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RESEARCH ARTICLE

## Malondialdehyde levels in canine appendicular osteosarcoma

Arayaporn Macotpet<sup>1\*</sup>, Ekkachai Pattarapanwichien<sup>2</sup>, Fanan Suksawat<sup>1</sup>

<sup>1</sup>Division of Small Animal Medicine, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

<sup>2</sup>Division of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand

\*Corresponding author's E-mail: arayama7972@gmail.com

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### Abstract

**Objective:** To compare serum malondialdehyde (MDA) levels between clinically healthy dogs and dogs suffering from spontaneous appendicular osteosarcoma.

**Materials and Methods:** Blood samples were obtained from 18 healthy dogs and 18 dogs with spontaneous appendicular osteosarcoma at Veterinary Teaching Hospital, Khon Kaen University. The final diagnosis of appendicular osteosarcoma was determined from histopathological findings. MDA levels were measured by thiobarbituric acid reactive substance assay. Packed cell volume, hemoglobin, red blood cell count and white blood cell count were measured using an automatic analyzer. Platelet estimation was done under the oil immersion lens of a microscope. Creatinine and ALT were measured using an automatic blood chemistry. Data are presented as mean  $\pm$  standard deviation. Parametric data were determined using the independent-sample T test. Values of  $P < 0.01$  were considered significant.

**Results:** Serum MDA levels were significantly higher in the appendicular osteosarcoma group than in clinically healthy dogs ( $P < 0.001$ ). Mean MDA level was  $10.64 \pm 1.98$   $\mu\text{mol/L}$  in the appendicular osteosarcoma group and  $4.30 \pm 0.49$   $\mu\text{mol/L}$  in the clinically healthy group. Packed cell volume ( $34.16 \pm 6.23\%$  vs  $46.66 \pm 6.08\%$ ), hemoglobin ( $11.92 \pm 2.08$  g% vs  $15.93 \pm 2.15$  g%) and red blood cells ( $5.55 \pm 1.70$  ( $\times 10^{12}/\text{L}$ ) vs  $8.35 \pm 1.04$  ( $\times 10^{12}/\text{L}$ )) were all significantly lower in cancer group than in control group. Platelet estimations were adequate in both groups. White blood cell count, creatinine and alanine aminotransferase levels were not significantly different between both groups.

**Conclusions:** This study sustains the assumption that oxidative stress is associated with osteosarcoma in dogs, as serum MDA levels were significantly higher in appendicular osteosarcoma-bearing dogs compared to clinically healthy dogs.

**Keywords:** Dog, Malondialdehyde, Osteosarcoma

## Introduction

A large amount of evidence indicates that reactive oxygen species (ROS) are involved in the etiopathogenesis of many diseases, one of which is cancer (Diehn et al., 2009; Szczubial et al., 2004). The imbalance between oxidative and antioxidative reactions leading to ROS excess is known as oxidative stress (Birben et al., 2012). Oxidative stress can be monitored using several markers, and levels can be determined in plasma or serum. Among such markers are antioxidative enzymes and the products of peroxidative processes involving lipids, proteins and nucleic acids (Szczubial et al., 2004). Lipid peroxidation has various deleterious effects on biological systems and has been implicated in several diseases, especially cancer (Gonenc et al., 2001). One of the most frequently used biomarkers providing an indication of the overall level of lipid peroxidation is the serum or plasma concentration of malondialdehyde (MDA), one of several byproducts of lipid peroxidation processes.

Osteosarcoma is the most common primary bone tumor in dogs. This locally invasive neoplasm shows a high rate of metastasis. Researchers are currently using canine osteosarcoma as a reliable model to study human osteosarcoma (Morello et al., 2011). Approximately 75% of osteosarcomas occur in the appendicular skeleton. In other canine studies, the incidence of osteosarcoma has been reported as 5-6% of all canine malignancies (Endicott, 2003). The incidence of osteosarcoma in all canine neoplasms was 1.39% in Bangkok, Thailand (Rungsipipat et al., 2003), and 8% at Khon Kaen Veterinary Teaching Hospital, Khon Kaen, Thailand, between January 2010 and December 2017. The average age for presentation of osteosarcoma in dogs is 7 years, but this pathology has been described in dogs as young as 3-months old (Ginel et al., 1996). Prognosis is poorer in older dogs. Large breed dogs are also at higher risk of osteosarcoma. The actual causes of osteosarcoma remain unclear. Many factors such as poor genetics, large breeds, fast growing breeds, bone infection, periostitis, osteomyelitis, bone ischemia, unfixed bone fracture and fixed bone fracture with complications such as virally infected unwelded bone are expected to be involved (Bennett et al., 1979; Gellasch et al., 2002; McNeill et al., 2007; Rosenberger et al., 2007; Selmic et al., 2014; Sinibaldi et al., 1976; Solov'ev et al., 1998; Stevenson, 1991; Stevenson et al., 1982). However, no studies have

clarified levels of oxidative stress in humans or dogs suffering from osteosarcoma. The objective of this study was to compare MDA levels between dogs with spontaneous appendicular osteosarcoma and clinically healthy dogs.

## Materials and Methods

### Study Design

#### *Animals*

All client-owned dogs treated at Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand between July and December 2017 were registered to this study. The final diagnosis of appendicular osteosarcoma was based on histopathological findings. Dogs undergoing chemotherapy, limb amputation or receiving antioxidant supplements were excluded. Clinically healthy dogs were more than 1 year old without any evidence of blood or intestinal parasites and had shown no abnormalities on physical examination at least 6 months before blood collection. Owner consent was obtained for all participating dogs.

#### *Blood sample collection and preparation*

Four milliliters of blood was drawn from the cephalic vein and divided into three parts. One milliliter was treated with ethylenediamine tetraacetic acid (EDTA) as an anticoagulant and used to determine a complete blood count and the presence of blood parasites. The remainder was centrifuged at 800×g at 4°C for 5 min to provide serum for blood chemistry tests (creatinine and alanine aminotransferase (ALT)) and MDA measurement.

Packed cell volume (PCV), hemoglobin (Hb), red blood cell count (RBC) and white blood cell count (WBC) were measured using an automatic analyzer (XT-2000iV; Sysmex, the United States). In addition, dried blood smears were fixed in methyl alcohol and stained with Wright-Giemsa stain and examined for blood parasites and platelet estimation under the oil immersion lens of a microscope. Creatinine and ALT were measured using an automatic blood chemistry analyzer (AU400; Olympus® the United State).

MDA concentrations were determined using serum samples kept at -80°C and the thiobarbituric acid reactive substance assay (Nielsen et al., 1997; Wong et al., 1987). One milliliter of serum diluted with normal saline was mixed

with 50 µL of 0.1 mmol/L butylated hydroxytoluene, 500 µL of 5 mmol/L EDTA, 1 mL of 8% sodium dodecyl sulfate, 1 mL of 10% trichloroacetic acid and 1.5 mL of 0.67% thiobarbituric acid. The reaction mixture was incubated at 95°C for 30 min, then centrifuged at 1500×g at room temperature for 10 min. Absorbance of the supernatant was measured at 532 nm and tetraethoxypropane was used to create a standard curve at concentrations over the ranges of the 0 - 35 µmol/L.

### Statistics Analysis

Data are presented as mean ± SD. Parametric data were determined using the independent-sample T tests whereas the Mann-Whitney U test was used for non-parametric data. All statistical analyses were performed by statistical software (SPSS version 17; SPSS Inc., Chicago, Ill.) and values of P<0.01 were considered significant.

### Ethics Statement

This study was approved by the Animal Ethics Committee at Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Record No. ACUC-KKU-40/60, Reference No. 0514.1.75/58).

### Results

Of a total of 45 dogs, 20 were diagnosed with spontaneous appendicular osteosarcoma and 25 were identified as clinically healthy. Two of the dogs with ap-

pendicular osteosarcoma did not fulfill the inclusion criteria and so were not included. Thus, 18 dogs with appendicular osteosarcoma were used for final analysis. Of 25 clinically healthy dogs, 7 dogs were excluded because they did not fulfill the inclusion criteria, and 18 clinically healthy dogs were therefore analyzed in this study.

Demographic characteristics of the dogs with appendicular osteosarcoma and clinically healthy dogs are shown in Table 1. Mean body weight was significantly higher in dogs with appendicular osteosarcoma (37.67±9.81 kg) than in clinically healthy dogs 24.33±9.62 kg, P<0.01) but no significant difference in age was evident between groups (P=0.425). From this study, the mean life expectancy of dogs with osteosarcoma was 7.50±2.91 years (range 2-12 years) and 77.78% were males. The proportion of pure breeds was higher among dogs with appendicular osteosarcoma (16/18 or 88.89%) than among clinically healthy dogs 6/18, 33.34%. Among dogs with appendicular osteosarcoma, the pure breeds comprised 8 Rottweilers, 2 Pit Bulls, 2 Saint Bernards, 1 Thai Ridgeback, 1 Great Dane, 1 Golden Retriever and 1 Labrador Retriever. Among clinically healthy dogs, the pure breeds were 2 Golden Retrievers, 2 Labrador Retrievers and 2 Rottweilers. The site of osteosarcoma in this study included the humerus (50%), tibia (27.78%) and radius (22.22%). Two cases showed lung metastasis (11.11%).

Serum MDA was significantly higher in dogs with appendicular osteosarcoma (10.64 ± 1.98 µmol/L) than in clinically healthy dogs (4.30 ± 0.49 µmol/L, P<0.001) (Table

**Table 1.** Demographic characteristics of *osteosarcoma-bearing dogs* and clinically healthy dogs.

Variables	Osteosarcoma-bearing dogs (n = 18)	Clinically normal dogs (n = 18)	P-value <sup>a</sup>
Age, mean ± SD (range), years	7.50±2.91 (2-12)	8.16±1.94 (5-12)	0.425
Weight, mean ± SD (range), kg	37.67±9.81 (4.2-49.2)	24.33±9.62 (4.8-40.4)	<0.01
Sex, (%)			
Male	14 (77.78%)	7 (38.89%)	
Female	4 (22.22%)	11 (61.11%)	
Breed, (%)			
Mixed	2 (11.11%)	12 (66.66%)	
Pure breed	16 (88.89%)	6 (33.34%)	
Site of osteosarcoma			
- Humerus	9 (50%)		
- Tibia	5 (27.78%)		
- Radius	4 (22.22%)		

<sup>a</sup>An independent-sample Students t-test was used for age and weight

**Table 2.** Malondialdehyde, complete blood counts and blood chemistry parameters for osteosarcoma-bearing dogs and clinically healthy dogs.

Variables	Osteosarcoma -bearing dogs (n = 18)	Clinically healthy dogs (n = 18)	P-value <sup>a</sup>	Normal ranges*
MDA, mean ± SD, μmol/L	10.64±1.98	4.30±0.49	<0.001	
PCV, mean ± SD, %	34.16±6.23	46.66±6.08	<0.001	37-55
Hemoglobin, mean ± SD, g%	11.92±2.08	15.93±2.15	<0.001	12-18
RBC, mean, 10 <sup>12</sup> /L	5.55±1.70	8.35±1.04	<0.001	5.5-8.5
WBC, mean, 10 <sup>9</sup> /L	16.29±6.62	14.82±4.35	0.436	6-17
Creatinine, mean, mg/dL	1.93±3.76	0.95±0.18	0.239	0.9-1.7
ALT, mean, U/L	34.20±31.77	53.72±26.69	0.054	10-120

Abbreviations: ALT, alanine aminotransferase; MDA, malondialdehyde; PCV, packed cell volume; RBC, red blood cell count; SD, standard deviation; WBC, white blood cell count.

<sup>a</sup>An independent-sample Students t-test was used for MDA, PCV, hemoglobin, RBC, WBC and ALT while the Mann-Whitney U test was used for creatinine.

2). RBC and WBC in both groups remained within normal ranges (Table 2), but RBC was significantly lower in dogs with appendicular osteosarcoma ( $5.55 \pm 1.70 \times 10^6/\mu\text{L}$ ) than in clinically healthy dogs ( $8.35 \pm 1.04 \times 10^6/\mu\text{L}$ ): ( $p < 0.001$ ). Platelet estimations were adequate in both groups. No blood parasites were found and ALT levels were within normal ranges, showing no significant difference between groups ( $P = 0.054$ ). Creatinine levels did not differ significantly between the appendicular osteosarcoma group and control group, but the cancer group tended to show creatinine level higher than the normal range.

## Discussion

Over recent years, a large number of studies have examined key factors associated with carcinogenesis and many contributing factors have been identified. One of the carcinogenetic factors that have long been studied is free radicals, a cause of oxidative stress (Paskalev, 2009). Oxidative stress is an imbalance between free radical production and antioxidant defenses. This may result from either overproduction of ROS or reduction of antioxidants, or both and/or presence of reactive metabolites. Oxidative stress significantly induces biohazardous effects leading to damage to DNA, RNA and protein and development of pathologies causing various diseases, among which the most important are cardiovascular diseases, neurologic diseases, inflammation associated diseases, human im-

munodeficiency, diabetes, kidney disease and cancer (Gu et al., 2003; Jacob and Burri, 1996; Kang and Hamasaki, 2005; Leiris, 2003; Szczubial et al., 2004; Valko et al., 2001; Winter et al., 2009). Many factors can affect oxidative stress and disease severity. In veterinary medicine, oxidative stress may be associated with carcinogenesis, affecting the mortality of cancers (Winter et al., 2009).

Many studies have supported the notion that oxygen free radicals are involved in the initiation and promotion of carcinogenesis in humans and animals (Cutler, 1991; Frenkel, 1992; Sharifi, 2009). In particular the incidence of cancers is usually increased in the elderly. This may be explained by free radical production increasing with age. The long-term battle of DNA with free radicals causes loss of normal cell function leading to cell death or mutation of DNA and the development of cancer from accelerating cell division rates (Karihtala and Soini, 2007; Kong et al., 2000). Moreover, chronic inflammation is associated with carcinogenesis and fatty acid oxidation caused by induction of oxygen free radical associated neoplastic transformation in veterinary medicine (Behrend et al., 2003). Oxidative stress may involve carcinogenesis leading to death (Winter et al., 2009). From a study in dogs with cancer, superoxide anion radicals were significantly higher than in normal dogs (Sato et al., 2003).

The presence of oxidative stress may be evaluated in 2 ways: 1) direct measurement of ROS which are

difficult to measure directly due to the many ROS with short half-lives (in the order of seconds) and the need for a special instrument: or 2) indirect measurement of damage to the biomolecules in the body, including substances produced from proteins, DNA, RNA, and lipids (Halliwell, 1997; Leiris, 2003). However, measurement of the damage to lipid peroxidation is more popular than use of DNA and proteins. MDA is the secondary product from lipid peroxidation, which involves the oxidation of polyunsaturated fatty acid (PUFA) in the cell membrane. MDA is commonly used as a biomarker for oxidative stress in various pathological conditions and diseases, including cancers. MDA may be involved in tumor promotion (Sies et al., 1992; Vaca et al., 1988). Many studies have examined the possibility of a connection between lipid peroxidation and cancer (Torun et al., 1995; Van der Paal et al., 2016).

This study clarified that MDA levels were significantly higher in dogs with appendicular osteosarcoma than in clinically healthy dogs (Table 2), representing an interesting finding given that the formation of free radicals and consequent lipid peroxidation may be related to the development of osteosarcoma. These results correspond to previous studies on humans with cancer (Chole et al., 2010; Gonenc et al., 2001; Peddireddy et al., 2012) and cancerous dogs (Macotpet et al., 2013). Moreover, free radicals are involved in the promotion phase of carcinogenesis (Gonenc et al., 2001). Increased flux of oxygen free radicals and loss of cellular redox homeostasis can be tumorigenic, and increased MDA levels in serum have been proposed to represent a sign of body carcinoma (Bekerecioglu et al., 1998). MDA levels in these dogs may result from this mechanism.

Osteosarcoma is the most common form of bone cancer in children and dogs (Fenger et al., 2014). In dogs, appendicular osteosarcoma most often affect the metaphyses of long bones. The fore limbs are affected twice as often as the hind limbs, with the radius and humerus representing the most frequent sites (Straw et al., 1990). Similar to this study, osteosarcoma occurred at the humerus (50%), tibia (27.78%) and radius (22.22%). The body weight of dogs with appendicular osteosarcoma was significantly higher in this study than in control dogs, because large and giant breeds are more commonly affected (Dernell et al., 2007). The eight breeds of dogs with appendicular osteosarcoma were Rottweiler, Pit Bull, Saint Bernard, Thai Ridgeback, Great Dane, Golden Retriever,

Labrador Retriever and mixed breed. However: size appeared more relevant than breed in dogs suffering from osteosarcoma (Rosenberger et al., 2007). The mean age of osteosarcoma suffering dogs was 7 years in this study compared to  $7.50 \pm 2.91$  years for dogs with appendicular osteosarcoma. RBC in dogs with appendicular osteosarcoma ( $5.55 \pm 1.70$ ) were lower than those of healthy dogs ( $8.35 \pm 1.04$ ), although they remained within the normal range (5.5-8.5) (Table 2). Anemia is one of the most common para-neoplastic syndromes in dogs, cats and humans (de Souza and Borges, 2018) and we conclude that dogs with appendicular osteosarcoma have a tendency to be anemic. Creatinine levels did not differ significantly the control group but the cancer group and appendicular osteosarcoma group, tended to show levels higher than the normal range for creatinine levels.

## Conclusions

In summary, this study supports the conclusions that: 1) oxidative stress was associated with canine appendicular osteosarcoma, as serum MDA levels were significantly higher in dogs with appendicular osteosarcoma compared to clinically healthy dogs; 2) canine appendicular osteosarcoma tended to be associated with anemia with significantly lower PCV, Hb and RBC; 3) large and giant breed dogs showed significantly greater incidences of appendicular osteosarcoma than small breed dogs; and 4) the forelimbs were affected as often as the hind limbs, with the humerus being the most frequent site, followed by the tibia and radius.

Veterinarians should pay attention to prevention of oxidative stress and resolutions that may prolong animal life, as studies in humans have indicated that prevention of oxidation stress and resolution could prolong the life span of cancer patients.

## Consent

All blood samples were taken after signed informed consent from dog's owners.

## Conflicts of Interest

The author declares that no conflicts of interest exist for this article.

## List of abbreviations

ALT: alanine aminotransferase; Hb: hemoglobin; MDA: malondialdehyde; PCV: packed cell volume; RBC: red blood cell counts; WBC: white blood cell counts.

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