Chronic Alcohol Consumption Alters Periodontal Health in Rats

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Background: The aim of this study was to assess the effects of chronic alcohol consumption on periodontitis development in rats.

Methods: Periodontal disease was experimentally induced by lipopolysaccharide (LPS; 2 mg/ml) injections into the gingival tissue around first upper and lower molar's neck, and into the interdental space between first and second molars. This protocol was repeated for 6 weeks on days 1, 3, and 5 of each week. Chronic alcohol consumption was induced by 20% ethanol (EtOH) as the only liquid source during 4 months.

Results: Chronic alcohol consumption by itself increased alveolar bone loss and biological mediators of periodontal disease such as prostaglandin E_2 (PGE_2) content on gingival tissue, and inducible nitric oxide synthase activity plus PGE_2 content in submandibular gland. Unexpectedly, alcohol consumption did not increase the damage evoked by the proved model of LPS injections for periodontitis induction.

Conclusions: Results suggest 20% alcohol consumption during 4 months generates differential effects on oral health of rats, depending on its pathophysiological state: It would exacerbate the inflammatory condition when periodontal damage is absent, but it would not when damage is installed.

Key Words: Alcohol Consumption, Periodontal Damage, Alveolar Bone Loss, Inflammatory Markers.

PERIODONTITIS IS AN oral disease comprising a group of inflammatory conditions affecting dentition's supporting structures, including periodontal ligament, alveolar bone, and gingival tissues (Giannobile et al., 2009). The host response plays a critical role in periodontal tissue breakdown, while the presence of periodontal pathogens is required but not sufficient for their commencement (Graves et al., 2011). Lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria (Medzhitov and Janeway, 1997), stimulating host cells to produce various pro-inflammatory cytokines: tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6). It also stimulates production of inflammatory mediators, such as prostaglandin E_2 (PGE_2) and nitric oxide (NO) (Graves et al., 2011; Ulevitch and Tobias, 1995), promoting metalloproteinase matrix release from host tissues, which is destructive for the extracellular matrix and alveolar bone (Birkedal-Hansen, 1993). LPS is thought to be a harmful factor in periodontitis, and it is known for its contribution to alveolar bone loss by enhancing osteoclastogenesis via CD14 protein and Toll-like receptor-4 expressed on target membranes (Palsson-McDermott and O'Neill, 2004). Soft and hard oral tissue maintenance is a complex biological process influenced primarily by the availability and chemical make-up of saliva (Slomiany et al., 1996). Submandibular gland (SMG) is one of the major salivary glands, together with the parotid and sublingual glands. While under physiological conditions, the protective potential is sufficiently maintained by salivary flow; this state seems to be disturbed in periodontitis. It has been demonstrated that in this disease, SMG salivary secretion diminishes and changes its composition (Amer et al., 2011).

Alcohol is historically the most commonly abused substance that maintained popularity from ancient Mesopotamian times to the 21st century. Although there is no formally accepted definition of alcoholism, the term is associated with alcohol dependence, which is a failure to control alcohol use and, in general, compulsive use of alcohol. The classic chronic disease resulting from alcoholism is cirrhosis of the liver, typically preceded by steatosis (Lieber, 1984). Chronic

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alcoholics are more prone to be infected with a variety of pathogens due to its diminished ability to fight against infections. Studies demonstrated that higher intake of alcohol induces cytokine production (Szabo and Mandrekar, 2009). Coincidentally, monocytic release of cytokines in gingival crevice is associated with periodontitis (Offenbacher, 1996). Ethanol (EtOH) intake is also associated with deleterious effects in different organs of the body, and recent reports demonstrated that its consumption increases periodontal inflammation (Dantas et al., 2012; Irie et al., 2008) and alters the normal function of the SMG (Prestifilippo et al., 2009; Proctor and Shori, 1995). Human studies evaluating the effects of alcoholism on oral tissues suggested that it may be associated with a greater risk for development of periodontal problems due to poor oral hygiene (Hornecker et al., 2003). However, there is evidence that persistent alcohol abuse affects periodontal disease's severity. Accumulating data of animal models indicate that chronic EtOH consumption leads to a marked enhancement in oral epithelial cell apoptosis triggered by changes in oral mucosal expression of TNF-α (Slomiany et al., 1997), and NO (Slomiany et al., 1998). Briefly, considering the scarce information available about alcoholism effects on alveolar bone loss and inflammatory markers in periodontitis, the aim of this study was to evaluate the effect of chronic alcohol consumption on the development of the periodontal disease.

MATERIALS AND METHODS

Animals

Wistar male adult rats (350 g) from our own colony were kept in group cages in an animal room having a photoperiod of 12 hours light (0700 to 1900), room temperature of 22 to 25°C and free access to rat chow and tap water until the beginning of the experiment. The experimental procedures were approved by the Animal Care Committee of the Dental School of the University of Buenos Aires, Argentina, and were carried out in accordance with the guidelines of the National Institutes of Health.

Experimental Design

Rats were divided into 6 groups (8 rats per group): (i) control rats receiving water as the only liquid source during 4 months; (ii) control rats receiving EtOH 20% (v/v) as the only liquid source during 4 months; (iii) rats receiving water as described for group 1 and gums injected with saline (vehicle of LPS); (iv) rats receiving EtOH as described for group 2 and gums injected with saline; (v) rats receiving water as described for group 1 and submitted to experimental periodontitis (gums injected with LPS); (vi) rats receiving EtOH as described for group 2 and submitted to experimental periodontitis. All experiences and assays were performed, at least, 3 times.

EtOH-treated rats received an aqueous EtOH solution as their only available liquid source for 4 months, based on previous works (Silva and Madeira, 2012; Silva et al., 2009). EtOH concentration was progressively increased, starting with a 5% (v/v) solution and rising by 1% per day to a final 20% (v/v) 2 weeks later. Food was freely available throughout the experiment.

Periodontitis was induced by injecting 10 μl of LPS (from *Escherichia coli* [serotype 055-B5]; Sigma-Aldrich, St. Louis, MO), 2 mg/ml, into the vestibular and oral gingival tissue around first

upper and lower molar's neck, and into the interdental space between the first and second upper and lower molars, under a CO_2 atmosphere. This protocol of injections was repeated for the last 6 weeks of the experiment to the rats of groups 5 and 6, on days 1, 3, and 5 of each week, based on a previously described method (Llavaneras et al., 2001; Ossola et al., 2012). Animals from groups 3 and 4 received injections of the saline solution (vehicle of LPS) in the same manner, while control rats (groups 1 and 2) received no injections throughout the experiments. Gingival injections were given with a 13-mm 27G microfine syringe. After the experimental period of 4 months, rats were sacrificed by decapitation; trunk blood was collected and centrifuged; SMG, liver, and gingival tissues were dissected from the neck of the first molar; and hemimandibles were immediately extracted.

Histological Analyses

Hemimandibles were extracted and fixed in formalin buffer. Three days later, they were decalcified in 10% EDTA pH 7, for 45 days. After this period, hemimandibles were dehydrated with EtOH and clarified with xylene. Finally, the sector containing the first molar of each decalcified hemimandible was embedded in paraffin at 56 to 58°C. Under a stereomicroscope (Stemi DV4 Stereomicro-scope; Carl Zeiss MicroImaging, Göttingen, Germany) and using a microtome (Jung AG, Heidelberg, Germany), sections oriented meso-distally of each first lower molar were obtained from paraffin blocks. Sections 7 mm in width were stained with hematoxylin 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: (i) bone volume (BV)/total volume (TV) (%) = fraction of TV corresponding to bone tissue; total volume was taken as bone tissue plus bone narrow and periodontal ligament, and (ii) height of the periodontal ligament (in μm); to measure the height of periodontal ligament, 10 equidistant points were marked on alveolar crest (AC) 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 (Vacas et al., 2008).

Microscopic Examination of Periodontal Bone Loss: Distance Method

Immediately after euthanasia, hemimandibles were resected and stained with 1% aqueous methylene blue to delineate cemento–enamel junction (CEJ) and AC (Crawford et al., 1978). A stereomicroscope (Stemi DV4 Stereomicroscope; Carl Zeiss MicroImaging) and a digital caliper (Digimess, Geneva, Switzerland) were used to measure 3 lingual/palatal distances (mesial, central, and distal) from the CEJ to the most apical area of the AC (Vacas et al., 2008). The sum of the 3 distances of each molar was used as a measure of the alveolar bone loss in millimeters.

Radioimmunoassay of PGE$_2$

To determine PGE$_2$ content, gingival tissue and SMG were homogenized in 500 μl of absolute EtOH, and, after centrifugation, supernatant was dried in a Speedvac at room temperature. The residues were then re-suspended with buffer. Antiserum (Sigma-Aldrich) was used as described in Mohn and colleagues (2011). The sensitivity of the assay was 12.5 pg per tube. The cross-reactivity of PGE$_2$ and PGE$_1$ was 100%, whereas cross-reactivity with other prostaglandins was 0.1%. The intra- and interassay coefficients of variation for PGE$_2$ were 8.2 and 12%,

respectively. Results were expressed in pg and fg of PGE per mg wet weight for gums and SMG, respectively, as the protocol of PGE extraction from the tissue includes homogenization in EtOH that interferes with protein determination. [^{3}H]PGE$_2$ was purchased from New England Nuclear Life Science Products (Boston, MA).

Measurement of Inducible Nitric Oxide Synthase Activity

Inducible nitric oxide synthase (iNOS) activity was measured by modifying the method of Bredt and Snyder (1989). In brief, gingival tissue and SMG were homogenized in 500 μl of ice-cold 20 mM HEPES (pH 7.4; Sigma-Aldrich) with EGTA (2 mM) and DL-dithiothreitol (1 mM; Sigma-Aldrich). After the tissue was homogenized, NADPH (120 μM; Sigma-Aldrich) and 200,000 dpm of [^{14}C]-arginine monochloride (297 mCi/mmol; Perkin-Elmer, Waltham, MA) were added to each tube and incubated for 10 minutes at 37°C in a Dubnoff metabolic shaker (50 cycles per minutes; 95% O$_2$/5% CO$_2$) at 37°C. The tubes were then centrifuged at 10,000×g for 10 minutes at 4°C. The supernatants were applied to individual columns containing 1 ml of Dowex AG 50 W-X8 Na$^+$ form mesh 200 to 400 (Bio-Rad Laboratories, Hercules, CA) and washed with 2.5 ml of double-distilled water. All collected effluent fluid from each column was counted for activity of [^{14}C]-citrulline in a liquid scintillation analyzer (TriCarb 2800TR; Perkin-Elmer). As NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as nmol of NO produced per min, per μg of protein for gums, and per mg of protein for SGM.

Determination of TNF-α

Trunk plasma was collected and centrifuged after decapitation. Plasma TNF-α concentration was determined using a sandwich ELISA according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). The data were expressed as pg TNF-α per ml.

Statistical Analysis

Statistical data were expressed as means ± SEM. Results were evaluated by 2-way analysis of variance (followed by Tukey post hoc test) or "t"-test, according to the groups included in the analysis. All results were conducted with Prism software (GraphPad Software, Inc., San Diego, CA). Differences with p-values <0.05 were considered statistically significant.

RESULTS

EtOH Consumption

No significant differences were observed among the 6 groups regarding liquid consumption during the experimental period (data not shown). The EtOH group exhibited moderate hepatic steatosis and scattered foci of inflammatory cell infiltration, as compared to control (data shown in Fig. S1).

TNF-α Content in Plasma

TNF-α analysis showed neither LPS-induced periodontitis nor saline injections altered its plasma concentration. Alternatively, alcohol consumption increased plasma cytokine content (Fig. 1).
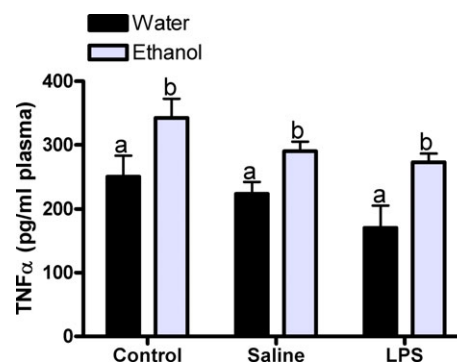

Fig. 1. Effect of chronic alcohol consumption, periodontitis induction, and the combination of both on tumor necrosis factor-α (TNF-α) plasma content. Data are reported as means ± SEM (8 rats per group). Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test. LPS, lipopolysaccharide.

Bone Loss Analyses

Evaluation of interradicular bone (Fig. 2A) showed that chronic alcohol consumption by itself produces bone resorption, revealed by increased periodontal ligament height (Fig. 2B) but not by alveolar bone volume (Fig. 2C). Besides, alcohol consumption increased CEJ-AC distance (Fig. 3). Saline injections did not induce significant changes as compared to controls, but alcohol consumption in this group increased CEJ-AC distance and periodontal ligament height. Significant bone resorption was obtained in rats with the LPS-induced periodontitis as revealed lower alveolar bone volume, higher CEJ-AC distance, and higher periodontal ligament height, compared with control rats. However, alcohol consumption did not alter bone status in rats treated with LPS.

iNOS Activity in Gingival Tissue

Increased gingival iNOS activity was found in rats receiving any kind of injections as compared to noninjected rats. However, no significant differences were observed in rats receiving saline or LPS applications despite alcohol consumption (Fig. 4).

PGE$_2$ Content in Gingival Tissue

Alcohol consumption by itself augmented gingival PGE$_2$ content. Besides, the LPS-induced periodontitis increased gingival PGE$_2$ content as compared to control. Unexpectedly, the increase was not seen in LPS group when it was submitted to alcohol consumption (Fig. 5).

iNOS Activity in SMG

Alcohol consumption by itself augmented iNOS activity compared with control. In addition, the LPS-induced periodontitis increased enzymatic activity in SMG. However, alcohol consumption did not significantly modify the activity of injected rats (Fig. 6).
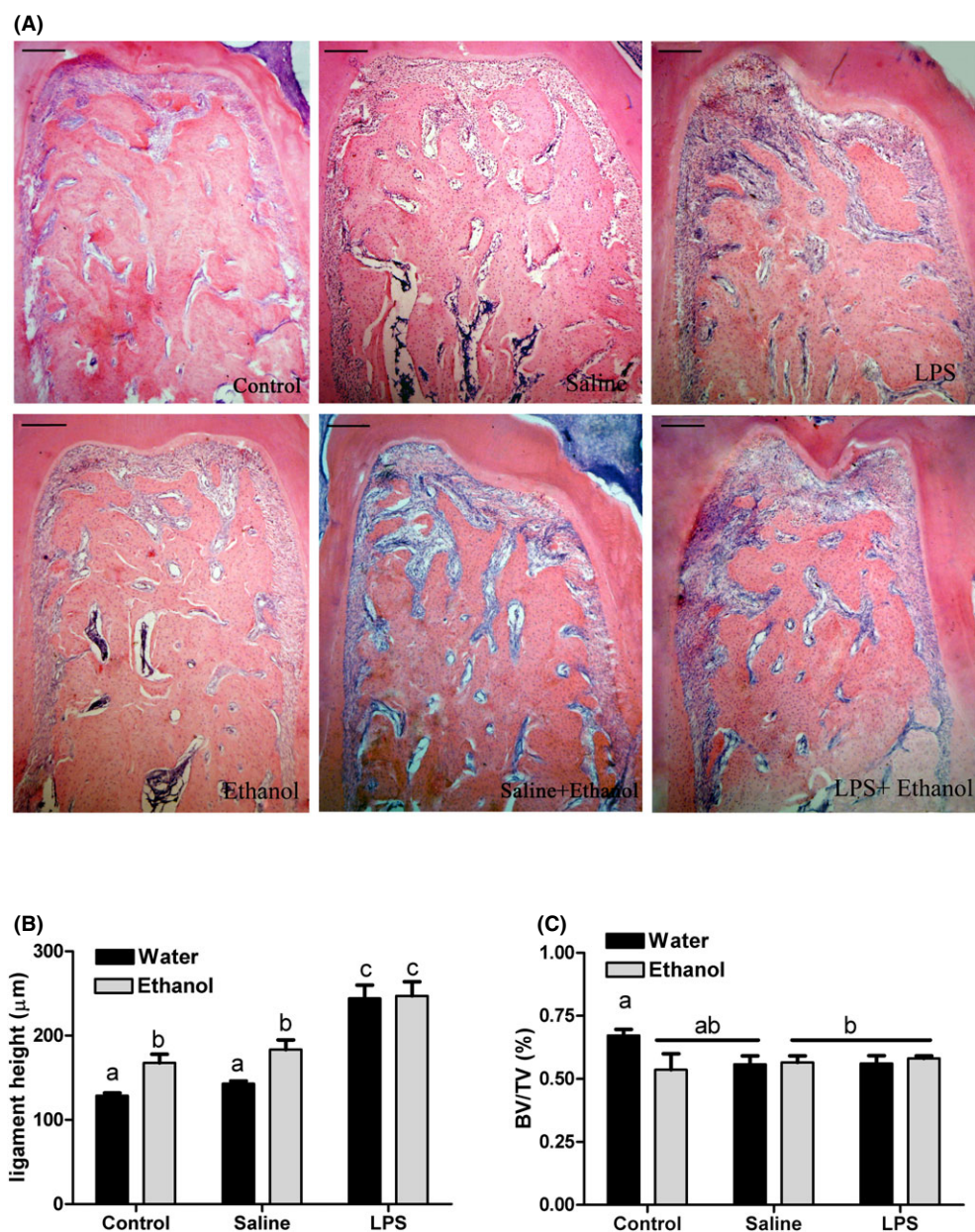
(A)

(B)

(C)

Fig. 2. Effect of chronic alcohol consumption, periodontitis induction, and the combination of both on static histomorphometry of interradicular bone. (**A**) Microphotographs showing interradicular bone in all groups (hematoxylin and eosin; original magnification 50×). Scale bar 200 μm. (**B**) Results of ligament height and (**C**) results of bone volume/total volume (BV/TV). Data are reported as means $\pm$ SEM (8 rats per group). Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test. LPS, lipopolysaccharide.

PGE_2 Content in SMG

LPS application did not significantly affect PGE_2 content in SMG. On the other hand, alcohol consumption significantly increased PGE_2 content in noninjected and saline-treated rats. Treatment with LPS apparently shadowed the effect of alcohol consumption, as no significant differences were found between LPS-treated groups (Fig. 7).

DISCUSSION

This work studies for the first time, to our knowledge, alveolar bone loss and inflammatory parameters in chronic alcoholic rats. We demonstrated that 20% alcohol consumption during 4 months induces alveolar bone loss and alters the status of inflammatory markers associated with periodontal disease. Conversely, alcoholic rats submitted to the proved model of LPS-induced periodontitis (Llavaneras et al., 1999; Ossola et al., 2012) did not exhibit an additional damage as compared to nonalcoholic rats submitted to LPS injections.

Several correlational studies in humans reported oral health alterations in chronic alcoholics. However, oral health reports in experimental animals submitted to chronic alcohol intake are scarce, and conclusions are contradictory between
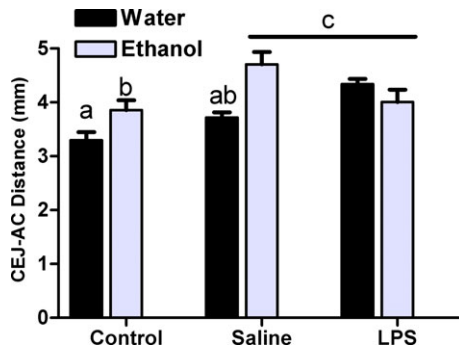
Fig. 3. Measurement of CEJ-AC distance in the first molar of rats with chronic alcohol consumption, periodontitis and the combination of both. Data are reported as means ± SEM (8 rats per group). Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test. CEJ, cemento–enamel junction; AC, alveolar crest; LPS, lipopolysaccharide.
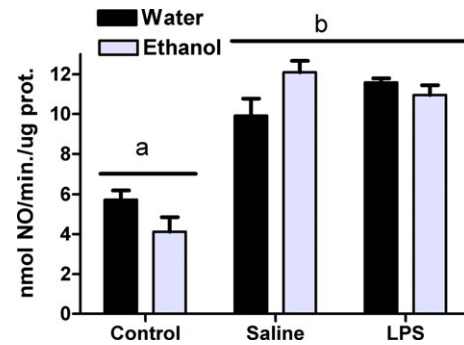

Fig. 6. Inducible nitric oxide synthase activity in submandibular gland of rats submitted to chronic alcohol consumption, periodontitis, and the combination of both. Data are reported as means ± SEM (8 rats per group). Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test. LPS, lipopolysaccharide.

Fig. 4. Gingival inducible nitric oxide synthase activity in rats submitted to chronic alcohol consumption, periodontitis, and the combination of both. Data are reported as means ± SEM (8 rats per group). Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test. LPS, lipopolysaccharide.
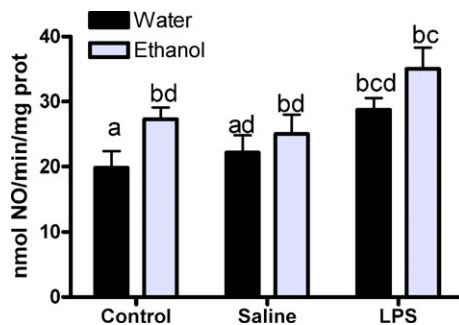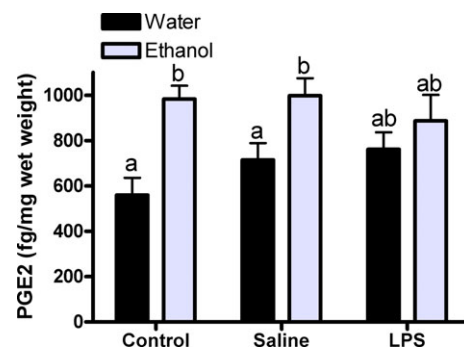

Fig. 7. Prostaglandin E_2 (PGE_2) content in submandibular gland of rats with lipopolysaccharide (LPS)-induced periodontitis, alcohol consumption, and the combination of both. Data are reported as means ± SEM. Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test.

Fig. 5. Effect of lipopolysaccharide (LPS)-induced periodontitis, alcohol consumption, and the combination of both on prostaglandin E_2 (PGE_2) concentration in gingival extracts. Data are reported as means ± SEM (8 rats per group). Different letters indicate significant difference ($p < 0.05$). Analysis made by 2-way analysis of variance followed by Tukey post hoc test.

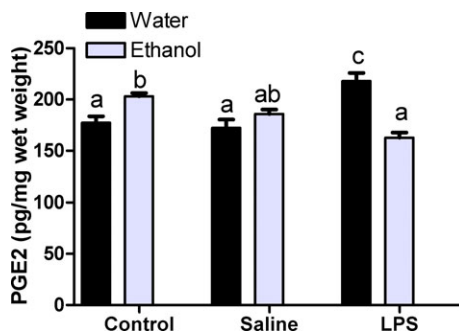different authors (Irie et al., 2008; Souza et al., 2009). The 20% alcohol consumption model used in this study has been reported in many studies (Hipólito et al., 2011; Silva and Madeira, 2012; Souza et al., 2009). Four months of alcohol ingestion was determined to guarantee a chronic condition. Liquid intake evaluation demonstrated that 20% alcohol did not produce changes in liquid consumption. Alcoholic rats

gained lesser weight than nonalcoholics and showed higher greasy content mainly at their tails (data not shown). These results together with hepatic alterations observed in alcoholic rats confirm its intake and validate the study. Chronic alcohol abuse has been associated with increased susceptibility to a variety of infections (Adams and Jordan, 1984; Szabo and Mandrekar, 2009). Alcohol abusers often exhibit elevated circulating levels of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 (McClain et al., 1993), the main factors involved in bone loss associated with periodontal disease. Reports of Bastos and colleagues (2014) showed that consumption of the Brazilian beverage cachaça during 100 days induces alveolar bone loss. In concordance with these reports, we demonstrated alveolar bone loss and increased plasma TNF-α after 4 months of alcohol intake, but we also showed increased PGE_2 in gums plus increased PGE_2 and iNOS activity in SMG of alcoholic groups as compared to nonalcoholics. It is important to remark that all parameters measured probably fluctuated along the experimental period, and we only measured their final values. Increases in these parameters in SMG have been reported to produce hyposialia, which in turn increases susceptibility to periodontal disease (Amer et al., 2011).

Also, it has been demonstrated one way to restore salivary gland function and to ameliorate gingival inflammation associated with periodontal bone loss, by blocking PGE_2 production (Lomniczi et al., 2001; Ossola et al., 2012). Rats submitted to saline injection were thought as controls of the mechanical damage induced by the injections of LPS. It seems like saline injection makes a very incipient damage, which is not enough to induce periodontitis (Ossola et al., 2012). Nevertheless, alcohol consumption together with saline application induced periodontal damage, observed by increased CEJ distance and increased periodontal ligament height. Those changes were not reflected in the final value of the inflammatory parameters assessed in the present work and may involve earlier fluctuations of them and other parameters not measured.

No significant differences were found between saline and LPS applications on CEJ-AC distance (outer measurement of bone loss) and alveolar bone volume (inner measurement) in alcoholic rats. However, periodontal ligament height showed significant variations between these groups. This lack of differences could be explained by compaction process of alveolar bone, favored by alcohol consumption plus oral inflammation leading to hyposialia induced by LPS, which in turn increases occlusal forces with consequent compaction and widening of the bone (Mavropoulos et al., 2004). This effect would impact on CEJ-AC distance and bone volume, masking bone loss induced by periodontitis, but producing no effect on the increased ligament height.

Some reports indicate that chronic alcohol consumption increases bone loss induced by experimental periodontitis (Bastos et al., 2014; Souza et al., 2009). However, those results are based in a different model of experimental periodontitis, called "traumatic," by placing a cotton ligature around the first molars. Although traumatic model is the most used and effective method for short-term experiments, at long terms, it presents limitations that could distort conclusions (Garcia de Aquino et al., 2009). Its main difficulty at long-term studies is that, while bone loss prospers, ligation losses contact with the periodontal tissue, ceasing to generate the trauma required to induce periodontitis. This does not occur in LPS induction model because injections are always applied near the gingival margin. Also, while in traumatic model bacteria are housed between the tooth and the ligature, and they release their own toxins, our study consists in repetitive application of exogenous LPS during 6 weeks into the gums.

We found that alcohol consumption diminishes the final gingival PGE_2 content increased by LPS. Besides, alcohol consumption did not induce additional deleterious effects in periodontal tissues of rats treated with LPS. Although a priori it is logical to suppose that susceptibility to periodontal disease would be increased in chronic alcoholics, studies suggested that alcohol intake produces tolerance to LPS-induced cytokine production and Toll-like receptors/CD14 clustering in peritoneal macrophages (Dai and Pruett, 2006). Additionally, 2-week EtOH feeding resulted in substantial impairment in the LPS-induced expression of TNF-α and macrophage

inflammatory proteins (MIP 1α and 2) in murine alveolar macrophages (Standiford and Danforth, 1997). In our study, after repetitive applications of LPS, the immune system appears to respond in a lesser degree in alcoholics than in nonalcoholics, as revealed in the biochemical parameters—PGE_2 and NO—measured in gums at the end of the experiments. Therefore, an explanation for these results could be tolerance to endotoxins induced by alcohol intake plus LPS application.

It is important to remark that histological differences founded in periodontal tissue of rats submitted to chronic alcohol intake may be explained by alterations in NO, PGE_2, and TNF-α concentration in plasma and oral tissues along the experimental period, in addition to other inflammatory mediators not assessed in this work. Therefore, periodontal bone status after 4 months of treatment is the final picture of long and complex processes in which periodontal markers may fluctuate.

Based on these results, we conclude that 20% alcohol consumption during 4 months generates differential effects on the oral health of rats, depending on its pathophysiological state. Although chronic consumption produces by itself periodontal damage compatible to periodontal disease, probably through systemic pathways, its intake does not produce significant deleterious additive effects in rats with periodontitis induced by local applications of LPS.

ACKNOWLEDGMENTS

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Liver specimens from rats in the control (A) and ethanol (EtOH) (B) groups stained with hematoxylin and eosin (600×).