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EFFECTS OF CHEMOTHERAPY ALONE AND CHEMOTHERAPY COMBINED WITH ANTIOXIDANTS IN DOGS WITH MAMMARY GLAND CARCINOMA

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Summary

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Most anticancer drugs (epirubucin, adriamycin, bleomycin, vincristin, cyclophosphamide) exert their cytotoxic effect by free radicals-mediated mechanisms. The purpose of this study was to establish whether the antioxidants co-administered with epirubicin and cyclophosphamide reduce the adverse side effects of cytostatic chemotherapy without affecting their efficacy in dogs with spontaneous mammary gland carcinomas. Studies were performed in 14 bitches aged 6–14 years, weighing 4–29 kg, with histologically confirmed mammary gland carcinoma. Ten days after the surgical removal of the tumour, animals were divided into two groups: group I, treated with epirubicin and cyclophosphamide alone and group II, treated with a combination of same anticancer drugs and antioxidants (vitamins C, E, and A). It was concluded that the inclusion of antioxidants as adjuvant to the standard chemotherapy protocol resulted in less adverse effects without reducing the efficacy of cytostatics and improved patients' tolerance to treatment.

Key words: antioxidants, cyclophosphamide, dogs, epirubicin, mammary tumours

INTRODUCTION

Mammary gland tumours are the second most common group of neoplasms in dogs (Moulton, 1999) – approximately 52% of all cases of malignant tumour growth in female dogs (Brodey *et al.*, 1983; Rutterman *et al.*, 2000). The average age of affected bitches is 10–11 years but there are reports in animals younger than 4 years of age (Rutterman *et al*., 2000). The high prevalence has motivated the extensive research on various aspects of tumour pathology in order to increase the survival rate and to improve the quality of life of oncological patients.

The treatment of malignant tumours is most frequently complex and includes surgery, radiation therapy and chemotherapy, but chemotherapy gives the best results in limiting metastasis development of the neoplasm.

Most anticancer drugs (doxorubicin, epirubucin, bleomycin, vincristin, cyclophosphamide) exert their cytotoxic effect by free radicals-mediated mechanisms (Hartley *et al*., 1988; Look *et al.*, 1994; Beinert *et al.*, 1999). Their interaction with antioxidants is still controversial. In the view of some authors (Greenberg, 1975; Teicher *et al.*, 1994; Labriola & Livingston, 1999) antioxidants may reduce the efficacy of chemotherapeutics and should not be administered with those

acting through a free radical mechanism. Other investigations provide proofs for reduced toxicity of doxorubicin and lack of inhibition after vitamin E application (Perez *et al.*, 1986). *In vitro* experiments have shown that vitamin E could improve the cytotoxic effect of doxorubicin upon prostatic cancer cells (Perez *et al*., 1986).

Antioxidants could be successfully used both solely and in combination with chemotherapy (Seifter *et al.*, 1984; Shimpo *et al*., 1991; Wells *et al.,* 1995).

Doxorubicin and cyclophosphamide are anticancer drugs, most commonly used for therapy of canine malignant mammary tumours (Sorenmo, 2003).

The purpose of the present study was to investigate the effect of antioxidants co-administered with chemotherapy on unwanted side effects and the efficacy of cytostatic drugs epirubicin and cyclophosphamide in dogs with spontaneous mammary gland carcinomas.

MATERIALS AND METHODS

Animals

The study was performed in 14 bitches, 6 to 14 years old, weighing from 4 to 29 kg, patients of the Small Animal Clinic to the Faculty of Veterinary Medicine, Stara Zagora with mammary adenocarcinoma confirmed by histopathology. The clinical stage of the neoplasm was determined by the TNM system (Philibert *et al.*, 2003).

Two groups of 7 dogs each were formed (Table 1). In the first group, the therapy consisted in surgical removal of the tumour and chemotherapy, whereas in the second group the surgical removal of the tumour was completed by chemotherapy associated with antioxidant therapy.

The surgical intervention consisted in removal of tumour masses by partial (regional and unilateral) or total (bilateral) mastectomy with or without removal of inguinal lymph nodes as required by oncosurgery principles. The choice of sur-

Table 1. Signalment of bitches with mammary adenocarcinoma selected to receive either chemotherapy alone (Group I, n=7) or chemotherapy plus antioxidants (Group II, n=7)

		Breed	TNM stage	Age (years)	Body weight, kg
Group I	Dog No 1	Mittelschnauzer	I	8	14.0
	Dog No 2	mixed-breed	IV	6	9.5
	Dog No 3	Bolognese	V	14	6.5
	Dog No 4	German shepherd	III	8	29.0
	Dog No 5	Dachshund	II	10	12.0
	Dog No 6	Bolognese	II	13	10.0
	Dog No 7	mixed-breed	III	7	14.5
Group II	Dog No 1	Bolognese	II	10	5.5
	Dog No 2	Miniature Pinscher	IV	8	3.6
	Dog No 3	Afghan hound	IV	9	17.0
	Dog No 4	Setter	IV	7	19.0
	Dog No 5	Bolognese	IV	13	4.0
	Dog No 6	Bolognese	II	13	7.0
	Dog No 7	Bolognese	IV	9	8.7

gical technique depended on tumour size and the number of affected mammary glands. The anaesthetic protocol was uniform. After catheterization (22G or 24G, according to dog's size) of the antebrachial cephalic vein, dogs were premedicated with 0.02 mg/kg 0.1% atropine sulfate (Sopharma, Bulgaria) subcutaneously. The induction of anaesthesia was done by slow intravenous injection of 0.5 mg/kg diazepam (Diazepam 0.5%, Sopharma, Bulgaria) and 10 mg/kg ketamine (Ketaminol, Intervet, Netherlands) 10 min after atropine administration. After endotracheal intubation, general anaesthesia was maintained with 1–1.5 vol% halothane (Narcotan, Leciva, Czech Republic) and oxygen flow 2–3 L/min.

The histological diagnosis was done according to the WHO classification of canine mammary gland tumours (Misdorp *et al.*, 1999; Misdorp, 2002). Thus, they were classified as 3 cases (21.4%) of complex carcinoma and 11 cases (78.6%) of simple carcinoma. Simple carcinomas were from the following subtypes: 4 (36.4%) solid carcinomas, 7 (63.6%) tubulopapillary carcinomas.

The chemotherapy in all dogs consisted in administration of the cytostatics epirubucin (Farmorubicin, Pharmacia & Upjohn, Italy) and cyclophosphamide (Endoxan, Asta Medica, Frankfurt) as followed: 1) intravenous injection of epirubicin at a dose of 20 (dogs weighing <20 kg) or 30 mg/m^2 (dogs > 20 kg) once weekly for 3 consecutive weeks; 2) intravenous injection of cyclophosphamide at 100 mg/m^2, once weekly, 3 days after epirubicin injection for 3 consecutive weeks.

During the entire course of the chemotherapy, the dogs from the second experimental group received the antioxidants vitamin C (Biovet, Bulgaria) as a daily subcutaneous dose of 50 mg/kg and vitamins A (3000 UI/kg), D (4000 UI/kg), and E (2 mg/kg) (vitamin AD3E, VetProm, Bulgaria) as a weekly intramuscular dose.

Blood analyses

Blood samples were obtained from the jugular vein at the following intervals: prior to the surgery; 10 days after the surgery prior to first epirubicin chemotherapy; 17 days after the surgery prior to second epirubicin injection; 24 days after the surgery prior to the third epirubicin injection and 39 days after the surgery.

Laboratory blood tests consisted in complete blood count (haemoglobin, haematocrit, red blood cell counts, total and differential blood cell counts) and a biochemistry panel (total protein, albumin, aspartate aminotransferase, alanine aminotransferase, urea, creatinine, uric acid, alkaline phosphatase, bilirubin).

Also, other tests were run to establish the condition of patients during the chemotherapy: 1) clinical tests: rectal body temperature, respiratory and heart rates, appetite, presence of vomiting, diarrhoea, weight loss, alopecia; 2) ECG – prior to and after the chemotherapy, six leads (3 standard and 3 augmented) were registered on a thermal paper at a speed of 500 mm/s in right lateral recumbency, by a single channel microcomputer electrocardiograph MAIMEX-ECG 1222 ASB (Bulgaria).

Radiographic surveys (lateral views; 55–60 kV, 10–16 mAs, 100 cm film-focus distance) were done immediately prior to surgery and after the end of chemotherapy in order to establish distant metastases.

Statistical analysis

The statistical analysis was performed with the non-parametric Friedman's test for two-way repeated measures analysis. In case of significant *P* values ($P<0.05$),

the non-parametric Tukey HSD test was then applied.

RESULTS

The distribution of dogs according to the TNM staging system (TNM stands for tumour, nodes, and metastases) was as followed: group I – 1 dog (14 %) in the first stage, 2 dogs (29 %) in the second stage, 2 dogs (29 %) in the third stage, 1 dog (14 %) in the fourth stage and 1 dogs (14 %) in the fifth stage; group II – 2 dogs (29 %) in the second stage and 5 dogs (71 %) in the fourth stage.

During the studied time intervals over the entire complex therapy, there were no significant deviations in rectal body temperature, heart and respiratory rate in both groups. In two dogs that received chemotherapy alone, there was intermittent fever that was treated with antipyretics – 10 mg/kg metamizole sodium (Analgin ®, Sopharma, Bulgaria).

Table 2 presents the incidence of side effects in dogs from both experimental groups. Gastrointestinal complaints – anorexia, vomiting and diarrhoea – were observed in 5 dogs from group I (71%). The other 2 dogs from this group exhibited only anorexia. In six patients (86%) 3.5–12.5% of the initial body weight was lost. Alopecia has occurred in one Bolognese (14%) 20 days after the beginning of the chemotherapy.

The dogs that received chemotherapy and antioxidants supported the therapy considerably better as could be seen from the incidence of unwanted gastrointestinal effects and weight loss. During the therapy of this group, three animals (43%) showed gastrointestinal signs: anorexia (over 3–4 days after the injection of cytostatics), vomiting (the day of cytostatic injection), but diarrhoea was not observed in any of bitches. Alopecia has occurred 30 days after the chemotherapy start in 3 dogs (43%), all Bolognese (Fig. 1) and it disappeared one month after chemotherapy cessation. Weight loss occurred only in one patient (14%).

The ECG performed showed a sinus rhythm in most dogs before and after the chemotherapy while some exhibited physiological respiratory arrhythmia. The heart rate varied considerably among patients, but was within the reference range during both studied periods. In one bitch from the first experimental group, a second-degree atrioventricular block Mobitz type II was observed (Fig. 2) whereas another two dogs showed atrial fibrillations after the chemotherapy. The deviations in measured ECG indices in the other studied dogs were not significant compared to baseline values. In the group

Table 2. Prevalence of side effects in bitches with mammary adenocarcinoma treated by either chemotherapy alone (group I, n=7) or chemotherapy plus antioxidants (group II, n=7)

	Group I	Group 2
	Number (%)	Number (%)
Gastrointestinal signs		
anorexia	7/7 (100%)	3/7 (43%)
vomiting	5/7 (71%)	3/7 (43%)
diarrhoea	2/7 (29%)	–
Weight loss	6/7 (86%)	1/7 (14%)
Alopecia	1/7 (14%)	3/7 (43%)

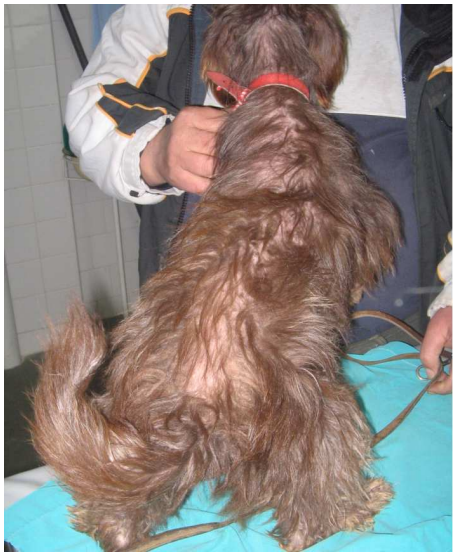

Fig. 1. Alopecia of the head and body in a Bolognese 30 days after the beginning of the chemotherapy.

where chemotherapeutics were co-administered with antioxidants, there were neither rhythm and conduction disorders, nor repolarization changes.

The chest radiography showed multiple disseminated shadows of a various size and intensity in the lungs of two dogs from group I and one dog from group II. No radiologically visible metastases (negative finding is possible for lesions <5 mm) were seen in the other patients from both groups.

The CBC results (Table 3) showed a statistically significant decrease in total leukocyte counts before the 2nd ($P<0.01$) and the 3rd ($P<0.05$) epirubicin injections compared to baseline in the first experimental group. The difference between leukocyte counts on the 17th and 39th post operative days was also considerable ($P<0.01$). With regard to differential leukocyte counts, an absolute neutropenia without left shift, monocytopenia and eosinopenia were observed.

Blood uric acid concentrations (Table 4) at the time of the 2nd epirubicin administration were statistically significantly higher vs both the first and fifth sampling ($P<0.05$). Blood urea levels decreased in the 2nd ($P<0.05$), 4th and 5th periods ($P<0.01$) as compared to initial values

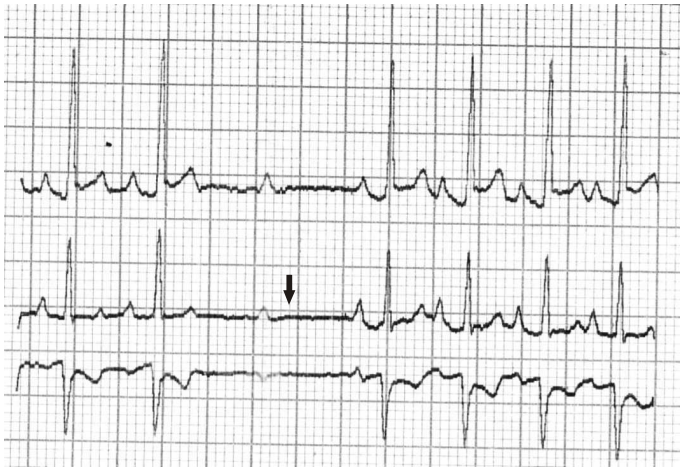

Fig. 2. ECG in a dog after chemotherapy without antioxidants. Second-degree atrioventricular block Mobitz type II –the QRS complex after the P wave is dropped (arrow).

Table 3. Time course of CBC in dogs from experimental group I (chemotherapy alone) and group II (chemotherapy + antioxidants). Data are presented as mean ± SEM, n=7

Parameter	Group	Baseline, before surgery	10 days after surgery, prior to 1st epirubucin injection	17 days after surgery, prior to 2nd epirubicin injection	24 days after surgery prior to the 3rd epirubicin injection	39 days after surgery
Haemoglobin, g/L	I	147±5	132±11	129±10	127±9	133±9
	II	157±7	139±7	129±7*	127±6**	139±3*
Haematocrit, L/L	I	0.45±0.02	0.4±0.03	0.4±0.03	0.38±0.03	0.38±0.03
	II	0.45±0.02	0.41±0.02	0.38±0,01*	0.37±0,01*	0.41±0.02
Erythrocytes, T/L	I	6.26±0.42	5.39±0.26	5.52±0.41	5.54±0.37	5.59±0.45
	II	6.81±0.56	6.03±0.5	5.71±0,40	5.41±0.28	5.71±0.19
Leukocytes, G/L	I	11.04±2.56	8.91±0.94	3.86±0.72**▲▲	5.44±1.11*	13.17±4.37
	II	10.71±2.33	11.66±2,21	4.46±0.85*▲	4.43±0.50*▲	10.21±2.94
Band neutrophils (G/L)	I	0.77±0.03	0.8±0.01	0.34±0.01**▲▲▲	0.38±0.01*▲▲	1.18±0.17*
	II	0.85±0.05	0.70±0.02	0.22±0.01***▲▲▲	0.22±0.01***▲▲▲	0.51±0.03***
Segmented neutrophils, G/L	I	6.73±0.1	5.08±0.08	1.78±0.05***▲▲▲	2.34±0.08***▲▲▲	7.9±0.04
	II	6.10±0.09	5.83±0.09	2.00±0.06***▲▲▲	2.35±0.02***▲▲▲	4.90±0.15***
Lymphocytes, G/L	I	3.2±0.1	2.76±0.03	1.62±0.06***▲▲▲	2.50±0.09**	3.82±0.26
	II	3.43±0.12	4.55±0.11***	2.05±0.05***▲▲▲	1.77±0.02***	4.39±0.15
Monocytes, G/L	I	0.22±0	0.18±0	0.08±0***	0.11±0**	0.13±0.04*
	II	0.21±0.01	0.23±0	0.09±0***▲▲▲	0.04±0***	0.20±0
Eosinophils, G/L	I	0.11±0.01	0.09±0	0.04±0▲	0.11±0	0.13±0
	II	0.21±0.02	0.23±0.02▲▲▲	0.09±0.01***▲▲▲	0.04±0***	0.10±0***

* P≤0.05, ** P≤0.01, *** P≤0.001 vs baseline; ▲ P≤0.05, ▲▲P≤0.01, ▲▲▲P≤0.001 vs 39 days after surgery.

Table 4. Time course of some blood biochemistry parameters in dogs from experimental group I (chemotherapy alone) and group II (chemotherapy + antioxidants). Data are presented as mean ± SEM, n=7

Parameter	Group	Baseline, before surgery	10 days after surgery, prior to 1st epirubucin injection	17 days after surgery, prior to 2nd epirubicin injection	24 days after surgery prior to the 3rd epirubicin injection	39 days after surgery
Uric acid, μmol/L	I	87±8	83±13	114±7*▲	124±21	66±14
	II	64±21	57±10	98±18	88±21	69±23
Urea, mmol/L	I	5.76±0.52	4.17±0.74*	5.39±0.56▲	4.11±0.34**	3.7±0.2**
	II	7.75±1.04	7.31±0.77	6.98±0.49	6.82±1.40	7.49±1.06
Creatinine, μmol/L	I	95±13	71±6	76±7	79±6	76±5
	II	118±22	98±13	87±5	102±11	106±7
ASAT, U/L	I	17±3	15±2	11±2	23±7	21±2
	II	13±2	9±2	12±2	10±1	11±1
ALAT, U/L	I	25±6	18±5	19±5	21±6	24±3
	II	21±1	14±3	16±2*	17±2	19±3
Alkaline phosphatase, U/L	I	83±7	216±80	144±48	102±20	128±36
	II	71±29	119±22	109±10*	94±17	77±10

* P≤0.05, ** P≤0.01, *** P≤0.001 vs baseline; ▲ P≤0.05, ▲▲P≤0.01, ▲▲▲P≤0.001 vs 39 days after surgery.

whereas levels in the 3rd period were higher than final detected concentrations ($P<0.05$).

The results from haematological analyses in group II showed statistically significant reductions in haemoglobin content during the last three samplings vs the initial one ($P<0.05$). Haematocrit was also decreased at the 2nd and 3rd epirubicin injections ($P<0.05$) compared to preoperative values.

The absolute leukopenia in group II was the most pronounced before the 2nd and the 3rd epirubicin infections as compared to both baseline and final values ($P<0.05$). The changes in leukocyte classes were similar to those in group I. Blood biochemistry results showed statistically significantly increased alkaline phosphatase and lower ALAT activities in the third period ($P<0.05$) vs baseline.

The survey periods of patients were 48 and 40 months for group I and II, respectively. There were no recurrences of the neoplastic growth at the site of the primary tumour in any dog. In two bitches from the first group, lung metastases were detected one and two years after the end of the chemotherapy. In the group treated with antioxidants, such metastases were observed in only one dog two years after the therapy.

In both groups, the shortest survival time was 8 months. The death of the two dogs with TNM stage II from the second group was caused by other illnesses (pyometra in one bitch and perforated stomach ulcer in the other). Until April 2010, four animals of each group had died. Bitches that survived so far are with TNM stage I, II and III (for group I) and TNM stage IV (for group II). They are submitted to control health examinations on a periodical basis.

DISCUSSION

Chemotherapy is accompanied by a number of unwanted effects. The most commonly observed complications are gastrointestinal signs, bone marrow toxicity and immunosuppression (Ahaus *et al*., 2000; Haskell, 1980; MacEven, 1980).

Anorexia, vomiting and diarrhoea could appear consequently to damage of gastrointestinal epithelium or to central nervous system effects. Other, less frequent signs, are diarrhoea, stomatitis, oesophagitis, gastroduodenal ulcerations (Harris, 1977). It should be noted that gastrointestinal signs occurred in all dogs from the group with chemotherapy alone whereas dogs that received antioxidants tolerated better the chemotherapy. As a result of gastrointestinal effects, weight loss had occurred in 86% of dogs from group I compared to 14% in group II. Therefore, the co-administration of antioxidants with cytostatics results in lower incidence of unwanted gastrointestinal effects.

Skin reactions and alopecia are more rarely seen in veterinary patients as compared to humans. Shorthair and curly coat breeds (Shepherd dogs, Poodles, Afghan hounds, Terriers) are more likely to develop such signs (Conroy, 1979). Alopecia in this study was observed only in Bologneses (1 out of 2 from group I and 3 out of 4 from group II). It could be therefore suggested that the inclusion of antioxidants in the chemotherapy protocol did not contribute to decrease of alopecia incidence.

A very serious problem related to chemotherapy is bone marrow toxicity resulting in leukopenia and humoral and cellular immunity suppression. It could affect all blood components. Anaemia and thrombocytopenia could be life-threatening. The primary and more frequent

problem is leukopenia due to the shorter half-life and smaller reserve of white blood cells. In the present study, a clear reduction of all myeloid line cells has occurred evidencing a total suppression of the bone marrow activity. The inhibition was strong in both experimental groups indicating that the application of antioxidants (vitamins A, C and E) during the chemotherapy did not influence the bone marrow toxic effects of cytostatics.

From blood biochemistry parameters, the effects of cytostatics were reflected by uric acid elevation in both groups, but a statistically significant difference vs baseline was present only in group I. The secondary hyperuricaemia developed consequently to the enhanced metabolism of purines accompanying the rapid degradation of neoplastic cells by cytostatics (Locatelli & Rossi, 2005). The lower increase rate of blood uric acid levels in group II was probably due to the application of vitamin C as antioxidant. Huang *et al*. (2005) have shown that vitamin C resulted in lower serum uric acid concentrations due to its more extensive renal excretion.

Anthracyclines are known to possess a dose-related cardiotoxicity whose mechanisms are not fully understood (Gianni & Myers, 1992; Gerwirtz, 1999; Hrdina *et al*., 2000). According to Hershko *et al*. (1993) and Schimmel *et al*. (2004) anthracycline cardiotoxicity is mediated by formation of free radicals, leading to oxidative stress. It could result in serious complications (Schimmel *et al*., 2004). Anthracycline-induced cardiac damage is a progressive myocardial degeneration, myocytolysis, vacuolization and fibrosis (Mauldin *et al*., 1992). The probability for cardiac failure increases parallelly to accumulation of doses although its beginning could be delayed by several weeks after the last dose. Arrhythmias and conductivity disorders occur rapidly but are not necessarily related to initial congestive heart failure (Mauldin *et al*., 1992).

The co-administration of doxorubicin and 2 mg/kg vitamin C resulted in lower cardiotoxicity as compared to treatment with doxorubicin only in mice and guinea pigs (Schimpo *et al*., 1991). In our investigation, a second-degree atrioventricular block Mobitz type II occurred in one dog from group I while another 2 dogs exhibited atrial fibrillations. The ECG study of bitches from group II did not show any rhythm or conduction disorders, or repolarization changes. Probably the application of antioxidants during the chemotherapy had a beneficial effect in counteracting anthracycline cardiotoxicity similarly to what was suggested by Schimpo *et al*. (1991).

Singh *et al*. (1998) provided evidence for the antioxidants' benefits in cancer therapy and their potential interactions with radiation and chemotherapy. Many investigations in humans have shown reduction of the side effects when chemotherapeutics are co-administered with antioxidants (Weijl *et al*., 1997), similarly to our findings. A question that remains open is whether exogenous antioxidants, applied simultaneously with chemotherapeutics could decrease their effect on malignant cells.

The survival time and the efficacy of chemotherapy depend on a number of factors as the stage of disease, the histological type and the degree of cancer differentiation, the tumour size, early diagnostics and timely therapy (Novosad, 2003; Sorenmo, 2003; Philibert *et al*., 2003). Several studies on chemotherapy with antioxidants have shown reduction of tumour size and longer survival time

(Seifter *et al*., 1984; Taper *et al.,* 1987). In the view of Schimpo *et al.* (1991), the treatment with vitamin C did not decrease doxorubicin efficacy and was related to higher survival times as compared to the independent application of doxorubicin in mice and guinea pigs.

The early detection of tumours and the rapidly initiated therapy in dogs and cats usually prevent local and distant metastases (Novosad, 2003). In a study on dogs with invasive malignant mammary tumours, Simon *et al.* (2006) found out that the average time for appearance of metastases after post operative treatment with doxorubicin or docetaxel was 10 months with average survival time 12 months. In our study, the survey periods were 48 and 40 months for group I and II respectively. No recurrences of the tumour were observed in both groups. Lung metastases were established in two bitches from group I and one from group II. However, an evaluation of the effect of applied therapeutic protocols could be hardly made because of the different stage of the disease and the two deaths in group II due to pyometra and perforated ulcer. In fact, the three dogs from the second group that remained in good condition were with TMN stage IV whereas the three patients from the first group – in TMN stages I, II and III.

In a study in Beagle dogs with malignant mammary neoplasms, treated by mastectomy, the average survival time was 10 months (Moulton *et al*., 1986). In another study, dogs whose death was directly attributed to the tumour have lived for 14 months on the average after the operative intervention (Philibert *et al*., 2003).

The inclusion of antioxidants in the standard chemotherapy protocol in dogs with mammary gland carcinoma led to lower incidence of unwanted side effects of cytostatics and better tolerance without reduction of their anticancer effects. This was confirmed by the fewer gastrointestinal effects, the lower weight loss and the lack of cardiac disturbances. At the same time, antioxidants did not influence drug-induced bone marrow toxicity as shown by the clear reduction of counts of all myeloid line blood cells. Alopecia, another side effect of chemotherapy, was neither influenced by antioxidant application, with Bologneses being the most susceptible breed.

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