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MARIO ROBERTO PONTES LISBOA

AVALIAÇÃO DOS EFEITOS DA ELETROACUPUNTURA NA PERIODONTITE INDUZIDA POR LIGADURA EM RATOS

FORTALEZA

2014

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Dissertação de Mestrado apresentada à coordenação do Programa de Pós-Graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Mestre em Odontologia

Área de concentração: Clínica Odontológica

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RESUMO

Tem sido relatado que a acupuntura é capaz de modular a resposta imunoinflamatória do hospedeiro. O objetivo deste estudo foi a avaliar os efeitos da eletroacupuntura (EA) na periodontite induzida por ligadura em ratos. Trinta e dois animais foram divididos nos grupos C (controle), PE (periodontite experimental), PE/EA-*sham* e PE/EA. Nos grupos PE, uma ligadura foi posicionada ao redor dos 1os molares inferiores direitos. Cinco sessões de EA ou EA-*sham* foram realizadas a cada dois dias, iniciando-se no dia seguinte à colocação da ligadura. Para o tratamento com EA, os acupontos IG4, IG11, E36 e E44 foram utilizados. A EA-*sham* foi realizada em pontos localizados fora de meridianos. Os animais foram submetidos à eutanásia 11 dias após a indução da periodontite. Análises histomorfométrica e microtomográfica foram realizadas. Expressões dos RNAm de interleucina (IL)-1β, metaloproteinase de matriz (MMP)-8, IL-6, fator de necrose tumoral (TNF)-α e ciclo-oxigenase (COX)-2 foram avaliadas por meio da reação em cadeia da polimerase da transcrição reversa em tempo real (qRT-PCR). Os dados foram estatisticamente analisados (ANOVA, $p<0,05$). As análises histomorfométrica e microtomográfica demonstraram que o grupo PE/EA apresentou perda óssea alveolar reduzida quando comparado ao grupo PE ($p<0,05$). O tratamento com EA diminuiu a expressão gênica de IL-1β e MMP-8 ($p<0,05$), aumentou a expressão do RNAm de IL-6 ($p<0,05$) e não modificou a expressão gênica de TNF-α e COX-2 em animais com PE ($p>0,05$). Dentro dos limites do presente estudo, pode ser concluído que a EA reduz a destruição tecidual periodontal e a expressão de alguns mediadores pró-inflamatórios na PE em ratos.

Palavras-chave: Eletroacupuntura; Periodontite; Reabsorção Óssea; Mediadores Inflamatórios.

ABSTRACT

Acupuncture has been reported as capable of modulating the host's immuno-inflammatory response. The purpose of this study was to evaluate the effects of electroacupuncture (EA) on ligature-induced periodontitis in rats. Thirty-two animals were divided into groups C (control), EP (experimental periodontitis), EP/EA-sham and EP/EA. On EP groups, a ligature was placed around right mandibular 1st molars. Five sessions of EA or EA/sham were assigned every other day, starting one day after ligature placement. For EA treatment, acupoints LI4, LI11, ST36 and ST44 were used. EA-sham was performed in off-meridian points. Animals were euthanized 11 days after the induction of periodontitis. Histomorphometric and microtomographic analyses were performed. Expressions of interleukin (IL)-1β, matrix metalloproteinase (MMP)-8, IL-6, tumor necrosis factor (TNF)-α and cyclooxygenase (COX)-2 mRNA were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Data were statistically analyzed (ANOVA, $p<0.05$). Histomorphometric and microtomographic analyses demonstrated that group EP/EA presented reduced alveolar bone loss when compared with group EP ($p<0.05$). EA treatment decreased the genic expression of IL-1β and MMP-8 ($p<0.05$), increased the mRNA expression of IL-6 ($p<0.05$) and did not modify the genic expression of TNF-α and COX-2 in animals with EP ($p>0.05$). Within the limits of the present study, it can be concluded that EA reduces periodontal tissue destruction and the expression of some pro-inflammatory mediators in EP in rats.

Key Words: Electroacupuncture; Periodontitis; Bone Resorption; Inflammation Mediators.

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1. INTRODUÇÃO GERAL

A doença periodontal é uma doença multifatorial que envolve biofilmes bacterianos e a geração de respostas inflamatórias.[1, 2] Sob certas condições, como predisposição genética, fumo e diabetes melito, a flora bacteriana patogênica pode exceder a capacidade de defesa do sistema imunológico do hospedeiro, o que pode levar à periodontite. Esta é caracterizada principalmente pela reabsorção óssea alveolar, perda de inserção e formação de bolsas periodontais.[3]

O conhecimento sobre a patogênese das doenças periodontais evoluiu consideravelmente nos últimos 50 anos, desde que foi relatado, pela primeira vez, que o biofilme bacteriano exerce uma função importante no estabelecimento e na progressão das doenças periodontais.[1] Sabe-se que a presença de uma flora oral patogênica (por exemplo, *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*)[4] pode induzir uma reação inflamatória levando à secreção de mediadores pró-inflamatórios, tais como Interleucina (IL) -1β, Fator de Necrose Tumoral-α (TNF-α), Prostaglandina E_2 (PGE_2) e Metaloproteinases de Matriz (MMPs) por células imunes (leucócitos e macrófagos) e também por fibroblastos gengivais.[5] Os mediadores pró-inflamatórios estimulam a reabsorção óssea alveolar mediada por osteoclastos e também a migração apical do epitélio juncional. A severidade e a progressão da doença são modificadas em indivíduos geneticamente suscetíveis e/ou na presença de fatores de risco imunorreguladores.[6] Para os pacientes que são suscetíveis, a maior parte da destruição periodontal pode ocorrer devido à resposta inflamatória do hospedeiro.[7]

Os pré-requisitos para um tratamento periodontal convencional bem-sucedido são a cooperação do paciente, uma adequada higiene oral[8] e o debridamento mecânico de todas as superfícies dentárias (Raspagem e Alisamento Radicular).[9, 10] Entretanto, recentemente tem sido estudada uma nova abordagem para o tratamento periodontal, envolvendo o controle das respostas do hospedeiro à agressão bacteriana.[7] Com base na premissa de que a resposta imunoinflamatória é um fator primordial na determinação da severidade da doença periodontal, alguns autores propuseram o conceito de "modulação da resposta do hospedeiro", com intervenções que visam modular essa resposta.[11-13]

A acupuntura é uma modalidade de terapia da medicina tradicional chinesa que tem seu fundamento na associação entre os sistemas nervoso e imunológico, tendo sido

primeiramente relatada em meados do século II a.C..[14] O método baseia-se na colocação e estimulação de agulhas na pele de determinadas regiões corpóreas, objetivando o ajuste da energia vital (Qi), que circula por meridianos situados ao longo do organismo.[14] As agulhas podem ser estimuladas manualmente, com movimentos rotacionais ou oscilatórios, e também eletricamente, com a ligação de uma fonte elétrica de baixa amperagem às agulhas, estimulando-as em frequências da ordem de 10 Hz.[15, 16] Embora em menor escala, outros métodos de acupuntura também foram utilizados, como a apipuntura, na qual veneno de abelha em pequenas concentrações é injetado nos pontos acupunturais, e a acupuntura a laser, na qual os pontos são estimulados por radiação a laser de baixa intensidade.[17, 18] Alguns estudos mostraram uma superioridade da eletroacupuntura (EA) em relação à estimulação manual, quando foram consideradas a fadiga muscular e a resposta cerebral à estimulação.[19, 20]

Em 1997, o NIH publicou um relatório de consenso (*National Institutes of Health Consensus Statement*, EUA) reconhecendo a eficácia da acupuntura para o tratamento de náuseas e vômitos, consequentes de quimioterapia ou intervenções cirúrgicas, e da dor dentária pós-operatória. O valor da acupuntura também foi reconhecido como um tratamento adjunto ou como uma alternativa de tratamento para a dor e/ou inflamação em uma variedade de condições, como: enxaquecas, cólicas menstruais, dores de cabeça, epicondilite, fibromialgia, dor miofascial, osteoartrite, dores lombares, síndrome do túnel carpal, asma e obesidade.[21, 22] O método ainda foi considerado promissor na reabilitação de pacientes viciados em álcool, tabaco e outras drogas e de pacientes que sofreram acidentes vasculares cerebrais.[21]

Uma evidência da relevância da acupuntura para as Ciências da Saúde foi o estabelecimento de um conjunto de regras-guia para o delineamento de ensaios clínicos com o método, chamado “Standards for Reporting Interventions in Clinical Trials of Acupuncture” (STRICTA), que teve como base o CONSORT (“Consolidated Standards of Reporting Trials”).[23] Publicado pela primeira vez em 2001 e com várias atualizações a partir desta data, o STRICTA tem como objetivo melhorar a integridade e a transparência do relato dos ensaios clínicos de acupuntura, para que esses sejam mais apuradamente interpretados e prontamente replicados. Desta maneira, os viéses e a tendenciosidade são evitados, oferecendo aos leitores uma visão clara da metodologia e dos resultados do tratamento.[24]

Experimentalmente, a acupuntura tem apresentado ação moduladora da resposta inflamatória em diversas condições induzidas em ensaios, como asma, lesão de medula

espinal, peritonite, doença pulmonar obstrutiva crônica, artrite da articulação temporomandibular, dor neuropática e lesões cerebrais por isquemia. [25-33] Após a aplicação da acupuntura, observa-se diminuição da dor, do edema, do infiltrado inflamatório e/ou da produção de citocinas, ocorrendo uma tendência de resolução do quadro inflamatório.[25-33]

A acupuntura pode agir no eixo neuroimunológico por meio de diferentes mecanismos. A inflamação aguda causada pela colocação e estimulação da agulha leva à secreção local de peptídeos, como substância P, histamina, bradicinina e enzimas proteolíticas, que ativam o sistema opioide, reduzindo a dor inflamatória.[34] Essas substâncias também ativam o sistema nervoso autônomo e o sistema endocanabinoide, regulando a expressão e a secreção de citocinas inflamatórias.[14, 35-37] O estresse local causado pela acupuntura induz à secreção central de catecolaminas, ativando o sistema nervoso simpático. A epinefrina, ligando-se ao receptor adrenérgico β_2 nas células imunes, culmina no decréscimo da produção de moléculas inflamatórias, como TNF-α, IL-1β, IL-6 e IL-18.[38] Todavia, alguns autores apontam que o sistema nervoso autônomo parassimpático apresenta um papel anti-inflamatório ainda mais relevante do que o do sistema nervoso simpático.[14, 38, 39] Com a ativação do sistema nervoso parassimpático, induz-se à secreção vagal de acetilcolina, que se liga a receptores nicotínicos α7 no sistema monócito-macrófago, inibindo a síntese de citocinas pró-inflamatórias, como TNF-α e IL-6, e também a ativação do sistema do Fator Nuclear κB (NF-κB).[39] Ademais, observou-se que receptores muscarínicos periféricos também mediam os efeitos anti-inflamatórios da acupuntura.[40]

Mais recentemente, evidenciou-se que a acupuntura apresenta ação antinociceptiva e anti-inflamatória também por meio do sistema endocanabinoide (SEC), aumentando a produção de agonistas canabinoides endógenos.[41, 42] O SEC é um sistema de sinalização endógena lipídica, formado pelos receptores canabinoides CB1 e CB2, dois agonistas principais, a anandamida e o 2-araquidonoil glicerol, e um aparato bioquímico responsável pela degradação desses agonistas.[43] Os receptores CB1 são encontrados principalmente em células do sistema nervoso, sendo responsáveis pela maioria das ações centrais dos agonistas canabinoides. Já os receptores CB2 são normalmente encontrados em células do sistema imunológico, como monócitos e macrófagos, embora também sejam expressos em células do sistema nervoso.[44] A antagonização dos receptores CB2 mostrou-se capaz de reverter os efeitos antinociceptivos e anti-inflamatórios da EA em modelos experimentais de edema de pata e artrite da articulação têmporo-mandibular em ratos,

aumentando a dor inflamatória, o edema e a secreção de citocinas pró-inflamatórias, como IL-1β, IL-6 e TNF-α.[29, 36, 41]

O SEC também parece exercer um importante efeito na modulação da resposta imunoinflamatória nos tecidos periodontais. Diversas células presentes no periodonto, como fibroblastos gengivais, células do ligamento periodontal, macrófagos e células endoteliais, expressam tanto receptores CB1 como CB2.[45-47] De fato, a ativação do SEC em modelos experimentais de periodontite foi capaz de diminuir a perda óssea alveolar,[48, 49] provavelmente agindo via modulação do sistema NF-κB.[45, 48] Além disso, a ativação de receptores CB1 mostrou-se capaz de diminuir a secreção de TNF-α e PGE_2 por fibroblastos gengivais.[49] Em modelos experimentais de periodontite em ratos, a administração de anandamida diminuiu a produção de IL-1β e TNF-α.[48, 50] Somado aos efeitos anti-inflamatórios, o SEC também pode diminuir o colapso do tecido conjuntivo via células do ligamento periodontal, aumentando a produção de fibronectina e de fator de transformação do crescimento-β (moléculas importantes para a produção de matriz extracelular) e diminuindo a produção e a atividade de MMPs -1 e -2.[51] Ademais, o SEC também parece exercer um efeito importante no reparo dos tecidos periodontais. Após cirurgia periodontal em humanos, os níveis de anandamida no fluido crevicular gengival encontram-se aumentados.[52] *In vitro*, a anandamida aumentou a proliferação de fibroblastos gengivais.[52] É importante considerar que os receptores canabinoides também são expressos no tecido ósseo. Os receptores CB1 são encontrados prioritariamente nas porções terminais de ramificações nervosas simpáticas no tecido ósseo, próximos a osteoblastos, e os receptores CB2 são encontrados nos osteoblastos, pré-osteoblastos e osteócitos.[53] Dessa forma, o SEC tem um papel importante na regulação do metabolismo ósseo, com uma ação pró-osteogênica por meio da sinalização cérebro-óssea intermediada por receptores CB1 e por estimulação mitótica celular dos osteoblastos intermediada por receptores CB2.[53]

Na Odontologia, a acupuntura ainda é pouco utilizada, mas tem sido aplicada como método adjunto no tratamento de disfunções têmporo-mandibulares, dores faciais e dor pós-operatória.[21, 54-57] A literatura é escassa no que diz respeito aos efeitos da acupuntura nos tecidos periodontais. Schoor e colaboradores[58] relataram o caso de uma paciente que apresentava um quadro de desconforto crônico na gengiva, diagnosticado como doença periodontal, e que desapareceu após tratamento com acupuntura. Outros autores afirmaram que a acupuntura, associada à raspagem e à moxabustão, é um tratamento rápido e confiável

para a periodontite.[59] Contudo, não há estudos clínicos ou experimentais que analisaram a influência da acupuntura na doença periodontal.

2. PROPOSIÇÃO

O objetivo deste estudo foi avaliar os efeitos eletroacupuntura nos tecidos periodontais, por meio de análises histomorfométrica e microtomográfica, e na expressão gênica dos mediadores inflamatórios IL-1β, metaloproteinase (MMP)-8, IL-6, TNF-α e ciclo-oxigenase-2 (COX-2), por meio da reação em cadeia da polimerase da transcrição reversa em tempo real, na periodontite induzida por ligadura em ratos.

3. DESENVOLVIMENTO

Esta dissertação de Mestrado baseia-se no Artigo 46º do Regimento Interno do Programa de Pós-Graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado. Este capítulo consta de uma cópia do artigo científico de autoria do candidato, redigido de acordo com as normas da revista científica escolhida para publicação ("Journal of Periodontology").

Por se tratar de pesquisa envolvendo animais, o projeto de pesquisa referente a esta dissertação foi submetido à apreciação da Comissão de Ética em Pesquisa Animal (CEPA) da Universidade Federal do Ceará, tendo sido aprovado sob número de protocolo 56/2012 (Anexo A).

Artigo Científico:

Effects of Electroacupuncture on Experimental Periodontitis in Rats.

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ABSTRACT

Background: Acupuncture has been reported as capable of modulating the host's immuno-inflammatory response. The purpose of this study was to evaluate the effects of electroacupuncture (EA) on ligature-induced periodontitis in rats.

Methods: Thirty-two animals were divided into groups C (control), EP (experimental periodontitis), EP/EA-sham and EP/EA. On EP groups, a ligature was placed around right mandibular 1st molars. Five sessions of EA or EA/sham were assigned every other day, starting one day after ligature placement. For EA treatment, acupoints LI4, LI11, ST36 and ST44 were used. EA-sham was performed in off-meridian points. Animals were euthanized 11 days after the induction of periodontitis. Histomorphometric and microtomographic analyses were performed. Expressions of interleukin (IL)-1β, matrix metalloproteinase (MMP)-8, IL-6, tumor necrosis factor (TNF)-α and cyclooxygenase (COX)-2 mRNA were evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Data were statistically analyzed (ANOVA, $p<0.05$).

Results: Histomorphometric and microtomographic analyses demonstrated that group EP/EA presented reduced alveolar bone loss when compared with group EP ($p<0.05$). EA treatment decreased the genic expression of IL-1β and MMP-8 ($p<0.05$), increased the mRNA expression of IL-6 ($p<0.05$) and did not modify the genic expression of TNF-α and COX-2 in animals with EP ($p>0.05$).

Conclusion: Within the limits of the present study, it can be concluded that EA reduces periodontal tissue destruction and the expression of some pro-inflammatory mediators in EP in rats.

Key Words: Electroacupuncture; Periodontitis; Bone Resorption; Inflammation Mediators.

INTRODUCTION

Periodontitis (PD) is a worldwide health problem[1-3] and affects almost half of the population aged 30 years or older in the United States.[2] It is estimated that 5 to 15% of the global population presents the severe forms of PD.[4] Its development is related to the formation of a periodontopathogenic biofilm, which induces a periodontal inflammatory response.[5, 6] Although it is known that the microbial challenge is necessary for the development of PD, the host's inflammatory response is the ultimate responsible for the appearance of its main clinical features, such as bone loss and periodontal tissue collapse.[7, 8]

Although the main prerequisites for a successful conventional periodontal treatment are patient's cooperation, an adequate oral hygiene regimen[9] and mechanical removal of dental plaque and plaque retentive factors,[10] researchers have been focusing on the modulation of the host's inflammatory response, a new periodontal approach.[11-13]

Acupuncture is a modality of the traditional Chinese medicine that relies on skin stimuli of specific points called acupoints by needles. Nowadays, acupuncture is considered an adjunct treatment or an acceptable alternative for a number of clinical conditions, such as addiction, stroke rehabilitation, headache, menstrual cramps, tennis elbow, fibromyalgia, low back pain, carpal tunnel syndrome and asthma.[14] With the rising of scientific evidence proving acupuncture to be an effective therapy, a set of guidelines called "Standards for Reporting Interventions in Clinical Trials of Acupuncture" (STRICTA), an official extension of CONSORT (Consolidated Standards of Reporting Trials),[15] started to be used, aiming to improve the integrity and transparence of clinical trials regarding acupuncture.[16]

The stimulation of acupoints leads to neuroendocrine inflammatory responses through many pathways, activating different systems, such as opioid, endocannabinoid and autonomous nervous systems.[17-19] Acupuncture has been proved to reduce the genic expression and the protein levels of certain inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, impairing pro-inflammatory reactions in arthritis and skin inflammation.[20, 21] It has been used as a treatment for the inflammatory aspects of many other experimental diseases, such as colorectal distention, neuropathic pain and asthma.[22-24] In addition, it was demonstrated that electroacupuncture (EA) was capable of influencing bone metabolism experimentally, enhancing cellular proliferation and BMD.[25-27]

To the best of our knowledge, very little data has been published linking acupuncture therapy to periodontal inflammation. Schoor et al.[28] reported a case of a patient that presented a chronic low-grade discomfort in the gingiva, diagnosed as PD, which disappeared after

acupuncture treatment. Other researchers stated that acupuncture, moxibustion therapy and scaling are a rapid and reliable treatment for PD.[29] However, there are no clinical trials or experimental studies analyzing the influence of acupuncture treatment on PD.

Based on the potentiality of acupuncture to modulate the inflammatory response, the purpose of this study was to evaluate the effects of EA on ligature-induced PD in rats.

METHODS

Sample

This study was conducted in compliance with the ethical principles of animal experimentation, as well as standards for the didactic-scientific practice of vivisection and the Universal Declaration of Animal Rights by United Nations Educational, Scientific and Cultural Organization (UNESCO). The present study was conducted only after review and approval by the Ethics Committee on Animal Research at Federal University of Ceara - UFC (protocol 56/2012).

Experimental Model

Thirty-two adult male rats (*Rattus norvegicus, albinus*, Wistar), weighing between 200 and 250 g, were used (Central Animal Facility, UFC). The rats were kept in a room with a 12-hour light/dark cycle and temperature between 22 and 24°C. Throughout the experiment, the animals were housed in plastic cages and fed with selected solid diet and water *ad libitum*. They were randomly assigned to one of 4 experimental groups (n = 8), according to the following protocol:

- Group C (control): Experimental Periodontitis (EP) was not induced and EA or EA-sham were not performed;
- Group EP: EP was induced with ligature. EA or EA-sham were not performed;
- Group EP/EA-sham: EP was induced with ligature and EA-sham was performed;
- Group EP/EA: EP was induced with ligature and EA was performed.

Induction of Experimental Periodontitis

All animals were anesthetized by an intra-muscular injection of ketamine (70 mg/kg body weight) and xylazine (6 mg/kg bodyweight). They were positioned on an operating

table, allowing the maintenance of the rats mouth opened, facilitating the access to the posterior mandibular teeth. A cotton ligature was placed around the cervical area of the right mandibular 1st molar of each animal, except for the ones of group C. The ligatures were knotted at the buccal surface of the tooth and remained in place for 11 days.

EA and EA-sham Procedures

The animals were not anesthetized nor sedated for these procedures. To reduce animals' stress during these events, they were adapted to a specially manufactured bed that allows the exposure of their tails and front and back paws. During the 5 days that preceded the EP induction, the rats remained in these beds for 10 minutes a day.

EA and EA-sham were performed with 0.18 mm in diameter and 8 mm in length stainless steel needles‡ inserted to a depth of 3 mm under the skin in predetermined points. The acupuncture point selection was based on Traditional Chinese Medicine meridian theory. In group EP/EA, the large intestine meridian points 4 and 11 (LI4 and LI11, respectively) and the stomach meridian points 36 and 44 (ST36 and ST44, respectively) were stimulated.[19, 30] The stimulation of these acupoints causes a local acute inflammation that targets the activation of opioid, sympathetic, parasympathetic and endocannabinoid systems.[17-19] In group EP/EA-sham, two sham-points located 5 mm laterally and 5 mm above the gallbladder meridian point 30 (GB30) were stimulated instead.[19, 30] When sham-points are stimulated, even though a local inflammatory response takes place, the activation of the anti-inflammatory pathways aforementioned is not observed.[31] The LI4 point is located in the front paw, between the 1st and the 2nd metacarpal bone. The LI11 point is the depression formed when the elbow is flexed at the lateral end of the transverse cubital crease near the lateral epicondyle of the humerus. The ST36 point is at the proximal 1/5 site of the craniolateral surface of the leg distal to the head of the tibia in a depression between the muscles of the cranial tibia and long digital extensor. The ST44 point is at the dorsum of the hind leg, proximal to the web margin between the 2nd and the 3rd metatarsal.[19, 30] The GB30 point is located at the junction of the lateral 1/3 and medial 2/3 distance between the prominence of the greater trochanter and the hiatus of the sacrum.[21]

The sessions of EA and EA-sham were performed every other day, starting from the day after the ligature placement. All acupoints or sham-points were stimulated bilaterally and simultaneously with low frequency and rectangular pulses (f=10 Hz, recurrence time=1 s, intensity=3 mA)[19, 30, 32, 33] during 20 minutes, using an electric stimulation device§. EA and EA-sham procedures were conducted by an experienced practitioner (D.V.G).[19, 30, 34]

The animals were euthanized under anesthesia with a final solution of xylazine (30 mg/kg body weight) and ketamine (240 mg/kg body weight) 11 days after the placement of the ligatures. Samples of gingival tissues around right mandibular 1st molars of each animal were collected and stored at -80 ºC. The right mandibles were excised, fixed in 4% paraformaldehyde for 24 hours and rinsed with water.

Microcomputed Tomography Analysis

Non-demineralized specimens were scanned by a cone beam micro-computed tomography (CT) system‖. The x-ray generator was operated at an accelerated potential of 50 kV with a beam current of 200 µA and an exposure time of 650 ms per projection. Images were produced with a voxel size of 6 x 6 x 6 µm.

Using an appropriated software¶, the generated 3 dimensional models were rotated into a standard position as the following criteria: (1) in transaxial plane, the mandibular 1st molar (M1) had its axis vertically positioned and (2) in coronal plane, the mandibular bone was vertically orientated, with the mesial root of the M1 in the upper position of the image. Linear measurements on alveolar bone level (ABL) were performed at 3 different sites: buccal, lingual and interproximal. For buccal and lingual sites, on the transaxial image passing through the distal root of the M1, the linear distances from cementoenamel junction (CEJ) to buccal/lingual alveolar bone crest (ABC) were measured (Figure 1A). For the interproximal site, coronal dataset was analyzed using appropriated software#. The distance between the last image showing the ABC, between mandibular 2nd molar (M2) and M1, and the first image showing the CEJ of M1, was measured (Figures 1B,1C).

Bone mineral density (BMD) was also analyzed. A volume of interest (prismatic section) was outlined from the apexes of all roots of M1 up to the roof of the furcation of M1, touching the roots surfaces, in all images of the coronal dataset. BMD was determined by comparing the volume of interest of the samples with a pattern that presented a known mineral density, using the same software applied for the analysis of the interproximal site (Figure 1D).

All micro-CT analyses were performed by one calibrated examiner (M.R.P.L.) who was blinded to the experimental groups and treatments rendered.

Histopathological and Histometric Analysis of Periodontal Tissues

The mandibles were decalcified in 4% Ethylenediamine tetraacetic acid (EDTA) solution. After complete decalcification, the specimens were processed and embedded in

paraffin. Serial sections, 4 μm thick, were obtained in a mesiodistal direction. The sections were stained with hematoxylin and eosin (H&E) for analysis by light microscopy.

The histopathological analysis was performed by a certified histologist (E.E.) using a light microscope**. The following parameters were evaluated: nature and degree of inflammation of periodontal tissues, influence of the inflammatory process on surrounding tissues, presence and extension of tissue necrosis, presence and extension of osseous sequestrum, presence and extension of root resorption, vascular status and cellularity pattern of epithelial, connective, bone and hematopoietic tissues.

For histometric analysis, sections representing the most central buccal-lingual portion of right mandibular 1st molars were selected. Microphotographies were captured using a digital camera†† connected to a light microscope‡‡ with an original magnification of x40. The generated images were analyzed with an adequate software§§. The furcation area not filled with bone or periodontal ligament (Area of No Bone or Periodontal Ligament-ANBL) was measured by outlining the region surrounded by the roof of the furcation, the most coronal portion of the ABC in furcation, the mesial and the distal roots of the 1st molar. Histometric analysis was performed by one calibrated examiner (M.R.P.L.) who was blinded to the experimental groups and treatments rendered.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The gingival samples were manually macerated under freezing with liquid nitrogen. The tissue was homogenized with TRIzol‖‖ (1 mL/0,1 mg of gingival tissue) as recommended by the manufacturer's protocol. Total RNA extraction was performed by an extraction kit¶¶ following manufacturer's recommendations and spectrophotometrically quantified##. Target genes, manufacturer's*** reference of the probes used and their predicted amplicon sizes are shown in Table 1.10 μM of each probe for detection of IL-1β, matrix metalloproteinase (MMP)-8, IL-6, TNF-α and cyclooxygenase (COX)-2 and 5 μL of complementary DNA (cDNA) were used in every reaction. The amplification was performed in a thermocycler††† for 40 cycles and according to the manufacturer's protocol. For mRNA analysis, the genic expression levels of IL-1β, MMP-8, IL-6, TNF-α and COX-2 were calculated by comparison with levels of β-actin mRNA expression in the same sample, using the cycle threshold method. The cycle threshold of the target genes was normalized to an endogenous reference (β-actin), relative to a calibrator group (group C), and was given by the ΔΔCT method using the formula $2^{-\Delta\Delta CT}$.[35]

Examiner Calibration

To estimate the intra and inter-examiner error, histometric and microtomographic analyses were performed by two examiners who were blinded to the experimental groups and treatments rendered. A second sample was measured again 48 hours after the first measurement. The paired t test was used to calculate the intraexaminer error. A Pearson correlation analysis between the data obtained by the two examiners was also performed. P values > 0.05 in paired t test and r > 0.90 values in the Pearson correlation test were considered to estimate the feasibility of the proposed method.

Statistical Analysis of the Data

The data obtained were grouped and presented as means and standard deviations. The significance of differences among groups were verified by analysis of variance (ANOVA) followed by *post-hoc* Tukey test. The significance level was set at 5% in all tests.

RESULTS

All animals tolerated the experimental procedures well and remained healthy throughout the experimental period. No significant differences regarding body weight variation were observed among groups (ANOVA, $p>0.05$).

Examiner Calibration

There were no significant differences between the measurements performed by the same examiner in all analyses performed when the first and the second evaluations were compared ($p>0.05$). There was also a significant correlation between the measurements obtained by the two examiners ($r>0.90$).

Micro-CT and Statistical Analyses

At lingual site, group EP/EA presented significant less alveolar bone resorption than groups EP and EP/EA-sham ($p<0.05$) and no significant difference when compared with group C ($p>0.05$; Figure 2A). At both buccal and interproximal sites, however, group EP/EA demonstrated ABL not statistically different from the other groups ($p>0.05$) (Figures 2B, 2C).

BMD assessment revealed that group EP/EA presented a reduced BMD when compared with group C, but a greater BMD in relation to groups EP and EP/EA-sham, although no significant differences were found when group EP/EA was compared with any of the other groups ($p>0.05$; Figure 2D).

Histopathological Analysis of Periodontal Tissues

In group C, periodontal ligament was comprised of a great amount of collagen fibers, fibroblasts and blood vessels. Collagen fibers were inserted both in cementum and in alveolar bone. Cementum surface was totally sound and covered with cementoblasts. The bone tissue of the interradicular septum presented a few irregularities on its surface and it was coated with osteoblasts or bone lining cells. At this site, bone trabeculae were considerably thick, limiting little medullary spaces (Figures 3A, 3B). Groups EP and EP/EA-sham presented a moderate inflammatory infiltrate predominantly composed of mononuclear cells and a small amount of neutrophils in the periodontal ligament. This ligament presented interstitial edema and a reduced amount of collagen fibers when compared with the periodontal ligament observed in group C. In the majority of the specimens, the cementum presented small areas of active resorption. The bone tissue in the interradicular septum presented thin trabeculae, with a very irregular contour and many active osteoclasts (Figures 3C, 3D, 3E, 3F). In group EP/EA, the mononuclear inflammatory infiltrate in the periodontal ligament was very slight. This tissue was much more fibrous and less edematous than the periodontal ligament present in groups EP and EP/EA-sham, which demonstrates minor alterations in collagen fibrilogenesis. The cementum was sound in the majority of the specimens, although some samples presented areas with active resorption. Bone tissue in the interradicular septum was comprised of bone trabeculae with an irregular external contour and it was covered with osteoblasts or bone lining cells. Few active osteoclasts were observed (Figures 3G, 3H).

Histometric and Statistical Analyses

Group EP/EA (0.158 ± 0.056 mm^2) presented lower ANBL than groups EP (0.312 ± 0.0844 mm^2, $p<0.05$) and EP/EA-sham (0.324 ± 0.0933 mm^2, $p<0.05$). There were no differences in ANBL when groups C (0.096 ± 0.021 mm^2) and EP/EA were compared ($p>0.05$; Figure 4).

qRT-PCR and Statistical Analyses

Regarding the expression of IL-1β mRNA, groups EP/EA and EP/EA-sham presented lower levels when compared with group EP (5.4-fold higher when compared with group C; $p<0.05$) and similar levels to the ones presented by group C ($p>0.05$; Figure 5A).

Group EP/EA presented a decreased expression of MMP-8 mRNA when compared with groups EP (3.47-fold higher in relation to group C; $p<0.05$) and EP/EA-sham (2.08-fold

higher when compared with group C; $p<0.05$). On the other hand, the expression of MMP-8 mRNA in group EP/EA was similar to the one found in animals not submitted to the induction of EP (group C; $p>0.05$; Figure 5B).

Group C presented higher expression of IL-6 mRNA than group EP ($p<0.05$). The animals treated with EA (group EP/EA) exhibited expression of IL-6 mRNA even greater than the presented by group C (1.30-fold higher; $p<0.05$; Figure 5C).

No significant differences were found among groups regarding the levels of TNF-α mRNA ($p>0.05$; Figure 5D). Group EP/EA-sham presented increased expression of COX-2 mRNA when compared with the other groups (2.11-fold higher in comparison to group C; $p<0.05$; Figure 5E).

DISCUSSION

Acupuncture has been reported as a therapy capable of modulating the inflammatory response.[17, 30] To the best of our knowledge, this is the first experimental study analyzing the influence of acupuncture treatment on PD. The aim of this study was to evaluate the effects of EA on ligature-induced PD in rats.

Notably, the present model of PD induction was effective. Significant bone loss, decrease in BMD and moderate inflammatory infiltrate in the periodontal ligament were observed in group EP, but not in group C. Moreover, some pro-inflammatory molecules, such as IL-1β and MMP-8, presented their genic expression up-regulated in group EP, when compared with group C. These mediators are commonly associated with PD.[36, 37] In fact, some studies have shown that the ligature model is one of the most representative experimental models of PD.[38-40]

Overall, the treatment with EA was able to decrease the amount of bone resorption in EP in the present study. It was noticeably observed when the bone tissue was analyzed at furcation and lingual sites (histologically and through micro-CT analysis, respectively). Although group EP/EA did not present significant differences in ABL when microtomographies of interproximal and buccal sites were analyzed, it can be observed a clear trend towards a reduction in bone resorption in this group when compared with group EP. The same was noticed in relation to the BMD measured at the furcation area of the specimens. In fact, radiographic analysis of ovariectomized rabbits' femurs demonstrated that EA treatment was capable of restoring their BMD towards what was observed in naive rabbits.[41] It has also been shown that EA positively influenced bone metabolism.[25] In ovariectomized rats, EA

prevented osteoporosis, enhancing the number of bone trabeculae as well as the trabecular area.[26] In addition, EA induced cellular proliferation in experimental bone fracture in rats, leading to an enhanced bone repair.[27]

In the present study, it was observed that animals treated with EA presented reduced mRNA expression of some inflammatory mediators, such as IL-1β and MMP-8, when compared with animals not treated with EA (group EP). These results are in accordance with other studies, which found a significant decrease in the mRNA expression and/or protein levels of IL-1β in experimental wound healing and paw inflammation in rats, when they were treated with acupuncture or EA.[21, 42] Acupuncture was capable of reducing mRNA expression levels of MMP-9 and MMP-13.[43, 44] However, to the best of our knowledge, this is the first assessment of the effects of EA on MMP-8, and a decrease in the genic expression of MMP-8 was demonstrated in the animals treated.

In this research, there was a decreased genic expression of IL-6 in group EP and an increased genic expression of IL-6 in group EP/EA, both in comparison with group C. While some reported the expression of IL-6 as an important biomarker of PD progression,[45, 46] others[47, 48] hypothesized that IL-6 might have an anti-inflammatory role in bone destruction. Knock-out mice for the IL-6 gene presented more severe bone destruction than wild type mice in experimental periapical lesion.[47, 48] In fact, Darowish et al.[49] reported that IL-6 plays an important role in bone protection, blocking the differentiation of early osteoclast cells. Therefore, the elevated genic expression of IL-6 observed in group EP/EA in this study might have favored a decrease in bone resorption.

The mRNA expression of TNF-α was not reduced in the animals treated with EA in the present study. Other studies found that acupuncture significantly decreased the mRNA expression and protein levels of TNF-α in inflammatory conditions.[21, 42, 50, 51] However, a lack in the reduction of TNF-α protein levels after acupuncture was demonstrated in peritonitis in mice and in rats.[52, 53] It is also important to consider that, in the present study, the levels of TNF-α were similar in all groups, including no differences between groups C and EP. It may point out for a lack of participation of TNF-α in the inflammatory reactions at this time of evaluation of the EP (after 11 days of the placement of the ligature) or even indicate that TNF-α might not be a powerful biomarker of the inflammatory process in PD.[54-56]

The levels of COX-2 mRNA expression were not statistically different between groups C and EP, though these levels were greater in group EP in this study. The expression of COX-2 in periodontal tissues is closely correlated to the amount of prostaglandin E_2 (PGE_2) produced by the oxidation of the arachidonic acid, catalyzed by the aforementioned

enzyme.[57] Although PGE_2 was initially recognized for its effects on bone resorption, it became evident that it also stimulates bone formation.[58] Understanding the role of prostaglandins in skeletal metabolism has been complicated because they act locally and transiently, are regulated at several levels, have multiple receptors, and can have opposing effects depending on the test system.[58] The treatment with EA did not reduce the COX-2 genic expression when compared with the animals not treated (group EP) in the present study. In fact, while it was found that acupuncture may decrease COX-2 and PGE_2 levels,[59, 60] it was also demonstrated that acupuncture effects might not be mediated by changes in prostaglandins levels.[61]

Although acupuncture is currently used worldwide to treat several conditions, its mechanisms of action are not totally elucidated.[60] Acupuncture may activate sympathetic, parasympathetic or endocannabinoid systems, leading to anti-inflammatory effects.[17, 19, 21, 62-64] The needle stimulation causes a secretion of catecholamines, which can lead to anti-inflammatory reactions through activation of the adrenergic receptors α_2 and β.[18, 62] Furthermore, systemic injection of atropine, a cholinergic antagonist, totally impaired the anti-inflammatory effects of acupuncture in a carrageenan-induced paw inflammation in rats.[64] In fact, vagal stimulation leads to an increase in acetylcholine production, which binds to both nicotinic and muscarinic receptors, inducing anti-inflammatory effects.[65] The anti-inflammatory effects of acupuncture through the endocannabinoid system were recently described.[19, 21, 63] Cannabinoid receptors CB1 and CB2 can be found both in gingival fibroblasts[66, 67] and in periodontal ligament cells.[68, 69] Moreover, the treatment with cannabinoid agonists, such as anandamide, cannabidiol, HU-308 and methanandamide, reduced the alveolar bone resorption in EP in rats,[70, 71] the activation of nuclear factor (NF)-κB pathway in periodontal cells[66, 68] and the expression of TNF-α, IL-1β, IL-6 and PGE_2 in periodontal tissues in vitro or in vivo.[66, 70-72] In addition, cannabidiol led to a dose-dependent reduction of the production of MMP-1 and MMP-2 and of the activity of MMP-2 in vitro.[73] It was reported that anandamide induces an up-regulation of COX-2 mRNA mainly via CB1, leading to an increased production of PGE_2.[74] Hypothesizing that EA treatment acted through the endocannabinoid system in this study, it is possible that the increased mRNA expression of COX-2 observed in group EP/EA was mainly due to the activation of the endocannabinoid system. Also, endocannabinoid system plays an important role on bone metabolism regulation, presenting a pro-osteogenic function mediated by brain-bone signaling through CB1 receptors, and a mitotic stimulatory function on osteoblasts through CB2 receptors.[75]

In studies evaluating the effects of the acupuncture, it is essential to carefully select the sites of sham acupuncture. Based on Chinese medicine theory, it is possible that the acupoints for other unrelated conditions or non-acupoints on the meridian can also exert a certain degree of therapeutic effects.[76] Therefore, in the present study, non-acupoints outside the channel of meridian were used for sham acupuncture, as recommended.[76] Although group EP/EA-sham presented worse results than the presented by group EP/EA in most of the analyses, it is intriguing that both groups presented similar expressions of IL-1β mRNA. In addition, the expression of MMP-8 mRNA was decreased in group EP/EA-sham when compared with group EP. A possible explanation for the down-regulation of those genes in the sham-treated group is the fact that the electrical stimulation itself may conduct to an anti-inflammatory reaction.[77-79] Transcutaneous electrical nerve stimulation is a non-acupoint electrical stimulation management that has been shown to reduce the production of IL-1β in experimental wound healing in rats.[78] In spite of the lack of evidence regarding the effects of electrical stimulation in genic expression or production of MMP-8, other MMPs, as well as the tissue inhibitor of metalloproteinase-1 (TIMP-1) may be influenced by electrical stimulus.[77, 79] Uemura et al.[77] reported that vagal electrical stimulation led to a decrease in MMP-9 activity and to an increase of TIMP-1 in a cardiac ischemia-reperfusion model in rabbits. In addition, annulus fibrosus cells exposed to an electrical field presented a reduction in the level of MMP-1.[79] However, it is important to emphasize that the inflammatory process is complex and involve many other aspects besides the roles played by IL-1β and MMP-8. This may explain why the decrease in their genic expression did not result in a reduced alveolar bone loss in sham-treated animals in this study.

Besides the fact that this study presents the inherent limitations of an experimental research in rats, many other investigations should be performed to understand the actual influence of the EA treatment on PD before a clinical application could be projected. The mRNA expressions of some inflammatory mediators were evaluated in this study, but since these assays are not necessarily correlated to the tissue expression of the target molecules,[80, 81] other analysis should be performed. Further studies are encouraged in order to clarify the possible participation of other inflammatory mediators. It is also necessary to elucidate the pathways by which EA influenced some inflammatory aspects in EP, for instance evaluating the expression of endocannabinoid receptors as well as the production of endocannabinoid agonists. In addition, even though the acupoints used in this study have been proved to present anti-inflammatory effects,[19, 30, 50, 82] many other acupoints that succeeded in modulating the immuno-inflammatory response should be investigated, such as the ones

located at the stomach, bladder, gallbladder and governing vessel meridians.[21, 24, 63, 83-85] Since some studies demonstrated that acupuncture may produce different effects when applied as a treatment or a pre-treatment for some inflammatory conditions,[30, 86] it might support additional studies with different treatment protocols for EP.[64, 87]

There are potential interesting implications regarding the study of the acupuncture in PD, since it may influence some systemic factors possibly associated with PD. Acupuncture is considered a valid treatment for psychological stress[88, 89] and there are evidences that stress is capable of influencing the development and progression of PD[90-92]. In addition, acupuncture is associated with changes in eating habits and with the resolution of metabolic syndrome in obese patients,[93] a condition that might also be associated with PD.[94, 95]

CONCLUSION

Within the limits of the present study, it can be concluded that EA reduces periodontal tissue destruction and the expression of some pro-inflammatory mediators in EP in rats.

FOOTNOTES

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‖Skyscan 1172, Bruker, Kontich, Belgium.
¶Data Viewer®, version 1.5.0, Bruker, Kontich, Belgium.
#CT-Analyser®, version 1.13.5.1+, Bruker, Kontich, Belgium.
**Axiovision 4.8.2, Carl Zeiss MicroImaging GmbH, Jena, Germany.
††DC300F, Leica Microsystems, Wetzlar, Germany.
‡‡DMLB, Leica Microsystems, Wetzlar, Germany.
§§ImageJ®, National Institutes of Health, Washington, DC, USA.
‖‖Invitrogen™, Life Tecnhologies, Carlsbad, CA, USA.
¶¶SV Total RNA Isolation System, PROMEGA, Fitchburg, WI, USA.
##NanoVue Plus, GE Healthcare Life Sciences, Fairfield, CT, USA.
***Life Technologies, Carlsbad, CA, USA.

†††StepOnePlus, Applied Biosystems, Foster City, CA, USA.

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FIGURE LEGENDS

Figure 1. Micro-CT images for linear measurements and BMD evaluation in the area of mandibular 1st molar. In transaxial plane, buccal and lingual alveolar bone levels (blue and red lines, respectively; A). In coronal dataset, the last image showing ABC (red arrowhead; B), the first image showing CEJ of M1 (red arrowhead; C) and a bidimensional representation of the prismatic area used for determining the BMD.

Figure 2. Micro-CT analysis. Means and standard deviations of the ABL, with comparisons among groups, at lingual (A), buccal (B) and interproximal (C) sites, and of the BMD assessment (D). Same letters indicate no significant differences among groups (ANOVA, Tukey, $p<0.05$). Abbreviations: C, control; EA, electroacupuncture; EP, experimental periodontitis.

Figure 3. Photomicrographs of the periodontal tissues in the furcation areas of mandibular 1st molars. Abbreviations and symbols: ab, alveolar bone; C, control; EA, electroacupuncture; EP, experimental periodontitis; pdl, periodontal ligament; Hematoxylin and Eosin staining.

Figure 4. Histometric analyses. Means and standard deviations of the ANBL of mandibular 1st molars, with comparisons among groups. Same letters indicate no significant differences among groups (ANOVA, Tukey, $p<0.05$). Abbreviations: C, control; EA, electroacupuncture; EP, experimental periodontitis.

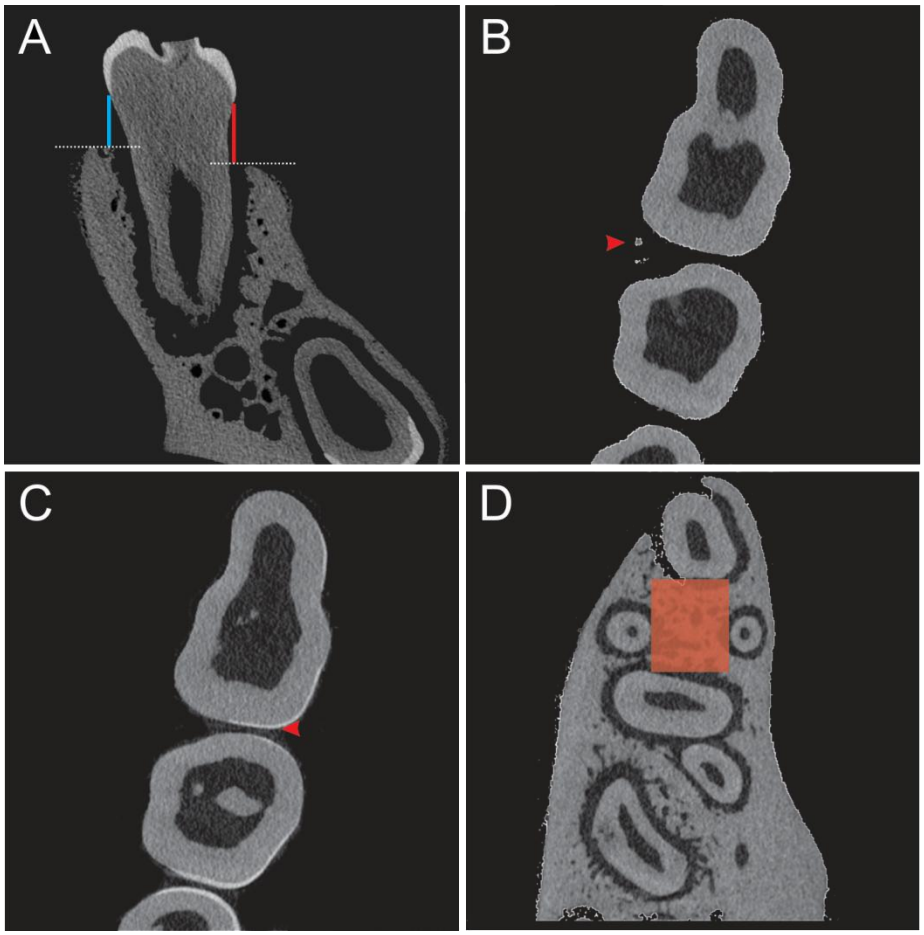
Figure 5. qRT-PCR analyses. Means and standard deviations of the relative genic expression of IL-1β (A), MMP-8 (B), IL-6 (C), TNF-α (D) and COX-2 (E), with comparison among groups. Same letters indicate no significant differences among groups (ANOVA, Tukey,

$p<0.05$). Abbreviations: C, control; COX, cyclooxygenase; EA, electroacupuncture; EP, experimental periodontitis; IL, interleukin; MMP, matrix metalloproteinase; TNF, tumor necrosis factor.

Table 1. Target genes analyzed, probe references and their amplicon sizes

Target gene	Manufacturer's*** Reference	Amplicon size
IL-1β	Rn00580432_m1	74 bp
MMP-8	Rn00573646_m1	92 bp
IL-6	Rn01410330_m1	121 bp
TNF-α	Rn01525859_g1	92 bp
COX-2	Rn01483828_m1	112 bp

bp, base pairs of amplicon sizes; COX, cyclooxigenase; IL, interleukin; MMP, matrix metalloproteinase; TNF, tumor necrosis factor.



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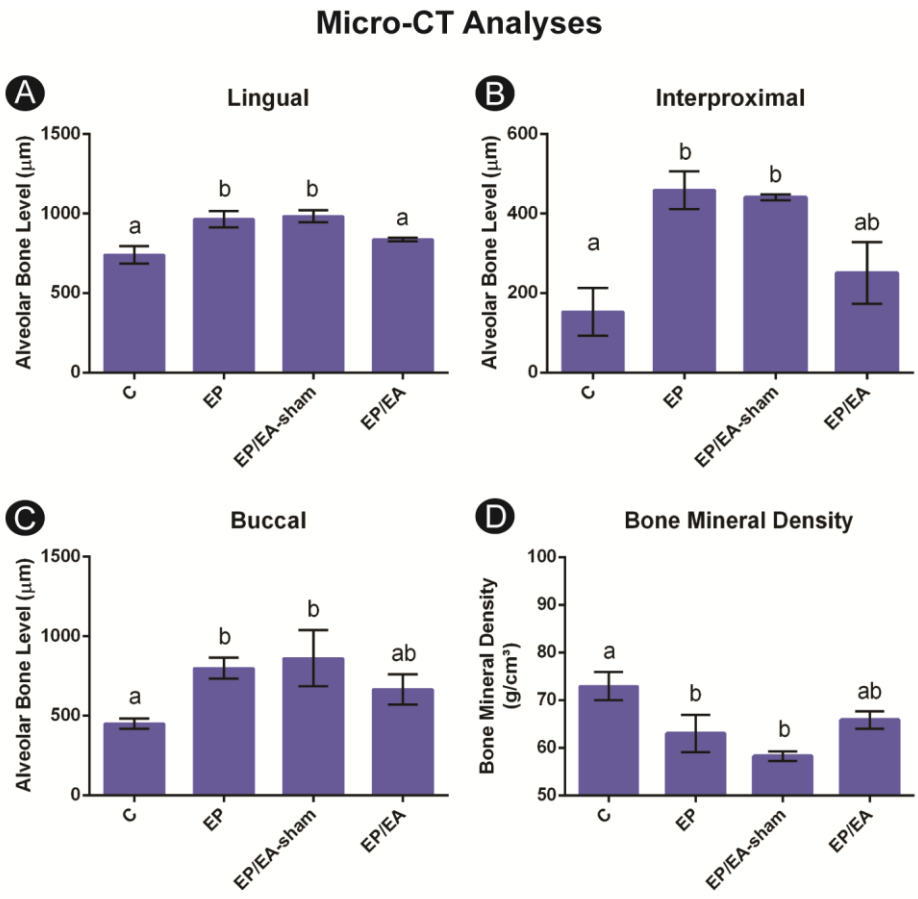

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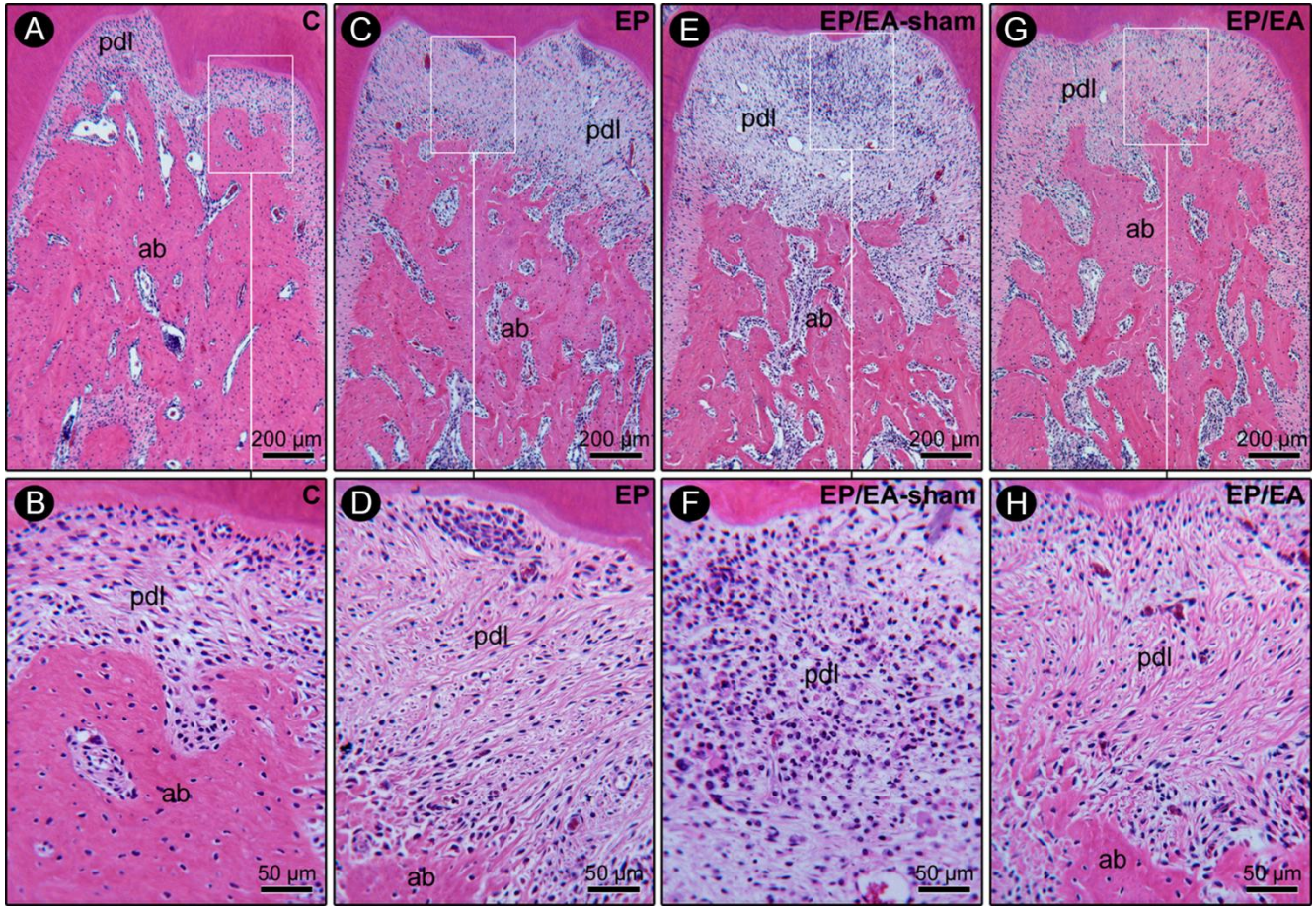

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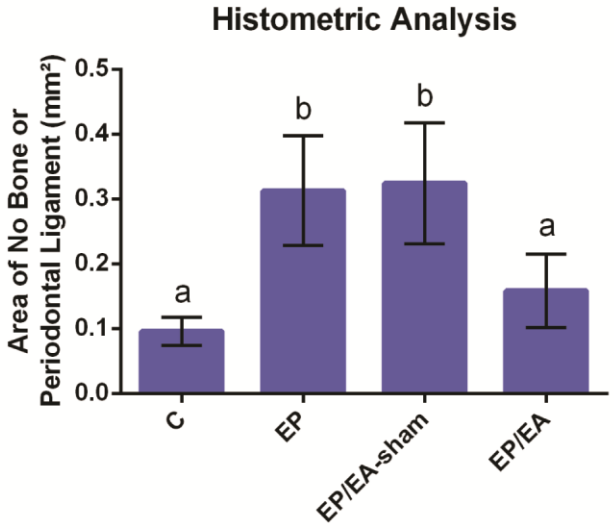

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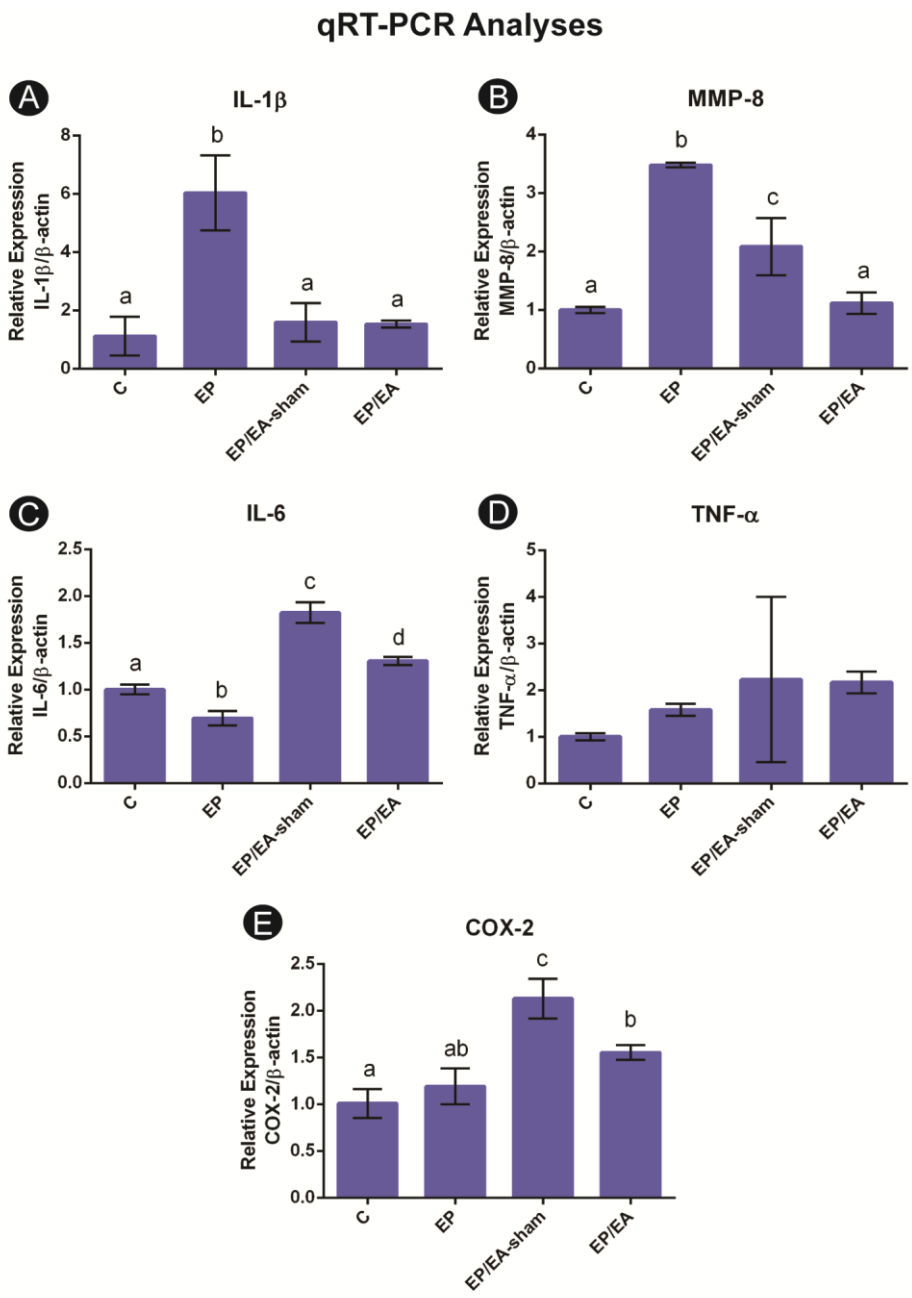

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4. CONCLUSÕES GERAIS

Dentro dos limites deste estudo, pode ser concluído que:

1. A aplicação de eletroacupuntura reduz a perda óssea alveolar na periodontite induzida por ligadura em ratos;
2. O tratamento com eletroacupuntura diminui a magnitude do infiltrado inflamatório e a destruição dos tecidos periodontais na periodontite experimental em ratos;
3. A eletroacupuntura reduz a expressão gênica de alguns mediadores pró-inflamatórios na periodontite induzida por ligadura em ratos.

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ANEXO A

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DECLARAÇÃO

Declaramos que o protocolo para uso de animais em experimentação n° 56/2012, sobre o projeto intitulado: **"AVALIAÇÃO DOS EFEITOS DA ELETROACUPUNTURA NA PERIODONTITE INDUZIDA POR LIGADURA EM RATOS."**, de responsabilidade de Flávia Aparecida Chaves Furlaneto e está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA).

Declaramos ainda que o referido projeto foi aprovado pela Comissão de Ética em Pesquisa Animal – CEPA – em reunião realizada em 28 de novembro de 2012.

Fortaleza, 29 de novembro de 2012

Profa. Dra. Nylane Maria Nunes de Alencar
Coordenadora da Comissão de Ética em Pesquisa Animal – CEPA