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Conditioned fear stress increases bone resorption in apical periodontitislesions in Wistar male rats

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ABSTRACT

Objective: Because the impact of conditioned fear stress on apical bone resorption is unknown, the aim of the current studywas to use a rat model to evaluate the impact of conditioned fear stress on the bone resorption of inflammatory apical periodontitis lesions.

Methods: Twenty-five animals were divided into two groups. They underwent a surgical procedure in the first left lower molar tooth to expose the dental pulp and induce inflammatory apical periodontitis lesions through the retention of contamination (bacterial infection) during a 56-day period. The animals in the case group were stressed daily by using electrical stimuli (1.10 mA), whereas the animals in the control group were absent from the stressful stimuli (shocks). The open field test was performed to validate the stress methodology. The jaws were removed and collected for histological and radiographic analyses.

Results: Stressed animals presented increased levels of bone loss and inflammatory cells in the root apex in comparison with the control group ($P = 0.0001$). However, no radiographic differences were observed between the groups ($P > 0.05$).

Conclusions: Our results demonstrated that conditioned fear stress could modify a periapical lesion by increasing the size of bone loss there. Conditioned fear stress also increased the total number of inflammatory cells compared with the control group. Studies evaluating the impact of conditioned fear stress on human periapical inflammatory lesions should be encouraged.

1. Introduction

Inflammatory apical periodontitis lesions often stem from bacterial infection in the root canal system, which promotes chronic inflammation and periapical bone resorption (Becconsall-Ryan, Tong, & Love, 2010; Kojima, Kumazaki, Ishii, & Miura, 1998). A bacterial stimulus induces a host response with the production of antibodies and cytokines, such as interleukin (IL) -1, IL-6, tumor necrosis factor (TNF), and IL-1β (de Queiroz et al., 2016; Fonseca-Silva et al., 2012; Krajewski et al., 2009). Osteoclast activators stimulate bone resorption and the destruction of periapical tissues, through an imbalance in the RANKL/RANK/OPG system, which is triggered by the inflammatory process and its products (Berar, Bondor, Matros, & Campian, 2016; Diegues, Colombo Robazza, Costa Hanemann, Costa Pereira, & Silva, 2011; Fan et al., 2011; Jiang, Zuo, Chen, & Holliday, 2003; Matsuo, Ebisu, Shimabukuro, Ohtake, & Okada, 1992). Recent studies demonstrated that bone biology is related to systemic factors (Aguiar et al., 2013; Gomes et al., 2013; Wippert, Rector, Kuhn, & Wuertz-Kozak, 2017).

Psychological Stress also influences the progression of periodontal disease in humans (Aguiar et al., 2013; Doyle & Bartold, 2012; Gomes et al., 2013; Semenoff-Segundo et al., 2012; Susin & Rosing, 2003). However, in clinical practice, the relationship betweenpsychological

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stress and bone has not been observed (Godinho et al., 2011).Conditioned fear stress is widely used to investigate the brain structures and neurotransmitter systems involved in aversive emotional learning and memory (Li, 2012). Conditioned fear stress might modulate the function of lymphocytes, neutrophils, and macrophages, thus affecting the defense of the organism, andpredisposing it to infections (Blalock, 1994; Mashaghi et al., 2016). Additionally,conditioned fear stress is a risk factor for the body's psychophysiological reactions, stimulating a variety of physical or emotional stimuli that interfere with the body's homeostasis (Chrousos & Gold, 1992).The mast cell is essential for the inflammatory process in neoplasia (Ribatti, 2013; Souza et al., 2010) and also in inflammatory dental lesions (Fonseca-Silva et al., 2012).Interestingly enough, psychological stress activates mast cells in the skin (Caruntu, Boda, Musat, Caruntu, & Mandache, 2014) and could be associated with the stress-induced initiation or exacerbation of cutaneous inflammatory processes (Theoharides & Cochrane, 2004).

To our knowledge,the mechanism related to conditioned fear stress and the pathophysiology of periapical bone resorption in endodontic research has not been previously investigated. Therefore, the present study aim was to use a rat model to evaluate the impact of conditioned fear stress on the bone resorption of inflammatory apical periodontitis lesions.

2. Materials and methods

Ethical approval for this study was obtained from the relevantInstitutional Animal Care and Use Committee (protocol #1512008).

2.1. Animals and experimental conditions

Twenty-five60-day-old Wistar male rats (*Rattus norvegicus Albinus*) weighing 280–350 g were used in this study. The animals were kept in an environmentwith a controlled temperature of 21 ± 2 °C and with a cycle of 12 h of light /12 h of dark (lights on from 12 h, and they were fed with rations and filtered water. Animals were randomly divided into two groups (13 animals in the case group and 12 in the control group). Inboth groups,periapical disease was induced, but only the case group received conditioned fear stress.

2.2. Open field test

Locomotor activity was assessed through an open field testto quantify animal conditioned fear stress.The test was performed in the box of an open square field (1 m^2) that had its floor divided into 25 equal areas (20 cm^2)(Aguiar et al., 2013; Gomes et al., 2013). The rats were individually placed in the central area and were allowed to explore an area freely for five minutes. The animal's trajectory was quantifiedin centimeters traveled using the Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD). The field was cleaned with 70% ethanol after each run, and the rats were then returned to their proper cages. Two open field tests were performed before the induction of conditioned fear stress and before the animals' sacrifice.

2.3. Induction of inflammatory apical periodontitis lesions

In the open field test, both groups presented similar locomotor activity. Periapical disease was induced in all animals. Inflammatory apical periodontitis lesions were induced in rats under general anesthesia with 70 mg/kg ketamine (Vallée, Montes Claros, MG, BR) and with10 mg/kg xylazine (Vallée). Pulp exposures were performed in the lower left first molar, using diamond tips # 2214 (Fava, Pirituba, SP, BR) coupled with a contra-angle (D700,Dabi Atlante, Ribeirão Preto, SP, BR) attached to an electric motor (Beltec LB 100, Araraquara, SP, BR). The drill was inserted into the distal fossa, located medially to the distal cusp on the occlusal face of the tooth, at a depth of about 1 mm, with caution to avoid perforating the furcation. The teeth were conditioned for pulp exposure with the aim of inducing bacterial contamination and the consequent development of inflammatory apical periodontitis lesions.

2.4. Induction of animal conditioned fear stress

The conditioned fear stress methodology used in this study has been previously described (Aguiar et al., 2013; Gomes et al., 2013). Briefly, beginning on the day after pulp exposure, rats were submitted to conditioning fear stress sessions for 50 consecutive days in a conditioning fear stress chamber (37 cm x 25 cm x 21 cm, Skinner Box, ELT-02, Eltrones, Joinville, SC, BR). In each stress section, animals received the presentation of a neutral conditioned stimulus (sound lasting two seconds) before a shock took place (five seconds of 1.10 mA). During the stress section, six shocks were delivered,and the interval between each shock was 25 s. Considering the procedures, each stress section lasted 185seconds.The animals in the control group were also placed individually in the chamber and submitted to the same experimental conditions, but the sound they heard was only the stimulus without shocks (Supplementary Material 1). The tests were performed in an experimental room with one animal at a time, to prevent other animals from hearing noises released by the animal that was subjected to the experiment. The chamber was cleaned with 70% ethanol before and after each rat entered it.

2.5. Sacrifice of animals

Animals were sacrificed via decapitation through a guillotine. Shortly after their sacrifices,the equipment was cleaned and sanitized with 70% alcohol so that the rats could not smell the blood of the animals previously euthanized. Immediately afterward, the heads of each animal were takenindividually to another experimental room, where the jaws were removed and separated into two hemi- mandibles with a scalpel (Bard-Parker®, Caledonia, MI). The material was placed in properly labeled containers and was fixed in 10% formalin solutionfor 48 h.

2.6. Radiographs and histological analyses

Following formalin fixation in 10% formalin solution for 48 h, periapical radiographs were obtained. Based on previous studies (Teixeira et al., 2011), an experimental model was created to permit the standardized positioning of samples, in which an acrylic box was used to insert a digital radiographic sensor (WYS, Softys Dental, France). An impression of the sensor was made with acrylic resin to permit the sensor to be inserted in the same position for all radiographs. The acrylic box was placed over a radiographic platform, always in the same position to yield standardized radiographs.The radiographic exposures were made with a dental X-ray unit(TIMEX 708981 Gnatus, Ribeirão Preto, SP, BR) using 70 Kvp, 8 Ma, 25 mm of distance between the tube and the sensor, as well as an exposition time of 0.04 s. ImageJ software (Schneider, Rasband, & Eliceiri, 2012) was used to measure the area and intensity of the radiopacity corresponding to the periapical lesion radiographically (De Rossi, Silva, Leonardo, Rocha, & Rossi, 2005).

2.7. Histological preparation and analyses

All laboratory techniques were performed following the protocol

previously described (Aguiar et al., 2013; Gomes et al., 2013). Briefly, after radiographs, jaws were submitted to decalcification in a 10% EDTA solution for 30 days, with daily solution changes. After demineralization, samples (hemi mandibular) were included in paraffin. The specimens were submitted to completed serial sections. Each section of 5 µm was obtained using a microtome (Easy Path EP-MR10). After all mandibles were sectioned, every fifth section (25 µm) was stained with Gomori trichrome. The perforation of the tooth was also a reference for analyses. The apical third of both the mesial and the distal root stained with Gomori trichrome was used to calculate the lesion size. The immediate posterior section ofthe Gomori trichrome chosen section was also stained with hematoxylin-eosin (HE) for double-checking purposes. The following posterior section to HE was stained with toluidine blue. The samples were covered with glass coverslips for the purposes of observation and histological quantification through microscopy (Olympus Fsx100, Center Valley, Palo Alto, CA) (Supplementary Material 2).

2.8. Quantifications

The quantification of the periodontal ligament was performed at 42x magnification in the apical third. Also in the apical third, inside the periodontal ligament (lesion), the number of inflammatory cells was evaluated. The morphology and cytochemistry in Gomori trichrome and HE were used to quantify inflammatory cells (Supplementary Material 3). Polymorphonuclearleukocytes (PMNs) or mononuclear leukocytes (MNLs) were counted using HE. On the other hand, the mast cells were quantified based on cell morphology and cytochemistry according to toluidine blue staining. Three fields on the hot spot ofthe inflamed region were photographedusing a microscope (FSX100, Olympus, Center Valley, PA, USA), with an increase of 200x being used to quantify the inflammatory cells. All morphological measurements were made using ImageJ software (Schneider et al., 2012), which was used previously(Aguiar et al., 2013; Gomes et al., 2013)

2.9. Statistical analysis

A statistical analysis was performed using the PASW Statistics18–SPSS software (IBM, Armonk, NY). Samples that had nonparametric distribution (Kolmogorov–Smirnov and Shapiro-Wilk tests) were subjected to an independent T-test, and samples that did not follow this distribution were subjected to a Mann-Whitney nonparametric test. Statistical analysis showing confidence above 95% ($P < 0.05$) was considered to be significant. Graphs were created using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Conditioned fear stress reduced locomotor activity

General locomotor activity measured conditioned fear stress. It was demonstrated that conditioned fear stress reduces animal general locomotor activity (Aguiar et al., 2013; Gomes et al., 2013). In the current study, it was observed that conditioned fear stress reduced the distance traveled ($p = 0.0001$, Fig. 1, and Supplementary Material 4). Moreover, a reduction of general locomotor activity as a consequence of freezing behaviors is observed (Supplementary Material 1).

3.2. Conditioned fear stress did not change radiographic features

Mandible radiographs were performed to evaluate the macroscopic aspect of the apical periodontitis lesions (Fig. 2A). The radiographic size of the periapical lesion was similar in the case and control groups (Fig. 2B).The intensity of the apical periodontitis lesions was also evaluated with mandible radiographs. No significant differences between the case and the control group were observed regarding the size of the bone loss area (Fig. 2C).

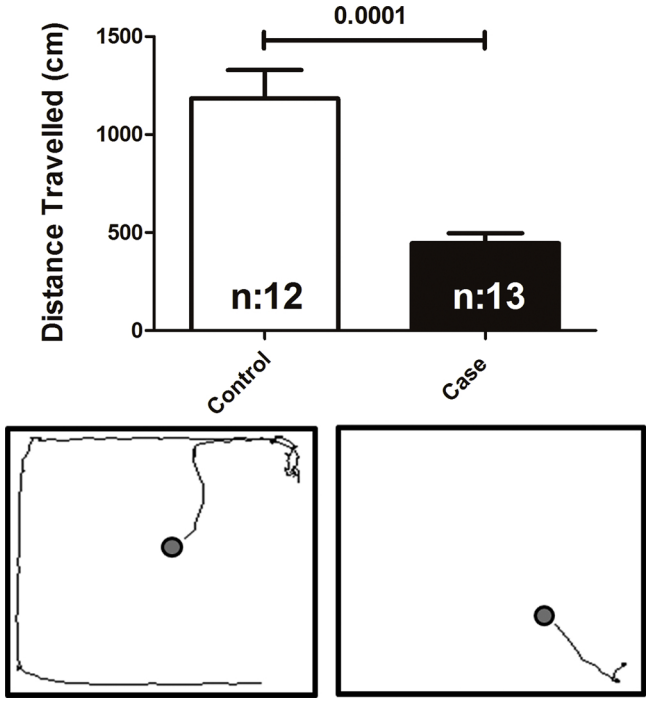

Fig. 1. Quantification of Conditioned fear stress by the animal behavior test: The induction of Conditioned fear stress promotes behavioral changes in stressed animals. Conditioned fear stress promoted a significant reduction in the traveled distance was observed in stressed animals (P = 0.0001). Bar charts represent means, and errors bars represent standard deviation.

3.3. Conditioned fear stress increased the histological bone loss

Histopathological analyses were performed to compare the inflammation profile of apical periodontitis lesions between the groups. The development of inflammatory apical periodontitis lesions is illustrated in Fig. 3A-D. Interestingly enough,conditioned fear stress increased the histological size of the periapical lesion in comparison with the control group ($p = 0.006$, Fig. 3A).

3.4. Conditioned fear stressincreased the total number of inflammatory cells but did not change the inflammation profile

Conditioned fear stress increased the total number of inflammatory cells compared with the control group ($p = 0.0004$, Fig. 4A). However, no differences in the ratio of PMNs/mononuclear leukocytes were observed (data not shown). Additionally, conditioned fear stress did not change the number of mast cells between the groups (Fig. 4B).

4. Discussion

Due to the limitations of conducting this type of research in humans (Poswar Fde et al., 2015), the present study was performed using an animal model, in which 60-day-old rats were used because they were young adult animals, showing active sexual maturity. The lower left first molar was chosen for the induction of apical periodontitis lesions due to the ease of endodontic and radiographic access, as well as anatomical similarity with the human tooth (Dammaschke, 2010).The use of the experimentally induced periapical disease model in rats makes it

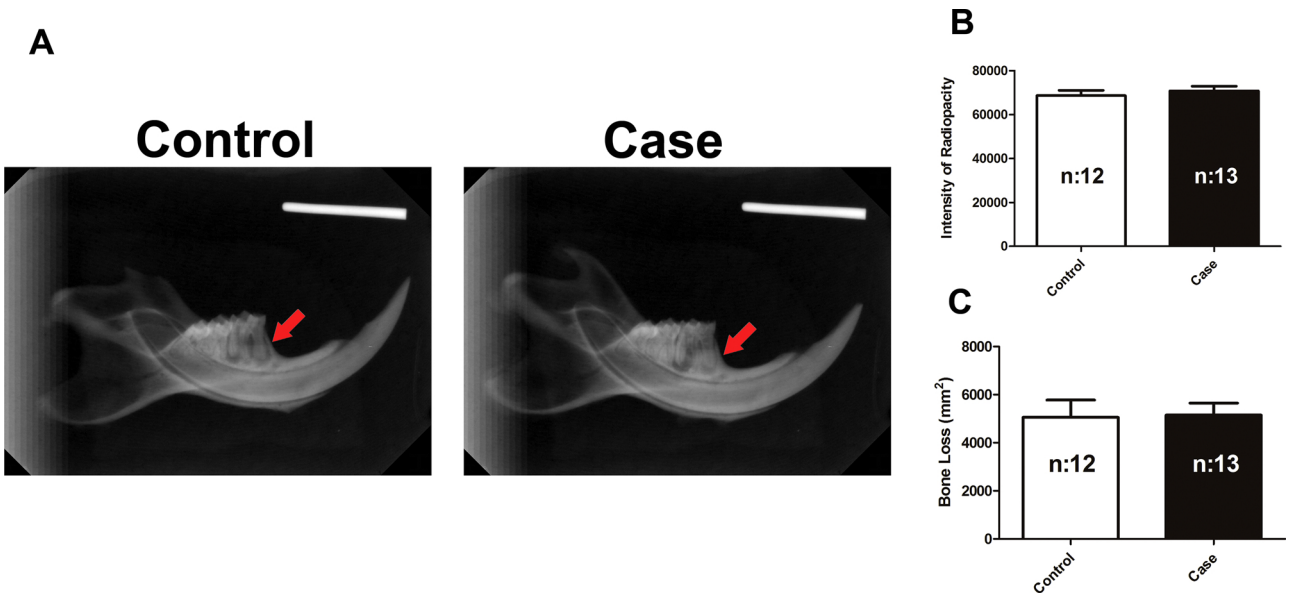

Fig. 2. Radiographic quantification of bone loss: A radiographic feature of periapical lesions in stressed and control group (Fig. 2A). There was no difference between case and control groups in radiographic lesion density (Fig. 2B). No significant differences between case and control group were observed regarding the size of bone loss area (Fig. 2C). Bar charts represent means, and errors bars represent standard deviation.

possible to study the dynamics of the evolution of endodontic lesions (Stashenko & Yu, 1989; Stashenko, Yu, & Wang, 1992; Stashenko, Wang, Tani-Ishii, & Yu, 1994), allowing for a histological and a radiographic quantification (Torabinejad, Corr, Buhrley, Wright, & Shabahang, 2011).

In the present study, the number of freezing behaviors was more evidentinthe animals subjected to shocks, suggesting that the conditioned fear stress was induced successfully. An open field test reveals changes in anxiety behavior, which is characterized by a decrease in locomotor activity in the evaluation performed through the trial, which is then used to quantify animal conditioned fear stress(Choleris, Thomas, Kavaliers, & Prato, 2001; Prut & Belzung, 2003; Ramos & Mormede, 1998).Our results showed that stressed rats exhibited increasedperiapical bone loss.It has been demonstrated that fluctuations in mood can influence inflammation by affecting cytokine production (Matsunaga et al., 2011). Polymorphonuclear cells, such as macrophages (Hofbauer et al., 1999); mononuclear cells, such as neutrophils (Morimoto, Yamasaki, Nakata, Tsuji, & Nakamura, 2008); and osteoclasts, represent the primary mechanism responsible for bone loss stemming from the cells and inflammatory mediators involved in bone destruction in the apical periodontitis lesions induced in rats (Palmqvist et al., 2006; Wang, Sun, Liu, & Peng, 2014). Proinflammatory cytokines activate common pathways associated with bone resorption (Solanki, Aminoshariae, Jin, Montagnese, & Mickel, 2013; Takeichi, Saito, Tsurumachi, Moro, & Saito, 1996). Cytokines such as IL-1β, IL-6, TNF-α, and PGE-2, among other proinflammatory ones, have been related toperiapical lesion pathogenesis(Gazivoda et al., 2009). In periodontics, the effect of conditioned fear stress on bone loss has been established(Aguiar et al., 2013; Branco-de-Almeida et al., 2012; Gomes et al., 2013). Likewise, our results suggested that conditioned fear stress can play a role in the advancement of apical periodontitis lesions.

In this study, experimentally induced inflammatory apical periodontitis lesions in rats promoted bone expansion and resorption, in agreement with other studies (da Silva, Ferreira, De Rossi, Nelson-Filho, & Silva, 2012; Dill et al., 2015; Kawashima et al., 2007; Menezes et al., 2008; Morimoto et al., 2008; Solanki et al., 2013; Takeichi et al., 1996; Zhang, Yu, & Miao, 2012). In general, inflammatory apical periodontitis is diagnosed based on the association of clinical and conventional radiographic findings (Diegues et al., 2011). To simulate how a clinician would perceive apical periodontitis, the current study used conventional radiographic. The radiographic analysis did not indicate differences in the bone loss for both stressed or non-stressed animals in the period of development (60 days) of the apical periodontitis lesions.Our results might be explained by the limitation of radiographic accuracy in detecting periapical changes until 90 days of evaluation (Carrillo et al., 2008).

On the other hand, histological evaluation is the gold standard for the diagnosis of inflammatory apical periodontitis lesions (Diegues et al., 2011), but in patients, it is not possible to perform routinely. No differences in the ratio of PMNs/mononuclear leukocytes were observed. Additionally, no significant difference between groups was verified in relation to some mast cells. These results corroborate previous studies that evaluated the effect of conditioned fear stress on periodontal diseases (Aguiar et al., 2013; Gomes et al., 2013). No quantitative morphologic studies exist regarding induced apical periodontitis lesions in rats subjected to conditioned fear stress. Interestingly enough, in previous studies with humans, the number of mast cells was related to the type of disease (Fonseca-Silva et al., 2012; Shiromany et al., 2014). However the development of the apical inflammatory lesion was demonstratedpreviously (Stashenko et al., 1992). The endpoint of the current study is bone resorption. Thus, the morphological screening techniques presented here did not reveal differences in pathological patterns. Future large-scale techniques might be useful for distinguishing if molecular differences exist (Khurshid et al., 2016).

In conclusion, our results demonstrated that conditioned fear stress could modify aperiapical lesion by increasing the size of the bone loss there. Conditioned fear stress also increased the total number of inflammatory cells compared with the control group. Studies evaluating the impact of conditioned fear stresson the human periapical inflammatory lesions should be encouraged.

Conflict of interest statement

The authors deny any conflicts of interest related to this study.

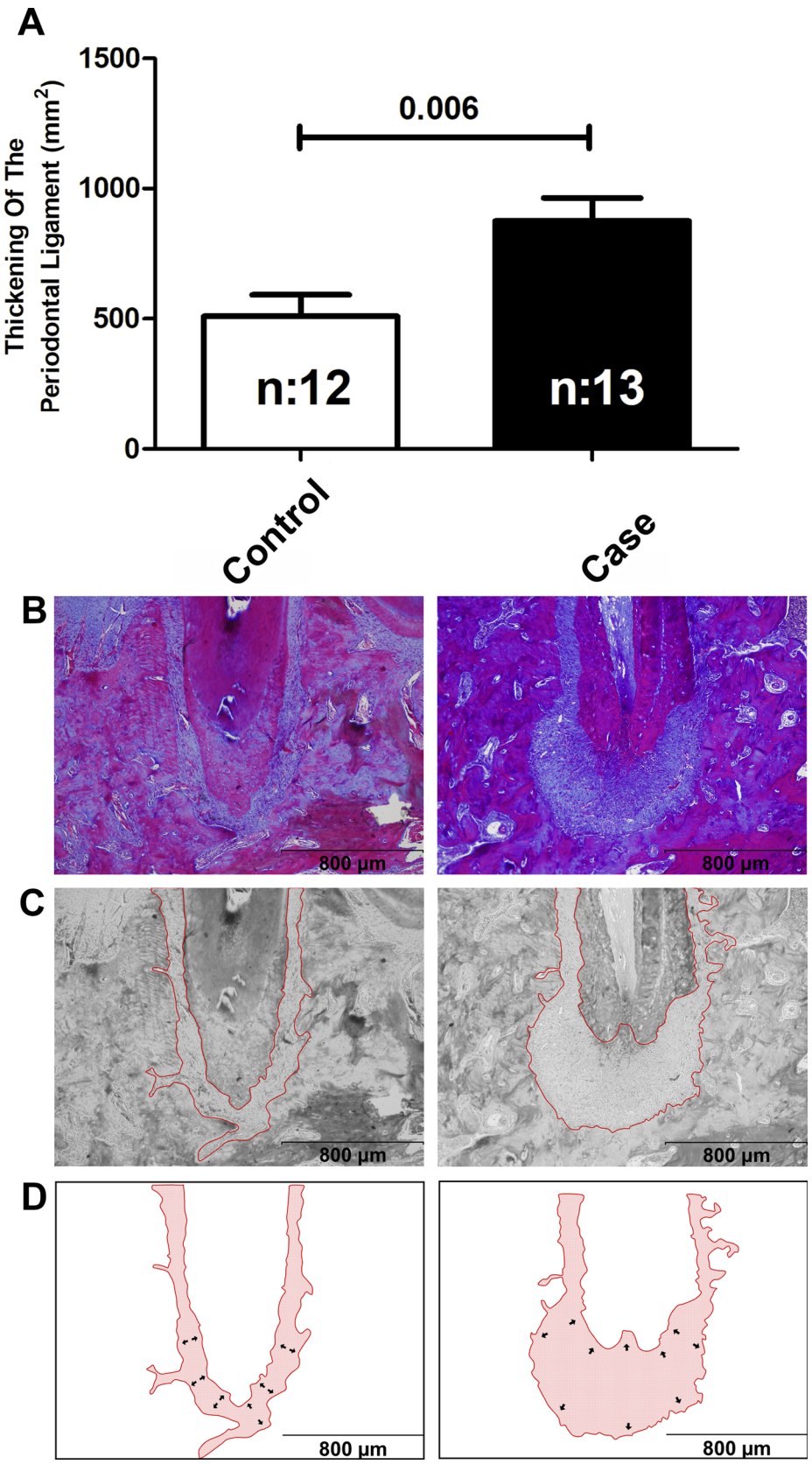

Fig. 3. Histological evaluation of periapical lesion: The comparison of bone loss between groups was shown in green arrows (Fig. 3A–D). Thickening of the periodontal ligament; where it shows a more significant bone loss in the case group than in the control group (P = 0.006) (Fig. 3A and B). Image used to quantify the area and the representation of the Region of Interest (ROI) of both groups (Fig. 3C and D respectively). Bar charts represent means, and errors bars represent standard deviation.

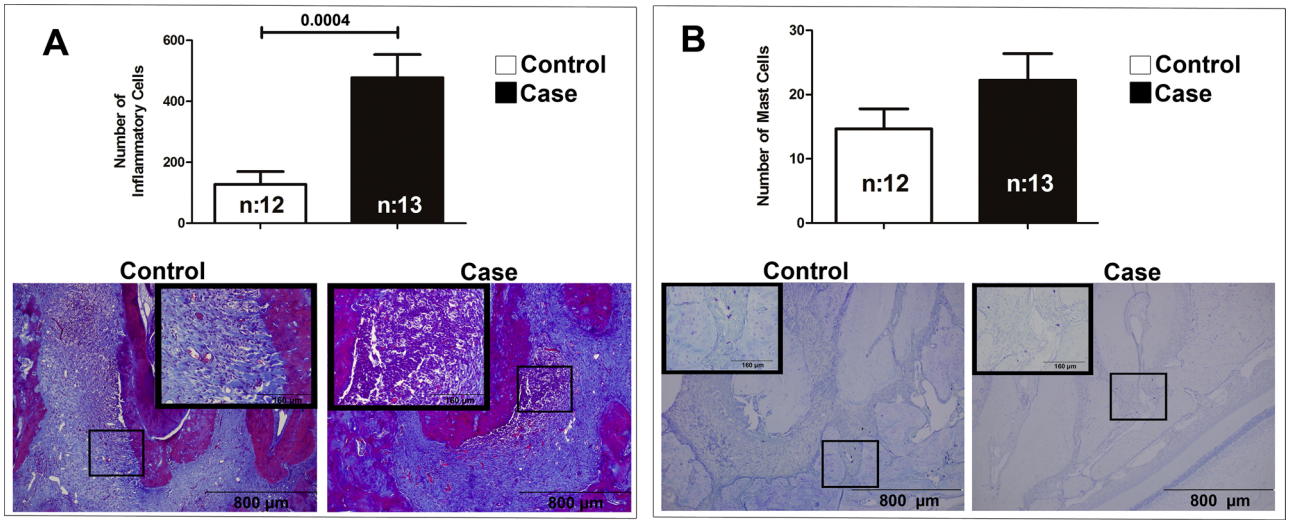

Fig. 4. Histological evaluation of inflammatory cells: Quantification of total inflammatory cells was presented in Fig. 4A; a significant difference between groups was demonstrated in the analyses, with the case group presenting a higher total number of inflammatory cells in the control group (P = 0.0004). Mastocyte counting was presented in Fig. 4B. No Mastocyte differences were observed between the groups (P = 0.114). Bar charts represent means, and errors bars represent standard deviation.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2018.10.004.

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