UNIVERSIDADE ESTADUAL DE MONTES CLAROS

Emisael Stênio Batista Gomes

# Impacto do estresse em lesões periapicais inflamatórias em ratos Wistas: foco na reabsorção óssea

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Orientador: Prof. Dr. André Luiz Sena Guimarães

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***"Pelo contrário, Deus escolheu as coisas absurdas do mundo para envergonhar os sábios; e escolheu as coisas fracas do mundo para envergonhar as fortes[27].***

***Ele escolheu as coisas insignificantes do mundo, as desprezadas e as que são nada para reduzir as que são[28], para que nenhum mortal se glorie na presença de Deus[29]"***

(1 Coríntios 1: 27-29)

# RESUMO

A lesão periapical inflamatória corresponde pela exposição pulpar e infecção bacteriana nos canais radiculares, promovendo a inflamação crônica como resposta e destruição dos tecidos periapicais como a reabsorção óssea. Estudos recentes revelam que o estresse tem uma associação positiva com o agravamento da doença periodontal, mas não há nenhum estudo que mostra que o estresse é um indicativo positivo para a evolução da lesão periapical inflamatória infecciosa. O presente estudo teve como objetivo avaliar a evolução da lesão periapical induzida e sua perda óssea, em ratos Wistar expostos ao estresse. Vinte e cinco animais foram divididos em dois grupos (caso e controle), onde os mesmos foram submetidos a um procedimento cirúrgico no primeiro dente molar inferior esquerdo para a indução da lesão periapical inflamatória através da exposição da polpa dentaria, por meio de retenção da contaminação bacteriana de um período de 56 dias. Os animais do grupo caso foram estressados diariamente, por meio de estímulos elétricos (1.10 mA), já os animais do grupo controle não foram expostos à estímulos estressantes (choques). No 55° dia, todos os animais foram submetidos ao teste de *open field*, e no dia seguinte os mesmos foram sacrificados. As mandíbulas foram retiradas e coletadas para analises histológicas e radiográficas. Os animais do grupo caso apresentaram maior nível de estresse e maior número total de células inflamatórias o que consequentemente exibiram maior perda óssea quando comparados aos animais do grupo controle. Houve também uma diferença entre as análises histológicas e radiográficas, nos revelando que o diagnóstico e quantificação através das análises histológicas são muito mais relevantes e fidedignas em relação as análises radiográficas. Concluímos que o estresse favorece a uma maior evolução no quadro clinico na lesão periapical inflamatória por desencadear um nível mais avançado na destruição dos tecidos periapicais e sua perda óssea.



Palavras-chave: Lesão periapical inflamatória, estresse, perda óssea.

ABSTRACT

The inflammatory periapical lesion corresponds to the pulp exposure and bacterial infection in the root canals, promoting chronic inflammation as a response and destruction of periapical tissues, such as bone resorption. Recent studies have shown that stress has a positive association with the worsening of periodontal disease, but studies correlating stress and the evolution of inflammatory periapical inflammatory lesion are absent in the literature. The present study aimed to evaluate the evolution of the induced periapical lesion and its bone loss in Wistar rats exposed to stress. Twenty-five animals were divided into two groups (case and control), where they underwent a surgical procedure in the first left lower molar tooth for the induction of inflammatory periapical lesion through exposure of the dental pulp, through retention of the contamination (bacterial infection) during a 56 days period. The animals of the case group were stressed daily by electrical stimuli (1.10 mA), whereas the animals in the control group were absent from the stressful stimuli (shocks). On day 55, all animals were subjected to the open field test, and the next day they were sacrificed. The jaws were removed and collected for histological and radiographic analysis. The animals in the group of patients present a higher level of stress and a greater number of inflammatory cells that cause greater weight loss when compared to the animals in the control group. There was also a difference between the histological and radiographic analyzes, revealing that the diagnosis and quantification through the histological analysis are much more relevant and reliable as compared to the radiographic analyzes. We conclude that stress favors a greater evolution of the inflammatory periapical lesion by triggering worsened levels in the periapical tissue destruction and its bone loss.

Keywords: Periapical inflammatory lesion, stress, bone loss.

LISTA DE ILUSTRAÇÕES

# LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| IL | Interleucina |
| LPS | Lipopolissacarídeo |
| NF-κB | Fator Nuclear Kappa B |
| OPG | Osteoprotegerina |
| PAMPs | Padrões Moleculares Associados a Patógenos |
| TLRs | Toll-Like |
| TNF | Fator de Necrose Tumoral |

SUMÁRIO

# 1 INTRODUÇÃO

## 1.1 Lesão Periapical inflamatória Infecciosa

A lesão periapical é um processo inflamatório em torno do ápice do dente causado pela infecção bacteriana do sistema radicular (1). Entretanto a exposição crônica bacteriana e suas toxinas é um pré-requisito para indução da inflamação e destruição dos tecidos periodontais; apenas a presença bacteriana não é suficiente para explicar os mecanismos que levam ao sua progressão e desenvolvimento (2). A patogênese resulta em sua interação entre fatores bacterianos e respostas do hospedeiro, sendo assim, implica a presença dos fatores hostis bacterianos com o resultado da ativação da resposta imune do organismo (2-5). As bactérias e seus produtos tóxicos dentro dos canais radiculares progridem através do forame apical em tecidos periapicais, desta forma, o processo continua com reações inflamatórias específicas, que incluem a produção de anticorpos, citocinas e uma série de mediadores inflamatórios com o propósito da disseminação da infecção e a proteção dos tecidos periapicais (6, 7).

Desafortunadamente, a inflamação desencadeia a destruição dos tecidos periapicais estimulando o desenvolvimento de lesões peripicais inflamatórias (8, 9). A lesão é uma das condições patológicas mais frequentes dentro do osso alveolar (10). A maior parte das lesões inflamatórias periapicais de origem endodôntica são, os granulomas e os cistos periapicais (11, 12). As lesões periapicais inflamatórias, sendo elas, os granulomas periapicais, cistos radiculares e abscesso periapical, apresentam uma etiologia comum, pois são comumente encontrados no ápice dos dentes com polpa necrótica (13-15).

As células epiteliais de Malassez da região apical dos dentes com necrose pulpar são estimuladas a se dividir e proliferar por mediadores inflamatórios como, citocinas pró-inflamatórias e fatores de crescimento liberados das células hospedeiras (3, 16-19). Durante a inflamação, os fatores angiogênicos envolvidos na angiogênese são liberados pela ativação de diferentes tipos de células (células endoteliais, macrófagos, fibroblastos). Os macrófagos têm um efeito pró-antigênico no local da inflamação; induzem angiogênese por estimulação da proliferação de vasos sanguíneos, o que provoca a infiltração secundária de macrófagos nos tecidos moles (3, 20).

Receptores como Toll-like (TLRs) desempenham um papel proeminente na indução de respostas inatas e inflamação após o reconhecimento de PAMPs (padrões moleculares associados a patógenos), estruturas comuns compartilhadas pela maioria dos patógenos. Os PAMPs desencadeiam a sinalização de TLR que leva à ativação de NF-κB (fator nuclear kappa B) e subsequente produção de citocinas pró-inflamatórias. TLR2 e TLR4 são os TLRs mais investigados, uma vez que TLR2 e TLR4 são críticos no reconhecimento de bactérias gram-positivas e negativas, respectivamente. O TLR2 reconhece a presença de PAMPs tais como o ácido lipoteicóico, peptidoglicano, e também lipoproteína, enquanto TLR4 é crucial no reconhecimento de LPS (lipopolissacarídeo), um representante dos PAMP de bactérias gram-negativas (21, 22).

A inflamação periapical está associada ao processo de síntese e liberação de citocinas ativadoras de osteoclastos (23), como interleucina (IL) -1 e 6, fator de necrose tumoral (TNF), e IL-1β (24, 25), o que estimula a liberação de prostaglandina-E2 nas células hospedeiras, afetando os osteoclastos (24, 26).

Nos últimos anos, o tratamento periapical tem sido realizado através do tratamento endodôntico, com uma taxa de sucesso de aproximadamente 90% (27-29). Mesmo quando um padrão adequado de tratamento é realizado, as falhas podem ocorrer, devido às características anatômicas dos sistemas de canais radiculares e à presença de fatores nocivos peculiares dentro do tecido inflamado (8, 9, 30, 31). O retratamento do canal radicular tem o mesmo objetivo do tratamento primário dos canais radiculares infectados: eliminação completa de microrganismos e vedação hermética com materiais biocompatíveis. Isto é feito através da remoção do material de enchimento do canal radicular, desinfecção do sistema de canais radiculares e vedação dos mesmos (31, 32).

## 1.2 Avaliação de perda óssea

Radiografias periapicais têm sido comumente utilizadas para avaliar o tamanho das lesões periapicais. No entanto, essas radiografias têm as seguintes limitações, uma vez que a informação é processada em apenas 2 dimensões: uma lesão periapical só pode ser detectada na radiografia quando a perda mineral do osso atingir de 30% a 50%; a extensão bucolingual da lesão não pode ser determinada com radiografias; e é difícil interpretar a radiografia

quando a lesão se sobrepõe com estruturas anatômicas vizinhas ou quando o padrão de fundo é complexo (33). Vários fatores podem influenciar a avaliação radiográfica bidimensional de lesões periapicais, como a espessura do osso cortical, ângulo de incidência dos raios X, localização e extensão da lesão e estruturas anatômicas vizinhas (27, 33, 34).

## 1.3 Impacto do estresse na reabsorção óssea

Os mecanismos moleculares subjacentes à reabsorção óssea são controlados principalmente pela interação entre o ativador do receptor do ligante NF-kB (RANKL) e a osteoprotegerina (OPG), que pertencem ao ligante do fator de necrose tumoral (TNF) e às superfamílias dos receptores, respectivamente (35, 36). A produção de RANKL e OPG, por vários tipos de células, é controlada por estímulos sistêmicos e locais, incluindo hormônios, mediadores inflamatórios e produtos bacterianos (37, 38). A eficiência global da atividade de RANKL na formação de osteoclastos e na reabsorção óssea está fortemente acoplada aos níveis do seu inibidor natural, OPG (39, 40).

Foi recentemente demonstrado que RANKL e OPG desempenham papéis críticos na destruição óssea periapical (36, 41-43). Durante a remodelação óssea em condições normais, os osteoblastos são estimulados para proporcionar RANKL que desencadeia em níveis moderados a reabsorção óssea. A ativação anormal do sistema imunológico leva a destruição exacerbada do tecido ósseo, através da alta intensidade dos níveis de RANKL em doenças tais como artrite reumatóide e periodontite (44, 45).

A infiltração periapical é caracterizada principalmente por macrófagos e linfócitos T e B; estas células podem ser estimuladoras importantes de osteoclastogenesis. Estudos mostram que RANKL está estreitamente relacionado com infiltrados inflamatórios periapicais, e a proporção relativa de RANKL / OPG pode ser um determinante chave da reabsorção óssea mediada por RANKL (36, 46).

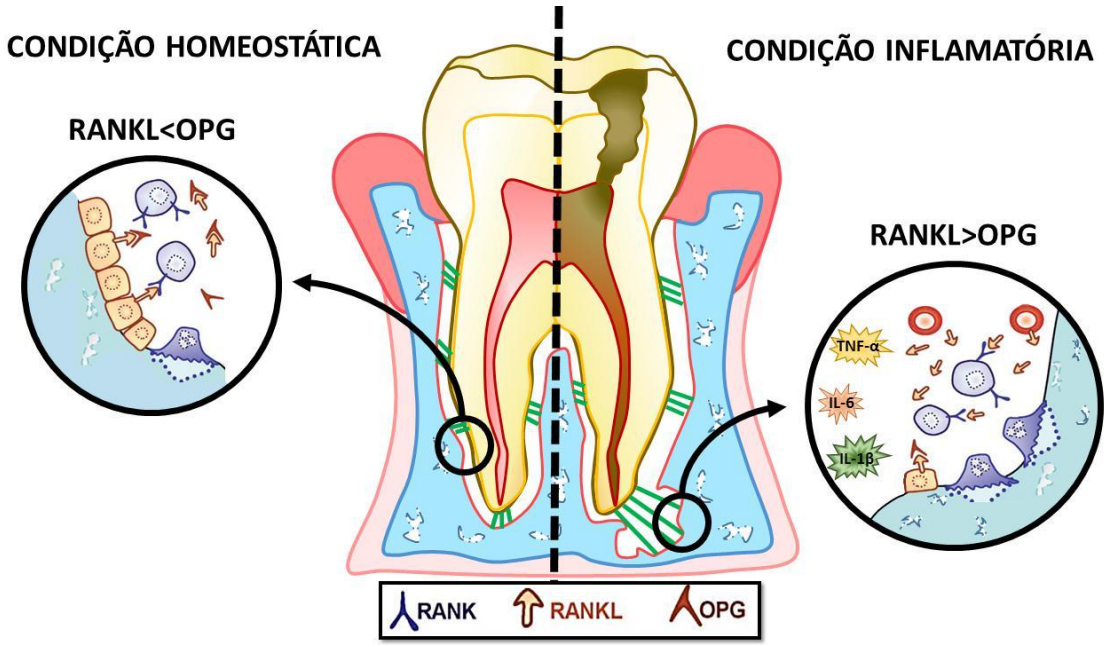


Figura 1: Representação esquemática da atuação das citocinas pró-inflamatórias sobre a reabsorção óssea via ativação da expressão da proteína RANKL

Em humanos, o estresse promove a ineficiência da resposta imunológica frente a agentes bacterianos, o que também desencadeia o desequilíbrio da RANKL e OPG, proporcionando um aumento na reabsorção óssea nos tecidos periapicais, constituindo uma via que participa da modelagem de alguns comportamentos que favorecem a manifestação na periodontite crônica (47-49). Contudo o estresse pode constituir um fator de risco para a progressão da doença periodontal em humanos, pois estudos em modelos animais mostram uma possível participação das contingências estressoras sobre o tecido periodontal, o que constatou perda óssea alveolar e destruição do ligamento periodontal em animais expostos a situações de estresse (50-54).

O estresse tende ao favorecimento de uma constelação de eventos que compreende um estímulo estressor, a uma reação precipitada do cérebro acerca deste estímulo (percepção do estresse) e ativação de mecanismos fisiológicos do corpo (resposta ao estresse) (55). Detalhadamente, o estresse é definido como um conjunto de reações psicofisiológicas do corpo a variedade de estímulos físicos ou emocionais que interferem na homeostase do organismo (56). Neste sentido, configura-se como uma reação ou resposta geral do organismo frente às situações ansiogênicas, difíceis ou inespecíficas, em que a harmonia do funcionamento dos órgãos é alterada, ocasionando desconforto tanto físico como emocional para o mesmo. Essas reações têm se mostrado exacerbadas em doenças

infecciosas, em modelos humanos e animais, uma vez que o estresse leva à modulação da função dos linfócitos, neutrófilos e macrófagos, afetando a defesa do organismo tornando-o predisposto a infecções (57, 58).

A mensuração ou quantificação dos níveis de estresse, ansiedade e depressão animal pode ser obtida pela ausência ou presença de alguns comportamentos. Existem testes comportamentais clássicos validados para tal finalidade, como o Teste de Campo Aberto, do inglês *Open Field* (fotografia 1). Trata-se de um teste mundialmente utilizado na quantificação do comportamental animal para a avaliação do quadro emotivo. O teste consiste em colocar o animal experimental em uma arena com a base subdividida em sessões. Cinco minutos de observação são considerados suficientes para a análise comportamental, no qual terá como alvo principal da avaliação seu comportamento locomotivo, pois em algumas situações em que apresentam sinais de perigo, porém com relativa distância, nota-se, geralmente, reações de imobilidade tensa que corresponde ao congelamento (freezing) ou inibição comportamental defensiva. Altos níveis de estresse aumentam consideravelmente comportamentos de freezing em estudos de modelo animal (59-64) (fotografia 2).

Embora estudos apontem uma influência importante do estresse sobre o desenvolvimento da doença periodontal, e sua relação com a absorção óssea (51, 52), nenhum estudo evidencia os mecanismos que envolvem a relação da fisiopatologia da reabsorção óssea com os eventos estressores na etiopatogênese da doença periapical. Isso sugere o desenvolvimento de um estudo, no qual há associação entre esses fatores, para que assim possa expandir os conhecimentos desta doença e seus possíveis agravantes no intuito de desenvolver novos tratamentos dessas desordens, o que irá constituir uma abordagem interessante na perspectiva terapêutica das lesões periapicais inflamatórias. Portanto, o presente trabalho tem como objetivo avaliar o impacto do estresse na reabsorção óssea em lesões periapicais inflamatórias induzidas em ratos Wistars (fotografia 3).

# 2 OBJETIVOS

## 2.1 Objetivo geral

- Analisar e quantificar a evolução da lesão periapical, e sua perda óssea, em seus aspectos radiográficos e histológicos, em ratos Wistar (Rattus norvegicus albinus) condicionados ao estresse.

## 2.2 Objetivos específicos

• Avaliar, e elucidar as questões relacionadas no presente trabalho no desenvolvimento da lesão periapical e sua perda óssea em ratos Wistar (*Rattus norvegicus albinus*) expostos ao estresse.

# 3 PRODUTOS

3.1 Produto 1: *Impact of stress on inflammatory periapical lesions in Wistar rats: focus on bone resorption*, formatado segundo as normas para publicação do periódico Journal of Endodontics

Artigo 1

# Impact of stress on inflammatory periapical lesions in Wistar rats: focus on bone resorption

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**Abstrat**

**Introduction:** The inflammatory periapical lesion corresponds to the pulp exposure and bacterial infection in the root canals, promoting chronic inflammation as a response and destruction of periapical tissues, such as bone resorption. The present study aimed to evaluate the evolution of the induced periapical lesion and its bone loss in Wistar rats exposed to stress. **Methods:** Twenty-five animals were divided into two groups, where they underwent a surgical procedure in the first left lower molar tooth for the induction of inflammatory periapical lesion through exposure of the dental pulp, through retention of the contamination (bacterial infection) during a 56 days period. The animals of the case group were stressed daily by electrical stimuli (1.10 mA), whereas the animals in the control group were absent from the stressful stimuli (shocks). On day 55, all animals were subjected to the open field test, and the next day they were sacrificed. The jaws were removed and collected for histological and radiographic analysis. **Results:** The animals in the group of cases presented higher levels of stress, consequently greater weight loss and greater total number of inflammatory cells in relation to the control group. There was also a difference between the histological and radiographic analyzes, revealing that the diagnosis and quantification through the histological analysis are much more relevant and reliable as compared to the radiographic analyzes. **Conclusions:** We conclude that stress favors a greater evolution of the inflammatory periapical lesion by triggering worsened levels in the periapical tissue destruction and its bone loss.

**Keywords:** Periapical inflammatory lesion, stress, bone loss.

## Introduction

Infective inflammatory periapical lesions are characterized by exposure of the pulp tissue and, subsequently, by bacterial contamination and oral infection of root canal systems, which causes etiopathology for the development of this disease (1-3, 65-67). Pulp exposures can be the result of iatrogenic procedures, fractures of the tooth structure, and caries development that dissolve the mineralized dental tissues, among others, which allow bacterial access in the dental pulp, which subsequently triggers pulp necrosis (1-3, 31, 65, 67, 68). Inflammation is usually a host response to bacterial stimulus aggressions, inducing some mediators such as the production of antibodies and cytokines such as interleukin (IL) -1 and 6, tumor necrosis factor (TNF), and IL-1β (24, 25), osteoclast activators, stimulating bone resorption and destruction of the periapical tissues, through the imbalance in the RANKL/OPG system triggered by the oral inflammatory process and its products (3, 10, 23, 36, 46).

The periapical lesions that are diagnosed at a higher frequency are granulomas and root cysts and, in a lower incidence, the periapical abscess, all have a common etiology since they are commonly found at the apex of the teeth with necrotic pulp (13, 15). The treatment of the periapical lesion has been performed through endodontic treatment, which involves the removal of infected or dead soft tissue (pulp) located in the inner part of the tooth (chamber and canal) for subsequent cleaning and insertion of a sealing material (27-29).

Studies have shown that stress has promoted an ineffectiveness of the immune response to bacterial agents, triggering changes in the RANKL/RANK/OPG system and destruction of the periapical tissues (43, 69), constituting evolution in the manifestation of chronic periodontitis, suggesting that stress constitutes a risk factor for the progression of periodontal disease in humans, because studies in animal models were able to evidence a possible participation of the stressor contingencies on the periodontal tissue, which verified alveolar bone loss and destruction of the periodontal ligament in animals exposed to stress situations (50-54).

These reactions have been evidenced in infectious diseases in human and animal models, since stress leads to the modulation of the function of lymphocytes, neutrophils and macrophages, affecting the defense of the organism making it predisposed to infections (57, 58), since stress has been a risk factor for the body's psychophysiological reactions,

stimulating a variety of physical or emotional stimuli that interfere with the body's homeostasis (56).

There is no study in the literature that evidences mechanisms involving the relationship of the pathophysiology of bone resorption with the stressor events in the etiopathogenesis of the periapical inflammatory periapical lesion. Therefore, the present work aims to evaluate the impact of stress on bone resorption in inflammatory periapical lesions in Wistars rats.

## Materials and methods

### Experimental design:

Wistar rats (*Rattus norvegicus albinus*) have been subjected to periapical disease through a dental drill and divided into two groups. Twelve animals were from the control group, and 13 animals from the case group, which were exposed to stress for 50 days. A field test was used for a quantification of the stress animal. After 56 days of induction of the periapical lesion, the animals were sacrificed. As jaws were removed for radiographic and histological analysis.

### Animals:

Twenty-five Wistar rats (*Rattus norvegicus albinus*), males weighing 280-350 g (60 days old) were used. The animals were kept in environmental conditions at a controlled temperature of 21 ± 2 ° C with a cycle of 12 hours of light / 12 hours dark (lights on from 7 to 7 o'clock) no Biotério da Universidade Estadual de Montes Claros - UNIMONTES Montes Claros, Minas Gerais, Brazil), and fed with ration and filtered water. All experiments were approved by Protocol Ethics Committee No. 1512008 (CETEA / UFMG).

### Induction of inflammatory periapical lesion:

The inflammatory periapical lesion was induced in rats under ketamine anesthesia (70 mg/kg, intraperitoneal) and xylazine (10 mg/kg, intraperitoneal). Once the anesthesia was applied, and after 3 to 5 minutes, the animals presented sedative status, which in turn were accommodated to a special operating table that allows the animal to be restrained with the open mouth, however without hurting them. The animals were then submitted to pulp exposure in the lower left first molar, using diamond tips 2214 (FAVA Pirituba, São Paulo, Brazil) coupled to a contra angle D700 (Dabi Atlante, Ribeirão Preto, São Paulo, Brazil) attached to a micro motor of prosthesis (Beltec LB 100, Araraquara, São Paulo, Brazil) in low concentration. The drill was inserted into the distal fossa, located mesially to the distal cusp on the occlusal face of the tooth, at depth of about 1 mm, taking care not to perforate the furcation. The teeth were conditioned to pulp exposure with the aim of inducing bacterial contamination and consequent development of inflammatory periapical lesion.

**Open field test:**

To evaluate a spontaneous locomotor activity to quantify the animal stress, it was performed after three days of periapical induction or open field test. One box consisted of an open square field (1 $m^2$), which had its floor divided into 25 equal areas (20 $cm^2$). The rats were individually placed in the central area and were allowed to explore an area freely for 5 min. The animal's trajectory was quantified by centimeters traveled by ImageJ software. The field was cleaned with 70% ethanol after each run, then the rats were returned to their proper cages.

**Induction of animal stress:**

Four days after the pulp exposure, the rats were submitted to brief sessions of stress induction, conditioned by fear for 50 days. Initially, rats were placed individually for 3 min in a chamber to be used in the apparatus (37 cm x 25 cm x 21 cm, Skinner Box, ELT-02, Eltrones, Joinvile, Santa Catarina, Brazil). Subsequently, the rats in the case group received a presentation of a neutral conditioned stimulus (light) for 5s followed immediately by a harmful unconditioned stimulus (shock of 1.10 mA), six times, for 5s each, with an intershock interval of 20s, Totaling 3 min. The animals of the control group were also placed individually in the chamber and submitted to the same experimental conditions, but they did not receive the stimulus, that is to say, they were unconditioned to the shock, the light being the only stimulus. The tests were performed in an experimental room where one animal was kept at a time, so that other animals did not hear the noises released by the animal that was subjected to the experiment. The chamber was cleaned with 70% ethanol before and after each rat.

**Sacrifice of animals and obtaining specimens**

Fifty-six days after the induction of the inflammatory periapical lesion, the animals were individually taken to a surgical room where they were sacrificed by decapitation through a guillotine, shortly afterward the equipment was cleaned and sanitized with 70% alcohol, a precaution taken to avoid that the rats could smell the blood of the animal previously euthanized. Immediately afterward, individually, the heads of each animal were taken to another experimental room where the jaws were removed and separated into two hemimandibles using a scalpel (Bard-Parker). The material was placed in properly labeled containers and fixed in 10% phosphate-buffered saline for 48 hours. Then the decalcification

of the jaws was carried out in 10% EDTA solution for 30 days, with daily solution changes. After demineralization, the samples (hemimandibulae) were included in paraffin using the histotechnique (LUPE PT 05), so that the blocks were cut into a microtome (Easy Path EP-MR10) a 5 µm. To be later adhered in common glass blade stained with hematoxylin-eosin (HE), gomori trichrome, toluidine blue, and protected with glass coverslips for observation and histological quantification through microscopy (Olympus Fsx100). All laboratory techniques were performed following the protocol described above (51, 52).

**Quantifications**

To quantify the cells, 3 microscopic fields of the inflamed region were photographed with an increase of 200x, and for the quantification of periodontal ligament thickening, only 1 microscopic field of one of the roots of the injured tooth was necessary, with a 42x magnification. All microscopic images were taken with the microscopic Olympus Fsx100. Cell counting and mensuration of periodontal ligament thickening were performed using the ImageJ program, the same was used to quantify the inflammatory periapical lesion of the mandibular radiographic images.

**Statistical analysis**

Statistical analysis was performed using the PASW Statistics18 - SPSS software, where the normality test of Shapiro-Wilk was used, samples that had normal distribution were applied independent T-test samples and samples that did not follow this distribution was applied the Nonparametric Test Of Mann-Whitney. For the construction of the graphs, he used the software GraphPad Prism 5.

**Results**

According to our results obtained through the behavior test (fig. 1), the number of freezes per animal increased after the stressful stimuli (shocks) in the case group, in relation to the control group, absent from the stimuli (p=0.0001), confirming that the stress induction method was successfully emphasized in the animals.

No significant difference was observed (p=0.703) in the quantification of bone loss between groups in the radiographic analysis (fig. 2), the same was observed in the analysis of intensity of the region of interest (periapical lesion) (p=0.504) (fig. 3), demonstrating that the radiographic analysis are not as specific as histological findings, which presented significant differences (p= 0.006) in periodontal ligament thickening (fig. 4), which results in a greater bone loss in the case group compared to the control group. A significant difference was also seen (p= 0.0004) in the total number of inflammatory cells in the case group compared to the control group (fig. 5), which gives us indications of a more intense inflammation in stressed animals. However, our data did not detect a significant difference (p=0,114) in the quantification of the number of mast cells between the groups (fig.6).

**Discussion**

Due to the limitation of this type of research in humans, the present study was carried out in an animal model. In our study, rats with 60 days of age were used because they were young adult animals, presenting active sexual maturity. The first lower left molar, more specifically its distal root canal, was chosen for the induction of the periapical lesion by the ease of endodontic and radiographic access, as well as by human-like anatomy (70).

The periapical reaction caused by the pulpal exposure follows, in the rat, the same evolution as in man, that is, the formation of inflammatory periapicopathy. The use of the experimentally induced periapical disease model in rats makes it possible to study the dynamics of the evolution of these lesions (71-73). Thus, the microscopic and radiographic analysis of the induced inflammatory periapical lesion allowed an improved study in the period of development of the periapical lesion, allowing us a histological and radiographic quantification.

In the present study, it was noticeable that the animals subjected to stress induction presented an increase in the number of freezing behaviors, suggesting that the shocks were able to induce stress in the animals successfully. An open-field test may reveal changes in anxiety behavior, which is characterized by a decrease in locomotor activity in the evaluation performed through the test, which is used to quantify animal stress (74-76).

It is of great relevance to highlight that in our study, the number of frozen rats after induction of animal stress was significantly higher in the case group compared to the control group absent from stressful stimuli (shock) (fig. 1).

In this study, it was possible to identify cell types present, such as mononuclear, polymorphonuclear, mast cells (fig. 6) and osteoclasts. Studies show that activities mediated by polymorphonuclear cells such as macrophages (77), mononuclear cells such as neutrophils (78), and osteoclast represent the primary mechanism responsible for bone loss caused by cells and inflammatory mediators involved in bone destruction in periapical lesions induced in rats (79, 80).

Cytokines such as IL-1β, IL-6, TNF-α and PGE-2, among other proinflammatory ones, have been emphasized in the pathogenesis of the periapical lesion (81), usually by activating common pathways associated with bone resorption (82, 83), however, it is expected that these same mechanisms are associated in the present study.

In view of our results, we could observe that experimentally induced inflammatory periapical lesions in rats promoted bone expansion and resorption (fig. 4). Our findings are in agreement with those of other authors (41-43, 78, 82-85). However, if we were trying to add one more variable, which was the stress, our results were consistent with studies with periodontal disease (52), where there was a significant difference in bone resorption between the case group compared to the control group. However, the present work has been the pioneer in evaluating the bone resorption in rats experimentally induced to inflammatory periapical lesion, having as a coadjuvant factor the exposure to stress.

In our study, we chose to perform conventional radiographs submitted to scanning for analysis and later to a radiographic quantification. By making a correlation between the microscopic and radiographic findings, we can affirm that some microscopic changes such as the presence of periapical lesion and bone resorption were also identified radiographically. In addition, the quantitative aspects showed significant differences between the groups, in histological analyzes, such as total number of inflammatory cells (fig. 5), and periodontal ligament thickening (fig. 4) , which was not noticeable this difference between groups in the radiographic quantifications, such as the size of the lesion, and its intensity (fig. 3), proving that sensitivity and specificity in the radiographs, in the detection and classification of the periapical inflammatory lesion (if it is in humans) were reduced when compared to the histology.

Histologically, our results showed no significant difference between groups, in the number of mast cells; in the literature, there are no quantitative studies of mast cells in induced periapical lesions in rats exposed to stress, in order to corroborate or refute the results. Only studies performed in humans, which show the higher number of mast cells in periapical granuloma in the radicular cyst

Our studies corroborate previous work (86-89), but we contributed reinforcing our results by adding the stress factor in periapical lesions induced in an animal model.

We conclude that stress favors and positively influences the more aggressive development of the inflammatory periapical lesion and, consequently, a greater progression in bone loss and periapical tissue destruction. In addition, our results suggest that stress may trigger an increase in pro-inflammatory cytokine levels, and an increase in osteoclastic activity through high RANKL levels. Together, these results suggest that individuals diagnosed with periapical lesions under stress conditions present a greater evolution in bone loss and destruction of periapical tissues in relation to non-stressed individuals. Finally, these findings lead us to classify stress as an aggravating factor in the development of the inflammatory periapical lesion, and suggest that a pro-inflammatory cytokine blockade therapy may present an efficiency in regression of bone loss and destruction of periapical tissues in individuals with diagnosis of periapical lesions.

More studies are needed, such as the quantification of some cytokines and markers responsible for bone resorption in the inflammatory periapical lesions in animals exposed to stress, so that they may strengthen our results.

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**Figura 1.** Open Field Animal Behavior Test. Quantification of stress by the animal behavior test. Significant difference in frozen behavior in the case group in relation to control (p= 0.0001).

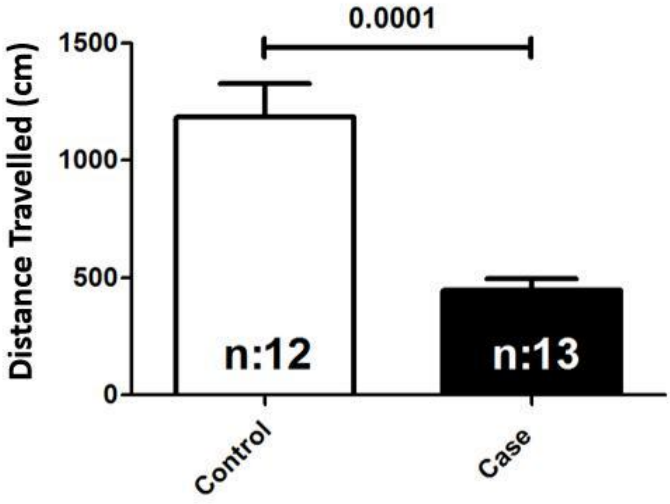


**Figura 2.** Radiographic Quantification of Bone Loss. There was no significant difference in the quantification of bone loss between groups in the radiographic analyzes (p = 0.703).

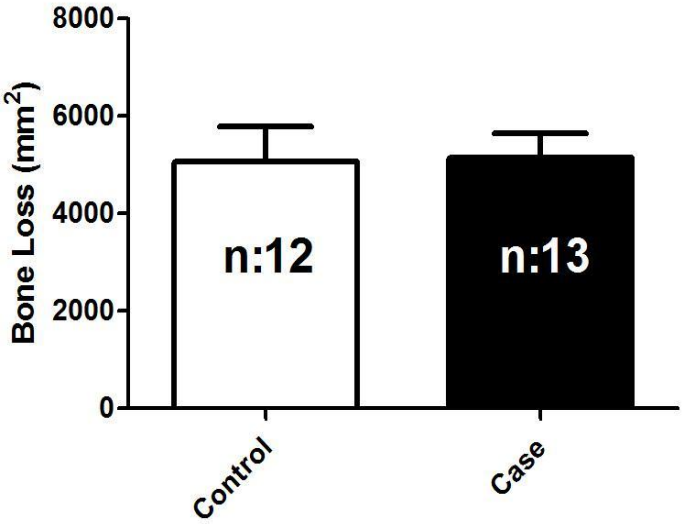


**Figura 3.** Intensity of the Periapical Lesion. The intensity of the periapical lesions in the radiographs did not present significant differences p = 0.504 between groups.

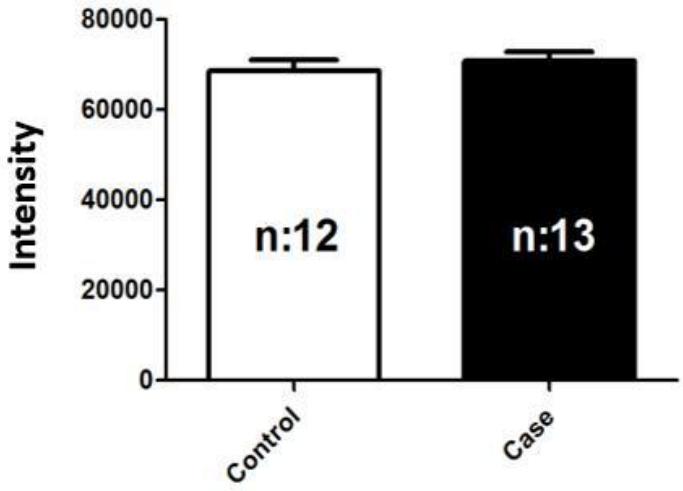


**Figura 4.** Analysis Thickening Of The Periodontal Ligament. According to the data, there is a greater bone loss in the case group in relation to the control group p = 0.006.

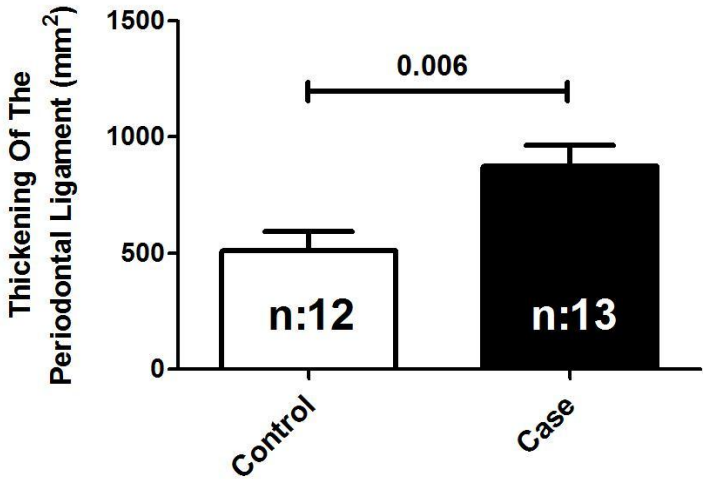


**Figura 5.** Quantification of Inflammatory Cells. A significant difference (p = 0.0004) between groups was demonstrated in the analyzes, and the case group presented a higher total number of inflammatory cells in relation to the control group.

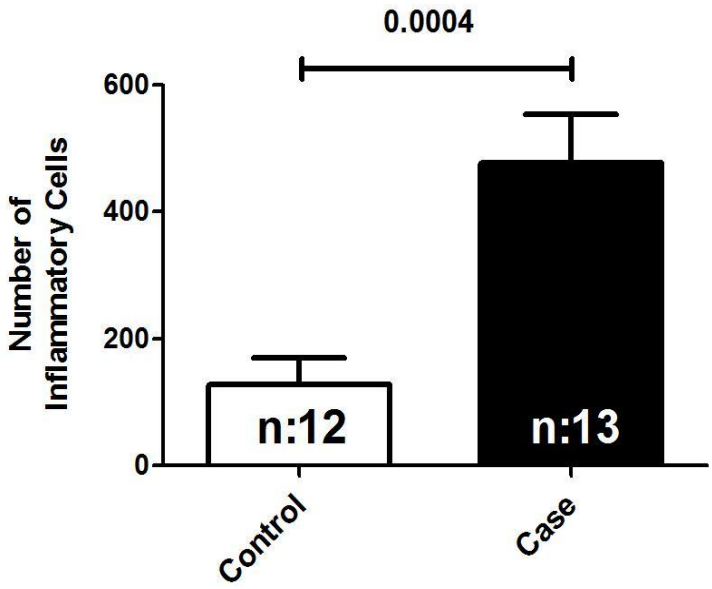


**Figura 6.** Quantification of mast cells. The data did not detect a significant difference (p = 0.114) in the quantification of the number of mast cells between the groups.

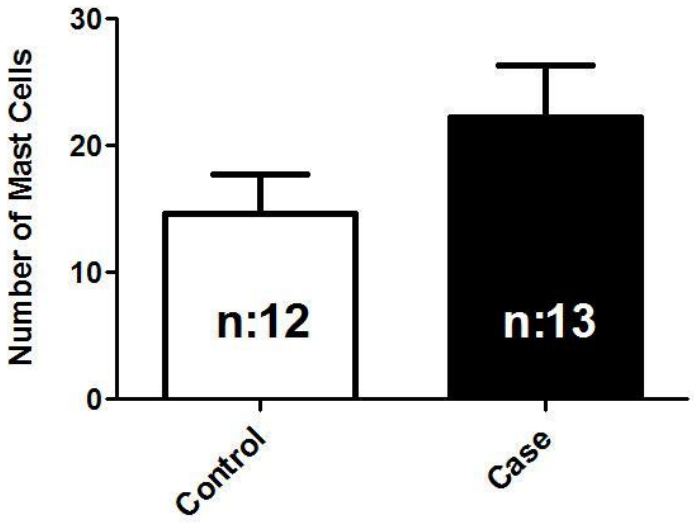
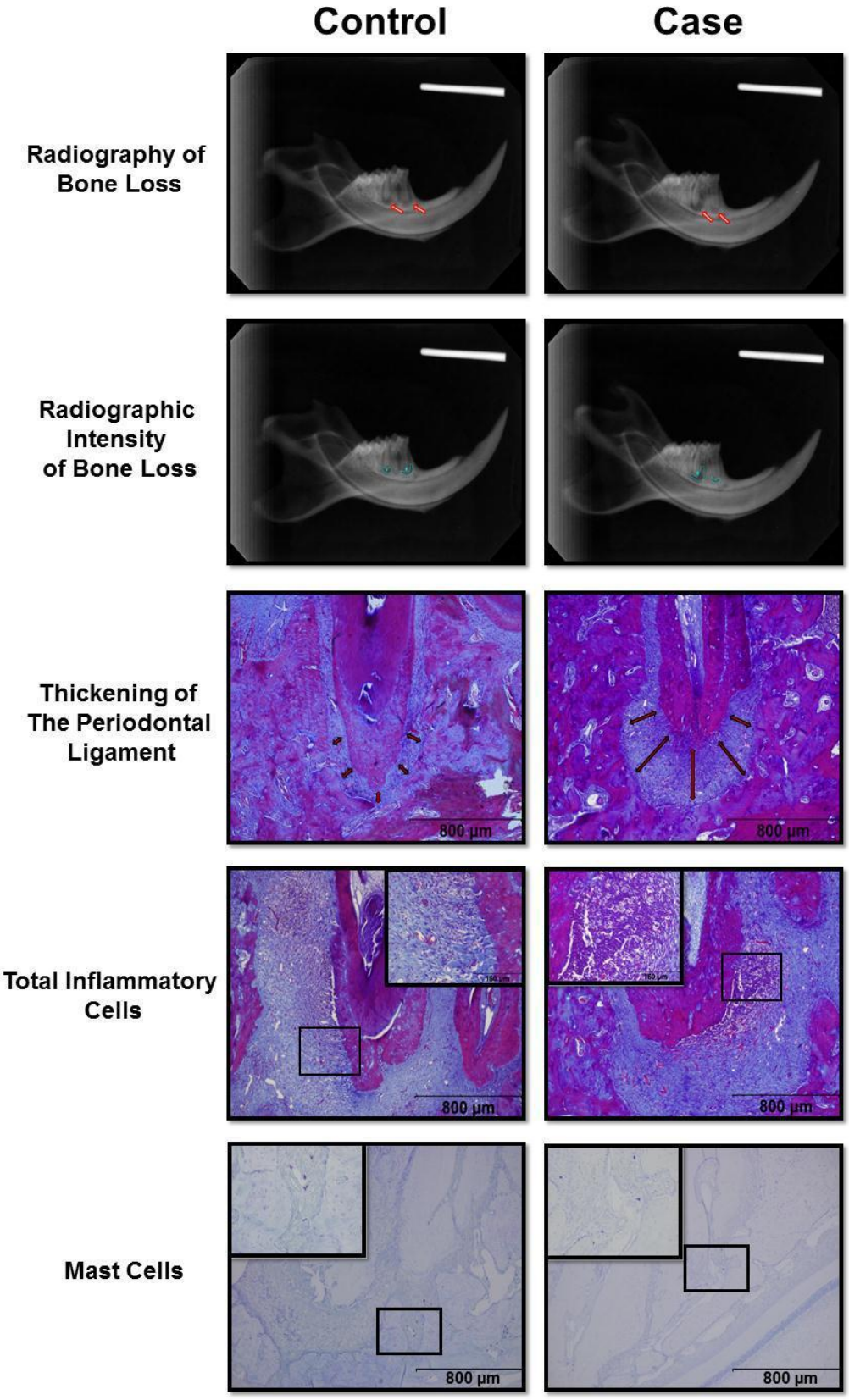


**Figura 7.** Illustrative Image of the Quantifications of Infectious Inflammatory Periapical Lesions



# 4 CONCLUSÕES

Conclui-se que o estresse favorece e influência positivamente no desenvolvimento mais agressivo da lesão periapical inflamatória e, por conseguinte, uma maior progressão na perda óssea e destruição dos tecidos periapicais. Além disso, nossos resultados sugerem que o estresse pode desencadear um aumento nos níveis de citocinas pró-inflamatórias e consequentemente pode acarretar o aumento na atividade osteoclástica através de altos níveis de RANKL. Juntos, esses resultados sugerem que os indivíduos diagnosticados com lesões periapicais sob condições de estresse apresentam uma maior evolução na perda óssea e destruição dos tecidos periapicais em relação aos indivíduos não estressados. Por fim, estes achados nos levam a classificar o estresse como fator agravante no desenvolvimento da lesão periapical inflamatória e sugere que a terapia pró-inflamatória de bloqueio de citocinas pode apresentar uma maior eficiência na regressão da perda óssea e destruição de tecidos periapicais em indivíduos com diagnóstico de lesões periapicais inflamatória.

São necessários mais estudos, como a quantificação de algumas citocinas e marcadores responsáveis pela reabsorção óssea nas lesões periapicais inflamatórias em animais expostos ao estresse, para que possam fortalecer os resultados.

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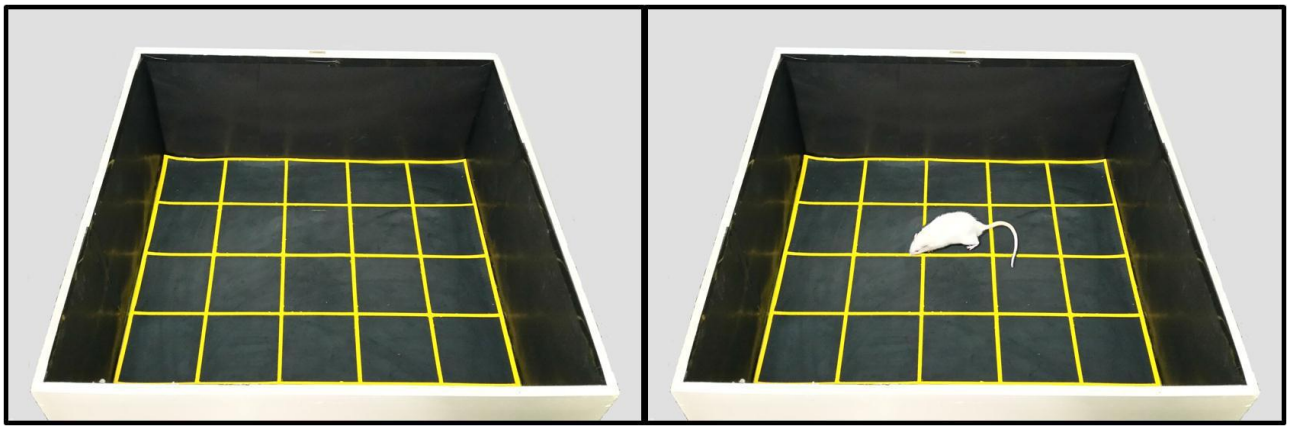
ANEXOS

ANEXO A – fotografia 1

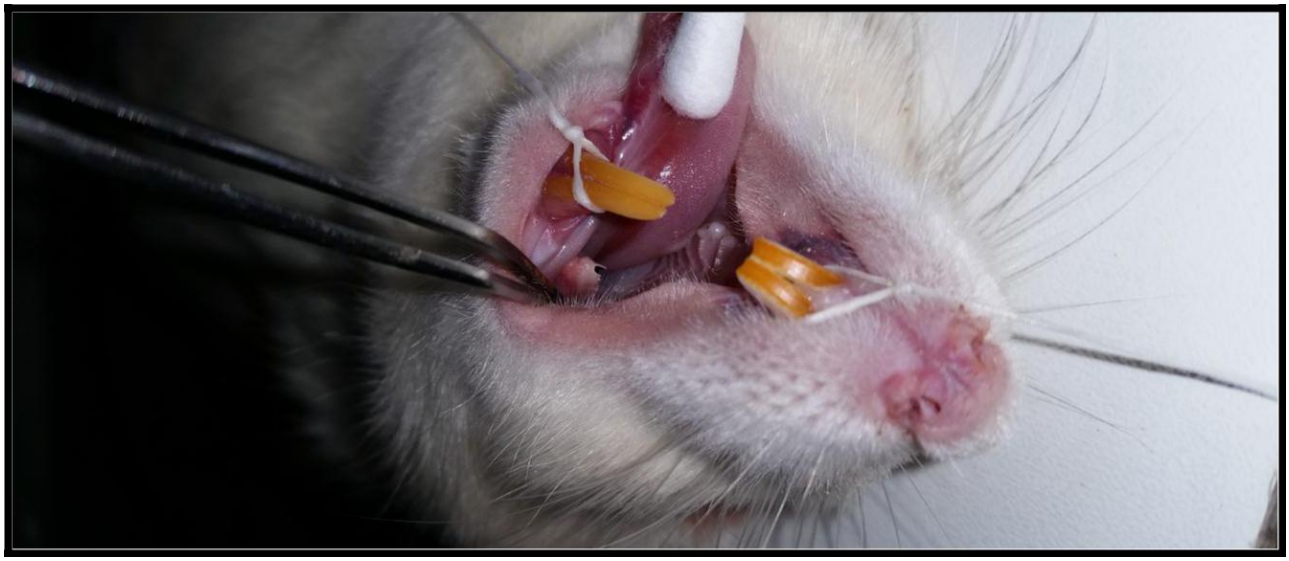


**Caixa de Campo Aberto (Open Field)**

ANEXO B – Fotografia 2


**Comportamento de Congelamento Animal**

ANEXO C – Fotografia 3



**Indução da Lesão Periapical Inflamatória Infecciosa**

ANEXO D – Parecer do Comitê de Ética e Pesquisa

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

**CERTIFICADO**

Certificamos que o **Protocolo nº 151/2008,** relativo ao projeto intitulado "***O Estresse na Etiopatogênese da doença periodontal em ratos Wistar***", que tem como responsável(is) **André Luiz Sena Guimarães** , 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 **8/ 10/2008.**

Este certificado expira-se em **8/ 10/ 2013.**

**CERTIFICATE**

We hereby certify that the **Protocol nº 151/2008,** related to the project entitled "***The stress on Etiopathol of periodontal disease in Wistar rats***", under the supervisiors of **André Luiz Sena Guimarães,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **October 8, 2008.**

This certificate expires in **October 8, 2013.**

Belo Horizonte, 13 de Outubro de 2008.

**Prof. Humberto Pereira Oliveira**
**Coordenador do CETEA/UFMG**

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