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Bull Vet Inst Pulawy 59, 417-423, 2015
DOI:10.1515/bvip-2015-0061

Total antioxidative capacity and zinc concentration in dogs suffering from perianal tumours

Adam Brodzki[1], Piotr Brodzki[2], Marcin R. Tatara[3], Krzysztof Kostro[4]

[1]Department and Clinic of Animal Surgery,
[2]Department of Andrology and Biotechnology of Reproduction
[3]Department of Animal Physiology,
[4]Department of Epizootiology and Clinic of Infectious Diseases,
Faculty of Veterinary Medicine, University of Life Sciences in Lublin, 20-612 Lublin, Poland
brodzkiadam@op.pl

Received: June 14, 2015 *Accepted: September 4, 2015*

Abstract

The aim of the study was to determine total antioxidative capacity (TAC) and zinc concentration in serum of dogs suffering from perianal tumours just before the start of the antihormonal treatment (AHT) and one and six months later. The study was performed on 45 dogs divided into two groups: control group suffering from non-malignant tumours (N = 24) and a group with malignant neoplastic changes (N = 21). Serum TAC and zinc concentrations were measured using photometric and atomic absorption spectrophotometric methods. Six months after the start of the AHT, TAC was significantly lower by 10.6% in dogs with malignant tumours when compared to controls (P = 0.03). In the non-malignant group, serum zinc concentration was higher before the treatment than in the malignant group, while the opposite results were observed six months later (P < 0.001). In the non-malignant group, gradually decreasing values of serum zinc concentration at each stage of the investigation were observed, while the opposite results were obtained in the malignant group (P < 0.05). The obtained results indicate that malignant neoplastic process is associated with significantly reduced TAC. Determination of serum zinc concentration in dogs with non-malignant and malignant perianal tumours may have practical diagnostic and prognostic values and may serve towards increasing the effectiveness of AHT monitoring.

Keywords: dog, perianal gland tumours, zinc, oxidative stress, total antioxidative capacity.

Introduction

Apart from typical apocrine glands of the perianal sinuses in dogs, skin tissue of the anal region consists of specific glands exclusive to this region, called hepatoid glands. They do not secrete and are classified as incomplete sebaceous glands. Hepatoid glands are located in 2–3 cm radius around the anus and within the foreskin, as well as in the skin of pelvic limbs and tail. The highest number of these glands is found within dorsal and ventral skin of the 9th tail vertebrae region. Neoplasms of perianal glands occur in males exceeding 8–9 years of life and are considered to be the most frequent skin neoplasms (8, 10). Surgical neutering or removal of perianal gland tumours in patients at an advanced age is relatively invasive and accompanied by high anaesthetic risk. It may cause anal sphincter lesions and disturb its proper function. Thus, effective non-surgical therapeutic procedures, such as antihormonal treatment (AHT), seem to be more advantageous in patients with neoplastic changes. Androgens and oestrogens are important causative factors responsible for perianal tumour development. In experimental studies on female dogs, significantly increased serum and tumour tissue concentrations of testosterone and 17-β-oestradiol were shown to be associated with malignant mammary tumours incidence, since their concentration was significantly higher in comparison to healthy controls or dogs suffering from benign mammary tumours (23). In prostate cancer, androgen receptors activation by androgens such as testosterone and 5α-dihydro-testosterone is associated with progression of neoplastic disease; however, androgens acting through the androgen receptors are necessary for physiological prostate development and function. Biological activity of hormones both during normal cell function and during neoplastic proliferation is exerted through

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binding to their receptors and inducing transcriptional activity (15). Free radicals (FR) appearing during physiological course of vital metabolic processes also play a very important role in development of neoplastic diseases (11). In a properly functioning organism, physiological balance between formation and elimination of FR from the body is constantly maintained. Disruption of such physiological balance results in increased FR production leading to oxidative stress (OS) on cellular and tissue levels. Oxidative stress induces cellular damage due to the effects of reactive oxygen species (ROS) on proteins, lipids, and intracellular organelle and DNA mutations. Negative changes within cellular structures and functions contribute significantly to neoplastic transformation and subsequent tumour growth (16, 28). Several defence mechanisms operate within human and animal bodies protecting them against negative consequences of ROS activity. The main protective role in such preventive mechanisms is ascribed to such enzymes as superoxide dismutase (SOD), catalase, peroxidase, glutathione peroxidase (GPx), and reductase (12, 18). SOD is present in all body tissues, participating in oxidative processes and being responsible for key protection against negative effects of FR. Chemical structure of SOD consists of zinc (Zn), copper (Cu), manganese (Mn), and iron (Fe) (9). Iron SOD is the enzyme of prokaryotes and some higher plants. Copper/zinc SOD and manganese SOD are the enzymes of prokaryotes and eukaryotes. Three unique and highly compartmentalised mammalian superoxide dismutases have been biochemically and molecularly characterised. SOD1 (CuZn-SOD) is a copper and zinc-containing homodimer that is found almost exclusively in intracellular cytoplasmic spaces. SOD2 (Mn-SOD) exists as a tetramer and is initially synthesised containing a leader peptide, which targets this manganese-containing enzyme exclusively to the mitochondrial spaces. SOD3 (EC-SOD) is a copper and zinc-containing tetramer, and is synthesised containing a signal peptide that directs this enzyme exclusively to extracellular spaces (3, 32). All the bioelements present in SOD play a significant role in metabolic processes occurring during the neoplastic disease. Accumulation of Cu within liver tissue was found to be associated with neoplastic process development (25). Elevated serum and urine concentrations of Cu and ceruloplasmin were stated in patients suffering from liver cancer when compared to healthy controls. Thus, it was suggested that evaluation of Cu and ceruloplasmin concentrations may serve as biological markers of neoplastic process in the liver (33). Zinc is considered a competitive element to Cu in the body and its supplementation stimulates cellular activity protecting the whole organism against bacterial infections, and neoplastic transformation and growth (26). Dietary Zn deficiency is associated with a higher risk of colorectal cancer (20). Investigations on women suffering from breast cancer revealed significantly lowered serum Zn content, while serum Cu concentration and Cu:Zn ratio were found to be significantly increased in comparison to healthy individuals (30). Moreover, experimental studies have shown that evaluation of serum Cu and Zn concentrations, as well as their ratio, may be useful for early diagnosis of cancer within the gastrointestinal tract (19).

The aim of the study was to determine total antioxidative capacity (TAC) and serum Zn concentration in dogs suffering from perianal tumours immediately prior to the start of the AHT as well as one and six months later. The study is original and the results obtained provide novel data on serum antioxidative capacity and zinc concentration in dogs with malignant and benign perianal tumours both before and after the AHT.

Material and Methods

Experimental design and sampling procedure. The study was performed on 45 male dogs of different breeds divided into two experimental groups. Clinical and laboratory examinations (haematological, biochemical, radiological, and ultrasound) did not reveal any concomitant systemic diseases or metastases to local lymphatic nodes in the dogs. The dogs were not castrated and possessed physiologically developed gonads. Body conditions of the dogs did not deviate from their normal range characteristic for particular age that varied between 8 and 17 years. All dogs were fed a standard diet *ad libitum* (well balanced commercially available diets) without any additional mineral supplementation. Dogs suffering from benign neoplastic changes of the perianal glands such as adenoma (hepatoid gland adenoma) constituted the control group (N = 24; mean age 12.1 ± 2.5 years). The experimental group (N = 21) consisted of dogs suffering from malignant tumours due to epithelioma (hepatoid gland epithelioma, N = 12; mean age 12.6 ± 2.9 years) and carcinoma (hepatoid gland carcinoma, N = 9; mean age 10.0 ± 1.6 years). Diagnostic differentiation of dogs was performed on the basis of initial histopathological examination performed at the Department of Pathological Anatomy, University of Life Sciences in Lublin (Figs 1–3). Tissue samples for histopathological examination (0.5 mm in diameter) were obtained from the patients treated in the Department and Clinic of Animal Surgery, University of Life Sciences in Lublin, as the result of perianal gland tumour biopsy. Tissue collection was performed on animals under sedation (*i.m.* injection of 2 mg/kg b.w. of xylazine (Sedazin®, Biowet Puławy, Poland) and local anaesthesia with 2% lignocainum gel (Lignocainum, Jelfa, Jelenia Góra, Poland). Evaluation of such sex hormones as serum 17-β-oestradiol and testosterone was performed prior to the antihormonal treatment. Elevated oestrogen level in dogs with perianal tumours was the criterion used in deciding whether or not to apply the AHT with tamoxifeni citras

(Tamoxifen, Polfa Kutno, Poland) at the daily dosage of 1 mg/kg b.w. Dogs with serum concentration of 17-β-oestradiol equal 7 ng/mL or higher were subjected to the treatment using tamoxifeni citras. Elevated serum testosterone concentration resulted in antiandrogen treatment with cyproteroni acetas (Androcur, Schering, Germany) at the dosage of 5 mg/kg b.w./day. Dogs with serum concentration of testosterone equal 150 ng/dL or higher were subjected to the antiandrogen treatment. Blood samples for TAC and Zn evaluation were collected to 9 mL tubes without anticoagulant (Medlab-Products, Poland) immediately prior to the start of the AHT and one and six months later.

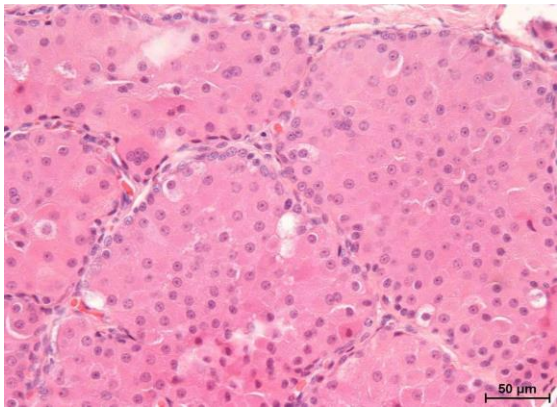


Fig. 1. Hepatoid gland adenoma as an illustration of benign neoplastic changes. Haematoxylin and eosin, 200×. Bar = 50 μm

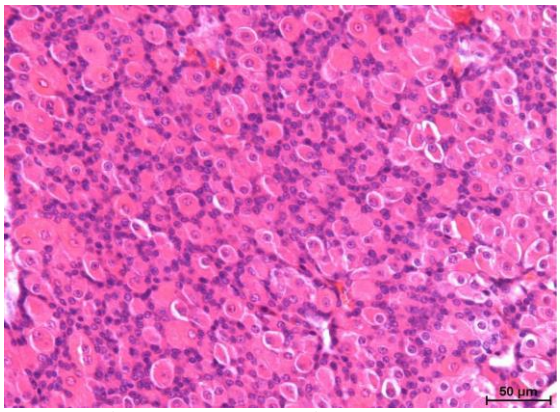


Fig. 2. Proliferating basaloid cells of hepatoid gland epithelioma as an illustration of malignant neoplastic changes. Haematoxylin and eosin, 200×. Bar = 50 μm

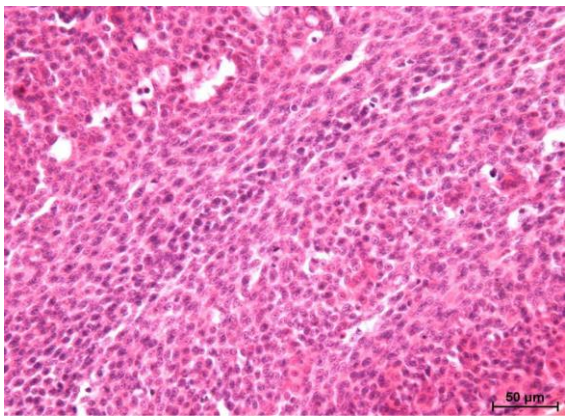


Fig. 3. Poorly-differentiated epithelial cells forming weaving of hepatoid gland carcinoma. Haematoxylin and eosin, 200×. Bar = 50 μm

Measurement of TAC and zinc concentration. Quantitative determination of TAC in serum was performed using commercial photometric test system (ImAnOx (TAS) Kit, Immundiagnostik, Germany). The measurement was performed with the use of Benchmark Plus microplate spectrophotometer supplied with Microplate Manager Software Version 5.2.1 (Bio-Rad Laboratories Inc., Hercules, USA).

To determine Zn concentration, serum samples were placed in quartz dishes and dried at 80°C for 72 h. After drying, the samples were mineralised by calcination at 450°C. The ash obtained was dissolved in spectrally clean hydrochloric acid (Merck, Germany), which had previously been mixed with deionised water at 1:1 ratio. Zinc concentration was measured directly from the water phase. Determination of Zn concentration was performed using atomic absorption spectrophotometric method and PYE UNICAM apparatus (Pye Unicam Ltd., UK). The wavelength used for determination of Zn was set at 213.9 nm and the electric current was set at 10 mA.

Statistical analysis. All data are presented as means ±SEM. Statistical analysis was performed using Statistica software and analysis of variance (ANOVA) with repeated measures and post-hoc Tukey's test (with time as the factor). Student's *t*-test for non-dependent variables was used to compare the significance of differences between the control and experimental groups at several periods during the study. The data were normally distributed in accordance to Kolomogorov-Smirnov test. Post-hoc Tukey's test was used to evaluate the significance of the differences of the evaluated parameters (TAS and Zn) prior to the treatment and one and six months later. The differences between mean values were considered as statistically significant at $P < 0.05$.

Results

Results of hormonal evaluation in dogs suffering from non-malignant and malignant tumours and the limiting thresholds are shown in Table 1. Routine AHT lasted four weeks; however, in case of incompletely effective therapy and the appearance of tumours with a significantly reduced volume, the treatment was prolonged for three more weeks until neoplastic changes disappeared completely. No side effects of the applied antioestrogen treatment were observed. Ginecomastia symptoms occurred in two dogs undergoing antiandrogen treatment. AHT was effective in all hepatoid gland adenomas. Hepatoid gland epitheliomas disappeared in five dogs (41.7%), while significant reduction of tumour size (by approximately 70%-80%) was obtained in response to the AHT in seven cases (58.3%). The obtained therapeutic effects were characterised by reduced tumour size within the range of 40%-70% in all dogs with hepatoid gland carcinoma.

Table 1. Serum concentration of 17-β-oestradiol and testosterone in dogs suffering from non-malignant (hepatoid gland adenoma) and malignant (hepatoid gland epithelioma and hepatoid gland carcinoma) tumours of the perianal glands and their limiting thresholds used for qualification of patients to antihormonal treatment with antioestrogen or antiandrogen

Hormone	17-β-oestradiol range (pg/mL)		Testosterone range (ng/dL)	
	Range	Limiting threshold	Range	Limiting threshold
Hepatoid gland adenoma (N = 24)	7.4-35.0	7.0	60.3-570.2	150.0
Hepatoid gland epithelioma (N = 12)	18.0-52.0	7.0	12.0-650.9	150.0
Hepatoid gland carcinoma (N = 9)	7.0-45.0	7.0	10.0-179.9	150.0

Table 2. Antioxidative capacity (μmol/L) and zinc (Zn) concentration (μmol/mL) in serum of dogs suffering from non-malignant and malignant tumours

Parameter	Non-malignant group (N = 24)	Malignant group (N = 21)
Before treatment		
Total antioxidative capacity	329.39 ± 6.06 [a]	336.29 ± 4.48 [a]
Zn	11.24 ± 0.44 [a]	6.72 ± 0.14* [a]
One-month treatment		
Total antioxidative capacity	360.33 ± 3.02 [b]	359.36 ± 5.27 [b]
Zn	8.96 ± 0.21 [b]	8.58 ± 0.12 [b]
Six-month treatment		
Total antioxidative capacity	337.15 ± 12.81 [ab]	301.41 ± 3.36* [c]
Zn	7.00 ± 0.25 [c]	11.08 ± 0.30* [c]

Values are expressed as means ±SEM.
*Statistically significant differences of means between non-malignant and malignant groups at particular stages of the study for $P < 0.05$ as compared by Student's *t*-test.
[abc]Different superscript letters within each column indicate statistically significant differences of the investigated parameter between different stages of the study for $P < 0.05$ as compared by ANOVA with repeated measures and post hoc Tukey's test.

Table 2 shows TAC and zinc concentrations in dogs immediately prior to the treatment and one and six months later. TAC did not differ between the groups suffering from non-malignant and malignant tumours of perianal glands of dogs before the beginning of the AHT ($P = 0.39$) and one month later ($P = 0.88$). Six months after the start of the AHT, TAC was significantly lower in dogs with malignant tumours and this value was lower by 10.6% when compared to those with non-malignant tumours ($P = 0.03$). Zinc concentration in dogs with malignant tumours was significantly lower before the beginning of the AHT when compared to that of the controls ($P < 0.001$). Zinc concentration in dogs from both groups was not significantly different one month after the start of the AHT ($P = 0.16$). Zinc concentration in dogs with malignant tumours was significantly higher six months after the start of the AHT when compared to the controls ($P < 0.001$). Time-related changes of TAC and Zn concentrations in dogs from both groups are shown in Table 2. In the group of dogs suffering from non-malignant tumours, TAC was significantly increased one month after the beginning of the AHT; this value was by 9.4% higher when compared to the baseline value of 329.39 ± 6.06 μmol/L ($P = 0.049$). However, six months after the start of the AHT, the value of TAC in dogs with non-malignant tumours did not differ from the values obtained five ($P = 0.17$) and six months earlier ($P = 0.81$). One month after the beginning of the AHT in the group of dogs suffering from malignant tumours, TAC was significantly higher by 6.8%, when compared to the baseline value ($P = 0.005$). Six months after the start of the AHT, the value of TAC in these dogs was found to be significantly lowered; this value decreased by 16.1% and 10.4% when compared to the values obtained five and six months earlier, respectively ($P < 0.001$). Serum concentration of Zn before the beginning of the AHT in dogs with non-malignant tumours was found to be significantly higher by 20.3% and 37.7% when compared to the values obtained one and six months later, respectively ($P < 0.001$). Six months after the beginning of the AHT in the non-malignant group, Zn concentration was significantly lowered; the value decreased by 21.9%, when compared to the value obtained five months earlier ($P < 0.001$). Zinc concentration in dogs from the malignant group was found to be significantly raised, with the values increased by 27.7% and 64.9% at 1 and 6 months from the beginning of the AHT, respectively, when compared to the baseline value ($P < 0.001$). Moreover, Zn concentration significantly increased by 29.1% in dogs with malignant tumours six months after the start of the AHT when compared to the values obtained five months earlier ($P < 0.001$).

Discussion

The results obtained in this study have shown that TAC in serum of dogs was generally maintained at a high level, exceeding 320 μmol/L. The only exception was found six months after the start of the AHT in dogs suffering from malignant perianal tumours, where TAC reached the values between 280-320 μmol/L, representing the range for middle level of TAC regarding the reference values provided by the supplier of the analytic kit. Reduced TAC in these dogs was associated with poor therapeutic

outcome, since constant progression of neoplastic disease was observed in the group of dogs with malignant perianal tumours in spite of continuing the AHT. It must be pointed out here that after initial inhibition of malignant neoplastic growth and visually reduced tumour size in dogs undergoing the AHT, cessation of antioestrogen and antiandrogen treatment induced neoplastic progression, decreasing clinical condition of the animal patients. In the group with malignant tumours, the most beneficial clinical outcome was observed in the initial period of the AHT, and this effect was combined with significantly increased TAC after one month of the AHT in comparison to the baseline value. However, six months after the beginning of the AHT, TAC has decreased dramatically, reaching values below the initial level. Moreover, at this point in time, the value of TAC in the malignant group was lower by over 10% in comparison to the control dogs with non-malignant tumours. All these results indicate that improvement of the effectiveness of the AHT in dogs with malignant tumours requires enhancement of antioxidative capacity. Such effects may be obtained by applying additional administration of constituents contributing to antioxidative capacity such as selenium, vitamin C, and vitamin E (21, 24). The analysis of time-related changes of TAC in serum of dogs from the non-malignant group demonstrated its significant improvement after one month of the AHT. This effect was combined with very positive clinical results of the treatment, since the perianal tumours disappeared in all dogs from this group and the animals have recovered effectively.

The observed negative changes of health status and TAC during progression of malignant neoplastic process were associated with a gradually increasing concentration of Zn in serum; the element has reached its highest value six months from the start of the AHT. In the malignant group, a progressive increase of Zn concentration in association with the neoplastic process development was confirmed by statistically significantly different values at each stage of the study. Contrary to the observations in dogs with malignant neoplastic disease, positive clinical effects of the AHT in the non-malignant group were associated with consequently decreasing values of serum Zn concentration at each stage of the investigation. The fact that significant differences of Zn concentration were observed between both groups of dogs at the initial and final stages of the study is also noteworthy. Before the treatment, serum Zn concentration in the non-malignant group was higher than in the malignant groups, while the opposite results were observed six months later, indicating different Zn metabolism during the development and treatment of benign and malignant tumours of the perianal glands. Thus, evaluation of serum Zn concentrations in dogs may have a practical value for the effectiveness of antihormonal therapy monitoring, as well as for a diagnostic evaluation of patients and further prognosis. It may be postulated that low Zn concentration is characteristic for dogs suffering from malignant perianal tumours, and its subsequent increase during antihormonal therapy course indicates neoplastic process progression and poor prognosis for patients. These observations are in accordance with previous reports on dogs showing effectiveness of evaluation of magnesium and amino acids in serum for diagnostic differentiation of non-malignant and malignant perianal tumours and further prognosis for patients (5, 6). The observed time-related changes of Zn concentration in dogs subjected to the antineoplastic therapy may be explained by differentiated tissue demand for this element. In the case of malignant tumour development which is characterised by a very intensive cellular proliferation and increased blood supply of Zn, the element may be essential for accelerated tumour tissue metabolic processes and its rapid growth. It was reported in experimental studies that increased Zn demand of neoplastic tissue is related to the acceleration of cellular metabolism and a higher activity of intracellular Zn-dependent enzymes (4, 13, 29). Furthermore, cellular accumulation of Zn inhibits the apoptosis of neoplastic cells (31). On the other hand, increased serum content of Zn may result from internal mobilisation of antioxidative defence with the use of enzyme SOD consisting of this element to protect tissues from negative DNA changes characteristic for neoplastic processes (14, 22). In dogs with non-malignant tumours, the application of a highly effective AHT leads to tumour tissue atrophy and decreasing neoplastic tissue demand for Zn and other elements. In such a case, the internal mobilisation of antioxidative mechanisms over standard level with Zn-dependent SOD does not seem to be essential. However, the confirmation of this hypothesis requires further studies.

The results obtained in the current study correspond to the data published previously by Szczubiał *et al.* (28), where the parameters describing oxidative stress of blood in bitches with mammary gland tumours were measured. It was shown that SOD and GPx activities in bitches with malignant tumours were not different in comparison to the group suffering from non-malignant changes. However, both these indices of TAC were found to be significantly higher in bitches with malignant tumours, when compared to the healthy controls. Unfortunately, concentrations of zinc and other trace elements and vitamins contributing to antioxidative capacity were not evaluated in the earlier study (27). In a previously published report by Brodzki and Tatara (7), increased tumour tissue concentrations of Zn and Cu were revealed in bitches suffering from malignant and non-malignant mammary gland tumours, when compared to control dogs without neoplastic disease. Contrary to the current study, in the previous experiment on bitches the differences in serum Zn and Cu concentrations between the investigated groups were not found (7). The significantly decreased TAC of serum at the last stage of the experiment in the malignant group of dogs observed in the current study

is in accordance with a previous investigation in humans. In the current study as well as in the study performed on women with breast cancer, the TAC of serum was significantly reduced in the case of non-metastatic and metastatic cancer when compared to the healthy age-matched controls (1). Although the healthy control group of dogs was not included in the current study, clinical examinations and observations following the AHT of animal patients from non-malignant group enabled to consider all these animals as healthy. Another study on humans demonstrated significantly lower values of erythrocyte SOD and GPx activity in patients suffering from various types of cancers when compared to the values observed in the controls (2). Moreover, the evaluation of erythrocyte lysate in women suffering from fibroadenoma and adenocarcinoma of the breast has also revealed poor antioxidative capacity expressed by a significantly decreased activity of SOD, GPx, catalase, and reduced glutathione when compared to age-matched healthy controls (17).

In conclusion, the results obtained in this study have revealed reduced TAC and increased serum Zn concentrations in dogs with malignant perianal tumours six month after the start of the AHT. These results were associated with a poor therapeutic outcome and a constant progression of the malignant neoplastic disease. These observations, as well as the data collected from humans, indicate that malignant neoplastic process is associated with a significantly reduced TAC. Thus, it may be postulated that improving antioxidative capacity during neoplastic process treatment, especially in malignant tumours, is crucial for obtaining more effective clinical outcome of the applied therapy. Significantly increased serum TAC in dogs from non-malignant and malignant groups after one month of the AHT compared to the baseline values indicates the participation of SOD and GPx in tumour growth inhibition and recovery processes. Considering the differences of initial and final serum Zn concentrations in the malignant and non-malignant groups, as well as the opposite time-related changes of this element concentrations in both these groups during the course of the AHT, the evaluation of Zn concentrations in serum of dogs may have a practical diagnostic and prognostic value, and may serve towards increasing the effectiveness of antihormonal therapy monitoring.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was supported by Grant No NN 308 29 59 37 from the Polish Ministry of Education and Science.

Animal Rights Statement: The experimental procedures used throughout this study were approved by the II Local Ethics Committee on Animal Experimentation of University of Life Sciences in Lublin, Poland.

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