ANTIOXIDANT STATUS IN CANINE CANCER PATIENTS

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The present study was conducted to investigate the antioxidant status in canine cancer patients. Patients with multicentric lymphoma, oral fibrosarcoma, mast cell tumour, malignant melanoma, appendicular osteosarcoma, nasal tumours and peripheral ameloblastoma were selected. Each group consisted of 6 patients. Total antioxidant capacity (TAC) and enzyme antioxidants: glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) were measured in serum and whole blood, respectively, and were compared to 31 healthy dogs.

The results of the study showed a significant increase of CAT activity in tumour groups except in the patients with nasal tumours. SOD activity increased significantly in malignant melanoma, mast cell tumour, multicentric lymphoma and oral fibrosarcoma patients. Appendicular osteosarcoma and multicentric lymphoma patients showed significantly increased levels of GPX and TAC, respectively. Activities of CAT and SOD were significantly higher comparing the all 42 cancer patients with healthy dogs. Tumor patients showed significantly lower levels of haemoglobin when compared to healthy dogs.

The increase of antioxidant enzyme activities and TAC in these animals suggest the activation of antioxidant defence mechanisms in different cancer diseases. Further studies involving more animals and other antioxidant parameters and oxidative stress markers are necessary.

Key words: cancer, catalase, dog, glutathione peroxidase, superoxide dismutase, total antioxidant capacity

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INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are constantly generated *in vivo* as an integral part of cell metabolism. Low levels of ROS are indispensable in intracellular messaging, cell differentiation, arrest of growth, apoptosis and defence against microorganisms. High doses and/or inadequate removal of ROS result in oxidative stress with consequential severe metabolic malfunction and damage to biological macromolecules: DNA, lipids and proteins (Halliwell and Gutteridge, 1999; Mates *et al*., 1999; Mates and Sanchez-Jimenez, 2000). These changes may activate or suppress signal pathways and gene expression and thus cause cell death (apoptosis) or activation of some proto-oncogenes and/or inactivation of tumour suppression genes (Mates and Sanchez-Jimenez, 2000). This dual effect of ROS, known as a "threshold concept" can be used for cancer treatment (Kong *et al*., 2000).

Mammals have evolved complex antioxidant strategies to protect cells from oxidation. Antioxidants within cells, cell membranes and extracellular fluids can be upregulated and mobilized to neutralize excessive and inappropriate ROS formation (Halliwell and Gutteridge, 1999; Mates *et al*., 1999, Mates and Sanchez-Jimenez, 2000; Kotur-Stevuljević *et al*., 2005). According to their mode of action they can be differentiated into three main groups:

− "preventative antioxidants", which prevent the formation of new ROS (caeruloplasmin, metallothionine, albumin, ferritin, transferrin, myoglobin),

− "scavenging antioxidants" remove ROS once formed, thus preventing radical chain reaction (reduced glutathione (GSH), vitamin E, vitamin C, β-carotene, uric acid, bilirubin) and

− "enzyme antioxidants" that function by catalysing the oxidation of other molecules (glutathione peroxidase (GPX), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD) (Chapple, 1997).

The last group acts on several levels. SOD destroys superoxide anions by converting them into less reactive hydrogen peroxide that can be destroyed by GPX or CAT reactions. CAT catalyses the transformation of H_2O_2 into water and molecular oxygen; and with hydrogen donors into methanol, ethanol, formic acid or phenols. GPX catalyses the reduction of various hydroperoxides using reduced glutathione (GSH) (Mates and Sanchez-Jimenez, 2000).

The extent of oxidative stress and hence lipid peroxidation increases with age in healthy individuals (Vajdovich *et al*., 1997; Inal *et al*., 2001, Öztürk and Gümüslü, 2004). Enlarged antioxidant expression is an adaptation mechanism that ensures protection from ROS stress. This compensation process has a limited capacity and compensation mechanisms may get exhausted (Pelicano *et al*., 2004) and hence lead to neoplastic transformation (Kong *et al*., 2000).

A lot of studies investigated free radical production and antioxidant status in human cancer patients. The results remain controversial, although increased ROS production with its by-products (most often malondialdehyde − MDA, a parameter showing lipid peroxidation) is a consistent finding in tumour tissues and/or erythrocytes and blood plasma (Zima *et al*., 1996; Ray *et al*., 2000; Yeh *et*

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al., 2005). Increased ROS production may be accompanied with significantly lower activities of GPX and SOD in erythrocytes of multiple myeloma (Zima *et al*., 1996) or cervical cancer patients (Manju *et al*., 2002), lower activity of CAT and increased levels of GPX and SOD in breast cancer patients (Ray *et al*., 2000), also increased levels of GPX and SOD in erythrocytes of acute myeloid leukemia patients (Er *et al*., 2007), or SOD, CAT and xanthine oxidase in lung cancer patients (Kaynar *et al*., 2005). Similar studies in veterinary medicine have not been performed to such extent.

Kumaraguruparan and colleagues (2005) looked at oxidant-antioxidant profiles in canine mammary tumours and found enhanced lipid peroxidation in tumour tissues when compared to uninvolved tissue, accompanied by significant elevation of various antioxidants. On the contrary, Vajdovich *et al*. (2005) found significantly lower values of SOD activities and GSH concentrations in red blood cell haemolysates in dogs with non-Hodgkin's lymphoma compared to controls.

Taking into account the lack of data, the aim of the study was to contribute to research efforts on oxidative stress in cancer in dogs in order to see how it can affect the level of antioxidant enzymes and total antioxidant capacity, and whether additional antioxidant therapy is needed. The hypothesis was that antioxidant status in cancer patients is reduced due to depletion of compensatory mechanisms and addition of antioxidant supplements could be beneficial.

MATERIALS AND METHODS

Study population

Forty-two dogs, 20 females (10 of them spayed) and 22 males (3 of them castrated), with an average age of 8.1 ± 2.8 years (ranging from 1 to 13 years), admitted as cancer patients to the Hofheim Veterinary Hospital (Germany), were divided into 7 groups (6 patients per group) depending on the type of tumour disease and treatment modality. Dogs with major concurrent diseases, such as chronic renal failure, hepatic failure and congestive heart failure were excluded from the study. Groups were formed from patients with multicentric lymphoma (ML), malignant melanoma (MMel), appendicular osteosarcoma (OSA), oral fibrosarcoma (FSA), mast cell tumour (MCT), different tumours of nasal cavities (Nose) and peripheral ameloblastoma (PAmel). Clinical examination of all dogs was performed. Samples for cytological and/or pathohistological examination were taken and staging of the disease was performed. None of them had evidence of macroscopic matastases detected by X-ray and/or ultrasound. The clinical stage of 6 canine lymphoma cases was: stage Ia for one dog, stage IIa for one dog, IIIb for two dogs, IVa for one dog and Va for one dog. Blood samples for the determination of haematological and antioxidant status parameters (GPX, CAT, SOD and TAC) were collected.

The group of healthy dogs consisted of 31 healthy dogs, 15 females (6 of them spayed) and 16 males (1 of them castrated), their average age was 6.1 ± 2.5 years (ranging from 2 to 11 years). They were admitted to the Clinic for Small Animal Medicine and Surgery, Veterinary Faculty, Ljubljana for elective procedures. The health status of these dogs was established on the results of

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clinical examination and haematological, biochemical and coagulation parameters (data not shown). Blood samples for the determination of antioxidant status were collected under the same conditions as for the study group. Owners signed a consent form before enrolling their dogs in the study.

The patients and controls did not receive any dietary antioxidant supplements.

Preparation of blood samples

Venous blood samples for the determination of haematological and antioxidant enzyme parameters were collected from the cephalic vein into blood collection tubes with anticoagulant K_3EDTA (Greiner Bio-One Vacuette). EDTA blood samples for complete blood count (CBC) and white cell differential count (WCDC) determination were stored at room temperature and analysed from 5 to 30 minutes after sampling with the automated haematology analyzer using laser flow cytometry (LaserCyte, IDEXX Laboratories, Inc., Westbrook, ME, USA) at the Hofheim Veterinary Hospital (oncologic patients) or with automatic laser haematology analyzer Bayer Technicon H*1 (Bayer Technicon, Germany) at the Clinic for Small Animal Medicine and Surgery, Veterinary Faculty, Ljubljana (healthy dogs).

EDTA blood samples for the determination of GPX, CAT and SOD were collected, divided into three aliquots, flash frozen in liquid nitrogen from 5 to 30 minutes after sampling and stored at -20°C for up to 2 months and then at -80°C until analysed. Whole blood lysates were assayed at the end of the study 3 to 12 months after the sampling in duplicate.

Blood samples for the determination of TAC were collected into tubes with coagulation activator and separation gel (Greiner Bio-One Vacuette), allowed to clot for 30 minutes and centrifuged at 1200g for 10 minutes at 4°C. Serum was separated, frozen in liquid nitrogen and stored at -20°C for up to 2 months and then at -80°C until analysed.

Measurement of GPX activity

Activity of GPX was measured using commercial Ransel kit (Randox Laboratories, Crumlin, UK) with an automated biochemical analyser Hitachi 917 (Hitachi, Japan). According to the method of Paglia and Valentine (1967) GPX activity is determined indirectly by measuring the rate of formation of oxidized glutathione (GSSG). GPX catalyzes the reaction of GSH with synthetic cumene hydroperoxide to GSSG. In the presence of NADPH and glutathione reductase GSSG is transformed to glutathione, and NADPH is oxidized to NADP. The rate of oxidation of NADPH was measured spectrophotometrically as reduced absorbance at 340 nm and is proportional to the activity of GPX in the specimen. Activity of GPX was expressed as μkat/g of haemoglobin (μkat/g Hgb).

Measurement of CAT activity

The method for determination of CAT activity (Bioxytech Catalase-520, OxisResearch, USA) is based on a two-stage reaction. The rate of dismutation of hydrogen peroxide (H_2O_2) to water and molecular oxygen is proportional to the

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concentration of CAT. A known amount of H_2O_2 was added to the specimen with CAT and incubated for exactly 1 minute. The reaction was quenched with sodium azide. The amount of H_2O_2 remaining in the reaction mixture was then determined by the oxidative coupling reaction of 4-amino-phenasone (PAP) and 3,5-dichlorhydroxybenzenesulfonic acid (DHBS), and the reaction was catalyzed by horseradish peroxidase. The resulting quinoneimine dye was measured at 520 nm with the biochemical analyzer Olympus AU600 (Olympus, Japan). The results were expressed as mkat/g Hgb.

Measurement of SOD activity

SOD activity was determined spectrophotometrically (550 nm) with an automatic biochemical analyser Hitachi 917, using commercially available Ransod kit (Randox Laboratories, Crumlin, UK). According to the method the superoxide radicals were generated by the xanthine and xanthine oxidase reaction. The amount of superoxide radical produced was determined by 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrasolium chloride (INT) as an indicator, which reacts with a superoxide radical to form formazan dye. The SOD activity was determined by the grade of inhibition of the described reaction. The standard calibration curve of percentage of inhibition by standard solutions and log concentrations (U/ml) was used to determine SOD activity in our specimens. Activity was expressed as μkat/g Hgb.

Measurement of TAC

Total antioxidant capacity was determined spectrophotometrically (600 nm) with the commercially available TAS kit (Total Antioxidant Status, Randox, Crumlin, UK) with an automatic biochemical analyser Technicon RA-XT (Bayer-Technicon, Germany) following the instructions of the kit. The assay is based on the reduction of free radicals 2,2'-azino-di-3-ethylbenzothiazoline-6-sulfonate (ABTS$^{\cdot+}$) measured as a decrease of absorbance at 600 nm at 3 min by antioxidants. The ABTS$^{\cdot+}$ radical cation is formed by the interaction of ABTS with ferrylmyoglobin radical species, generated by the activation of metmyoglobin with hydrogen peroxide. The results were expressed as mmol/l of Trolox equivalents.

Statistical analysis

For each variable the data were examined for normality. Mean and median were calculated for each variable in each group. When the hypothesis of the normal distribution was not rejected, mean and standard deviations were used for further calculations. Otherwise the median and a 95 % confidence interval were used.

Means were compared using the Student's t-test. For non normal distributions the Mann-Whitney test was used.

The minimum level of significance was defined at $p < 0.05$. All analyses were performed using the SPSS 12.0 computer program.

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RESULTS

Haemoglobin (Hgb), antioxidant enzyme activities and TAC of the total group of cancer patients were compared to the group of healthy dogs and resulted in significantly higher activity of CAT, SOD and significantly lower levels of haemoglobin (Table 1).

Table 1. Comparison of haemoglobin and antioxidant status parameters of 31 healthy dogs and 42 canine cancer patients

Parameter	Healthy		Cancer patients		p
	Median	Mean ± SD	Median	Mean ± SD	
Hgb (g/l)	172	174 ± 15	146	145 ± 20	0.000*
GPX (μkat/g Hgb)	5.29	5.52 ± 0.78	5.78	5.70 ± 1.36	0.078
CAT (mkat/g Hgb)	0.613	0.625 ± 0.200	0.839	0.863 ± 0.211	0.000*
SOD (μkat/g Hgb)	11.4	11.9 ± 1.5	13.3	13.3 ± 1.8	0.000*
TAC (mmol/l)	0.87	0.89 ± 0.14	0.90	0.88 ± 0.16	0.880

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$p < 0.05$ in comparison with healthy dogs

Table 2 shows haemoglobin and levels of antioxidant status of the various groups of cancer patients compared to the same parameters in the control group.

Patients in all tumour groups showed significantly lower levels of haemoglobin when compared to healthy dogs (Tables 1 and 2). With the exception of dogs with nasal tumours, all the others had significantly increased levels of CAT activity when compared to healthy dogs. SOD activity was significantly increased in patients with malignant melanoma, mast cell tumour and multicentric lymphoma (Table 2) when compared to healthy dogs. There was also a rise in the SOD activity in other groups, but the changes were not statistically significant, which was the same for the increased CAT activity in nasal tumour patients. Additionally, dogs with appendicular osteosarcoma showed statistically significant higher GPX activity and dogs with multicentric lymphoma showed higher TAC when compared to healthy dogs.

Table 2. Comparison of haemoglobin and antioxidant status parameters of 31 healthy dogs and 7 groups of different cancer patients

Parameter	Hgb (g/l)		GPX (μkat/g Hgb)		CAT (mkat/g Hgb)		SOD (μkat/g Hgb)		TAC (mmol/l)	
	Mean±SD	p	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p	Mean ± SD	p
Healthy	174 ± 15		5.52 ± 0.78		0.625 ± 0.200		11.9 ± 1.5		0.89 ± 0.14	
Nose	143 ± 17	0.000*	5.01 ± 1.78	0.984	0.792 ± 0.125	0.058	13.0 ± 1.7	0.061	0.76 ± 0.23	0.180
ML	146 ± 24	0.001*	5.90 ± 1.23	0.510	0.877 ± 0.120	0.006*	12.8 ± 1.0	0.048*	1.02 ± 0.09	0.016*
MMel	139 ± 28	0.027*	5.83 ± 2.81	0.284	0.921 ± 0.321	0.005*	13.7 ± 2.0	0.041*	0.80 ± 0.09	0.063
OSA	149 ± 11	0.000*	6.04 ± 0.53	0.039*	0.891 ± 0.225	0.006*	13.2 ± 2.1	0.117	0.84 ± 0.15	0.695
MCT	154 ± 14	0.005*	5.94 ± 011	0.070	0.869 ± 0.284	0.036*	13.4 ± 1.6	0.027*	0.93 ± 0.16	0.375
FSA	141 ± 16	0.000*	5.56 ± 0.52	0.510	0.888 ± 0.263	0.008*	13.6 ± 1.6	0.034*	0.88 ± 0.11	0.853
PAmel	141 ± 27	0.029*	5.64 ± 1.07	0.821	0.804 ± 0.125	0.043*	13.4 ± 3.1	0.180	0.94 ± 0.14	0.509

*p<0.05 in comparison with healthy dogs

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DISCUSSION

It is well known that excess generation of ROS with compromised antioxidant production can cause oxidative stress and that ROS are involved in cancer initiation and promotion (Mates and Sanchez-Jimenez, 2000). Common ways to asses oxidative stress is to measure the formation of thiobarbituric acid-reactive substances (TBARS), conjugated dienes (CD), and MDA (indirect methods), or by measuring ROS directly, which is more difficult, because of their highly reactive nature (Halliwell and Whiteman, 2004). Many studies in human and veterinary medicine demonstrated increased oxidative stress in tumour tissues using either method (Inci *et al*., 2003; Kumaraguruparan *et al*., 2005; Er *et al*., 2007), accompanied with significantly higher (Inci *et al*., 2003; Kumaraguruparan *et al*., 2005) or lower activities of antioxidant enzymes when compared to normal tissues (Er *et al*., 2007). Similarly, higher levels of ROS were noticed on a systemic level in the blood of different tumour patients (Manju *et al*., 2002; Yeh *et al*., 2005). The assessment of oxidative stress in our study was incomplete, as only activities of intracellular antioxidant enzymes and serum TAC were determined. Nevertheless, we noticed increased activities of different antioxidant enzymes in the blood, mostly induction of CAT and to a minor degree SOD enzymes, which provide the first line of defence against ROS induced damage (Kumaraguruparan *et al*., 2005). In comparison with healthy dogs significantly higher levels of GPX in appendicular osteosarcoma patients were found. These findings are consistent with studies performed on small cell and non-small cell lung cancer (Kaynar *et al*., 2005), breast cancer (Yeh *et al*., 2005) and acute myeloid leukemia patients in humans (Er *et al*., 2007) and on mammary tumour patients in veterinary medicine (Szczubial *et al*., 2004).

Upregulation of antioxidants may not only be the result of higher systemic ROS production but could develop due to excess leakage of $O_2^{\bullet-}$ from tumour tissues, which after crossing the membrane of erythrocytes serve as a signal to activate antioxidant enzyme production, as proposed by Er *et al*. (2007).

Antioxidants may get depleted due to increased scavenging of lipid peroxides, as well as sequestration by tumour cells (Manju *et al*., 2002), which in our study was not observed in any group, though it was reported by many studies in humans (Zima *et al*., 1996; Manju *et al*., 2002; Bakan *et al*., 2003) and also by Vajdovich *et al*. (2005) on canine lymphoma patients, potentially due to more advanced stage of the disease compared to our patients.

TAC measures the total antioxidant effect of all antioxidant defence systems in circulation. The antioxidative status of an individual is very much dependant on the oral intake of dietary antioxidants (Serafini and Del Rio, 2004). Thus, the depletion of TAC may represent the consumption of antioxidants in plasma by high generation of ROS or by the reduced intake of dietary antioxidants (Ghiselli *et al*., 2000). However, the present study shows that TAC levels in the serum specimens of canine cancer patients (except multicentric lymphoma patients) were comparable to those of healthy dogs, probably because the enzyme antioxidants removed redundant ROS. Since none of the dogs was supplemented with dietary antioxidants, the rise of TAC in multicentric lymphoma patients is

believed to be due to increased antioxidant production, also proven by the increased levels of CAT and SOD.

No reference values for antioxidant enzymes in dogs were found in the literature. We compared our results in healthy dogs with GPX, CAT and SOD activities published for three healthy Beagles (Nakamura *et al*., 1999) and found significantly higher activities of all the enzymes in our dogs, probably due to differences in the methodologies used. Furthermore, in the study by Nakamura *et al*. (1999) the age of the Beagles is not reported, which is known to significantly affect the antioxidant levels (Vajdovich *et al*., 1997). A second study (Nemec *et al*., 2000) reported TAC values in serum of healthy, one to three year old Beagles. Mean and median TAC values (1.08 and 1.10 mmol/l, respectively) were higher than the TAC values of healthy dogs in our study, most likely due to higher age of dogs and the use of an automated biochemical analyser in our study compared to the use of a manual photometer in the study by Nemec *et al*. (2000).

Comparing GPX activities of our healthy dogs with those in the study from Stowe *et al*. (2006) in the age group comparable to the average age of our healthy dogs (6 years), we found significantly higher GPX activity. However, different manipulation, storage at 2°C until assay and different feeding protocols could contribute to the lower GPX activity in the mentioned study. The authors are aware of only one study that reported stability of GPX and SOD enzymes when stored at 4°C and -20°C. Minimum stability of SOD at these temperatures was 56 days in contrast to GPX, which was stable for 3 days at 4°C and only 1 day at -20°C (Jones, 1985). To overcome this problem, we used quick freezing in liquid nitrogen and storage at -20°C and -80°C as proposed by OxisResearch, the manufacturer of catalase test kit (Bioxytech Catalase-520, OxisResearch, USA).

Abiaka and colleagues (2000) demonstrated that CuZn-SOD and GPX in washed erythrocyte haemolysates are stable at -80°C for almost 2 years.

It is known that haematological, biochemical and also antioxidant status parameters change during the lifetime (Bush, 1991; Vajdovich *el al*., 1997), with higher haemoglobin, GPX and SOD levels in older animals. Higher enzyme antioxidant levels in older people and animals (Vajdovich *et al*., 1997; Inal *et al*., 2001) probably occur in order to compensate for the increased ROS production and lipid peroxidation process (Vajdovich *et al*., 1997). To overcome the age effect, we selected a group of sex-matched adult healthy patients to compare them with our study population. They were 2 years younger than cancer patients, but Stowe *et al*. (2006) showed very similar levels of GPX in these two age groups.

Anaemia is one of the most common paraneoplastic syndromes in veterinary oncology (Bergman, 2007) and is a possible explanation for significantly decreased haemoglobin levels (although within the reference range) in oncologic patients when compared to normal dogs in all groups (Bush, 1991).

Although we showed that there is an activation of antioxidant defence system in different canine malignancies, further studies with more patients and the use of additional oxidative stress markers are needed to elucidate the exact mechanisms of activation, which until today are unknown.

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ANTIOKSIDATIVNI STATUS PASA SA MALIGNITETOM

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SADRŽAJ

Predstavljena studija je bila izvedena sa namjerom da istraži antioksidantni status kod pasa sa tumorskom bolešću. Izabrali smo pacijente sa multicentričnim

limfomom, fibrosarkomom usne šupljine, mastocitomom, malignim melanomom, apendikularnim osteosarkomom i perifernim ameloblastomom. Svaka grupa je sadržavala šest pacijenata. U serumu smo izmjerili totalni antioksidantni kapacitet (TAC) i u punoj krvi aktivnosti enzimskih antioksidanata: glutation peroksidaze (GPX), katalaze (CAT) i superoksi dismutaze (SOD). Ove rezultate smo usporedili sa grupom od 31 zdravog psa.

Rezultati studije su ukazali signifikantan porast aktivnosti CAT u svim grupama onkoloških pacijenata osim pacijenata sa tumorima nosnih i obnosnih šupljina. Signifikantan porast aktivnosti SOD otkrili smo u grupama pacijenata sa malignim melanomom, mastocitomom, multicentričnim limfomom i fibrosarkomom. Pacijenti sa apendikularnim osteosarkomom imali su signifikantno višu aktivnost GPX i pacijenti sa multicentričnim limfomom višu vrijednost TAC.

U usporedbi sa grupom zdravih pasa u grupi svih 42 onkoloških pacijenata utvrdili smo signifikantno višu aktivnost CAT i SOD. U usporedbi sa grupom zdravih pasa u svim grupama onkoloških pacijenata utvrdili smo signifikantno niže vrijednosti hemoglobina.

Porast aktivnosti antioksidantnih enzima i vrijednosti TAC kod ovih životinja ukazuju na aktivaciju antioksidantnih obrambenih mehanizama kod različitih onkoloških pacijenata.