Experimental periodontitis promotes transient vascular inflammation and endothelial dysfunction

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\textbf{A B S T R A C T}

Objectives: This study aimed to evaluate the systemic inflammatory response and cardiovascular changes induced by experimental periodontitis in rats.

\textit{Design:} Experimental periodontitis was induced by placing a cotton ligature around the cervix of both sides of mandibular first molars and maxillary second molars in each male rat. Sham-operated rats had the ligature removed immediately after the procedure. Seven, 14 or 28 days after procedure, the effects of acetylcholine, sodium nitroprusside and phenylephrine were evaluated on blood pressure, aortic rings and isolated and perfused mesenteric bed. The blood was obtained for plasma Interleukin-6 (IL-6), C-reactive protein (CRP) and lipid evaluation. The mesenteric vessels were obtained to evaluate superoxide production and nitric oxide synthase 3 (NOS-3) expression.

\textit{Results:} Ligature induced periodontitis reduced endothelium-dependent vasodilatation, a hallmark of endothelial dysfunction. This effect was associated with an increase in systemic inflammatory markers (IL-6 and CRP), worsens on lipid profile, increased vascular superoxide production and reduced NOS-3 expression. It is interesting to note that many of these effects were transitory.

\textit{Conclusion:} Periodontitis induced a transient systemic and vascular inflammation which leads to endothelial dysfunction, an initial step for cardiovascular diseases. Moreover, the animal model of periodontitis used here may represent a valuable tool for studying the relationship between periodontitis and endothelial dysfunction.

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1. Introduction

Cardiovascular disease is a major public health problem in many societies, accounting for 17 million deaths each year.\textsuperscript{1} A large body of epidemiologic studies have clearly demonstrated a link between certain risk factors such as high cholesterol levels, smoking, sedentary lifestyle and diabetes and the development of cardiovascular diseases.\textsuperscript{2} However, these risk factors do not fully explain why some people may experience a

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cardiovascular event and others do not. Therefore, inflammation has emerged as an integrative cardiovascular disease factor, and novel risk factors such as fibrinogen and inflammatory markers have been introduced.

Interestingly, individuals with severe chronic periodontitis have been reported to have a significantly increased risk of developing cardiovascular disease, after adjusting for many traditional risk factors. Although the mechanisms accounting for such a relationship have not been fully defined, it has been proposed that bacteria can access systemic circulation, leading to the invasion of vascular cells and increased levels of circulating cytokines.

The endothelium is the active inner monolayer of the blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. It is well established that systemic inflammatory factors activate the endothelium, leading to its dysfunction. One of the hallmarks of endothelial dysfunction is an altered response to endothelial-dependent stimuli, such as acetylcholine. Acetylcholine stimulates endothelial nitric oxide synthase (NOS-3) to generate NO, that diffusing to the underlying smooth muscle cell and induces relaxation by increasing the production of cGMP. On the other hand, response to the endothelium-independent vasodilator sodium nitroprusside, a nitric oxide donor, remains intact during endothelial dysfunction. Additionally, it has been shown that endothelial dysfunction enhances vasoconstriction response to agents like phenylephrine by reduction of endothelial nitric oxide buffering capacity.

Endothelial dysfunction is an early event in the development of cardiovascular disease. A number of studies have shown that patients with cardiovascular risk factors but no clinical signs of atherosclerosis have endothelial dysfunction. Emerging evidence has shown an association between periodontitis and endothelial dysfunction in humans. These findings suggest that periodontitis is associated with endothelial dysfunction through decreased nitric oxide (NO) bioavailability and that systemic inflammation may be, at least in part, a cause of endothelial dysfunction.

Despite the link between periodontitis and endothelial dysfunction in humans, more knowledge of this association is needed. The studies that show this relationship use flow-mediated dilation of brachial artery as a clinical marker of endothelial function, a method that is reproducible and closely correlated with invasively measured endothelial function, but that fail to provide more detailed information about the vascular changes. Thus, the objective of the present work was to evaluate the vascular reactivity changes in isolated vessels and specific vascular beds as well as the systemic inflammatory response induced by periodontitis in rats. The use of animal models may provide a better understanding and improved strategies to prevent and treat the cardiovascular changes induced by periodontitis.

2. Materials and methods

2.1. Animals

Male Wistar rats (200–250 g; 10 weeks old) were housed in a temperature- and light-controlled room with free access to water and food. All of the procedures are in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and were approved by the University Institutional Ethics Committee (Protocol number 23080.034301/2009-36).

2.2. Ligature-induced periodontitis

To induce periodontitis, rats were first anesthetised with an intraperitoneal injection of ketamine and xylazine (90 and 15 mg/kg, respectively). A cotton ligature (4/0) was placed around the cervixes of both sides (right and left) of mandibular first molars and maxillary second molars in each animal. Hence, four ligatures were placed at each animal. The ligature was knotted on the vestibular side, so that it remained subgingival on the palatal side. Placement of ligatures induces periodontal disease by facilitating bacterial invasion of gingival. Sham-operated rats had the ligature removed immediately after the procedure.

2.3. Mean arterial pressure (MAP) measurement

Forty eight animals were randomly distributed into two groups of 24 animals each to be submitted to ligature or sham procedure. Seven, 14 and 28 days after ligature or sham procedure, 8 rats per group were anaesthetised, and heparinised PE-20 and PE-50 polyethylene catheters were inserted into the left femoral vein for drug injections and into the right carotid artery to record the mean arterial pressure (MAP). The animals were allowed to breathe spontaneously via a tracheal cannula. Body temperature was monitored and maintained at 37 ± 1 °C. The blood pressure data were recorded with a catheter pressure transducer coupled to a Powerlab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia) running LabChart 7c software.

Dose-response curves to intravenously acetylcholine, sodium nitroprusside and phenylephrine were obtained. At the end of the experiment, the animals were sacrificed with pentobarbital overdose. The results are expressed as the mean ± SEM of the peak changes in the MAP (mmHg) relative to baseline.

2.4. Rat thoracic aorta rings

Forty eight animals were randomly distributed into two groups of 24 animals each to be submitted to ligature or sham procedure. Seven, 14 and 28 days after ligature or sham procedure, thoracic aorta rings from 8 rats per group were isolated as described previously. The rings were mounted using two wires inserted through the lumen of the vessel in an organ chamber in Krebs-Henseleit solution (composition in mM: NaCl, 113; KCl, 4.7; CaCl₂, 2.5; KH₂PO₄, 0.9; NaHCO₃, 25; MgCl₂, 1.1; glucose, 11; pH 7.4) continuously gassed with 95% O₂/5% CO₂ at 37 °C and under a resting tension of 1 g. The mechanical activity was recorded isometrically by a force transducer connected to an amplifier and chart recorder (Soft and solutions-KITCAD8, São Paulo, SP, Brazil).

After an equilibration period of 60 min, the rings were precontracted with phenylephrine (1 μmol/L), and the presence of functional endothelium was assessed by the ability of
acetylcholine (1 μmol/L) to induce greater than 75% relaxation. After testing the functional endothelium, cumulative concentration–response curves for phenylephrine were obtained. Then, the rings were pre-contrasted with a submaximal concentration of phenylephrine (1 μmol/L); upon reaching a plateau, a cumulative concentration–response curve for acetylcholine was obtained. The phenylephrine response is expressed as the percentage of the maximal response (in grams) recorded for the control curve (sham), and the vasodilator effect of acetylcholine is expressed as the percentage of vasodilation.

2.5. Rat-isolated mesenteric arterial bed

Forty eight animals were randomly distributed into two groups of 24 animals each to be submitted to ligature or sham procedure. Seven, 14 and 28 days after ligature or sham procedure, the mesenteric arterial bed (MAB) from 8 rats per group were isolated and perfused via the superior mesenteric artery.25 The preparations were dissected and mounted on a stainless steel grid in a humid chamber and perfused with Krebs-Henseleit at a constant flow rate of 4 mL/min, gassed with 95% O2/5% CO2 and maintained at 37 °C. The responses were measured as changes in the perfusion pressure (mmHg) using a pressure transducer coupled to acquisition hardware and software (PowerLab 8/30 running LabChart 7®).

After equilibration, a concentration–response curve for phenylephrine was obtained. Then, a submaximal concentration of phenylephrine (750–1500 μg) was added to the perfusion fluid to increase the perfusion pressure of the preparations by 70–150 mmHg above baseline. When the pressor effect of phenylephrine reached a plateau, acetylcholine (200 nmol/L) was injected to test endothelial functionality before the concentration–response curves for acetylcholine were obtained. The contractile response to phenylephrine is expressed in mmHg, and the vasodilatory effect of acetylcholine is expressed as a percentage decrease in relation to the pressor effect of phenylephrine.

2.6. Fluorescence microscopy for oxidised dihydroethidium

Eight animals were randomly distributed into two groups of 4 animals each to be submitted to ligature or sham procedure. Twenty-eight days after ligature, three alternating sections (8-μm thick, with an individual distance of ~100 μm) of the mesenteric arteries were obtained of each animal of each group using a cryostat (Leica, Germany). The vascular sections were placed on glass gelatin-coated slides and incubated with dihydroethidium (DHE, 1 μM; Molecular Probes, Invitrogen, NY, USA) in a dark, humidified chamber at 37 °C for 30 min. In the presence of superoxide anions, DHE is oxidised to ethidium, which intercalates within DNA strands, resulting in a red fluorescence. After washing with PBS, the coverslips were mounted on the slides using Gel Mount™ aqueous mounting medium (Sigma–Aldrich Co. LLC, St. Louis, MO, USA) and visualised by fluorescence microscopy (Olympus BX41; Olympus, Tokyo, Japan), and images were captured using Q-capture Pro 5.1 (Q-imaging).

2.7. Fluorescence microscopy for NOS-3 staining

The mesenteric arteries harvested for fluorescence microscopy for oxidised dihydroethidium (Section 2.6) were also used to NOS-3 staining. The vascular sections were fixed with acetone, incubated with PBS/0.5% Tween (20 min) and subsequently blocked with 5% bovine serum albumin and PBS/0.1% Tween (60 min). Then, the slices were incubated overnight at 4 °C with rabbit polyclonal anti-NOS3 (1:100; Santa Cruz Biotechnology, CA, USA). After washing three times, the slides were incubated for 60 min with Alexa 488-conjugated, anti-rabbit IgG (1:1000; Invitrogen, UK) at room temperature. After washing, the coverslips were mounted on the slides using Gel Mount™ aqueous mounting medium (Sigma–Aldrich Co. LLC, St. Louis, MO, USA) and visualised by fluorescence microscopy (Olympus BX41; Olympus, Tokyo, Japan), and the images were captured using Q-capture Pro 5.1 (Q-imaging).

2.8. Quantification of fluorescence intensity

Briefly, the relative quantification of fluorescence intensity was achieved through densitometry analysis, using the ImageJ® imaging software (NIH, Bethesda, MD, USA). The same microscope settings were used to acquire all images. The threshold value for the optical density that better discriminated staining from the background was obtained, and the settings of this threshold were recorded using Plugins Macro. All images were analyzed by the recorded Macro in order to dispose of any subjectivity. The results were expressed as fluorescence intensity (arbitrary units).

2.9. Measurement of leucocyte counts, plasma lipid profile, interleukin-6 (IL-6) and C-reactive protein (CRP)

Immediately before the withdrawal of the aorta (Section 2.4), whole blood samples were obtained in fresh vials containing heparin by cardiac puncture. The total leucocyte count was determined by Cell Dyn 1400 (Abbott Diagnostics, Abbott Park, Illinois, USA). Plasma lipid analyses were performed with an automated chemistry analyser (Vital Scientific, Netherlands) using a cholesterol oxidase method. Plasma CRP was quantified using a highly sensitive, rat enzyme-linked immunosorbent assay (ELISA) kit (Immunology Consultants Laboratory Inc, Newberg, USA). Plasma IL-6 was measured using an ELISA assay kit (RayBiotech, Inc, Norcross, USA).

2.10. Measurement of alveolar bone loss

After blood pressure experiments (Section 2.3), the withdrawal of the aorta (Section 2.4) and mesenteric arterial bed (Section 2.5) the mandible and maxilla were dissected. The mandible was split in half along the midline and between the central incisors. The defleshed mandible and maxilla were stained with aqueous 1% methylene blue to identify the cemento-enamel junction (CEJ). Standardised pictures were taken of each specimen with a digital camera (Sony Cybershot DSC 707, São Paulo, SP, Brazil). A minimal focal distance was used, and the samples were placed with the occlusal surface parallel to
the horizontal plane and a millimetre ruler was used as a scale reference. Pictures were taken from the lingual aspect of the specimens. The images were measured with a computer software (Image Pro Plus™ Version 4.5, Media Cybernetics, Silver Spring, USA). The distance of alveolar bone loss was measured between the CEJ and the alveolar bone crest. For evaluating average alveolar bone height, six points were measured on the buccal and lingual parts. The average alveolar height was calculated for each molar.

2.11. Statistical analysis

Data are expressed as the mean ± SEM of n rats. Statistical significance was analysed by two-way ANOVA, followed by Bonferroni’s post hoc t test, except for quantifying fluorescent intensity where Student’s t test was used. A P value less than 0.05 was considered to be significant. When necessary the values had been transformed into logarithms in order to achieve normality and homogeneity of variances. These conditions had been proved by the Shapiro–Wilk and Bartlett test, respectively. Agonist concentration–response curves were fitted using a nonlinear regression. Agonist potencies and maximum responses are expressed as the negative logarithm of the molar concentration of agonist producing 50% of the maximum response (pEC50) and the maximum effect elicited by agonist (EMax), respectively.

3. Results

3.1. Alveolar bone loss

The ligature was placed around the second maxillary molars and the first mandibular molars on both sides (right and left). However, for the sake of clarity, we pooled the results from the right and left maxilla and mandibles (Fig. 1). Alveolar bone loss was observed in the maxillary and mandible molars in the ligated rats when compared to matched sham group (Fig. 1). Interestingly, in mandible, there is no difference between 14 and 28 days ligated rats, indicating a stabilisation of bone loss (Fig. 1a). On the other hand, in maxilla, alveolar bone loss is progressive (Fig. 1b).

3.2. Effect of ligature-induced periodontitis on the mean arterial pressure

To evaluate endothelial function in rats with experimental periodontitis, we used endothelium-dependent and endothelium-independent vasodilators (acetylcholine and sodium nitroprusside, respectively). The reduction in the mean arterial pressure induced by sodium nitroprusside in rats with the ligature was similar to that of the sham rats. In contrast, the effect of the higher dose of acetylcholine was reduced in the rats submitted to ligature 14 days earlier (Fig. 2b). The pressor response to phenylephrine was similar in both groups at each time point (Fig. 2a–c).

3.3. Effect of ligature-induced periodontitis on vascular reactivity in isolated aortic rings

The response to acetylcholine (pEC50) was reduced in the periodontitis rats 14 days after the procedure, but the maximum (EMax) response was comparable to that of the sham group (supplementary Table 1; Fig. 3b). The acetylcholine dose–response curve was similar in both groups at 7 and 28 days after the procedure (Fig. 3a, c). The relaxation induced by sodium nitroprusside was not different when comparing the groups (data not shown). No differences between the groups were observed on the phenylephrine concentration–response curve (supplementary Table 1, Fig. 3a–c).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2013.03.009.

3.4. Effect of ligature-induced periodontitis on vascular reactivity in isolated mesenteric arterial beds

The maximal vasoconstrictive response (EMax) to phenylephrine in the ligature group did not change at any evaluated time (supplementary Table 2; Fig. 4a–c); however, the half-maximum response (pEC50) increased in the periodontitis animals on day 28 (supplementary Table 2; Fig. 4c).
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2013.03.009.

No significant reduction in pEC_{50} after acetylcholine administration to the mesenteric bed was found in the groups (supplementary Table 2; Fig. 4a–c). However, acetylcholine induced-relaxation was impaired in the mesenteric bed on day 28 post-procedure, as demonstrated by a reduction of the maximum response (supplementary Table 2; Fig. 4c).

3.5. Vascular superoxide production

Increased fluorescence was observed in the mesenteric arteries from ligature rat 28 days after procedure (Fig. 5b, d) compared to the sham rats (Fig. 5a, c), which reflects increased superoxide anion generation. Ethidium fluorescence was prominent in all three layers of the mesenteric arterial segments. The quantification of fluorescence intensity clearly shows the differences between the groups (supplementary Fig. 1a).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2013.03.009.

3.6. Fluorescence microscopy for NOS-3 staining

In the sham mesenteric arteries, a marked fluorescence to NOS-3 staining was observed (Fig. 6b, e). In contrast, in the vessels from the ligature rats, a weak NOS-3 immunopositivity was detected (Fig. 6c, f). The white arrows indicate NOS-3 staining, located primarily in endothelial cell layer. Control staining by omission of the primary antibody shows the auto fluorescence for collagen (Fig. 6a, d). Interestingly, the quantification of fluorescence intensity of the immunostainings, which excludes the background, shows a reduction on NOS-3 immunopositivity on ligature rats (supplementary Fig. 1b).

3.7. Effect of ligature-induced periodontitis on leucocyte counts, plasma lipid profile and serum IL-6 and CRP

Fourteen days after procedure, ligature group shows higher LDL-cholesterol levels than time-matched sham and 28 days ligature group (Fig. 7c). C-reactive protein levels increase at 14 days and return to basal level thereafter (Fig. 7e). IL-6 was increased 14 and 28 days after ligature when compared to...
time-matched control (Fig. 7f). The total leucocyte count did not change, but 14 days after the procedure there was a neutrophilia when compared to time-matched sham and 28 days ligature group (Table 1).

No differences between the groups were found for plasma total cholesterol (Fig. 7a), HDL-cholesterol (Fig. 7b), VLDL-cholesterol (Fig. 7d) and triglycerides (Table 1).

4. Discussion

In the last two decades, several epidemiological studies have pointed to a relationship between periodontitis and cardiovascular disease.26,27 However, the mechanistic relationship between oral disease and cardiovascular disorders remains unclear. In this study, we evaluated endothelial function in a rat periodontitis model.

Mainly due to easy handling, low cost and similarity to human disease, ligature-induced periodontitis in rats is among the most widely used experimental models of periodontitis. Alveolar bone loss is well-established 7 days after ligature placement, and it was reproduced in our conditions.23 Because the main goal of the majority of the studies using this model is to assess only the local consequences of the lesion, a time period of one week is rarely exceeded. However, we reasoned that the systemic consequences would be most likely slower in onset; therefore, we studied the animals for up to four weeks after the procedure. Systemic changes did indeed take more time to develop, and this report showed that, from two weeks, ligature-induced periodontitis reduced endothelium-mediated vessel relaxation in rats. This effect was observed in the whole animal, in isolated conductance vessels (aorta) and in microcirculation vascular bed (mesenteric bed). The vascular reactivity changes induced by periodontitis were associated with systemic and vascular inflammation.

Regarding blood pressure, endothelial dysfunction 14 days after the procedure was evident by the reduced response to
acetylcholine, which stimulates NO production by endothelial cells. No alterations in the blood pressure response to sodium nitroprusside were observed, indicating that smooth muscle cGMP-mediated signalling remained intact.

The endothelial dysfunction observed in the whole animal was matched with a reduction in acetylcholine-induced relaxation in isolated aortic rings. These results coincide with those of previous studies demonstrating that the aortas from ligature induced-periodontitis rats displayed lipid peroxidation, which may impair vascular reactivity.28

The presence of arterioles, which are important resistance vessels, makes the mesenteric bed an important sample for cardiovascular research. The mesenteric circulation receives approximately 20% of the cardiac output29 and contributes significantly to total peripheral resistance.30 Interestingly, endothelium dysfunction in the mesenteric bed seems to be of even slower onset because it was found 28 days after the procedure. The reduction in endothelium-dependent relaxation was followed by an increase in the constriction response to phenylephrine. This finding agrees with well-documented literature, which shows that the response to vasoconstrictive agents is enhanced under conditions of decreased vascular NO, as in endothelial dysfunction.13

Because a consistent endothelial dysfunction was observed in the mesenteric bed 28 days after the procedure, in this time point we evaluated the reactive oxygen species production in mesenteric artery, which is an important resistance vessel.31 It is known that systemic inflammation increases reactive oxygen species in the vessel wall and impairs endothelium-dependent relaxation by scavenging NO, thereby reducing NO bioavailability.32 Interestingly, we observed an increased superoxide anion production in the mesenteric arteries 28 days after ligature, and a reduction in NOS-3 content. Although we did not evaluate NOS-3 activity or measure NO production, this result agrees with the observed reduction in endothelium-dependent relaxation. Interestingly, a recent work demonstrated that superoxide anion generation is, at least in part, responsible for reduced NOS-3 levels.33 Nitric oxide plays a crucial role in regulating a wide spectrum of functions in the cardiovascular system, and

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***Fig. 4 – Effect of ligature-induced periodontitis on vascular reactivity in isolated mesenteric arterial bed. Seven (a), 14 (b) or 28 days (c) after the sham or ligature procedure the arterial mesenteric bed were mounted. Cumulative concentration curves for acetylcholine and phenylephrine were determined. Results are expressed as mean ± SEM (n = 8).** *P < 0.05 compared to the time-matched sham group.***
reduced endothelial NO production is associated with several cardiovascular disorders. Altogether, these vascular changes induced by an experimental model of periodontitis provide important insight into the relationship between oral infection and cardiovascular risk.

In addition to endothelial dysfunction, we have also shown that ligature-induced periodontitis increased LDL-cholesterol. Recently, it has been demonstrated that orally infect mice with Porphyromonas gingivalis showed a decrease in serum HDL without changes in LDL levels.34 Endothelial dysfunction and

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**Fig. 5** – Effect of periodontitis on superoxide generation in the rat mesenteric artery. Mesenteric artery sections were labelled with the oxidative dye dihydroethidium, which, in the presence of superoxide, is oxidised and gives red fluorescence. The images show fluorescence in the rat mesenteric artery harvested at day 28 after the sham (a, c) or ligature (b, d) procedure. Images are representative of at least two experiments.

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**Fig. 6** – Representative images of endothelial nitric oxide synthase (NOS-3) immunofluorescence. Mesenteric arteries from the 28 days sham (b, e) or periodontitis (c, f) rats are shown. The white arrows indicate NOS-3 staining, located primarily in endothelial cell layer. Control staining by omission of the primary antibody is shown in panels (a) and (d). Images are representative of at least two experiments.
an altered plasma lipid profile may play a synergistic role in developing cardiovascular disease. However, it is important to emphasise that the vascular changes as well as lipid profile alteration were transient and therefore the conclusions regarding the relationship of these effects and cardiovascular risk may be limited.

IL-6 is a proinflammatory cytokine that is crucial in regulating osteoclast activity and bone resorption. Additionally, IL-6 is an important prognostic factor for the future occurrence of major cardiovascular events. IL-6 production, in turn, induces the expression of hepatic acute-phase proteins, including CRP, which is measured clinically to

Table 1 – Effect of ligature-induced periodontitis on physiological parameters in rats.

<table>
<thead>
<tr>
<th></th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
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<td></td>
<td>Sham</td>
<td>Ligature</td>
<td>Sham</td>
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<tr>
<td>MAP</td>
<td>102 ± 3.6</td>
<td>98 ± 2.7</td>
<td>100 ± 3.3</td>
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<tr>
<td>Triglycerides</td>
<td>32 ± 4.9</td>
<td>33 ± 5.9</td>
<td>34 ± 5.5</td>
</tr>
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</table>

The variables are the mean arterial pressure (MAP, mmHg), leucocyte count, neutrophil count, lymphocytes count (cells K/μL) and triglyceride level (mg/dL). Results are expressed as mean ± SEM (n = 8).

* P < 0.05 compared to the time-matched sham group.

# P < 0.05 compared to the 7 day ligature group.

& P < 0.05 compared to the 28 day ligature group.
assess atherosclerotic risk. High CRP levels have been shown to be associated with endothelial dysfunction, and there is currently strong evidence that plasma CRP is elevated in periodontitis. Here, we showed an elevation of serum CRP and IL-6 in rats with ligature-induced periodontitis. Our results also showed that high levels of IL-6 and CRP are associated with neutrophilia and increased LDL-cholesterol. Interestingly, a recent work has shown that IL-6 positively correlates with a worsening lipid profile in patients with periodontitis, which supports previous work showing that increased IL-6 leads to increased hepatic fatty acid synthesis.

Interestingly, some cardiovascular and systemic inflammatory markers returned to basal levels at day 28 after ligature, while other changes became apparent at day 14 or 28 after the procedure. We do not have a good explanation why some markers were returned to basal levels at day 28; however we believe that this may be a consequence of rat resistance to infections and inflammatory stimulus compared to human. Most laboratory animals, including rats, have a great ability to adapt front of inflammatory stimuli. Therefore, the interpretation of these data should be done carefully. Anyway, these results not only demonstrate that the systemic changes induced by periodontitis are a complex, dynamic process but also point to the importance of temporal analysis.

A recent work has shown an increase of cardiac nitrotyrosine seven days after ligature-induced periodontitis. Additionally, has been demonstrated an increased IL-6 expression as well as an enhanced lipid deposits in the aorta of ligature rats. These works besides corroborate our results, point to an important relationship between systemic inflammation induced by periodontitis and cardiovascular changes.

An important difference between our work and others that use this experimental model is the number of ligatures used to induce periodontitis. To induce a generalised process, we used four ligatures, while the majority of studies use only one or two. Usually in human periodontitis, several teeth are affected, so that although the use of one ligature is enough to study local effects, like bone loss, our model with four ligatures produce a widely inflammatory periodontal process with systemic effects. Likewise, to investigate the association of periodontitis with histological changes in aorta and uterus, a recent work has performed two, three or six ligatures in rats. Interestingly, the main changes were observed in periodontitis rats with three or six ligatures. Thus, although some studies show systemic effects with one or two ligatures, or more ligatures are more consistent when more than three ligatures are placed.

In summary, we temporally characterised systemic inflammatory and endothelial dysfunction in an experimental model of periodontitis. This may provide insight into a pathogenic mechanism by which periodontitis may increase the risk of cardiovascular diseases. Furthermore, our results extend the data obtained from subjects with periodontitis, illustrating that this model can be a valuable tool for studying the relationship between periodontitis and cardiovascular diseases.

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**Competing interests**

The authors declare no conflicts of interest.

**Ethical approval**

The experimental protocols were executed following ethical principles for laboratory animal use in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and they were approved by Institutional Ethical Committee of Animal Research (Protocol number 23080.034301/2009-36).

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