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Ethanol consumption enhances periodontal inflammatory markers in rats

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ABSTRACT

Objective: The aim of this study was to assess the short term effect of ethanol administration on periodontal disease in rats.

Design: Rats received either ethanol 2 g/kg or water by gastric gavage twice a day. On the fifth day ligatures were tied around the molars of half of the rats to induce periodontitis. After 7 days gingival tissue was removed and assayed for inflammatory markers. Finally, hemi-mandibles were extracted to evaluate bone loss by histomorphometrical techniques. **Results:** The experimental periodontitis increased significantly the mRNA expression ($p < 0.001$) and activity ($p < 0.001$) of inducible nitric oxide synthase (iNOS) in the gingival tissue, whilst short time ethanol administration increased iNOS activity ($p < 0.05$) and produced an additive effect on iNOS mRNA expression augmented by periodontitis ($p < 0.01$). The short time ethanol administration also potentiated the periodontitis stimulatory effect on the mRNA expression of interleukin (IL)-1 β ($p < 0.01$ and $p < 0.001$, in semi-quantitative and real time PCR, respectively) and on the height of periodontal ligament ($p < 0.05$). However, the ligature-induced periodontitis, but not ethanol administration, increased the prostaglandin E₂ content ($p < 0.05$) and, diminished the alveolar bone volume ($p < 0.05$), as compared to sham rats.

Conclusion: The present results suggest that ethanol consumption could represent a risk indicator for periodontal disease since augments the expression of inflammatory markers, in healthy rats, and increases them, at short term, during the illness. However, scale longitudinal investigation and more case-control studies are needed to confirm this statement.

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1. Introduction

Alcohol has been shown as one of the modulators of host defence. Chronic alcoholics are more prone to be infected with

a variety of pathogens due to its diminished ability to fight against infections. Although moderate alcohol intake reduces the production of pro-inflammatory cytokines by monocyte, such as tumour necrosis factor (TNF)- α , interleukin (IL)-1 and IL-6, possibly allowing microbial proliferation,¹ higher intakes

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produces more cytokine production.² Precisely, the monocytic release of these cytokines in the gingival crevice is associated with periodontitis.³

It is likely that bacteria contribute to the pathogenesis of periodontal disease directly by producing products that are toxic or harmful to surrounding cells. Furthermore, once the main elements of periodontal protection are overcome by the mechanism of bacterial virulence, begin the destructive processes mediated by the host itself. Bacterial lipopolysaccharides, are known to stimulate the production of cytokines and inflammatory mediators in turn to promote the release of metalloproteinase matrix from the host tissues, which are destructive to the extracellular matrix and alveolar bone.⁴ Interleukin 1 and TNF- α are found to be associated with periodontitis, enabling the ingress of inflammatory cells into sites of infection, promotes bone resorption and stimulates eicosanoid release, specially prostaglandin E₂, by monocytes and fibroblast.^{5,6} An increased concentration of prostaglandin E₂ has been found in gingival crevicular fluid of patients with periodontitis,⁷ and in periodontal tissue following stimulation with interleukin IL-1.⁸ Moreover, prostaglandin E₂ is considered a potent stimulator of bone resorption associated with loss of periodontal attachment tissue.⁹ Although IL-1 β was reported as the main cytokine involved in periodontal disease,¹⁰ IL-6 and TNF- α are also increased in this pathology.¹¹

Nitric oxide derived from inducible nitric oxide synthase (iNOS) is known to play an important role in host defence, as well as in inflammation induced tissue lesions. It has been shown to have important effects on several inflammatory events, including the cell migration, observed in periodontitis.¹² Several reports have shown that nitric oxide may promote osteoclast maturation and enhance bone resorption induced by cytokines.^{13,14}

Rats have been used as experimental model to assess the influence of potential risk indicators¹⁵ and the effect of different treatments on periodontal disease.¹⁶ Ethanol intake is associated with deleterious effects in different organs of the body and a recent report demonstrated that its chronic consumption increased periodontal inflammation.¹⁷ Therefore, the aim of the present paper was to assess the short term effect of ethanol administration on the oral health of rats subjected or not to experimental periodontitis.

2. Materials and methods

2.1. Animals

Wistar male adult rats (250 g) from our own colony were kept in group cages in an animal room having a photoperiod of 12 h of light (0700 to 1900), room temperature at 22–25 °C and free access to rat chow and tap water. The experimental procedures performed were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Council of Scientific and Technical Research of Argentina and were carried out in accordance with the guidelines of the National Institute of Health (NIH).

2.2. Experimental design

The rats were divided in four groups (6 rats per group): (1) rats receiving water (1.5 ml/100 g of body weight) by gastric gavage twice a day (at 12 pm and 18 pm) during 12 days; (2) rats receiving ethanol 2 g/kg (4.5 ml of ethanol 25%) by gastric gavage twice a day in the conditions described above; (3) rats submitted to experimental periodontitis and receiving water as described for group 1; and (4) rats submitted to experimental periodontitis and receiving ethanol as described for group 2. All experiences and assays were performed, at least, three times.

2.3. Induction of periodontal disease

The rats were anaesthetized by intramuscular administration of 2% xylazine hydrochloride (König Laboratories SA, Buenos Aires, Argentina) (5 mg/kg body weight) and ketamine hydrochloride (Holliday-Scott SA, Buenos Aires, Argentina) (50 mg/kg body weight). The bilateral periodontitis was induced as described by Vacas et al. by placing a cotton thread ligature around the neck of lower first right and left molars. Rats without ligature were used as controls.¹⁸ The cotton thread ligature was pushed into the gingival sulcus and was left in place until the sacrifice (7 days post surgery). At the end of these periods, the gingival tissue of the first lower molars and the hemi-mandibles were extracted for quantification of inflammatory markers and alveolar bone loss, respectively.

2.4. Measurement of iNOS activity

The activity of inducible NOS (iNOS) was measured by modifying the method of Bredt and Snyder.¹⁹ In brief, gingival tissue was homogenized in 500 μ l of ice-cold 20 mM HEPES (pH 7.4) with EGTA (2 mM) and DL-dithiothreitol (DTT, 1 mM). After the tissue was homogenized, NADPH (120 μ M) and 200,000 dpm of (¹⁴C)-arginine (297 mCi/mmol, Perkin-Elmer, Waltham, MA, USA) were added to each tube and incubated for 10 min at 37 °C in a Dubnoff metabolic shaker at 37 °C. Then, the tubes were centrifuged at 10,000 \times g for 10 min at 4 °C. The supernatants were apply to individual columns containing 1 ml of Dowex AG 50 W-X8 Na⁺ form, and washed with 2.5 ml of double distilled water. All collected effluent fluid from each column were counted as activity of (¹⁴C)-citrulline in a scintillation counter. Since NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as nmol of NO produced per min per mg of protein.

2.5. Radioimmunoassay

The determination of corticosterone plasma level was performed by RIA as described Etches et al. with minor modifications.²⁰ The samples were incubated in buffer [0.05 M Tris HCl containing 0.1 M NaCl, 0.1% BSA, and 0.1% sodium azide (pH 8)] with rabbit anti-corticosterone antibody (Sigma Chemicals, St. Louis, MO, USA) for 30 min at room temperature. Then, [³H]corticosterone (Perkin-Elmer, Waltham, MA, USA) was added as a tracer and incubated for 1 h at 37 °C. The reaction was stopped by the addition of cold dextran-coated charcoal suspension and after the incubation for 10 min at

0 °C; the tubes were centrifuged at 2000 × *g* for 15 min at 4 °C. The scintillation mixture was added to the supernatant, and the amount of radioactivity was determined in a beta counter. The sensitivity of the assay was 30 pg per tube.

To determine prostaglandin E₂ content, the gingival tissue was homogenized in 500 µl of absolute ethanol and after centrifugation, the supernatant was dried in a Speedvac at room temperature. The residues were resuspended with buffer afterward; antiserum from Sigma was used as described in Mohn et al.²¹ The sensitivity of the assay was 12.5 pg per tube. The crossreactivity of PGE₂ and PGE₁ was 100%, but the crossreactivity of other prostaglandins was 0.1%. The intra- and interassay coefficients of variation for PGE₂ were 8.2% and 12%, respectively.

2.6. RNA isolation and reverse transcription

Gingival fragments from treated and untreated rats were homogenized in 0.5 ml of Trizol[®] (Invitrogen, CA, USA). Total RNA was extracted according to manufacturer recommendations, dissolved in RNA storage solution (Ambion, TX, USA), UV quantified by a Nano Drop[®] (Eppendorf, Hamburg, Germany), and stored to –80 °C. RNA aliquots (4 µg) were digested by RNase-free DNase I (Invitrogen deoxyribonucleic acid [DNA]-free[™] kit) in a 20-µl final volume reaction mixture, to remove contaminating genomic DNA. After DNase digestion, concentration and purity of RNA samples were evaluated by the RNA-6000-Nanodrop[®], using a 2100 Bioanalyzer[®] equipped with a 2100-Expert-Software[®] (Agilent Technologies Inc., CA, USA), following the manufacturer instructions. For all samples tested, the RNA integrity number was greater than 6 (relative to a 0–10 scale). Four micrograms of total RNA were retro-transcribed in a 25-µl reaction mixture containing: 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM deoxyribonucleotide triphosphate, 20 U of RNase inhibitor, 0.125 A260 units of hexanucleotide mixture for random priming and 200 U of MoMuLV Superscript[®] III reverse transcriptase (Invitrogen, CA, USA). The reaction mixture was incubated in a TPersonal thermocycler (Biometra[®], Goettingen Germany) for 2 min at a 37 °C step, followed by a rapid chilling for 2 min at 4 °C. The protocol was stopped at this step and the MoMuLV reverse transcriptase was added to the samples, excepting the negative controls (–RT). The incubation was resumed by three thermal steps: 10 min at 25 °C followed by 50 min at 37 °C. Finally, the reaction was terminated by heating at 70 °C for 15 min.

2.7. iNOS and IL-1β mRNA expression by semi-quantitative RT PCR analysis

2.5 µl of each cDNA sample was mixed with 5 µl nuclease free water, 2.5 µl sense primers, 2.5 µl antisense primers and 12.5 µl Gotaq (Promega), and amplified in the TPersonal thermocycler. To determine the optimal conditions, allowing the signal to be in the linear portion of the amplification curve, experiments were performed under conditions in which cycle number and template's concentrations were altered. A negative control lacking template RNA or reverse transcriptase was

included in each experiment. Aliquots of PCR products were run on a 2% agarose gels containing ethidium bromide and visualized under UV light (UVP Systems).

2.8. IL-1β mRNA expression by quantitative (real-time) RT PCR (qPCR) analysis

Quantitative real-time PCR was performed by CR Corbett Research Cycloer[®] in a 25-µl reaction mixture containing: 1 × SYBR[®]-Green-Supermix (Bio-Rad), 20 ng of complementary DNA (cDNA; calculated on the basis of the retro-transcribed RNA), and 330 nM for each primer. The amplification profile consisted of an initial denaturation of 2 min at 94 °C and 40 cycles of 30 s at 94 °C, annealing for 30 s at optimum annealing temperature and elongation for 45 s at 68 °C. The data of fluorescence were collected during the elongation step. A final extension of 7 min was carried out at 72 °C, followed by melt-curve data analysis. Optimized primers for SYBR[®]-Green analysis were designed by the REST[®] 08 Rotor-Gene 6000 software 1.7 version (Corbett Life Science, Qiagen Ltd., UK) and were synthesized (high-performance liquid chromatography purification grade) by Invitrogen, CA, USA. Assays were performed in triplicate (maximum ΔCt of replicate samples less than 0.5), and a standard curve from fivefold consecutive dilutions (100–0.10 ng) of a cDNA pool, representative of all samples was included, for PCR efficiency determination. A relative expression analysis was corrected by PCR efficiency and was normalized with respect to reference gene β-actin, was performed by REST[®] 08 for group wise comparison and statistical analysis. The following primers were used in sqRT-PCR and/or in qRT-PCR:

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iNOS
Sense, 5'-ACC ACT CGT ACT TGG GAT GC-3'
Anti-sense 5'-CAC CTT GGA GTT CAC CCA GT-3'
IL-1β
Sense, 5'-TGA GTG ACA CTG CCT TCC TG-3'
Anti-sense, 5'-AGG CTT CCT TGT GCA AGT GT-3'
β-Actin
Sense, 5'-AGC CAT GTA CGT AGC CAT CC-3'
Anti-sense 5'-CTC TCA GCT GTG GTG GTG AA-3'
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2.9. Histomorphometric evaluation of alveolar bone loss and periodontal ligament

After sacrifice, hemi-mandibles were extracted and fixed in formalin buffer. Two days later, they were decalcified in 10% EDTA pH 7, for 25 days. After this period, hemi-mandibles were dehydrated with ethanol and were clarified with xylol. Finally, the sector containing the three lower molars of each decalcified hemi-mandible was embedded in paraffin at 56–58 °C. Under a stereomicroscope (Stemi DV4 Stereomicroscope, Carl Zeiss MicroImaging, Göttingen, Germany) and by using a microtome (Jung AG, Heidelberg, Germany), sections oriented meso-distally of each first lower molar were obtained from paraffin blocks. Sections 7 µm in width were stained with haematoxylin and eosin, and histomorphometrical evaluation was performed on digitized microphotographs using imaging software (Image Toll, University of Texas Health Science Center at San Antonio, San Antonio, TX). The following static parameters were evaluated on the interradicular bone: (1)

bone volume (BV)/total volume (TV) (%) = fraction of TV corresponding to bone tissue; total volume was taken as bone tissue plus bone marrow and periodontal ligament, and (2) height of the periodontal ligament (in micrometres); to measure the height of periodontal ligament, ten equidistant points were marked on alveolar crest on the interradicular bone, and a line was drawn from each of the points to the bone. The length of the lines was measured, and the mean value was calculated to obtain the height of the periodontal ligament of each section.¹⁸

2.10. Statistic

Data are expressed as means \pm SEM. The results were evaluated by Two Way ANOVA followed by the Tukey multiple comparison test for unequal replicates. All analyses were conducted with the Prism software (GraphPad Software, Inc.). Differences with p values <0.05 were considered statistically significant.

3. Results

3.1. Plasma corticosterone

Rats submitted to ethanol administration showed increased plasma corticosterone concentration, around of 100% when compared to rats receiving water (from 26.4 ± 4.1 ng/ml to 59.9 ± 11.0 ng/ml in sham rats, $p < 0.05$; and from 35.7 ± 6.8 ng/ml to 71.1 ± 16.3 ng/ml in rats with periodontitis, $p < 0.05$), whilst ligature-induced periodontitis did not modify significantly plasmatic corticosterone, neither when was applied alone nor when was applied concomitantly with ethanol administration, compared with respective controls without periodontitis (Fig. 1).

3.2. Inducible NOS mRNA quantification and protein activity

The ligature-induced periodontitis increased iNOS mRNA expression in gingival tissue when compared with rats

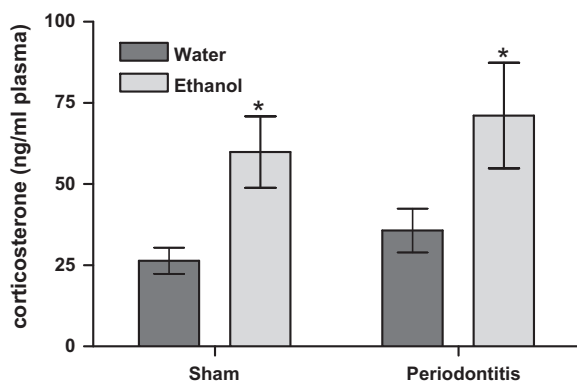


Fig. 1 – Effect of ethanol short administration, periodontitis induction and the combination of both on plasma corticosterone concentration. Data are reported as means \pm SEM (6 rats per group). * $p < 0.05$ versus water (two way ANOVA followed by Tukey's post-test).

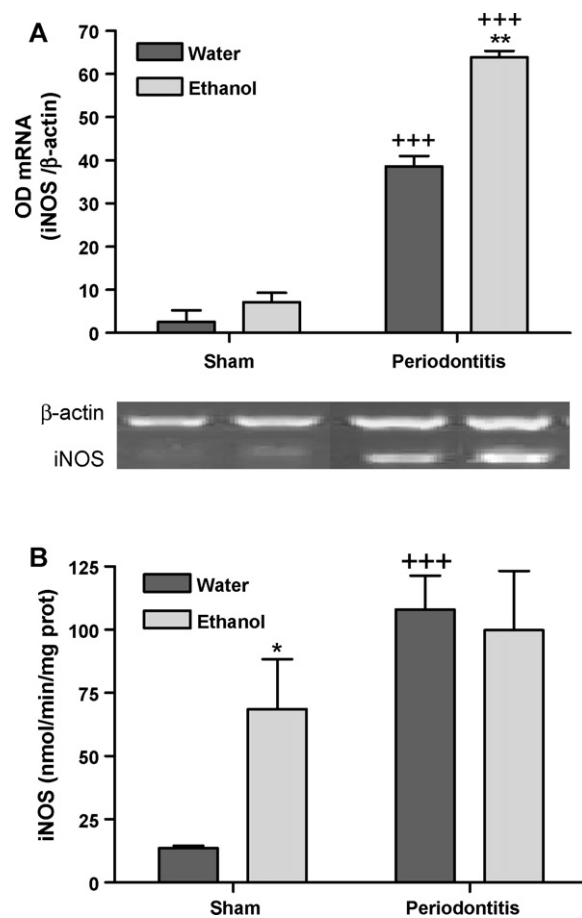


Fig. 2 – Effect of ethanol short administration, periodontitis induction and the combination of both on (A) relative expression of iNOS mRNA, analysed by RT-PCR and (B) iNOS activity in gingival tissue. Data are reported as means \pm SEM (6 rats per group). * $p < 0.05$, ** $p < 0.01$ versus water, ⁺⁺⁺ $p < 0.001$ versus Sham (two way ANOVA followed by Tukey's post-test).

without periodontitis ($p < 0.001$). Additionally, ethanol administration in rats with periodontitis has produced a significant additive effect on its expression ($p < 0.01$) (Fig. 2A). Also, both periodontitis and ethanol administration increased iNOS activity (from 13.6 ± 0.9 nmol NO/min/mg prot in sham rats drinking water to 68.6 ± 19.8 in sham rats with ethanol administration, $p < 0.05$; and to 108.0 ± 13.4 in rats with periodontitis drinking water, $p < 0.001$) (Fig. 2B). However ethanol administration did not show additive effect on periodontitis-increased iNOS activity.

3.3. IL-1 β mRNA quantification

The experimental periodontitis augmented the relative expression of IL-1 β mRNA, analysed by semi-quantitative RT-PCR (sq-PCR) in gingival tissue extracted 7 days post induction ($p < 0.05$). In rats with periodontitis, ethanol administration has produced high significant additive effect on its expression ($p < 0.01$) (Fig. 3A). Moreover, this result was confirmed by quantitative real time RT-PCR (q-PCR) analysis, where the

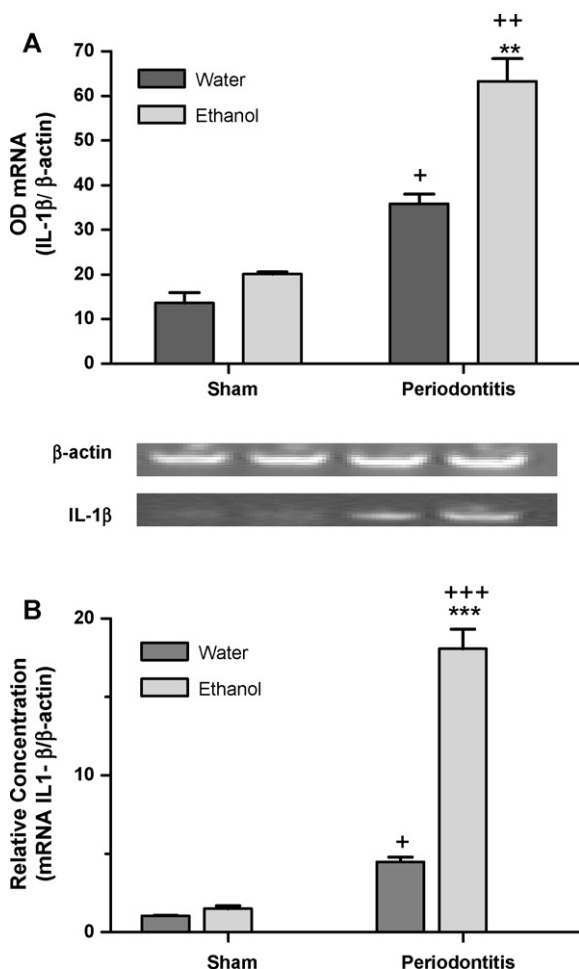


Fig. 3 – Effect of ethanol short administration, periodontitis induction and the combination of both on IL-1β mRNA expression, analysed by (A) semi-quantitative RT PCR (sqPCR) and by (B) quantitative real time RT PCR (q-PCR) in gingival tissue. Data are reported as means ± SEM (6 rats per group). *p* < 0.01, ****p* < 0.001 versus water, +*p* < 0.05, ***p* < 0.01 and ****p* < 0.001 versus Sham (two way ANOVA followed by Tukey’s post-test).**

effect of ethanol administration showed a potentiation of periodontitis-stimulatory effect on IL-1β mRNA relative expression (*p* < 0.001)(Fig. 3B).

3.4. Prostaglandin E₂ content

Prostaglandin E₂ content was significantly higher in the gingival tissue extracted from rats with the ligature-induced periodontitis as compared to rats without ligature (from 234.0 ± 50.0 pg/mg tissue to 933.8 ± 277.6, *p* < 0.05). However, ethanol administration did not alter PGE₂ content neither in rats without ligature nor in rats with the ligature-induced periodontitis (Fig. 4).

3.5. Bone loss

Histomorphometrical evaluation of interradicular bone showed significant bone resorption in rats with the ligature-induced

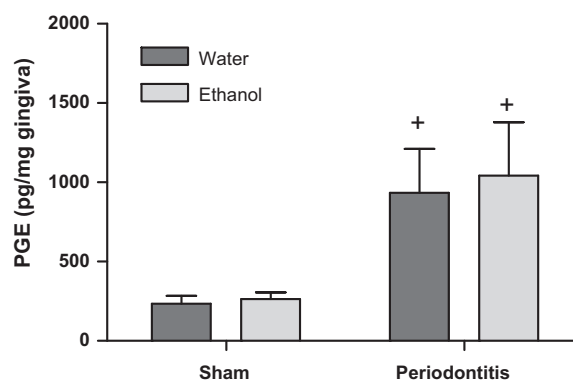


Fig. 4 – Effect of ethanol short administration, periodontitis induction and the combination of both on PGE content in gingival tissue. Data are reported as means ± SEM (6 rats per group). +*p* < 0.05 versus Sham (two Way ANOVA followed by Tukey’s post-test).

periodontitis as revealed the lower alveolar bone volume (*p* < 0.05 in rats drinking water and *p* < 0.01 in rats with ethanol administration) (Fig. 5A and B) and the periodontal ligament’s higher height comparing to sham rats (404.9 ± 29.7 mm in sham rats and 594.4 ± 36.9 mm in rats with periodontitis drinking water, *p* < 0.05) (Fig. 5A and C). Moreover, rats with periodontitis that received ethanol showed higher ligament height (929.3 ± 145 mm) than rats with periodontitis that received water (*p* < 0.05), but these differences were not observed by analysing alveolar bone volume.

4. Discussion

Our results demonstrated that short time ethanol administration increased the expression of parameters linked to periodontal breakdown in the gingival tissue of rats. Additionally, its administration increased the length of the periodontal ligament in rats submitted to ligature induced periodontitis, which suggest that alcohol consumption promotes the progression of this disease in the short term.

The present results showed short term additive effects of ethanol on IL-1β and iNOS mRNA expression induced by periodontitis. Moreover, iNOS activity was increased after short time ethanol administration. Accordingly, it was reported that chronic alcohol consumption also increases periodontal inflammation, oxidative damage, and cytokines production, increasing the severity of periodontal inflammation in the ligature model.¹⁷

The increase in plasma corticosterone concentration observed in rats that consumed ethanol for 12 days, agrees with the literature reports describing that chronic consumption of ethanol produces a sustained increase in stress hormones, such as corticosterone and epinephrine.^{22,23} However, the ligature-induced periodontitis during 7 days did not alter neither the basal plasma corticosterone, nor the induced by ethanol.

In concordance with previous reports,^{12,24} we observed that rats subjected to bilateral periodontitis showed higher prostaglandin E₂ content, higher relative expression of iNOS

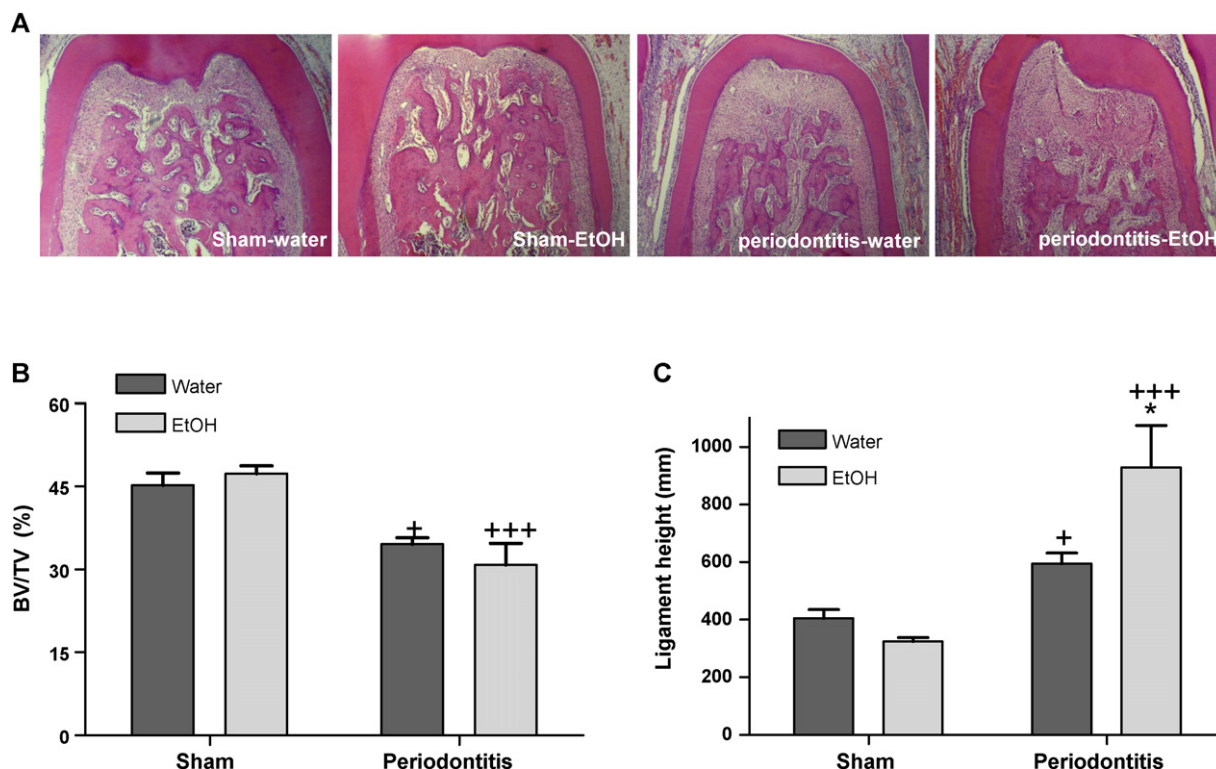


Fig. 5 – Effect of ethanol short administration, periodontitis induction and the combination of both on static histomorphometry of interradicular bone. (A) Microphotographs showing interradicular bone in all groups (haematoxylin and eosin; original magnification 50 \times), (B) results of bone volume/total volume and (C) results of ligament height. Data are reported as means \pm SEM (6 rats per group). * p < 0.05, versus water, † p < 0.05 and +++ p < 0.001 versus Sham (two way ANOVA followed by Tukey's post-test).

mRNA and of protein activity, as well as higher relative expression of IL-1 β mRNA in the gingival tissue, as compared to controls. Moreover, the ligature-induced periodontitis produced higher alveolar bone loss, as revealed by histomorphometrical techniques, comparing to sham rats. The increase of IL-1 β mRNA expression and prostaglandin E₂ content in the gingival tissue after periodontitis induction agrees with previous reports demonstrating that gingival fibroblasts treated with LPS of *Porphyromonas gingivalis* show increments in the release of IL-1 β and prostaglandin E₂.²⁵

Also, it was reported that ligature-induced periodontitis causes other kind of deleterious effects related to oxidative stress, such as increase of plasma lipid peroxide and augmentation of ethanol-induced lipid peroxidation in the liver.²⁶

The literature shows conflicting results regarding the effects of alcohol consumption vary according to time of administration and dose used in each experimental model. It was reported that a daily liquid diet of ethanol 20% during 8 weeks did not alter bone loss in rats without ligature, but increased significantly bone loss in rats submitted to periodontitis from the fourth week of alcohol diet until the end of the experiment.¹⁵ A recently published epidemiological work in humans concludes that occurrence of periodontitis amongst alcohol users was high and the frequency of alcohol consumption increased the odds of periodontitis.²⁷ On the other hand, it was reported that low concentration alcohol intake (5%) inhibited spontaneous alveolar bone loss but did

not affect the alveolar bone loss in ligature induced periodontal breakdown.²⁸ Although our results showed that ethanol consumption increased distinct inflammatory markers induced by periodontitis and periodontal ligament height augmented by periodontitis, not changes were showed on alveolar bone volume nor in gingival content of prostaglandin E₂, both increased by periodontitis. Therefore, short term effects of ethanol (20%) are enough to increase the inflammatory process, and advance the onset of bone loss processes; but should be insufficient to show the additive effects on bone loss induced by periodontitis as observed by chronic ethanol administration.¹⁵ Taking into account our results and those consulted in the literature, we conclude that the effect of ethanol intake on periodontal breakdown depends on the dose and time of consumption.

Needless to say, that unlike the experiments described previously, our experimental design do not contemplates local effects of alcohol in the oral tissues since it is administrated in the pit of the stomach, directly.

In summary, the present study suggests that short time ethanol consumption may promote an inflammatory state in the gingival tissues, especially in rats with the ligature-induced periodontitis. Twelve days of ethanol consumption seems to be not enough time to produce additive significant effects on the ligature-induced alveolar bone loss, however, proved to be enough time to start exercising additive effects on the resorptive processes induced by periodontitis. In order to

establish the effect of alcohol as a risk factor for periodontal disease, larger scale longitudinal and case–control studies are needed to confirm the relationship between alcohol and periodontitis.

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Competing interests

None declared.

Ethical approval

Not required.

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