Oxidant–Antioxidant Status in Canine Multicentric Lymphoma and Primary Cutaneous Mastocytoma

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Received: 9 June 2020; Accepted: 5 July 2020; Published: 8 July 2020

Abstract: Oxidative stress is a prominent event in several acute and chronic diseases including neoplasia. Although its direct involvement in carcinogenesis still remains to be clearly defined, a deeper knowledge of oxidative stress in oncologic patients could help to monitor their clinical outcome and to develop new therapeutic approaches. Therefore, the present study was undertaken to explore redox status in blood of neoplastic dogs affected either by multicentric lymphoma or by primary cutaneous mastocytoma. Superoxide anion (O$_2$•$^-$), nitric oxide (NO) and hydroperoxides (ROOH) were measured. Detoxifying enzyme superoxide dismutase (SOD) and total non-enzymatic antioxidant capacity (ferric reducing-antioxidant power (FRAP)) were assessed. The oxidative stress index (OSi) both for enzymatic (OSi$_E$) and non-enzymatic (OSi$_NE$) scavengers were evaluated. Both pathologies, showed a reduced NO generation, while O$_2$•$^-$ levels were decreased only in mastocytoma. The oxidative stress indexes showed a significant decrease in mastocytoma patients, only for OSi$_E$.

Keywords: dog; lymphoma; mast cell tumors; oxidative stress; redox status

1. Introduction

Oxidative stress derives from an imbalance between oxidizing and antioxidant agents, whose persistence induces degenerative processes and the emergence of further pathological conditions. The major targets of oxidative stress are DNA, proteins, and lipids [1–3]. In dogs, numerous physiological and pathological conditions are characterized by an increase of the oxidative stress levels [4,5]. In general, the assessment of oxidative stress levels in blood samples comprises the evaluation of lipid peroxidation derivatives, the measurement of reactive oxygen species (ROS), and the activity of enzymes, such as superoxide dismutase and glutathione peroxidase, that are involved in their inactivation. ROS play an important role in microvascular blood flow modulation, vascular endothelial growth factor (VEGF) gene expression, and endothelial cell proliferation [6]. Nitric oxide (NO), the main reactive nitrogen species (RNS), is a prominent intra- and intercellular messenger, active against bacterial infections, as well as, by promoting apoptosis, against cancer cell proliferation and metastasis. Moreover, at high concentration levels, NO is a potent cellular killer. Nevertheless, its positive effect on angiogenesis sometimes can be responsible for dichotomous roles [6].

Oncogenesis is a complex process characterized by “key” mechanisms that can be influenced by an imbalance of the redox status. In general, high levels of oxidants can cause both DNA mutations and gene expression modifications that may give rise to the carcinogenic process [7]. In particular, it has been shown that the chemical activities of the different ROS can fragmentate single-ended double-strand DNA and hydroxilate DNA bases, thus causing genetic mutations and affecting transcription [8].
However, cancer cells have been found to be in a state of redox imbalance, where an alteration in the homeostasis between oxidants and antioxidants takes place. To date, the role played by oxidative stress in veterinary oncology has not yet been deeply investigated, especially in the lymphoma and mastocytoma [9,10], which are the most common hematological and solid tumors affecting the canine species [5,11].

Aiming to shed light on the possible role of oxidative stress in these cancer forms, we quantified reactive oxygen species (ROS) in plasma samples, namely superoxide anion (O$_2^{-}\bullet$), nitric oxide (NO), and hydroperoxides (ROOH) in dogs affected by lymphoma or by mastocytoma. In addition, we evaluated the activity of the detoxifying enzyme superoxide dismutase (SOD) and the total non-enzymatic antioxidant capacity as determined by ferric reducing-antioxidant power (FRAP) assay. Moreover, additional integrated information was achieved by calculating oxidative stress index (OSi) both for enzymatic (OSi$_E$) and non-enzymatic (OSi$_{NE}$) scavengers [12,13].

2. Materials and Methods

The dogs enrolled (privately owned) were presented for a clinical examination to the Veterinary Teaching Hospital (University of Parma, Parma, Italy), and divided into three groups: healthy (as assessed by history, physical examination, complete blood count, biochemistry profile, and urinalysis) or affected either by multicentric lymphoma (stage III–IV) or by mast cell tumor (MCT) at clinical stage I–II (Table 1). This study plan was submitted to the Committee for Animal Ethics of the University of Parma (approval number 09/CE/2019), and the experiments were conducted in accordance with the approved guidelines.

Table 1. Animals used in this study: number, breed, sex, age and body weight.

| Animal Groups         | Breed                                      | Age (years) | Sex | Body Weight (kg) |
|-----------------------|--------------------------------------------|-------------|-----|------------------|
| Multicentric lymphoma | Bernese Mountain Dog (2)                   | 7.9         | F (5)| 25.9             |
|                       | Mongrel (2)                                 | (4-13)      | M (6)| (8-37,8)         |
|                       | Riesenschnauzer (1)                         |             |     |                  |
|                       | Setter inglese (1)                          |             |     |                  |
|                       | West Highland White terrier (1)             |             |     |                  |
|                       | Tosa Inu (1)                                |             |     |                  |
|                       | Golden retriever (1)                        |             |     |                  |
|                       | American staffordshire terrier (1)          |             |     |                  |
| Mastocytomas          | Mongrel (3)                                 | 11.2        | F (9)| 28.0             |
|                       | German shepherd dog (2)                    | (8-15)      | M (5)| (14-46)          |
|                       | Labrador retriever (2)                      |             |     |                  |
|                       | Boxer (2)                                   |             |     |                  |
|                       | American staffordshire terrier (1)          |             |     |                  |
|                       | Rottweiler (1)                              |             |     |                  |
|                       | Cane corso italiano (1)                     |             |     |                  |
|                       | Dobermann (1)                               |             |     |                  |
|                       | English Setter (1)                          |             |     |                  |
| Control group         | Mongrel (16)                                | 9.5         | F (9)| 18.8             |
|                       | Beagle (3)                                  | (7-12)      | M (15)| (7-38)          |
|                       | Poodle, miniature (1)                       |             |     |                  |
|                       | Dachshund (1)                               |             |     |                  |
|                       | English Setter (1)                          |             |     |                  |
|                       | Labrador retriever (1)                      |             |     |                  |
|                       | Riesenschnauzer (1)                         |             |     |                  |

F = female; M = male.

Diagnosis of multicentric lymphoma and MCT was performed by a baseline complete hematological and biochemical profile, urinalysis, thoracic radiographs, abdominal ultrasonography, fine needle aspiration of the interested lymph nodes in patients with multicentric lymphoma and fine needle aspiration of sentinel lymph nodes, and cytological/histopathological examination of cutaneous...
lesions for MCT patients. There were 11 dogs (4 to 13 years old) with generalized multicentric lymphoma, 14 dogs (8 to 14 years old) with primary cutaneous MTC, and 24 healthy dogs (6 to 12 years old) (mean 9.5 years) housed at the owners’ residence that were selected for the study. None of the dogs with MCT had evidence of macroscopic metastases, as detected by X-ray and/or ultrasound exams. Since the oxidative status is largely dependent on the oral intake of dietary antioxidants, all the animals were fed with a high-quality dry pet food formulated for adults. Dogs that received any medical treatment within 30 days prior consultation, or with pre-existing diseases over the previous 1 year, were excluded from the study. All samples were obtained prior to the initiation of chemotherapy and/or surgery. Dogs were fasted for 12 h before blood sampling. Blood was collected from the cephalic or from the jugular vein. Plasma (lithium heparin) was separated by centrifugation within 15 min of collection and stored in cryotubes at −80 °C until analyses were performed. Superoxide anion was determined by the WST-1 test (WST-1 Assay Reagent, Sigma Chemical Co Lt, St. Louis, MO, USA) according to the guidelines of the manufacturer. The assay is based on the reducing capacity of superoxide for highly water-soluble tetrazolium salt, giving rise to a soluble formazan that can be quantitatively evaluated by a colorimetric assay [14,15]. NO was assessed by a colorimetric assay measuring nitrite levels; the method based on the formation of a chromophoric compound after reaction with the Griess reagent [16,17]. ROOH levels were quantified by using the “d-ROMs test” (d-ROMs Assay, Diacron s.r.l. Grosseto, Italy). SOD levels were assessed by using a commercial enzymatic activity assay (SOD Assay Kit, Sigma Chemical Co Lt, St. Louis, MO, USA). Scavenging non-enzymatic activity was evaluated determining the reducing ability of the plasma samples by the so-called FRAP assay [18]. FRAP assay measures the change in absorbance at 620 nm due to the formation of a blue-coloured Fe**-tripyridyltriazine (TPTZ) compound from a colourless oxidised Fe**+ form, which is determined by the action of electron donating antioxidants. Enzymatic stress index (OSiE) was calculated for each dog by the ratios between the values of O2 •− and those of SOD, while non-enzymatic stress index (OSiNE) was determined by dividing the values of d-ROMs with those of FRAP [12,13].

Experimental data are presented as mean ± SEM; statistical differences (p < 0.05) were calculated with Student’s t-test using Statgraphics package (STSC Inc., Rockville, MD, USA).

3. Results

3.1. Determination of Superoxide Anion (O2 •−)

In comparison with the healthy patients, O2 •− generation was significantly (p < 0.05) reduced in plasma samples from cutaneous mastocytoma patients while it was unaffected in plasma from those affected by multicentric lymphoma (Figure 1).

![Figure 1. Superoxide anion (O2 •−) levels expressed as milliabsorbance units (mAbs). Healthy dogs (control, n = 24), multicentric lymphoma (lymphoma, n = 11), primary cutaneous MCT (mastocytoma, n = 14). * p < 0.05 vs. control.](image-url)
3.2. Determination of Nitric Oxide (NO)

NO production was inhibited ($p < 0.05$), approximately at the same extent, both in plasma samples from patients affected by lymphoma and in plasma from those affected by mastocytoma (Figure 2).

![Figure 2](image2.png)

**Figure 2.** Nitric oxide (NO) levels expressed as µM. Healthy dogs (control, $n = 24$), multicentric lymphoma (lymphoma, $n = 11$), primary cutaneous MCT (mastocytoma, $n = 14$). *$p < 0.05$ vs. control.

3.3. Determination of Hydroperoxides (-ROOH)

Hydroperoxides level were measured in plasma from healthy and neoplastic dogs. As a difference to the data collected for nitric oxide, no significant variation could be found for the levels of hydroperoxides in plasma from healthy and pathological subjects (Figure 3).

![Figure 3](image3.png)

**Figure 3.** Hydroperoxide levels expressed as Carratelli Units (CARR U). Healthy dogs (control, $n = 24$), multicentric lymphoma (lymphoma, $n = 11$), primary cutaneous MCT (mastocytoma, $n = 14$).

3.4. Determination of Superoxide Dismutase (SOD) Activity

Although not statistically significant, SOD activity in plasma from lymphoma patients was higher, in comparison with the controls, while dogs affected by mastocytoma exhibited approximately the same level of plasma SOD activity of the controls (Figure 4).

![Figure 4](image4.png)

**Figure 4.** Superoxide dismutase (SOD) activity expressed as units/mL (U/mL). Healthy dogs (control, $n = 24$), multicentric lymphoma (lymphoma, $n = 11$), primary cutaneous MCT (mastocytoma, $n = 14$).
3.5. Determination of Ferric Reducing Antioxidant Power (FRAP)

FRAP values, although not statistically significant in comparison with those detected in the control group, were lower in plasma from lymphoma patients, while were unaffected in plasma from those presenting mastocytoma (Figure 5).

![Figure 5](image)

**Figure 5.** Ferric reducing-antioxidant power (FRAP) levels expressed as µM. Healthy dogs (control, n = 24), multicentric lymphoma (lymphoma, n = 11), primary cutaneous MCT (mastocytoma, n = 14).

3.6. Determination of Oxidative Stress Indexes

The determinations of the oxidative stress indexes showed a statistically significant (p < 0.05) variation—only for OSiE of the mastocytoma patients whose mean in consequence of the reduced O2•− values resulted lower than that of the controls (Tables 2 and 3).

| Animal Groups | Mean  | SEM  | t-Test vs Controls |
|---------------|-------|------|-------------------|
| Controls      | 5.75  | 0.90 |                   |
| Lymphoma      | 3.68  | 0.79 | 0.09              |
| Mastocytoma   | 3.39  | 0.41 | 0.05              |

**Table 2.** OSiE (O2•−/SOD)×100

| Animal Groups | Mean  | SEM  | t-Test vs Controls |
|---------------|-------|------|-------------------|
| Controls      | 1.06  | 0.13 |                   |
| Lymphoma      | 1.43  | 0.26 | 0.23              |
| Mastocytoma   | 0.98  | 0.09 | 0.59              |

**Table 3.** OSiNE (DROMS/FRAP)×1000

4. Discussion

Oxidative stress, chronic inflammation, and cancer are closely linked [19]. All the animals of the present investigation belonging to the ‘lymphoma’ group were affected by multicentric lymphoma (stage III–IV) type B that, according to the WHO classification, is of intermediate malignancy (grade 2) [9]. In our study, the dogs enrolled that were of different breeds, were of medium to large size with an average age of 7.9 years. In the literature, it is reported that the detection of redox status alterations in plasma could be useful to either orient or confirm a diagnosis of lymphoma in dogs. The data collected in the present study, in agreement with Plavec et al. [20], show a trend to higher SOD activity in dogs affected by lymphoma compared to the healthy ones and no alterations in O2•− and −ROOH levels. The lack of significance of SOD determinations, in consequence of the high variability of the single measurements could be probably overpassed by analyzing the plasma samples of a higher number of clinical cases. Nevertheless, our data on SOD activity, which are in accordance with those by Guven and coworkers [21] who reported significantly higher levels in human patients with Hodgkin’s Diseases, drive to different conclusions with respect to those of Vajdovich et al. [9],
who observed exactly the opposite in dogs. Moreover, our study highlights a lower, although not significant, non-enzymatic scavenger activity of ROS, as measured by FRAP, while no changes in total antioxidant status based on FRAP were found between control lymphoma affected dogs by Vajdovich et al. [9]. In addition, in the case of FRAP, the lack of significance of its mean value might reflect the high variability of the different measurements. Our data of FRAP in plasma from lymphoma patients are in contrast with those of Bottari et al. [22] and Vajdovich et al. [9] who report that an increase in FRAP levels might be a response that protects host cells against exacerbated damage. Moreover, the present investigation, is in disagreement with those of Vajdovich et al. [9] and Winter et al. [23], who measured plasma levels of isoprostanes (isoP) and MDA (malondialdehyde) respectively; here the oxidative activity was evaluated by the d-ROMs test that detects the hydroperoxides before they induce lipid peroxidation [24]. However, the variations found for d-ROMs did not affect the significance of the OSiNE indexes, whose mean values for both pathologies were similar to those of the controls. Instead, the OSi OSI levels were significantly higher in diffuse large B-cell lymphoma of human patients with stage III or IV disease [13]. The results of Wang et al. support the hypothesis of an active role of the oxidative stress for the induction of lymphomagenesis in the non-Hodgkin lymphoma [25]. With regards to the role of SOD, it remains unclear as a deficient activity of this enzyme has also been reported in malignant lymphoma. Some animal studies have demonstrated a correlation between reduced MnSOD activity, DNA damage, and cancer incidence. It has been reported in humans that patients with malignant lymphomas have free radical hematic levels different from those of normal subjects due to lower oxidative burst capacity of their leukocytes. In the presence of oxidative stress, human B-lymphoma cells are unable to undergo apoptosis and die instead by a form of necrosis driven by the presence of oxidants that can modify cell death pathways [26]. With regards to the mastocytoma patients, our data show a significant reduction of $O_2^{-}$ and NO, while the levels of hydroperoxides and FRAP were unmodified. Our results are in disagreement with the findings of Macotpet et al. [27] who report that the dogs with grade II MCTs had increased oxidative stress (MDA and protein hydroperoxides) and decreased antioxidants (GSH, retinol, and alpha-tocopherol). The significant reduction of the superoxide levels found here resulted into a correspondent significant decrease of the OSiE index. Since the SOD level results were unchanged, it is plausible to hypothesize that the higher activity the enzyme might be responsible for the reduced superoxide levels. It is known that metal ions ($\text{Cu}^{2+}, \text{Zn}^{2+}, \text{Fe}^{2+}$, and $\text{Mn}^{2+}$) and sugars (maltose, sucrose, lactose, trehalose, glucose, d-fructose, d-trehalose, d-xylose) play a crucial role on the activity of SOD. Therefore, on this basis, it is possible that in the plasma of dogs affected by mastocytoma, the biologically available levels of some of these components might determine and enhance the activity of the enzyme in-vivo, which cannot be evaluated by the enzymatic assay adopted in the present investigation. Nitric oxide (NO) is a reactive radical that acts as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense mechanisms, smooth muscle relaxation, and immune regulation. Data collected in our study show a significant reduction of NO levels both in lymphoma and MCTs-affected dogs compared to the controls. In general, NO is a signaling molecule with pleiotropic physiological roles in normal cells and pathophysiological roles in cancer. NO facilitates paracrine signaling, mediates immune responses, and triggers angiogenesis. Therefore, several studies are now aimed to assess the perspective of co-targeting NO metabolism with first-line therapies for improved outcome [28,29]. The results of this study indicate that certain redox status biomarkers are altered in dogs with neoplastic disease with differences, like in the case of the OSiE index of the mastocytoma patients, that can be related to a specific type of tumor. Therefore, a better knowledge of redox status in tumor-affected dogs could be useful to evaluate the benefits of supplementation treatments with antioxidants. Several pieces of evidence at present support a preventive effect of this approach both in humans and in animals; however, it should be noted that other studies demonstrate an increased risk for specific tumors due to antioxidant molecules (i.e., beta-carotene in lung cancer). In particular, Lawenda et al. [30] point out that the use of antioxidant treatment during chemotherapy and radiation therapy should be
discouraged because it can favor both tumor progression and reduced survival. Further studies are needed to address these important topics.

**Author Contributions:** A.C. data curation and writing—original draft preparation; S.B. data curation; R.R. and G.B. writing—original draft preparation and review and editing; F.Q. conceptualization, methodology, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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