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Complete List of Authors:	Ossola, Cesar Angel; Faculty of Dentistry, University of Buenos Aires, Buenos Aires, Argentina , Physiology Surkin, Pablo Nicolas; Faculty of Dentistry, University of Buenos Aires, Buenos Aires, Argentina , Physiology; National Council for Scientific and Technical Research, Argentina Mohn, Claudia; School of Dentistry, University of Buenos Aires, Physiology Department; National Council for Scientific and Technical Research, Argentina Buenos Aires, Buenos Aires, AR Elverdin, Juan Carlos; Facultad de Odontología, Universidad de Buenos Aires,, Cátedra de Fisiología Fernandez-Solari, Javier; Faculty of Dentistry, University of Buenos Aires, Buenos Aires, Argentina , Physiology; National Council for Scientific and Technical Research (CONICET), Argentina

Key Words:	Anti-inflammatory agents, Saliva, Periodontitis

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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,[*] Javier Fernández-Solari[*,†]

[*]Department of Physiology, Faculty of Dentistry, University of Buenos Aires, Buenos Aires, Argentina

[†]National Council of Scientific and Technical Research (CONICET), Argentina

Correspondence to: Dr. Javier Fernandez-Solari, Faculty of Dentistry, University of Buenos Aires, Department of Physiology, Marcelo T de Alvear 2142, floor 3A, Buenos Aires (C1122 AAH), Argentina. Fax: (5411) 4508-3958. Email: javierfsolari@yahoo.com.ar.

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Key findings: This study demonstrates the anti-inflammatory, osteoprotective and pro-homeostatic effects of the cannabinoid receptor type 2 agonist HU-308 in oral tissues of rats with lipopolysaccharide -induced periodontitis.

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Abstract

Background: Anti-inflammatory and immunological properties of cannabinoids have been reported in several tissues. Also, cannabinoid receptors type 2 (CB2) were reported to be expressed in osteoblast and osteoclast, suggesting a key role in bone metabolism. The aim of the present study was to assess the effect of the treatment with the cannabinoid-2 receptor agonist HU-308 in the oral health of rats subjected to lipopolysaccharide (LPS)-induced periodontitis. Methods: Periodontitis was induced by LPS (1 mg/ml) injected into the gingival tissue of the first upper and lower molars, and into the inter-dental space between the first and second molars, three days a week, during 6 weeks. HU-308 (500 ng/ml) was applied topically daily on the gingival tissue. Results: Alveolar bone loss resultant from LPS-induced periodontitis was significantly attenuated with HU-308 treatment, measured by macroscopic and histological examination. The treatment also reduced the gingival production of inflammatory mediators augmented in LPS-injected rats, such as inducible nitric oxide (iNOS) activity (LPS: 90.18±36.51 vs. LPS+HU: 16.37±4.73 pmol/min/mg prot., $p<0.05$), tumor necrosis factor alpha (TNFα) (LPS: 185.70±25.63 vs. LPS+HU: 95.89±17.47 pg/mg prot., $p<0.05$) and prostaglandin E_2 (PGE_2) (LPS: 159.20±38.70 vs. LPS+HU: 71.25±17.75 pg/mg w.w., $p<0.05$). 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 the anti-inflammatory, osteoprotective and pro-homeostatic effects of HU-308 in oral tissues of rats with LPS-induced periodontitis.

Keywords: Anti-inflammatory agents, Periodontitis, Saliva.

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Introduction

Periodontal disease, also called periodontitis, is a disease developed by a change in the oral microbiota, characterized by inflammation of tooth-surrounding tissues and of 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 its consequent loss, evidencing the development of a chronic progressive disease.[3] Destruction of periodontal tissue is mainly due to complex interactions between the pathogenic bacteria and the host-derived mediators generated during the immunoinflammatory response.[1,2] Exposure to bacterial products such as lipopolysaccharides (LPS), originated in gram-negative organisms, can trigger a sequence of inflammatory events.[2,4] LPS is known to stimulate the production of cytokines and other inflammatory mediators, in turn promoting the release of matrix metalloproteinases from the host tissues, which degrades extracellular matrix and alveolar bone.[5] Among other factors, interleukin (IL)-1β and tumor necrosis factor alpha (TNFα) are found to be associated with periodontal inflammation, enabling the entry of inflammatory cells into sites of infection, which promotes bone resorption and stimulates 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] Moreover, the increase of these mediators of inflammation was reported to be involved in the imbalance between alveolar bone formation and resorption, by favoring the resorption mediated by the receptor activator of nuclear factor-κB ligand (RANKL), its receptor RANK, and a decoy receptor osteoprotegerin (OPG).[9,10] Likewise, nitric oxide (NO) is a free radical involved in various pathophysiological 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 parotid and sublingual glands.[13] While under physiological conditions the protective potential is sufficiently maintained by salivary flow, this state seems to be disturbed in periodontitis as reported by Amer et al., (2011), who demonstrated that in rats subjected to ligature-induced periodontitis, SMG salivary secretion diminishes and changes in composition.[14] On the other hand, alteration of salivary flow and composition was shown to aggravate periodontitis.[15]

The endocannabiniod system (ECS) is a signaling network that modulates a diverse spectrum of physiological processes including nociception, behaviour, appetite, motor control, memory formation and inflammation.[16] It comprises endogenous ligands such as anandamide (AEA) and 2-arachidonoylglycerol; a series of mechanisms for their synthesis

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and degradation; and classical G-protein-coupled membrane receptors, being CB1 and CB2 the main specific cannabinoid receptors and transient receptor potential vanilloid type 1 (TRPV1) the main unspecific one.[17] Although cannabinoid receptors are present in different kind of cells and tissues, CB1 is highly expressed in central nervous system and to a lower extent in peripheral tissues, whilst CB2 is expressed mainly in immune cells such as monocytes, macrophages, lymphocytes 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 the suppression of cytokine production, inhibition of cell proliferation, induction of apoptosis and induction of T-regulatory cells.[20] On the other hand, there is extensive evidence showing that endocannabinoids and their receptors are involved in bone metabolism by regulating bone mass, bone loss and bone cells function.[21,22] Even more, endocannabinoids are synthesized in bone tissue.[21] Cannabinoids also promote the proliferation of human gingival fibroblasts (HGFs) via CB1/CB2 receptors in periodontal healing, and therefore, the ECS may have an important modulatory role in such process.[23] In turn, CB2 receptors have been reported to be expressed in osteoblasts, osteocytes and osteoclasts.[21]

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

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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 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 Dental School of the University of Buenos Aires, Argentina and were carried out in accordance with the guidelines of the National Institutes of Health (NIH).

Experimental design

Rats were distributed in four groups (6 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 first upper and

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lower molars, and into the inter-dental space between the first and second upper and lower 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 remained intact along the experiments. This protocol of injections was executed during 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 G microfine insulin syringe.

Topical treatment with HU-308

HU-308 was prepared, first by dissolving the powder drug in 100% ethanol and then diluted in saline, obtaining a final concentration of 500 ng/ml to treat the animals, containing 1% ethanol, approximately. The volume of each topical application was 200 µl per tooth, resulting in 800 µl per animal whenever the treatment is performed. Except for the intact controls, the remaining 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 the sites of LPS/saline injections, throughout the six weeks of the experiment. The optimal dose of HU-308 was obtained based on our 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 preliminary studies.

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Macroscopic examination of periodontal bone loss: distance and width methods

Immediately after rats sacrifice, hemi-mandibles 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 a 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 the alveolar bone loss in millimeters.

On the other hand, mandibular alveolar process width was measured in the first lower molar area. The distance between a point located at the central root level of the buccal face and other equally located at the lingual face was obtained from the lower jaw bones, with a digital caliper in millimeters[§].

Histological analysis

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[‡] and using a microtome[ǀ], sections oriented mesial-distally of each first lower molar were obtained from paraffin blocks. Sections 5 mm in width were stained with hematoxylin and eosin, and histomorphometrical evaluation was performed on digitized microphotographs using imaging software[¶]. Interradicular bone loss was evaluated by measuring the periodontal space height, plotting ten equidistant lines between the alveolar crest and the cementum of the furcation zone. The length of the lines was measured and the mean value was calculated. Additionally, alveolar bone loss 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 narrow and periodontal ligament.

Biochemical analysis

Measurements of iNOS activity

The activity of inducible nitric oxide synthase (iNOS) was measured in gingival tissue and SMG by modifying the method of Bredt and Snyder, 1989 .[31] In brief, gingival tissues were homogenized in 500 µl of cold 20mM HEPES (pH 7.4) with EGTA (2 mM) and DL-dithiothreitol (DTT, 1 mM). A similar procedure was used for the SMG, but this time the tissue was homogenized in 600 µl of HEPES. After the tissue homogenates were achieved, NADPH (120µM) and 200,000 dpm of [14C]-arginine were added to each tube and incubated for 10 min at 37ºC in a Dubnoff metabolic shaker (50 cycles per min; 95% O2/ 5% CO2) at 37°C. Then, the tubes were centrifuged at 10,000 x g for 10 min at 4ºC. The supernatants were applied 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 Liquid Scintillation Analyser TriCarb 2800TR[#]. Since NOS converts arginine into equimolar quantities of NO and citrulline, the data were expressed as pmol of NO produced per min per mg of protein.

Radioimmunoassay of PGE_2

To determine PGE_2 content, gingival tissue and SMG were homogenized in 500 µl and 1000 µl of absolute ethanol, respectively, and after centrifugation, the supernatants were dried in a Speedvac at room temperature. Thereafter, the

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residues were resuspended with buffer; antiserum from Sigma-Aldrich[**] was used as described in Mohn et al., 2011.[32] The sensitivity of the assay was 12.5 pg per tube. The crossreactivity of PGE_2 and PGE_1 was 100%, but the crossreactivity of other prostaglandins was 0.1%. The intra- and interassay coefficients of variation for PGE_2 were 8.2% and 12%, respectively. The results were expressed in pg of PGE per mg of wet weight, since the protocol of PGE extraction from the tissue includes homogenization in ethanol that interferes with protein determination. [3H]PGE_2 was purchased from New England Nuclear Life Science Products[††].

Determination of TNFα

For TNFα preservation after extraction, the gingival tissue was immediately homogenized in PBS buffer containing protease inhibitory cocktail for mammalian tissue extracts[**]. Concentration of TNFα was determined using a sandwich ELISA according to the manufacturer´s instructions[‡‡]. Data were expressed as pg TNFα/ml.

Stimulated salivary secretion assessment

Rats were removed from cages one week before their sacrifice and anesthetized with intraperitoneal injection of ketamine hydrochloride (70 mg/kg body weight) and 2 % xylazine hydrochloride (10 mg/kg body weight). Then, a dose of pilocarpine (0,5mg/kg body weight) was administered intraperitoneally to induce salivation and a cotton ball was immediately placed under the rat's tongue to take up the total salivary secretion, which was determined as the difference in weight of the cotton ball before and after collection. The procedure of saliva collection with the cotton ball was done at 30-min intervals after pilocarpine was administered, during 90 minutes.[33]

Chemicals

LPS serotype 055-B5 from *E.coli* (lyophilized powder, purified by trichloroacetic acid extraction, 1-10% of protein impurities), PGE_2 standard and antiserum, DLL-DTT, NADPH, HEPES and pilocarpine were purchased from Sigma-Aldrich[**], Dowex AG 50W-X8 Na+ form mesh 200–400 was obtained from Bio-Rad Laboratories[§§], and [14C]-arginine monochloride (297 mCi/mmol) was from Perkin–Elmer[#]. [3H] PGE_2 was purchased from New England Nuclear TMLife Science Products[ll]. HU-308 was purchased from Tocris[¶¶]. Xylazine hydrochloride and ketamine hydrochloride were purchased from König Laboratories SA[##] and Holliday-Scott SA[***], respectively.

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Statistical analysis

Data are expressed as means ± SEM. The results were evaluated by One Way ANOVA followed by the Newman-Keuls multiple comparisons 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.

Results

Alveolar bone loss: distance method

The alveolar bone loss produced as a result of LPS-induced periodontitis, measured at lingual/palatal and buccal side by the distance method (Fig. 1A), was lessened by the treatment with HU-308 (500 ng/ml). This effect was observed both in the maxilla, at buccal side (Fig. 1B), as in the jaw, at lingual side (Fig. 1C). The buccal side of jaws showed lower differences than the lingual side among groups, perhaps as a consequence of the thicker bone plate. Even more, HU-308 group showed no significant changes compared to LPS group in buccal side of jaws. Moreover, a significant impact caused by saline application was noticed at the lingual side on jaws (Fig. 1C); perhaps due to a traumatic effect generated just by the needle puncture on the gingival tissue during six weeks.

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Alveolar bone loss: width method

Alveolar bone level measured in buccal-lingual direction of first lower molars (Fig. 2) was reduced in rats subjected to LPS, while HU-308 treatment prevented that decrease, also showing a bone preservation effect (Table 1).

Histological analysis

In the interradicular area, LPS injected rats showed a higher periodontal space (Fig. 3A and B) and lesser alveolar bone area represented as BV/TV (%) (Fig. 3A and C) than control and vehicle groups, while HU-308 treated rats, showed a prevention of these deleterious effects. Additionally, the interradicular bone in LPS injected rats had erosive surfaces with presence of osteoclasts, and active bone remodeling with predominance of reversal lines. Conversely, in HU-308 treated rats, the bone showed a less active bone remodeling, aimed at repairing, osteogenesis with active osteoblasts and osteoid (Fig. 3A).

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Inflammatory markers in gingival tissue

The activity of iNOS in gingival tissue, which was increased in rats with LPS-induced periodontitis, exhibited a reduction when the animals were also treated with HU-308 (Fig. 4A). Gingival TNFα content was lesser in rats with LPS-induced periodontitis treated with HU-308 as compared to rats with LPS-induced periodontitis but treated daily only with HU-308 vehicle (Fig. 4B). Prostaglandin E_2 content was significantly higher in the gingival tissue of rats with LPS-induced periodontitis as compared to controls. Once more, treatment with HU-308 decreased PGE_2 content compared to untreated rats (Fig. 4C).

Salivary secretion evaluation

The collected total salivary secretion was not significantly different between groups after 30 minutes of pilocarpine stimulation; however an unexpected tendency of lower salivation in intact controls can be observed respect to all other groups. This difference could suggest a lower response to pilocarpine in intact animals, although the observed tendency have no statistical significance due to the high deviations (Fig. 5). After 30-60 minutes of pilocarpine stimulation, rats with LPS-induced periodontitis showed a reduced salivary response as represented by lower levels of saliva compared to control and vehicle groups, with this effect of LPS being prevented in animals treated with HU-308. A similar effect was observed in the period 60-90 min, where HU-308 also restored the diminished response of salivary glands to pilocarpine, caused by LPS-induced periodontitis.

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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 gingival tissue (Fig. 6A and B). Unexpectedly, the rats treated with HU-308 showed reduced levels of iNOS activity compared to controls.

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Discussion

There are many studies in the literature showing the influence of LPS both on the increase of bone loss as well as on inflammatory mediators.[34-37] Recently, Jin *et al*., 2014, have worked with local injections of LPS in rats, showing that induced periodontitis increases alveolar bone loss and stimulates leukocyte infiltration to the tissue and the expression of osteoclastogenic molecules.[36] RANKL is essential for the 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 TNFα potently increases osteoclast proliferation/differentiation in the presence of RANKL.[39] Besides, IL-1 and LPS were reported to stimulate osteoclastogenesis through two parallel events: direct enhancement of RANKL expression and suppression of OPG expression, which is mediated by PGE_2 production.[40] In concordance, we demonstrated that LPS-induced periodontitis increased alveolar bone loss as well as gingival inflammatory mediators involved in such disease including PGE_2, nitric oxide and TNFα. These factors are usually linked to periodontal pocket formation, insertion tissue loss, gingival bleeding and tooth mobility.[37] In these installed conditions, we evaluated the effect of a synthetic cannabinoid applied locally on the area of experimental periodontitis induction. Our results showed that HU-308 causes a clear reduction of alveolar bone loss and inflammatory mediators in gingival tissues, which are increased by LPS-induced periodontitis in the absence of treatment. While the main effect of the treatment with HU-308 is supposed to be local, we cannot discard the presence of a systemic effect due to some swallowing of the formulation.

Based on all mentioned reports, it is clear that the attenuation of gingival inflammatory parameters, after the treatment with HU-308, leads to bone metabolism normalization. In accordance with these results, Qian et al., 2013 have demonstrated that LPS increases the concentration of IL-1β, IL-6, TNFα and RANKL in human periodontal ligament cells treated *in vitro*, and that all these parameters were attenuated by HU-308.[37] This evidence supports the importance of the CB2 receptors activation in inflammatory and immune responses as well as in bone formation and its resorption in order to control periodontal disease. Even more, in a study of osteoporosis, a non-infectious disease, CB2 receptor has been postulated as a bone mass regulator.[41] Apparently, CB2 receptors activation would stimulate osteoblastic cells differentiation and would mitigate osteoclastogenesis, suggesting that the CB2 receptor signaling produces blockage of bone loss via a direct action on bone cells and, at the same time, through the inhibition of the expression of proresorptive cytokines.[42] Independently of bone loss, CB2 receptor activation could also be able to reduce pain and substances released during inflammation through inducing apoptosis in activated T cells, reducing the number of activated T cells, and suppressing induction of mast cells, NK cells, and neutrophils at sites of inflammation.[43]

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CB1 receptor has been classically associated with the central and peripheral nervous system effects of cannabinoids, while CB2 receptor has been linked to immune cells and inflammatory processes, along with the bone remodeling boosted by them.[41] Our present results support these reports. Nevertheless, in a previous study, we 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 (GPR55), has been studied in recent years as a nonspecific target for cannabinoids, showing similarities with 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 over-expressed in response to LPS.[44]

In another vein, rats with LPS-induced periodontitis exhibited lesser total salivary response to pilocarpine and higher values of PGE_2 and nitric oxide in the SMG. It is well known that SMG is the major contributor to total saliva production in oral cavity, and that salivary secretion is an essential factor in the statement and progression of oral infectious processes.[30,45] In this line, the influence of periodontitis on the pathophysiology of the SMG was evidenced in the present work. In concordance with our results, PGE_2 and nitric oxide were augmented in SMG of rats subjected to the ligature-induced model of periodontitis.[14] Additionally, the increase of these parameters was demonstrated to be involved in the consequent reduction of salivary secretory function.[46] These results are in agreement with our findings where, interestingly, HU-308-treated animals showed attenuated levels of PGE_2 and nitric oxide in SMG, and a recovery of salivary function, suggesting that both the periodontal damage progression and its recuperation, alter the pathophysiology of salivary glands. Furthermore, since our evaluation of wet and dry weight of submandibular and sublingual glands did not show significant differences between groups (data not shown), we believe that LPS-induced periodontitis could alter salivary secretion function by modifying signaling pathways, rather than affecting glandular parenchyma.

On the other hand, despite the obvious indirect effect of cannabimimetic agents on salivary function due its preventive effect of LPS-induced periodontitis, we cannot discard its direct influence on salivation, since CB1 and CB2 receptors have already been found in acinar and ductal cells of SMG.[28] Additionally, it has been demonstrated that endocannabinoids production is increased in SMG of rats with endotoxemia, induced by intraperitoneal injections of LPS, while salivary secretion is decreased.[47,48] All this exposed evidence might suggest that the ECS, first, takes a significant part in the periodontal disease control mechanisms, and second, acts through more than one pathway to its anti-inflammatory role.[49,50]

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Conclusion

In conclusion, based on our results, the beneficial effect of HU-308 on alveolar bone, gingival tissue and salivary function, evidences the participation of CB2 receptor signaling in the control of periodontal damage and its associated oral alterations caused by inflammatory processes, supporting the participation of the endocannabinoid system in homeostasis recovery under pathophysiological conditions.

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Figures Legend

Figure 1. **(A) Photographs showing alveolar bone loss, measured by the distance method on the mesial, central and distal roots of the first molar.** Each bar represents one millimeter. **Below, the effect of LPS-induced periodontits and its treatment with HU-308 on the alveolar bone loss covering the roots of (B) upper and (C) lower first-molars**. *ρ<0,05 and †ρ<0,001 vs. control, ‡ρ<0,05 and §ρ<0,01 vs. vehicle, ¶ρ<0,05 vs. LPS. Results are means ± SD.

Figure 2. Picture showing the lower molars and the site of the alveolar bone width measurement.

Table 1. Alveolar bone width evaluated in the zone of the central root of the lower first molar. *ρ<0,001 vs. Control, †ρ<0,01 vs. LPS. Results are means ± SD.

Figure 3. (A) Photomicrographs (H&E) showing the histological features of the first lower molar interradicular area of rats subjected to the different experimental conditions (control, vehicle, LPS, LPS plus HU-308). **OC** marks osteoclasts and erosive surfaces; **OB** marks osteoblasts and osteoide formation. (**B) Periodontal space height evaluation.** (**C) Percentage of interradicular bone measured as BV/TV (%).** *ρ<0,05 and †ρ<0,01 vs. control, ‡ρ<0,05 vs. vehicle, §ρ<0,05 and ¶ρ<0,001 vs. LPS. Results are means ± SD.

Figure 4. Effect of LPS-induced periodontits and its treatment with HU-308 on (A) iNOS activity, (B) TNFα content, and (c) PGE_2 content, in the gingival tissue. * ρ<0,05 vs. control, †ρ<0,01 vs. vehicle, ‡ρ<0,05 vs. LPS. Results are means ± SD.

Figure 5. Effect of LPS-induced periodontits and its treatment with HU-308 on salivary response to pilocarpine. * ρ<0,05 vs. control, †ρ<0,05 vs. vehicle , ‡ρ<0,05 vs. LPS. Results are means ± SD.

Figure 6. Effect of LPS-induced periodontits and its treatment with HU-308 on (A) iNOS activity and (B) PGE_2 content in the SMG. *ρ<0,05 and †ρ<0,01 vs. control, ‡ρ<0,05 and §ρ<0,01 vs. vehicle, ¶ρ<0,05 and #ρ<0,001 vs. LPS. Results are means ± SD.

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Footnotes

*Department of Physiology, Faculty of Dentistry, University of Buenos Aires, Buenos Aires, Argentina

†National Council of Scientific and Technical Research (CONICET), Argentina

[‡]Stemi DV4 Stereomicroscope, Carl Zeiss MicroImaging, Göttingen, Germany

[§]Digimess, Geneva, Switzerland

[ǁ]Jung AG, Heidelberg, Germany

[¶] Image Toll, University of Texas Health Science Center, San Antonio, TX, USA

[#] PerkinElmer, Waltham, MA, USA

[**] Sigma-Aldrich St. Louis, MO, USA

[††] New England Nuclear Life Science Products, Boston, MA, USA

[‡‡]BD Pharmingen, San Diego, CA,USA

[§§] Bio-Rad Laboratories, Hercules, CA, USA

[ǁǁ] New England Nuclear TMLife Science Products, Boston, MA, USA

[¶¶] Tocris, Ellisville, MO, USA

[##]König Laboratories SA, Buenos Aires, Argentina

[***]Holliday-Scott SA, Buenos Aires, Argentina

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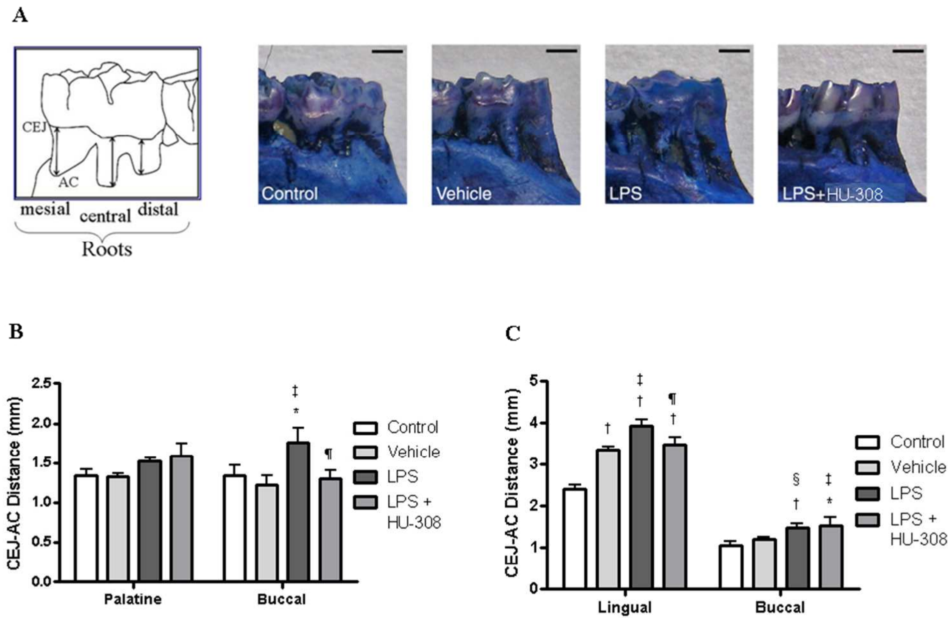

Figure 1. (A) Photographs showing alveolar bone loss, measured by the distance method on the mesial, central and distal roots of the first molar. Each bar represents one millimeter. Below, the effect of LPS-induced periodontits and its treatment with HU-308 on the alveolar bone loss covering the roots of (B) upper and (C) lower first-molars. *$\rho<0,05$ and †$\rho<0,001$ vs. control, ‡$\rho<0,05$ and §$\rho<0,01$ vs. vehicle, ¶$\rho<0,05$ vs. LPS. Results are means ± SD.
254x190mm (96 x 96 DPI)

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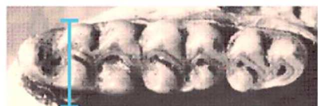

Figure 2. Picture showing the lower molars and the site of the alveolar bone width measurement.
254x190mm (96 x 96 DPI)

Review

Alveolar bone	Control	Vehicle	LPS	LPS + HU-308
Width (mm)	2.62 ± 0.07	2.54 ± 0.05	2.45 ± 0,08*	2.61 ± 0.07[†]

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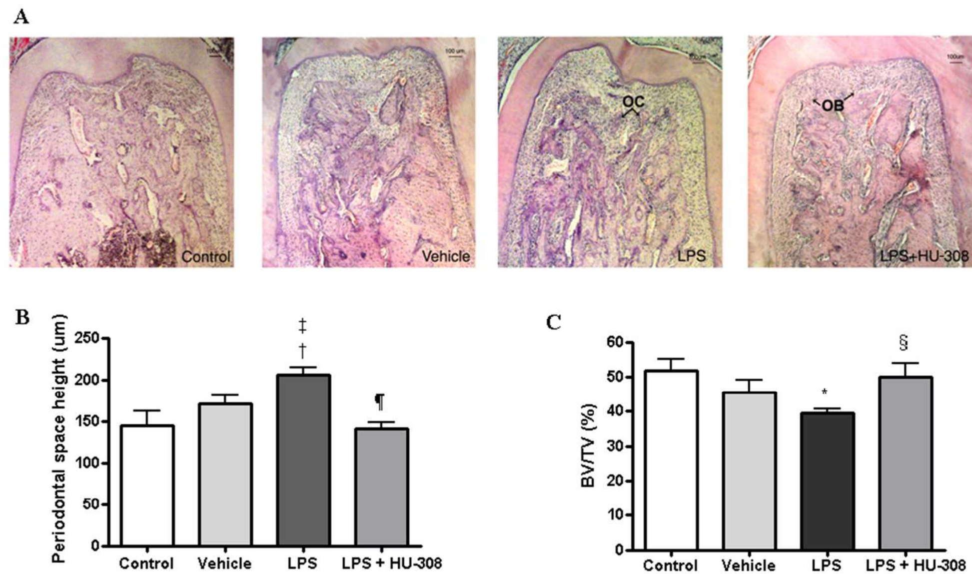

Figure 3. (A) Photomicrographs (H&E) showing the histological features of the first lower molar interradicular area of rats subjected to the different experimental conditions (control, vehicle, LPS, LPS plus HU-308). OC marks osteoclasts and erosive surfaces; OB marks osteoblasts and osteoide formation. (B) Periodontal space height evaluation. (C) Percentage of interradicular bone measured as BV/TV (%). *ρ<0,05 and †ρ<0,01 vs. control, ‡ρ<0,05 vs. vehicle, §ρ<0,05 and ¶ρ<0,001 vs. LPS. Results are means ± SD.
254x190mm (96 x 96 DPI)

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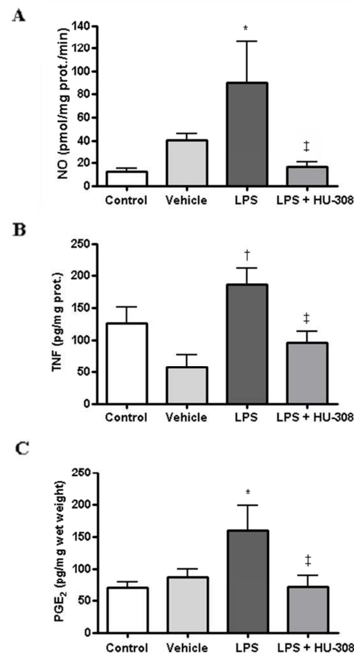

Figure 4. Effect of LPS-induced periodontits and its treatment with HU-308 on (A) iNOS activity, (B) TNFα content, and (c) PGE2 content, in the gingival tissue. * ρ<0,05 vs. control, †ρ<0,01 vs. vehicle, ‡ρ<0,05 vs. LPS. Results are means ± SD.
330x247mm (96 x 96 DPI)

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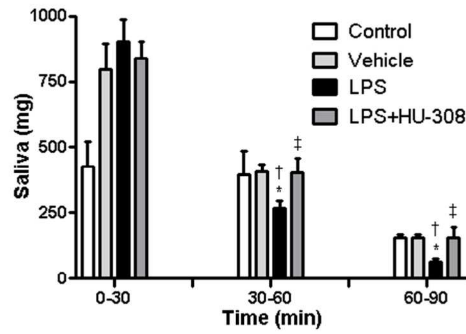

Figure 5. Effect of LPS-induced periodontits and its treatment with HU-308 on salivary response to pilocarpine. * $\rho<0,05$ vs. control, †$\rho<0,05$ vs. vehicle , ‡$\rho<0,05$ vs. LPS. Results are means ± SD.
254x190mm (96 x 96 DPI)

eview

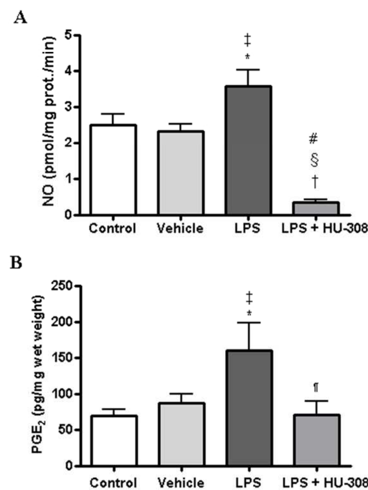

Figure 6. Effect of LPS-induced periodontits and its treatment with HU-308 on (A) iNOS activity and (B) PGE2 content in the SMG. *ρ<0,05 and †ρ<0,01 vs. control, ‡ρ<0,05 and §ρ<0,01 vs. vehicle, ¶ρ<0,05 and #ρ<0,001 vs. LPS. Results are means ± SD.
293x190mm (96 x 96 DPI)

Review