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Decrease of Pericytes is Associated With Liver Disease Caused by Ligature-Induced Periodontitis in Rats

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Abstract

Background: Damage caused by periodontitis not only affects periodontal tissues, but also increases the severity of various illnesses such as rheumatoid arthritis, diabetes, and liver diseases. The aim of this study is to investigate the association between induced periodontitis and damage caused through its systemic effects on the liver.

Methods: Twenty rats were divided into two groups: control and periodontitis. The following parameters were evaluated: gingival bleeding index (GBI), probing depth (PD), myeloperoxidase (MPO) activity, alveolar bone loss (ABL) for periodontal tissues; histopathologic examination of gingival and liver tissues; immunohistochemistry to cells positive for neural/glial antigen 2 (NG2) expressed in hepatic pericytes, glutathione (GSH), and malondialdehyde (MDA) concentrations in liver; and serum levels of alanine aminotransferase and aspartate aminotransferase.

Results: GBI, PD, MPO, ABL, and histopathologic examinations demonstrated the development of periodontitis. There was a significant increase in microvesicular steatosis accompanied by a

marked reduction in NG2+ pericytes in the periodontitis group compared with the control group. The periodontitis group had significantly lower GSH and higher MDA concentration in the liver compared with the control group.

Conclusions: The present study results link the systemic effects of induced periodontitis with changes in hepatic tissues such as microvesicular steatosis, likely caused by an increase in oxidative stress and lipid peroxidation. The findings from the present study implicate an association between a decrease of pericytes and liver disease caused by ligature-induced periodontitis in rats.

Keywords

Fatty Liver; histology; non-alcoholic fatty liver; oral medicine; oxidative stress; risk factors

Periodontitis is an infectious disease of the oral cavity with a prevalence of 4.2% in Oceania to 20.4% in Latin America.¹ The disease destroys periodontal supporting and protecting tissues of the teeth.² This process is complex and involves several components such as a host response influenced by genetics, cells, extracellular matrix, overproduction of free radicals, deregulation of cytokine production, and presence of periodontal pathogens.³⁻⁵ Damage caused by periodontitis affects not only periodontal tissues, but also increases the severity of different illnesses such as rheumatoid arthritis,⁶ diabetes,⁷ and liver diseases.^{8,9}

Liver disease may be non-alcoholic fatty liver disease (NAFLD), characterized by fatty liver accumulation with a spectrum of liver damage ranging from simple steatosis to non-alcoholic steatohepatitis or severe cases of cancer.¹⁰ In Hong Kong, prevalence of NAFLD was 42.9%.¹¹ Exacerbating the situation, 55.7% of NAFLD patients had normal liver enzyme tests.¹¹ Steatosis is associated with metabolic syndrome in the general population and can be considered a multisystem disease associated with inflammation and overproduction of reactive oxygen species, whose byproduct is lipid peroxidation, such as malondialdehyde (MDA) and reduction of antioxidant molecules like glutathione (GSH).¹²

To evaluate liver disease, histologic analysis is the golden standard, which allows the detection of minimal changes in hepatic structure. Hepatic biology has shown the importance of mast cells that are increased in chronic C virus-related hepatitis.¹³ In addition, pericytes associated with portal vessels may be niches of cells with a high potential for cell differentiation, as has been demonstrated recently.¹⁴

Moreover, regarding liver disease, in previous years, studies^{8,9} have demonstrated an association with periodontitis. An animal study¹⁵ investigating this relationship used *Escherichia coli* lipopolysaccharide and *Streptomyces griseus* proteases applied into the gingival sulcus to induce periodontitis and the occurrence of steatosis. *Porphyromonas gingivalis*, an important periodontal pathogen, presented in the liver of patients with hepatic fibrosis and influenced progression of the liver disease.¹⁶

Other studies were conducted associating periodontitis with the progression of viral liver disease,⁸ with alcohol ingestion or not.¹⁷ Corroborating this relationship, recent studies have

demonstrated that periodontal therapy may decrease levels of oxidative stress^{5,18} in the periodontium, saliva, and crevicular fluid and also reduce liver fibrosis.⁸

Although there are studies showing effects of periodontitis on liver disease,^{8,9,15–18} there are few studies on oxidative hepatic stress,^{9,15} and none, to the best knowledge of the authors, investigated lipid peroxidation through MDA levels. Furthermore, and again according to the best knowledge of the authors, there are no studies investigating the influence of mast cells and pericytes on hepatic tissues associated with periodontitis. To elucidate the association, a rat periodontitis model was used in this study.

MATERIALS AND METHODS

Animals

Twenty 6-week-old adult female Wistar rats (*Rattus norvegicus*), weighing 152.8 ± 17.8 g, were used. The rats were maintained under standard conditions, with a 12-hour light-dark cycle, temperatures at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$, and free access to water and food. All animal treatments and procedures were in accordance with both the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, Maryland) and the guidelines of the Institutional Animal Ethics Committee (protocol number 0061/14).

Experimental Design

Rats were randomly divided into two groups ($n = 10$): 1) control (no ligature) and 2) periodontitis (ligated teeth). Periodontitis was induced under general intramuscular anesthesia by injection with a solution of 15 mg/kg of 2% xylazine hydrochloride[#] and 35 mg/kg of ketamine.^{**} The ligatures were placed on the first day of experiment, using nylon 3–0,^{††} around the cervical region of the mandibular right first molar of each rat and then tightly knotted.¹⁹ Nylon ligature was selected to be more resistant, thereby avoiding replacing the ligature during the experiment. Twenty days later, samples of peripheral blood were collected from the retroorbital plexus for biochemical examinations. Animals subsequently were sacrificed, and total weights, as well as liver weights, were measured.

Gingival Bleeding Index (GBI)

The periodontal pockets or gingival sulcus of the mandibular first molar were probed for 10 seconds and graded in accordance with Liu et al.²⁰ with scores of 0, 1, 2, 3, 4, and 5.

Probing Depth (PD)

PD was performed using a round-ended probe with a 0.4-mm-diameter tip. Three points (mesio-buccal, disto-buccal, and mid-buccal) were evaluated in the ligated mandibular right first molar, and the mean of the values was used.²⁰

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Myeloperoxidase (MPO) Activity

MPO activity was evaluated through neutrophil accumulation in the gingival tissue around the mandibular first molar. Briefly, 45 mg of gingival tissue was homogenized at 55 mg/mL in potassium buffer containing 0.5% hexadecyltrimethylammonium bromide. The homogenate was centrifuged at $39,000 \times g$ for 6 minutes at 4°C . The pellet was resuspended, and MPO activity was assayed by measuring the change in absorbance at 450 nm using *o*-dianisidinedihydrochloride and 1% hydrogen peroxide. MPO activity is reported as units/mg of tissue. A unit of MPO activity was defined by the conversion of 1 μmol hydrogen peroxide to water in 60 seconds at 23°C , as described by Chaves et al.²¹

Measurement of Alveolar Bone Loss (ABL)

To define the cemento-enamel junction (CEJ), mandibles were stained with aqueous methylene blue (1%) after the gingiva was dissected. A stereomicroscope equipped with a 30 \times objective was used to capture the image of alveolar bone height for each hemimandible. For evaluation of average alveolar bone height, three points were measured on the buccal and lingual parts. Measurements were made along the axis of the root²⁰ by measuring the height from the CEJ to the alveolar crest in: 1) ABL-1, the anterior portion (mesial) of the mandibular first molar; 2) ABL-2, the mesial root of the mandibular first molar; 3) ABL-3, the intermediate root of the mandibular first molar; and 4) ABL-4, the distal root of the mandibular first molar.

These images, as well as the histopathologic assessment, were measured using an image analysis system.^{‡‡}

Histopathologic Assessment of Gingival Tissue and Liver

Specimens were removed, cut into sections, and fixed in 10% buffered formaldehyde. After dehydration in increasing concentrations of alcohol and immersion in xylol, specimens were embedded in paraffin. The 6- μm -thick sections were stained with hematoxylin and eosin (H&E) as well as toluidine blue for mast cell density before assessment with light microscopy.^{§§}

The polymorphonuclear leukocytes (PMNs) and blood vessels were quantified in two typical zones (50 \times 50 μm each) of the connective tissue (CT) subjacent to the apical portion of the junctional epithelium (JE) under light microscopy^{|||} at 400 \times magnification. Additionally, the PMNs were counted in the JE, and its density per area was calculated in three sections (50 \times 50 μm each) according to Tomofuji et al.¹⁵

Histologic assessment of liver fat and inflammation was scored for steatosis (the percentage of liver cells containing fat): 1+: <25%; 2+: <50%; 3+: <75%; and 4: >75%.²² In addition, for density of mast cells (DMCs) the number of positive cells on toluidine blue/ mm^2 was counted.

^{‡‡}ImageJ v.1.48, US National Institutes of Health, Bethesda, MD.

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Immunohistochemistry for Pericytes

Pericytes appear as cells positive for neural/glial antigen 2 (NG2) expressed in hepatic tissues; NG2-positive cells were used to count the number of pericytes. After the previous routine histologic proceedings, sections were deparaffinized, and endogenous peroxidase activity was blocked with 4% hydrogen peroxide in water. After blocking non-specific reactivity with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), sections were reacted with each goat polyclonal primary antibody diluted in 1% BSA/PBS: antirat NG2,^{¶¶} 25 µg/mL, overnight at 4°C. After washing in PBS, sections were incubated with biotinylated secondary antibody,^{###} followed by streptavidin–peroxidase complex^{***} for 30 minutes at 37°C. Brown staining was made by treating sections with diaminobenzidine solution (3,3 diaminobenzidine),^{†††} and counter-staining was produced with hematoxylin. NG2 positive cells (pericytes) were counted in five sections (50 × 50 µm each). As a negative control, 1% BSA/PBS was used instead of primary antibodies.

GSH Levels

Liver samples were homogenized in 5 mL of cold 0.02-M EDTA solution (1 mL/100 mg tissue). Aliquots (400 µL) of tissue homogenate were mixed with 315 µL distilled water and 85 µL 50% (wt/vol) trichloroacetic acid in glass tubes and centrifuged at 3,000 rpm for 14 minutes. Four hundred microliters of each supernatant was mixed with 800 µL of Tris buffer (0.4 M, pH 8.8) and 25 µL 0.01-M 5,5-dithio-bis (2-nitrobenzoic acid). After shaking the preparation, absorbance was measured at 412 nm on a spectrophotometer. GSH concentration was determined via a reduced GSH standard curve, produced in parallel.²³

MDA Concentration

Fragments of hepatic tissue weighing from 90 to 160 mg were homogenized with cold 1.2% KCl to prepare 10% homogenates. In summary, 260 µL of each homogenate was added to 1.5 mL of 1% H₃PO₄ and 0.5 mL of 0.6% *tert*-butyl alcohol (aqueous solution). This mixture was agitated and heated, for 50 minutes, in a boiling water bath. The preparation was cooled instantaneously in an ice water bath, followed by addition of 3.5 mL of *n*-butanol.^{†††} This mixture was shaken, and the butanol layer was separated by centrifugation at 1,200 × g for 9 minutes. Optical density was determined to be 535 and 520 nm, and the optical density difference between the two determinations was calculated as the *tert*-butyl alcohol value.²⁴

Serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Determination

Serum levels of ALT and AST were measured with a commercial enzyme-linked immunosorbent assay kit.^{§§§}

¶¶Sigma-Aldrich, St. Louis, MO.

###LSAB2 system, DAKO, Glostrup, Denmark.

***LSAB2 system, DAKO.

†††LSAB2 system, DAKO.

†††Dinamica, Diadema, Brazil.

§§§Labtest, Lagoa Santa, Brazil.

Statistical Analyses

Data are expressed as mean \pm standard error of the mean (SEM) and/or median. Distribution of the data was tested through Shapiro–Wilk test. Differences between the two groups were analyzed using Mann–Whitney *U* tests for non-parametric data and unpaired Student *t* tests for parametric data.

RESULTS

GBI, PD, and MPO Activity

There were significant changes in the gingival tissues of the periodontitis group compared with the control group (Figs. 1A and 1B). Gingival papillae in the periodontitis group showed alterations in color, severe edema, ulceration, and intensive bleeding compared with controls. There were also significant differences in GBI scoring ($P=0.001$) (Fig. 1C).

Mean PD value for the periodontitis group was 2.7 ± 0.8 mm versus 0.5 ± 0.1 mm in the control group ($P=0.001$; Figure 1D).

MPO activity in the periodontitis group was twice that in the control group (6.1 ± 0.6 U/mg of tissue versus 2.7 ± 0.4 U/mg of tissue, $P<0.001$), shown in Figure 1E.

Histopathologic Assessment of Gingival Tissue

The control group exhibited healthy periodontal tissues (Fig. 1F). The periodontium of the periodontitis group revealed intense inflammatory cell infiltration within the JE and CT, as shown by H&E staining (Fig. 1G). In addition, the periodontitis group showed an increase in blood vessel density (Table 1 and Fig. 1G).

Measurement of ABL

Figures 2A and 2B represent the changes in alveolar bone in the control and periodontitis group, respectively. There are statistically significant differences ($P<0.05$) in the four parameters evaluated (ABL1: $P=0.002$; ABL2: $P=0.04$; ABL3: $P=0.001$; and ABL4: $P<0.001$) between the control and periodontitis groups (Fig. 2C).

Values for GBI, PD, MPO, and ABLs, as well as the histopathology of the gingival tissue, were strong evidence for the presence of induced periodontitis, enabling subsequent systemic evaluations.

Animal Total and Liver Weights

There was no significant difference in liver weight between the control and periodontitis groups ($P>0.05$), as shown in Table 2.

Histologic and Immunohistochemistry Assessment of Liver

Figure 3A shows the normal liver structure of the control group. Hepatic tissues in the periodontitis group showed a slight constriction of the central vein, partial loss of the radiating organization of hepatocytes in cordons, and degeneration and size variability of hepatocytes (Fig. 3B). Prevalence of steatosis in the periodontitis group, assayed by

histopathologic scoring, was significantly higher compared with the control group (Table 1). In addition, extensive microvesicular steatosis was seen around the central vein (Fig. 3B). There was no fibrosis in the portal and sinusoidal areas of animals. In addition, there was no difference ($P>0.05$) in DMCs in the periodontitis and control groups (Table 1, Figs. 3C and 3D). The immunohistochemistry assessment of the liver, shown in Figures 3E and 3F, demonstrated that the induced periodontitis was associated with a significant ($P<0.05$) reduction of NG2-positive cells (pericytes) (4.9 ± 0.37 , 1.2 ± 0.29 NG2-positive cells per $2,500 \mu\text{m}^2$ of the liver for control and periodontitis groups, respectively) compared with the control group (Table 1).

GSH and MDA for Liver

As shown in Figure 3G, induced periodontitis was associated with a significant ($P= 0.02$) decrease in liver GSH content (461.6 ± 38.1 and $186.8 \pm 76.7 \mu\text{g/g}$ of tissue for control and periodontitis groups, respectively) compared with the control group.

There was also increased lipid peroxidation in the liver, as shown by MDA levels in the periodontitis group compared with the control group ($128.3 \pm 14.4 \text{ mmol/g}$ of tissue versus $78.6 \pm 3.8 \text{ mmol/g}$ of tissue, $P= 0.006$) (Fig. 3H).

Serum ALT and AST

Serum levels of ALT (control group: $45.3 \pm 17.3 \text{ U/L}$; periodontitis group: $40.0 \pm 24.9 \text{ U/L}$; $P>0.05$), and AST (control group: $91.0 \pm 13.1 \text{ U/L}$; periodontitis group: $88.0 \pm 11.1 \text{ U/L}$; $P>0.05$) were not significantly different between the two groups.

DISCUSSION

In recent years there has been emerging evidence for an association between periodontitis and liver disease.^{8,9,15,25} It has been demonstrated that liver transplant candidates have significantly higher prevalence of apical periodontitis than healthy control patients.²⁶ Earlier data has suggested the inflammatory condition present in periodontal disease contributes to the release of inflammatory cytokines, such as interleukin (IL)-1b, IL-6, and IL-17a,^{27,28} and increases oxidative stress and lipid peroxidation,²⁹ not only in the periodontium, gingival fluid,³⁰ blood serum, and saliva,^{4,5} but also in the liver. These findings have been, however, largely based on association studies in vitro or indirect findings from unrelated disease models. Hence, the mechanism underlying the association between periodontitis and liver disease had remained unclear. In the present study, the effect of induced periodontitis on liver parameters was examined in an in vivo induced periodontitis model.

GBI, PD, MPO activity, ABL, and gingival histopathologic findings were all indicative of a robust model of periodontal disease. Using this animal model it was possible to demonstrate that induced periodontitis can result in microvesicular steatosis. The induced periodontitis was also associated with altered structural organization of hepatocytes in cordons and degeneration and size variability of the hepatocytes, similar to injuries observed in response to long-time exposure to commercial chemicals.³¹ These changes are considerable, given the short period of induction. There was no difference in DMCs between periodontitis and control groups. This is in contrast to a previous study that observed an increase in

DMCs in hepatic steatosis.¹³ Induced periodontal disease in the current model was also not associated with any alterations in body weights and liver function. Furthermore, some other features of non-alcoholic steatohepatitis,²² such as liver fibrosis or Mallory body formation, were absent in the current model. Overall, the hepatic histologic changes found in the periodontitis group were not severe. It is, however, conceivable that prolonged periodontitis could have potentially increased the risk for advanced liver disease.

Interestingly, the number of pericytes (NG2-positive cells) was decreased in the hepatic vasculature of the animals with induced periodontitis. To the best knowledge of the authors, this is the first study that demonstrates a correlation between pericyte number and steatosis induced by periodontitis. A reduction in pericytes in the kidney has been shown to be a critical step in the development of fibrosis.³² It has been suggested that NG2 positive cells may act as repository cells with great potential for differentiation.¹⁴ Similarly, findings from the current study suggest that reduction of hepatic pericytes is associated with microvesicular steatosis. Future studies are needed to clarify this association.

Liver diseases begin with accumulation of lipid into hepatocytes.^{12,15} These injuries may occur because of an overproduction of free radicals, nitrogen, and reactive oxygen species, commonly produced as byproducts of cell metabolism. Under physiologic conditions, GSH protects the liver from oxidative stress, whereas its depletion has been associated with lipid accumulation in hepatocytes.³³ In the current periodontal disease model, there was a significant reduction of GSH compared with control animals. This finding is consistent with a previous report,⁹ which showed induced periodontitis increases 8-hydroxydeoxyguanosine by lipid peroxidation. In addition, the levels of malondialdehyde, which disrupts liver GSH, were significantly increased in the liver of the animals with periodontitis.

MDA is one end-product of lipid peroxidation; thus, increased MDA content in the current periodontitis model compared with controls reflects increased oxidative stress. Moreover, it is well known that MDA plays a pivotal role in liver injury.^{12,33} Reduced GSH and increased MDA may, in part, explain changes observed in hepatic tissues in animals with periodontitis. Serum AST and ALT levels, two enzymes that indicate hepatocellular injury,³⁴ were not increased in mice with induced periodontitis. This result is consistent with previously published data.¹⁵

The treatment of periodontal disease also has been linked to favorable local and systemic effects. Accordingly, the therapy for periodontal disease has been shown to be associated with both reduction of oxidative stress in the periodontum^{5,18} and reduced liver fibrosis.⁸ Periodontitis may be an important risk factor for liver disease, but further studies in animals and humans are necessary to consolidate this hypothesis.

CONCLUSIONS

The current study results link the systemic effects of induced periodontitis with changes in hepatic tissues such as microvesicular steatosis, likely caused by an increase in oxidative stress and lipid peroxidation. The current findings implicate that a decrease of pericytes is associated with liver disease caused by ligature-induced periodontitis in rats.

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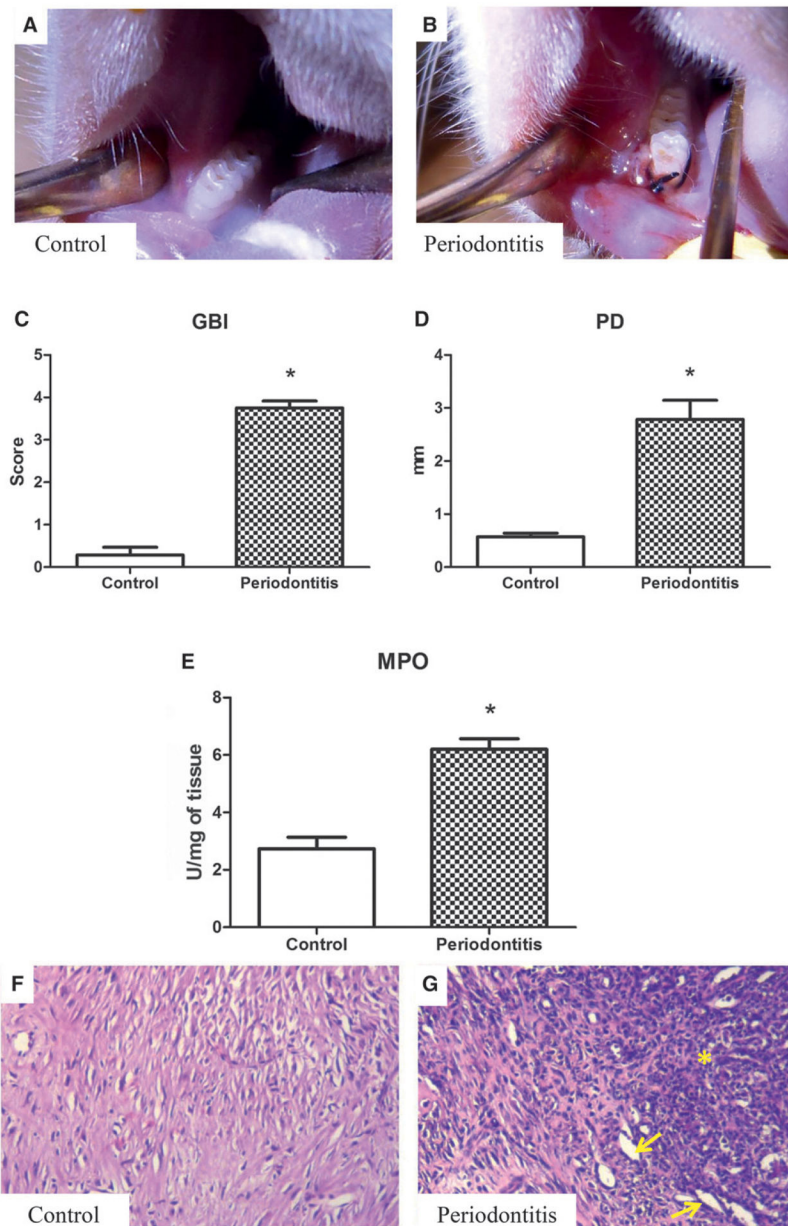


Figure 1. Clinical appearance of the control (A) and periodontitis (B) groups, with the latter showing changes in color, severe edema, ulcers, and bleeding after slight probing. C) GBI scores were significantly higher for the periodontitis group compared with the control group. D) PD is increased in the periodontitis group compared with the control group. E) MPO activity, with the periodontitis group showing significantly higher levels of MPO than the control group. C through E) Results are expressed as mean \pm SEM. * $P < 0.05$, periodontitis versus control group. F) Gingival tissue of the control group. G) Intense inflammatory infiltration of the PMNs (indicated by *) in the gingival CT, and increase in the blood vessel density (arrows). F and G) (H&E; original magnification $\times 300$.)

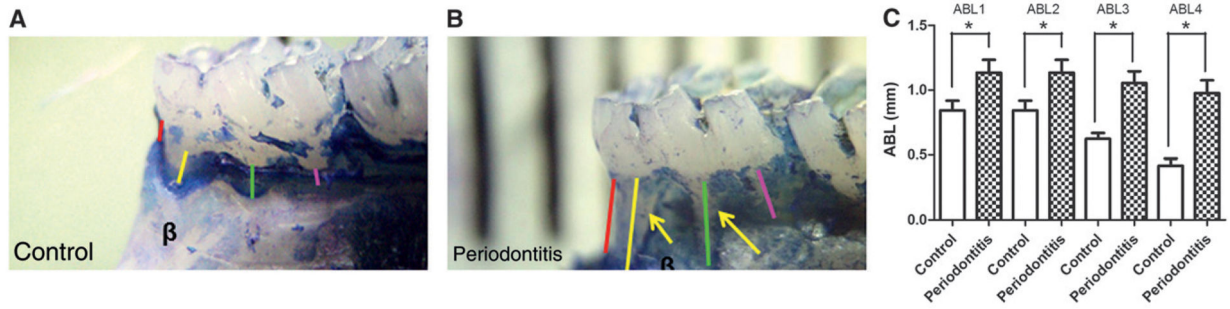


Figure 2.

Clinical and statistical differences between alveolar bone in control and periodontitis groups. **A** and **B**) The clinical difference between the alveolar bone (β) in the control and periodontitis groups, respectively; arrows indicate ABL. Red line = ABL1; yellow line = ABL2; green line = ABL3; pink line = ABL4. **C**) Illustration of the significant difference between the four evaluated sites (ABL1, ABL2, ABL3, and ABL4), comparing the periodontitis group with the control group, with an increase in ABL for the periodontitis group (ligated teeth). Results are expressed as mean \pm SEM. * $P < 0.05$, periodontitis versus control group.

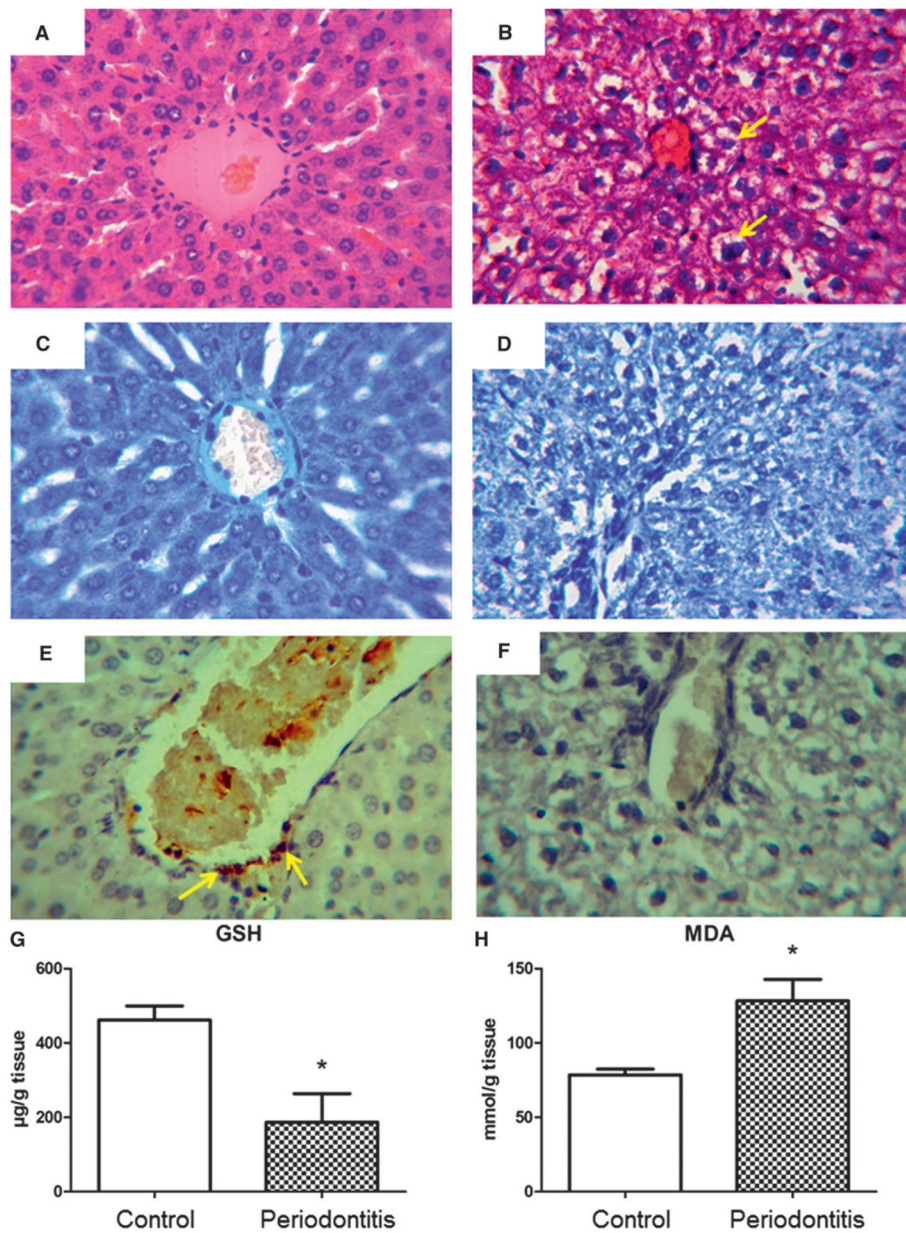


Figure 3.

A and C) Liver of the control group, which did not show histologic changes, and the normal architecture of hepatocytes radiating from the central vein. **B and D)** Liver of the periodontitis group, presenting a slight constriction of the central vein and partial loss of radiating organization of hepatocytes in cordons, degeneration, and size variability of the hepatocytes, as well as microvesicular steatosis (arrows) around the vein area. **A and B)** (H&E; original magnification $\times 600$.) **C and D)** (Toluidine blue; original magnification $\times 600$.) **E)** NG2-positive cells (pericytes; arrows) in control group. **F)** Reduction in hepatic pericytes in the periodontitis group. **E and F)** (Immunostaining; original magnification $\times 600$.) **G)** Liver GSH levels, with a significantly higher concentration for the control group compared with the periodontitis group. **H)** MDA liver concentration shows significantly

higher lipid peroxidation for the periodontitis group compared with the control group. G and H) Results are expressed as mean \pm SEM. *P <0.05, periodontitis versus control group.

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Table 1.

Histopathologic Assessment of Gingiva and Liver

Parameters	Control	Periodontitis
Gingival		
PMN density within the JE [†] (per 2,500 μm^2)	3.1 \pm 0.5	8.3 \pm 0.8*
PMN density within the CT [†] (per 2,500 μm^2)	2.1 \pm 0.6	9.5 \pm 0.6*
Blood vessel density [†] (per 2,500 μm^2)	1.3 \pm 0.3	3.8 \pm 0.7*
Liver		
Liver median score [‡]	0 (0–1)	3 (2–3)*
Mast cell density [†] (per 2,500 μm^2)	1.9 \pm 0.8	2.1 \pm 0.4
Pericytes [†] (equal to unity)	4.9 \pm 0.37	1.2 \pm 0.29*

Pericytes were counted by immunohistochemistry for NG2-positive cells. Results were expressed as mean \pm SEM (*t* test) or median and range (Mann–Whitney *U* test).

* Statistical difference ($P < 0.05$) between periodontitis and control groups.

[†] Unpaired *t* test.

[‡] Mann–Whitney *U* test.

Table 2.

Body Weight and Absolute and Relative Liver Weight

Group	Body Weight (g)	Liver Weight	
		Absolute (g)	Relative (%)
Control	192.6 ± 4.4	6.4 ± 0.9	3.35 ± 0.2
Periodontitis	189.3 ± 3.1	6.5 ± 0.8	3.49 ± 0.3

Relative weights are organ weight/body weight (g), mean of individual ratios ± SEM for values, measured at time of sacrifice. Comparison between the weights of organs in the control and periodontitis group showed no statistically significant difference in any parameter ($P>0.05$).

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