

Preventive anti-inflammatory and osteoprotective effects of the FAAH inhibitor URB597 in a rat model of LPS-induced periodontitis

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Research Article

Keywords: experimental periodontitis, alveolar bone loss, endocannabinoids, anti-inflammation

Posted Date: October 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11120122/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background: The endocannabinoid system plays a crucial role in maintaining periodontal tissue homeostasis. Fatty acid amide hydrolase (FAAH) is the main enzyme responsible for degrading the endocannabinoid anandamide (AEA). This study aimed to evaluate the effects of URB 597 (20 µg/mL), a FAAH inhibitor, in a rat model of experimental periodontitis (EP). **Methods:** EP was induced by injecting 20 µL of LPS (1 mg/mL) into the gingiva and interdental spaces of the upper and lower first molars, three times per week for six weeks. URB 597 was topically applied daily to the LPS-affected teeth (EP+URB group). **Results:** URB 597 significantly attenuated alveolar bone loss associated with LPS-induced periodontitis, as evidenced by macroscopic, histological, and microtomographic analyses. The preventive intervention reduced gingival levels of inflammatory mediators, including TNFα (EP+URB: 118.52 ± 1.2 vs. EP: 505.43 ± 2.73 ng/mg protein; $P < 0.05$) and PGE₂ both in gingiva (EP+URB: 27.72 ± 4.82 vs. EP: 54.55 ± 6.88 pg/mg; $P < 0.05$) and dental attachment tissue (EP+URB: 191.66 ± 13.66 vs. EP: 291.72 ± 16.66 pg/mg; $P < 0.05$). URB 597 also reduced LPS-induced gelatinolytic activity of MMP9 (EP+URB: 5629.64 ± 285.94 vs. EP: 16297.69 ± 322.04 intensity pixel area; $P < 0.05$) and MMP2 (EP+URB: 7218.47 ± 301.52 vs. EP: 17015.60 ± 132.86 intensity pixel area; $P < 0.05$). **Conclusion:** Topical treatment with the FAAH inhibitor URB 597 exerts preventive osteoprotective and anti-inflammatory effects in rats with LPS-induced periodontitis, supporting further investigation of FAAH inhibition as a potential modulatory strategy in periodontal diseases.

Introduction

Periodontitis (P) is a progressive oral infection that causes the deterioration of the periodontal tissues of protection (gingiva) and dental attachment (cementum, periodontal ligament and alveolar bone), due to a combined effect between products released by bacteria and substances produced by the host during the subsequent inflammatory response. This disease is initiated by the accumulation of bacterial plaque, by changes in its composition, and by the action of its harmful products including hydrolytic enzymes and toxins, but its pathogenesis also involves the host's immune response, which causes deleterious effects on periodontal tissues triggered by inflammatory mediators [1]. A key factor in the development and progression of periodontitis is dysbiosis, an imbalance in the composition of the subgingival microbiota, characterized by an overgrowth of Gram-negative and anaerobic microorganisms that disrupt the homeostatic relationship between the host and the microbial community, thereby exacerbating tissue destruction and inflammation [2].

Lipopolysaccharide (LPS) is an endotoxin from Gram-negative bacterial biofilm that leads to an infiltrate of leukocytes in the mucogingival tissue. As a primary signaling receptor for these bacterial endotoxins, Toll-like receptor 4 (TLR4) on immune cells triggers the activation of nuclear factor kappa B (NF-κB) [3–5]. This pathway plays a crucial role in periodontal disease, as NF-κB is activated by these pathogenic stimuli [6] to regulate the expression of proinflammatory genes (such as cytokines, chemokines, and adhesion molecules) which recruit and activate immune cells at the site of infection. Ultimately, the chronic activation of NF-κB leads to a sustained production of these inflammatory mediators,

perpetuating inflammation and contributing to the destruction of periodontal tissues, including the alveolar bone and periodontal ligament. All this, increases the synthesis and release of proinflammatory cytokines such as IL-1, IL-6 and TNF- α , prostaglandins, especially PGE₂, oxidative stress markers and hydrolytic enzymes with proinflammatory and catabolic activity such as matrix metalloproteinases (MMPs) [7]. Experimental evidence points to prostaglandins as mediators of bone resorption [8]. Elevated levels of PGE₂ detected in the crevicular fluid of patients with periodontitis have been related to an increase in the severity of the disease [9]. In periodontitis, MMPs play a role in the initiation of bone resorption and the destruction of periodontal soft tissues. There is a positive correlation between the severity of periodontal disease and MMP concentrations in crevicular fluid [10]. Increased expression of MMP9 has been shown to promote osteoclast formation and subsequent alveolar bone resorption. For its part, MMP2 degrades type IV collagen, causing loss of attachment tissues [11, 12].

The endocannabinoid system (ECS) is an intercellular signaling network that modulates a diverse spectrum of physiological processes including nociception, behavior, appetite, motor control, memory formation, salivary secretion and inflammation [13]. It comprises endogenous ligands such as anandamide (AEA) and 2-arachidonylglycerol (2-AG); a series of mechanisms for their synthesis and degradation; and G protein-coupled membrane receptors (CB_r), with CB1_r and CB2_r being the main cannabinoid-specific receptors and TRPV1, one of the most studied non-specific ones [14]. N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) are the main enzymes responsible for the synthesis and degradation of AEA, respectively, while diacylglycerol lipase (DAGL) and monoacylglycerol lipase (MAGL) are responsible for 2-AG. Although CB_r are present in different cell types and tissues, CB1_r is highly expressed in nervous tissue, whereas CB2_r is mainly expressed in immune cells such as monocytes, macrophages, lymphocytes and in bone cells [13]. In recent years, drugs have been developed to modulate the function of components of the ECS. URB 597 is a compound developed as a selective inhibitor of FAAH. The inhibition of this enzyme leads to the accumulation of AEA in both the central nervous system and the periphery, increasing its activity on the receptors of the system [15].

In previous studies we have shown that treatment with CB1 and CB2 selective agonists, such as methanandamide and HU 308, respectively, prevented experimental periodontitis (EP)-induced damage [16–18]. What is innovative about URB 597 preventive intervention is that, instead of acting as an exogenous cannabinoid receptor agonist or antagonist, it acts by increasing the functional availability of an endogenous cannabinoid.

While conventional treatments such as antibiotics, antiseptics and scraping aim to control bacterial growth and inflammation, researchers are exploring new therapeutic avenues, including the use of cannabinoid receptors ligands as well as endocannabinoid modulators that have potential anti-inflammatory and analgesic properties. This article delves into the potential effects of URB 597 in treating periodontal disease.

Materials and methods

Animals

Three month old adult male Wistar rats (300–350 g) from the authors' own laboratory colony were kept in group cages in an animal room with a 12-hour light photoperiod (0700–1900), room temperature maintained at 22°C–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 (CICUAL-ODON/FOUBA N° 01/2019), and were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Only male Wistar rats were used to minimize hormonal variability that could influence inflammatory and bone remodeling responses. Likewise, all animal experiments comply with the ARRIVE guidelines.

Design

Twenty one rats were distributed in the following three groups containing seven rats per group: 1) control rats, 2) rats subjected to experimental periodontitis (EP), 3) rats subjected to EP and treated with URB 597 (EP + URB).

In groups 2 and 3, periodontitis was induced by injecting 20 µl of LPS (1 mg/ml) from *Escherichia coli* (serotype 055:B5, Sigma-Aldrich, St. Louis, Missouri, USA) into the buccal and palatal/lingual gingiva of the right and left upper and lower first molars and into the interdental space between the first and the second molars (60 µl of LPS per tooth and 240µl per rat per treatment session) under inhalational sevoflurane anesthesia, while control rats did not receive any 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 [16]. Gingival injections were placed with a 13-mm 27-gauge microfine insulin syringe. Animals were monitored daily for signs of distress or discomfort throughout the experimental period. All the experiments were performed at least twice. The graphs represent the results obtained from one of them.

URB 597 [(3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate] (Cayman Chemical, Ann Arbor, Michigan, USA) was prepared by dissolving the powdered drug in 100% ethanol and subsequently diluting it in saline solution to obtain a final concentration of 20 µg/mL, with 1% ethanol as the vehicle. The concentration of URB 597 was selected based on preliminary dose-response experiments performed in the same experimental model. This approach was adopted considering the high pharmacological potency of URB 597 as a selective inhibitor of fatty acid amide hydrolase (FAAH), and the objective of achieving a sustained local pharmacological effect at the gingival tissue while minimizing unnecessary systemic exposure. At the selected concentration, each topical application delivered 4 µg of URB 597 per affected tooth, corresponding to 200 µL of solution per tooth. Thus, when all four affected teeth were treated, each animal received a total of 800 µL of the URB 597 solution (16 µg URB 597/animal/day).

To control for potential effects of the vehicle application, animals in the periodontitis group (EP) received the same volume of the vehicle solution (1% ethanol in saline solution) following an identical topical

administration protocol. Except for intact controls, all animals received daily topical applications at the sites affected by LPS injections throughout the 6-week experimental period. Accordingly, the EP and EP-URB groups were exposed to the same LPS-induced periodontal challenge, treatment procedure, application volume, frequency, and vehicle composition, with the presence or absence of URB 597 representing the experimental treatment variable.

Microscopic examination of alveolar bone loss: distance and width method

Immediately after killing the rats, the hemi-mandibles were resected, defleshed and stained with 1% aqueous methylene blue to delineate the cemento-enamel junction (CEJ) and the alveolar crest (AC) [19]. A stereomicroscope (Stemi DV4 Stereomicroscope, Carl Zeiss MicroImaging, Göttingen, Germany) and a digital caliper (Degimess, Derby, UK) were used to measure three lingual and three buccal distances (mesial, central and distal) from the CEJ to the most apical area of the AC. The sum of the six distances of each molar was used as a measure of the total alveolar bone loss per tooth in millimeters (Fig. 1A and B). Lingual and buccal alveolar bone loss was also measured independently, in the lower molars, considering the sum of the three corresponding distances.

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 (Fig. 1C and D)..

Histomorphometric analysis

Hemimandibles were extracted and fixed in a formalin buffer solution. After 3 days, they were decalcified in 10% EDTA at pH 7 for 45 days. Subsequently, the hemimandibles were dehydrated with ethyl alcohol and cleared with xylene. The portion containing the first molar of each decalcified hemimandible was then embedded in paraffin at 56°C to 58°C. Mesial-distal oriented sections of each mandibular first molar were obtained from the paraffin blocks using a Jung microtome (Leica Biosystems, Nussloch, Germany) under a stereomicroscope. Sections, 5 µm in thickness, were stained with hematoxylin and eosin (H&E), and a histomorphometric evaluation was performed on digitized microphotographs using imaging software (ImageTool, University of Texas Health Science Center, San Antonio, TX). Bone histomorphometric analysis was carried out by determining the interradicular periodontal space height and bone area. The periodontal space, expressed as tissue width (T.Wi), was determined by drawing 10 equidistant lines between the AC and the cementum of the furcation zone, and the mean value was calculated. The bone area was evaluated by obtaining the ratio bone area (B.Ar)/tissue area (T.Ar) (%), which represents the fraction of tissue area corresponding to bone tissue. T.Ar was calculated as bone tissue plus bone marrow and periodontal ligament. Histomorphometric analyses were performed by an examiner blinded to the experimental groups.

Micro-computed tomography (μ CT) assessment

After a 48 hour-period of fixation in 4% paraformaldehyde solution and 10% 0.1M phosphate buffer (pH 7.4), hemimandible samples were scanned using a high-resolution micro-computed tomography system (SkyScan 1172, Bruker microCT, Kontich, Belgium) at 90 kV, 111 μ A, with a 0.5 aluminium + 0.038 copper filter and 36% beam hardening correction, ring artifacts reduction 13, 180 degrees of rotation and exposure range of 0.4 degree. Images were captured with 1304x1024 pixels and a resolution of 13.2 μ m pixel size. Projection images were reconstructed using NRecon software (version 1.7.1.0, Bruker microCT, Kontich, Belgium), and three-dimensional images were obtained using CT-Vox software (Bruker microCT, Kontich, Belgium). Morphological parameters of trabecular bone microarchitecture were assessed employing CTAn software (Bruker microCT, Kontich, Belgium), in accordance with Bouxsein et al recommended guidelines [20].

One region of interest was confined to the first molar-interradicular trabecular bone and limited by its four roots. Tomograms were oriented in the axial direction, considering the most coronal part of the interradicular bone and the last tomogram which showed the four first molar roots, as the upper and lower borderlines, respectively. Working area was drawn among these four roots and redefined as tomograms progress. Measurements of axial images included bone volume fraction (bone volume/total volume, BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular separation (Tb.Sp, mm) and trabecular number (Tb.N, 1/mm). On the other hand, interradicular bone was also assessed through tomograms oriented in the parasagittal direction. Images for this evaluation were selected so that whole mesial and distal roots and bone located between them appeared in tomograms, while central (lingual and buccal) roots remained out of them. In the parasagittal images, the periodontal space height was evaluated by obtaining the average of three equidistant lines between bone surface and dental surface (BS-DS) in millimeters.

Determination of TNF- α

After extraction, the gingival tissue from upper and lower molars was promptly homogenized in phosphate-buffered saline containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA) suitable for mammalian tissue extracts to preserve TNF- α . The concentration of TNF- α was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) following the manufacturer's guidelines. In summary, a 96-well ELISA plate was used, where capture antibody, standards, and samples, detection antibody, streptavidin-peroxidase (SAv-HRP) enzyme (BD Pharmingen, San Diego, CA), and tetramethylbenzidine (TMB) (BD Pharmingen, San Diego, CA) substrate were added in sequence. Proteins concentration was determined by following the Bradford method [21]. The results were expressed as nanograms of TNF- α per mg of protein.

Radioimmunoassay of PGE

To determine the PGE₂ content, gingival or dental attachment tissues, from upper and lower molar, were homogenized in 500 ml of absolute ethanol. After centrifugation, the supernatants were dried using a centrifugal vacuum concentrator (SpeedVac, Thermo Fisher Scientific, Waltham, MA) at room temperature. The residues were then suspended in buffer, and PGE₂ antiserum (Sigma-Aldrich, St. Louis, Missouri, USA) was used as described by Mohn et al. [22]. The assay's sensitivity was 12.5 pg per tube. The cross-reactivity between PGE₂ and PGE₁ was 100%, while the cross-reactivity with other prostaglandins was 0.1%. The intra-assay and inter-assay coefficients of variation for PGE₂ were 8.2% and 12.0%, respectively. The results were expressed in picograms of PGE per milligram of wet weight.

Metalloproteinases by gelatin zymography

The enzymatic activity of MMP-2 and MMP-9 in gingival tissue, from upper and lower molars, was assessed using gelatin zymography. Briefly, a homogenate of gingival tissue from the unilateral upper and lower first molar was prepared and introduced into a 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) and 1 mg/ml purified gelatin (G8150, Sigma-Aldrich, St. Louis, Missouri, USA) as the MMP substrate. This gel was then placed in an electrophoretic cell with running buffer. After electrophoresis, the gels were washed and incubated in an appropriate buffer for 24 hours at 37°C to allow proteases to degrade the gelatin. The gelatin was stained with Coomassie Blue R-250 solution (Imperial Chemical Industries plc, London, UK) to visualize the clear bands corresponding to enzymatic activity, and then subjected to a bleaching solution. MMP-2 and MMP-9 were identified based on the molecular weight of the proteolytic bands, compared with a commercial standard (Rainbow Marker, Amersham ECL, Buckinghamshire, UK). The intensity of the stained bands was determined by densitometry using Image J software, and results were expressed in arbitrary densitometry units. Protein concentration was determined using the Bradford method [21]. Salivary gland homogenates, known for their high concentration of MMP-2 and MMP-9, were used as positive controls (data not shown).

Salivary secretion assessment

Rats were removed from cages one week before euthanasia and anesthetized with an intraperitoneal injection of ketamine hydrochloride (Holliday-Scott SA, Buenos Aires, Argentina) (70 mg/kg body weight) and 2% xylazine hydrochloride (König Laboratories SA, Buenos Aires, Argentina) (10 mg/kg body weight). Then, a sole dose of pilocarpine (Sigma-Aldrich, St. Louis, Missouri, USA) (0.5 mg/kg body weight) was administered intraperitoneally to induce salivation, and a cotton ball was immediately 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. When the amount of saliva exceeded the absorption capacity of a cotton ball, it was replaced with another similar ball, and both were evaluated. Saliva collection was carried out at 30-minute intervals after the administration of pilocarpine, over a 90-minute total period.

Statistical Analyses

Data are expressed as means $\pm$ standard error of the mean. Results were evaluated by one-way analysis of variance followed by the Tukey's 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

Alveolar Bone loss (ABL): Distance Method

Total alveolar bone loss, measured as the sum of the six distances described in Materials and Methods between the cemento-enamel junction and the alveolar crest (CEJ-AC) and resulting from LPS-induced EP in the mandibular first molars, was reduced by preventive intervention with URB 597 (20 μ g/mL). Furthermore, when CEJ-AC distance was measured separately in lingual and buccal sides in each jaw, the same preventive effect of URB 597 was observed (Fig. 2 and Table 1).

ABL: Width Method

Alveolar bone width measured in a buccal-lingual direction in mandibular first molars was reduced in rats subjected to EP, while URB 597 treatment prevented that reduction, showing a bone preservation effect (Table 1).

Histological Analysis

In the interradicular area, rats subjected to EP showed a greater periodontal ligament space (T.Wi.) and less alveolar bone area represented as B.Ar/T.Ar. (%) compared with the control group. However, EP rats treated with URB 597 showed no signs of these deleterious effects, exhibiting a smaller periodontal ligament space and a larger alveolar bone area than the EP rats (Fig. 3).

Micro-computed tomography (μ CT) assessment

In the interradicular region, the Control and EP + URB 597 groups showed lower periodontal space height compared to the EP group (Fig. 4) and greater bone volume fraction (BV/TV) (Table 2). Additionally, EP rats exhibited reduced trabecular number (Tb.N) and trabecular thickness (Tb.Th), and higher trabecular separation (Tp.Sp) relative to both Control and EP + URB 597 groups, indicating a preventive effect of the treatment on bone damage (Table 2)

Inflammatory Markers in gingival and dental attachment tissues

The elevated gingival levels of TNF- α and PGE₂ produced by the induction of periodontitis were prevented by URB 597 treatment (Fig. 5A and B). Likewise, PGE₂ levels, increased in dental attachment tissues of rats with EP, remained at control values after the treatment with URB 597 (Fig. 5C).

Additionally, EP rats showed greater gingival activity of MMP9 and MMP2 compared to controls. In both cases, URB 597 preventive intervention maintained, at least partially, enzymatic activity levels close to those of controls (Fig. 6).

Salivary secretion assessment

After 30 minutes of pilocarpine stimulation, the hyposalivation induced in the group of animals subjected to EP was prevented with URB 597 (Fig. 5). During the following time periods, 30–60 and 60–90 minutes, salivary secretion tended to decrease in all groups and no differences were found between them.

Discussion

Before discussing the potential benefits of URB597, it is important to consider the mechanisms underlying periodontal disease. Periodontitis typically originates from the accumulation of dental plaque, a bacterial biofilm on tooth surfaces, which triggers a host inflammatory response. The interaction between bacterial products and the immune system promotes inflammation and progressive destruction of periodontal tissues, including the gingiva, periodontal ligament, and alveolar bone, potentially leading to tooth mobility and tooth loss.

Although LPS derived from *E. coli* is not a typical component of the oral biofilm, it has been widely used as an experimental tool to reproduce key inflammatory features of periodontal disease. As a Gram-negative bacterial product, *E. coli* LPS activates innate immune signaling pathways, including NF- κ B, and promotes the release of pro-inflammatory cytokines in periodontal tissues. While differences in Toll-like receptor activation may exist compared with LPS from specific periodontal pathogens, the resulting inflammatory response shares important mechanistic characteristics with those involved in periodontal disease progression. Moreover, the *E. coli* LPS-induced periodontitis model has been extensively characterized and validated in rodents, including in previous studies conducted by our group [16–18, 35, 42].

The exploration of therapeutic strategies targeting the endocannabinoid system (ECS) to treat inflammatory diseases, including periodontitis, is actively progressing [23–25]. Topical administration of cannabinoid receptor agonists has demonstrated beneficial effects in experimental periodontal inflammation. In previous studies, we demonstrated the beneficial effects of daily topical treatment with cannabinoid receptor agonists in rats subjected to experimental periodontitis. Meth-AEA, a selective CB1 receptor agonist, exerted osteoprotective and anti-inflammatory effects in rats with LPS-induced periodontitis, reducing alveolar bone loss and gingival inflammatory mediators [16]. Subsequently, daily topical treatment with the selective CB2 receptor agonist HU 308 improved alveolar bone, gingival health, and salivary function in the same experimental model [17]. Both treatments reduced gingival TNF- α and PGE₂ levels and attenuated endotoxin-induced alveolar bone loss, while HU 308 additionally reduced inducible nitric oxide synthase activity.

In the present study, we investigated a preventive intervention aimed at increasing the functional availability of anandamide through FAAH inhibition in rats subjected to LPS-induced experimental periodontitis. FAAH is a homodimeric serine amide hydrolase associated with the endoplasmic reticulum membrane. URB597 binds covalently to FAAH near its catalytic region, resulting in sustained inhibition of anandamide hydrolysis [26]. Following topical application to periodontal tissues, URB597 significantly prevented LPS-induced alveolar bone resorption. This protective effect was supported by morphometric, histological, and microtomographic analyses. URB597 prevented the increase in the CEJ-AC distance induced by LPS and the reduction in alveolar bone width at the lower first molar. Histomorphometric analysis further showed that URB597 prevented the reduction in alveolar bone area relative to total area observed in EP animals. Similarly, the increase in periodontal space height observed in EP rats was prevented by URB597 treatment. Microtomographic analysis confirmed these findings, showing that URB597 prevented the increase in periodontal ligament space. In addition, the ratio of interradicular bone volume to total volume and the three trabecular parameters evaluated, all of which were altered by EP, showed values comparable to those of controls following URB597 treatment.

Although topical administration of URB597 to periodontal tissues has not previously been characterized, increasing evidence supports the feasibility of local drug delivery strategies in periodontitis, allowing high local drug concentrations while potentially reducing systemic exposure [16–18]. Several physicochemical and biological characteristics may support URB597 penetration into periodontal tissues. Its high lipophilicity facilitates diffusion across biological membranes and distribution through gingival epithelial and connective tissues. In addition, the inflammatory microenvironment induced by LPS is associated with increased vascular permeability and disruption of epithelial barrier integrity, which may further facilitate tissue penetration. Because FAAH is localized in the endoplasmic reticulum, effective inhibition requires intracellular access. Previous pharmacological studies have shown that URB597 rapidly reaches intracellular compartments and irreversibly inhibits FAAH through covalent carbamylation of its catalytic serine residue, thereby increasing local endocannabinoid availability [27, 28]. Nevertheless, although the anti-inflammatory and osteoprotective effects observed here are compatible with enhanced endocannabinoid signaling following FAAH inhibition, the present study did not directly assess AEA concentrations, FAAH activity, or cannabinoid receptor activation. Therefore, the mechanisms underlying these effects remain inferential, and future studies should address receptor-specific participation, downstream signaling pathways, and local endocannabinoid concentrations in periodontal tissues.

There is substantial evidence that endocannabinoids and their receptors participate in bone metabolism and remodeling [25]. CB2 receptors have been identified in osteoblasts, osteocytes, and osteoclasts, and both CB1 and CB2 receptors have been detected in gingival tissue [29]. AEA has been reported to reduce excessive periodontal inflammation, whereas cannabidiol has been shown to attenuate bone loss associated with EP by reducing TNF- α and IL-1 β production [30, 31]. However, contradictory effects have also been reported for different endocannabinoids, with AEA showing anti-inflammatory effects and 2-AG exhibiting pro-inflammatory effects in studies involving periodontal ligament cells [31].

Cannabinoids can also enhance the proliferation of human gingival fibroblasts through CB1 and CB2 receptors, suggesting a role for the ECS in periodontal healing [32]. In addition, treatment of human periodontal ligament cells with the CB2 agonist HU 308 increased the expression of osteogenic genes while decreasing the expression of osteoclastogenic genes, and enhanced mineralization [33]. Other CB2 receptor agonists have likewise been shown to reduce bone loss associated with osteoporosis and bone cancer [34]. Together, these findings support a role for ECS modulation in oral pathological processes, although relatively few studies have specifically investigated its pharmacological manipulation in periodontal disease.

Regarding inflammatory parameters associated with periodontitis, daily application of URB597 significantly prevented the increase in gingival TNF- α in rats subjected to EP. Similarly, the increase in PGE₂ levels in gingival and dental attachment tissues induced by EP was prevented by URB597 treatment. Extracellular MMP9 and MMP2 activity also increased in the gingiva of rats with LPS-induced periodontitis, but this enzymatic activity was only partially affected by URB597. Overall, these findings indicate that topical URB597 exerted both anti-inflammatory and osteoprotective effects in this model.

Periodontitis has been associated with an imbalance in the ECS, and restoration of this balance through cannabinoid administration or modulation of endocannabinoid signaling has been proposed as a potential strategy for controlling periodontal inflammation [35]. Given the central role of NF- κ B in periodontal inflammation, AEA may contribute to the anti-inflammatory effects associated with FAAH inhibition by modulating NF- κ B activity. Previous studies have shown that AEA can inhibit NF- κ B activation, including its nuclear translocation and DNA-binding activity, thereby reducing the expression of NF- κ B-dependent pro-inflammatory genes [36, 37]. AEA has also been associated with increased expression of the anti-inflammatory cytokine IL-10, which may further contribute to the attenuation of inflammatory responses [38]. Thus, the anti-inflammatory effects observed after URB597 treatment in the present study may, at least in part, result from increased local AEA availability and subsequent modulation of NF- κ B-dependent pathways. However, because neither AEA levels nor NF- κ B activity was directly assessed, this interpretation should be considered a mechanistic hypothesis rather than a demonstrated pathway in the present model.

Supporting our findings, a previous study demonstrated that PF-3845, another FAAH inhibitor, reduced alveolar bone loss and local osteoclast numbers in a one-week ligature-induced periodontitis model in mice, through suppression of ERK and NF- κ B signaling and downstream NFATc1 expression, a major transcriptional regulator of osteoclast differentiation [39]. Interestingly, the same study found that URB597 did not directly inhibit RANKL-induced osteoclastogenesis *in vitro*. In contrast, our *in vivo* findings demonstrate a clear protective effect of local topical URB597 against periodontal bone loss. This apparent discrepancy suggests that FAAH inhibition may exert different effects depending on the experimental context. In particular, the chronic six-week LPS-induced inflammatory environment used in the present study, together with local topical administration, may involve complex interactions among immune, periodontal, and bone-remodeling cells that are not reproduced in short-term *in vitro* monocultures or acute one-week ligature models. Consistent with this interpretation, the protective

effect observed in our study does not necessarily imply a direct inhibitory action of URB597 on osteoclast differentiation, but may instead reflect modulation of the inflammatory microenvironment that regulates periodontal bone remodeling.

In a recent publication from our group, we demonstrated that CB1 and CB2 receptor gene expression is upregulated in the gingival tissue of rats subjected to LPS-induced periodontitis, likely representing an allostatic response aimed at controlling oral inflammation and tissue damage under these conditions [40]. This finding, together with the present results, further supports the potential relevance of ECS modulation as a strategy to reduce inflammation and protect periodontal tissues.

Additional evidence supports the anti-inflammatory potential of FAAH inhibition in other tissues. In human intestinal mucosal tissue, inflammation increased the secretion of granulocyte-macrophage colony-stimulating factor, IL-12, IL-13, and IL-15, whereas *ex vivo* treatment with URB597 and JZL184, a monoacylglycerol lipase inhibitor that increases 2-AG availability, prevented these changes. This effect was abolished by CB1 receptor blockade [41]. These findings indicate that endogenous AEA and 2-AG, together with CB1 receptor activation, may contribute to the regulation of tissue responses under inflammatory and hypoxic conditions. Although such findings cannot be directly extrapolated to periodontal tissues, they provide additional support for the concept that FAAH inhibition may modulate inflammatory responses through endogenous cannabinoid signaling.

URB597 has also shown anti-inflammatory effects in other experimental models. Topical administration of URB597 reduced acute inflammation in mice with monoiodoacetate-induced osteoarthritis, decreasing osteoarthritis pain and preventing joint nerve damage during the chronic degenerative phase [42]. In another mouse model, URB597 was the most active compound in suppressing carrageenan-induced edema compared with capsazepine, a TRPV1 blocker, and AA-5-HT, which combines TRPV1-blocking and FAAH-inhibitory properties [43]. These findings, together with the present results, suggest that the anti-inflammatory actions of URB597 may extend beyond periodontal tissues.

Experimental models of periodontitis also show alterations in salivary secretion [44]. In a previous study from our group, administration of a CB2 receptor agonist prevented the hyposalivation induced by LPS-associated periodontitis [17]. A similar preventive effect was observed in the present study following FAAH inhibition and increased AEA availability. However, because stimulation of CB1 and CB2 receptors has also been associated with reduced salivary secretion through local and central mechanisms [45, 46], and because periodontal disease and hyposalivation may reciprocally influence one another in experimental models [47], the preservation of salivary secretion observed here may represent an indirect consequence of attenuated periodontal damage rather than a direct effect of enhanced ECS signaling within the salivary glands or autonomic nervous system.

It is important to note that URB597 has not been approved for clinical use in humans, and further studies are required to determine its safety and efficacy in inflammatory diseases, including periodontitis. Because modulation of the ECS may produce systemic effects, potential adverse effects and limitations must be carefully evaluated before considering clinical applications. The present study also has several

limitations. First, URB597 administration began simultaneously with disease induction; therefore, the experimental design evaluates preventive rather than therapeutic efficacy after periodontal destruction has been established. Second, a URB597-only group was not included, which would have allowed assessment of possible baseline effects of the compound on periodontal tissues independently of inflammation. This decision was deliberate, as preliminary studies conducted by our group in a small number of animals indicated that URB597, similarly to CB1 and CB2 agonists, did not produce significant effects in rats without LPS-induced inflammation. However, the study was not expanded with additional animals for ethical reasons. Finally, because only male rats were included, potential sex-dependent differences in endocannabinoid signaling and inflammatory responses could not be evaluated.

In conclusion, topical URB597 administration prevented alveolar bone loss and attenuated inflammatory changes in rats with LPS-induced experimental periodontitis. These findings support the potential of FAAH inhibition as a strategy to modulate endocannabinoid signaling and protect periodontal tissues. However, because AEA levels, FAAH activity, and cannabinoid receptor activation were not directly assessed, the precise mechanisms underlying these effects remain to be established. Further studies should determine whether the observed protection depends on increased local AEA availability, specific cannabinoid receptor activation, and modulation of inflammatory and bone-remodeling pathways. In addition, therapeutic studies in established periodontitis and further safety assessments will be necessary to determine the translational potential of URB597 as an adjuvant approach to periodontal therapy.

Declarations

Conflict of interest:

No competing financial interests or conflicts of interest exist for any of the authors of the present article.

Funding:

The study was supported by research grants PIP CONICET 11220200100262CO from the National Council for Scientific and Technological Research (Argentina), and UBACYT 20020190100009BA of the University of Buenos Aires, Argentina.

Author Contribution

JRR, GRT, JIA, FN, CEM, CAO and JFS have made substantial contributions to conception and design of the study; and have been involved in data collection and data analysis. JRR, CAO and JFS have been involved in data interpretation, drafting the manuscript and revising it critically and have given final approval of the version to be published. All authors reviewed the manuscript.

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Figures

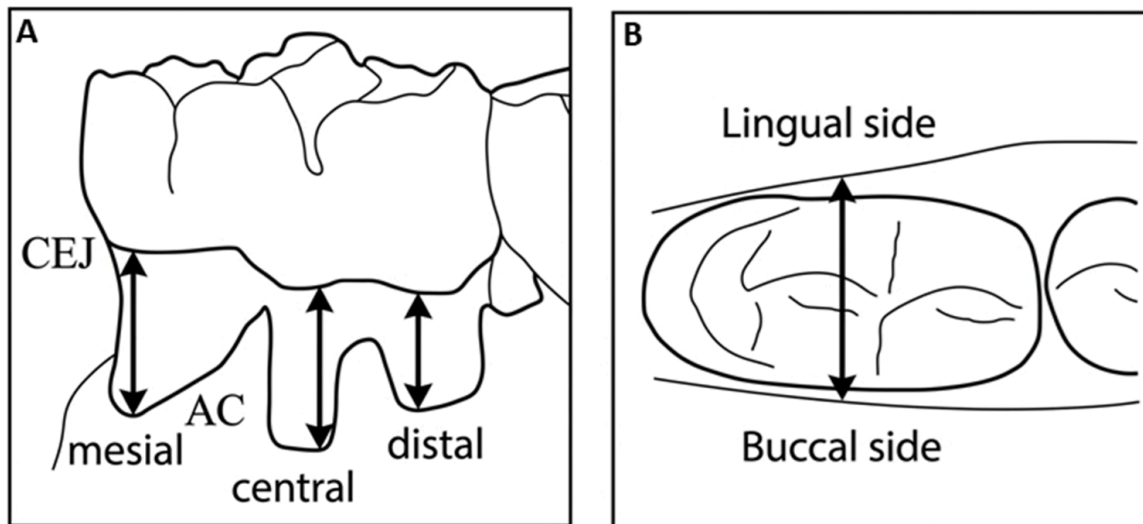


Figure 1

Methods for assessing alveolar bone loss on the first lower molar: (A) diagram of the CEJ-AC distance method and (B) diagram of the width method.

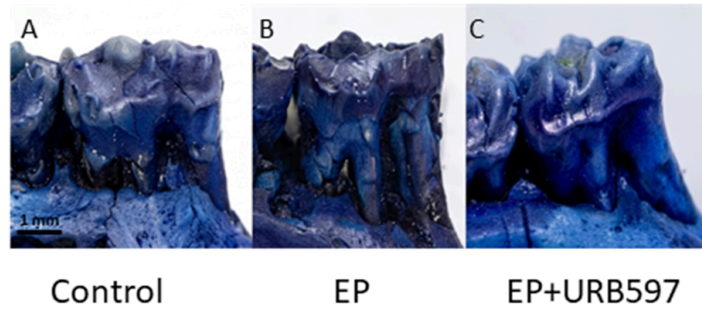


Figure 2

Images of representative teeth of each experimental group, showing alveolar bone loss on the lingual face of the first lower molar.

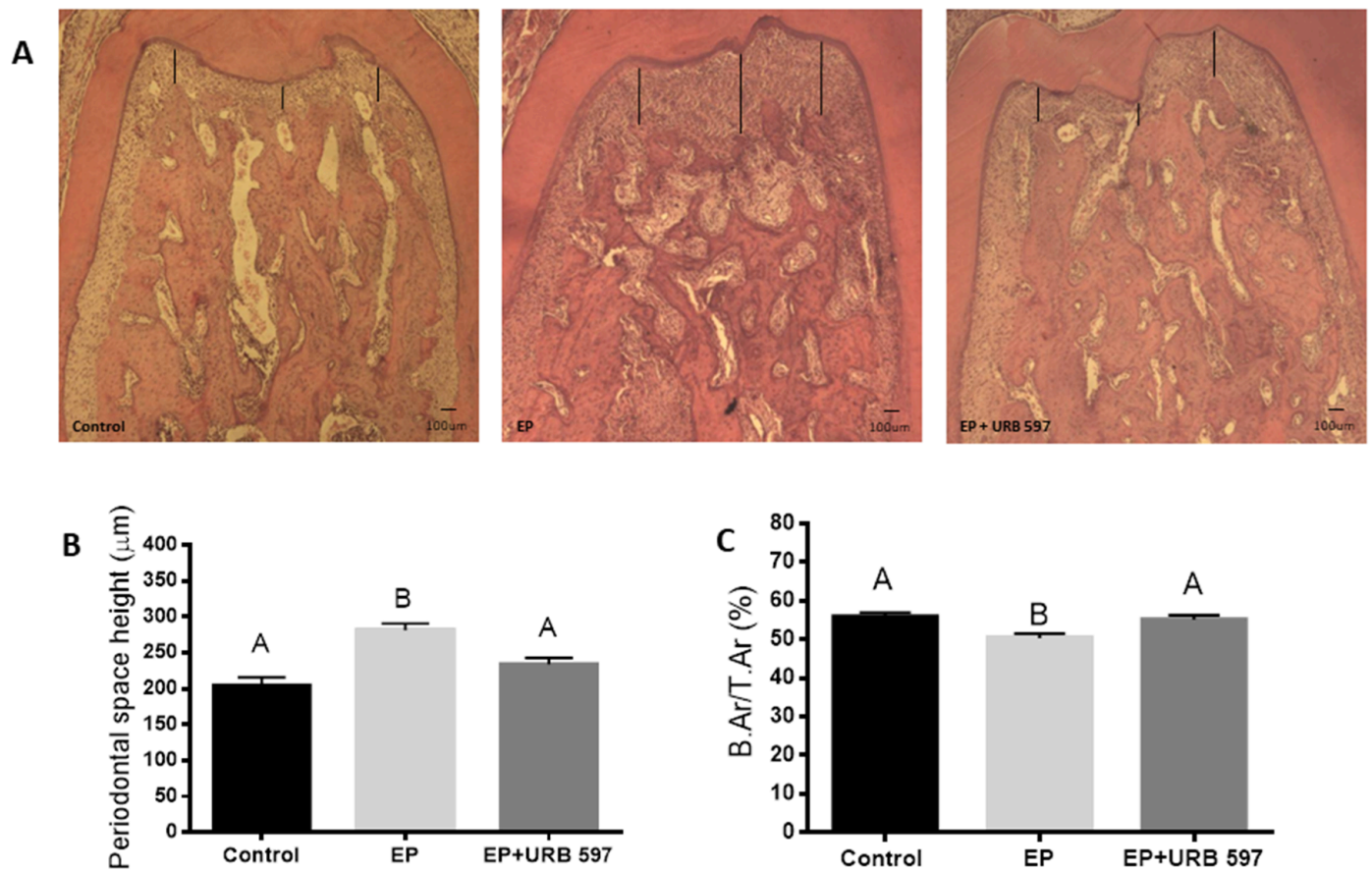


Figure 3

(A) Photomicrographs (H&E stain; original magnification $\times 40$) showing histologic features of the mandibular first molar interradicular bone with mesio-distal orientation, of rats subjected to different experimental conditions (control, EP, EP+URB 597). Vertical lines represent the method used to measure the periodontal space. (B) Periodontal space height evaluation (μm). (C) Interradicular bone measured as B.Ar/T.Ar (%). Results are presented as mean $\pm$ SEM. A p-value < 0.05 was considered statistically significant and is indicated by different letters among groups.

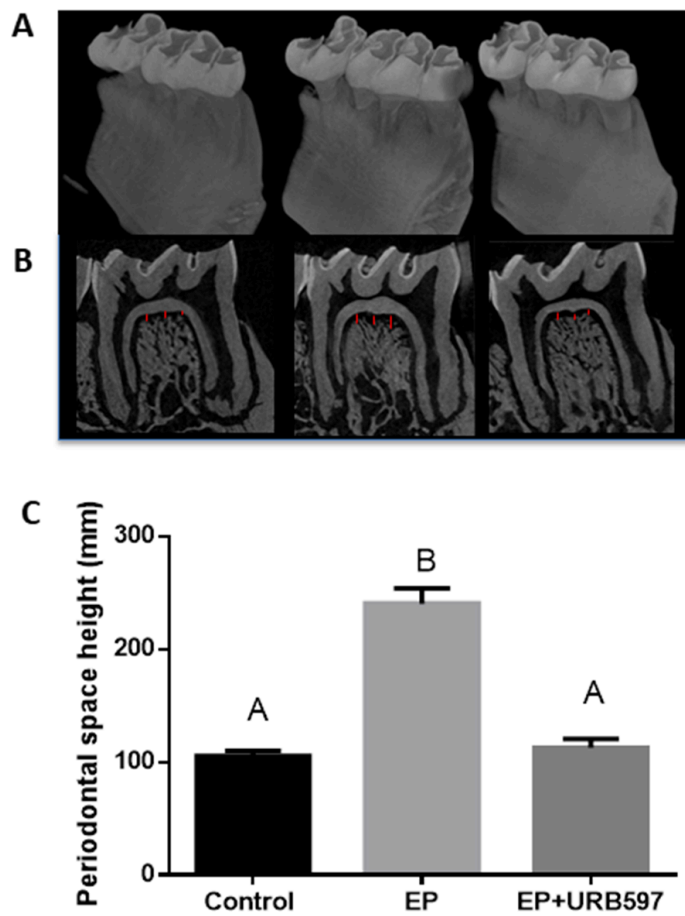


Figure 4

(A) Three-dimensional reconstruction via micro-computed tomography (micro-CT) of the mandibular first molar and surrounding alveolar bone in rats from the control, EP, and EP+URB 597 groups. (B) Representative mesiodistal micro-CT images of the mandibular first molar for each group. (C) Height of the periodontal ligament space measured on mesiodistal tomographic images.

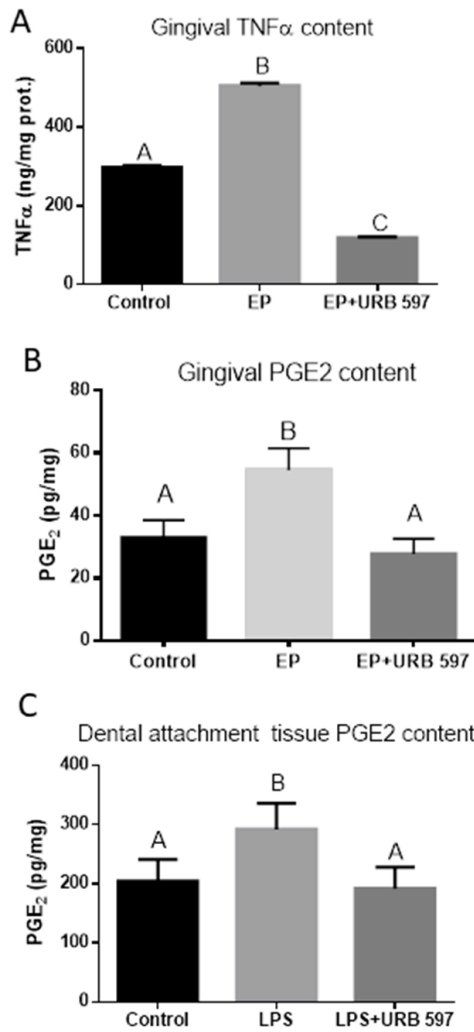


Figure 5

Inflammatory mediators in periodontal tissues: (A) gingival TNF α content, (B) gingival PGE₂ content and (C) dental attachment tissues PGE₂ content. Results are presented as mean $\pm$ SEM. A p-value < 0.05 was considered statistically significant and is indicated by different letters among groups.

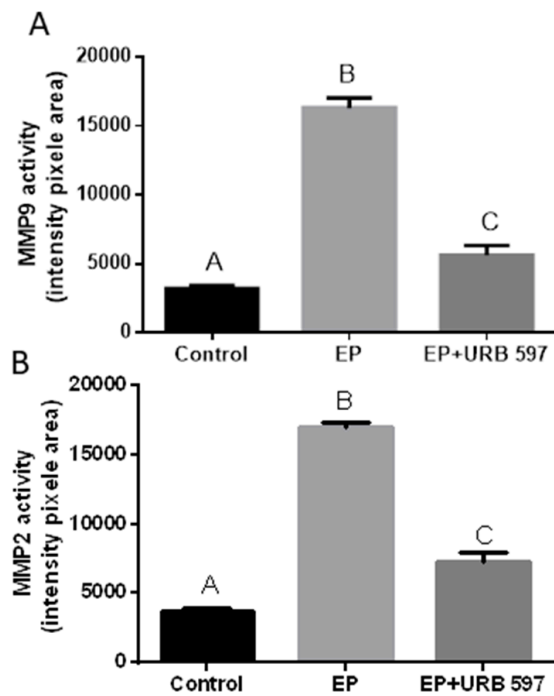


Figure 6

(A) Extracellular matrix metalloproteinase 9 (MMP9) and (B) 2 (MMP2) activity. The unit of measurement is the intensity of the pixel area. Results are presented as mean $\pm$ SEM. A p-value < 0.05 was considered statistically significant and is indicated by different letters among groups.

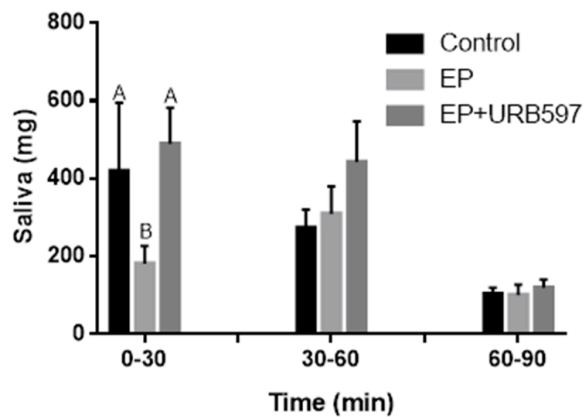


Figure 7

Assessment of salivary secretion following administration of a single dose of pilocarpine. Results are presented as mean $\pm$ SEM. A p-value < 0.05 was considered statistically significant and is indicated by different letters among groups.

Supplementary Files

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