animals. The levels increased from preoperative to the day of surgery and increased on 7th postoperative day and decreased on 14th day which may be due to a depletion of antioxidant defense mechanisms as a result of excess utilization of GSH-Px to neutralize ROS produced during preoperative stage.

The results of the present study was in accordance to Szczubial and Dabrowski (2009) [25]. The reduced activity of GSH-Px indicate impaired cellular antioxidative protection mechanism in bitches with pyometra. This also impose the risk of insufficient neutralization of hydrogen peroxide, the main substrate for the production of hydroxyl radical and other toxic ROS. However the activity of GSH-Px was increased post operatively at 7th day and decreased at 14 days after ovariohysterectomy compared to preoperative and on the day of surgery values which may a suggest a compensatory mechanism. The results were in accordance with SczubiaL *et al.* (2015) who recorded the activity of GSH-Px to be markedly increased although not significantly at 14 days after ovariohysterectomy and then significantly decreased on 30th day after ovariohysterectomy. The post-operative period state is accompanied by a series of metabolic changes, involving a compensatory response to the stress of surgery. In the present study the activity of GSH-Px increased on 7th postoperatively day indicating the restoration of tissue homeostasis

Superoxide Dismutase (SOD)

In pyometra there was a significant inhibition of antioxidant defense system. Oxidative stress in pyometra coexists with a reduction in the antioxidative capacity, which can increase the deleterious effects of the free radical. (Pigeolet *et al.* 1990) [17]

The activities of antioxidant enzyme plays an important role in culminating the complications of pyometra and which was postulated to be associated with increased SOD, CAT, and GSH-Px activities. (Edge *et al.* 1997) [6].

In mammals superoxide dismutase (SOD) occurs in three distinct forms varying in a cofactor required for their proper functioning and location (Fridrovich, 1995). The dismutase dependent on zinc and copper ions (ZnCu - SOD) will be present in the cytoplasm and its activity constitutes about 70 % of the total SOD activity The manganese-dependent dismutase (MN-SOD) is located in the mitochondria. The third form of SOD present in the plasma, is the extracellular superoxide dismutase (EC – SOD). The mechanism of SOD – catalyzed reaction is dismutation of superoxide anion radicals to hydrogen peroxides. SOD is the first line of defense against ROS and is active in catalyzing and detoxification of superoxide radical (O_2) (Gonzales *et al.* 1984). Generated H_2O_2 in this reaction is restored to water in the presence of Catalase and Glutathione peroxidase. Alternatively CAT can catalyze the peroxidases reaction. (Fridovich, 1995)

SOD are considered as critical antioxidative enzymes that act as a scavenger of oxygen anion to form hydrogen peroxide and thereby diminishing the toxic effects due to the free radical. The decrease in the SOD concentration in the present study could have resulted from excessive usage of this enzyme to neutralize ROS or by glycation of this enzyme which are known to occur during pyometra. (Gutteridge *et al.* 1986) [9].

In the present study, SOD concentration in the plasma were found to be significantly reduced ($P < 0.05$) in pyometra affected bitches on preoperatative day. As pyometra produces substantial alteration in the intracellular metabolism in the liver and kidney, resulting in elevated free radicals in these organs. (Szczubial and Dabrowski, 2009) [25]. This increase in the ROS production may result in the excess usage of SOD. Hence there is a reduction in the SOD concentration during the 14th postoperative day of pyometra affected bitches.

Pyometra affected bitches showed concomitant decrease in SOD activity during the preoperative period, to handle superoxide radicals the highly reactive hydrogen peroxide formed as a result of dismutation, which was further degraded by CAT and GSH-Px.

The significant increase in SOD activity from the preoperative values to the day of surgery may suggests removal of toxin liberated by the microorganisms in the uterus of the pyometra affected bitches by inhibition of induced superoxide formation by NAD(P)H oxidase or by down regulating mRNA expression of NAD(P) oxidase and inhibition of GTPase translocation from the cytosolic compartment to the cell membrane which is required for its activation. (Halliwell and Whiteman, 2004) An slight decrease on 7th postoperative day and 14th postoperative day may suggest that their activities were reduced as they continuously work to neutralize ROS produced in increasing amounts. (Sczubial *et al.* 2015).

Kankofer *et al.* (2007) showed that the SOD activity decreased at 4th week after ovariectomy when compared with the 1st week after ovariectomy which was similar to the present findings.

The results of the present findings shows that pyometra causes decrease in the concentration of SOD. The present findings are in accordance with the Szczubial *et al.* (2015) [26], Toydemirkarabulat *et al.* (2016) found significant difference in SOD activity after 15th day of ovariohysterectomy.

Catalase (CAT)

Catalase is a tetramer composed of four subunits, about 60 kDa each in mammalian cells, catalase occurs predominantly in peroxisomes (Nordberg, 2001). Together with glutathione peroxidase, it prevents the accumulation of hydrogen peroxide in the cell by degrading it to water and molecular oxygen (Urshini *et al.* 1987). Catalase is believed to play an essential role at high concentration of hydrogen peroxide. In normal conditions, it is the most adaptive antioxidant enzyme to play a significant role in cell defense against oxidative damage in the presence of oxidative stress (Mates, 1999).

Pyometra affected group showed decrease in the plasma CAT concentration. The decrease in CAT activity may be due to inactivation of this enzyme by glycation which renders structural abnormality thereby limiting the activity of this enzyme. The increase in the concentration of CAT on 7th postoperative day may be because of inhibition of glycation of this enzyme and thereby promoting the enzyme action (Poli *et al.*, 2004) [18]. However a significant decrease was noticed in the 14th postoperative day which may be due to trauma of the surgical procedure and also due to tissue healing process, these activates the inflammatory response resulting in high oxidative stress in pyometra affected bitches. Hence there will be excess consumption of CAT which is reflected in the results as reduction in their concentration on 14th day after surgery (Naziroglu and Gunay 1999, Baines and Shenkin, 2002, Alva *et al.*, 2006; Gunay *et al.*, 2011 and Prajwalita *et al.*, 2013) [16, 5, 2, 19].

Azvedo *et al.* (2001) observed that the ovariectomy markedly reduced CAT activity in intraperitoneal macrophages in rats one month after surgery.

The present findings are in accordance with Szczubial *et al.* (2015) [26] whereas Toydemikarabulat *et al.* (2016) did not find significant difference in CAT activity postoperatively.

Thiobarbituric Acid Reactive Substance (TBARS)

Oxygen free radicals have been shown to catalyze the oxidative modification of lipids resulting in lipid peroxidation. Lipid peroxides are formed by the autoxidation of polyunsaturated fatty acids primarily present on all cell

membranes resulting in membrane damage (Kawamara *et al.*, 1992) [13] Lipid peroxidation occurs as a consequence of increased oxidative stress primarily due to disruption of pro-oxidant/antioxidant balance. Increased oxidative stress has been associated with increased lipid peroxidation in many diseases (Gunay *et al.*, 2011).

The thiobarbituric acid reactive substance level was numerically increased on the preoperative to the day of surgery and increased from seventh postoperative day and on fourteenth day. However it did not differ statistically between the preoperative and postoperative groups. Amounts of ROS produced in during the late postoperative period after ovariohysterectomy may exceed the capacity of antioxidant defense for their neutralization. This leads to lipid peroxidation and increases the end products of lipid peroxidation, such as MDA, which can be measured by TBARS concentration. Previous studies in bitches have shown an increase in lipid peroxidation intensity based on plasma MDA concentration during 24 hrs after ovariohysterectomy. (Serin *et al*., 2008 and Gunay *et. al.*, 2011) [23].

The peritoneum is an organ of high metabolic activity connected with reactive oxygen species (ROS) generation and participating in the healing process. ROS generated after trauma caused by surgical intervention leads to damage of cellular components and lipid peroxidation.

Conclusion

The present study shows that pyometra causes pronounced oxidative stress in the affected bitches. After ovariohysterectomy the causative agent for pyometra was removed which caused the reduction in reactive oxygen species production. This shows ovariohysterectomy will reduce the oxidative stress caused by pyometra.

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