Original Article

Oxidative stress and antioxidant in canine cutaneous mast cell tumors

Arayaporn Macotpet[1*] Ekkachai Pattarapanwichien[2] Fanan Suksawat[1] Patcharee Boonsiri[3]

Abstract

Oxidative stress can result from either the excessive production of reactive oxygen species (ROS) or an impaired antioxidant system, or both. It causes damage to lipids, proteins and DNA. Therefore, oxidative stress may be involved in carcinogenesis, and is associated with many types of cancer in dogs. The objective of this study was to compare the levels of malondialdehyde, protein hydroperoxides, glutathione, retinol and alpha-tocopherol between dogs with mast cell tumors and clinically healthy controls. Blood samples were obtained from eighteen clinically healthy dogs and fourteen dogs with spontaneous mast cell tumors. Malondialdehyde and protein hydroperoxides levels were measured by the thiobarbituric acid reactive substance assay, and the ferric-xylenol orange assay, respectively. Glutathione level was determined using spectrophotometric assay. Retinol and alpha-tocopherol levels were measured using the high performance liquid chromatographic method. Dogs with mast cell tumors had significantly higher levels of malondialdehyde ($P<0.01$) and protein hydroperoxides ($P<0.05$) compared with the clinically healthy controls. When considering antioxidants, dogs with mast cell tumors had significantly lower levels of glutathione ($P<0.01$), retinol ($P<0.05$) and alpha-tocopherol ($P<0.01$) compared with the clinically healthy controls. Mast cell tumors in dogs are associated with oxidative stress and antioxidant status. Further studies on oxidative stress and antioxidant activity in dogs should be conducted to guide and plan the complementary treatment of canine cancer.

Keywords: dogs, mast cell tumors, malondialdehyde, protein hydroperoxides, glutathione, retinol, alpha-tocopherol

[1]*Division of Small Animal Medicine, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand*
[2]*Division of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand*
[3]*Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand*
***Correspondence**: arayama7972@gmail.com*

Introduction

Mast cell tumors (MCTs) are one of the most common malignant cutaneous tumors in dogs, and often demonstrate aggressive biological behavior (Jark et al., 2017). However, they can be found in extracutaneous locations, such as the oral cavity, nasopharynx, salivary gland, etc. (Patnaik et al., 1982; Carberry et al., 1987). MCTs may spread to nearby lymph nodes, spleen, liver, other skin sites and distant organs. MCTs can be classified into 3 grades. Grade I MCTs develop slowly and persist for years without increasing in size, whereas grade III MCTs show aggressive growth and have a high recurrence potential. Grade II MCTs are intermediate between the other two forms. More than 80% of grade III MCTs metastasize and frequently cause death (Welle et al., 2008). The prognosis for grade II MCTs is variable and they may cause death in 17-56% of cases due to the failure of treatment or metastatic status (Blackwood et al., 2012).

There is much evidence that reactive oxygen species (ROS) are involved in the aetiopathogenesis of many diseases such as cancer (Waris and Ahsan, 2006; Choudhari et al., 2014; Katakwar et al., 2016). ROS can seriously alter the structure of biomolecules such as lipids, proteins and deoxyribonucleic acid (DNA). The imbalance between the rates of ROS production and ROS removal is known as oxidative stress, and this is involved in carcinogenesis in dogs (Macotpet et al., 2013). Malondialdehyde (MDA) is one of the lipid peroxidation products commonly used as a biomarker of oxidative stress (Gawel et al., 2004). Oxidative damage of proteins yields protein hydroperoxides, which are occasionally utilized for the evaluation of oxidative stress (Gebicki, 1997).

Antioxidants act as "free radical scavengers" by preventing and repairing damages caused by ROS, and can improve immune protection and lower the risk of cancer and other diseases (Valko et al., 2006). Glutathione (GSH) is an antioxidant which participates in the body's defense against free radicals and carcinogens. A retrospective study of natural and synthetic retinoids has demonstrated an inhibition of the growth and the development of different types of cancers in human and dogs (Hong et al., 2000b; Ohashi et al., 2001; Pinello et al., 2009; Tang and Gudas, 2011). Alpha-tocopherol is the most naturally occurring compound of vitamin E and is an effective antioxidant that prevents the incidence of some cancers by protecting cells and DNA from free radicals (Traber and Atkinson, 2007; Rizvi et al., 2014).

The purpose of this study was to compare some biomarkers of oxidative stress (MDA and protein hydroperoxides) and antioxidant (glutathione, retinol and alpha-tocopherol) levels between dogs with grade II MCTs, and clinically healthy controls. The knowledge from this study may introduce a complementary method, such as the supplementation of food rich in antioxidants, for the prevention or treatment of Grade II MCTs in dogs.

Materials and Methods

Animals: Dogs were registered in this study between July-November 2017 at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, Thailand. Of 65 dogs, 19 dogs were identified with grade II MCTs and 46 dogs were detected as being clinically healthy. Five of the dogs with grade II MCTs and 28 clinically healthy dogs were excluded from the study, as they did not achieve the inclusion criteria. Therefore, 14 dogs with grade II MCTs and 18 clinically normal dogs were included in the present study.

There were 18 clinically healthy dogs: 7 males and 11 females with an average age of 8.17 years (5-12 years old), and 14 dogs with grade II MCTs: 7 males and 7 females with an average age of 8.87 years (5-12 years old). For inclusion criteria, grade II MCTs-bearing dogs had to be diagnosed by a histopathology test according to the classical Patnaik grading system (Patnaik et al., 1984) and were classified as stage III disease (The World Health Organization (WHO) classification scheme for canine mast cell tumors). Dogs receiving chemotherapy or antioxidant supplements were excluded from the study. Clinically healthy dogs had to be aged 5-12 years old without blood parasites or intestinal parasites and they had to have had a normal physical examination at least six months before the blood collection. Owner consent was obtained for all participating dogs, and the study protocol was approved by the Animal Ethics Committee of Khon Kaen University.

Blood sample collection and preparation: Four milliliters of blood was drained from the cephalic vein and separated into 2 parts. One ml was treated with ethylenediamine tetraacetic acid (EDTA), for a complete blood count analysis and the presence of blood parasites. The remainder was centrifuged at 3000 rpm at 4°C for 10 min to provide serum for blood chemistry tests.

Complete blood count and blood chemistry analysis: Packed cell volume (PCV), hemoglobin (Hb), red blood cell counts (RBC) and white blood cell counts (WBC) were measured using an automatic analyzer (XT-2000iV: Sysmex®, USA). Creatinine and alanine aminotransferase (ALT) were assayed using an automatic blood chemistry analyzer (AU400; Olympus®, USA).

Determination of oxidative stress marker and antioxidant levels: Oxidative stress markers (MDA and protein hydroperoxides) and antioxidant (retinol, alpha-tocopherol and GSH) were determined using serum samples preserved at -80°C. MDA and protein hydroperoxides were measured by the thiobarbituric acid reactive substance assay (TBARS) (Nielsen et al., 1997) and the ferric–xylenol orange peroxide assay (Gay et al., 1999), respectively. GSH level was determined using 5-5′dithiobis [2-nitrobenzoic acid] (DTNB) reagent (Shigesawa et al., 1992). Retinol and alpha-tocopherol, were measured using a high performance liquid chromatographic (HPLC) method modified from Thurnham (Thurnham et al., 1988).

Statistical analysis: Data is presented as mean ± SD. Parametric data was determined using the independent-sample t test 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 $P<0.05$ were considered significant.

Results

There was no significant difference in age and body weight between groups (P=0.36 and 0.42, respectively). The proportion of pure breeds in the dogs with grade II MCTs and the clinically healthy dogs showed no difference between groups (5/14 or 35.71% vs 6/18 or 33.34%, respectively). In the dogs with grade II MCTs, the pure breeds were Rottweiler (n=1), Thai Ridgeback (n=2) and Golden Retriever (n=2). In the clinically healthy dogs, the pure breeds were Golden Retriever (N=2), Labrador Retriever (n=2), Poodle (n=1) and Rottweiler (n=1).

The numbers of red blood cells and white blood cells in both groups remained within normal ranges; however, red blood cell counts were significantly lower in dogs with grade II MCTs than in clinically healthy dogs ($P<0.001$). No significant differences in white blood cell counts between clinically healthy and grade II MCTs groups (P=0.28) were found. Notably, an upward trend in white blood cell counts was observed in dogs with grade II MCTs. Creatinine and ALT levels were within normal ranges and not significantly different between the two groups (P=0.054 and P=0.14, respectively).

The oxidative stress marker levels show that serum MDA was significantly higher in dogs with grade II MCTs than in clinically healthy dogs (Table 1). Similarly, serum protein hydroperoxides were significantly higher in dogs with grade II MCTs than in clinically healthy dogs. The results in Table 1 also show that antioxidant levels including serum retinol, alpha-tocopherol and GSH were significantly lower in dogs with grade II MCTs than in clinically healthy dogs.

Table 1 MDA, protein hydroperoxides, GSH, retinol and alpha-tocopherol for dogs with grade II mast cell tumors and clinically healthy dogs.

Parameters	dogs with grade II mast cell tumors (N = 14)	clinically healthy dogs (N = 18)	P-value[a]
MDA, mean ± SD, µmol/L	6.14 ± 1.67	4.30 ± 0.49	<0.01
protein hydroperoxides, mean ± SD, µmol/L	63.84 ± 62.52	8.64 ± 3.14	<0.05
GSH, mean ± SD, mg/dL	6.73 ± 1.71	12.24 ± 1.39	<0.01
retinol, mean ± SD, µmol/L	2.58 ± 1.81	3.68 ± 1.10	<0.05
alpha-tocopherol, mean ± SD, µmol/L	10.64 ± 5.28	19.33 ± 6.94	<0.01

Abbreviations: GSH: glutathione; MDA: malondialdehyde

Discussion

The imbalance between the rates of reactive oxygen species (ROS) production and removal is known as oxidative stress, which leads to the degradation of lipids, proteins and nucleic acids. Lipid peroxidation products, including MDA, are often used as biomarkers for oxidative stress status (Macotpet et al., 2013). In the present study, serum MDA levels in dogs with grade II MCTs were significantly higher than those of the control group (Table 1). This implies that the formation of free radicals and consequent lipid peroxidation may be related to mast cell tumors. Moreover, protein hydroperoxides, major products of hydroxyl and singlet oxygen attack on the body proteins during the burden of oxidative stress, are also found in those with oxidative stress (Gebicki, 1997). These hydroperoxides can cause oxidative damage and may contribute to the changing of cellular redox signaling and reducing antioxidants in the body (Davies, 2005; Gracanin and Davies, 2007). The results in Table 1 show significantly higher serum protein hydroperoxides in dogs with mast cell tumor than in the control group. In this study, the standard deviation of protein hydroperoxides in the cancerous group is quite high, and this may result from the extremely high protein hydroperoxide level in 2 dogs. This may be explained by noting that hydroxyl and singlet oxygen in these 2 dogs was high (Gebicki, 1997). The results of oxidative stress marker levels as shown in Table 1, therefore, reflect that lipid and protein damage occur in dogs with grade II MCTs.

The physiological level of ROS is usually regulated by antioxidant defense mechanisms. GSH acts as a powerful antioxidant by working synergistically with the other antioxidants to neutralize and scavenge ROS and prevent oxidative stress (Li et al., 2004; Kerksick and Willoughby, 2005). In this study, the GSH levels were significantly lower in the grade II MCTs group than in the clinically healthy group ($P<0.001$) (Table 1). Our results agree with a previous study (Kumar et al., 1995), which reported a significantly lower level of GSH in grade III cervical intraepithelial neoplasia and invasive cancer compared to the controls. Furthermore, reduced GSH levels of the normal tissues were found to be higher than those in the tumoral tissues which may be a consequence of increased detoxification activity in the tumor cells (Saydam et al., 1997). The quantitative changes occurring in serum GSH is a useful finding and it might represent a systemic biochemical marker for MCTs.

Nowadays, antioxidant vitamins are being studied for use in the treatment and prevention of diseases related to oxidative stress, including cancer. Most studies of antioxidant vitamins are conducted in humans rather than in animals. In this study, dog

serum retinol and alpha-tocopherol, which are commonly known as vitamin A and E respectively, were determined by HPLC. At the present time, the results of a study on vitamin A and its provitamin are still being debated between researchers about the effectiveness of antioxidants (Lewicka et al., 2017). In contrast, the effectiveness of antioxidants for vitamin E are irrefutably confirmed by several studies (Traber and Atkinson, 2007).

Retinol, a derivative form of vitamin A, has cytotoxic effects and the induction of apoptosis against cancer (Bushue and Wan, 2010). Retinoic acid, a derivative of retinol, can inhibit tumor growth by tumor suppressor genes regulation (Connolly et al., 2013) and it has been used successfully for the treatment and chemoprevention of acute promyelocytic leukemia and solid cancers (Hansen et al., 2000; Lengfelder et al., 2000; Lengfelder et al., 2005). Retinoic acid, in combination with surgery, can extend the survival rate of thyroid carcinoma and decrease the time to tumor recurrence (Castillo et al., 2016). In dogs, retinoids encourage morphological differentiation and growth inhibition in cell lines of canine osteosarcoma. Thus, retinoids may have the ability to act as an adjunctive treatment for osteosarcoma and MCTs in dogs (Hong et al., 2000a; Ohashi et al., 2006). In vitro, retinoic acid may be a potential chemotherapeutic agent for the treatment of canine MCTs (Pinello et al., 2009). However, retinoids do not induce differentiation, apoptosis or growth inhibition of melanoma cell lines in dog (Ohashi et al., 2001).

In this study, retinol levels were significantly lower in dogs with grade II MCTs than in clinically healthy dogs (Table 1). The possible explanation is that these dogs had diets with low retinol. A similar study reported that dogs with low vitamin A in their meals had low blood vitamin A level (Schweigert and Bok, 2000). Retinol supplementation for dogs with MCTs during a cancer protocol might reduce MTCs risk factors. A further study on retinol supplementation in dogs with cancer to encourage longer-term survival, including prevention of cancer in dogs is warranted.

Vitamin E may be advantageous in the inhibition of carcinogenesis and cancer metastasis as well as in the enhancement of immunity against the cancer (Ricciarelli et al., 2001). In humans, vitamin E has been suggested to reduce the risk of cancer. In addition, vitamin E supplementation in dog food can prevent lethal ventricular arrhythmias associated with ischemia and reperfusion (Sebbag et al., 1994). Alpha-tocopherol, one form of vitamin E in the body, acts as a cellular antioxidant defense system (Rizvi et al., 2014) and it may decrease oxidation during exercise and improve performance or recovery in dogs (Scott et al., 2001).

In this study, alpha-tocopherol levels were significantly lower in dogs with grade II MCTs than in clinically healthy dogs (Table 1). The results are supported by a previous study that alpha-tocopherol levels decreased in dogs with lymphoma when compared to those of the control dogs (Winter et al., 2009). Dogs with mammary cancer had decreased alpha-tocopherol levels in their neoplastic tissues compared with the normal tissue of an adjacent mammary gland (Karayannopoulou et al., 2013) which indicated a greater utilization of the vitamin. However, there has been a report that alpha-tocopherol did not differ between healthy and dogs undergoing chemotherapy for malignant neoplasms (Galler et al., 2015).

Conclusion: MCTs in dogs may be enhanced or developed if there is an imbalance between oxidative stress and antioxidant status. The dogs with grade II MCTs in this study had increased oxidative stress (MDA and protein hydroperoxides) and decreased antioxidants (GSH, retinol, and alpha-tocopherol). Further studies on oxidative stress and antioxidant activity in dogs should be conducted to obtain clinical guidelines for the prevention or treatment of canine cancer.

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บทคัดย่อ

ภาวะเครียดออกซิเดชันและสารต้านอนุมูลอิสระในสุนัขที่เป็นเนื้องอกมาสต์เซลล์ที่ผิวหนัง

อารยาพร มคธเพศ[1*] เอกชัย ภัทรพันธ์วิเชียร[2] ฟ้าน่าน สุขสวัสดิ์[1] พัชรี บุญศิริ[3]

ภาวะเครียดออกซิเดชันเกิดจากอนุมูลอิสระกลุ่มออกซิเจนมีปริมาณมากเกินไป ระบบของสารต้านอนุมูลอิสระเกิดความเสียหาย หรือทั้งสองภาวะร่วมกัน ซึ่งทำให้เกิดความเสียหายแก่สารชีวโมเลกุลประเภทไขมัน โปรตีน และดีเอ็นเอ การเปลี่ยนแปลงเหล่านี้อาจมีส่วนเกี่ยวข้องกับกระบวนการเกิดโรคมะเร็ง และมีความสัมพันธ์กับมะเร็งหลายชนิดในสุนัข วัตถุประสงค์ของการศึกษานี้เพื่อเปรียบเทียบระดับของมาลอนไดอัลดีไฮด์ โปรตีนไฮโดรเปอร์รอกไซด์ กลูตาไธโอน เรตินอล และอัลฟาโทโคฟีรอล ระหว่างสุนัขที่เป็นเนื้องอกมาสต์เซลล์ที่ผิวหนังและสุนัขสุขภาพดี โดยการเก็บตัวอย่างเลือดจากสุนัขสุขภาพดีจำนวน 18 ตัว และสุนัขที่เป็นเนื้องอกมาสต์เซลล์จำนวน 14 ตัว จากนั้นนำตัวอย่างเลือดไปตรวจหาระดับมาลอนไดอัลดีไฮด์ด้วยวิธีไทโอบาบิทูริค และระดับของโปรตีนไฮโดรเปอร์รอกไซด์ด้วยวิธีเฟอริค-ไซลินอล ออเรนจ์ การวัดระดับกลูตาไธโอนใช้วิธีการวัดค่าการดูดกลืนแสง ส่วนการตรวจวัดระดับเรตินอลและอัลฟาโทโคฟีรอลใช้วิธีโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่าสุนัขที่เป็นเนื้องอกมาสต์เซลล์มีระดับของมาลอนไดอัลดีไฮด์และระดับของโปรตีนไฮโดรเปอร์รอกไซด์สูงกว่าสุนัขสุขภาพดีอย่างมีนัยสำคัญ ($P<0.01$ และ $P<0.05$ ตามลำดับ) แต่มีระดับของกลูตาไธโอน ระดับของเรตินอล และระดับของอัลฟาโทโคฟีรอลมีค่าต่ำกว่าสุนัขสุขภาพดีอย่างมีนัยสำคัญ ($P<0.01$, $P<0.05$ และ$P<0.01$ ตามลำดับ) ดังนั้นสุนัขที่ป่วยเป็นเนื้องอกมาสต์เซลล์อาจมีการพัฒนาหรือเกี่ยวข้องกับภาวะเครียดออกซิเดชัน เนื่องมาจากมีภาวะความไม่สมดุลกันระหว่างภาวะเครียดออกซิเดชันและความสามารถของสารต้านออกซิเดชันในร่างกาย ทั้งนี้ควรมีการศึกษาเพิ่มเติมในเรื่องของภาวะเครียดออกซิเดชันและฤทธิ์ของสารต้านอนุมูลอิสระในสุนัข เพื่อเป็นแนวทางที่อาจเป็นไปได้ในการวางแผนสำหรับเป็นการรักษาเสริมในสุนัขที่เป็นมะเร็งต่อไปในอนาคต

คำสำคัญ: กลูตาไธโอน โปรตีนไฮโดรเปอร์รอกไซด์ เนื้องอกมาสต์เซลล์ มาลอนไดอัลดีไฮด์ เรตินอล สุนัข อัลฟาโทโคฟีรอล

[1]กลุ่มวิชาอายุรศาสตร์สัตว์เลี้ยง คณะสัตวแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น ประเทศไทย 40002

[2]กลุ่มวิชาพยาธิชีววิทยา คณะสัตวแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น ประเทศไทย 40002

[3]ภาควิชาชีวเคมี คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น ประเทศไทย 40002

****ผู้รับผิดชอบบทความ** E-mail: arayama7972@gmail.com*