

## EXAMINATION OF OXIDATIVE/ANTIOXIDATIVE STATUS AND TRACE ELEMENT LEVELS IN DOGS WITH MAMMARY TUMORS

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### Abstract

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The aim of this study was to detect differences in oxidative/antioxidative status in dogs with and without mammary tumors by monitoring the extent of lipid peroxidation (TBARS), activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), and reduced glutathione (GSH) as well as trace elements required for antioxidative activities such as copper (Cu), zinc (Zn), selenium (Se), and manganese (Mn). Samples of mammary tumors were obtained from the experimental group by surgery, and healthy mammary glands, from the control group by surgical biopsy. Blood samples were collected before the surgery (day 1) and on 15<sup>th</sup> day post-surgery, while mammary tissue samples were collected after the surgery for the detection of antioxidant status, levels of trace elements, and histopathology. Antioxidant activities and levels of trace elements were compared between days 1 and 15 for serum samples, as well as between the control and experimental groups for tissue samples using repeated measures ANOVA and Student's *t* test, respectively. Significantly higher activities of antioxidative enzymes SOD/CAT and GST were detected in serum samples (day 1) and tissue homogenates (tumor tissue), respectively, from dogs with mammary tumors compared to the control group; however, significantly higher SOD activity was observed in tissue homogenates from the control group, suggesting host defense against the tumor. The levels of trace elements also showed variations dependent on the occurrence of mammary tumors. In conclusion, higher plasma and tissue antioxidant activities in dogs with mammary tumors suggests the importance of considering antioxidants while deliberating future treatment options for canine mammary tumors. Moreover, higher level of copper in tumor tissue suggests that it functions as a co-factor for antioxidant complexes.

**Keywords:** antioxidant, blood, canine mammary tumor, general anesthesia, mastectomy

**List of abbreviations:** Malondialdehyde - MDA; Thiobarbituric acid reactive substances - TBARS; Glutathione - GSH; Glutathione peroxidase - GPx; Glutathione S - transferase GST; Superoxide dismutase - SOD; Catalase - CAT; Reduced glutathione - GSH; Copper - Cu; Zinc - Zn; Manganese - Mn; Selenium - Se; Aluminium - Al; Rubidium - Rb; Lithium - Li

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## Introduction

Mammary tumors are the most common type of tumor in female dogs (Sorenmo, 2003). Mammary epithelial cells are responsive to xenoestrogens, which are found in water, food, and air; the resultant constant exposure to these xenoestrogens leads to their accumulation in mammary tissues and generation of reactive oxygen products with very high metabolic toxicity (Olea et al., 1998; Kumaraguruparan et al., 2005). Aerobic metabolism in living organisms generates reactive oxygen species (ROS), which need to be inactivated; any imbalance in this process leads to oxidative stress (Sies, 1993). ROS induce cytotoxicity, membrane damage, lipid peroxidation, mutagenesis, and carcinogenesis by stimulating the transformation of normal cells into cancerous cells (Kumaraguruparan et al., 2005). Increased levels of ROS by-products constitute an important characteristic of neoplastic tissues (Zima et al., 1996; Ray et al., 2000; Yeh et al., 2005). A decrease in antioxidative defense mechanisms of living organisms contributes to oxidative stress; therefore, the levels of antioxidants can be employed for evaluating oxidative stress in biological samples. Malondialdehyde (MDA) is an end product of lipid peroxidation and has been widely employed as a biomarker for oxidative stress; MDA is measurable by assays monitoring thiobarbituric acid-reactive substances (TBARS; Macopet et al., 2013). Glutathione (GSH) is the most common non-protein thiol found in eukaryotic cells (Ortega et al., 2011), and is involved in the maintenance of cellular balance along with glutathione peroxidase (GPx) and glutathione S-transferase (GST) through detoxification of reactive intermediates generated during the biotransformation of environmental chemicals; it is also involved in the defense against free radicals, peroxides, carcinogens, and a large number of xenobiotics (Mates et al., 1999; Datta et al., 2000; Kumaraguruparan et al., 2005). GST is a member of the phase II detoxification enzyme family and protects cellular macromolecules against reactive electrophiles (Turkoglu, 2008). Kumaraguruparan et al. (2005) reported the detection of lipid peroxidation through the formation of TBARS, and of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), GPx, and GST, as well as non-enzymatic antioxidants including reduced GSH levels in canine mammary tumors.

Several important nutritionally derived minerals are components of antioxidant enzymes, including essential trace elements such as manganese (Mn), copper (Cu), and zinc (Zn) for SOD as well as selenium (Se) for GPx (Miller and Brzezinska-Slebozinska, 1993). Manganese is a component of mitochondrial Mn-SOD (Evans and Halliwell, 2001) and of several other enzymes involved in the biosyntheses of fatty

acids and cholesterol. Copper is essential for ceruloplasmin-mediated loading of iron onto transferrin and the formation of CuZnSOD (Evans and Halliwell, 2001). The minerals Cu, Zn, and Mn are essential for the activity of two types of SOD, CuZnSOD and Mn-SOD, respectively (Evans and Halliwell, 2001). Selenium functions as an antitumor agent, and is a component of GPx, an enzymatic antioxidant with an important role in the decomposition of hydrogen peroxide and lipid peroxides (Bock et al., 1991; Machlin and Bendlich, 1987; Huang et al., 1999). Neoplastic tissue is characterized by the accumulation of certain elements such as Cu, aluminum (Al), rubidium (Rb), Se, or lithium (Li) (Skibniewska et al., 2010).

The current study aims to detect variations in the levels of lipid peroxidation (TBARS), activities of SOD, CAT, GPx, GST, and reduced GSH, as well as trace elements such as copper, zinc, selenium, and manganese between dogs with and without mammary tumors.

## Materials and Methods

### *Animals and study design*

Dogs with mammary tumors and of 5–15 years age ( $n = 20$ ) as well as clinically healthy female dogs of 9–12 years age ( $n = 10$ ), which were presented to Istanbul University, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, were included in the experimental and control groups, respectively, in the current study. The following breeds of dogs were included: four Cocker, seven Terrier, two Boxer, two Setter, one Rottweiler, one Dachshund, one Pinscher, one Pit bull, two Turkish Shepherd, and nine mixed breed. The dogs were fed commercial dog food and not prescription food for at least 6 months prior to the surgery. Twenty female dogs with malignant mammary tumors were subjected to surgery, and tumor samples were obtained from the largest tumoral lesion. Ten normal mammary gland samples were obtained by surgical biopsy from left inguinal mammary lobes of dogs in the control group; the absence of any tumoral lesions (examined by palpating the mammary glands) and of a history of mammary or endocrine disease was verified for this group. All the collected tissues were divided into three groups post-surgery, and were 1) immediately frozen in liquid nitrogen and stored at  $-86^{\circ}\text{C}$  until analysis of antioxidant levels in the tissue homogenates, 2) stored at  $-20^{\circ}\text{C}$  until trace element analysis, and 3) sent to the Pathology department for histopathology analysis.

General anesthesia was employed for the surgery. Fasting blood samples (8 ml) were collected from all the animals before the surgery (day 1) and on the 15<sup>th</sup> day post-surgery (day 15) from the jugular vein and placed into lithium-heparin tubes for analysis of antioxidant levels.

The mammary tissue samples of group 1 were washed with PBS (pH 7.4), followed by the addition of 5–10 ml of cold buffer. Homogenization was carried out using homogenizer (MICCRA-D1, ART Prozess and Labortechnik GmbH and Co. KG., Germany), and the homogenates were centrifuged at 4°C; the resulting supernatant was stored at –86°C for a maximum of 1 month until further analysis.

### **Analyses of antioxidant activities**

Antioxidant activities in tissue homogenates and plasma samples were estimated using  $\mu$ Quant ELISA System. Assay kits for SOD (Item no. 706002), CAT (Catalog no. 707002), TBARS (Catalog no. 10009055), GPx (Item no. 703102), GSH (Catalog no. 703202), and GST (Item no. 703302) obtained from CAYMAN Chemical Co. (USA) were employed for the analyses. For the detection of TBARS levels in tissue homogenates for evaluating the extent of lipid peroxidation, the tissue samples were subjected to sonication (Sonopuls HD 2200, BANDELIN Electronic GmbH and Co. KG., Germany) at 40 V.

### **Trace element analysis**

Five milliliters of the blood samples collected on days 1 (before surgery) and 15 (15<sup>th</sup> day post-surgery) were centrifuged at 3000  $\times$  g for 10 min, serum samples obtained and were employed for trace element analysis. The samples of serum and mammary tissues (collected post-surgery) were stored at –20°C and sent to the Department of Biophysics, Cerrahpasa Medical Faculty, Istanbul University, for trace element analysis. The concentrations of Cu, Zn, Se, and Mn in serum and tissue (malignant mammary tumor and normal mammary gland) samples were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES-6000, Thermo). The wavelengths of the various elements in ICP-OES measurements were (327, 393) nm for Cu; (257, 610) nm for Mn; (196, 090) nm for Se; and (206, 200) nm for Zn, respectively. The appropriate database and method for the measurements were incorporated in ICP-OES spectrophotometer. Calibration graph was drawn for each element, and measurements were recorded using standard solutions and deionized water as blind.

### **Histopathology**

The mammary tissue samples were fixed with 10% buffered formalin, embedded in paraffin wax, and sections of 2–3  $\mu$ m were cut for histopathological evaluations. For the reassessment of conventional diagnosis, morphological features of cells, tumor growth pattern, mitotic activity, necrosis, edema, and inflammation were evaluated on the basis of criteria established by the World Health Organization in the Inter-

national Histological Classification of Tumors of Domestic Animals (Misdorp et al., 1999).

### **Ethical approval**

This study was approved by the “Istanbul University Animal Research and Ethics Committee” (verdict number: 2011/76).

### **Statistical analyses**

Statistical analysis of plasma activities of GPx, CAT, GST, SOD, and TBARS as well as serum levels of Cu, Mn, Se, and Zn compared between two sampling times (day 1, before the surgery; day 15, 15<sup>th</sup> day post-surgery) was carried out using repeated measures ANOVA (Ekiz et al., 2013). The comparison of these parameters (day 1 of tissue homogenate values) between the control and experimental groups was carried out using Student’s *t* test.

## **Results**

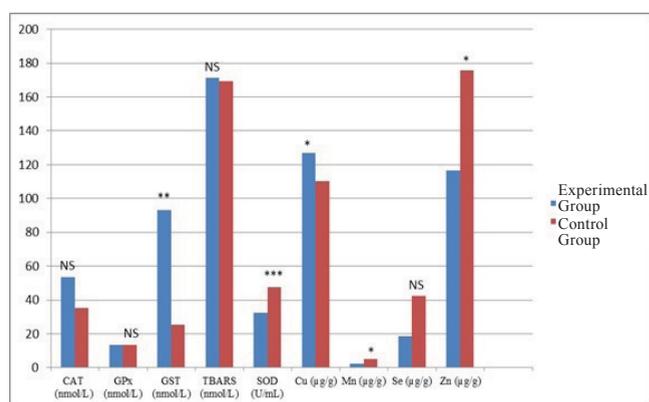
Mean plasma antioxidant activities, the extent of lipid peroxidation, and serum trace elements on days 1 and 15 are shown for the experimental and control groups (Table 1). Differences in the GST and GPx activities in blood samples were not found to be statistically significant. Higher serum TBARS levels were detected in the experimental group than in the control group using plasma samples from day 15, with the differences being statistically significant ( $P < 0.01$ ). Significantly higher values in the experimental group than in the control group were also found for mean plasma SOD activity on days 1 ( $P < 0.01$ ) and 15 ( $P < 0.05$ ), and for mean serum CAT activity ( $P < 0.01$ ) on day 1. In contrast, significantly higher mean serum levels of Mn ( $P < 0.05$ ) and Se ( $P < 0.001$ ) on day 15 post-surgery and of Zn ( $P < 0.05$ ) on day 1 were found in the control group than in the experimental group (Table 1). Reduced GSH activity could not be determined in both blood and mammary tissue homogenate samples, and was therefore not considered for statistical analysis.

For the tissue homogenate samples, statistically significant differences were not observed in the activities of GPx and CAT or the levels of TBARS and Se. Significantly higher levels ( $P < 0.05$ ) of Mn and Zn were detected in the control group than in the experimental group, while the exact opposite was observed for Cu. Significantly higher GST activity ( $P < 0.01$ ) was observed in the experimental group (tumor tissue) than in the control group, whereas SOD activity was found to be significantly higher ( $P < 0.001$ ) in the control group (healthy tissue) than in the experimental group (Figure 1). Mammary tissue samples were subjected to histopathological evaluation (Figure 2), and the results are summarized in Table 2.

**Table 1**  
Mean plasma antioxidant activity, lipid peroxidation, and levels of serum trace elements on days 1 and 15 in the experimental and control groups

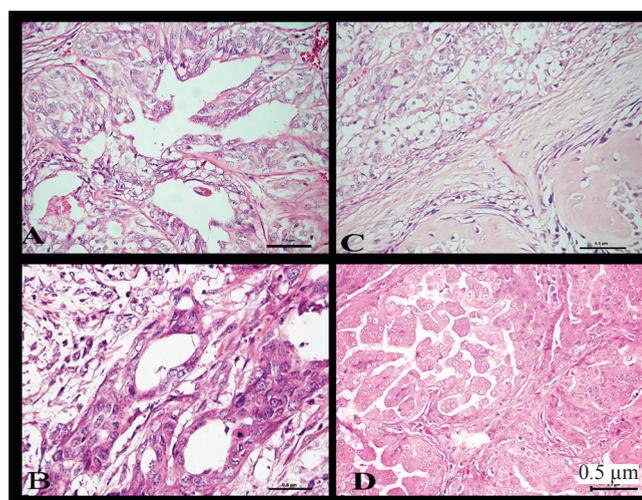
Parameter	Sampling Time	Group		Statistical significance
		Experimental (Mean ± SE)	Control (Mean ± SE)	
GST (nmol/L)	1	8.46 ± 2.76	3.15 ± 0.32	NS
	15	6.54 ± 1.64	8.57 ± 5.62	NS
TBARS (nmol/L)	1	921.94 ± 334.62	596.96 ± 370.53	NS
	15	283.03 ± 115.40	208.97 ± 398.6	**
SOD (U/mL)	1	57.65 ± 2.11	43.10 ± 3.97	**
	15	52.68 ± 3.30	39.35 ± 5.24	*
CAT (nmol/L)	1	32.21 ± 3.79	15.57 ± 2.07	**
	15	35.12 ± 6.19	19.86 ± 2.24	NS
GPx (nmol/L)	1	15.44 ± 3.10	12.32 ± 1.27	NS
	15	15.45 ± 3.49	23.55 ± 9.31	NS
Cu (µg/dL)	1	100.81 ± 8.38	94.62 ± 14.33	NS
	15	126.86 ± 6.25	110.06 ± 7.44	NS
Mn (µg/dL)	1	3.41 ± 0.63	2.40 ± 0.51	NS
	15	2.47 ± 0.73	5.23 ± 0.69	*
Se (µg/dL)	1	42.86 ± 4.90	29.06 ± 5.68	NS
	15	18.63 ± 1.93	42.24 ± 7.06	***
Zn (µg/dL)	1	119.190 ± 12.91	169.16 ± 18.02	*
	15	116.61 ± 7.26	175.56 ± 16.90	NS

\*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; NS, not significant



**Fig. 1.** Antioxidant profiles, lipid peroxidation and trace element measurements in tissue samples obtained from the experimental and control groups on day 1.

NS, Not significant; \*, \*\*, and \*\*\* indicate statistically significant differences at P < 0.05, P < 0.01, and P < 0.001, respectively



**Fig. 2.** Histopathological evaluation of Tubulopapillary carcinoma (grade I) (a), Complex carcinoma (grade III) (b), Carcinosarcoma, and (c) Solid carcinoma (grade II) (d)

**Table 2**  
**Histological grading and types of canine mammary tumors**

Histological type of mammary tumors	Clinical grade of the mammary tumors			Total number
	Grade I	Grade II	Grade III	
Complex carcinoma	4	2	2	8
Tubulopapillary carcinoma	5	-	-	5
Carcinosarcoma	-	-	4	4
Basosquamous carcinoma	1	-	-	1
Solid carcinoma and mastitis	-	1	-	1
Complex and tubulopapillary carcinoma	-	-	1	1

## Discussion

In the current study, we sought to compare the extent of lipid peroxidation (TBARS) and the activities of enzymatic (SOD, CAT, GPx, and GST) and non-enzymatic (reduced GSH) antioxidants and trace elements (Cu, Zn, Se, and Mn) in dogs with and without mammary tumors.

A comparison of the serum samples from the experimental and control groups revealed that TBARS concentration and SOD activity decreased after the surgery in both groups, albeit significantly higher values for both TBARS concentration ( $P < 0.01$ ) and SOD activity ( $P < 0.05$ ) were found in the experimental group than in the control group on day 15. In addition, significantly higher ( $P < 0.01$ ) serum CAT activity was observed in the experimental group than in the control group on day 1.

The serum Se levels showed decrease post-surgery in the experimental group, but increased in the control group with statistically significant differences between the two groups at day 15 ( $P < 0.001$ ). In line with the observations in the current study, Askar et al. (2009) reported higher mean serum Cu and lower mean Zn levels in dogs with mammary tumors. Moreover, the observations of Szczubial et al. (2004) that higher plasma SOD activity is found in female dogs with malignant tumors compared to healthy ones is also reflected in the current study, as observed from CAT and SOD activities in plasma samples collected at day 1.

The current study revealed significantly higher ( $P < 0.05$ ) Cu levels in malignant mammary tissue as opposed to higher Mn and Zn levels in healthy mammary tissue. These observations mirror those of Skibniewska et al. (2010), who found significantly higher Cu content in neoplastic tissue compared to healthy mammary glands in dogs. Moreover, Askar et al. (2009) reported lower levels of serum Zn in the experimental group than in the control group, which is in line with the observations of the current study. However, significantly higher serum Se levels in the experimental group than in the control

group was reported by Huang et al. (1999), which agree with the observations of the current study with respect to day 1 samples, albeit statistically significant differences were not observed between the two groups.

According to Kumaraguruparan et al. (2005), SOD and CAT activities show an initial increase in tissue homogenate samples due to ROS-induced damage. Accordingly, in the current study, significantly higher SOD activity from mammary tissue homogenates was found in the control group than in the experimental group. Turkoglu (2008) reported significantly higher GST enzyme in healthy tissues compared to tumor samples from the human mammary gland in a comparison of the malignant tumors and their healthy surrounding tissue. This is in contrast with the current study, and is attributable to differences between the two species. GST activity and Cu levels were found to be significantly higher ( $P < 0.01$  and  $P < 0.05$ , respectively) in the experimental group than in the control group, while SOD activity and levels of Mn and Zn were significantly higher ( $P < 0.001$  and  $P < 0.05$ , respectively) in the control group in tissue homogenate samples at day 1.

In the current study, complex carcinoma ( $n = 8$ ) and tubulopapillary carcinoma ( $n = 5$ ) were the most frequently diagnosed types of tumor. Complex carcinoma was also reported as the most frequently observed type of tumor ( $n = 6$ ) in the study by Szczubial et al. (2008) on oxidative/antioxidative status of plasma in female dogs with mammary tumors.

The increased activities of antioxidative enzymes and levels of trace elements in the blood of animals in the experimental group compared with those in the control group at day 1 suggests the activation of antioxidative defense mechanisms in mammary tumors, possibly reflecting host defense against tumourigenesis.

## Conclusions

In conclusion, the significantly higher activities of SOD and CAT in plasma and GST in tissue homogenates in dogs

with mammary tumors supports the importance of considering antioxidants while deliberating future treatment options for canine mammary tumors. In addition, the significantly higher level of Cu in tumor tissue homogenates in dogs suggests that it is used as a co-factor in antioxidant complexes; however, further studies are required for confirming this hypothesis.

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