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Electroacupuncture increases immunoexpression of CB1 and CB2 receptors in experimental model of inflammatory bone loss

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ABSTRACT

This study evaluated the participation of CB1 and CB2 receptors in the antiresorptive effect of electroacupuncture (EA) on an experimental model of inflammatory bone loss in rats. 30 rats were divided into five groups: C (control); EP (experimental periodontitis); EA (C+ EA); EP-EA (EP+ EA in the acupoints LI4, LG11, ST36, ST44); EP - EA-sham (EP+ EA in sham acupoints). For the EP groups, a ligature was placed around the right mandibular first molars at day 1. Sessions of EA or EA-sham were assigned every other day. Animals were euthanized at day 11. Histometric analysis was performed to evaluate the percentage of bone area in the furcation area. Immunolabeling patterns in the periodontal tissues and immunofluorescent staining in the trigeminal ganglia and in the trigeminal spinal tract for CB1 and CB2 receptors were performed. It was observed increased bone loss in the furcation in the EP and EP-EA-sham groups, in comparison to the other groups ($p < 0.05$). Enhanced CB2 immunolabeling was observed in the periodontal tissues in the EP-EA group, when compared to the EP and EP-EA-sham groups ($p < 0.05$). Increased CB1 immunofluorescent staining was observed in the neural tissues in the EA treated group in comparison with the other groups ($p < 0.05$), while no expression of CB2 was observed in those regions. Our study showed that in the presence of inflammatory bone disease, EA treatment reduced bone erosion and increased the immunoexpression of CB1 in the neural tissues and CB2 in the periodontal tissues.

1. Introduction

Chronic periodontitis is an inflammatory condition that affects the tissues surrounding the teeth, reducing their bone support. It is mainly initiated by bacteria, but the progress of the disease depends on the host's immune response [1], which can be modified by the genetical susceptibility and/or by the presence of immunoregulatory risk factors [2]. Hence, for these individuals, the greater fraction of periodontal destruction may be due to the inflammatory response of the host [3]. Based on these observations, a new management for periodontal treatment involving the modulation of the immune-inflammatory response has been widely stimulated [4–6].

The endocannabinoid system (ECS) is an endogenous lipid signaling system comprised of two main receptors coupled to the G protein (CB1 and CB2), agonist neurotransmitters, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and a biochemical apparatus responsible for the synthesis, the reuptake and the degradation of these transmitters [7]. Although CB1 receptors may also be expressed in non-neuronal tissues, they are more commonly found in nervous cells and they are responsible for the majority of the neural cannabinoids actions. CB2 receptors are present in peripheral tissues, where they are more commonly expressed in cells of the immune system [8].

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The stimulation of CB1 and CB2 receptors inhibit the adenylate cyclase, decreasing the concentration of intracellular cyclic adenosine monophosphate, leading to a reduction of transcription factors for the cellular DNA [9]. It may diminish the expression of immunoregulatory genes, suppressing the secretory function of T cells and macrophages [9]. Moreover, the binding of agonists to CB1 and CB2 receptors generates many cellular responses by different types of leucocytes, resulting in immunoregulation by the suppression of cellular activation, inhibition of the production of pro-inflammatory cytokines, apoptosis via nuclear factor κB (NF-κB) and modulation of the functions of T-helper cells [10].

In this point of view, the immunomodulatory effect of endocannabinoid agonists is associated with anti-inflammatory action in experimental models induced by lipopolysaccharides, decreasing pro-inflammatory mediators, such as tumor necrosis factor alfa (TNF-α), interleukin (IL)-6, IL-8, IL-12, induced nitric oxide synthase (iNOS), prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (COX-2) [11]. In addition to this effect, increase of oxytocin and IL-10 levels, which exert anti-inflammatory effects, have been reported [12–14]. Due to the cellular immunoregulatory and anti-inflammatory effects of the endocannabinoid system, it is possible that its activation.

plays an important role in the modulation of the host's inflammatory response in periodontal diseases.

In a previous study, we have evaluated the effects of the electroacupunture (EA) in the periodontal disease in rats [6]. We observed that the treatment with EA produced an anti-inflammatory effect on experimental periodontitis, reducing bone loss and the expression of pro-inflammatory cytokines [6]. In this way, we hypothesized that the endocannabinoid system may be involved in the anti-inflammatory effects of EA in an experimental model of periodontitis, since the mechanisms involved in these effects have not been investigated yet. Thus, since it is known that EA acts through the stimulation nociceptive pathway [15], the present study aimed to investigate if the anti-inflammatory effect of EA in experimental periodontitis (EP) in rats was related on the peripheral and central immunoexpression of CB1 and CB2 receptors.

2. Material and methods

2.1. Animals

This study was conducted in compliance with the ethical principles of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications n° 8023, revised 1978) and it was conducted after the approval by the Ethics Committee on Animal Research at the Federal University of Ceará (protocol 48/2017). Moreover, this report followed the ARRIVE guidelines [16]. Thirty Wistar male rats, weighing 200–250 g, were used (Central Animal Facility, Federal University of Ceará). The rats were kept in a 12-h light/dark cycle with temperature between 22 °C and 24 °C and were housed in plastic cages with food and water ad libitum. They were randomly assigned to one of the five experimental groups ($n = 6$): control (C); EP; EA-treated (EA); EP + EA sham (EP-EA-sham); and 4) EP and EA (EP-EA).

2.2. Induction of alveolar bone loss induced by ligature

All animals were anesthetized by an intramuscular injection of ketamine (70 mg/kg body weight; Dopalen®, Agribands, Paulinia, SP, Brazil) and xylazine (6 mg/kg body weight; Rompum®, Bayer Saude Animal, Sao Paulo, SP, Brazil). A ligature-induced periodontitis model was used to produce inflammatory bone loss [6,17]. Briefly, a cotton ligature was placed around the right mandibular first molars [4,6], knotted at the buccal surface of the tooth and kept in place for 11 days [6]. C and EA groups did not receive the ligature. The induction of EP was conducted by the same experienced experimenter (MRPL).

2.3. EA treatment

The EA treatment was based on Gondim et al. [18], which consists of bilateral insertion of stainless steel needles (0.7 mm diameter; Dongbang, Dongbang AcuPrime, Ungcheon, Korea) to a depth of 3 mm at predetermined acupuncture points (LI4, LI11, ST36, and ST44) or sham points in the gluteal region (5 mm lateral and 5 mm above the GB30 acupoint) [18]. For this, sham and acupuncture points were bilaterally stimulated with low-frequency rectangular pulses (frequency 10 Hz, recurrence time 1 s, intensity 3 mA) using an EA equipment (NKL EL530, NKL Produtos Eletrônicos, Santa Catarina, Brazil). EA was applied for 20 min and it was performed every other day at 8:00 am, beginning in the day after the ligature placement, until the end of the experimental period, totaling 5 sessions of EA treatment, as described by Lisboa et al. [6]. EA and EA-sham procedures were conducted by an experienced practitioner (DVG).

2.4. Histomorphometry

The animals were anesthetized (240 mg/kg ketamine and 30 mg/kg xylazine, i.p.) and euthanized 11 days after the induction of the inflammatory bone loss. The mandibles were excised and decalcified in EDTA solution (10%) and they were processed and embedded in paraffin. Serial sections, 4 μm thick, were obtained in a mesio-distal direction and were stained with hematoxylin and eosin. Sections representing the most central bucco-lingual portion in the furcation area of the right first molar were selected for histometric analyses. Photomicrographs were captured using a digital camera (DFC295, Leica Mycrosystems, Wetzlar, Germany) connected to a light microscope (DM2000, Leica Microsystems, Wetzlar, Germany) using an original magnification of 40 X. The images were analyzed with a proper software (FIJI Image J software, National Institute of Health, Bethesda, USA). The bone loss evaluation was performed by measuring the percentage of bone area in the furcation. The histometric analysis was performed by one masked and calibrated examiner (LCVA).

2.5. Immunohistochemical assay

The samples were excised and fixed in 4% paraformaldehyde for 24 h. They were dehydrated and paraffin-embedded. Immunohistochemistry for CB1 and CB2 was performed using the streptavidin–biotin peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 μm thick) mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated using xylene and a graded series of alcohol. After antigen retrieval with citrate buffer (pH 6.0) at 95 °C (15 min), endogenous peroxidase was blocked twice (10 min) with 3% (v/v) hydrogen peroxide and washed in PBS. To block unspecific bindings, the slides were incubated with goat serum during 30 min. The sections were incubated overnight at 4 °C with rabbit anti-CB1 (1:300 dilution; ab23703, Abcam, San Francisco, USA) or rabbit anti-CB2 antibody (1:300 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS plus bovine serum albumin (BSA; 5%).

The slides were then incubated with biotinylated donkey anti-goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:400 in PBS–BSA 5%. After PBS washing, the slides were incubated with streptavidin horseradish peroxidase conjugate (StrepABC complex; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min according to the manufacturer's protocol. Immunostaining was visualized with the chromogen 3,3′-diaminobenzidine (DAB; ABC staining system, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Negative control sections were simultaneously processed as described above but with the primary antibody incubation replaced by 5% PBS–BSA. None of the negative controls showed immunoreactivity to CB1 or CB2. The slides were counterstained with Mayer's hematoxylin, dehydrated in a graded series of alcohol, cleared in xylene, and coverslipped.

Each group consisted of six animals. Semiquantitative analyses of the immunolabeling of CB1 and CB2 were conducted in the entire furcation region at 400× magnification by an optic microscope (DM2000, Leica Microsystems, Wetzlar, Germany) coupled with digital camera (DFC232, Leica Mycrosystems, Wetzlar, Germany). The coronal limit of this area was the roof of furcation, from which the region analyzed extended apically, comprising the periodontal ligament and the alveolar bone. The scores of immunolabeling were given similarly to Lisboa et al. (2015) as following: 0, no immunoreactivity; 1, ≤¼ of immunoreactive area (low pattern of immunolabeling); 2, between ¼ and ½ of immunoreactive area (moderate pattern of immunolabeling); 3, between ½ and ¾ immunoreactive area (high pattern of immunolabeling); and 4, up to completely immunoreactive area (extremely high pattern of immunolabeling) [6].

2.6. Trigeminal ganglia and trigeminal spinal tract extractions

The rats were perfused transcardiacally with 0.9% saline solution, followed by perfusion with 4% paraformaldehyde in phosphate buffer under deep anesthesia. The structures of trigeminal via – trigeminal ganglia (TG) from the right side and trigeminal spinal tract (subnucleus caudalis trigeminal) – were removed for immunofluorescence assay. Right TG was then dissected, keeping the three branches of the trigeminal nerve. The trigeminal nuclei area was localized and the sections used in this study were cut −14.04 mm to – 15.00 mm from bregma [19].

2.7. Immunofluorescence assay

The specimens collected were kept in 4% paraformaldehyde during 2 h at room temperature. After this time, the tissues were cryoprotected (30% sucrose in 0.2 M PBS during 3 days and changed every 24 h) and frozen in Tissue-Tek (OCT compound 4583; Sakura Finetechnical Co. Ltd., Tokyo, Japan). Sections at thickness of 10 µm were obtained using a cryostat (Leica CM3050; Leica Microsystems, German). Then, the sections were blocked with glycine 0.1 M in 5% BSA for 1 h, washed and incubated overnight at 4 °C with rabbit primary antibody anti-CB1 (1:50 dilution; ab23703, Abcam, SP, Brazil) or anti-CB2 (1:400 dilution; sc-25,494, Santa Cruz Biotechnology, CA, USA). Next, the slides were washed in 0.2 M PBS and incubated at room temperature for 1 h with a secondary antibody goat anti-rabbit conjugated with Alexa Fluor 568 (1:400 dilution; Invitrogen®, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA). Anti-NeuN antibody conjugated with Alexa Fluor 488 (1:400 dilution; ABN78A4, Merck Millipore, Darmstadt, Germany) was used to label neuronal soma. Sections were viewed using a confocal microscope (Zeiss LSM700, Carl Zeiss MicroImaging GmbH, Jena, Germany). Fluorescence intensity was analyzed using FIJI-Image J software (FIJI Image J software, National Institute of Health, Bethesda, USA), and the number of pixels in the selected area was estimated from the total number of pixels in the NeuN fluorescent image for quantification of CB1 or CB2 expression.

2.8. Statistical analysis

Data were grouped and presented as means and standard deviations (continuous variables) or medians, interquartile ranges, and maximum and minimum values (ordinal variables). Normality and homoscedasticity of the data were verified. The significance of the differences among the groups in relation to the immunolabeling pattern of CB1 and CB2 was determined by Kruskal-Wallis tests, followed by Dunn's multiple comparison post hoc test. The data from the other analyses were assessed by analysis of variance (ANOVA), followed by post hoc Tukey test. The significance level was set at 5% in all tests.

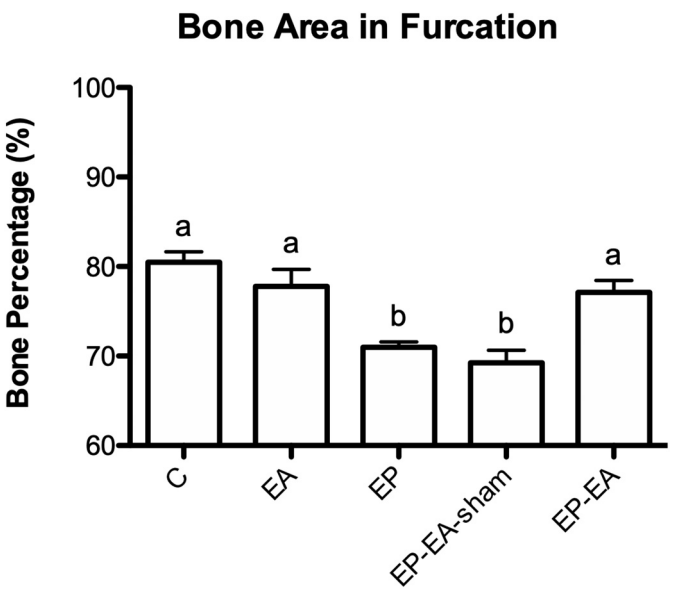

Fig. 1. Bone area in furcation. Bone percentage (%) analysis shows the means and standard deviations of the furcation bone area of the specimens compared among the groups. Experimental periodontitis alone or combined with EA-sham treatment reduced bone percentage in the furcation area, which was reverted by the EA treatment. Same letters indicate no significant differences among the groups ($p < 0,05$, ANOVA, Tukey test).

3. Results

3.1. Histometric analysis

The animals of the EP and EP-EA-sham groups showed a significant lower furcation bone area when compared to the other groups ($p < 0.05$). The treatment with EA, but not with EA-sham, significantly reverted the reduction in the bone percentage, when compared to the EP group ($p < 0.05$). There was no statistical difference between EP and EP-EA-sham groups or among C, EA and EP-EA groups ($p > 0.05$; Fig. 1).

3.2. Immunohistochemical analyses

Since the group EA presented similar bone levels to the group C, this group was not considered for these analyses. The photomicrographs showed immunolabeling to CB1 and CB2 in periodontal ligament fibroblasts, macrophage-like cells, endothelial cells, osteoblasts, osteoclasts and osteocytes (Figs. 2 and 3).

The immunolabeling pattern of CB1 was significantly greater in EP and EP-EA group when compared to C group ($p < 0.05$). There was no significant difference between the EP groups ($p > 0.05$). In the reaction for CB2, the EP-EA group presented a significantly higher immunolabeling pattern when compared to the other groups ($p < 0.05$) (Fig. 4).

3.3. Immunofluorescence assay to CB1 and CB2 in the trigeminal ganglia and trigeminal spinal tract

Since the sham treatment did not exert any effects in bone resorption nor in CB1 and CB2 periodontal expression when compared to the EP group, the EP-EA-sham group was not used for further analyses. Moreover, since acupuncture acts through the neural nociceptive pathway [15,20], the EA treated group was considered for these essays in order to evaluate the central and peripheral effects of sole EA in the neural tissues. Immunofluorescence staining showed a positive expression of CB1 (red) in the neuronal body cells (green) of the TG and

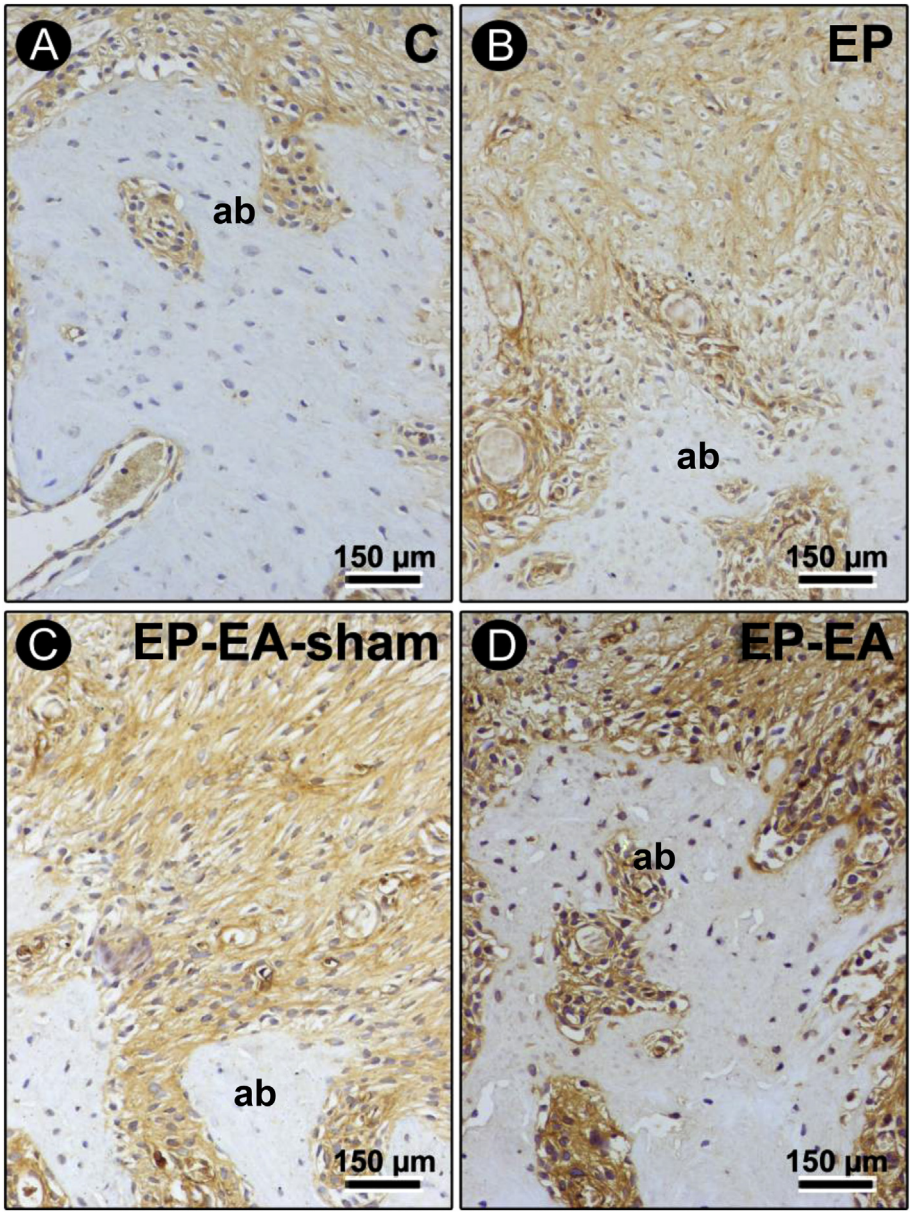

Fig. 2. Photomicrographs showing immunolabeling for CB1 in the periodontium. ab = alveolar bone.(Hematoxylin counterstaining).

the trigeminal spinal tract. It was observed a greater expression of CB1 staining in the EP-EA group when compared to the other groups ($p < 0.05$) in the TG (Figs. 5A and 6A) and in the subnucleus caudalis of the trigeminal spinal tract (Figs. 5B and 6B). It was not verified immunofluorescence staining for CB2 neither in the trigeminal ganglia nor in the trigeminal spinal tract of all experimental groups (data not shown).

4. Discussion

Acupuncture is a part of the Traditional Chinese Medicine and it uses the stimulation of specific points of the body called acupoints. These points are distributed along meridians. EA is a modality of acupuncture that uses pulsating electric stimuli to acupuncture needle. The stimulus of the acupoints promotes neuroendocrine effects [15,21,22]. Many studies related theses effects to the activation of the opioidergic, serotoninergic, GABAergic, cholinergic, adrenergic and endocannabinoid systems [15,22,23].

The endocannabinoid system is an endogenous signaling system that modulates inflammation by the activation of the neuro-endocrine-immune axis and participates in diverse physiological processes, such as nociception, memory, behavior and motor control [23]. It has been shown that periodontal inflammation seems to increase the production of endocannabinoid agonists [24]. These agonists bind to the cannabinoid receptors located in the immune cells and in gingival fibroblasts and periodontal ligament cells, modulating the production of pro-inflammatory mediators, such as TNF-α, IL-β and PGE-2 [25]. Cannabinoid agonists reduced bone loss in experimental models of periodontitis, probably due to NF-κB pathway inhibition [24], and the local application of cannabinoid receptor agonists may attenuate the excessive inflammatory reaction associated with periodontitis [26].

Even though CB1 receptor is mainly expressed in the central nervous system [27] and CB2 is more related to immune cells in the periphery [28], it has been shown that inflammatory bone resorption in experimental periodontitis is prevented by the activation of either CB1 [29] or CB2 [30] receptors. Ossola et al. [29] used methanandamide, a selective CB1 agonist, in a dose that ensures a high response of the receptor. In this study, it was observed that the administration of

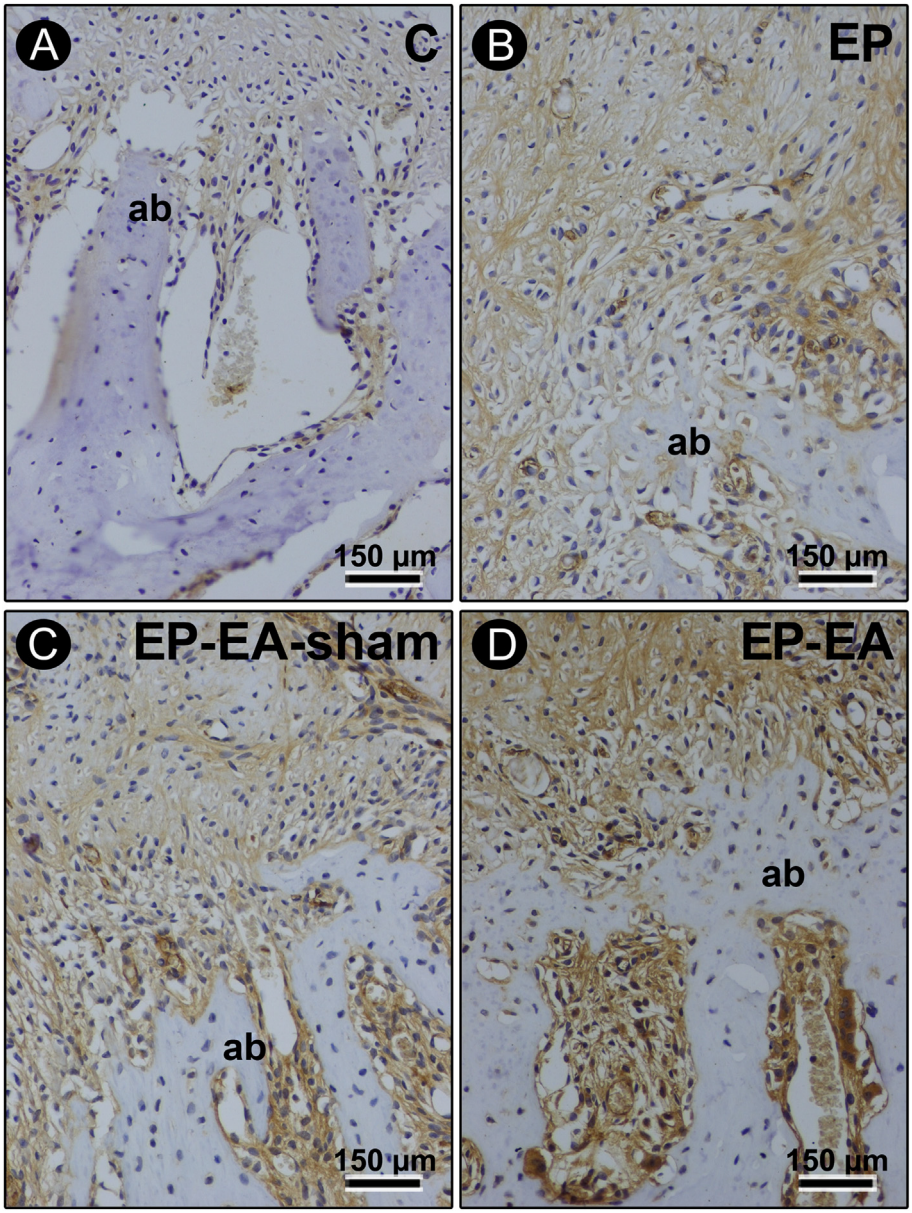

Fig. 3. Photomicrographs showing immunolabeling for CB2 in the periodontium. ab = alveolar bone.(Hematoxylin counterstaining).

methanandamide reduced the alveolar bone loss and the inflammatory parameters in an experimental model of periodontitis in rats [29]. In another study, Ossola et al. [30] used the CB2 agonist HU-308 in rats with periodontal disease and showed the participation of CB2 signaling in the control of periodontal damage.

Our group, in previous studies, showed that EA reduced inflammation and mechanical hipersensitivity in temporomandibular joint arthritis in rats [18] and that CB1 and CB2 receptors participate in

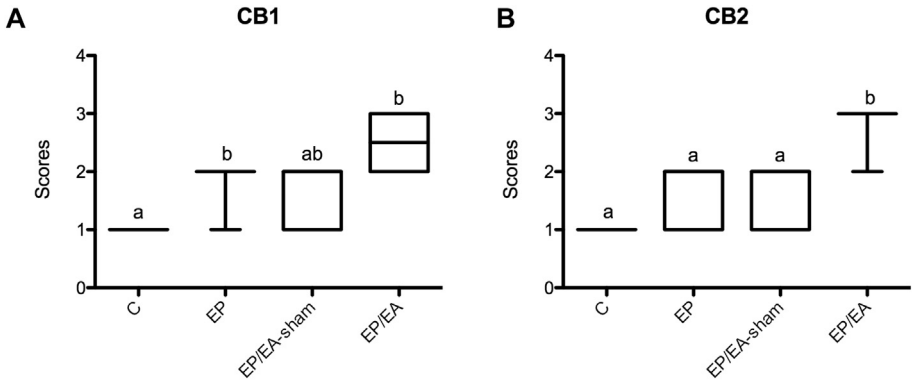

Fig. 4. Immunohistochemical analyses. Medians, interquatile ranges, and maximum and minimum values of the immunolabeling scores for CB1 (A) and CB2 (B). (A) EP alone or associated to EA treatment increased the immnunoexpression of CB1 when compared to control group. (B) Only the animals with EP and treated with EA showed a higher immunoexpression of CB2 when compared to others groups. Same letters indicate no significant differences among the groups ($p < 0.05$; Kruskal-Wallis, Dunn's tests).

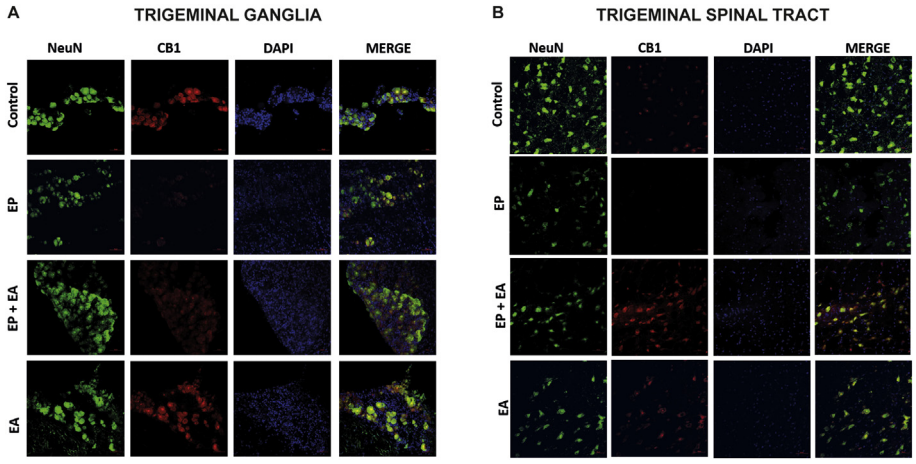

Fig. 5. CB1 expression in the trigeminal ganglia (A) and trigeminal spinal tract (B) in rats with periodontitis induced by ligature. Green: neuronal marker (NeuN); red: CB1; blue: DAPI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

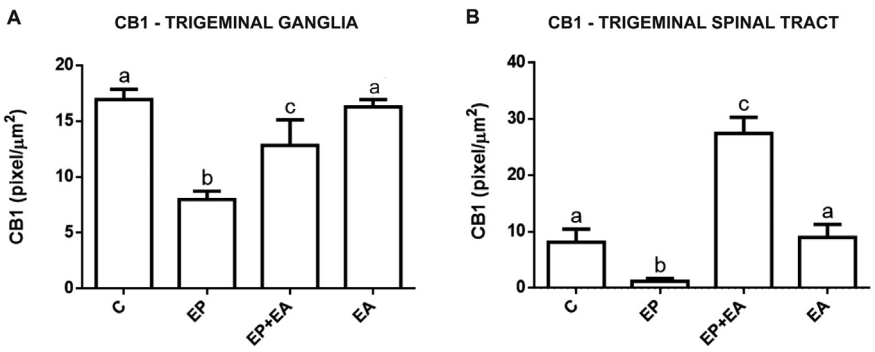

Fig. 6. Immunofluorescence analyses shows the means and standard deviations of CB1 immunolabeling in the trigeminal ganglia (A) and in the trigeminal spinal tract (B). (A) There was a lower immunoexpression of CB1 in the animals with EP, when compared to the control group ($p < 0.05$), which was partially reverted by the EA treatment ($p < 0.05$). (B) The group of animals with EP showed a lower immnunoexpression of CB1 when compared to the other groups ($p < 0.05$). The EA treatment increased the immunoexpression of CB1 only in the presence of the EP, compared to the other groups ($p < 0.05$). Same letters indicate no significant differences among the groups (ANOVA,Tukey test).

these responses [15]. Recently, we also showed that EA produced anti-inflammatory effects on EP in rats, reducing alveolar bone loss and the production pro-inflammatory cytokines [6]. Thus, the present study sought to investigate if the anti-inflammatory effect of EA in experimental periodontitis involved the activation of cannabinoid receptors in the periodontal tissue and in regions of the trigeminal system.

In this study, we verified that EA reduced alveolar bone loss in EP, as previously showed [6]. We also observed that EA increased the immunoexpression of CB1 and CB2 receptors in the periodontium, when compared to the control group. However, regarding the EA-treated group, only the CB2 immunoexpression was statistically different from the EP and EP + EA-sham groups.

Qian et al. [31] demonstrated that the activation of CB2 is able to enhance osteogenic differentiation of the periodontal ligament cells. Additionally, it has been shown that the expression of CB2 receptors has been reported in osteoblasts, osteocytes and osteoclasts, whereas expression of CB1 was very low or not observed [32]. Two mechanisms are suggested for bone mass maintenance through CB2 receptor activation. The first one is the stimulation of stromal cells/osteoblasts and the other is by inhibition of osteoclasts, reducing the availability of RANKL, which is a critical osteoclastogenic factor [33,34]. As for CB1, it has been shown that central CB1 stimulation might lead to peripheral bone formation modulated by the adrenergic system [35]. In fact, our data showed an increased CB2 expression in peripheral tissues and an increased CB1 immunolabeling in the nervous system, which might have led to the observed protection of inflammatory bone resorption. Moreover, CB2 receptor might play an important role in bone metabolism, as well as inflammation [22]. Some studies have shown that EA treatment reduced the expression of pro-inflammatory mediators through the activation of the CB2 receptor [15,36].

It is important to emphasize that bacterial products can activate nociceptors and contribute to inflammatory pain [37]. Bacteria or bacterial byproducts directly activate nociceptive neurons trough toll like receptor 4 (TLR4), a receptor that shows a physiological role in the transduction of neurogenic regulation of the inflammatory process in the periodontium [38]. Vindis et al. [38] also hypothesized that local tissue inflammation activates the sensory neurons of the peripheral nervous system to upregulate the expression of TLR4 and accelerate its transport from the cell soma to the nerve endings innervating the inflamed tissue. In another study, an increased expression of calcitonin gene related peptide (CGRP), and, to a lesser extent, substance P, in the neurons of the ipsilateral TG, projecting to the inflamed area were showed one week after ligature-induced periodontitis in rats [39]. These authors have demonstrated that some central processes of TG neurons project to the contralateral trigeminal nuclear complex in the brainstem of the rat, through transmedian fibers that provide the opportunity for a crosstalk between the ipsilateral and the contralateral TG neurons and could explain the changes of neuropeptides expression in the contralateral TG. It is important to observe that, in this study, EA solely increased the expression of CB1 in the trigeminal ganglia, but not in the trigeminal spinal tract. This novel data leads us to believe that the central neuro-immune-modulation exerted by EA depends on the presence of inflammation. Reduction of responses to inflammatory and nociceptive stimuli can occur at multiple levels of the nervous system by the cannabinoid neuromodulatory action [40]. Studies have suggested that during inflammation, microglia, resident macrophages, astrocytes and neurons produce high levels of endocannabinoids, which bind to CB1 and CB2 receptors and attenuate neuronal damage by protecting the nervous system against excitoxicity [41,42].

The activation of CB1 and CB2 represent an important link between the nervous and immune systems, providing neuroprotection and immunomodulation [42]. The EA treatment in periodontal disease in rats

may be a pathway to stimulate neuroimmunomodulatory responses through the endocannabinoid system, increasing the immunoexpression of CB2 receptors in periodontal tissue and of CB1 receptors in neurons of the trigeminal pathway. In this way, EA is an interesting approach for treating inflammatory diseases, including inflammatory bone loss, due to EA's action in the control of the inflammatory response. The usage of this therapy may improve the clinical outcomes of the standard periodontal therapy (scaling and root planning), by reducing the levels of inflammatory cytokines and preventing bone erosion, probably via the endocannabinoid system.

Besides the inherent limitations of experimental studies, specific blockage of the cannabinoid receptors or studies in vivo silencing of the CB1/CB2 receptor with antisense oligonucleotide would better clarify if the bone protective effect of EA is dependent on the either or both CB1 and CB2 receptors. Moreover, further studies evaluating bone mechanical characteristics and specific mineral composition should be considered.

In conclusion, EA reduced bone loss in experimental periodontitis and increased the immunoexpression of the CB1 receptor in the neural tissues and CB2 in the periodontal tissues.

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Declaration of Competing Interest

None.

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