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# Long-term treatment with methanandamide attenuates LPS-induced periodontitis in rats

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**Abstract**
*Objective* Evidence exists of the anti-inflammatory and immunological properties of endocannabinoids in various tissues; the aim of the present study was therefore to assess the effect of long-term treatment with the synthetic cannabinoid methanandamide (Meth-AEA) on the progression of periodontitis in rats.
*Materials and methods* Periodontitis was induced by injecting LPS (1 mg/ml) into the gingiva around the neck of the first upper and lower molars, and into the inter-dental space between the first and second molars. This protocol was repeated for 6 weeks on days 1, 3, and 5 of each week.
*Results* Long-term treatment with topical Meth-AEA (500 ng/ml), applied daily to gingival tissue of rats induced with periodontitis, significantly diminished the alveolar bone loss, measured as the distance between the cemento-enamel junction and the alveolar crest, in both maxillary and mandibular first molars, compared to rats without treatment ($P < 0.05$). The treatment also reduced the production of some biological mediators of periodontal disease augmented by LPS, such as tumor necrosis factor alpha (from 119.4 ± 9.9 pg/mg protein to 75.1 ± 10.8, $P < 0.05$) and nitric oxide produced by inducible nitric oxide synthase (from 507.7 ± 107.1 pmol/min/mg protein to 163.1 ± 53.9, $P < 0.01$).
*Conclusion* These results demonstrate the beneficial effects of treatment with Meth-AEA on gingival tissue of rats with periodontitis.

**Keywords** Oxygen radicals · Bone injury · Prostaglandins · Tumor necrosis factor · Lipopolysaccharide

## Introduction

Periodontitis is an infectious disease understood as a response to challenge of the bacterial subgingival dental biofilm, and characterized by inflammation of tooth-supporting tissues and by periodontal pocket formation, which results in alveolar bone resorption and loss of periodontal attachment tissue [1]. Periodontitis can cause teeth loss and in most cases is also a disease with a chronic progression. The destruction of periodontal tissue is mainly due to complex interactions between the pathogenic bacteria and the host-derived mediators generated during the immuno-inflammatory response [1, 2]. Exposure to lipopolysaccharide (LPS) and other bacterial products can trigger the sequence of inflammation events [1, 3]. LPS is known to stimulate the production of cytokines and other inflammatory mediators, in turn promoting the release of metalloproteinase matrix from the host tissues, which is destructive to the extracellular matrix and alveolar bone [4, 5]. Interleukin (IL)-1 and tumor necrosis factor alpha (TNFα) are found to be associated with periodontitis, enabling the ingress of inflammatory cells into sites of infection, promoting bone resorption and stimulating eicosanoid release, especially prostaglandin (PG) $E_2$, by

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monocytes and fibroblasts [6–8]. $PGE_2$ is known to be a potent stimulator of bone resorption associated with loss of periodontal attachment tissue [9]. LPS has also been shown to stimulate host immune cells to produce pro-inflammatory molecules via CD14 protein and Toll-like receptor-4 expressed on the target membranes [4]. These pro-inflammatory molecules are believed to induce several cellular reactions which eventually lead to the inflammatory status of the periodontal tissue. It is likely that bacteria also contribute to the pathogenesis of periodontal disease by producing enzymes that could alter surrounding tissues of the periodontium, and products that are toxic to surrounding cells. Additionally, once the major protective elements in the periodontium have been overwhelmed by the bacterial virulence mechanism, destructive processes mediated by the host are initiated.

The endocannabinoid system (ECS) is an intercellular communication network. It includes two classical cannabinoid receptors, a variety of lipid ligands, such as anandamide (AEA) and 2-arachidonoyl glycerol, and a series of enzymes that are responsible for ligand synthesis and breakdown. Synthetic cannabinoids have been created for research, such as methanandamide (Meth-AEA), a stable selective agonist for cannabinoid receptor type 1 [10]. The ECS has been implicated in multiple regulatory functions in health and disease. Endocannabinoids and their receptors have been implicated in the control of a wide variety of physiological processes ranging from appetite regulation and pain perception to motor function and regulation of the immune response [11]. The anti-inflammatory and immunological effects of the ECS have been observed in different organs and tissues [12, 13]. The ECS has also been shown to be involved in the repair of various tissues [14–16]. There is accumulating evidence to suggest that cannabinoids and their receptors play important roles in bone metabolism by regulating bone mass, bone loss, and bone cell function [17]. The cannabinoid receptors CB1 and CB2 are also expressed in human gingival fibroblasts, and markedly upregulated under pathological conditions. Cannabinoids also promote the proliferation of gingival fibroblasts via CB1/CB2 receptors in periodontal healing [18]. Increased levels of AEA in periodontal wounds and upregulation of the expression of cannabinoid receptors in healing granulation tissue has also been demonstrated, suggesting that the ECS may have an important modulatory role in periodontal wound healing. Cannabinoid receptors have also been reported to be expressed in osteoblasts, osteocytes, and osteoclasts [17]. Although both CB1 and CB2 receptors are involved in periodontal health, in the present work we decided to study exclusively the effects mediated by CB1. For this purpose, we selected a synthetic analogue of anandamide selective for CB1, Meth-AEA, which has a four-fold higher affinity for this receptor than anandamide itself and which additionally has a high resistance to enzymatic hydrolysis [10]. Based on all the reports mentioned above, the aim of the present work was to assess the effect of long-term treatment with Meth-AEA on the progression of periodontitis in rats.

## 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 light (0700–1900), room temperature of 22–25 °C and free access to rat chow and tap water. The experimental procedures were approved by the Animal Care Committee of the Dental School of the University of Buenos Aires, Argentina, and were carried out in accordance with the guidelines of the National Institutes of Health (NIH).

### In-vitro experiments

Attached gums were dissected from intact rats and divided into four groups: (1) control, (2) LPS [from *Escherichia coli* (serotype 055-B5); Sigma-Aldrich, St. Louis, MO, USA], (3) LPS plus Meth-AEA (Tocris, Ellisville, MO, USA), and (4) Meth-AEA alone (6 per group). They were then pre-incubated in Krebs–Ringer bicarbonate-buffered (KRB) medium (pH 7.4) containing 0.1 % glucose for 15 min before replacement with fresh medium or medium containing the substances to be tested: LPS (10 µg/ml, primed with interferon-gamma, 100 ng/ml) and LPS plus Meth-AEA ($10^{-9}$ mol/l). The optimal concentration of Meth-AEA used was obtained from dose–response curves performed from $10^{-11}$ to $10^{-7}$ mol/l (data not shown). The incubation was continued for 90 min followed by removal of the medium and storage of samples at −20 °C before assays. All incubations were carried out in a Dubnoff shaker (50 cycles per min; 95 % $O_2$/5 % $CO_2$) at 37 °C. $PGE_2$ content and TNFα released from the tissues to the medium were determined, as a measure of the potential anti-inflammatory effect of Meth-AEA on the gingival tissue.

### In-vivo experiments

#### *Experimental model of periodontitis induction*

The rats were divided into four groups (6 rats per group): (1) control rats; (2) sham rats; (3) rats induced with experimental periodontitis; (4) rats induced with experimental periodontitis and treated with Meth-AEA. Periodontitis was

induced by injecting 20 µl of LPS, 1 mg/ml, into the vestibular and oral gingiva around the neck of the first upper and lower molars, and into the inter-dental space between the first and second upper and lower molars, under a $CO_2$ atmosphere. This protocol of injections was repeated for 6 weeks on days 1, 3, and 5 of each week, based on a previously described method [19, 20]. Sham animals received injections of the vehicle (physiological solution) in the same manner while control rats remained intact throughout the experiments. Gingival injections were given with a 13-mm 27G microfine insulin syringe. In previous publications, we used the experimental model of ligature-induced periodontitis especially to assess risk factors for the disease such as alcoholism or hyposialia. However, we believe that for the study of potential therapies, the model of LPS-induced periodontitis is more appropriate, since the presence of the foreign body in the ligation model could prevent the attenuation of the inflammatory process after the treatments.

*Topical treatment with Meth-AEA*

Treatment with Meth-AEA (500 ng/ml) consisted of a daily topical application to the sites of LPS injection throughout the 6 weeks of each experiment. The dose of Meth-AEA finally tested was based on our previous reports using anandamide on oral tissues in vivo, concomitantly with dose–response preliminary studies (data not shown) [21, 22]. The rationale for this selection is also based on the $K_i$ of Meth-AEA to CB1, whose value is reported to lie in the range 17.9–28.3 nM [23, 24]. It should be noted that 500 ng/ml Meth-AEA represents a molarity in the order of picomolar, three orders of magnitude greater than its $K_i$ to CB1. Therefore, the selected dose ensures a high response of the CB1. The rats treated with Meth-AEA showed neither adverse effects nor signs of toxicity after treatment.

*Microscopic examination of periodontal bone loss: distance 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) [25]. A stereomicroscope (Stemi DV4 Stereomicroscope, Carl Zeiss MicroImaging, Göttingen, Germany) and a digital caliper (Digimess, Geneva, Switzerland) were used to measure three lingual/palatal and three buccal distances (mesial, central and distal) from the CEJ to the most apical area of the AC [26]. The sum of the three distances on each side of each molar was used as a measure of the alveolar bone loss in millimeters.

*Measurement of iNOS activity*

The activity of inducible nitric oxide synthase (iNOS) was measured by modifying the method of Bredt and Snyder [27]. In brief, gingival tissue was homogenized in 500 µl of ice-cold 20 mM HEPES (pH 7.4; Sigma-Aldrich) with EGTA (2 mM) and DL-dithiothreitol (DTT, 1 mM; Sigma-Aldrich). After the tissue was homogenized, NADPH (120 µM; Sigma-Aldrich) and 200,000 dpm of [$^{14}$C]-arginine monochloride (297 mCi/mmol; Perkin–Elmer, Waltham, MA, USA) were added to each tube and incubated for 10 min at 37 °C in a Dubnoff metabolic shaker (50 cycles per min; 95 % $O_2$/5 % $CO_2$) at 37 °C. The tubes were then centrifuged at 10,000*g* for 10 min at 4 °C. The supernatants were apply to individual columns containing 1 ml of Dowex AG 50 W-X8 $Na^+$ form mesh 200–400 (Bio-Rad Laboratories, Hercules, CA, USA), and washed with 2.5 ml of double-distilled water. All collected effluent fluid from each column was counted for activity of [$^{14}$C]-citrulline in a liquid scintillation analyser (TriCarb 2800TR, Perkin-Elmer). 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, the gingival tissue was homogenized in 500 µl of absolute ethanol and, after centrifugation, the supernatant was dried in a Speedvac at room temperature. The residues were then resuspended with buffer; antiserum (Sigma-Aldrich) was used as described in Mohn et al. [28]. The sensitivity of the assay was 12.5 pg per tube. The crossreactivity of $PGE_2$ and $PGE_1$ was 100 %, whereas 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 wet weight, since the protocol of PGE extraction from the tissue includes homogenization in ethanol that interferes with protein determination. [$^3$H]$PGE_2$ was purchased from New England Nuclear Life Science Products (Boston, MA, USA)

*Determination of TNFα*

In-vitro experiments: the incubation media were collected and protease inhibitory cocktail added to measure TNFα released from the gingival tissue.

In-vivo experiments: for TNFα preservation after extraction, the gingival tissues were immediately homogenized in PBS buffer containing protease inhibitory cocktail for mammalian tissue extracts (Sigma-Aldrich). The concentration of rat TNFα was determined using a sandwich ELISA according to the manufacturer's instructions (BD Pharmingen, USA).

*Statistical analysis*

Statistical 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 Prism software (GraphPad Software, Inc.). Differences with $P$ values $<0.05$ were considered statistically significant.

## Results

In-vitro experiments

*Inhibitory effects of Meth-AEA in vitro on LPS-induced TNFα release and $PGE_2$ production from gingival tissue*

In-vitro studies demonstrated that LPS (10 μg/ml)-induced TNFα release and $PGE_2$ production from gingival tissue extracts of intact rats were prevented, at least partially, by Meth-AEA ($10^{-9}$ mol/l) added to the incubation media (Fig. 1a, b).

Assessments after in-vivo treatments with Meth-AEA

*Alveolar bone loss*

Meth-AEA (500 ng/ml) daily topical application attenuated LPS-induced bone loss in the first mandibular (Fig. 2a) and maxillary (Fig. 2b) molars, at lingual/palatal and buccal sides, measured as the sum of the three distances between the CEJ and the AC of the mesial, central and distal roots (Fig. 2c–d).

*Inducible NOS activity in gingival tissue*

Meth-AEA (500 ng/ml) daily topical application attenuated LPS-induced iNOS activity in gingival tissue dissected from the neck of the first lower molar (Fig. 3).

*TNFα content in gingival tissue*

Meth-AEA (500 ng/ml) daily topical application attenuated LPS-induced TNFα content in gingival tissue dissected from the neck of the first lower molar (Fig. 4).

*Prostaglandin $E_2$ content in gingival tissue*

$PGE_2$ concentration was significantly higher in the gingival tissue extracted from rats with LPS-induced periodontitis than in that from sham or control rats (Fig. 5). However, Meth-AEA (500 ng/ml) daily topical application did not alter the $PGE_2$ content in rats with LPS-induced periodontitis.

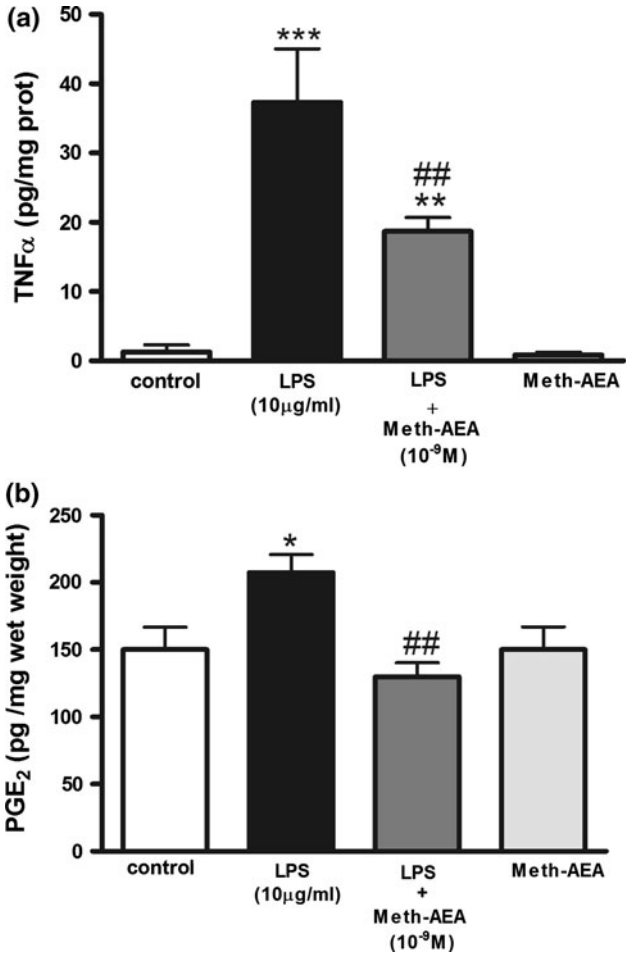


**Fig. 1** Effect of Meth-AEA ($10^{-9}$ mol/l) on LPS (10 μg/ml)-induced TNFα release (**a**) and $PGE_2$ production (**b**) from gingival extracts incubated in vitro. Data are reported as means ± SEM (6 extracts per group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control; $^{\#\#}P < 0.01$ versus LPS (one-way ANOVA followed by Newman–Keuls post-hoc test)

## Discussion

Cannabinoid receptors are expressed in osteoblasts, osteocytes, and osteoclasts in mammals, as well as in gingival fibroblasts [17, 18]. Gingival fibroblasts are essential for maintaining oral homeostasis, since they participate in tissue repair and contribute to tissue remodeling at wound connective tissue after various dental procedures such as removal of dental calculus or periodontal surgery [18]. Therefore, we propose the ECS as a possible therapeutic target for periodontal disease. Previous reports demonstrating that endocannabinoids are produced in bone and in synovial joints, and preclinical studies showing the effectiveness of cannabinoids in the treatment of inflammatory arthritis and in the prevention of ovariectomy-induced bone loss, support this hypothesis [29, 30]. Moreover, these reports are consistent with our

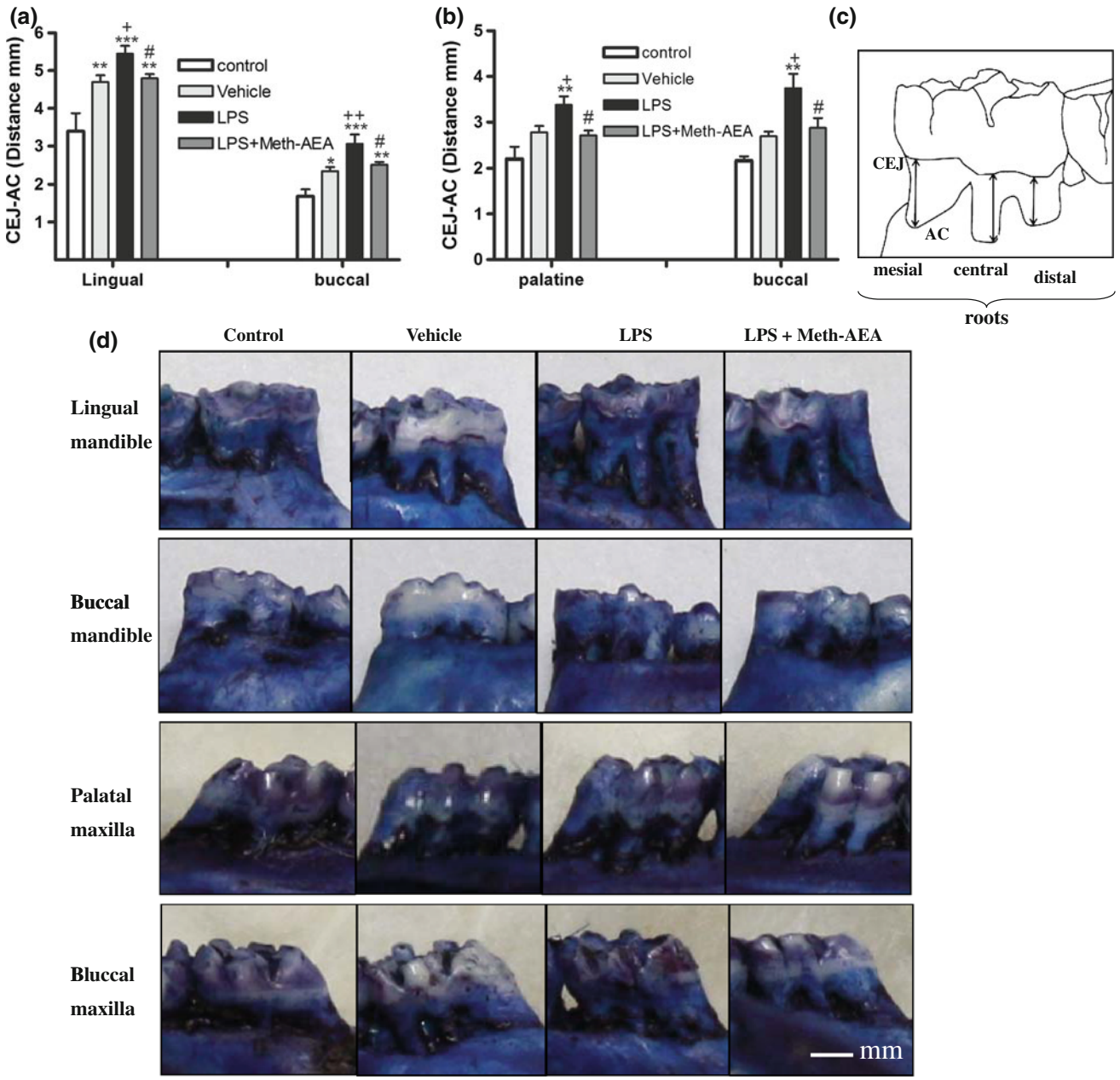


**Fig. 2** Examination of periodontal bone loss by the distance method after Meth-AEA treatment in rats with LPS-induced periodontitis. **a**, **b** Measurements of mandible (**a**) and maxillary (**b**) first molars. Data are reported as means ± SEM (six rats per group). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control; $^{+}P < 0.05$ and $^{++}P < 0.001$ versus vehicle; $^{\#}P < 0.05$ versus LPS (one-way ANOVA followed by Newman–Keuls post-hoc test). **c** Distance method: diagram of a section of first molar. Three distances (*arrows*) were measured from the CEJ to the most apical area of the AC. **d** Mandibular and maxillary photographs showing lingual/palatal and buccal sections of first molars in controls, rats injected with vehicle, rats with LPS-induced periodontitis (*LPS*), and rats with periodontitis plus treated with Meth-AEA (*LPS+Meth-AEA*). *Scale bar* 1 mm

initial studies showing that Meth-AEA had an inhibitory effect on the production of $PGE_2$ and the release of TNFα induced by LPS from gingival tissues in vitro. Several reports have demonstrated the safety of treatments with cannabinoid receptor ligands in patients, showing their potential clinical use [31, 32].

In short, the results of this study are that long-term treatment with Meth-AEA significantly diminished the alveolar bone loss which is increased by LPS-induced periodontitis, and reduced the production of some biological mediators of periodontal disease, such as TNFα and nitric oxide produced by iNOS. Therefore, the present

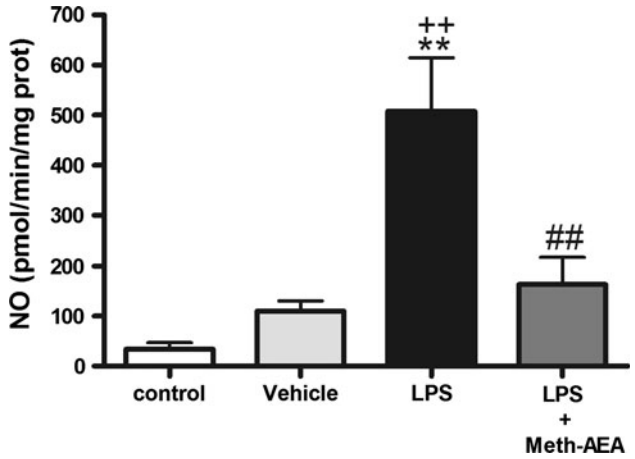


**Fig. 3** Effect of topical Meth-AEA (500 ng/ml) applied daily on iNOS activity in gingival extracts from rats with LPS-induced periodontitis. Data are reported as means ± SEM (six extracts per group). $^{**}P < 0.01$ versus control; $^{++}P < 0.01$ versus vehicle; $^{\#\#}P < 0.01$ versus LPS (one-way ANOVA followed by Newman–Keuls post-hoc test)

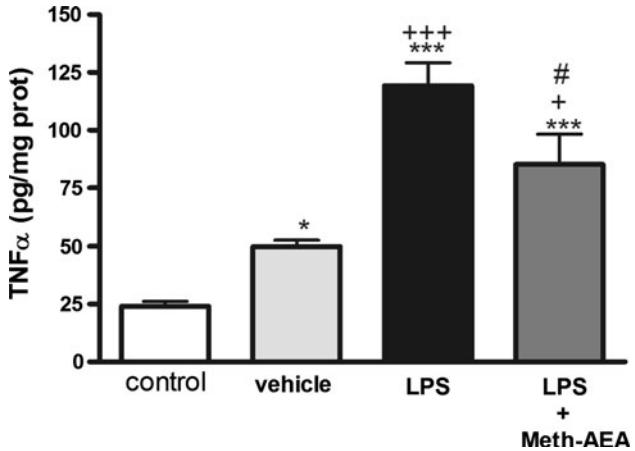


**Fig. 4** Effect of topical Meth-AEA (500 ng/ml) applied daily on TNFα concentration in gingival extracts from rats with LPS-induced periodontitis. Data are reported as means ± SEM (six extracts per group). $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control; $^{+}P < 0.05$ and $^{+++}P < 0.001$ versus vehicle; $^{\#}P < 0.05$ versus LPS (one-way ANOVA followed by Newman–Keuls post-hoc test)

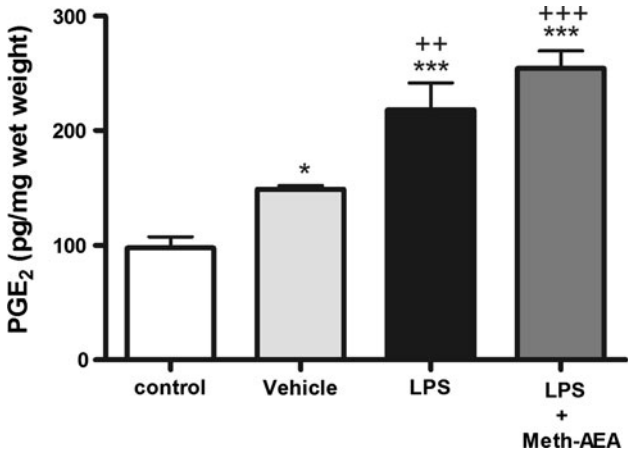


**Fig. 5** Effect of topical Meth-AEA (500 ng/ml) applied daily on $PGE_2$ concentration in gingival extracts from rats with LPS-induced periodontitis. Data are reported as means ± SEM (6 extracts per group). $^{*}P < 0.05$ and $^{***}P < 0.001$ versus control; $^{++}P < 0.01$ and $^{+++}P < 0.001$ versus vehicle (one-way ANOVA followed by Newman–Keuls post-hoc test)

results suggest for the first time, to our knowledge, the role of cannabinoid receptors as possible targets for future therapeutics against periodontal disease. In this regard, it has already been demonstrated that AEA significantly reduces the production of pro-inflammatory mediators (IL-6, IL-8, and monocyte chemotactic protein-1) induced by LPS of *Porphyromonas gingivalis* in human gingival fibroblasts, and that this effect is attenuated by AM251 and SR144528, selective antagonists of CB1 and CB2, respectively [16]. In addition, AEA levels are increased in gingival crevicular fluid after periodontal surgery in human patients with periodontitis. Furthermore, the proliferation of human gingival fibroblasts in vitro by AEA was significantly attenuated by AM251 and AM630 [18]. Our results also agree with previous reports that AEA may regulate periodontal inflammation through inhibition of the NF-κB pathway via the cannabinoid receptors CB1 and CB2 [33].

As in our study, $PGE_2$ concentration is increased in gingival tissues of patients suffering periodontitis [34]. However, treatment with Meth-AEA did not reduce the $PGE_2$ concentration in the gingival tissue measured at the end of the experiment. These results contradict the inhibitory effects of Meth-AEA on $PGE_2$ observed in vitro. This difference can be explained by the absence of nervous and vascular regulation in the in-vitro model.

Periodontitis also causes oxidative stress, whose consequences occur in the oral cavity [35] and in most distant organs [36, 37]. Nitric oxide derived from iNOS is known to play an important role in host defense, as well as in inflammation-induced tissue lesions. It has been shown to have important effects on several inflammatory events, including cell migration, observed in periodontitis [38]. Several reports have shown that nitric oxide may promote osteoclast maturation and enhance bone resorption induced by cytokines [39, 40]. Therefore, the attenuation of iNOS activity induced by periodontitis which was produced by the Meth-AEA treatment could be directly related to the observed protective effect of endocannabinoids on alveolar bone. These results were to be expected since the ECS acts as a homeostatic system that attempts to recover the balance of biological systems after disturbances, such as those occurring during inflammation and infection [41].

Nevertheless, there are several cannabis-associated oral effects of importance to the dental practitioner, including xerostomia, with predisposition to caries and periodontitis, severe gingivitis, and oral mucosal disease [42]. Moreover, it was recently reported that smoking cannabis for 8 min per day impacts alveolar bone by increasing bone loss resulting from ligature-induced periodontitis [43]. Although experimental studies have shown that endocannabinoids and some cannabinoids from *Cannabis sativa*, such as cannabidiol, produce similar beneficial effects on the organism [44–46], marihuana consumption can produce adverse effects such as alteration of cognitive function, drowsiness, anxiety, and paranoia, due to the presence of additional compounds in the natural extract [47]. In any case, harmful effects have been reported from continuous activation of cannabinoid receptors, since endocannabinoids are produced on demand when required to restore homeostasis, and are then rapidly degraded to avoid adverse side effects [48].

In summary, the results presented demonstrate the beneficial effects of treatment with Meth-AEA of rats with periodontitis, since it attenuates alveolar bone loss and reduces the levels of inflammatory mediators in gingival tissues, and therefore lay the foundations for future studies in humans based on the endocannabinoid system as a therapeutic target in periodontal disease.

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