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Short communication

ANTIOXIDANT ENZYMES IN CANINE MAMMARY TUMORS

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Spontaneous mammary tumors are very common in bitches. The involvement of oxidative stress and the function of antioxidant enzymes in cancerogenesis have been studied in depth in human medicine, while data in veterinary medicine are still fragmentary. The main aim of this study was to evaluate the activity and the expression of superoxide dismutases (Cu-ZnSOD and MnSOD) and the activity of catalase (CAT) in canine mammary tumors in comparison with the adjacent healthy tissue. Six female dogs (mean age 10.4 years) were included in this study. After surgery, fresh tumor and healthy tissue samples were immediately frozen in dry ice and stored at -80°C for biochemical analyses, while the remaining parts were used for histopathological analysis. Enzyme activity was measured by spectrophotometric assays and protein expression by western blotting. In canine mammary tumors, Cu-ZnSOD activity and expression increased significantly compared with healthy control tissues ($p=0.03$). MnSOD showed a significantly lower activity in tumoral tissues at stage 2 ($p<0.05$), while a significant increase of expression was measured in tumors. CAT activity was significantly higher in healthy tissues respect to tumors ($p=0.015$). These variations of antioxidant enzymes activities and expression could be related to an increase of oxidative stress in breast cancer tissues and could be considered as biomarker candidates for neoplastic transformation.

Key words: cancer; dog; superoxide dismutase; catalase

INTRODUCTION

In veterinary medicine, mammary tumors represent the most common malignant neoplasms in the bitch [1]; furthermore, they share a wide pathological and clinical heterogeneity with their human counterparts [2]. Investigations aimed at discovering clinical and pathologic parameters with prognostic and/or therapeutic significance for these neoplasms are considered an important field of study. The dog is also considered as a suitable animal model for research on human breast cancer [3].

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Despite advances in the identification of genes involved in tumor growth, progression and resistance to drugs, the way these genes interact with one another and the pathogenesis of cancer development remain still unclear. An excessive production of reactive oxidative species (ROS) could interfere with the physiological functions of the antioxidant system which is implicated in the pathogenesis of different cancer types, including mammary tumors. In this way, an excessive generation of ROS can induce cytotoxicity, membrane damage, lipid peroxidation, mutagenesis and carcinogenesis with transformation of normal to neoplastic cells [4].

The aims of this preliminary study were to evaluate the activity and the expression of antioxidant enzymes in canine mammary tumors in comparison to the adjacent healthy tissue and to determine whether these biochemical parameters could be correlated to the histological stage and neoplasm grade.

MATERIAL AND METHODS

Reagents

The reagents utilized for enzyme assays were purchased from Sigma Aldrich (St Louis, MO USA), Roche Diagnostics (Basel, CH) and Merck & Co (White House Station, NJ USA). Protein determination was performed with a Bio-Rad DC Protein Assay kit (Hercules, CA, USA). Materials used for electrophoresis and Western blotting were purchased from Invitrogen (Paisley, UK), while PVDF membranes were from Bio-Rad (USA); the primary antibodies were obtained from Stressgen (Victoria, British Columbia, CAN).

Biological samples

Six bitches of different breeds aged 9 to 13 years (mean value 10.4 years) were included in this study. The animals attended the Veterinary Teaching Hospital "Giuseppe Gentile" (University of Bologna) for surgery excision of mammary gland tumor. No other pathologies were detected. Metastasis suspicions were solved by thoracic radiographs and abdominal ultrasonographs before surgery. Dogs were anesthetized and surgically treated; all patients underwent either nodulectomy or mastectomy and none of the patients had experienced preoperative systemic chemotherapy or radiotherapy. After surgery fresh tumor and normal tissues were in part immediately frozen in dry ice and stored at -80°C for biochemical analyses and the remaining parts were used for histopathological analysis. All procedures were performed under informed consent of the owners for diagnostic and/or therapeutic purposes.

Histopathological analysis

Tissues were fixed in 10% neutral buffered formalin, paraffin embedded and 3-4 μm thick sections were obtained and stained with haematoxylin and eosin and histologically

evaluated. Diagnosis was achieved accordingly with the literature data [5]. For each case the histological stage (S) (to assess the invasiveness of the tumors) and histological grade (G) (to assess the malignancy of the tumors) were determined [6,7].

Tissue extraction for enzyme assay

200-300 mg wet tissue were homogenized in 10 volumes of 50 mM phosphate buffer (pH 7.6) containing 0.1 mM EDTA. Tissues were homogenized for 2 min (13,000 rpm) on ice in an UltraTurrax and then centrifuged at 500g for 10 min at 4°C in a Beckman TJ25 Centrifuge. After centrifugation, the supernatants were centrifuged again at 11000g for 15 min at 4°C in a Beckman LE80 Ultracentrifuge. The obtained supernatants were stored at -80°C for subsequent analysis.

Superoxide dismutase activity assay

Total superoxide dismutase (SOD) activity was determined at 25°C with the xanthine oxidase-cytochrome c method according to Crapo et al. [8]. The superoxide anion produced by the xanthine oxidase reaction reduces cytochrome c, which can be monitored spectrophotometrically at 550 nm. MnSOD was determined in the presence of 1 mM KCN, which inhibited the activity of Cu-ZnSOD, whose activity was determined by subtracting from total SOD the activity of MnSOD. Each sample was tested in quadruplicate using a Beckman DU 530 UV-Vis spectrophotometer. SOD activity was expressed as specific activity (U/mg protein), where one Unit of SOD is defined as the enzyme activity which causes 50% inhibition of the xanthine oxidase reaction.

Catalase assay

Catalase (CAT) activity was determined at 25°C by following the decrease of absorbance at 240 nm according to Aebi [9], using a 50 mM phosphate buffer, pH 7.6 and 20 mM H_2O_2. Each sample was tested in quadruplicate using a Beckman DU 530 UV-Vis spectrophotometer. CAT activity was expressed as specific activity (U/mg protein), where one Unit was defined as the enzyme activity required to decompose 1 μmol of H_2O_2 per min.

Protein assay

Soluble protein concentration in supernatants was measured according to Lowry et al. [10]. Bovine serum albumin (BSA) was used as a standard. Each sample was tested at 750 nm in triplicate using 96-well microtiter plates in a MultisKan EX spectrophotometer.

Electrophoresis and Western blotting

SDS-PAGE was performed in an Invitrogen Xcell SureLock Mini-Cell using 12% Bis-Tris mini gels with MES running buffer at pH 7.3, under reducing conditions.

Appropriate volumes of cytosolic extracts in order to obtain 25 μg protein/lane were loaded to each well. After electrophoresis, proteins were transferred for 1 h to PVDF membranes in an Invitrogen Xcell SureLock Blot Module using transfer buffer (pH 7.2). After blotting, the PVDF membranes were treated with the chromogenic Western blot immunodetection kit (Invitrogen). Polyclonal rabbit anti-human Cu-ZnSOD, diluted 1:7000 and polyclonal rabbit anti-rat MnSOD, at dilution 1:5000 were used. The blots were visualized using a chromogenic substrate containing BCIP (5-bromo-4-chloro-3-indolyl-1-phosphate) and NBT (nitro blue tetrazolium). No cross reactivity between the two SOD isoforms nor with other tissue extract proteins were observed. The intensity of the bands was determined using the Quantity One Software (Biorad).

Statistical analysis

Data were analyzed with statistical software R version 2.15.1 and expressed as mean ± standard deviation (SD). Data were checked for normal distribution with Shapiro-Wilk test. The comparison between control (n=6) and tumoral tissues (n=6) was made by paired t-test. The tumoral group was further divided into two sub-groups based on the histological stage (S1, n=3 and S2, n=3) and grade (G1, n=3 and G2-3, n=3). The comparison among the three groups was made by ANOVA for repeated measures followed by post-hoc test pairwise t-test. $p < 0.05$ was considered as significant.

RESULTS

Specific histological diagnosis and tumor grade and stage are reported in Table 1.

Table 1. Dog clinical history and histological data of canine mammary tumors included in the study.

Breed	Age	Ovario hysterectomy	Diagnosis	Histological Stage(S)	Histological Grade(G)
Westy Terrier	13	yes	Ductal carcinoma	S1	G1
Mixed breed	9	yes	Tubulopapillary carcinoma	S1	G2
Mixed breed	12	no	Tubular carcinoma	S1	G1
Maremma sheepdog	9	no	Complex carcinoma	S2	G1
German shepherd	9	no	Tubular carcinoma	S2	G2
Pitbull	12	no	Mucinous carcinoma	S2	G3

Data on enzyme activities are reported in Table 2. In healthy tissues adjacent to tumors Cu-ZnSOD activity showed a wide variability (range 0-3 U/mg protein) and was lower than MnSOD and CAT activities. Cu-ZnSOD activity in mammary tumors increased significantly compared with healthy control tissues ($p=0.03$), while CAT activity

was significantly higher in the healthy mammary gland respect to tumors (p=0.015). MnSOD and total SOD did not show significant variations.

Table 2. Enzyme activities in canine mammary tissues. Data are expressed as specific activity (U/mg protein) and reported as mean±SD. CuZnSOD activity was calculated as difference between total SOD and MnSOD activities. * indicates significant difference between normal and tumoral tissues. Different lower cases in the row indicate significant difference between normal tissue and tumoral S1 and S2 tissues; different Greek letters in the row indicate significant difference between normal tissue and tumoral G1 and G2-3 tissues.

Enzyme	Normal tissue (n=6)	Tumoral tissue (n=6)	Tumoral tissue S1 (n=3)	Tumoral tissue S2 (n=3)	Tumoral tissue G1 (n=3)	Tumoral tissue G2-3 (n=3)
Cu-ZnSOD	$0.75\pm1.3^{a\alpha}$	$5.1\pm2.8^{*}$	6.4 ± 0.6^{b}	3.8 ± 3.8^{ab}	$4.9\pm3.1^{\alpha}$	$5.4\pm3.2^{\alpha}$
MnSOD	$17.6\pm3.8^{a\alpha}$	10.9 ± 4.3	13.2 ± 3.1^{ab}	8.6 ± 4.6^{b}	$9.3\pm3.9^{\alpha}$	$12.6\pm4.8^{\alpha}$
Total SOD	$18.2\pm3.6^{a\alpha}$	16.0 ± 5.5	19.6 ± 2.2^{a}	12.4 ± 5.4^{a}	$14.6\pm7.2^{\alpha}$	$17.9\pm4.1^{\alpha}$
CAT	$22.2\pm6.6^{a\alpha}$	$10.3\pm3.1^{*}$	12 ± 3^{ab}	7.9 ± 0.3^{b}	$13.7\pm0.8^{\alpha\beta}$	$8.1\pm0.4^{\beta}$

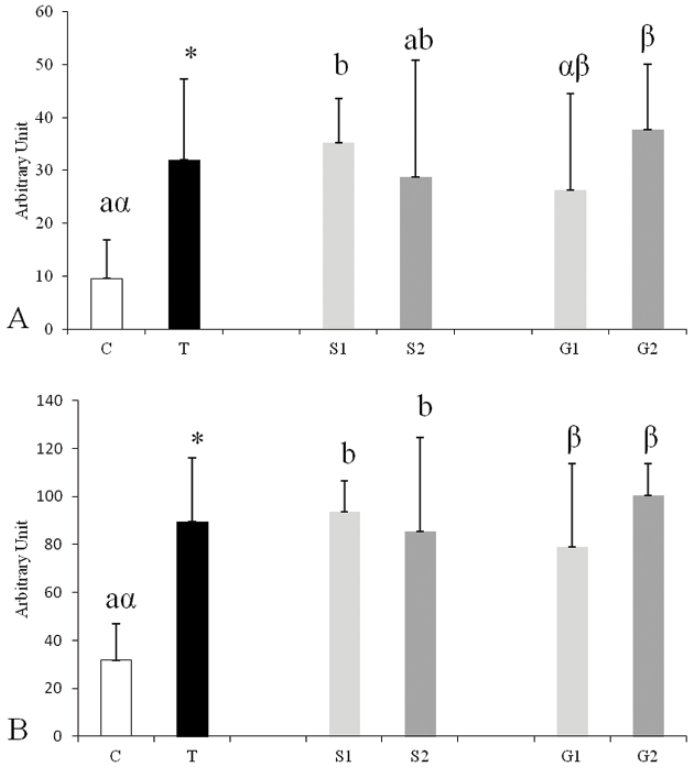

Figure 1. Results of Western blots (A. Cu-Zn SOD expression; B. Mn SOD expression) expressed as mean ± SD (arbitrary unit); control tissues (C, n=6) and tumoral tissues (T, n=6) of different stage (S1, n=3; S2, n=3) or grade (G1, n=3; G2-3, n=3) are reported. *indicates significant difference between normal and tumoral tissues; different lower cases indicate significant difference beetwen normal tissue an tumoral S1 and S2 tissues; different Greek letters indicate significant difference between normal tissue and tumoral G1 and G2-3 tissues.

Considering the histological stage, MnSOD and CAT showed a significantly lower activity in tumors at stage 2 ($p<0.05$) in comparison with normal tissue. In addition CAT showed a significantly lower activity ($p<0.05$) in tumors at grade 2-3 in comparison with normal tissue. Total SOD activity did not show significant variations related to stage and grade. Western blot analysis evidenced the presence of a band with an apparent molecular weight of 16 kDa corresponding to the monomer of Cu-ZnSOD and a band with an apparent molecular weight of 21 kDa corresponding to the subunit of MnSOD. The expression of the two SODs was significantly higher ($p<0.01$) in tumors than in control tissues (Fig. 1), with a predominant expression of MnSOD respect to Cu-ZnSOD in all the examined samples.

DISCUSSION

In the present research we reported data on antioxidant enzymes Cu-ZnSOD, MnSOD and CAT in canine mammary tumors in comparison with the normal adjacent mammary gland. The low Cu-ZnSOD activity in healthy tissues may be related to the age of the animals and to the physiological involution of the mammary gland, according to data reported by Kumaraguruparan et al. [11]. Differently, the higher activity of MnSOD could be maintained by the positive action of oextradiol, at least in entire bitches. It has been reported by Borrás et al. [12] that oestrogens protect females against aging by up-regulating the expression of antioxidant genes such as glutathione peroxidase and MnSOD. CAT specific activity determined in normal mammary tissues is similar to the values reported by Schogor et al. [13] in dairy cows.

Increasing evidence suggests that cancer cells produce high levels of ROS that facilitate tumor growth and metastasis and it is well known that breast cancer is characterized in women by oxidative stress [14]. Increased ROS production can in turn stimulate SOD gene up-regulation via ARE sequences. The increase of CuZnSOD activity and expression with the concomitant decrease of CAT activity reported in the present paper in canine mammary tumors can determine higher intracellular concentrations of H_2O_2 leading to a stimulation of signaling pathways resulting in cell proliferation and metastasis formation. This hypothesis is in accordance with data reported by Sen et al. [15] that in breast cancer cells intracellular concentrations of H_2O_2 are found to be higher than normal cells and with experimental evidence that cancer cells produce high levels of ROS favoring tumor growth and metastasis. Moreover, the high concentration of H_2O_2 during tumor progression can promote the translocation of CAT to the outside of the cell membrane and a local increase of the membrane-associated enzyme lowering its intracellular activity [16]. In the present research the significant decrease of intracellular CAT activity in tumoral tissues is in accordance with this hypothesis. At the transcriptional level, Glorieux et al. [17] reported that CAT is down-regulated in human and rodent tumor tissues compared to normal tissues of the same origin.

MnSOD overexpression in canine mammary tumors reported in the present paper is in accordance with data presented by Zamani-Ahmadmahmudi et al. [2]. The MnSOD overexpression in cancer cells compared to normal cells is a common finding in studies on different carcinomas [18], including also breast cancer [19]. The reduced activity of MnSOD found in this research in neoplastic mammary glands is in accordance with the increase of a nonfunctional enzyme in high-grade prostatic cancer [20]. This apparent discrepancy could be related to a sophisticated MnSOD regulation mechanism based on p53. It has been proposed that the activation of p53 and its translocation into the mitochondrial matrix could result in the interaction between MnSOD and p53 with subsequent partial inhibition of enzyme activity [21]. The inactivation of the enzyme could be related also to the presence of autoantibodies against MnSOD. This hypothesis is supported by the identification of serum autoantibodies against MnSOD in women [22] and bitches with mammary gland tumors [2].

Regarding the possible correlation between antioxidant enzymes and histological stage and grade of the neoplasms, data on canine mammary gland tumors are fragmentary. Zamani-Ahmadmahmudi et al. [23] detected a significant correlation between MnSOD immunoreactivity and histological grade of tumors. Accordingly, we detected a numerical increase of MnSOD and CuZnSOD expression, though not significant, in grade 2-3 tumors. Moreover, the significant reduction of CAT activity in both stage 2 and grade 2-3 determined in the present study is in agreement with the correlation between the increase of oxidative stress and stage or grade of breast cancer in human patients reported by Rezk et al. [24].

In conclusion, though preliminary and obtained on a limited number of animals, the data reported in this study show variations of antioxidant enzyme activity and expression suggesting an increase of oxidative stress and could provide additional evidence on the involvement of antioxidant enzymes and ROS in carcinogenesis of the canine mammary gland. Further research is needed to confirm these findings and to demonstrate the usefulness of the enzymes as clinical biomarkers for transformation of normal cells to neoplastic cells and to determine the mechanism of ROS-mediated canine carcinogenesis. This knowledge may be essential in evolving a new approach to cancer therapy in veterinary medicine.

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Authors' contributions

AG participated in assay of antioxidant enzymes analysis and drafted the manuscript. AG carried out the histopathological analysis. FE participated in the design of the study and performed the statistical anaysis. IG conceived of the study, and participated

in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ANTIOKSIDANTIVNI ENZIMI KOD TUMORA MLEČNE ŽLEZDE PASA

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Spontani tumori mlečne žlezde kod pasa se često pojavljuju. U medicinskim naukama, dobro je opisana povezanost oksidativnog stresa i funkcije antioksidativnih enzima u kancerogenezi kod ljudi. Međutim, u veterinarskoj medicini nema podataka ili su oni delimični. Osnovni cilj studije je bio evaluacija aktivnosti i ekspresija superoksid dismutaze (Cu-ZnSOD i MnSOD) kao i aktivnost katalaze (CAT) kod tumora mlečne žlezde u poređenju sa okolnim zdravim tkivom. Studijom je obuhvaćeno šest kuja, prosečne starosti 10,4 godine. Neposredno posle hirurške operacije, tkiva tumora i okolnog zdravog tkiva su zamrzavana u suvom ledu i ostavljena na -80°C, a radi biohemijske analize. Ostatak tkiva je upotrebljavan za histopatološku analizu. Aktivnost enzima je merena spektrofotometrijski, a ekspresija proteina je određivana Western blot metodom. Kod tumora mlečne žlezde kuja, aktivnost CuZnSOD kao i ekspresija, bile su značajno povećane u poređenju sa zdravim okolnim tkivom ($P=0,03$). MnSOD je pokazala značajno manju aktivnost u tkivima tumora u fazi 2 ($p<0,05$), uz značajno povećanu ekspresiju u tkivima tumora. CAT aktivnost je bila značajno povećana u zdravom tkivu u odnosu na tkivo tumora ($p=0,015$). Ove varijacije u aktivnostima antioksidantnih enzima kao i ekspresije, mogu da budu u vezi sa povećanjem oksidativnog stresa u tkivima mlečne žlezde. Istovremeno, varijacije ovih parametara ukazuju da oni mogu da budu parametri koji bi se ispitivali i ukazivali na neoplastičnu transformaciju.