EVALUATION OF SYSTEMIC INFLAMMATION PARAMETERS IN DOGS WITH PERIODONTITIS

KURTDEDE Efe1*, ARALAN Gizem1, CENGIZ Remzi Soner2, KILINÇ Ayten Aşkın3, COŞKUN Çağlar4, SALMANOĞLU Berrin1

1Ankara University Faculty of Veterinary Biochemistry Department, Ankara, Turkey; 2University of Kayseri, Yeşilhisar Vocational Collage, Department of Animal Science, Kayseri, Turkey; 3Republic Of Turkey Ministry Of Agriculture And Forestry Poultry Research Institute, Ankara, Turkey; 4Veterinary Practice Dost Pati, Ankara, Turkey

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Periodontal diseases are the most common diseases in veterinary medicine. The first clinical finding is chewing difficulty, saliva flow and bad oral odor. It further develops into plaque and tartar formation, gingival inflammation and hemorrhagic appearance of the gingiva, periodontal pockets formation, alveolar bone resorption and tooth loss.

In this study an evaluation has been made to determine which degree reflects on the parameters of systemic inflammatory reaction with special attention to IL-6 (Interleukine-6), CRP (C-reactive protein), osteopontin, superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GPx) and Ig (Immunglobulins = Total protein – Albumin) and hematological parameters in dogs with periodontitis.

Two groups have been defined in this study. The first group included 10 healthy and owned dogs as a control group. The second group consisted of 10 owned dogs with moderate-severe periodontitis.

The difference between monocyte (p <0.001) and neutrophil (p <0.05) counts was found to be significant. In addition, the difference between SOD, MDA, glutathione peroxidase, CRP, IL-6 measurements in group 1 and group 2 was significant. (p <0.001).

The level of osteopontin in moderate-severe periodontitis cases was found significantly higher than the level measured in the healthy group.

Measured values in the moderate-severe periodontitis cases are higher than the healthy group in terms of CRP, IL-6, and osteopontin levels. Increasing severity of periodontitis was associated with changes in oxidative stress parameters: increased MDA, decreased SOD and glutathione peroxidase levels. These differences provide important information about the evaluation of the cellular responses. There is a need for continued research into the systemic impact of periodontal disease.

Key words: Canine periodontitis, CRP, IL-6, SOD, MDA, glutathione peroxidase
INTRODUCTION

Periodontal diseases are one of the most common diseases in veterinary medicine. Periodontitis varies from mild to moderate and severe forms. When the owner identifies the disease, patients are usually diagnosed at the clinic with periodontitis ranging from moderate to severe. It is known that plaque formation, bacterial growth and immune response play a role in the formation of periodontitis. The immune response involves the activation of polymorphic nuclear neutrophils and macrophages that progress against periodontal pathogenic microorganisms. Beginning from the start of periodontitis, the increase in neutrophil count (a marker of systemic inflammation) and increase in CRP and IL-6 levels catch the attention. These parameters can be used to grade the severity of the disease and to evaluate its prognosis. Pathological changes and systemic inflammatory reaction in patients with periodontitis cause oxidative stress. Measurements of SOD, MDA and glutathione peroxidase are important in the determination and evaluation of oxidative stress [1-4].

It has been determined that primates and dogs can be used as a research model on the decision of implementations in periodontitis cases. Due to limited applications on primates, the experiences that can be obtained from periodontitis in dogs are often used [4-6].

Periodontitis can be seen with an inflammatory response in the host. This inflammatory response results in tissue destruction [7,8]. The inflammation and tissue injuries occur through IL-6 proteases which are released as a result of immune response, osteoclast activity and methylation changes [9, 10]. Thus, an interaction between the immune response of bacteria and host takes place [11].

Interleukin-6 (IL-6) is a cytokine that is produced in response to conditions such as infection and trauma. Cytokines are soluble proteins. They are responsible for the initiation of the inflammatory and immune responses, as well as the continuation of these reactions [12]. Various cells such as macrophages, neutrophils, fibroblasts and endothelial cells play a role in the production of IL-6 [13].

One of the strongest indicators of inflammation is CRP (C-Reactive Protein). CRP production occurs in response to tissue injury and infection in periodontitis [14]. In the previous studies, increased CRP values were found in human patients with periodontitis. Decrease in CRP was observed after treatment of the patients [15].

In contrast to this study, increased levels of CRP in periodontitic patients were not found at the same time in the study done by Buttke et al. in 2005 [16]. A difference between hematological parameters before and after treatment was found in a study done on dogs. When considering the CRP values, a decrease was observed after treatment, but this change was not significant [17].

Once again, an increase in CRP levels was determined in the experimental model of dogs in the study conducted by Yu et al, 2010 [18]. When these studies are taken into account, it is seen that local inflammation of the periodontal tissue reflects on
systemic inflammation parameters depending on the severity of the inflammation. In this study, we aimed to determine to what extent moderate to severe periodontitis affects the systemic parameters of inflammation. CRP and IL-6 levels, which are valuable blood parameters were measured.

As a result of both in vivo and in vitro studies, it was proven that periodontal pathogens and endotoxins have an effect on systemic inflammation and oxidative stress [19].

Inflammation and injury in the gingival tissue occur with plaque formation. In this case, host cells start to release proinflammatory cytokines such as IL-1, IL-6. Cytokines stimulate the infiltration of polymorphonuclear cells against the pathogens in the gingival space as the initial response of cellular host defense. Polymorphonuclear cells produce proteolytic enzymes and O$_2$. As a result of the communication between bacteria and oxygen consumption, reactive oxygen species (ROS) production increase. The purpose of the increased ROS production is to kill bacteria that may cause periodontal tissue destruction [1-3,20].

Under normal conditions, there is a balance between ROS and antioxidant levels. Oxidative stress comprises unbalanced levels. As a result of the deterioration of this balance, the antioxidant system cannot neutralize the high level of ROS production [21].

Studies have shown that high TBARS levels were found in the blood sera and erythrocytes in patients with periodontitis [22].

According to the obtained results on humans, SOD and GPx activities decrease in the saliva of patients with periodontitis [23,24]. In another study, it was demonstrated that enzymatic antioxidant levels such as SOD, CAT in plasma, erythrocytes, and gingival tissue increased as well. Besides, it was found that the non-enzymatic antioxidant levels decreased [22].

Lipid peroxidation destroys the structural integrity of the cell membrane and changes its function. That is why it is one of the most important reactions of free radicals. Malondialdehyde (MDA) is the end product of lipid peroxidation. It causes tissue destruction through the formation of oxidative stress. Therefore, MDA is used as a marker for oxidative stress [2].

Ahmadi-Motamayel F. et al. measured MDA levels in the serum and saliva in patients with chronic periodontitis [23]. Within this scope, 55 patients with chronic periodontitis and 55 healthy people, 30-50 years of age, were involved in the study. MDA levels in the serum and saliva of the patients with periodontitis were found to be extremely high compared to the control group.

When all those studies are taken into account, it was concluded that pathological changes and systemic inflammation reactions, which are present in patients with periodontitis, cause circulatory oxidative stress. Measurements of SOD, MDA and glutathione peroxidase are important in the determination and evaluation of oxidative
stress. In this study, it was aimed to specify the levels of SOD, MDA and glutathione peroxidase used for grading the oxidative stress condition.

MATERIAL AND METHODS

In this study, 2 groups were defined. The first group included 10 clinically healthy and owned dogs as the control group. The second group consisted of 10 owned dogs. A detailed oral examination with gingival scoring, and radiographic survey were performed and clinically diagnosed moderate-severe periodontitis. Cooperation with Ankara Dost Pati Veterinary Clinic was arranged to get blood samples from the relevant dogs. The owners approved blood sampling signed an “Informed Consent Form”. The study was conducted with the approval of Ankara University Ethics Committee dated 21.06.2017 and numbered 2017/13/110.

Probe examination of loss of gum connection (25-50 %), determination of the increase in pocket depth (>3 mm), inflammation on the gingiva and related hemorrhagic appearance, evaluation of the tooth root depth with the determination of the cement-tooth enamel junction through dental radiography and the changes on tooth root and bony tissue were specifically performed. These indicators were used for the determination of the patients with periodontitis ranging from moderate to severe levels and for evaluation of the disease [24,25]. Dogs with these characteristics included the moderate-severe periodontitis group in the study. Dogs that were given any anti-inflammatory drugs and/or antibiotics, or underwent sedation/anesthesia 3 months and periodontal treatment, were excluded from the study.

Blood samples (5 ml) were collected from each control and periodontitis group of dogs into non-anticoagulant tubes. And also 2 ml of blood samples were taken from each animal into tubes with EDTA. Blood was taken in standard tubes for hematology and biochemistry analysis under aseptic conditions.

Cloud Clone Elisa Kits were purchased for IL-6 (SEA079Ca, double-antibody Sandwich), osteopontin (SEA899Ca, double-antibody Sandwich) analysis. SOD, MDA, GPx were analyzed with Cayman Elisa Kits in the laboratory of Ankara University, Faculty of Veterinary Medicine, Department of Biochemistry. Duplication of the samples and standards was done with Elisa kits. The analyses were carried out according to the procedures that are specified in the kit manuals. GPx level was measured using the commercial test kit (Cayman, 703102) according to the method specified by Paglia and Valentine 1967 [26]. The SOD level was determined accordingly to Sun et al. [27], with the aid of the commercial test kit (Cayman, 706002). MDA level was determined as described by Ohkawa et al. [28] using the MDA test kit (Cayman, 100009055).

Total protein and albumin levels were measured by automatic instrument “Random Access XL-600 clinical biochemistry auto analyzer” with Erba Turkey kit. CBC counts were detected with the instrument “Double Exigo Vet Hematology analyze 52285” CRP measurements were done by “Fuji film Nx 500” analyzer instrument.
Hematological analysis was performed with blood counting device (Bouble Exigo Vet Hematology Analyzer pr393).

All data were evaluated according to the parametrical test assumptions. The data distribution was assessed using the Shapiro-Wilk test in terms of normality, and Levene test in terms of homogeneity of variance. Student t-test was used in the healthy and periodontitis groups. Differences were considered significant at p< 0.05 and p<0.001. All exploratory data analyses and statistical tests were performed with the aid of SPSS 14.01 package program.

**RESULTS**

Our study included 10 healthy dogs and 10 dogs with moderate-severe periodontal disease. The mean age (±SD) was 6.1±0.73 years in the control group and 6.6±1.06 years in the periodontitis group. The total of 20 dogs of both sexes (10 males, 10 females) were of the small-medium breeds. According to anamnesis, it was stated that the dogs were fed with commercial food. IL-6 (Interleukine-6), CRP (C-reactive protein), osteopontin, superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GPx) and hematological parameters were used as indicators of the systemic inflammatory reaction level.

The levels of CRP, IL-6 and osteopontin in dogs with periodontitis have been found to be higher than the levels in the healthy group. The results are statistically significant (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>CRP (mg/L)</th>
<th>IL-6 (pg/mL)</th>
<th>Ig (Total protein-Albumin) (g/dl-g/dl)</th>
<th>Osteopontin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy dogs</td>
<td>7.4 ±3.09</td>
<td>144.96±41.99</td>
<td>3.18±1.11</td>
<td>1.04±0.36</td>
</tr>
<tr>
<td>Dogs with periodontitis</td>
<td>18.7±8.02*</td>
<td>216.96±60.78*</td>
<td>3.21±1.06</td>
<td>2.16±0.75*</td>
</tr>
</tbody>
</table>

Values are given as average ± standard deviation.

* The differences of CRP, IL-6, Ig (Total Protein-Albumin) and osteopontin levels between healthy and periodontitis group are found significant (p<0.001).

The levels of CRP, IL-6 and osteopontin in dogs with periodontitis have been found to be higher than healthy group's levels. The results have been significant (Table 1).

**DISCUSSION**

It was determined that plaque formation, increase in gingival pocket depth and gingival inflammation occurred in the observed periodontitis cases. It has been reported that neutrophil and macrophage infiltration occurred in response to this increases and
reactive oxygen species (ROS) increased in gingival and bone tissues. Therefore, SOD, MDA and glutathione peroxidase levels, which are used in grading the oxidative stress situation, have increased. In addition, it can be indicated that IL-6 and CRP, used in the determination of systemic inflammation, have an important diagnostic value in periodontitis cases. In this context, we are of the opinion that these parameters can be used for comparison with other infectious diseases [20-24].

According to the results of the study that was done by Buttke et al. in 2005 [16], reported CRP levels did not increase in dogs with periodontitis. On the other hand, in the study that was carried out by Negro et al. in 2015 [17], the differences in the haematological parameters between the pre-treatment and post-treatment were found in dogs. It was reported that CRP levels that were measured in dogs with periodontitis decreased after treatment. But, it was not specified as significant. In the study that was done by Yu et al. in 2010 [18], CRP levels in the periodontitis group were higher than in the control group. Accordingly, it can cause changes in systemic parameters depending on the severity of the inflammation in the periodontal tissue. Systemic serum CRP and IL-6 levels were determined in order to reveal to what extent local inflammation can effect systemic inflammation parameters in patients with periodontitis ranging from moderate to severe.

Kumar et al. [29] found that WBC levels are increased in the case of periodontitis in humans. Rawlinson et al. [30] stated that the chronic inflammatory process may or may not be associated with an increase in the number of WBCs. The number of WBCs is higher in humans with periodontitis, although this has not been determined in dogs. Hematological indicators as leukocyte, lymphocyte, monocyte, neutrophil and eosinophil levels were measured in our study. The differences between neutrophil (p < 0.05) and monocyte (p < 0.001) measurements of healthy and affected groups were found to be significant (Table 2). Neutrophil and monocyte levels in the periodontitis group ranging from moderate to severe were found to be different than the control group although they were not described as indicators of cellular reaction. The described differences were considered to be statistically significant (Table 2). These findings contrast with the study of periodontal disease in dogs [30]. Our results suggest that the severity and duration of plaque formation in small-medium breeds might be the probable cause of increased neutrophil and monocyte levels.

Table 2. Levels of hematological parameters in healthy and sick dogs (leukocyte, lymphocyte, monocyte, neutrophil and eosinophil)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Leukocyte 10^9/L</th>
<th>Lymphocyte 10^9/L</th>
<th>Monocyte 10^9/L</th>
<th>Neutrophil 10^9/L</th>
<th>Eosinophil 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy dogs</td>
<td>9.89±1.82</td>
<td>2.75±0.79</td>
<td>0.42±0.13</td>
<td>6.35±1.74</td>
<td>0.34±0.18</td>
</tr>
<tr>
<td>Dogs with periodontitis</td>
<td>12.25±3.67</td>
<td>2.09±1.04</td>
<td>0.8±0.27*</td>
<td>8.8±3.03**</td>
<td>0.46±0.28</td>
</tr>
</tbody>
</table>

Values are given as average ± standard deviation. * The differences of monocyte levels between healthy and periodontitis groups are found significant (p<0.001) **The differences of neutrophil levels between healthy and periodontitis groups are found significant (p<0.05).
Considering the studies evaluating the parameters of oxidative stress in dogs, Petelin et al. [31] indicated that periodontal inflammation and oxidative stress, which were created experimentally, decreased with the subgingival implementation of SOD. Sakallioglu et al [32] observed the findings of mucoperiostal recovery and they specified the increase of glutathione peroxidase and SOD levels in case of the periodontitis in their study. They evaluated the cases where the parameters of systemic inflammation decreased with recovery. In our study, SOD, MDA and glutathione peroxidase levels of the dogs with periodontitis were found significantly different than the control group. SOD, and glutathione peroxidase decreased and MDA increased (p < 0.001) (Table 3). The results confirmed the inflammatory response in periodontitis with an increased oxidative stress status compared with the control group.

Table 3. Levels of oxidative stress parameters in healthy and sick dogs (SOD, MDA and Glutathione peroxidase)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Oxidative stress parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOD</td>
</tr>
<tr>
<td>Healthy dogs</td>
<td>144.25±33.79</td>
</tr>
<tr>
<td>Dogs with periodontitis</td>
<td>71.09±19.01*</td>
</tr>
</tbody>
</table>

Values are given as average ± standard deviation. * The differences of SOD, MDA, Glutathione peroxidase levels between healthy and periodontitis group are found significant (p<0.001).

The mechanism of periodontal disease is considered to be the immune response in the periodontal tissue against bacterial growth and the biological active substances that are released. IL-6 proteases, which are released as a result of the immune response, cause inflammation and tissue damage through osteoclast activity and methylation changes. Also, it was specified that it could be a significant marker of periodontal diseases [9,10]. IL-6 is a parameter that is commonly used in periodontitis in humans. Li et al. [33] created periodontitis cases experimentally in their study. They indicated the increase of IL-6 at a level of mRNA in gingival tissue of the second molar with the ligament that was also involved, and they revealed the existence of local inflammation. Oliveira et al. [34] also created periodontitis cases experimentally in their study. The results of their study revealed an increase in the levels of IL-6 and IL-10 cytokines in the blood from patients with periodontitis, which confirmed the inflammation. On the other hand, it was specified that their levels decreased after treatment. In our study, IL-6 levels in dogs with periodontitis were compared with healthy dogs and it was found they were increased. (p<0.001) (Table 1). The immune response of periodontitis and periodontal tissue destruction, has led to increase IL-6 levels.

Dong et al. [35] evaluated the role of osteopontin on bone metabolism in periodontal diseases. They determined a decrease in osteoblast activity by osteoclast differentiation and proliferation following periodontitis progress. Christgau et al. [36] reported that the level of osteopontin was related with the inflammatory reaction and injury stabilization. Similarly, the level of osteopontin in moderate-severe periodontitis
cases was found significantly higher than the level of the healthy group in our study (p<0.001) (Table 1). Our study findings were similar with other previous publications. In general, changes in CRP were determined due to the severity and duration of the inflammation and also the response of the metabolism to inflammation. According to the studies done, it was seen that this situation causes systemic inflammation [30]. According to the result of our study, when the blood analysis from 10 dogs with periodontitis was evaluated, it indicated that the local inflammation could cause the increase on systemic levels. (p<0.001) (Table 1).

It is specified that differentiation of albumin and globulin levels between healthy dogs and dogs with periodontitis can be related with the severity, prevalence and level of inflammation and also cellular response of the metabolism to inflammation [3,37]. In the result of our study, changes in the levels of albumin-globulin are not significant. Changes in neutrophil and monocyte counts were correlated with the alteration of the cellular reaction to the degree of local periodontitis in unhealthy dogs. CRP, IL-6, osteopontin, neutrophil, monocyte, SOD, MDA and glutathione peroxidase levels in moderate-severe periodontitis cases differ from the levels of these parameters in healthy dogs. These differences offer important information about the occurrence of the changes on the statistical evaluation in terms of systemic disease and also about cellular response and periodontal status. In the study that was done by Silva et al. [38], it was determined that local inflammation relocated to systemic levels by means of oxidative metabolism that increases in parallel with the rise of neutrophil counts [38]. As a result, our study revealed that the increase in antioxidant enzymes such as glutathione peroxidase and oxidative stress markers such as MDA, SOD in the serum levels during periodontitis, which is a local inflammation, and also the increase in the level of CRP, which rises with the IL-6 effect, reflect on the general system. Also the decrease in SOD and GPx levels and increased MDA response suggest that oxidative balance is affected. Therefore, we think that it is important to evaluate the local and systemic response rates in determining the prognosis of periodontitis. Evaluation of whole systemic inflammatory responses and the cellular pathways need to be further studied.

Authors’ contributions
KE performed immunoassay. SB, CRS, AG, KAA, KE and SB design, coordination and helped to draft the manuscript. CC performed the statistical analysis. All authors read and approved the final manuscript.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
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PROCENA PARAMETARA SISTEMSKE INFLAMACIJE KOD PASA SA PERIODONTITISOM

KURTDĐEDE Efe, ARALAN Gizem, CENGIZ Remzi Soner, KILINÇ Ayten Aşkın, COŞKUN Çağlar, SALMANOĞLU Berrin

Periodontalna oboljenja su najčešća oboljenja u veterinarskoj medicini. Prvi klinički nalaz predstavlja otežano žvakanje, curenje pljuvačke i loš zadah iz usta. Dalje dovode do nastanka plaka i tartara, upale desni i hemoragičnog izgleda gingive, nastanka periodontalnih džepova, resorpcije alveolarne kosti i gubitka zuba.

U ovoj studiji urađena je procena kako bi se odredio stepen do kojeg se ogleda na parametre sistemske inflamatorne reakcije sa posebnom pažnjom na IL-6 (interleukin-6), CRP (C-reaktivni protein), osteopontin, superokсид dizmutaza (SOD), malonildialdehid (MDA), glutation peroksidaza (GPx) i Ig (Imunoglobulini – ukupni proteini – albumini) i hematološke parametare pasa sa periodontitisom.

U ovom radu su definisane dve grupe. U prvu grupu je uključeno 10 zdravih vlasničkih pasa koji predstavljaju kontrolnu grupu. Drugu grupu čine 10 vlasničkih pasa sa umerenim do teškim periodontitismom.

Razlika u broju monocita (p <0,001) i neutrofila (p <0,05) je signifikantna. Pored toga, razlika između SOD, MDA, GPx, CRP, IL-6 grupe 1 i grupe 2 je bila signifikantna (p <0,001). Nivo osteopontina kod umerenog-teškog periodontitisa je bio signifikantno viši u poređenju sa kontrolnom grupom. Teži slučajevi periodontitisa su bili u vezi sa promenama parametara oksidativnog stresa: povećanim MDA, smanjenom SOD i smanjenom aktivnošću GPx. Ove razlike pružaju važne informacije u proceni čelijskog odgovora. Postoji potreba za daljim ispitivanjem sistemskog efekta periodontalnih bolesti.