Universidade Federal de Minas Gerais
Faculdade de Odontologia

Jasílio Vilela Bastos

ATIVAÇÃO DE RECEPTORES OPIOIDES NA DOENÇA PERIODONTAL EXPERIMENTAL: EFEITOS E MECANISMOS

Belo Horizonte
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Dissertação apresentada ao Colegiado do Programa de Pós-Graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial para obtenção de título de Mestre em Odontologia.

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Ata da Comissão Examinadora para julgamento da Dissertação de Mestrado em Odontologia, área de concentração em **Periodontia**, do candidato **Jasílio Vilela Bastos**

Aos 22 de julho de 2010, às 14:00 h, na sala de Pós-Graduação (3403) da Faculdade de Odontologia, reuniu-se a Comissão Examinadora, composta pelos professores Dra. Cínthia Mara da Fonseca Pacheco, Dr. Rodrigo Villamarim Soares e Dr. José Eustáquio da Costa. A Professora Dra. Cínthia Mara da Fonseca Pacheco, Co-Orientadora da Dissertação, na qualidade de Presidente da sessão, apresentou a Comissão Examinadora e declarou abertos os trabalhos. Ao candidato foi dado o tempo de até 50 (cinquenta) minutos para fazer a exposição oral sobre o seu trabalho **"Ativação de receptores opióides na doença periodontal experimental: efeitos e mecanismos"**. Encerrada a exposição, foi iniciada a argüição, dentro do limite de tempo de 30 (trinta) minutos, pelos Professores Dr. Rodrigo Villamarim Soares, Dr. José Eustáquio da Costa e Dra. Cínthia Mara da Fonseca Pacheco, com limite de 30 (trinta) minutos para a resposta. Terminadas as argüições, a Presidente suspendeu os trabalhos por 10 minutos para que os examinadores pudessem decidir pelo resultado a ser dado ao candidato. A Comissão Examinadora opta pela ...aprovação... do candidato. Para constar, lavrou-se a presente ata, que vai assinada por mim Dra. Cínthia Mara da Fonseca Pacheco, Presidente e pelos demais membros desta comissão examinadora. Belo Horizonte, 22 de julho de 2010.

Dra. Cínthia Mara da Fonseca Pacheco
NEWTON PAIVA - Co-Orientadora

Dr. Rodrigo Villamarim Soares
PUC-MG

Dr. José Eustáquio da Costa
FO-UFMG

Homologado pelo Colegiado do
Programa de Pós-Graduação em Odontologia
Em reunião de 09/12/2010

À Rita e Vinícius, razões maiores de meu viver.

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A Deus, que me permitiu a grata oportunidade de viver e fazer parte de sua imensa criação.

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Aos meus irmãos e irmãs, pelos laços profundos de amizade e amor que nos unem.

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"Conseguir que as gerações futuras sejam mais felizes que a nossa será o maior prêmio a que se possa aspirar. Não haverá valor comparável ao cumprimento desta grande missão, que consiste em preparar para a humanidade futura um mundo melhor".

(Carlos Bernardo González Pecotche, criador da Logosofia)

Resumo

Os efeitos benéficos do agonista kappa opioide U-50,488 na prevenção da progressão da doença periodontal (DP) em ratos já foi descrito na literatura, embora seus mecanismos ainda não tenham sido totalmente esclarecidos. O presente estudo avaliou a expressão das seguintes citocinas: fator de necrose tumoral alfa (TNF-α), interleucina 6 (IL-6), interleucina 8 (IL-8) e interleucina 10 (IL-10) em tecidos periondontais de ratos com DP induzida e tratados com o U-50,488. Adicionalmente, correlacionou-se os efeitos do agonista kappa opioide com a atividade da enzima mieloperoxidase (MPO) — um marcador de neutrófilos ativados — e com a presença de osteoclastos nos tecidos periodontais. Ratos machos da linhagem Holtzman foram divididos em quatro grupos: *naïve* (N), ligadura (L), salina (S) e kappa (K). A DP foi induzida por meio da colocação de um fio de sutura estéril (ligadura) em volta do segundo molar superior esquerdo, exceto nos animais do grupo N. Os animais do grupo L não receberam qualquer tratamento farmacológico após a colocação da ligadura e os dos grupos S e K foram tratados, respectivamente, com a administração local de solução salina e do agonista kappa opioide U-50,488. Após cinco e 11 dias de indução da doença, os animais passaram pela eutanásia e as amostras dos tecidos periodontais foram coletadas para análises histológica e morfométrica, avaliação da produção de TNF-α, IL-6, IL-8, IL-10 e MPO. A DP experimental caracterizou-se pela formação de infiltrado inflamatório, reabsorção da crista óssea alveolar e aumento no número de osteoclastos no periodonto de ratos com ligadura. Verificou-se também, por intermédio do Teste de Elisa, uma concentração maior nos níveis de TNF-α, IL-6, IL-8 e MPO nos tecidos periodontais destes animais. A administração do agonista kappa opioide diminuiu a perda óssea e o número de osteoclastos sem alterar o número de células do inflamatório e a atividade de MPO. O U-50,488 reduziu os níveis de IL-6 e aumentou os de IL-10 de maneira significativa, mas não alterou a produção de TNF-α e de IL-8. Tais resultados sugerem que a administração local do agonista kappa opioide U-50,488 modula as respostas inflamatória e imune na DP experimental em ratos ao diminuir a expressão de IL-6 e aumentar a de IL-10.

Palavras-chave: Doença Periodontal. Agonista Kappa Opioide. Fator de Necrose Tumoral Alfa. Interleucina. Mieloperoxidase.

Abstract

Beneficial effects of the kappa opioid agonist U-50,488 in preventing periodontal disease (PD) progression in rats were already described, but the mechanisms by which it ocurred were unknwon. The present study evaluated the expression of TNF-α, IL-6, IL-8 and IL-10 in periodontal tissues of rats with ligature-induced PD, treated with U-50,488. It also correlated the effects of such agonist with myeloperoxidase activity and the presence of osteoclasts in periodontal tissues. Male Holtzman rats weighing 250-300g were divided into four groups: (1) Naïve, (2) Ligature, (3) Ligature + Saline and 4) Ligature + Kappa agonist. Experimental PD was induced by placing a sterile silk ligature around the 2nd left upper molar. Animals from groups 3 and 4 were locally administered with either saline or U-50,488, respectively, from the 3rd to the 5th day following ligation. After 5 or 11 days of ligature, animals were euthanized and periodontal tissue samples were collected for histological and morphometric analysis, for evalution of TNF-α, IL-6, IL-8, IL-10 and MPO. Ligature placement induced a significant alveolar bone loss, inflammatory infiltrate, number of osteoclasts, MPO activy, IL-6, IL-8 and TNF-α expression in periodontal tissues. U-50,488 decreased bone loss and the number of osteoclasts in periodontal tissues, although it did not alter histological inflammatory infiltrate and MPO activity. Furthemore, U-50,488 significantly reduced IL-6 and increased IL-10 levels, but it did not affect TNF-α and IL-8. Lowering the levels of IL-6 and increasing IL-10 are important mechanisms by which U-50,488 decreases alveolar bone loss in ligature-induced periodontal disease.
Keywords: alveolar bone loss, opioids, IL-6, IL-1

LISTA DE ABREVIATURAS E SIGLAS

DP - Doença Periodontal

IL – Interleucina

K - Kappa

L - Ligadura

LPS - Lipopolissacarídeos

MPO - Mieloperoxidase

N - *Naïve*

OPG - Osteoprotegerina

PGE_2 - Prostaglandina E_2

TNF-α – Fator de necrose tumoral alfa

RANK - Receptor ativador do fator nuclear kappa B

RANKL - Ligante do receptor ativador do fator nuclear kappa B

S - Salina

SUMÁRIO

1 INTRODUÇÃO

A doença periodontal (DP) é uma das patalogias ósseas mais prevalecentes em humanos e considerada uma das principais causas de perda dental em adultos (PIHLSTROM; MICHALOWICZ; JOHNSON, 2005). Caracteriza-se por um desequilíbrio entre os mecanismos de defesa do hospedeiro e os periodontopatógenos que colonizam o sulco gengival, podendo levar à completa destruição dos tecidos periodontais de suporte (PAGE *et al*., 1997; PAGE; KORNMAN, 1997). Embora esteja claro o papel das bactérias como agente primário e iniciador da doença (SOCRANSKY; HAFFAJEE, 1997), sabe-se que as respostas imune e inflamatória do hospedeiro determinam o grau de comprometimento dos tecidos de inserção e ósseo (TAUBMAN *et al.*, 2005) por meio da liberação de diversos mediadores químicos inflamatórios como as metaloproteinases da matriz, prostaglandinas e citocinas (PRESHAW, 2008).

Nas últimas décadas, uma variedade de agentes farmacológicos tem sido estudada com o objetivo de modular essa resposta (PRESHAW, 2008). Especialmente, o agonista opioide U-50,488, que age via receptores kappa, foi capaz de reduzir as perdas de inserção e óssea de maneira estatiscamente significativa, porém sem alterar o número de células inflamatórias dos tecidos afetados (PACHECO *et al*., 2008).

Os opioides são substâncias conhecidas por suas propriedades analgésicas no tratamento da dor aguda pós-cirúrgica e da dor crônica decorrente de diversas enfermidades como o câncer (SACERDOTE, 2008). Sua utilização tem sido limitada em função de efeitos sistêmicos colaterais adversos como depressão respiratória, náusea, constipação, e pelo fato de criar tolerância e dependência. Exercem seu efeito por intermédio da ligação a um ou mais receptores celulares expressos não só em células dos sistemas nervoso e periférico (STEIN; LANG, 2009), como também em sítios não neuronais, como células endoteliais vasculares (CADET *et al*., 2000), queratinócitos (BIGLIARDI; BUCHNER; RUFLI, 2002) e diversas células do sistema imune, como neutrófilos, monóciots, macrófagos e células T (WYBRAN *et al*., 1979; SIBINGA; GOLDSTEIN, 1988; BIDLACK, 2000; BIDLACK *et al*., 2006). A ativação de receptores epioides em leucócitos, com posterior alteração na secreção de diversas citocinas como IL-1, TNF-α, IL-6 e IL-8, é descrita como um importante mecanismo pelo qual os epioides interferem nas respostas imune (ALICEA *et al*., 1996) e inflamatória, conforme já demonstrado em diferentes modelos de inflamação *in vivo* e *in vitro* (FLORES; WAHL;

BAYER, 1995; GRIMM *et al.*, 1998; POURPAK; AHMADIANI; ALEBOUYEH, 2004; RAO *et al*., 2004). Técnicas de clonagem molecular identificaram três tipos principais de receptores opioides: mü (μ), kappa (κ) e delta (δ), todos pertencentes à superfamília de receptores com sete domínios transmembrana, aclopados à proteína G (DHAWAN *et al.*, 1996). Em uma revisão publicada em 2008, Finley *et al*. afirmaram que além de induzir analgesia, os opioides modulam a função das células envolvidas com a resposta imune mediante a regulação da expressão de citocinas, quimiociocinas e seus receptores.

As citocinas são importantes mediadores que interferem na patogênese da DP. Estudos em humanos demonstraram que os níveis de IL-β, IL-8 e TNF-α, no fluido do sulco gengival e nos tecidos periodontais de indivíduos com DP, estavam aumentados quando comparados com indivíduos saudáveis ou tratados (ZHONG *et al*., 2007; GAMONAL *et al*., 2001; IKEZAWA *et al*., 2005). A aplicação local de antagonistas de IL-1 e TNF-α resultou em menor perda óssea alveolar em um modelo experimental animal de DP (OATES; GRAVES; COCHRAN, 2002; DELIMA *et al*., 2002).

Da mesma forma, ao avaliar camundongos deficientes para IL-6, Baker *et al*. (1999) verificaram uma perda óssea menor após infecção com *Porphyromonas gingivalis*, confirmando o envolvimento dessa citocina na progressão da DP.

Por outro lado, a IL-10, considerada uma citocina anti-inflamatória, foi capaz de inibir a síntese de várias citocinas pró-inflamatórias, como IL-1, IL-6, IL-8 e TNF-α (CASSATELLA *et al*., 1993; PULITI *et al*., 2002). Altos níveis de ILK-10 nos tecidos periodontais foram também relacionados com a redução na expressão do ligante do receptor ativador do fator nuclear kappa (RANKL), um dos componentes da superfamília de proteínas do receptor do fator de necrose tumoral, responsável pela reabsorção óssea, que caracteriza doenças inflamatórias como a DP, a artrite reumatoide e a osteoporose (GARLET *et al*., 2006; PETTIT *et al*., 2006; FILI; KARALAKI; SCHALLER, 2009; ALLAM *et al*., 2010).

Diante do exposto, levantou-se a hipótese deste estudo: os opioides previnem a perda óssea e a perda de inserção associadas à DP experimental ao modular a expressão de citocinas envolvidas diretamente na patogênese desta condição.

2 JUSTIFICATIVA

Alguns estudos demonstraram que agonistas opioides — especialmente, o agonista de receptor kappa opioide U50,488 — são capazes de diminuir as perdas de inserção e óssea no periodonto de ratos com doença periodontal induzida por ligadura, sem, no entanto, alterar o número de células no infiltrado inflamatório dos tecidos após a indução da doença.

Entender o mecanismo pelo qual essas substâncias atuam pode levar ao desenvolvimento de novas terapias para o tratamento das doenças periodontais.

Um dos mecanismos propostos para explicar o efeito do agonista kappa opioide seria a sua ligação a receptores específicos, localizados em leucócitos e células residentes, com a consequente regulação da produção de diversas quimiocinas e citocinas envolvidas na perda dos tecidos periodontais.

3 OBJETIVOS

3.1 Objetivo geral

- Avaliar os mecanismos pelos quais a administração local do agonista de receptor kappa opioide diminui a reabsorção óssea e a perda de inserção em ratos com doença periodontal experimental induzida por ligadura.

3.2 Objetivos específicos

- Verificar a expressão de TNF-α, IL-6, IL-8 e IL-10 em tecidos de ratos que tiveram a doença periodontal experimental induzida por ligadura e foram tratados com o agonista kappa opioide U-50,488;
- Averiguar a atividade da enzima mieloperoxidase em tecidos de ratos que tiveram a doença periodontal experimental induzida por ligadura e foram tratados com o agonista kappa opioide U-50,488;
- Avaliar a presença de osteoclastos em tecidos de ratos que tiveram a doença periodontal experimental induzida por ligadura e foram tratados com o agonista kappa opioide U-50,488.

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Peripheral kappa opioid receptors activation reduces alveolar bone loss in rats by modulating interleukin-6 and -10

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ABSTRACT

Objective: The beneficial effects of kappa opioid agonist U-50,488 in preventing periodontal disease (PD) progression in rats have already been described, but its mechanism of action is unknown. The present study evaluated the expression of TNF-α, IL-6, IL-8 and IL-10 in the gingival tissues of rats with ligature-induced PD, treated with U-50,488. It also correlated the effects of this agonist with myeloperoxidase (MPO) activity and the presence of osteoclasts.
Design: Male Holtzman rats weighing 250–300 g were divided into four groups: (1) control, (2) ligature, (3) ligature + saline and (4) ligature + kappa agonist. Experimental PD was induced by placing a sterile silk ligature around the 2nd left upper molar. Rats from groups 3 to 4 were locally administered with either saline or U-50,488, respectively, from day 3 to day 5 following ligation. After 5 or 11 days, the rats were euthanized and periodontal tissue samples were collected for histological and morphometric analysis and for determination of TNF-α, IL-6, IL-8, IL-10 and MPO.
Results: Ligature placement induced significant alveolar bone loss. The number of osteoclasts, degree of MPO activity, IL-6, IL-8 and TNF-α expression were also increased by PD. U-50,488 reduced both bone loss and the number of osteoclasts, but did not alter histological inflammatory infiltrate or MPO activity. U-50,488 significantly reduced IL-6 and increased IL-10 levels, but did not affect TNF-α and IL-8.
Conclusion: Lowering the levels of IL-6 and increasing IL-10 are important mechanisms by which U-50,488 reduces alveolar bone loss in ligature-induced periodontal disease.

1. Introduction

Periodontal disease (PD) is one of the most prevalent forms of alveolar bone pathology in humans. It is characterized by an unbalanced host response to periodontopathogens, which leads to destruction of tooth-supporting tissues.[1,2] In order to prevent such conditions many strategies have been tried and a promising contribution is the recently described beneficial effects of opioid agonists in an experimental model of ligature-induced PD in rats.[3,4] These drugs, especially those that activate kappa receptors in the periphery, have been shown to reduce alveolar bone and fibre attachment loss, the hallmarks

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of PD, without altering the number of leukocytes in inflamed connective tissues.[4] However, the mechanisms by which activation of opioid receptors produced these effects have yet to be elucidated.

Activation of opioid receptors present in leukocytes with further reduction in cytokines release, particularly interleukin (IL)-1, tumour necrosis factor-α (TNF-α), IL-6 and IL-8, are described as important mechanisms by which opioids interfere with inflammatory responses, as demonstrated in different models of inflammation.[5–8] On the other hand, cytokines play an important role in PD development. It has been demonstrated that the levels of IL-1, IL-8 and TNF-α were increased in gingival crevicular fluid and in periodontal tissues of PD subjects when compared with healthy controls.[9–11] Moreover, antagonists of IL-1 and TNF-α were able to reduce alveolar bone loss in experimental models of the disease, confirming their pivotal role in this condition.[12,13] Additionally, IL-6-deficient mice presented decreased alveolar bone loss when compared with their wild-type counterparts.[14]

Conversely, IL-10 is considered an antiinflammatory cytokine that is able to inhibit the synthesis of several proinflammatory cytokines, such as IL-1, IL-6, IL-8 and TNF-α,[15,16] and higher levels of IL-10 in periodontal tissues have already been correlated with a reduction in the expression of the osteoclastogenic factor RANKL (receptor activator of nuclear factor κB-ligand) and matrix metalloproteinases in an experimental model of PD in mice.[17]

Since PD pathogenesis is marked by the release of several cytokines from host leukocytes,[10,11,14,15] our hypothesis is that opioids prevent PD-associated bone loss by interfering with cytokine expression.

Therefore, the main goal of the present study was to evaluate the expression of the cytokines TNF-α, IL-6, IL-8 and IL-10 in gingival tissues and periodontal ligament of rats with ligature-induced periodontal disease and treated with the kappa opioid receptor agonist U-50,488. The study also aimed to correlate the beneficial effects of this agonist with myeloperoxidase activity and the presence of osteoclasts in periodontal tissues.

2. Materials and methods

2.1. Animals

Male Holtzman rats weighing 250–300 g were obtained from the Animal House of the Institute of Biological Sciences, Universidade Federal de Minas Gerais (UFMG), Brazil, and were used throughout the experiments. The rats were maintained under a 12/12 h light/dark cycle (lights on 7:00 a.m.) at 23–25 °C with water and food *ad libitum*. The Animal Ethics Committee of the UFMG approved the handling of the rats throughout this study (protocol number: 46/2008).

2.2. Ligature placement

The experimental model of PD in rats, which was used in this study, was based on earlier publications.[3,18] Briefly, rats were anaesthetized with a mixture of ketamine and xylazine (60 and 9 mg/kg, respectively). A sterile silk ligature (4-0, 1.5 metric, Ethicon®, Johnson & Johnson, São José dos Campos, Brazil) was tied around the cervix of the second left upper molar and served as a retention device for subgingival oral microorganism accumulation. The ligature remained around the tooth until the end of the experiments, when rats were euthanized. The impaction of materials other than indigenous microbiota in rat interdental areas was avoided by keeping them in individual suspended cages after ligature placement.

2.3. Drug treatment

The rats were divided into the following groups: (1) control (C), which were comprised of rats with no signs of alveolar bone loss, fibre attachment retraction or inflammatory infiltrate ($n = 5$ rats for histology, morphometric analysis and TRAP; $n = 6$ rats for ELISA and MPO); (2) ligature-induced periodontal disease group, without treatment (L) ($n = 5$ rats for histology, morphometric analysis and TRAP; $n = 5$ rats (5 days) + $n = 5$ rats (11 days) for ELISA and MPO); (3) saline group (S) ($n = 4$ rats for histology, morphometric analysis and TRAP; $n = 4$ rats (5 days) + $n = 4$ rats (11 days) for ELISA and MPO); (4) Kappa opioid agonist group (K) ($n = 5$ rats for histology, morphometric analysis and TRAP; $n = 5$ rats (5 days) + $n = 5$ rats (11 days) for ELISA and MPO). Groups S and K were comprised of rats in which a ligature was placed and either sterile physiological saline or the kappa opioid agonist U-50,488 was locally administered, respectively, after being anaesthetized. Both sterile physiological saline (0.1 mL) and the kappa opioid agonist U-50,488 (250 μg/site/day, 0.1 mL; Tocris, Bristol, UK) were injected into the buccal gingivomucosal tissue in the region of the ligated tooth, as shown in Fig. 1, from day 3 to day 5 after ligature placement. Such schedules for drug treatment and the kappa agonist concentration have proven to be effective in reducing the main signs of ligature-induced PD in rats, i.e. alveolar bone loss, fibre attachment retraction and inflammatory infiltrate, and to have a local rather than a systemic effect.[3,4]

2.4. Measurement of alveolar bone loss by morphometric analysis

On either day 5 or day 11 after the ligature placement, rats were euthanized by CO_2 inhalation in a gas chamber. The left and right maxillae halves were excised, fixed in 10% buffered formalin solution, pH 7.2, for 48 h, washed in water and demineralized in 10% ethylenediaminetetraacetic acid (EDTA) for 30 days. At the end of demineralization period, each hemimaxilla was washed in tap water for 24 h, dehydrated in serial alcohols, cleared and embedded in paraffin. The blocks were cut in serial 4 μm sections in a mesiodistal direction. Haematoxylin and eosin staining was performed on the most central section of each tooth, i.e. the one showing the centre of the dental pulp. Images from experimental (ligated) and control (unligated) sites were obtained using a JVC TK-1270/RGB camera (Victor Company of Japan, Yokohama, Japan) adapted to a microscope. The distance from the cementoenamel junction (CEJ) to the most coronal level of alveolar bone crest (ABC) was measured in the mesial and in the distal regions of the second upper molars using KS300 software (CarlZeiss, Oberkochen, Germany) built into a Kontron Elektronick/CarlZeiss image analyzer. This software permitted all

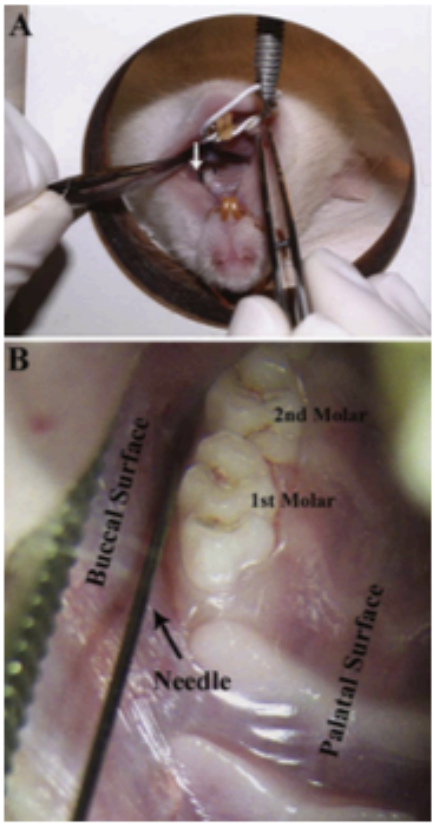

Fig. 1 – Photographs of the rat mouth showing the site of injection of drugs. Rats were anaesthetized and positioned in an experimental surgical apparatus, as described in Section 2, in order to allow complete visualization of the upper molars. The injection of saline or the kappa opioid agonist U-50,488 was made into the buccal gingivomucosal tissue in the region of the left upper second molar, as shown by the arrow in A. Panel B shows the positioning of the needle in the corresponding buccal region, in a higher magnification (7×).

measurements to be made strictly perpendicularly to a line drawn from the cementoenamel junction of the second to the first and third molars to obtain the measurements on the mesial and distal sides, respectively. Alveolar bone loss (mm) is reported as the difference between values at the experimental and unligated-control sites.[3,4] An observer who was unaware of the nature of tissue samples made the measurements.

2.5. TRAP

Tissue sections were also stained for tartrate resistant acid phosphatase (TRAP 387A-1KT, lot 108 K 4344; Sigma–Aldrich, Saint Louis, MO, USA), counterstained with haematoxylin and used for histological examination. Samples were analysed, comparing the presence of osteoclasts in the coronal two-thirds of the mesial and distal crestal alveolar bone adjacent to the left upper 2nd molar from ligature and kappa opioid agonist-treated rats. Osteoclasts were identified as TRAP-positive, multinucleated cells situated on the crestal alveolar bone. The total number of TRAP-positive cells was determined in five consecutive microscopic fields (400×) per section. For each rat, four maxillae sections, at a distance of 50 μm one from another, were analysed and the mean value of these four sections represented the number of osteoclasts (in five fields) present on the alveolar bone of that rat.

2.6. Assessment of cytokine levels

Immediately after euthanization of the rats, the left maxillae half was excised and the gingival tissue and periodontal ligament surrounding the second left upper molar were harvested, weighed (15–25 mg/sample), and placed in plastic vials containing a buffer solution (0.4 mM NaCl, 10 mM $NaPO_4$, pH 7.4) with inhibitors of proteases (0.1 mM PMSF – phenylmethylsulfonyl fluoride – 0.1 mM benzethonium chloride, 10 mM EDTA and 0.01 mg/mL aprotinin A) and Tween 20 (0.05%), pH 7.4 (normalization: 1000 μl of solution for 100 mg of wet tissue). These samples were mechanically homogenized, centrifuged at 10,000 rpm at 4 °C and the supernatants were further used for the immunoenzymatic assay.

Determination of cytokine quantities in the gingival tissue and periodontal ligament was performed by the immunosorbent assay ELISA. Samples were assessed quantitatively for the expression of the following cytokines: TNF-α, IL-6, IL-8 and IL-10, using commercially available kits (R&D Systems, Minneapolis, MN, USA). The concentrations of cytokines in the samples were determined by ELISA assays performed according to the manufacturer's instructions. The results were expressed as cytokine picograms (±SEM), adjusted for 100 mg of tissue and the absorbance was obtained in an ELISA reader at 492 nm.

2.7. Myeloperoxidase activity

Myeloperoxidase (MPO) activity was assessed on days 5 and 11 after ligature placement to quantify the extent of neutrophil accumulation in whole tissue samples. Immediately after rats euthanization, gingival tissues and periodontal ligaments of the upper left second molars were harvested, weighed, mechanically homogenized in cooled (4 °C) phosphate buffer (0.1 M NaCl, 0.02 M Na_3PO_4, 0.015 M NaEDTA, pH 4.7) and centrifuged at 4 °C for 10 min at 10,000 rpm. The pellet was then subjected to hypotonic lysis: 0.2% NaCl solution for 30 s followed by addition of an equal volume of a solution containing 1.6% NaCl and 5% glucose. After further centrifugation, the pellet was resuspended in 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% hexa-1.6-bisdecyltrimethylammonium bromide (HTAB, Sigma–Aldrich, Saint Louis, MO, USA). The suspensions were freeze-thawed three times and finally centrifuged at 10,000 rpm for 10 min at 4 °C. MPO activity in the resulting supernatant was assayed by

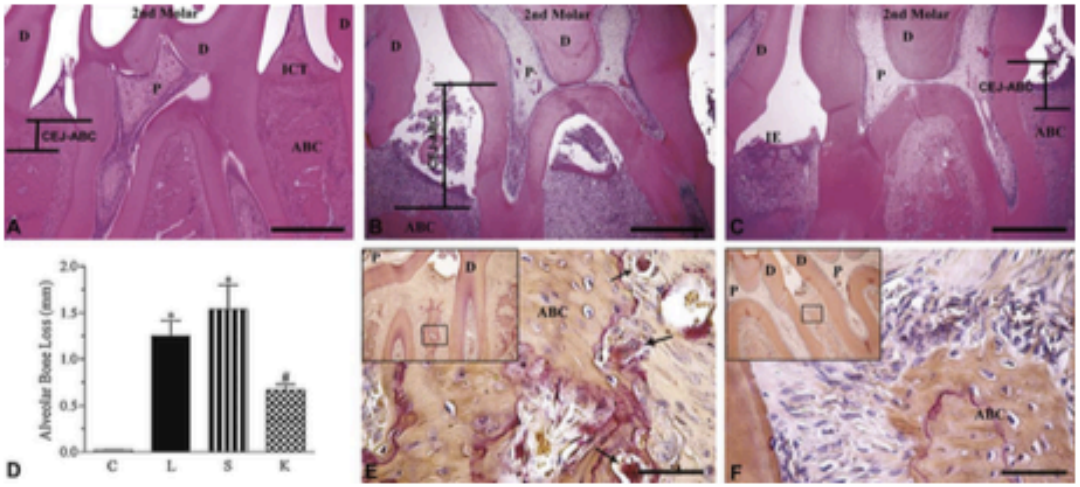

Fig. 3 – Effect of the kappa opioid agonist U-50,488 on ligature-induced alveolar bone loss and osteoclast number. Photomicrographs from transversal histological sections of maxillary molar teeth from control (A) and ligated rats treated with either saline (B) or U-50,488 (C), respectively. Observe in (C) a smaller retraction of periodontal attachment and alveolar bone crest when compared with (B); original magnification: 50×. Scale bars: 500 μm. IE: interdental epithelium; ICT: interdental connective tissue; D: dentine; P: dental pulp; ABC: interdental alveolar bone crest; CEJ-ABC: distance between the cementoenamel junction and the alveolar bone crest. (D) Quantification of bone loss 11 days after experimental PD induction. Kappa opioid agonist administration significantly prevented interdental crestal alveolar bone loss (K) compared with ligated, nontreated (L) or saline-treated (S) groups. In tartrate resistant acid phosphatase (TRAP) stained samples a difference in the interdental crestal alveolar bone of rats treated with saline (E) or U-50,488 (F) can be observed, regarding TRAP+ cells and bone morphology. The arrows show TRAP-stained osteoclasts. Magnification: 400×. Insert magnification: 50×. Scale bars: 50 μm; number of rats/group: C ($n = 5$), L ($n = 5$), S ($n = 4$), K ($n = 5$). *Significant difference in relation to control group (C) and #significant difference between ligated, nontreated (L) and saline-treated (S) groups using one-way ANOVA followed by Student–Newman–Keuls test.

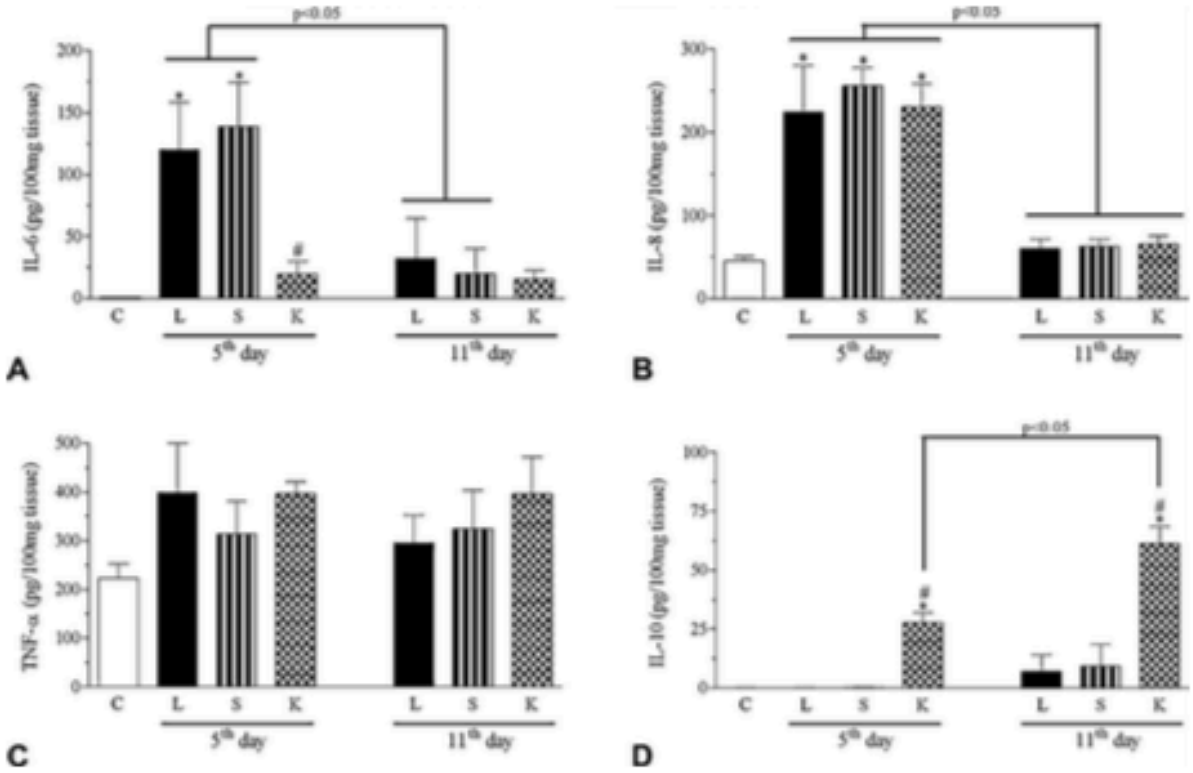

Fig. 4 – The kappa opioid agonist U-50,488 modulates the production of cytokines during the course of experimental periodontal disease. Gingival tissues and periodontal ligament were harvested and processed on days 5 and 11 after ligature placement following rats sacrifice. The control group (C) was left unligated. Measurements of cytokines concentrations (pg/100 mg tissue) were performed through the immunosorbent assay ELISA, as described in Section 2. (A) IL-6 concentration (pg/100 mg tissue); (B) IL-8; (C) TNF-α; (D) IL-10. Number of rats/group: C ($n = 6$); L 5 d ($n = 5$); S 5 d ($n = 4$), K 5 d ($n = 5$), L 11 d ($n = 5$), S 11 d ($n = 4$), K 11 d ($n = 5$). *Significant difference in relation to control group (C) and #Significant difference between the ligature-induced periodontal disease, nontreated (L) and saline-treated (S) groups using one-way ANOVA followed by Student–Newman–Keuls test.

3.1.2. Myeloperoxidase activity

In relation to MPO, the ligature-induced PD caused a significant increase in MPO activity at the onset of the disease, which spontaneously diminished as the inflammatory process progressed (Fig. 2D). Local administration of the kappa opioid agonist U-50,488 also did not affect the activity of this inflammation marker, as observed in Fig. 2D.

3.1.3. Osteoclast analysis

Analysis of tartrate resistant acid phosphatase (TRAP) stained samples verified that the periodontal tissues of ligated rats frequently exhibited more osteoclasts (16.5 ± 1.5 TRAP-positive cells, Fig. 3E) and an irregular bone morphology than kappa opioid agonist-treated rats (3.8 ± 1.5 TRAP-positive cells, Fig. 3F).

3.2. Kappa opioid agonist effects on cytokine expression in gingival tissues

Gingival tissues of ligated nontreated (L) and saline-treated rats (S) presented higher levels of the cytokines IL-6 (Fig. 4A) and IL-8 (Fig. 4B) 5 days after ligature-induced PD compared to the control group (C, $p < 0.05$). The levels of both cytokines were statistically lower ($p < 0.05$) during the peak of the experimental disease (day 11). Local administration of the kappa opioid agonist U-50,488 (K), from day 3 to day 5 after ligature placement, reduced IL-6 concentrations ($p < 0.05$, Fig. 4A), but did not reduce the increased concentration of IL-8 ($p > 0.05$, Fig. 4B).

TNF-α expression also tended to increase in gingival tissues of ligated rats (L and S) compared with tissues from rats with no signs of ligature-induced PD (C), although the differences were not statistically significant. Likewise, no difference was observed in TNF-α concentrations at the onset (day 5) and peak (day 11) of ligature-induced PD (Fig. 4C). The kappa opioid agonist U-50,488 did not alter the concentration of TNF-α (Fig. 4C).

Different from TNF-α, IL-6 and IL-8, the concentrations of IL-10 were undetectable in tissues of control (C) and ligated nontreated (L) and saline-treated (S) rats on day 5 of disease, but IL-10 increased significantly by day 11. Local administration of U-50,488 induced an enormous increase in the concentrations of IL-10 both on day 5 and day 11 after ligature placement (Fig. 4D).

4. Discussion

Although the analgesic properties of opioids have been known for centuries, only during the last three decades studies have shown their immunomodulatory effects in various diseases.[19] Our research group has previously demonstrated the beneficial effects of opioid agonists in reducing bone and fibre attachment loss, the main signs of periodontal disease, by placing a ligature around rat molars.[3,4] The effect of opioids activating kappa receptors in the periphery is of special interest, since the most unwanted side effects of these drugs are related to the activation of opioid receptors in the central nervous system. Nevertheless, the mechanisms by which a kappa opioid agonist reduced periodontal tissue loss remained unanswered.

In the present study, ligature-induced PD induced a significant migration of leukocytes to gingival tissues that was not affected by the administration of the kappa opioid receptor agonist U-50,488, confirming previous findings.[4] Our initial hypothesis was that since the kappa agonist was unable to affect inflammatory cell infiltration, it would affect the quality of this infiltrate, changing the pattern of leukocytes and, consequently, the mediators released by them. In this regard, myeloperoxidase (MPO) activity was also not affected by U-50,488. Since MPO has been applied as a useful method to estimate neutrophil content in inflamed tissues,[20,21] it can be affirmed that, under the present experimental conditions, these leukocytes persist during the course of disease, even when the destruction of alveolar bone and fibre attachment decreased, as observed in rats treated with the kappa opioid agonist. During PD development, a marked accumulation of neutrophils was observed in compromised periodontal tissues and this event is not only related to host response to bacterial invasion, but also to periodontal tissue destruction.[22,23] Since opioid receptors have already been identified in neutrophils,[24–26] it is possible that the engagement of U-50,488 with such receptors interferes with the pattern of mediators released by these cells, especially cytokines.

Activation of kappa opioid receptors seems to reduce classic inflammatory signs through the downregulation of proinflammatory cytokines. Studies using murine fibroblast and monocyte-like cell cultures have already demonstrated that the kappa opioid receptor agonist U50,488 significantly reduced IL-1, IL-6 and TNF-α expression by these cells.[27,28] Therefore, determining the role of kappa opioid agonist in the expression of the main cytokines involved in PD development was another goal of the present work.

Under the experimental conditions studied, proinflammatory cytokines TNF-α, IL-6 and IL-8 presented higher concentrations in ligated rats and IL-6 and -8 showed reduced concentrations during the course of connective tissue loss. This event occurred concurrently with increased IL-10 concentrations in rat gingival tissues. In fact, modulation of the inflammatory process is a characteristic of chronic inflammatory diseases and had already been observed in PD.[17]

Local administration of U-50,488 significantly diminished the concentrations of IL-6 at the onset of connective tissue loss, but did not affect the concentrations of TNF-α and IL-8. It has been demonstrated that the production of IL-6 contributes to bone resorption in a model of PD in mice.[14] Moreover, this cytokine was able to regulate osteoclast progenitor cell differentiation into mature osteoclasts[29] and enhanced the production of prostaglandins,[30] an important mediator of bone resorption.[31–33] Interleukin-6 also directly boosts RANKL mRNA expression in other inflammatory conditions, such as in rheumatoid arthritis.[34,35] RANKL is a ligand of the TNF family that induces differentiation and activation of osteoclasts through its receptor RANK. This event is prevented by ligation of RANKL to its decoy receptor, osteoprotegerin (OPG). Integrity of bone tissues is maintained by the equilibrium of this system and increasing RANKL/OPG ratios have been implied in numerous conditions in which bone loss is present, including PD.[36,37] In this context, the lower concentrations of IL-6 induced by U-50,488 could have reduced the RANKL/OPG ratio with consequent lower bone resorption. This hypothesis

is supported by the present findings of a smaller number of osteoclasts in the interdental crestal alveolar bone of ligated rats treated with the kappa opioid agonist than that observed in saline-treated rats.

Also of particular interest was the huge increase in IL-10 concentrations induced by the kappa opioid agonist at the onset of ligature-induced connective tissue loss. It is known that IL-10 suppresses the production of metalloproteinases and the differentiation of haemopoietic progenitors in cells with a resorptive function, representing an important matrix protective factor during inflammation.[38,39] Furthermore, the expression of IL-10 has already been shown to be lower in progressive periodontitis lesions than in the gingiva of healthy individuals in human studies.[40,41] Finally, IL-10 is able to reduce the concentrations of proinflammatory cytokines, including IL-6.[35,38] It is probable that not only direct regulation of IL-6 expression by migrated and resident cells, but increased IL-10 concentrations are important mechanisms by which the kappa opioid agonist modulates ligature-induced periodontal tissue loss.

In conclusion, lowering the concentrations of IL-6 and increasing those of IL-10 seem to be important mechanisms by which the kappa opioid agonist U-50,488 reduces the alveolar bone loss in ligature-induced periodontal disease. A clearer understanding of the role played by opioids in periodontal disease will lead to new therapeutic approaches for dealing with such conditions.

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Ethical approval

The Animal Ethics Committee of the UFMG approved the handling of the rats throughout this study (protocol number: 46/2008).

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Conflict of interest

There is no conflict of interest.

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5 CONSIDERAÇÕES FINAIS

As propriedades analgésicas dos opioides são conhecidas há séculos e a ideia de que modulam a resposta imune não é recente. Entretanto, somente nas últimas décadas, estudos têm demonstrado como seus efeitos na resposta imune alteram o curso de várias doenças crônicas (VALLEJO; LEON-CASASOLA; BENYAMIN, 2004; SACERDOTE, 2008).

No presente estudo, a DP experimental induzida por ligadura levou à formação de um infiltrado inflamatório de leucócitos mono e polimorfonucleares nos tecidos periodontais, destruição das fibras de ligamento periodontal e reabsorção da crista óssea alveolar. A administração local do agonista de receptor kappa opioide U-50,488 e a reabsorção óssea no periodonto dos animais tratados sem alterar o infiltrado inflamatório, confirmando resultados anteriores (PACHECO *et al.*, 2007; PACHECO *et al.*, 2008).

Uma das proposições para se explicar os efeitos do agonista opioide observados na DP seria a modulação de citocinas com consequente diminuição da perda dos tecidos periodontais. Tem sido demonstrado que um dos fatores determinantes da destruição tecidual na DP é o desequilíbrio entre os níveis de citocinas pró e anti-inflamtórias nos tecidos acometidos (PRESHAW, 2008). Nesse sentido, este trabalho evidenciou que a indução da DP experimental resultou em aumento na expressão de IL-6, TNF-α e IL-8, cinco dias após a colocação da ligadura, o que confirma os relatos de que a reabsorção óssea relaciona-se com o aumento nos níveis de citocinas pró-inflamatórias, dentre elas IL-6 e TNF-α, que estimulam a diferenciação e ativação de osteoclastos (LERNER, 2006). Baker *et al.* (1999) demonstraram a participação da IL-6 na reabsorção óssea em um modelo de DP experimental em camundongos. Além disso, essa citocina foi capaz de estimular a diferenciação de osteoclastos maduros (MANOLAGAS; JILKA, 1995) e aumentar a produção de prostaglandina (PGE_2), um importante mediador da reabsorção óssea (LIU *et al*., 2005; OKA *et al*., 2008; HIKIJI *et al*., 2008). IL-6 aumentou também a expressão do RNA mensageiro de RANKL na artrite reumatoide (PALMQVIST *et al*., 2002; WONG *et al*., 2006). RANKL é uma citocina da família TNF, que induz a diferenciação e ativação de osteoclastos por intermédio da ligação a seu receptor RANK. Essa ligação pode ser inibida pela osteoprotegerina (OPG), seu inibidor natural. Um desequilíbrio no eixo RANK/RANKL/OPG está relacionado à reabsorção óssea não só na

DP como também em outras enfermidades sistêmicas (PETTIT *et al*., 2006; FILI, 2009). Neste estudo, os baixos níveis de IL-6 observados após o tratamento local pelo U-50,488 provavelmente alteraram a proporção RANKL/OPG com redução da reabsorção da crista óssea alveolar. Tal fato é coerente com o menor número de osteoclastos observados nos animais com DP tratados com esse agonista de receptor kappa opioide. Embora a administração local do agonista kappa opioide U50,488 tenha reduzido significativamente os níveis de IL-6 e aumentado os de IL-10 no início da DP experimental (quinto dia após a colocação da ligadura), não houve alteração nos níveis de TNF-α e IL-8 durante todo o período da observação experimental. Estudos *in vitro* indicaram que a ativação de receptores kappa opioide expressos por fibroblastos e por células precursoras de macrófagos diminuiu a secreção de IL-1, IL-6 e TNF-α por estas células (ALICEA *et al*., 1996; PARKHILL; BIDLACK, 2006). Da mesma forma, em estudos *in vivo*, ao regular a expressão de diversas citocinas, agonistas de receptores kappa opioide atenuaram a atrite reumatoide (WILSON; CARMODY; WALKER, 2000; BUSH; KIRKHAM; WALKER, 2001), uma condição inflamatória crônica cuja patogênese assemelha-se à da DP (Mercado et al., 2003; Bartold et al., 2005).

Os níveis de TNF-α permaneceram inalterados mesmo após a administração local do agonista de receptor kappa opioide, sugerindo que essa citocina, embora seja pró-inflamatória e diretamente relacionada à reabsorção óssea, não esteja envolvida na modulação da DP pelos opioides. Essas observações estão de acordo com aquelas de Parkhill e Bidlack (2006) que demonstraram, *in vitro*, que células derivadas de monócitos de camundongos, estimuladas com lipopolissacarídeos (LPS), quando pré-tratadas com o agonista kappa opioide U-50,488, tiveram uma redução na expressão de IL-6 sem alterar os níveis de TNF-α.

O aumento da expressão da IL-10 no início da doença, após a administração local do agonista kappa opioide, sugere um mecanismo de modulação do opioide sobre a DP, considerando que se trata de uma citocina anti-inflamatória, capaz de suprimir a produção de metaloproteinases (LACRAZ *et al*., 1995) e a diferenciação de células clásticas (OWENS; GALLANGER; CHAMBERS, 1996), além de diminuir a expressão de citocinas pró-inflamatórias como IL-6 (PULITI *et al*., 2002). Em pesquisas realizadas em humanos, baixos níveis de IL-10 foram correlacionados com sítios ativos da DP quando comparados a sítios saudáveis (HIROSE *et al*., 2001).

Finalmente, é interessante salientar a não diminuição do número de células do infiltrado inflamatório após a administração do U-50,488, confirmando os achados de Pacheco *et al*. (2007; 2008). Utilizando o ensaio para MPO, um marcador da presença de neutrófilos (GOMES *et al*., 2009; QUEIROZ-JUNIOR *et al*., 2009), observou-se que administração local de U-50,488 não alterou o número dessas células, visto que não houve modificação na atividade de tal enzima. Tal achado é coerente com o fato de o agonista kappa opioide utilizado não ter sido capaz de reduzir a expressão de IL-8, uma quimiocina relacionada à migração de neutrófilos (YOSHIMURA *et al*., 1987; SILVA *et al*., 2007).

Pelo exposto, é pertinente sugerir que a diminuição na expressão de IL-6 e o aumento na expressão de IL-10 são importantes mecanismos pelos quais o agonista de receptores kappa opioide U-50,488 modula a DP induzida por ligadura em ratos. Entretanto, novos estudos são necessários para se avaliar o efeito desses agonistas na expressão de outras citocinas e nos diversos tipos celulares envolvidos com a perda óssea e com a perda de inserção na doença periodontal.

6 CONCLUSÕES

Com base nos resultados deste trabalho, conclui-se que:

- As citocinas IL-6, TNF-α e IL-8 estão envolvidas na doença periodontal induzida por ligadura em ratos;
- A administração local do agonista kappa opioide U-50,488 foi capaz de reduzir a perda óssea e de inserção na doença periodontal induzida por ligadura em ratos;
- O agonista de receptor kappa opioide U-50,488 modulou a expressão de citocinas em animais com DP induzida por ligadura, pela diminuição da expressão de IL-6 e aumento de expressão de IL-10, sem, entretanto, alterar a expressão de TNF-α e IL-8 nos tecidos periodontais.

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ANEXO 1 - CERTIFICADO DE APROVAÇÃO DO PROTOCOLO Nº 46/2008, DE ACORDO COM OS PRINCÍPIOS ÉTICOS DE EXPERIMENTAÇÃO ANIMAL

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 46/2008,** relativo ao projeto intitulado "***Ativação de receptores opióides e doença periodontal: Efeitos e mecanismos***", que tem como responsável(is) **Kátia Lucy de Melo Maltos** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **28/ 03/2008.**

Este certificado expira-se em **28/ 03/ 2013.**

CERTIFICATE

We hereby certify that the **Protocol nº 46/2008,** related to the project entitled "***Activation of opioid receptors and periodontal disease: Effects and mechanisms***", under the supervisiors of **Kátia Lucy de Melo Maltos,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **March 28, 2008.**

This certificate expires in **March 28, 2013.**

Belo Horizonte, 30 de Novembro de 2009.

Prof. Humberto Pereira Oliveira
Coordenador do CETEA/UFMG

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(Mod.Cert. v1.0)

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