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[AQ1] This article and the abstract have been edited for clarity and flow throughout with the intent to retain original meaning. This includes text body and headings, all footnotes (affiliation and otherwise), any figures and their legends, and any tables and their legends, and any acronyms that have been spelled out per journal style. Please read carefully for completeness and definition, and confirm changes made or amend as needed.

[AQ2] Please review the author byline and running heads to ensure that all author surnames are spelled correctly after typesetting and that the author order is as meant.

[AQ3] Information was added to the Methods section of the abstract to clarify the experimental methods of the study. Please review carefully and confirm or revise further if needed.

[AQ4] Only *exact matching* (meaning terms that have "[Mesh term]" following the term in the search details) terms and phrases are used. Please provide additional MeSH terms (**total of 6 in all**) as needed, if desired (see http://www.nlm.nih.gov/mesh/MBrowser.html for common MeSH terms and phrases).

[AQ5] Please provide age of rats used.

[AQ6] Please clarify "from our own colony" in the sentence starting 'Wistar male rats…' – per style, the use of "we/our/us" is avoided in text.

[AQ7] Please confirm expansion of HU-308 as set in text as correct.

[AQ8] Expansion of EGTA in the Biochemical Analysis section correct?

[AQ9] brand names are prohibited in the text. Generic descriptors were set for: Dubnoff metabolic shaker, Dowex AG 50 W-X8 Na+ form, Liquid Scintillation Analyzer TriCarb 2800TR, and Speedvac. Please confirm generic descriptions and manufacturer details provided.

[AQ10] The "Chemicals" section has been removed as the information has been footnoted at first mention (where it is clear it refers to this study) of each of the products. Please check these footnotes carefully, and ensure all information is provided and correct.

[AQ11] Please note that in the section ABL: Distance Method, the term "jaw" has been replaced with "mandible" in all instances for clarity. Please confirm or revise as needed.

[AQ12] Please confirm that supplementary materials have been provided for online publication.

[AQ13] Per style, the use of "we/our/us" is avoided in text. Please review the rewording provided carefully throughout.

[AQ14] Please confirm expansion of NK cells to natural killer cells.

[AQ15] Please provide to whom the grants were given and grant numbers if possible in Acknowledgment section.

[AQ16] Please check and confirm the journal name - Medline cannot find this reference or journal "Open J Stomatol" (in reference 37). A similar reference was found: Qian H, Zhao Y, Peng Y, et al. Activation of cannabinoid receptor CB2 regulates osteogenic and osteoclastogenic gene expression in human periodontal ligament cells. *J Periodontal Res* 2010;45:504-511.

[AQ17] For the symbols in the figures, because the symbols were already changed according to journal style in the legends, but not in the figures, it was difficult for me to know how to mark the symbols to be changed in the figures. Please carefully check my changes against the author original to make sure the symbols are matching up correctly to the given P values.

[AQ18] Please provide original magnification for Fig2.

[AQ19] For manufacturer footnotes ¶ and #, please confirm changes are correct, and if possible provide name/description of digital calipers for ¶.

[AQ20] All the provided figures are in low resolution. Could you please check and provide the high resolution images.

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Anti-Inflammatory and Osteoprotective Effects of Cannabinoid-2 Receptor Agonist HU-308 in a Rat Model of Lipopolysaccharide-Induced Periodontitis

Cesar A. Ossola,* Pablo N. Surkin,*† Claudia E. Mohn,*† Juan C. Elverdin,* and Javier Fernández-Solari*†

[AQ2] **Background:** Anti-inflammatory and immunologic properties of cannabinoids have been reported in several tissues. Expression of cannabinoid receptor Type 2 was reported in osteoblasts and osteoclasts, suggesting a key role in bone metabolism. The aim of this study is to assess the effect of treatment with cannabinoid-2 receptor agonist HU-308 in the oral health of rats subjected to lipopolysaccharide (LPS)-induced periodontitis.

[AQ3] **Methods:** Twenty-four rats were distributed in four groups (six rats per group): 1) control rats; 2) sham rats; 3) rats submitted to experimental periodontitis (LPS); and 4) rats submitted to experimental periodontitis and treated with HU-308 (LPS+HU). In groups LPS and LPS+HU, periodontitis was induced by LPS (1 mg/mL) injected into the gingival tissue (GT) of maxillary and mandibular first molars and into the interdental space between the first and second molars, 3 days per week for 6 weeks. In group LPS+HU, HU-308 (500 ng/mL) was applied topically to the GT daily.

Results: Alveolar bone loss resulting from LPS-induced periodontitis was significantly attenuated with HU-308 treatment (LPS+HU), measured by macroscopic and histologic examination. Treatment also reduced gingival production of inflammatory mediators augmented in LPS-injected rats, such as: 1) inducible nitric oxide (iNOS) activity (LPS: 90.18 ± 36.51 pmol/minute/mg protein versus LPS+HU: 16.37 ± 4.73 pmol/minute/mg protein; $P<0.05$); 2) tumor necrosis factor alpha (LPS: 185.70 ± 25.63 pg/mg protein versus LPS+HU: 95.89 ± 17.47 pg/mg protein; $P<0.05$); and 3) prostaglandin E_2 (PGE_2) (LPS: 159.20 ± 38.70 pg/mg wet weight versus LPS+HU: 71.25 ± 17.75 pg/mg wet weight; $P<0.05$). Additionally, HU-308 treatment prevented the inhibitory effect of LPS-induced periodontitis on the salivary secretory response to pilocarpine. Moreover, iNOS activity and PGE_2 content, which were increased by LPS-induced periodontitis in the submandibular gland, returned to control values after HU-308 treatment.

Conclusion: This study demonstrates anti-inflammatory, osteoprotective, and prohomeostatic effects of HU-308 in oral tissues of rats with LPS-induced periodontitis. *J Periodontol 2016;87:■■■-■■■.*

KEY WORDS

[AQ4] **Anti-inflammatory agents; periodontitis; saliva.**

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Periodontal disease, also called periodontitis, is a disease developed by a change in oral microbiota, characterized by inflammation of tooth-surrounding tissues and periodontal pocket formation.[1] These alterations lead to alveolar bone resorption and loss of periodontal attachment tissue.[2] Advanced periodontitis can cause tooth mobility and consequent tooth loss, evidencing development of chronic progressive disease.[3] Destruction of periodontal tissue is mainly due to complex interactions between pathogenic bacteria and host-derived mediators generated during the immunoinflammatory response.[1,2] Exposure to bacterial products such as lipopolysaccharides (LPS), originating in gram-negative organisms, can trigger a sequence of inflammatory events.[2,4] LPS is known to stimulate production of cytokines and other inflammatory mediators, in turn promoting release of matrix metalloproteinases from host tissues, which degrades extracellular matrix and alveolar bone.[5] Among other factors, interleukin (IL)-1β and tumor necrosis factor (TNF)-α are found to be associated with: 1) periodontal inflammation, enabling entry of inflammatory cells into sites of infection; 2) promotion of bone resorption; and 3) stimulation of eicosanoid release by monocytes and fibroblasts, especially prostaglandin E_2 (PGE_2).[6-8] It is known that PGE_2 is a prominent mediator of periodontal inflammation, making it a potent stimulator of bone resorption, and its production is associated with loss of periodontal attachment tissue.[7,8] Increase of these mediators of inflammation was reported to be involved in the imbalance between alveolar bone formation and resorption, by favoring resorption mediated by: 1) receptor activator of nuclear factor-kappa B ligand (RANKL), 2) its receptor RANK, and 3) a decoy receptor osteoprotegerin.[9,10] Nitric oxide (NO) is a free radical also involved in various pathophysiologic processes. Whereas NO, produced by the endothelium, is believed to play a protective role in the microvasculature, excessive NO production is associated with tissue injury.[11,12]

The submandibular gland (SMG) is one of the major salivary glands, together with the parotid and sublingual glands.[13] Under physiologic conditions, the protective potential is sufficiently maintained by salivary flow; however, this state seems to be disturbed in periodontitis, as reported by Amer et al.[14] It was demonstrated that in rats subjected to ligature-induced periodontitis, SMG salivary secretion diminishes and changes in composition, and the alteration of salivary flow and composition was shown to aggravate periodontitis.[15]

The endocannabinoid system (ECS) is a signaling network that modulates a diverse spectrum of physiologic processes, including: 1) nociception; 2) behavior; 3) appetite; 4) motor control; 5) memory formation; and 6) inflammation.[16] It comprises endogenous ligands, such as anandamide and 2-arachidonoylglycerol, and a series of mechanisms for their synthesis and degradation as well as classic G-protein-coupled membrane receptors. Cannabinoid receptor Type 1 (CB1) and Type 2 (CB2) are the main specific cannabinoid receptors, and transient receptor potential vanilloid Type 1 is the main unspecific receptor.[17] Although cannabinoid receptors are present in different kinds of cells and tissues, CB1 is highly expressed in the central nervous system and, to a lower extent, in peripheral tissues. CB2 is expressed mainly in immune cells such as 1) monocytes, 2) macrophages, 3) lymphocytes, and 4) and bone cells.[18,19] The ECS has been implicated in multiple regulatory functions in health and disease. Cannabinoids may act as potent anti-inflammatory agents, exerting their effects through: 1) suppression of cytokine production; 2) inhibition of cell proliferation; 3) induction of apoptosis; and 4) induction of T-regulatory cells.[20] There is extensive evidence showing that endocannabinoids and their receptors are involved in bone metabolism by regulating bone mass, bone loss, and function of bone cells[21,22] and are synthesized in bone tissue.[21] Cannabinoids also promote proliferation of human gingival fibroblasts via CB1/CB2 receptors in periodontal healing, and therefore, the ECS may play an important modulatory role in such processes.[23] CB2 receptors have been reported to be expressed in osteoblasts, osteocytes, and osteoclasts.[21]

In continuation of the above-mentioned reports,[20-23] the aim of the present work is to assess the effect of treatment with HU-308, a synthetic and highly selective agonist for CB2 receptors,[24] in the oral health of rats subjected to LPS-induced periodontitis.

MATERIALS AND METHODS

Animals

Twenty-four adult male Wistar rats (350 g) from the [AQ5] authors' own colony were kept in group cages in an [AQ6] animal room with a 12-hour light photoperiod (0700 to 1900), room temperature maintained at 22°C to 25°C, and free access to rat chow and tap water. The experimental procedures performed were approved by the Animal Care Committee of the Dental School of the University of Buenos Aires, Buenos Aires, Argentina and were carried out in accordance with guidelines of the National Institutes of Health.

Experimental Design

Twenty-four rats were distributed in four groups (six rats per group): 1) control rats; 2) sham rats; 3) rats submitted to experimental periodontitis; and 4) rats submitted to experimental periodontitis and treated with HU-308. Periodontitis was induced by injecting

20 μl of LPS‡ (1 mg/mL) from *Escherichia coli* into the vestibular and lingual gingiva of the maxillary and mandibular first molars and into the interdental space between the first and second maxillary and mandibular molars (60 μL of LPS per tooth and 240 μL per rat each time of treatment). Sham animals were injected in the same manner with the same volume of the vehicle of LPS, saline solution (20 μL each injection), while control rats received no injections during the experiments. This protocol of injections was executed for a period of 6 weeks on days 1, 3, and 5 of each week, based on a previously described method.[25-27] Gingival injections were placed with a 13-mm 27-gauge microfine insulin syringe.

Topical Treatment With CB_2 Receptor Cannaboid Agonist (HU-308)

HU-308 ([(1R,2R,5R)-2-[2,6-dimethoxy-4-(2-methyloctan-2-yl)phenyl]-7,7-dimethyl-4-bicyclo[3.1.1] [AQ7] hept-3-enyl] methanol)§ was prepared by dissolving the powder drug in 100% ethanol and diluting it in saline to obtain a final concentration of 500 ng/mL to treat animals, containing approximately 1% ethanol. Volume of each topical application was 200 μL per tooth, resulting in 800 μL per animal when treatment was performed. Except for controls, animals received a daily topical application of HU-308 (group 4) or its vehicle (1% ethanol in saline solution) (groups 2 and 3) in each affected tooth, on sites of LPS/saline injections, during the 6 weeks of experiment. Optimal dose of HU-308 was obtained based on previous reports using anandamide,[28] but fundamentally using methanandamide, a selective synthetic agonist of CB1 receptor,[25] on oral tissues in vivo concomitantly with the dose response curves in these preliminary studies.

Macroscopic Examination of Periodontal Bone Loss: Distance and Width Methods

Immediately after sacrifice of the rats, hemimandibles were resected, defleshed, and stained with 1% aqueous methylene blue to delineate the cemento-enamel junction (CEJ) and the alveolar crest (AC).[29] A stereomicroscope‖ and digital caliper¶ were used to measure three buccal and three lingual/palatal distances (mesial, central, and distal), from the CEJ to the AC.[30] The sum of the three distances of each side of molars was used as a measure of alveolar bone loss (ABL) in millimeters.

Mandibular alveolar process width was measured in the mandibular first molar area. Distance between a point located at the central root level of the buccal surface and another equally located on the lingual surface was obtained from the mandibles with the digital caliper, in millimeters.

Histologic Analysis

Hemimandibles were extracted and fixed in formalin buffer. After 3 days, they were decalcified in 10% EDTA pH 7 for 45 days. Following this, hemimandibles were dehydrated with ethyl alcohol and clarified with xylene. Finally, the sector containing the first molar of each decalcified hemimandible was embedded in paraffin at 56°C to 58°C. Under the stereomicroscope and using a microtome,# sections oriented mesial-distally of each mandibular first molar were obtained from paraffin blocks. Sections 5 mm in width were stained with hematoxylin and eosin (H&E), and histomorphometric evaluation was performed on digitized microphotographs using imaging software.** Interradicular bone loss was evaluated by measuring the periodontal space height, plotting 10 equidistant lines between the alveolar crest and the cementum of the furcation zone. Length of the lines was measured, and the mean value was calculated. Additionally, ABL was assessed by the following parameter: bone volume (BV)/total volume (TV) (%) = fraction of TV corresponding to bone tissue. TV was taken as bone tissue plus bone marrow and periodontal ligament.

Biochemical Analysis

Measurements of inducible nitric oxide (iNOS) activity. Activity of iNOS was measured in gingival tissue (GT) and SMG by modifying the method of Bredt and Snyder.[31] GTs were homogenized in 500 μL of cold 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4)†† with ethylene glycol-bis [AQ8] (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (2 mM)‡‡ and DL-dithiothreitol (DTT, 1 mM).§§ A similar procedure was used for the SMG, but the tissue was homogenized in 600 μL of HEPES. After the tissue homogenates were achieved, nicotinamide adenine dinucleotide phosphate (120 μM)‖‖ and 200,000 dpm of [14C]-arginine¶¶ were added to each tube and incubated for 10 minutes at 37°C in a metabolic shaker## (50 cycles [AQ9] per minute; 95% O_2/5% CO_2) at 37°C. The tubes were then centrifuged at 10,000 × g for 10 minutes at 4°C. Supernatants were applied to individual 1-mL resin

‡ LPS serotype 055-B5 from *Escherichia coli* (lyophilized powder, purified by trichloroacetic acid extraction, 1% to 10% of protein impurities), Sigma-Aldrich, St. Louis, MO.
§ Tocris Bioscience, Ellisville, MO.
‖ Stemi DV4 Stereomicroscope, Carl Zeiss MicroImaging, Göttingen, Germany.
¶ Digimess, Derby, UK. [AQ19]
# Jung Microtome, Leica Biosystems, Nussloch, Germany.
** ImageTool, University of Texas Health Science Center, San Antonio, TX.
†† HEPES, Sigma-Aldrich.
‡‡ EGTA, Sigma-Aldrich.
§§ DL-dithiothreitol (DL-DTT), Sigma-Aldrich.
‖‖ NADPH, Sigma-Aldrich.
¶¶ [14C]-arginine monochloride (297 mCi/mmol), PerkinElmer, Waltham, MA.
Dubnoff metabolic shaker, Thermo Fisher Scientific, Waltham, MA.

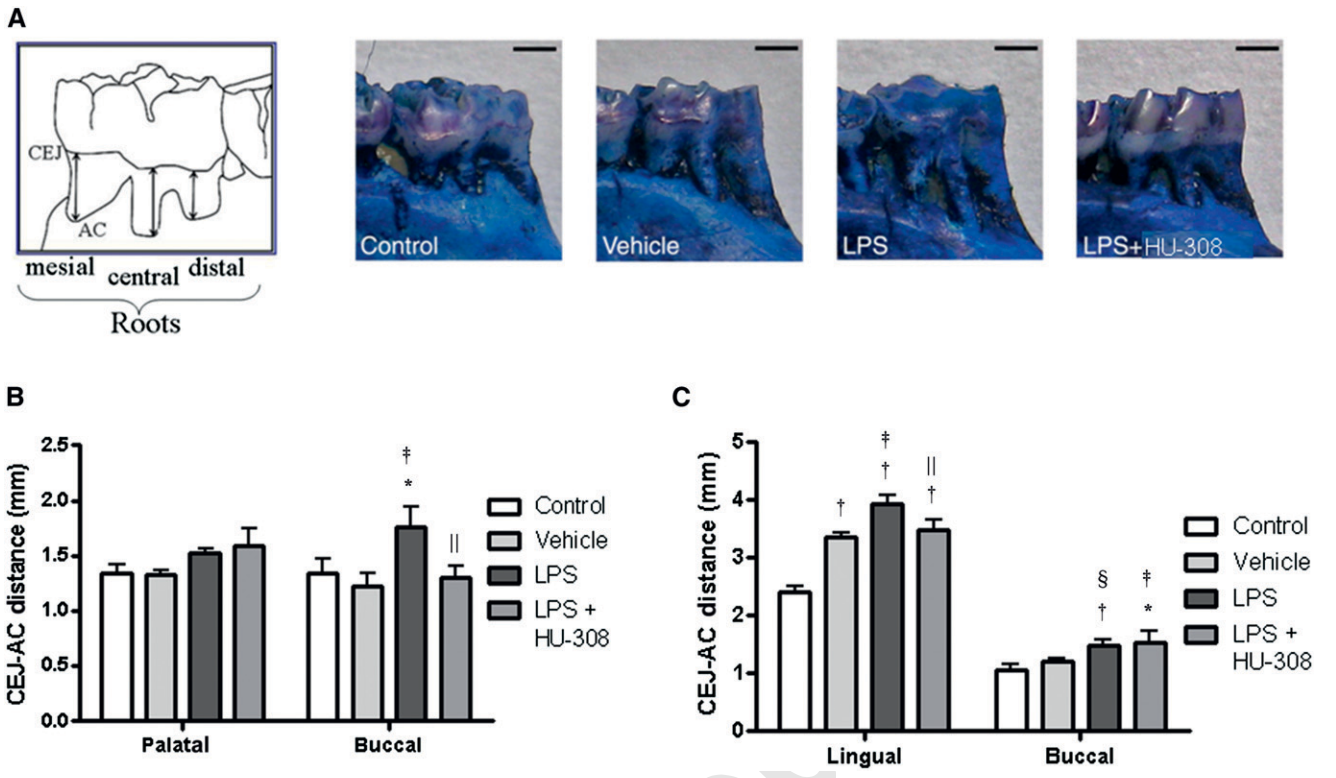

Figure 1.
A) *Images showing ABL, measured by the distance method on the mesial, central, and distal roots of a first molar. Scale bars = 1 mm. Effect of LPS-induced periodontitis and its treatment with HU-308 on ABL covering roots of maxillary* ***(B)*** *and mandibular* ***(C)*** *first molars. Results are presented as mean ± SD.* *P <0.05 *and* †P <0.001 *versus control;* ‡P <0.05 *and* §P <0.01 *versus vehicle;* ||P <0.05 *versus LPS.*

columns (200-400 mesh, Na+ form)*** and washed with 2.5 mL of double-distilled water. The collected effluent fluid from each column was counted as activity of [14C]-citrulline in a liquid scintillation analyzer.††† Since NOS converts arginine into equimolar quantities of NO and citrulline, data were expressed as picomoles of NO produced per minute per milligram of protein.

Radioimmunoassay of PGE_2. To determine PGE_2‡‡‡ content, GT and SMG were homogenized in 500 μL and 1,000 μL of absolute ethanol, respectively, and after centrifugation, supernatants were dried in a centrifugal vacuum concentrator§§§ at room temperature. Residues were then resuspended with buffer; antiserum‖‖‖ was used as described in Mohn et al.[32] Sensitivity of the assay was 12.5 pg per tube. The cross-reactivity of PGE_2 and PGE_1 was 100%, but the cross-reactivity of other prostaglandins was 0.1%. The intra- and interassay coefficients of variation for PGE_2 were 8.2% and 12.0%, respectively. The results were expressed in picograms of PGE per milligrams of wet weight, since the protocol of PGE extraction from the tissue includes homogenization in ethanol that interferes with protein determination.

Determination of TNF-α. For TNF-α preservation after extraction, GT was immediately homogenized in phosphate-buffered saline containing protease inhibitory cocktail for mammalian tissue extracts.¶¶¶ Concentration of TNF-α was determined using a sandwich enzyme-linked immunosorbent assay according to the manufacturer's instructions.### Data were expressed as picograms TNF-α per milliliter.

Stimulated salivary secretion assessment. Rats were removed from cages 1 week before sacrifice and anesthetized with an intraperitoneal injection of ketamine hydrochloride**** (70 mg/kg body weight) and 2% xylazine hydrochloride†††† (10 mg/kg body weight). Then, a dose of pilocarpine‡‡‡‡ (0.5 mg/kg body weight) was administered intraperitoneally to induce salivation, and a cotton ball was immediately

*** Dowex AG 50W-X8 Na+ form mesh 200-400, Bio-Rad Laboratories, Hercules, CA.
††† Tri-Carb 2800TR Liquid Scintillation Analyzer, PerkinElmer.
‡‡‡ [3H]PGE2, New England Nuclear Life Science Products, Boston, MA.
§§§ SpeedVac, Thermo Fisher Scientific.
‖‖‖ Sigma-Aldrich.
¶¶¶ Sigma-Aldrich.
BD Pharmingen, San Diego, CA.
**** Holliday-Scott SA, Buenos Aires, Argentina.
†††† König Laboratories SA, Buenos Aires, Argentina.
‡‡‡‡ Sigma-Aldrich.

Table 1.

Alveolar Bone Width (mm) Evaluated in Zone of Central Root of Mandibular First Molar

Control	Vehicle	LPS	LPS+HU-308
2.62 ± 0.07	2.54 ± 0.05	2.45 ± 0.08*	2.61 ± 0.07†

Results are given as mean ± SD.
* P <0.001 versus control.
† P <0.01 versus LPS.

placed under the tongue of the rat to take up the total salivary secretion. This was determined as the difference in weight of the cotton ball before and after collection. Saliva collection was carried out at 30-minute intervals after the administration of pilocarpine, over [AQ10] a 90-minute period.[33]

Statistical Analyses

Data are expressed as means ± standard error of the mean. Results were evaluated by one-way analysis of variance followed by the Newman-Keuls multiple comparisons test for unequal replicates. All analyses were conducted with appropriate software[§§§§] and differences with P values <0.05 were considered statistically significant.

RESULTS

[AQ11] ### *ABL: Distance Method*

ABL produced as a result of LPS-induced periodontitis, measured at the lingual/palatal and buccal sides by the distance method (Fig. 1A), was reduced by treatment with HU-308 (500 ng/mL). This effect was observed both in the maxilla, at the buccal side (Fig. 1B), and in the mandible, at the lingual side (Fig. 1C). The buccal side of the mandibles showed fewer differences than the lingual side among groups, perhaps as a consequence of a thicker bone plate. Additionally, the HU-308 group showed no significant changes compared with the LPS group in the buccal side of mandibles. A significant impact caused by saline application was noticed at the lingual side on mandibles (Fig. 1C), perhaps due to a traumatic effect generated by needle puncture on the GT during the 6-week study period.

ABL: Width Method

Alveolar bone level measured in a buccal-lingual direction on mandibular first molars (see supple- [AQ12] mentary Fig. 1 in online *Journal of Periodontology*) was reduced in rats subjected to LPS, while HU-308 treatment prevented that reduction, showing instead a bone preservation effect (Table 1).

Histologic Analysis

In the interradicular area, LPS-injected rats showed a higher periodontal space (Figs. 2A and 2B) and lesser alveolar bone area represented as BV/TV (%) (Figs. 2A and 2C) than control and vehicle groups. HU-308-treated rats showed no indication of these deleterious effects. Additionally, interradicular bone in LPS-injected rats had erosive surfaces with the presence of osteoclasts as well as active bone remodeling with predominance of reversal lines. Conversely, in rats treated with HU-308, bone showed less active remodeling and was aimed at repairing through osteogenesis with active osteoblasts and osteoid (Fig. 2A).

Inflammatory Markers in GT

Activity of iNOS in GT, which was increased in rats with LPS-induced periodontitis, exhibited a reduction when animals were also treated with HU-308 (Fig. 3A). Gingival TNF-α content was less in rats with LPS-induced periodontitis treated with HU-308 compared with rats with LPS-induced periodontitis but treated daily with only the HU-308 vehicle (Fig. 3B). PGE_2 content was significantly higher in GT of rats with LPS-induced periodontitis compared with controls. Again, treatment with HU-308 decreased PGE_2 content compared with untreated rats (Fig. 3C).

Salivary Secretion Evaluation

Total collected salivary secretion was not significantly different among groups after 30 minutes of pilocarpine stimulation; however, an unexpected tendency of lower levels of salivation in controls was observed with respect to all other groups. This difference could suggest a lower response to pilocarpine in intact animals, although the observed tendency has no statistical significance due to high deviations (Fig. 4). After 30 to 60 minutes of pilocarpine stimulation, rats with LPS-induced periodontitis showed a reduced salivary response as represented by lower levels of saliva compared with control and vehicle groups, with this effect of LPS being prevented in animals treated with HU-308. A similar effect was observed in the 60- to 90-minute period, where HU-308 also restored the diminished response of salivary glands to pilocarpine, caused by LPS-induced periodontitis.

Inducible NOS Activity and PGE_2 Content in SMG

LPS-induced periodontitis also increased iNOS activity and PGE_2 content in the SMG, and these effects were significantly blocked by HU-308 treatment on GT (Fig. 5). Unexpectedly, the rats treated with HU-308 showed reduced levels of iNOS activity compared with controls.

DISCUSSION

There are many studies in the literature showing the influence of LPS both on increase of bone loss and inflammatory mediators.[34-37] Recently, Jin et al.[36]

§§§§ Prism Software (GraphPad Software), La Jolla, CA.

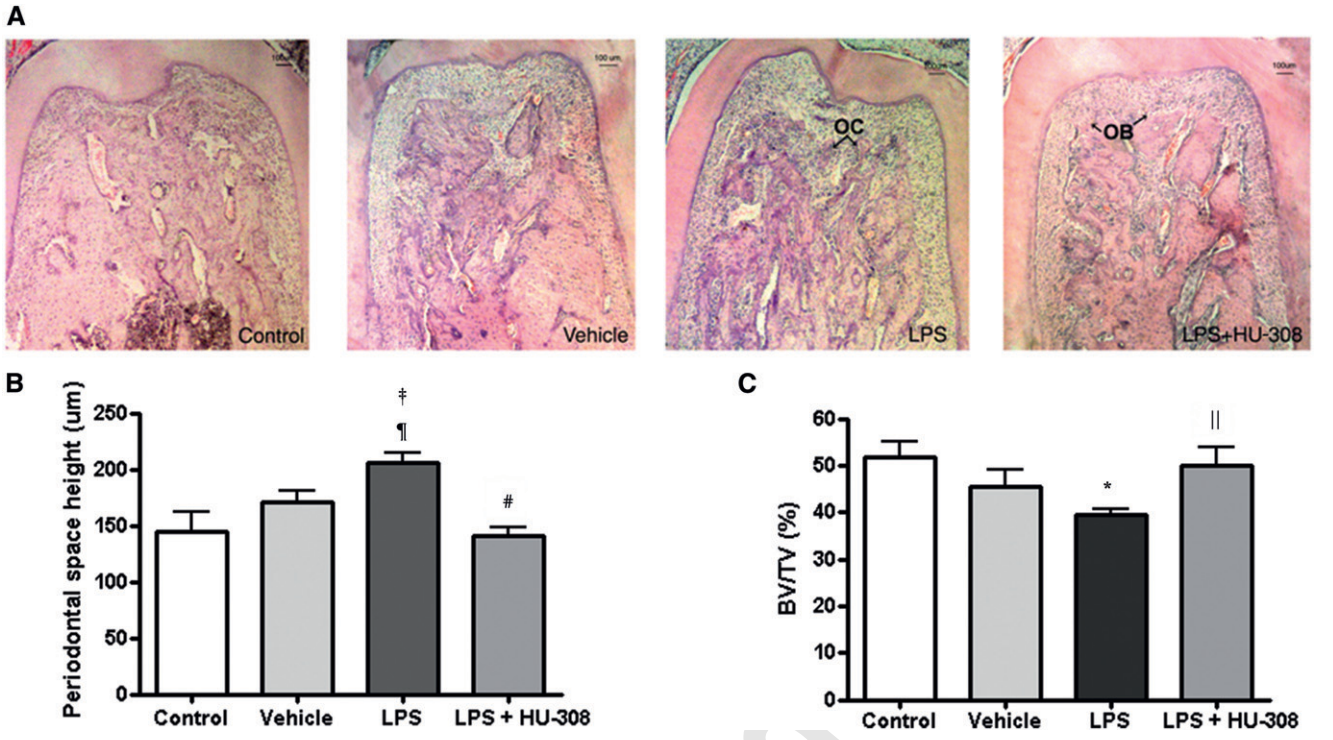

Figure 2.

A) *Photomicrographs (H&E stain; original magnification x**XX**) showing histologic features of the mandibular first molar interradicular area of rats subjected to different experimental conditions (control, vehicle, LPS, LPS+HU-308). OC = osteoclasts and erosive surfaces; OB = osteoblasts and osteoid formation.* ***B)*** *Periodontal space height evaluation.* ***C)*** *Interradicular bone measured as BV/TV (%). Results are presented as mean ± SD.* *P <0.05 *and* ¶P <0.01 *versus control;* ‡P <0.05 *versus vehicle;* ||P <0.05 *and* #P <0.001 *versus LPS.*

worked with local injections of LPS in rats, showing that induced periodontitis increases ABL and stimulates leukocyte infiltration to the tissue and expression of osteoclastogenic molecules. RANKL is essential for complete differentiation of osteoclast precursor cells and plays a critical role in periodontal bone resorption.[38] A study performed in cultures of osteoclast precursors concluded that TNF-α greatly increases osteoclast proliferation/differentiation in the presence of RANKL.[39] IL-1 and LPS were reported to stimulate osteoclastogenesis through two parallel events: direct enhancement of RANKL expression and suppression of osteoprotegerin expression, which is mediated by PGE_2 production.[40] In concordance, it was demonstrated that LPS-induced periodontitis increased ABL as well as gingival inflammatory mediators involved in the disease, including PGE_2, NO, and TNF-α. These factors are usually linked to: 1) periodontal pocket formation; 2) insertion tissue loss; 3) gingival bleeding; and 4) tooth mobility.[37] In these conditions, the effect of a synthetic cannabinoid applied locally to the area of experimental periodontitis induction was evaluated. Results showed that HU-308 causes a clear reduction of ABL and inflammatory mediators in GT, which are increased by LPS-induced periodontitis in the absence of treatment. Although the main effect of treatment with HU-308 should be local, the presence of a systemic effect due to some swallowing of the formulation cannot be disregarded.

Based on previously mentioned reports, it is clear that attenuation of gingival inflammatory parameters, after treatment with HU-308, leads to bone metabolism normalization. In accordance with these results, Qian et al.[37] demonstrated that LPS increases the concentration of IL-1β, IL-6, TNF-α, and RANKL in human periodontal ligament cells treated in vitro, and that these parameters were attenuated by HU-308. This evidence supports the importance of the CB2 receptors' activation in inflammatory and immune responses, as well as in bone formation and its resorption to control periodontal disease. In a study of osteoporosis, a non-infectious disease, the CB2 receptor has been postulated as a bone mass regulator.[41] CB2 receptor activation is thought to stimulate osteoblastic cell differentiation and mitigate osteoclastogenesis, suggesting that CB2 receptor signaling produces blockage of bone loss via direct action on bone cells and, simultaneously, through inhibition of expression of proresorptive cytokines.[42] Independently of bone loss, CB2 receptor activation could also reduce pain and the amount of substances

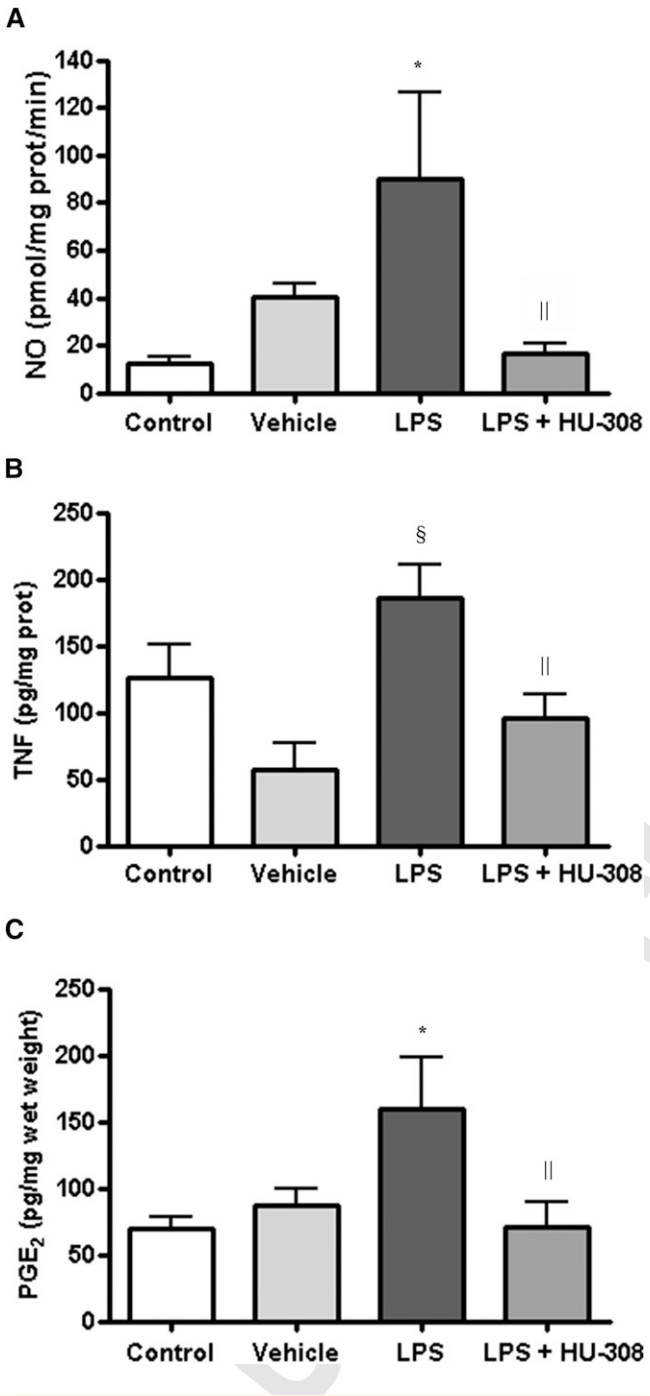

Figure 3.
Effect of LPS-induced periodontitis in GT and its treatment with HU-308 on ***A)*** *iNOS activity;* ***B)*** *TNF-α content; and* ***C)*** *PGE_2 content. Results are presented as mean ± SD. prot = protein; min = minute.* *P <0.05 *versus control;* §P <0.01 *versus vehicle;* ||P <0.05 *versus LPS.*

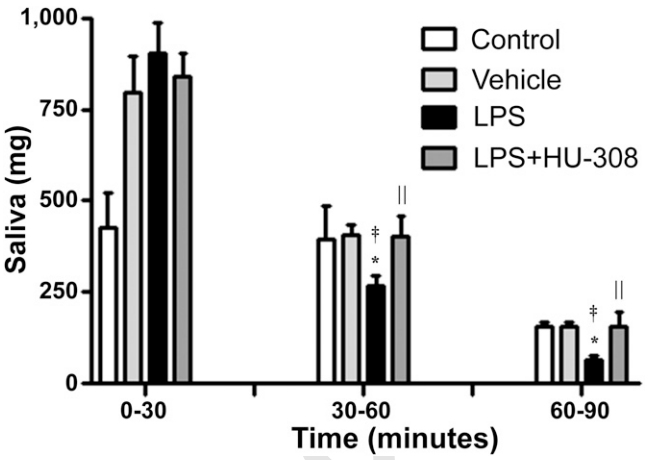

Figure 4.
Effect of LPS-induced periodontitis and its treatment with HU-308 on salivary response to pilocarpine. Results are presented as mean ± SD. *P <0.05 *versus control;* ‡P <0.05 *versus vehicle;* ||P <0.05 *versus LPS.*

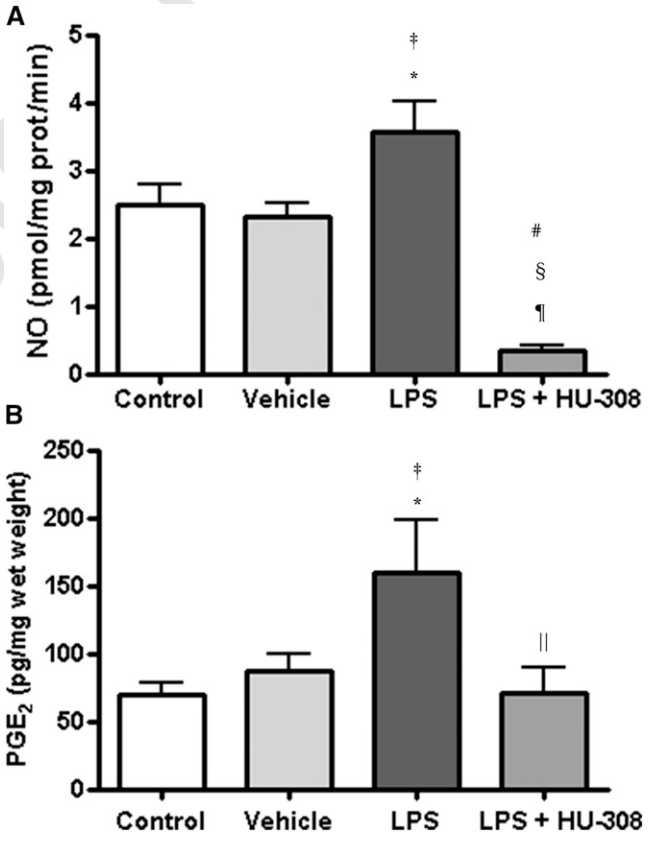

Figure 5.
Effect of LPS-induced periodontitis and its treatment with HU-308 on ***A)*** *iNOS activity; and* ***B)*** *PGE_2 content in the SMG. Results are presented as mean ± SD. prot = protein; min = minute.* *P <0.05 *and* ¶P <0.01 *versus control;* ‡P <0.05 *and* §P <0.01 *versus vehicle;* ||P <0.05 *and* #P <0.001 *versus LPS.*

[AQ20]

released during inflammation by inducing apoptosis in activated T cells. This would reduce the number of activated T cells and suppress induction of mast [AQ14] cells, natural killer cells, and neutrophils at sites of inflammation.[43]

The CB1 receptor has been classically associated with the central and peripheral nervous system effects of cannabinoids, whereas the CB2 receptor has been linked to immune cells and inflammatory processes, with bone remodeling boosted by them.[41] Results from this study support these reports. However, in a previous study it was demonstrated that the deleterious effect of LPS on periodontal tissues was prevented both in vivo and in vitro by using the selective CB1 receptor agonist, methanandamide.[25] Furthermore, the G protein-coupled receptor 55 has been studied in recent years as a nonspecific target for cannabinoids, showing similarities to CB1 and CB2 receptors. This receptor could be activated by natural, endogenous, and some synthetic cannabinoids, becoming particularly important in cancer and cell death and overexpressed in response to LPS.[44]

Rats with LPS-induced periodontitis exhibited a reduced total salivary response to pilocarpine and higher values of PGE_2 and NO in the SMG. It is well known that SMG is the major contributor to total saliva production in the oral cavity and that salivary secretion is an essential factor in the establishment and progression of oral infectious processes.[30,45] The influence of periodontitis on the pathophysiology of the SMG was evident in the present study. In concordance with results obtained here, PGE_2 and NO were augmented in the SMG of rats subjected to ligature-induced periodontitis.[14] Additionally, increase of these parameters was demonstrated to be involved in the consequent reduction of salivary secretory function.[46] These results are in agreement with findings presented here, where, interestingly, HU-308-treated animals showed attenuated levels of PGE_2 and NO in the SMG and a recovery of salivary function, suggesting that both the progression of periodontal damage and its recuperation alter pathophysiology of salivary glands. Furthermore, since evaluation in this study of wet and dry weights of the SMG and sublingual glands did not show significant differences between groups (data not shown), it is believed that LPS-induced periodontitis could alter salivary secretion function by modifying signaling pathways, rather than affecting glandular parenchyma.

Despite the obvious indirect effect of cannabimimetic agents on salivary function due to its preventive effect of LPS-induced periodontitis, its direct influence on salivation cannot be disregarded, as CB1 and CB2 receptors have already been found in acinar and ductal cells of the SMG.[28] Additionally, it has been demonstrated that endocannabinoid production is increased in the SMG of rats with endotoxemia, induced by intraperitoneal injections of LPS, while salivary secretion is decreased.[47,48] This evidence might suggest that the ECS plays a significant part in periodontal disease control mechanisms and acts through more than one pathway in its anti-inflammatory role.[49,50]

CONCLUSIONS

In conclusion, the beneficial effect of HU-308 on: 1) alveolar bone, 2) GT, and 3) salivary function demonstrates participation of CB2 receptor signaling in the control of periodontal damage and its associated oral alterations caused by inflammatory processes. This supports participation of the endocannabinoid system in homeostasis recovery under pathophysiologic conditions.

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