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Instituto de Ciência e Tecnologia

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O ESTRESSE CRÔNICO E A VIA DE SINALIZAÇÃO ADRENÉRGICA NO DESENVOLVIMENTO DA LESÃO PERIAPICAL

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Orientadora: Profa. Tit. Marcia Carneiro Valera

Coorientadora: Prof. Dra. Renata Falchete do Prado

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" Coração de estudante
Há que se cuidar da vida
Há que se cuidar do mundo
Tomar conta da amizade
Alegria e muito sonho
Espalhados no caminho
Verdes, plantas, sentimento
Folhas, coração, juventude e fé ".

Milton Nascimento

SUMÁRIO

LISTA DE ABREVIATURAS E SIGLAS

α1-AR	Receptor α1-Adrenérgico
α2-AR	Receptor α2 Adrenérgico
β-AR	Receptor β2-Adrenérgico
HPA	Hipotálamo-hipófise-adrenal
IL-1β	Interleucina 1β
IL-6	Interleucina 6
OPG	Osteoprotegerina
RANK	Receptor ativador do fator nuclear kappa-β
RANKL	Ligante do receptor do fator nuclear kappa β
SNS	Sistema Nervoso Simpático
TNF	Fator de necrose tumoral

Khoury RD. O estresse crônico e a via de sinalização adrenérgica no desenvolvimento da lesão periapical [dissertação]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2017.

RESUMO

Os objetivos deste estudo são: 1) Esclarecer a possível associação entre o estresse crônico e a estimulação do Sistema Nervoso Simpático (SNS) e investigar sua interferência no desenvolvimento e progressão da lesão periapical; 2) Avaliar a quantidade de receptores para os neurotransmissores na região periapical; 3) Elucidar uma via farmacológica de modulação inflamatória através do uso de bloqueadores adrenérgicos. Trinta e dois ratos Wistar foram submetidos à modelo animal de lesão periapical através da exposição da cavidade pulpar e em seguida foram aleatoriamente divididos em 4 grupos: sem estresse (NS); estresse + solução salina (SS); estresse + β-bloqueador (Sβ); estresse + α-bloqueador (Sα). Os grupos SS, Sβ e Sα foram submetidos à modelo animal de estresse crônico durante 28 dias e receberam injeções diárias de solução salina, propranolol (β bloqueador adrenérgico) e fentolamina (α bloqueador adrenérgico), respectivamente. Após 28 dias os animais foram eutanasiados e procedeu-se as seguintes análises: a) dos níveis séricos de corticosterona através de Radioimunoensaio; b) histomorfométrica por coloração com hematoxilina e eosina; c) da estrutura óssea periapical através de microtomografia computadorizada (micro-CT); d) expressão de receptores β e α adrenérgicos; e) da atividade osteoclástica através de histoquímica para fosfatase ácida resistente ao tartarato (TRAP). Os resultados obtidos mostram um aumento do nível sérico de corticosterona dos animais do grupo SS sendo estatisticamente significante comparados aos animais do grupo NS (sem estresse) ($p<.05$). Nenhuma diferença estatística foi observada a nível histológico uma vez que todos os animais apresentaram infiltrado inflamatório moderado e área de lesão periapical similares. A análise por micro-CT também mostrou similaridade da área e volume da lesão periapical em todos grupos. Através da histoquímica para TRAP verificou-se uma quantidade significativamente menor de osteoclastos nos grupos que receberam bloqueadores adrenérgicos (Sβ e Sα) ($p<.05$). Conclui-se que não houve influência significativa do estresse crônico no desenvolvimento e progressão da lesão periapical e a administração de bloqueadores adrenérgicos apesar de não ter sido capaz de modular a resposta inflamatória, diminuiu significativamente o número de osteoclastos na região periapical.

Palavras-chave: Estresse crônico. Lesão periapical. Bloqueadores adrenérgicos. Propranolol. Fentolamina.

Khoury RD. Chronic stress and the adrenergic signaling pathway on the development of periapical lesion [dissertation]. São José dos Campos (SP): São Paulo State University (Unesp), Institute of Science and Technology; 2017.

ABSTRACT

The objectives of this study are: 1) To clarify the possible association between chronic stress (CS) and stimulation of the Sympathetic Nervous System (SNS) and to investigate its interference in the development and progression of periapical lesion; 2) To evaluate the amount of receptors for neurotransmitters in the periapical region; 3) To elucidate a pharmacological pathway of inflammatory modulation through the use of adrenergic blockers. Thirty- two Wistar rats were submitted to animal model of periapical lesion through exposure of the pulp cavity and were then randomly divided into 4 groups: no stress (NS); stress + saline solution (SS); stress + β-blocker (Sβ); stress + α-blocker (Sα). The SS, Sβ and Sα groups were submitted to animal model of CS for 28 days and received daily injections of saline solution, propranolol (β blocker adrenergic) and phentolamine (α adrenergic blocker), respectively. After 28 days the animals were euthanized and the following analysis were carried out: a) serum corticosterone levels through Radioimmunoassay; b) histomorphometric by staining with hematoxylin and eosin; c) periapical bone structure through micro computed tomography; d) expression of β and α adrenergic receptors; e) osteoclast activity by histochemistry for tartrate resistant acid phosphatase (TRAP). The results obtained show an increase in the seric corticosterone level of the animals of the SS group being statistically significant compared to the NS group animals (without stress). No statistical difference was observed histologically since all animals had moderate inflammatory infiltrate and similar periapical lesion area. Micro-CT analysis also showed similarity of the area and volume of the periapical lesion in all groups. Through histochemistry for TRAP, a significantly lower amount of osteoclasts was observed in the groups receiving adrenergic blockers (Sβ and Sα). It was concluded that there was no significant influence of chronic stress on the development and progression of the periapical lesion and the administration of adrenergic blockers despite not being able to modulate the inflammatory response, significantly decreased the number of osteoclasts in the periapical region.

Keywords: Chronic stress. Periapical lesion. Adrenergic blockers. Propranolol. Phentolamine.

1 INTRODUÇÃO

A periodontite apical é uma doença inflamatória caracterizada pela resposta imune como consequência de uma infecção bacteriana e polpa dentária necrótica que resulta em reabsorção óssea periapical. A exposição da polpa dentária às bactérias e seus subprodutos pode provocar respostas inflamatórias inespecíficas, bem como reações imunológicas específicas nos tecidos perirradiculares, levando a destruição dos tecidos periapicais (Liapatas et al., 2003; Ricucci et al., 2006). A destruição dos tecidos moles e mineralizados é desencadeada por uma série de mediadores químicos que modulam de forma independente ou cooperativa a atividade proteolítica e a ativação dos mecanismos de reabsorção óssea (Graves et al., 2011; Fukada et al., 2008; Menezes et al., 2006). Portanto, a periodontite apical é originada da defesa imunológica do hospedeiro (Stashenko et al., 1992; Kawashima, Stashenko, 1999) frente a ação dos microrganismos e seus produtos.

Na área da Periodontia, diversas pesquisas têm sido realizadas nas últimas décadas a fim de esclarecer a etiologia da doença periodontal bem como os diversos fatores que podem interferir nas respostas imunoinflamatórias. Alguns fatores sistêmicos como diabetes mellitus, tabagismo, osteopenia, polimorfismos genéticos e maus hábitos de saúde foram associados as formas mais graves de doença periodontal sendo apontados como componentes importantes na estimulação da resposta inflamatória crônica da doença periodontal (Grossi et al., 1994, 1995; Genco et al., 1999; Preshaw, Bissett, 2013).

Além disso, fatores psicossociais como o estresse, ansiedade e depressão, têm sido relacionados à doença periodontal, e vem sendo frequentemente identificados como fatores de risco adicionais que podem afetar a progressão da doença periodontal (Boyapati, Wang, 2000; Rosania et al., 2009; Sabbah et al., 2011). Apesar de alguns estudos observacionais apoiarem este tipo de correlação, os mecanismos de influência dos fatores psicológicos ainda não foram devidamente elucidados na literatura (Gupta et al., 1993; Monteiro Da Silva et al., 1996; Moss et al., 1996).

Sendo assim, embora seja bem estabelecido que as bactérias são os principais agentes causadores da doença periodontal, sugere-se que a resposta

individual seja determinante para sua progressão, uma vez que, fatores de risco como os supracitados podem modificar não só a resposta imune do hospedeiro, como também influenciar na progressão, severidade e evolução da doença periodontal (Peruzzo et al., 2007). Até o momento não existem estudos disponíveis na literatura que investigam a possível influência do estresse crônico sobre a progressão da lesão periapical.

O estresse crônico resulta da pressão emocional vivida por um longo período de tempo, sobre a qual o indivíduo não possui controle (Gaspersic et al., 2002). Indivíduos que sofrem de estresse apresentam anormalidades de comportamento, como humor deprimido e sono prejudicado, juntamente com desregulação dos sistemas nervoso neuroendócrino e simpático que são por sua vez, vias eferentes críticas na regulação cerebral da imunidade. Segundo Joels e Baram (2009), o padrão e a magnitude da resposta do hospedeiro frente ao estresse parecem ser influenciados por diversos fatores, como a duração da exposição ao estresse (agudo ou crônico), o tipo de estresse (físico ou psicológico), gênero, entre outros.

Os eixos hipotálamo-hipófise-adrenal (HPA) e o Sistema Nervoso Simpático (SNS) são as principais vias neurais ativadas por fatores de estresse físicos psicológicos (Ader et al., 1995; Black, 1994; Chrousos, 1995). Durante o estresse ocorre a ativação simpática pelo eixo HPA, durante a qual existe um aumento na liberação dos hormônios neuroendócrinos, como os glicocorticoides (cortisol e corticosterona) e catecolaminas (epinefrina e norepinefrina) nos tecidos (Thaker et al., 2006) e plasma (Kondo, Togari, 2003). A síntese de noradrenalina (norepinefrina), bem como de molécula semelhante, a adrenalina (epinefrina) é catalisada pela enzima tirosina hidroxilase (Nagatsu et al., 1964; Nagatsu, 1995; Katori et al., 2012). Através da liberação desses hormônios diante da atividade excessiva do eixo autônomo simpático, o estresse pode ter efeitos prejudiciais sobre as funções imunológicas (Webster Marketon, Glaser, 2008; Dhabhar, 2009).

Modelos animais experimentais sugerem que o estresse crônico provoca o estado de inflamação através do aumento na liberação de citocinas pró-inflamatórias circulantes que por sua vez respondem a sinais específicos (Lu XT et al., 2013). As citocinas são proteínas sinalizadoras que transmitem informações entre as células imunológicas e também entre o sistema imunológico, o cérebro e o sistema

endócrino (Cannon, 2000).

Dentre as principais citocinas envolvidas no quadro de estresse associado à inflamação crônica podemos citar a interleucina-1β (IL- β) fator de necrose tumoral-α (TNF-α), interleucina-6 (IL-6), que são por sua vez conhecidas por induzirem atividade osteoclástica. A expressão dessas citocinas são reguladas pelo fator nuclear kappa-β que é portanto o mediador responsável pelo estabelecimento deste quadro (Black, 2006; Pahl, 1999).

Uma das vias comuns de secreção de moléculas pró-inflamatória é através de receptores ligantes presentes em células secretadoras de citocinas (Hofbauer et al., 2001; Tay et al., 2004). A reabsorção óssea pode ser regulada via ativação do sistema RANK/RANKL/OPG (receptor ativador do fator nuclear kappa β/ ligante do receptor do fator nuclear kappa β/ osteoprotegerina). O RANK e seu ligante (RANKL) foram recentemente identificados e desempenham papel fundamental no desenvolvimento dos osteoclastos. A ligação do RANKL ao seu receptor RANK, expressado na superfície dos osteoclastos, impulsionam sinais de diferenciação dos pré-osteoclastos, promovendo assim a reabsorção óssea. A osteoprotegerina (OPG) por sua vez, é uma proteína secretada principalmente pelas células osteoblásticas, que atua como receptor “isca” do RANKL impedindo a ligação deste ao seu receptor verdadeiro RANK, preservando a integridade óssea (Teitelbaum, 2000; Theill et al., 2002; Alliston, Derynck, 2002).

Diversos estudos apontam a expressão de RANKL e OPG na periodontite apical, o que sugere o papel dessas moléculas durante o desenvolvimento da lesão periapical (Sabeti et al., 2005; Menezes et al., 2006; Vernal et al., 2006; Kawashima et al., 2007). Em geral, o equilíbrio entre a expressão de RANKL e OPG é determinante para a resposta biológica (Tay et al., 2004; Garlet GP et al., 2006; Jin et al., 2007, Dunn et al., 2007; Garlet TP et al., 2007). No entanto, até o momento não existem estudos que correlacionam a expressão de RANKL/OPG com a atividade real de reabsorção óssea e os perfis de expressão de RANKL/OPG em lesões periapicais permanecem desconhecidos.

A via de sinalização adrenérgica é também considerada uma outra via importante na secreção de moléculas pró inflamatórias. Essa por sua vez, é mediada pelos receptores alfa e beta-adrenérgicos que se ligam à norepinefrina, que por sua vez pode estar aumentada nos estados de estresse devido a ativação do sistema

simpato-adrenal (Nance, Sanders, 2007). A ligação dos receptores α e β adrenérgicos à norepinefrina ativa a secreção de citocinas pró-inflamatórias.

Lu H et al. (2014) demonstraram in vivo, que a indução de estresse crônico acelerou a progressão da doença periodontal por excitação do SNS, resultando na liberação da catecolamina neurotransmissora epinefrina, a qual se liga ao receptor α1 na superfície das células do ligamento periodontal causando massiva liberação de mediadores inflamatórios que interferem na patogênese da doença periodontal.

Li et al. (2015) observaram que a norepinefrina pela via de ativação adrenérgica, apresenta efeito imunossupressor na função de macrófagos aumentando a síntese de IL-6 por estas células. Assim, Breivik et al. (2005) ao pesquisarem sobre a influência da via de sinalização adrenérgica em roedores com doença periodontal experimental, submetidos à simpatectomia, verificaram regeneração óssea e inibição da progressão da doença periodontal.

Os receptores adrenérgicos ou adrenoreceptores são alvos das catecolaminas, em especial a epinefrina e norepinefrina que se ligam a eles de acordo com a maior concentração e disponibilidade de cada tipo de receptor. Os receptores adrenérgicos são subdivididos em dois grandes grupos: α e β, e numerosos subgrupos (Furchgott, 1959; Rasmussen et al., 2011). Tecidos distintos exibem proporções diferentes de receptores α1, α2, β1, β2, entre outros (Brodde et al., 2001; Lu H et al., 2014). No entanto, dentre os diversos subgrupos de adrenoreceptores, destaca-se os receptores α1-adrenérgico (α1-AR) e β2 adrenérgico (β2-AR), que são geralmente considerados como integrantes na comunicação cérebro-imunitária (Siniscalchi et al., 2010; Marvar et al., 2011).

Evidências demonstram que o SNS regula de forma positiva e negativa a formação e reabsorção óssea, respectivamente, através da sinalização de β2-AR (Ducy et al., 2000; Togari, 2002; Takeda et al., 2002; Togari et al., 2005; Takeuchi et al., 2001; Harada, Rodan, 2003; Elefteriou et al., 2005).

No entanto, apesar da literatura apontar uma íntima relação entre o estresse crônico e a exacerbação da resposta inflamatória mediada por receptores adrenérgicos, pouco se sabe sobre os efeitos do estresse e o consequente estímulo do SNS na progressão da periodontite apical.

Os β-bloqueadores adrenérgicos, constituem uma classe terapêutica que apresentam como mecanismo de ação o bloqueio dos receptores β-adrenérgicos.

Esse tipo de medicação tem sido indicada formalmente como coadjuvante no tratamento de pacientes hipertensos com cardiopatia associadas, uma vez que os receptores adrenérgicos são mais conhecidos pelo seu papel na regulação das funções do músculo liso cardiovascular (ESH/ESC Task Force for the Management of Arterial Hypertension, 2013). No entanto, após a demonstração histológica de fibras de norepinefrina no osso, estudos farmacológicos mostraram que ambas as células osteoblásticas e osteoclásticas possuem receptores para norepinefrina. Desde que os β2-AR foram identificados em células osteoblásticas e osteoclásticas humanas (Moore et al., 1993; Takeda et al., 2002), levantou-se a hipótese de uma regulação simpática do metabolismo ósseo (Togari, 2002). Além disso, Fonseca et al. (2011) sugeriram que os β2-AR não são os únicos adrenoreceptores envolvidos na regulação da remodelação óssea e mostraram que a sinalização pelos receptores α2-adrenérgico também pode mediar as ações do SNS.

A fentolamina representa um bloqueador α-adrenérgico utilizado em diversos estudos (Fonseca et al., 2011; Lu H et al., 2014) e que possui como principal mecanismo de ação mecanismo de ação a inibição da capacidade das catecolaminas, como norepinefrina e epinefrina, em estimular a contração vascular (Grover et al., 2015).

Diante do exposto, fundamenta-se a hipótese não somente da modulação da resposta inflamatória na patogênese da periodontite apical via SNS como também a modulação da sinalização desse sistema através da administração de bloqueadores adrenérgicos.

Portanto, a presente pesquisa foi realizada a fim de avaliar os efeitos do estresse crônico no desenvolvimento da periodontite apical além de verificar a ação de bloqueadores α e β adrenérgicos na modulação da resposta inflamatória uma vez que não existe até o momento nenhum trabalho na literatura relacionado tais efeitos na área da Endodontia.

2 ARTIGOS

2.1 Artigo – Khoury RD, Matos FS, Prado RF, Carvalho CAT, Valera MC. Efeito do estresse crônico na progressão da lesão periapical / *Effect of chronic stress in the progression of periapical lesion**

RESUMO

O objetivo deste estudo foi verificar o possível efeito do estresse crônico no desenvolvimento e progressão da lesão e avaliar a modulação da resposta inflamatória através da administração de bloqueadores adrenérgicos. Trinta e dois ratos Wistar foram submetidos à modelo animal de lesão periapical através da exposição da cavidade pulpar e em seguida foram aleatoriamente divididos em 4 grupos: sem estresse (NS); estresse + solução salina (SS); estresse + β-bloqueador (Sβ); estresse + α-bloqueador (Sα). Os grupos SS, Sβ e Sα foram submetidos à modelo animal de estresse crônico durante 28 dias e receberam injeções diárias de solução salina, propranolol (β bloqueador adrenérgico) e fentolamina (α bloqueador adrenérgico), respectivamente. Após 28 dias os animais foram eutanasiados e procedeu-se as seguintes análises: a) dos níveis séricos de corticosterona através de Radioimunoensaio; b) histomorfométrica por coloração com hematoxilina e eosina; c) da estrutura óssea periapical através de microtomografia computadorizada (micro-CT). Os resultados obtidos mostram um aumento do nível sérico de corticosterona dos animais do grupo SS sendo estatisticamente significante comparados aos animais do grupo NS (sem estresse) ($p<.05$). Nenhuma diferença estatística foi observada a nível histológico uma vez que todos os animais apresentaram infiltrado inflamatório moderado. A análise por micro-CT mostrou similaridade no volume da lesão periapical em todos grupos. Conclui-se que não houve influência significativa do estresse crônico no desenvolvimento e progressão da lesão periapical e a administração de bloqueadores adrenérgicos não foi capaz de modular a resposta inflamatória.

Palavras-chave: Estresse crônico. Periodontite apical. Propranolol. Fentolamina.

ABSTRACT

The objective of the present study was to verify the possible effect of chronic stress (CS) in the development and progression of periapical lesion and to evaluate the modulation of the inflammatory response through administration of adrenergic blockers. Thirty-two Wistar rats were submitted to animal model of periapical lesion through exposure of the pulp cavity and were then randomly divided into 4 groups: no stress (NS); stress + saline solution (SS); stress + β-blocker (Sβ); stress + α-blocker (Sα). The SS, Sβ and Sα groups were submitted to animal model of CS for 28 days and received daily injections of saline solution, propranolol (β blocker adrenergic) and

phentolamine (α blocker adrenergic), respectively. After 28 days the animals were euthanized and the following analyzes were carried out: a) serum corticosterone levels through Radioimmunoassay; b) histomorphometric by staining with hematoxylin and eosin; c) of the periapical bone structure through micro computed tomography. The results obtained show an increase in the serum corticosterone level of the animals of the SS group being statistically significant compared to the NS group animals (without stress) (p<.05). No statistical difference was observed histologically since all the animals presented moderate inflammatory infiltrate. Micro-CT analysis showed similarity in periapical lesion volume in all groups. It was concluded that there was no significant influence of CS on the development and progression of the periapical lesion and the administration of adrenergic blockers was not able to modulate the inflammatory response.

Keywords: Chronic stress. Apical periodontitis. Propranolol. Phentolamine.

INTRODUCTION

Apical periodontitis (AP) results from an endodontic infection causing an inflammation and consequent necrosis of dental pulp (1). Pathogenic microorganisms disseminate through root canal systems, increasing the local levels of inflammatory cells and osteoclasts activity, leading to destruction of periapical bone tissue (2). Besides that, the process of bone resorption is also regulated by several other factor such as systemic conditions and host inflammatory response (3).

In recent years, increasing evidence has emerged from epidemiologic studies associating oral infections to stress, depression and anxiety (4, 5). Chronic stress (CS) is an organic response, which involves the Sympathetic Nervous System (SNS) activation the hypothalamic-pituitary-adrenal (HPA) axis that results in the release of neuroendocrine hormones (cortisol and corticosterone) and catecholamine neurotransmitters (epinephrine and norepinephrine) (6). The synthesis of these molecules is catalyzed by the enzyme tyrosine hydroxylase and the resultant adrenaline binds to adrenergic receptors present in several cells throughout the body causing a greater intracellular signal transduction (7).

Adrenergic receptors (AR) are subdivided into two major groups: α and β (8). However, among the several subgroups of adrenoreceptors, the α1- adrenergic (α1-AR) and β2-adrenergic (β2-AR) receptors are prominent, which are generally considered as integrants in brain-immune communication (9). Previous studies have

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demonstrated that bone remodeling is under control of the SNS through the signaling of β2-AR (10). Since β2-adrenergic receptors have been identified in human osteoblastic and osteoclast cells (11, 12), the hypothesis of sympathetic regulation of bone metabolism has been raised (13).

Many therapies have been studied aiming to treat and prevent the conditions resulting from CS through the blockade of β-adrenergic receptor. Propranolol is a β-adrenergic blocker and its effects on bone have been highlighted (14). In addition, it is demonstrated that β2-AR is not the single adrenoceptor involved in bone remodeling regulation, suggesting that α-AR signaling also mediates the SNS actions (15, 16). Phentolamine, a non-selective α-adrenergic blocker, have showed as a potential inhibitor of sympathetic catecholamine release caused by CS (15).

Therefore, for the first time, the present study aimed to evaluate the effect of CS in the progression of periapical lesions and if propranolol or phentolamine as adrenergic blockers would modulate the inflammatory response.

METHODS

Animals

Animal care and study protocols were approved by the Institute of Science and Technology of São José dos Campos (UNESP) Ethics Committee (CEUA - nº 01/2016). Experiments were conducted in accordance with the relevant guidelines.

Sample size estimates were calculated based on the data of a study by Lu et al. (16). Considering a significant level of 0.05 and a beta error of 0.20, a minimum number of 6 animals per group was considered necessary. Thus, it was established a number of 8 animals per group to perform our research.

Thirty-two male Wistar rats (Botucatu, SP, Brazil) with an average initial weight of ~180g were selected. The animals were acclimatized to the housing conditions over the following 4 weeks at 20–24 °C and 50–70% relative humidity with a 12-h light/dark cycle. Food and water were available ad libitum, except for those times when the stressors were applied. The animals were assigned randomly to four experimental groups after weight stratification as follows: no stress group (NS),

stress + saline solution group (SS), stress + propranolol group (Sβ), stress + phentolamine group (Sα). In all groups was induced an animal model of periapical lesion.

Periapical Lesion Induction

Periapical lesion was induced in each rat by occlusal exposure of the pulp on left mandibular first molars as described previously (17). Animals were anesthetized with ketamine (75 mg/kg, Dopalen; Ceva Saúde Animal Ltda, São Paulo, Brazil) and xylazine (50 mg/kg, Anasedan; Ceva Saúde Animal Ltda, São Paulo, Brazil) by intramuscular injection. The crowns of the mandibular first molars were drilled using a steel round bur (#1/4) and remained exposed to the oral cavity for lesion induction. These procedures were performed 4 days before CS induction.

Chronic Stress induction

CS was applied daily to the SS, Sβ and Sα rats from the 4th day after the induction of periapical lesion according to Willner et al. (18) with slight modifications. Each week of the 28-day CS regimen consisted of the following stresses: two periods of food or of water deprivation (overnight); two periods of 45° cage tilt (overnight); two periods of cage soiling (250ml water in sawdust bedding) (overnight); two periods of stroboscopic light (from 8:00 to 10:00 am or from 16:00 to 18:00 pm); two periods of 4 °C or 45 °C swimming (3 min); two periods of tail clamping (1min); and two periods of no stress. All of these stresses were applied randomly. This model has been shown to induce CS, as demonstrated by neuroendocrine activation, weight loss and anxiety-like behaviors (19). The treatments were administered daily as follows: SS – subcutaneous/intraperitoneal injection of saline solution; Sβ – subcutaneous injection of propranolol (Propranolol hydrochloride – Sigma, St. Louis MO, EUA) diluted in saline solution (0,1mg/Kg/day); and Sα – intraperitoneal injection of phentolamine (Phentolamine hydrochloride - Sigma, St. Louis MO, EUA) diluted in saline solution (0,1mg/kg/day).

Sample Collection

Animals were anesthetized with ketamine (75 mg/kg, Vetaset; Fort Dodge Animal Health Ltd, São Paulo, Brazil) and xylazine (50 mg/kg, Coopazine; Coopers

Ltd Brazil, São Paulo, Brazil) by intramuscular injection and killed after 32 days periapical lesion induction. The levels of corticosterone were determined by collecting blood samples from via inferior vena cava. The blood samples were centrifuged in heparin-containing tubes (MP Biomedicals: LLC) and the plasma was frozen at −20°C until the assay was carried out. The technique used for the analysis was radioimmunoassay (RIA). The mandibles were dissected out and the samples were fixed with 4% paraformaldehyde at 4 °C for 48 h. Then, they were then transferred into sealed containers filled with 0.5% paraformaldehyde for microcomputed tomography (micro-CT) scanning. After micro-CT scanning, the mandibles were decalcified with 10% EDTA solution for 2 months, rinsed, dehydrated and then embedded in paraffin. Semi-serial sections (4 µm thick) were prepared in the laterolateral direction, allowing sectioning of the mandibular first molar in its longitudinal axis. Sections were submitted to histomorphometric analysis as described below.

Histomorphometric Analysis

Whole slide images were obtained with Pannoramic Scan (3DHISTECH, Budapest, Hungary). Analysis were performed by a single calibrated operator who was blind to the specimen group affiliation using Pannoramic Viewer 1.14.50 (3DHISTECH). The inflammatory infiltrate was evaluated for intensity and extension. The average number of cells per field and the extension that went beyond the apical foramen were considered. For each experimental group, the number of cells was calculated as the average of 10 separate areas (x40 magnification).

The intensity of the inflammatory infiltrate was graded as absent (0 to 25 inflammatory cells: score 1), mild (25 to 50 cells: score 2), moderate 50–75 cells: score 3), and severe (>75 cells: score 4).

The extension of the inflammatory infiltrate was graded as absent (0 to few inflammatory cells: score 1), mild (cells occupying up to a 300-mm length: score 2), moderate (cells occupying up to a 600-mm length: score 3), and severe (cells occupying >a 600-mm length: score 4) (20).

For histometric analysis of the size of the periapical lesion, the periapical lesion area associated with the apical third of mesial root of mandibular first molar was measured by using Leica Microsystems Software (Leica, Wetzlar, Germany).

The area of the periapical lesion was calculated by rounding up the lesion boundary, considering outer external surface of the cementum, periodontal ligament, and the outer surface of the alveolar bone, and it was expressed in squared millimeters. The measurement was conducted in 3 equidistant sections to the root canal, and just the largest area was selected.

Micro-CT Scanning

After fixation, 08 hemimandibles of each experimental group were prepared for micro-CT scanning (SkyScan 1176 in vivo, Skyscan, Kontich, Belgium). The scanning was performed at 80 kV, 300 mA, with an increment of 17.4 mm, 0.5 mm aluminium filter and 300-ms integration time. Micro-CT images were acquired in the sagittal plane over the entire length of the mandible. The CTAnv.1.11.8 program (Skyscan) was used to select the micro-CT images that showed both the root canal and apical foramen of the mesial root to measure the periapical lesion volume. The images in the sagittal plane with standard orientation were exported to CTAn software (Skyscan, Kontich, Belgium), and the region of interest (ROI) was selected using personalized ROI tool involving the periapical lesion of the mesial root (Fig.1). Then, the tissue volume (TV), alveolar bone volume (BV) and BV/TV ratio were measured using CTan software (Skyscan, Kontich, Belgium). All measurements were performed by one trained examiner who had no previous identification information about the groups assessed, and the measurements were carried out twice at different times.

Statistical Analysis

Data obtained in parametric tests were registered and presented as the means observed in each group. Statistical differences between groups were stated by analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. The histologic analysis scores were analyzed using the Kruskal-Wallis test ($p<.05$). All statistical tests were performed using the software GraphPad Prism 5 (GraphPad Software Inc., California, USA).

RESULTS

Body Weight and Corticosterone Levels

All animals survived the whole experimental period. At the beginning of the experiment, the groups had a similar mean body weight (p>.05). In all groups, the body weights increased during the experimental period. There was a significant difference in body weights between NS and SS group after periapical lesion and CS induction indicating a less gain of weight in the stressed group (p<.05). However, no differences were observed regarding the body weights of Sβ and Sα. Corticosterone levels examined on the last day of experiment revealed an increased corticosterone level in the SS group being statistically significant different from the NS and Sα groups. However, no statistical difference was found between Sβ and all other groups (Table 1).

Histomorphometric Analysis

Evidence of inflammation was observed in all groups (Fig. 2 A–H). Periapical lesions were mostly restricted to the periapical region, and showed characteristics of periapical granuloma. All groups presented established periapical lesion composed predominantly of a chronic inflammatory infiltrate graded as moderate. Therefore, CS did not produce significant inflammatory changes during the experimental period. No statistical difference was observed regarding the extent of inflammatory infiltrate and histometrically, all groups exhibited similar periapical lesions area (Table 2).

Micro-CT Analysis

No statistical difference was observed on TV and BV among the experimental groups (Table 3). CS did not cause any change in periapical lesion size in the stressed animals. This was confirmed by detailed micro-CT analysis of BV/TV ratio of the periapical area surrounding the mesial root of the first molars, which showed that all animals presented similar periapical lesion size including the non-stressed group (Fig. 3).

DISCUSSION

Apical periodontitis (AP) is an inflammatory disorder characterized by a host response as a consequence of bacterial infection and pulp necrosis resulting in periapical bone resorption (21). The process of inducing AP by exposure of the pulp is widely used in animal studies (22, 23). These conditions are favorable for the occurrence of bacterial infection and consequent pulp necrosis, which induces an inflammatory response, thereby developing a periapical lesion. The reliability of the AP induction method was confirmed histologically in the present study by pulp images that showed total necrosis and periapical lesions.

Different animal models have been developed in order to investigate the relationship between CS and periodontal disease (16, 24). However, no study was conducted to evaluate the influence of CS on periapical lesion. Thus, the animal model of CS performed in this study was conducted according to Willner et al. (18) with minor modifications. The experimental procedures was conducted over a 32-day period because this amount of time is sufficient to observe the development of periapical lesions and complete pulpal necrosis (30–32) and induce established CS (16). Our results confirmed that periapical lesions have developed in the mandibular molar after infection, and CS was effectively induced confirmed by the increased corticosterone levels in SS control group.

All animals subjected to this study presented similar body weights. In the end of experimental procedures, all groups showed an increase in their body weight during the experimental period. However, the stressed animals gained less weight during medication intake, which was statistically significant compared with non-stressed rats, similarly to the results of previous studies (25).

In the present study, the data obtained from RIA suggested a greater role for the SNS in regulating stress-induced changes contributing to higher corticosterone levels in chronic stressed animals. Animals exposed to CS receiving placebo injections of saline solution (SS group) showed higher corticosterone level when compared to the NS group, suggesting that the animal model of CS was effectively induced in this study. These data are in accordance with previous findings (26),

which associated corticosterone responses to CS demonstrating enhanced SNS activation.

Histologic examination revealed inflammatory infiltrate in all groups. Therefore, the induction of CS did not induce a greater inflammation of periapical tissues during the study period (32 days). In addition, the intensity and extent of the inflammatory infiltrate presented similar scores in all different groups. Up to now, there is no study that have evaluated endodontic infection associated with CS. According to our results, there was no significantly increased infection-stimulated bone resorption compared to non-stressed animals. These findings suggest that CS, as induced by the present study, has no influence over the development and progression of periapical lesion.

However, it must be considered the different types of animal models of stress described in the scientific literature which may vary according to some characteristics that refer to the nature (psychological, chemical or physical), intensity (acute or chronic) and duration of stress (16, 24). According to some authors, the physiological effects on organism caused by stress may vary according to the type of stress applied, leading to different reponses regarding the hormonal release and immune functions. This reinforces the complexity of this physiological phenomenon on the organism and its possible influence in the development of different diseases (27, 28). Although several studies have shown an association between psychological factors and periodontal disease, the mechanisms involving this association to apical periodontitis remains unclear.

One relevant factor to consider in this field is the accuracy diagnosis of periapical lesions size. In our study, in addition to conventional histologic analysis, the size of periapical lesion was also measured by means of micro-CT analysis. In a recent study by Kalatzis-Sousa et al. (29), the authors address the failures of defined ROIs (i.e. rectangle, polygonal or round) since they include structures that are not part of the periapical lesion such as periodontal ligament, root pulp and medullary region. Therefore, in the present study the personalized ROI was selected to measure the periapical lesion since it allows to manual delineate the periapical lesion providing a more accurate size of bone resorption.

Based on our results, the comparison of volume and area between the groups were not similarly augmented after lesion induction. This can be explained by

a weak correlation between the histomorphometric measurement and the 3D micro-CT, as demonstrated by de Oliveira et al. (30). The histological sections are two-dimensional projections of three-dimensional structures and thus this technique usually do not provide an adequate representation of the region of interest, producing a lack of quantification accuracy (31). However, it is important to emphasize that histologic staining can not be completely replaced by micro-CT analysis, remaining an essential pathologic tool to provide valuable information of cell types and tissue morphology, allowing to count the inflammatory infiltrate in periapical lesions (23).

Some authors have demonstrated the presence of β-adrenergic receptors in osteoblastic and osteoclastic human cells suggesting the possible regulation of bone remodeling by the sympathetic nervous system (11–13). In our research, propranolol was selected as the β-adrenergic blocker medication since it has been widely used in previous studies aiming to inhibit the adrenergic signaling pathway in order to avoid the bone resorption (14). In addition, a study by Fonseca et al.(32) suggested that the α-adrenergic receptors also mediates the sympathetic nervous system and thus phentolamine was used since its capacity to inhibit cathecolamines activity has been shown in previous study (15, 16). Interestingly, phentolamine prevented the greater corticosterone response in chronic stressed animals of Sα group, showing similar results to the non-stressed group (NS), which statistically differed from the SS control group. This data indicates that SNS activation may be mediated by α- adrenergic blockers and thus should be more closely examined.

Some studies have demonstrated that CS was found to accelerate periodontal disease development and progression (4, 5). In contrast, an experimental animal model study conducted by Soletti et al. (33), and some clinical studies (34, 35) failed to demonstrate the connection between periodontal disease and depression. According to some authors, there is a lack of an adequate animal models including difficulty to quantify the amount and duration of stress, and a disregard for relevant common mediators, which turns uncertain the direct association between periodontal disease and stress (27). Our study did not found a positive correlation between CS and apical periodontitis.

The exposed findings are very important because in contrast to several studies on periodontal disease linked to CS, our results suggested that the influence of these factors on apical periodontitis remains to be proven, since the development

and progression of AP was not influenced by the sympathetic nervous system activation induced by CS. Nonetheless, further investigations are needed, not only to prove the effective influence of CS on AP, but also to elucidate the mechanisms of possible association between psychological factors and periodontal disease, which is widely published in the periodontal literature. Likewise, more studies including different experimental models of chronic stress taking into account the type, intensity and time of chronic stress should be considered.

According to the present study, it can be concluded that CS, as induced in the present research, although it increased the corticosterone levels and caused a reduced body weight gain, it did not affect the progression of apical periodontitis. However, the results and observations described above should not be directly compared to humans. Moreover, although periodontal disease associates with CS, corresponding links regarding apical periodontitis (AP) remains to be clarified. Nonetheless, the present study may be considered the first step to obtain evidence in order to study patients who suffers from concurrent CS.

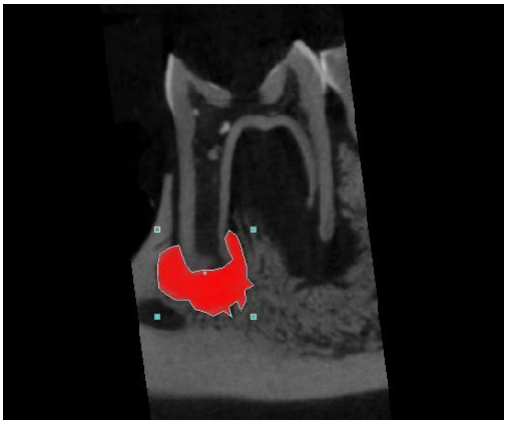

Figure 1. *Image of the original periapical lesion in the left mandibular first molar of a specimen. Illustration of the use of personalized ROI involving the periapical lesion of the mesial root*.

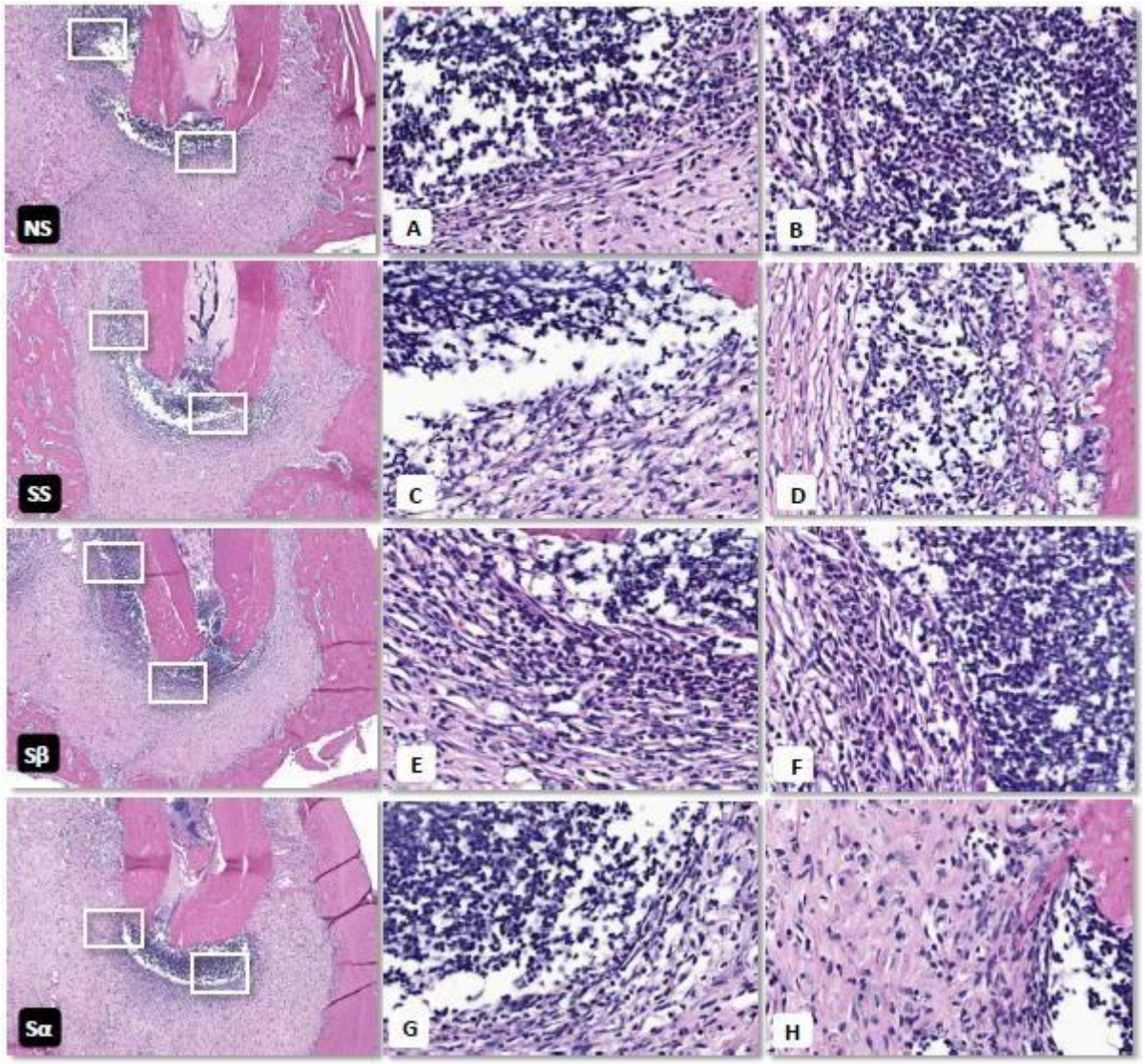

Figure 2. *Histologic aspects of periapical lesions after 32 days of after pulp exposure to the oral environment. Moderate chronic inflammatory cell infiltration in NS (A and B), SS (C and D), Sβ (E and F) and Sα (G and H). Hematoxylin-eosin staining, x40 in Pannoramic Viewer Software*

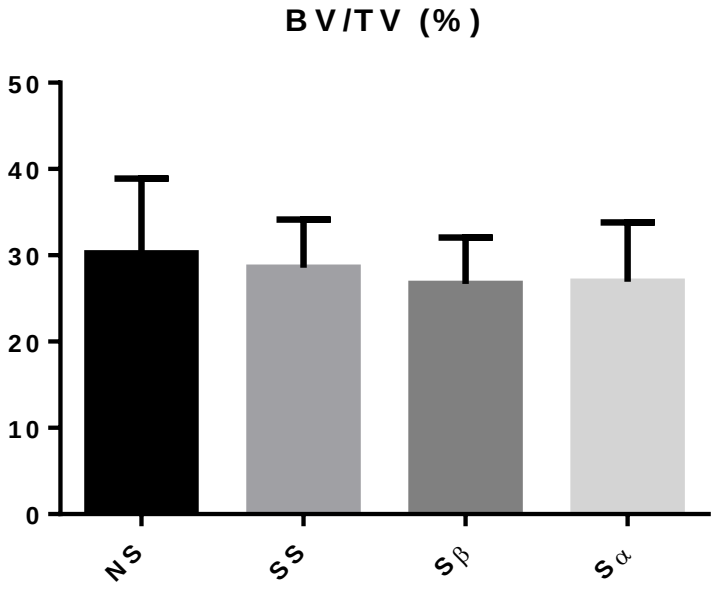

Figure 3. *Graph bar showing Bone Volume/Tissue Volume proportion measured by micro-CT analysis*

TABLE 1. *Weight (g) and Corticosterone Levels (ng/dL) in animals from all Groups (Mean and Standard Deviation [SD*])*

Groups	***Weight (g) ± SD****		***Corticosterone Levels (ng/mL) ± SD***
	Day 0	Day 32	
NS	*281.7 ± 21.8^{a}*	*333.9 ± 47.7^{a}*	*302.5 ± 142.6^{b}*
SS	*270.6 ± 9.3^{a}*	*288.3 ± 24.7^{b}*	*568 ± 133.6^{a}*
Sβ	*281 ±15.3^{a}*	*311.9 ± 17.9^{ab}*	*461.1 ±158.5^{ab}*
Sα	*274.1 ± 12.5^{a}*	*307.8 ± 20.5^{ab}*	*277.5 ± 117.4^{b}*

NS, no stress; SS, stress + saline solution; Sβ, stress + propranolol; Sα, stress + phentolamine.
**Different letters in the columns indicate statistical differences, P < .05.*

TABLE 2. *Scores, Median, Mean, and Standard Deviation (SD) of Histologic Findings in the Rats from All Groups*

Histologic Parameters	***Scores***	***Groups***			
		NS	***SS***	***Sβ***	***Sα***
Inflammatory Infiltrate	*1*	*0/8*	*1/8*	*0/8*	*0/8*
	2	*1/8*	*2/8*	*2/8*	*3/8*
	3	*7/8*	*5/8*	*5/8*	*4/8*
	4	*0/8*	*0/8*	*1/8*	*1/8*
	*Median**	*3[a]*	*3[a]*	*3[a]*	*3[a]*
Inflammation extent	*1*	*1/8*	*0/8*	*1/8*	*2/8*
	2	*6/8*	*6/8*	*7/8*	*4/8*
	3	*1/8*	*2/8*	*0/8*	*2/8*
	4	*0/8*	*0/8*	*0/8*	*0/8*
	*Median**	*2[a]*	*2[a]*	*2 [a]*	*2[a]*
Periapical Lesion Area ($mm^{2)}$	*Mean ± SD*	*1.56 ± 0.38[a]*	*1.45 ± 0.38[a]*	*1.56 ± 0.46[a]*	*1.55 ± 0.39[a]*

NS, no stress; SS, stress + saline solution; Sβ, stress + propranolol; Sα, stress + phentolamine.
**Different letters in the columns indicate statistical differences, P < .05.*

TABLE 3. *Tissue Volume, Bone Volume, Resorption Volumes and Areas of*

Groups		Tissue Volume (mm^3)	Bone Volume (mm^3)	BV/TV (%)	Periapical Lesion Volume (mm^3)
NS	**Mean ± SD**	3.40 ± 0.69[a]	1.01 ± 0.35 [a]	30.21 ± 8.67 [a]	2.33 [a]
SS		3.04 ± 0.42 [a]	0.85 ± 0.11 [a]	28.55 ± 5.57 [a]	2.12 [a]
Sβ		3.67 ± 0.62 [a]	0.96 ± 0.14 [a]	26.70 ± 5.34 [a]	2.38 [a]
Sα		3.40 ± 0.79 [a]	0.87 ± 0.09 [a]	26.85 ± 6.87 [a]	2.61 [a]

Periapical Lesions (Mean and Standard Deviation [SD])*

NS, no stress; SS, stress + saline solution; Sβ, stress + propranolol; Sα, stress + phentolamine.
**Different letters in the columns indicate statistical differences, P < .05.*

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2.2 Artigo – Khoury RD, Matos FS, Prado RF, Carvalho CAT, Valera MC. Expressão de receptores adrenérgicos e osteoclastos em lesões periapicais sob condições de estresse crônico: um estudo *in vivo* / *Expression of adrenergic receptors and osteoclasts in periapical lesions under chronic stress conditions: an in vivo study**

RESUMO

O objetivo do presente estudo foi investigar se a periodontite apical (AP) associada ao estresse crônico (CS) influenciou a expressão de receptores β e α adrenérgicos (AR) e se os bloqueadores adrenérgicos são eficazes na prevenção da reabsorção óssea periapical em ratos. Trinta e dois ratos Wistar foram divididos em 4 grupos: sem estresse (NS); Estresse + solução salina (SS); Etresse + β-bloqueador adrenérgico (Sβ); Estresse + α-bloqueador adrenérgico (Sα). A AP foi induzida em todos os grupos, expondo o tecido pulpar ao ambiente oral. Os grupos SS, Sβ e Sα foram submetidos ao modelo animal de CS por 28 dias e receberam injeções diárias de solução salina, propranolol (β bloqueador adrenérgico) e fentolamina (α bloqueador adrenérgico), respectivamente. Os animais foram sacrificados após 32 dias e foram realizadas as seguintes análises: a) peso corporal e níveis séricos de corticosterona através do radioimunoensaio; b) microcotomografia computadorizada (micro-CT); c) imunohistoquímica; c) histoquímica enzimática. Os resultados obtidos mostram um aumento no nível sérico de corticosterona dos animais do grupo SS sendo estatisticamente significativo em comparação com os animais do grupo NS (não estressado). A análise de micro-CT mostrou similaridade na área de lesão periapical em todos os grupos. Não houve diferença estatística entre os grupos quanto à expressão de β-AR e α-AR. O número de células TRAP positivas foi significativamente menor nos grupos que receberam injeções diárias de bloqueadores adrenérgicos. Concluiu-se que não houve influência significativa do CS no desenvolvimento e progressão da lesão periapical e a administração de bloqueadores adrenérgicos foi eficaz na redução do número de osteoclastos na região periapical.

Palavras-chave: Estresse crônico. Periodontite apical. Bloqueadores adrenérgicos. Propranolol. Fentolamina.

ABSTRACT

The aim of the present study was to investigate if apical periodontitis (AP) associated with chronic stress (CS) influenced the expression of β and α adrenergic receptors (AR) and if adrenergic blockers are effective in preventing periapical bone resorption in rats. Thirty-two Wistar male rats were divided into 4 groups: no stress (NS); stress + saline solution (SS); stress + β-blocker (Sβ); stress + α-blocker (Sα). AP was induced in all groups by exposing the pulpal tissue to the oral environment. The SS, Sβ and Sα groups were submitted to animal model of CS for 28 days and received daily injections of saline solution, propranolol (β blocker adrenergic) and

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phentolamine (α blocker adrenergic), respectively. The animals were euthanized after 32 days and the following analyzes were carried out: a) body weight and serum corticosterone levels through Radioimmunoassay; b) micro computed tomography c) immunohistochemistry; c) enzyme histochemistry. The results obtained show an increase in the serum corticosterone level of the animals of the SS group being statistically significant compared to the NS group animals (non-stressed). Micro-CT analysis showed similarity in periapical lesion area in all groups. No statistical difference was observed between the groups regarding the expression of β-AR and α-AR. The number of TRAP-positive cells was significantly lower in the groups which received daily injections of adrenergic blockers. It was concluded that there was no significant influence of CS on the development and progression of the periapical lesion and the administration of adrenergic blockers was effective in reducing the number of osteoclasts in periapical region.

Keywords: Chronic stress. Apical periodontitis. Adrenergic blockers. Propranolol. Phentolamine.

INTRODUCTION

Apical periodontitis (AP) is an inflammatory disease caused by an endodontic bacterial infection and its development and persistence is characterized by periapical bone resorption regulated by the host immune/inflammatory response (1).

It is known that chronic periodontal disease (PD) and AP share some similarities regarding their origin and microbiota composition, both are chronic infections from oral cavity with prevalence of Gram-negative anaerobic bacteria (2). In addition, acute and chronic manifestations of both conditions have been shown to elevate the concentration of inflammatory mediators (3). Likewise, one might deduce that AP is associated with the same systemic disorders that are linked to PD (4).

The scientific literature has shown reliable evidence for an association between chronic stress (CS) and PD (5, 6). Accordingly, several studies have demonstrated the role of stress hormones in the immune host response (7), which explains the detrimental effects of CS on development of PD.

In general, CS enhances the release of neuroendocrine hormones (e.g., corticosterone) and catecholamine neurotransmitters (i.e., epinephrine and norepinephrine) (8). Tyrosine hydroxylase (TH) is the enzyme responsible for catalyzing the synthesis of these molecules and the resultant adrenaline binds to

adrenergic receptors (AR) expressed in different types of cells leading to an increased intracellular signal transduction (9). Since, β2-AR were identified in human osteoblastic and osteoclastic cells (10), one might assume that bone metabolism is regulated by sympathetic modulation (11). Moreover, previous studies have suggested that α1-AR may also mediates the inflammatory response through regulation of sympathetic neurotransmissions (12).

Therefore, an intimate relationship between CS and exacerbation of the inflammatory response mediated by AR is verified in the scientific literature. However, the relationship between CS and AP has never been investigated. Therefore, ascertaining whether the presence of isolated AP or AP under CS conditions enhances the expression of biological markers associated with adrenergic signaling pathway, such as β2-AR and α1-AR, would be of great interest, both from a scientific point of view and from a public health perspective.

This understanding will also aid in the development of new therapies aiming to modulate inflammatory responses through the blockade of adrenergic receptors. For this reason, the aim of the present study was to investigate the expression of adrenergic receptors on bone under chronic stress conditions and evaluate the effectiveness of adrenergic blockers using propranolol (β-adrenergic blocker) and phentolamine (α-adrenergic blocker) as systemic medications to treat AP in a CS status.

METHODS

Animals

Animal care and study protocols were approved by the Institute of Science and Technology of São José dos Campos (UNESP) Ethics Committee (CEUA - nº 01/2016).

A minimum number of 6 animals per group was considered necessary, considering a significant level of n alpha error of 0.05 and a beta error of 0.20 The sample size was estimated based on the data of a study by Lu et al. (13). Thus, it was established a number of 8 animals per group to perform our research.

A total of thirty two male Wistar rats (Botucatu, SP, Brazil) with an average initial weight of ~ 180g were selected. The animals were acclimatized to the housing conditions over the following 4 weeks at 20–24 °C and 50–70% relative humidity with a 12-h light/dark cycle. Food and water were available ad libitum, except for those times when the chronic stress was applied. The animals were assigned randomly to four experimental groups after weight stratification as follows: no stress group (NS), stress + saline solution group (SS), stress + propranolol group (Sβ), stress + phentolamine group (Sα). In all groups were induced an animal model of periapical lesion.

Animal Model of Periapical Lesion

Periapical lesion was induced in each rat by pulp chamber exposure of the left mandibular first molars as described previously (14). The animals were anesthetized with ketamine (75 mg/kg, Vetaset; Fort Dodge Animal Health Ltd, São Paulo, Brazil) and xylazine (50 mg/kg, Coopazine; Coopers Ltd Brazil, São Paulo, Brazil) by intramuscular injection), the crowns of the mandibular first molars were drilled using a steel round bur (#1/4) and remained exposed to the oral cavity for periapical lesion development.

Animal Model of Chronic Stress

After 4 days of periapical lesion induction procedure the chronic stress protocol was initiated. The stressors were applied daily to the SS, Sβ and Sα animals according to Willner (15) with slight modifications. Each week of the 28-day CS regimen consisted of the following stresses: two periods of food or of water deprivation (overnight); two periods of 45° cage tilt (overnight); two periods of cage soiling (250ml water in sawdust bedding) (overnight); two periods of stroboscopic light (from 8:00 to 10:00 am or from 16:00 to 18:00 pm); two periods of 4 °C or 45 °C swimming (3 min); two periods of tail clamping (1min); and two periods of no stress. All of these stresses were applied randomly. This model has been shown to induce chronic stress, as demonstrated by neuroendocrine activation, weight loss and anxiety-like behaviors (16).

The treatments were administered daily as follows: SS –

subcutaneous/intraperitoneal injection of saline solution; Sβ – subcutaneous injection of propranolol (β-adrenergic blocker) (Propranolol hydrochloride – Sigma, St. Louis MO, EUA) diluted in saline solution (0,1mg/Kg/day); and Sα – intraperitoneal injection of phentolamine (α-adrenergic blocker) (Phentolamine hydrochloride - Sigma, St. Louis MO, EUA) diluted in saline solution (0,1mg/kg/day).

Sample Collection

Animals were anesthetized with ketamine (75 mg/kg, Vetaset; Fort Dodge Animal Health Ltd, São Paulo, Brazil) and xylazine (50 mg/kg, Coopazine; Coopers Ltd Brazil, São Paulo, Brazil) by intramuscular injection and killed after 32 days periapical lesion induction. Right before euthanasia, blood samples were collected from via inferior vena cava in order to determine the levels of corticosterone. The blood samples were centrifuged in heparin-containing tubes (MP Biomedicals: LLC) and the plasma was frozen at −20°C until the assay was carried out. The technique used for the analysis was radioimmunoassay (RIA).

The mandibles were dissected out and the samples were fixed with 4% paraformaldehyde at 4 °C for 48 h. Then, they were then transferred into sealed containers filled with 0.5% paraformaldehyde for microcomputed tomography (micro-CT) scanning. After micro-CT scanning, the mandibles were decalcified with 10% EDTA solution for 2 months, rinsed, dehydrated and then embedded in paraffin. Semi-serial sections (4 µm thick) were prepared in the laterolateral direction, allowing sectioning of the mandibular first molar in its longitudinal axis. Sections were submitted to enzyme histochemistry and immunohistochemistry analysis as described below.

Micro-CT Scanning

After fixation, 08 hemimandibles of each experimental group were prepared for micro-CT scanning (SkyScan 1176 in vivo, Skyscan, Kontich, Belgium). The scanning was performed at 80 kV, 300 mA, with an increment of 17.4 mm, 0.5 mm aluminium filter and 300-ms integration time. Micro-CT images were acquired in the sagittal plane over the entire length of the mandible. The CTAnv.1.11.8 program (Skyscan) was used to select the micro-CT images that showed both the root canal and apical foramen of the mesial root to measure the periapical lesion volume. The

images in the sagittal plane with standard orientation were exported to CTAn software (Skyscan, Kontich, Belgium), and the region of interest was selected using personalized ROI involving the periapical lesion of the mesial root. Then, the periapical lesion area (mm^2) was measured using CTAn software (Skyscan, Kontich, Belgium). All measurements were performed by one trained examiner who had no previous identification information about the groups assessed, and the measurements were carried out twice at different times.

Immunohistochemistry

The sections were deparaffinized in xylene for 15 minutes, rehydrated through graded alcohols for 2 minutes, and finally washed in water. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 20 minutes. The sections were then washed in running tap water for 10 minutes and deionized water for 5 minutes twice. Rabbit polyclonal antibodies against β2 adrenergic receptor (ab137494 Abcam Cambridge, MA USA), and α1 adrenergic receptor (ab202936 Abcam Cambridge, MA USA) at dilution of 1:100 each, were used as primary antibodies for 24 hours at 4ºC. The sections were then counterstained with hematoxylin (Figure 1A), mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Immunohistochemical staining was examined with the aid of Leica Microsystems Software (Leica, Wetzlar, Germany). The amount of area stained was calculated in μm^2 in order to quantify the β-AR and αAR expression (Figure 1B).

Enzyme Histochemistry

To identify osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed as previously described (17). Sections of the periapical region were selected and submitted to TRAP activity examination using a TRAP kit (Sigma387A, SigmaAldrich®, St Louis, MO, USA). Briefly, the sections were rehydrated, rinsed, incubated in a solution of naphthol AS-BI phosphoric acid and Fast Garnet GBC for 1 h at 37 °C and then stained with haematoxylin. Sections incubated in substrate-free medium to serve as TRAP activity controls.

For each specimen, the osteoclast amount was analyzed by counting TRAP-positive cells in the periapical tissues; counting was done by a blinded observer using

Pannoramic Viewer 1.14.50 (3DHISTECH). The area (mm^2) and perimeter (mm) of periapical lesion was calculated by contouring the boundary of the periapical lesion with the aid of Leica Microsystems Software.

Statistical Analysis

Data obtained in parametric tests were registered and presented as the means observed in each group. All analysis were analyzed using the Kruskal-Wallis test (P<.05). Statistical differences between groups were stated by analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. Pearson's chi-squared test was performed to investigate the correlation between area measurement by micro-CT and enzyme histochemistry sections. The positive correlations was graded according to the value of the "Correlation Coefficient" (r^2), such as: 1: perfect; .7 to .9: strong; .4 to .6: moderate; .1 to .3: weak; 0: no correlation (18). All statistical tests were performed using the software GraphPad Prism 5 (GraphPad Software Inc., California, USA).

RESULTS

Body Weight and Corticosterone levels

All animals survived the whole experimental period. At the beginning of the experiment, the groups had a similar mean body weight (P > .05). In all groups the body weights increased during the experimental period. There was a significant difference in body weights between NS and SS group after periapical lesion and chronic stress induction indicating a less gain of weight in the stressed group (P < .05). However, no differences were observed regarding the body weights of Sβ and Sα (data not shown).

For corticosterone levels analysis all samples were tested in duplicate and the average value of the two tests was considered for the statistical analysis. The inferior limit of sensitivity was 1.52 ng/ml and the intra assay coefficients of variation were 3.24% and 5.93%, respectively (data not shown). Corticosterone levels examined on the last day of experiment revealed an increased corticosterone level in

the SS group being statistically significant different from the NS and Sα groups. However, no statistical difference was found between Sβ and all other groups (data not shown).

Micro-CT Analysis

No statistical difference was observed between the groups regarding the periapical lesion area measured by Micro CT analysis (Figure 2A). Pearson's chi-squared test showed a weak correlation between the analysis by 2D micro-CT and enzyme histochemistry sections regarding the periapical lesion area (r^2= .269). The Figure 2B presents the correlation graph between these two parameters.

Immunohistochemistry

The expression of β-AR and α-AR is illustrated in Figure 1C and Figure 1D, respectively. No statistical significance was shown between the groups, but comparing the medicated groups a larger area of α-AR was detected in Sβ.

Enzyme Histochemistry

Periapical lesion perimeter in mm is represented in Figure 3A, but no statistical difference was observed between the groups. The number of TRAP-positive cells per millimeter on the periapical lesion perimeter in NS and SS group was significant higher than that observed in Sβ and Sα (Figure 3B). The aspects of TRAP immunohistochemistry are shown in Figure 3C-F.

DISCUSSION

It is well established that sympathetic nervous system (SNS) and the hypothalamic--pituitary--adrenal (HPA) axis responses plays a significant role in immune regulation and for the outcome of infections and inflammatory disorders (19). The excessive activity of the autonomic sympathetic axis causes the release of cathecolamines and glucocorticoids and thus stress can have detrimental effects on

immune functions (20, 21).

Although there is a high prevalence of patients suffering from concurrent chronic stress, there are no indications of its negative influence in periapical lesions. The data obtained in the present study did not show a clear relationship between this health condition and periapical lesion phenotype. The periapical lesion size and adrenergic blockers expressions from periapical lesion were similarly independent of the presence of chronic stress.

Several experimental animal models of stress have been developed to investigate the effect of this chronic condition on the development of periodontal disease (13, 22, 23). Stress status can be considered acute and chronic according to its intensity and time (24). In scientific literature, the experimental models may vary according to some characteristics regarding the nature (psychological, chemical or physical), intensity (acute or chronic) and duration of stressors that may result in different responses (25 –27).

The animal model of periapical lesion through exposure of the pulp chamber is widely used in several studies (28, 29). Such condition favors the occurrence of bacterial infection and consequent pulp necrosis, which causes an inflammatory response, and as a consequence, the development of the periapical lesion occurs in 21 days.

Although there is evidence demonstrating the responses to chronic stress leading to significant immunosuppression (30), it is important to consider that there is a certain divergence in the scientific community regarding the type, duration and intensity of stress (24). In addition, it is demonstrated that effects of different stressors applied successively may interfere in body responses and that chronic stress may eliminate the effects of acute stressors, reinforcing the complexity of this physiological phenomenon on the organism and consequently the development of periapical lesions. Therefore, based on the results obtained in the present study, it is suggested to perform further investigations considering different experimental models of CS taking into account the type, intensity and mainly the time of duration of stress protocol.

In the present study, the animal model of chronic stress was performed based on the protocol of Willner et al. (15) , with some changes. The experimental procedures lasted 32 days, since this period is demonstrated to be enough time to

observe the establishment of both conditions, periapical lesion and chronic stress status. Considering the animal weights, all groups submitted to the study had similar body weight initially and when evaluated at the end of the experiment, all groups showed an increase in weight gain, however, the gain was significantly lower in the stressed groups when compared to non-stressed animals. These results corroborate previous studies (31, 32).

Despite the weight evaluation, the efficacy of chronic stress induction protocol in this study was also confirmed by the seric levels of corticosterone, since non stressed rats from NS group showed significantly lower levels than rats in the SS group. Curiously, one should notice that rats in Sα group presented similar corticosterone levels to non-stressed rats (NS), which indicates a block potential of phentolamine regarding the release of glucocorticoids by the hypothalamic-pituitary-adrenal (HPA) axis. In contrast, the Sβ group, which received daily injections of propranolol, was unable to significantly prevent the corticosterone release, as its been shown in previous study (33).

Interestingly, the groups treated with adrenergic blockers, Sβ and Sα, presented almost half the number of differentiated osteoclast compared with NS group and SS group. Since the osteoclasts are responsible for bone resorption, the effect of metabolic disorders regulated by adrenergic signaling pathway on bone must be considered. However, as the SS control group, the non-stressed group NS also showed a high number of TRAP-positive cells. From this, we can infer that the inflammation of periapical tissue for itself, present in both control groups, was sufficient to increase the number of osteoclasts and the chronic stress condition, as induced in the present study, did not interfere.

Immunohistochemical analysis were also presented as an immunostaining of α and β adrenergic receptors, and as observed, there was no significant difference of α and β–AR expression between the groups. *These results are conflicting with the study by Lu et al. (13), which also evaluate the expression of adrenergic receptors in periodontal disease under stress conditions. The authors reported an increase in the levels of adrenergic receptors after a stress induction. However, it must be considered that immunohistochemical analysis was performed without soft tissue, which may explain the contradictory results with the present study.*

Moreover, a previous study showed an increased expression of β2-AR in differentiated osteoblasts stimulated by glucocorticoids. This finding has proposed an intense responsiveness of these cells to sympathetic system activation characterized by the release of catecholamines, and thus leading to bone loss (34). Therefore, the reduced corticosterone levels in Sα group may explains the low expression of β-AR in the Sα group, observed in immunohistochemical staining.

On the other hand, the periapical lesion area measured thorugh histochemistry sections did not show any significant difference between the groups. Nonetheless, because of the weak correlation between histologic measurement and the 2D micro-CT analysis reported by de Oliveira et al. (18), the present study also evaluated the area of the periapical lesion by means of micro-CT, which, despite confirming the similarity between the groups, also showed a weak correlation with the histologic analysis, corroborating previous findings (18).

Among the major cytokines involved in stress associated with chronic inflammation, interleukin-1β (IL-β) tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) are known to have osteoclastic activity. The expression of these cytokines is regulated by the kappa-β nuclear factor and thus, this is another mediator mentioned as a central regulator of stress responses (35). Therefore, it must be considered the complexity in the activation of this network of communication between nervous and immune systems due to the involvement of numerous factors such as cytokines, neurotransmitters, receptors and intracellular transcription factors, which requires more elucidation about their mechanisms of action and different functions under specific conditions.

Considering the results of this study, it is observed how this relationship between chronic stress and apical periodontitis can be complex and undoubtedly intriguing. Accordingly, it is essential to understand the functionality of the organism during a stress status and its impact on the inflammatory immune response. In this way, new studies can be performed in order to seek new therapies to aid in the treatment of the periapical lesion.

Although the effects of the medications used in the present study did not present congruent results in the development of the periapical lesion, the present study represents a trigger for a better understanding of the mechanisms of the stress response on apical periodontitis, which up to now have not been addressed in the

endodontic literature. In addition to the progression of the apical periodontitis, the repair time of periapical lesions can also undergo intervention under stress conditions and hence should be considered for investigation. It has been shown that stressed individuals present longer injury repair times due to increased cortisol, which in turn prevents the healing process by inhibiting cell communication and production of proinflammatory cytokines at the site of injury (20).

Future investigations must take into account the type of stress and the intervals between the cessation of a chronic variable stressor and the new stressor, which can influence the hormonal release and immune functions on the body (25 – 27).

In conclusion, despite the fact that no differences could be observed regarding the periapical lesion size and adrenergic receptors expressions under the chronic stress condition tested, the groups treated with adrenergic blockers showed a reduced number of osteoclast differentiation. This finding proposes a possible influence of adrenergic signaling pathway in the endodontic treatment outcome under chronic stress condition, but further investigations are needed to elucidate all pathways involved in this relationship.

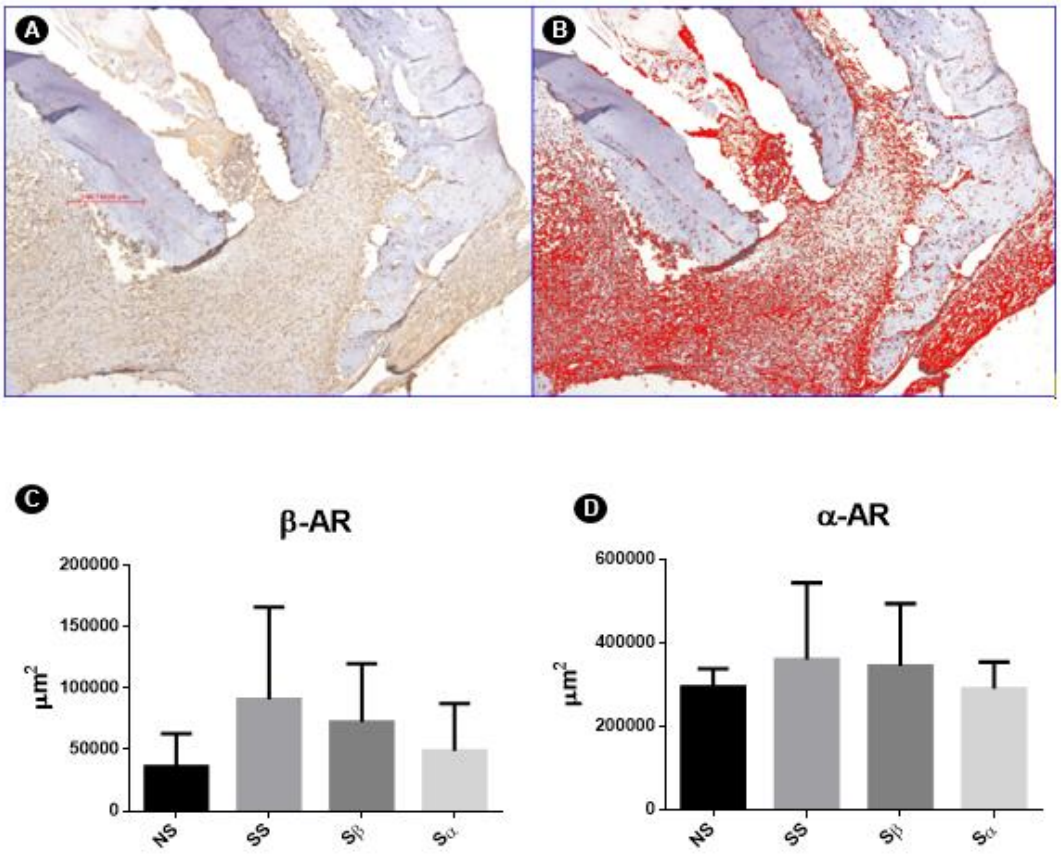

Figure 1. *(A) Image representing immunohistochemical staining with hematoxylin (B) Red staining by Leica Microsystems Software (Leica, Wetzlar, Germany). The amount of area stained in red was calculated in µm² in order to quantify the β-AR and α-AR expression (C) Expression of β-adrenergic receptor in periapical region in µm² (D) Expression of α-adrenergic receptor in periapical region in µm².*

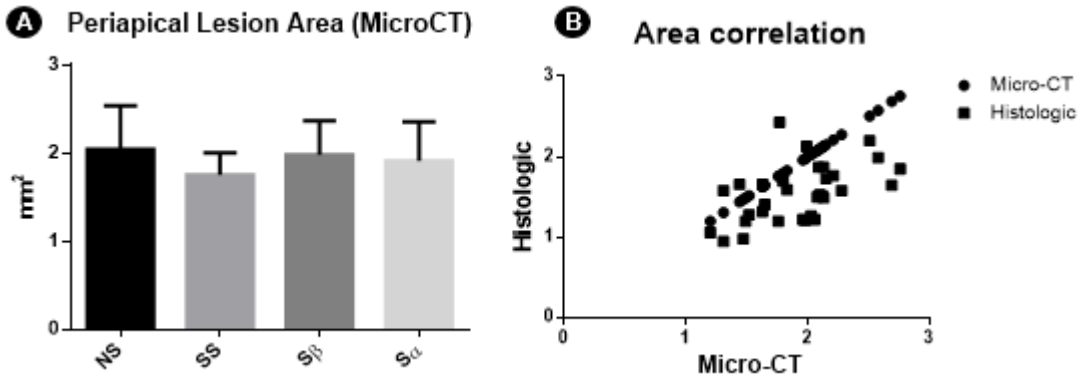

Figure 2. *(A) Periapical lesion area (mm²) in mesial root of mandibular first molar in different treatment groups measured by Micro-CT analysis. (B) Correlation between micro-CT and histologic measurement of periapical lesions area.*

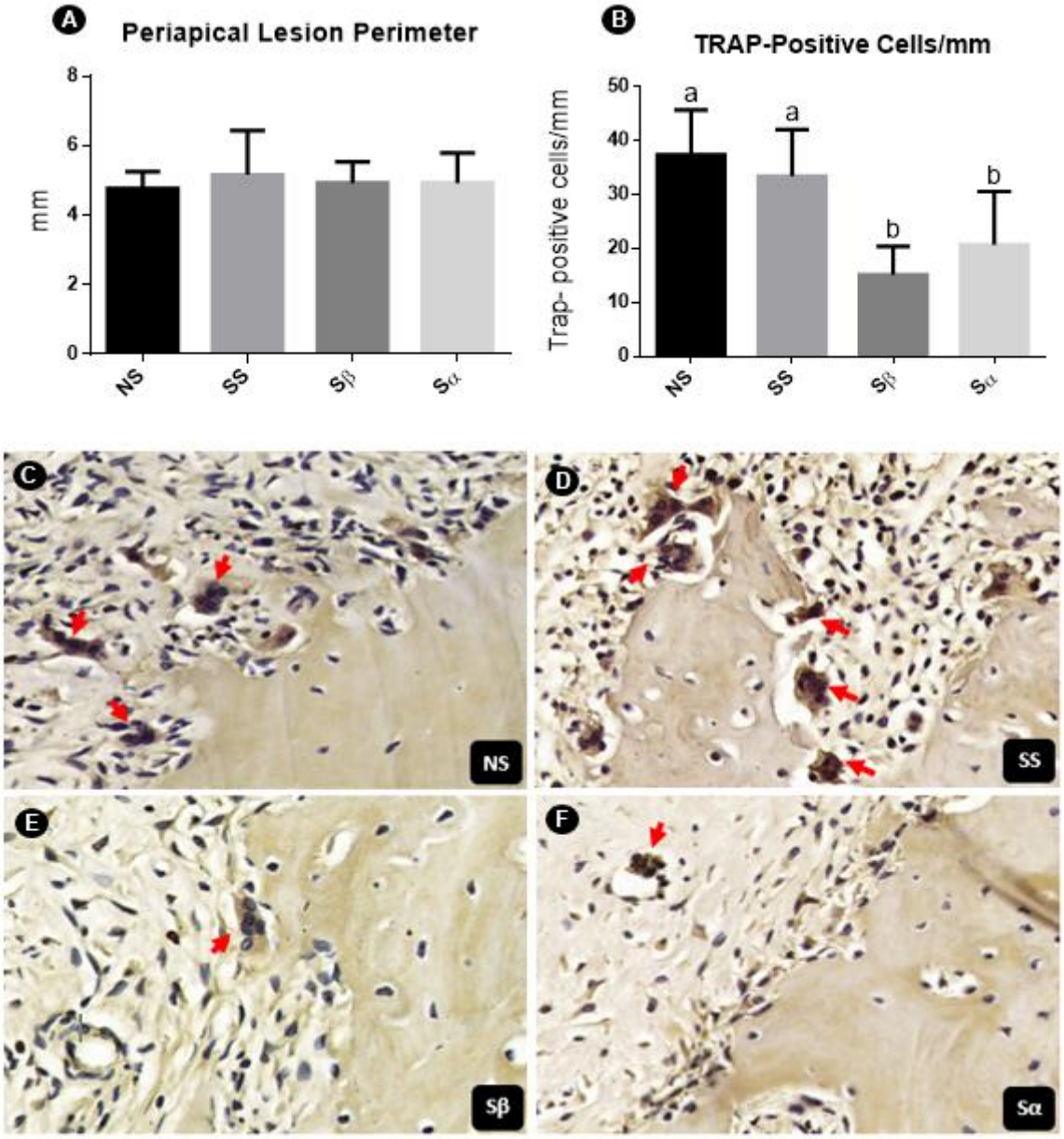

***Figure 3**. (A) Periapical lesion perimeter in mm (B) Number of TRAP-positive cells per mm in the perimeter of periapical lesion of mesial root of mandibular first molar in different groups. (C)(D)(E)(F) Photomicrographs showing TRAP-positive cells (arrowheads) in groups NS, SS, Sβ and Sα respectively.*

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3 CONSIDERAÇÕES GERAIS

Fatores estressores de âmbito psicológico e emocional, como o pânico, a ansiedade e depressão estão cada vez mais presentes na vida humana moderna. Diante da grande diversidade de condições estressantes, juntamente com as dificuldades de adaptação do ser humano a certos agentes estressores, as condições de estresse e a sua complexa relação com o sistema imunológico têm recebido atenção especial por parte dos pesquisadores na área da saúde (Black, 1994; Dhabhar, 2002; Black, 2006; Kemeny, Schedlowski, 2007; Dhabar, 2009).

A modulação do sistema imune pelo Sistema Nervoso Central é bem estabelecida na literatura. De forma mais específica, estudos realizados no campo da Psiconeuroimunologia demonstraram que a resposta ao estímulo de estresse é mediado pela ativação do eixo HPA e também pelo SNS, que em desequilíbrio podem resultar em alterações do sistema imune. Diante desse quadro, algumas enfermidades podem se estabelecer como depressão, câncer, doenças inflamatórias crônicas, além de maior susceptibilidade a infecções causadas por microrganismos (Ostrander et al., 2006; Rosania et al., 2009; Lamkin, 2015; Dumitrescu, 2016).

Na Odontologia, a área da Periodontia tem se dedicado cada vez mais às investigações que esclareçam a complexa interação bidirecional entre o SNS e a resposta imune inflamatória. Estudos ao longo dos anos têm demonstrado a influência do estresse crônico, tão presente nos dias atuais, na progressão da doença periodontal (Boyapati, Wang, 2000; Rosania et al., 2009; Sabbah et al., 2011; Goyal et al., 2013; Lu H et al., 2014; Dumitrescu, 2016).

Sabe-se que a doença periodontal e a periodontite apical compartilham algumas semelhanças em termos de origem e composição da microbiota, que é em sua maior parte composta de microrganismos anaeróbios Gram-negativos (Siqueira, Rôças, 2014). Além disso, estudos têm demonstrado que manifestações agudas e crônicas, tanto da doença periodontal quanto da periodontite apical, elevam os níveis de mediadores inflamatórios (Caplan D, 2004; Caplan DJ et al., 2006). Dessa forma, supõe-se que a periodontite apical pode também estar associada as mesmas doenças sistêmicas relacionadas a doença periodontal (Segura-Egea et al., 2015).

A periodontite apical que por sua vez também é uma doença inflamatória, é

caracterizada pela resposta imune como consequência de uma infecção bacteriana e necrose pulpar que resulta em reabsorção óssea periapical (Stashenko et al., 1998; Zhang, Peng, 2005). No entanto, de forma contrária ao campo da Periodontia, até o momento não existem estudos na literatura que relacionam a influência do estresse no desenvolvimento e progressão da lesão periapical. Portanto, o presente estudo pode ser considerado como o primeiro passo para se obter evidências dessa relação a fim de buscar novas terapias que levam em consideração o estado emocional e psicológico do paciente.

O modelo animal de lesão periapical através da exposição da câmara pulpar é amplamente utilizado em diversos estudos (Azuma et al., 2017; Prieto et al., 2017; Samuel et al., 2017). Tal condição favorece a ocorrência de infecção bacteriana e consequente necrose pulpar, que provoca uma resposta inflamatória, e como consequência, ocorre o desenvolvimento da lesão periapical. A confiabilidade dessa metodologia foi confirmada nesse estudo através das imagens histológicas e microtomográficas que mostraram evidente estabelecimento da lesão periapical dentro do período experimental.

Diversos modelos animais experimentais de estresse têm sido desenvolvidos a fim de investigar os efeitos do estresse no desenvolvimento da doença periodontal (Shapira et al., 1999, 2000; Lu H et al., 2014). Na literatura os modelos podem variar de acordo com algumas características que se referem à natureza (psicológico, químico ou físico), intensidade (agudo ou crônico) e duração do estresse. Além disso, segundo alguns autores, os efeitos do estresse no organismo podem variar conforme o tipo de estresse em questão, exercer diferentes efeitos em relação a liberação hormonal e funções imunes, o que reforça a complexidade desse fenômeno fisiológico sobre o organismo e o desenvolvimento de enfermidades, como por exemplo, a lesão periapical (Ostrander et al., 2006; Goyal et al., 2013; Dumitrescu, 2016). Por exemplo, em um estudo realizado por Ostrander et al. (2006), nos animais submetidos a 7 dias de estresse crônico variável foi observado uma diminuição na atividade do eixo HPA quando submetidos a um novo estressor. Em outro estudo, também foi demonstrado que os efeitos de diferentes estressores aplicados sucessivamente podem interferir e que o estresse crônico pode eliminar os efeitos de estresse agudo (Cancela et al., 1995; Ferret et al., 1995; Aloisi et al., 1998). Diante disso, podemos dizer que estudos futuros a fim

de investigar a relação entre o estresse crônico e a periodontite apical, devem levar em consideração o intervalo entre a cessação do estresse crônico variável e o novo estresse, e também à natureza desse estímulo, ou até mesmo a junção desses dois fatores.

O estresse pode ser considerado agudo e crônico conforme a sua intensidade e tempo (Zelena et al., 1999). O estresse agudo é caracterizado por uma resposta imediata e de curto prazo podendo variar de segundos a poucos minutos (Sapolsky et al., 2000). Além disso, de acordo com Pruett (2003) o estresse relativamente agudo parece favorecer as funções imunológicas podendo induzir a resposta imune celular, através do aumento do número de células natural killer (NK) e de granulócitos (Kemeny, Schedlowski, 2007). O estresse crônico por sua vez, persiste por vários dias, semanas ou meses (Dhabhar, 2002). Existem evidências de que as respostas ao estresse crônico levam a uma expressiva imunossupressão (Jacobs et al., 2001), embora haja uma certa divergência na comunidade científica em relação ao tipo, duração e intensidade do estresse (Zelena et al., 1999).

No presente estudo, o modelo animal de estresse crônico foi realizado de acordo com o protocolo de Willner et al. (1992), com algumas alterações. Os procedimentos experimentais foram realizados durante 32 dias, uma vez que esse período é comprovadamente suficiente para observar o estabelecimento tanto da lesão periapical quanto do quadro de estresse crônico. Diante dos diversos modelos experimentais existentes, alguns parâmetros indicadores são utilizados a fim de comprovar a eficácia de tais modelos, como por exemplo a concentração de corticosterona, glicemia, aparecimento de úlcera, determinação da presença do hormônio adrenocorticotrófico e até mesmo avaliação comportamental. A determinação de cortisol é utilizada como indicador de estresse em seres humanos, enquanto que a de corticosterona é utilizada no caso de roedores (Besedovsky, del Rey, 1996).

Neste estudo, a concentração da corticosterona e avaliação do ganho de peso dos animais foram selecionados como parâmetros para determinar se o estresse crônico foi eficazmente aplicado. Nos resultados obtidos observamos uma diferença significativa entre os grupos controle NS e SS, uma vez que o grupo SS apresentou níveis séricos elevados de corticosterona quando comparado aos demais grupos, este dado está de acordo com estudos previamente realizados

(Lowrance et al., 2016). Curiosamente, o grupo Sα apresentou níveis de corticosterona similares ao grupo controle não estressado (NS). Este dado sugere um efeito positivo da fentolamina na liberação de glicocorticoides através do eixo HPA.

Levando em consideração os pesos dos animais, todos os grupos submetidos ao estudo apresentaram peso corporal similares inicialmente e quando avaliados no final do experimento, todos os grupos mostraram um aumento no ganho de peso, no entanto, o ganho foi significativamente menor nos grupos estressados quando comparados ao animais não-estressados. Estes resultados corroboram com estudos previamente realizados (Simch et al., 2008; Soletti et al., 2009).

O SNS quando ativado induz a liberação de catecolaminas que por sua vez são responsáveis pelo aumento da frequência cardíaca, da glicemia, do metabolismo celular, que são mecanismos de defesa do organismo frente ao estímulo de estresse (Webster Marketon, Glaser, 2008). A resposta adaptativa do estresse ativa rapidamente o SNS enquanto que o eixo HPA responde de forma mais lenta e estimulando a liberação de glicocorticoides, que dependendo dos níveis secretados podem deprimir o sistema imunológico (De Vente et al., 2003).

Na tentativa de impedir ou reverter os efeitos prejudiciais do estresse sobre o equilíbrio do sistema imune, tem sido considerada a busca por novas terapias que incluem a administração de antagonistas ou agonistas de receptores dos hormônios supracitados liberados durante o estresse. Primeiramente as pesquisas são realizadas em animais para que posteriormente, diante de resultados favoráveis in vivo, as alternativas terapêuticas possam ser testadas clinicamente com o objetivo de verificar a sua efetividade em seres humanos. Visando conter os efeitos do estresse sobre o sistema imune, o nosso estudo incluiu duas classes de bloqueadores adrenérgicos recentemente identificados na superfície óssea, o β-bloqueador propranolol e o α-bloqueador fentolamina (Moore et al., 1993; Takeda et al., 2002; Togari, 2002; Fonseca et al., 2011; Lu H et al., 2014; Grover et al., 2015). Os fármacos foram administrados por via subcutânea (propranolol) e intraperitoneal (fentolamina).

No presente trabalho, o efeito do estresse, bem como o uso de bloqueadores adrenérgicos foram avaliados através de análise microtomográfica,

histológica, imunohistoquímica.

A presença de infecção persistente resulta em cronificação da inflamação, tornando o infiltrado inflamatório da lesão periapical predominantemente mononuclear (Scales, Huffnagle, 2013). Através do exame histológico foi observado infiltrado inflamatório em todos os grupos. A intensidade e extensão do infiltrado foram avaliados através de scores e depois de aplicada a estatística, concluiu-se não haver diferença entre os grupos em termos de quantidade de células inflamatórias. Além do infiltrado inflamatório também avaliou-se o tamanho da lesão periapical através da análise histológica. No entanto, um fator importante a ser considerado nesses tipos de estudo é a precisão diagnóstica do tamanho da lesão periapical. de Oliveira et al. (2015), alertam para a baixa correlação existente entre a medida histomorfométrica e microtomográfica. Essa falta de correlação pode ser justificada uma vez que os cortes histológicos representam projeções bidimensionais de estruturas tridimensionais e podem portanto falhar na precisão da quantificação da medida (von Stechow et al., 2003). Por isso, além da avaliação histológica, o tamanho da lesão periapical também foi avaliado através de microtomografia computadorizada. No entanto, não foi observado diferença estatística nos valores volumétricos da lesão periapical entre os grupos.

As análises imunohistoquímica também mostraram que a imunomarcação dos receptores alfa e beta adrenérgicos não foram significativamente diferente entre os grupos. Esses resultados são conflitantes com o estudo de Lu H et al. (2014) que também avaliou a expressão de receptores adrenérgicos na doença periodontal sob condições de estresse. Os autores relataram um aumento dos níveis de receptores adrenérgicos após a indução do estresse, valendo ressaltar que a análise foi realizada no tecido mole, o que pode explicar os resultados contraditórios com o presente estudo.

De forma geral, podemos dizer que na presente pesquisa, verificou-se que o bloqueio adrenérgico não influenciou na reabsorção da lesão periapical dos animais submetidos à indução de estresse crônico, apesar de diminuir significativamente o número de osteoclastos. Os resultados inconclusivos apresentados corroboram com estudos prévios que não encontraram influência do bloqueio adrenérgico sobre a perda óssea, inclusive na doença periodontal induzida em ratos (Marenzana et al., 2007; Martins, 2011). Segundo Bonnet et al. (2008), os estudos que investigam a

influência da sinalização adrenérgica no metabolismo ósseo alertam para a complexidade da regulação da modelação óssea pelo SNS.

Apesar dos efeitos dos medicamentos utilizados no presente estudo, não apresentarem resultados congruentes diante do desenvolvimento da lesão periapical, podemos dizer que o nosso estudo representa um gatilho para uma melhor compreensão dos mecanismos da resposta do estresse sobre a periodontite apical que até o momento não foram abordados na literatura endodôntica.

Além da progressão da lesão periapical, pode-se ressaltar a necessidade de investigação quanto ao tempo de reparação das lesões periapicais que também pode sofrer intervenção sob condições de estresse. Foi demonstrado que indivíduos estressados apresentam maior tempo de reparo de lesões em geral devido ao aumento de cortisol, que por sua vez impede o processo de cura inibindo a comunicação celular e a produção de citocinas pró-inflamatórias no local da injúria (Webster Marketon, Glaser, 2008).

Por fim, durante a realização desse estudo verificamos que o campo de pesquisa sobre os efeitos do estresse e suas consequências no percurso de doenças inflamatórias, apesar de amplo, ainda é inexistente na área da Endodontia. A complexidade existente na ativação dessa rede de comunicação entre os sistemas nervoso e imune se deve ao envolvimento de inúmeros fatores como citocinas, neurotransmissores, receptores e fatores de transcrição intracelular, que por sua vez necessitam de maior elucidação sobre seus mecanismos de ação e diferentes funções em condições específicas.

Diante dos resultados desse estudo podemos observar o quanto essa relação entre o estresse crônico e a periodontite apical pode ser complexa e sem dúvida, intrigante. Desta forma, é imprescindível o entendimento da funcionalidade do organismo durante o estresse e seu impacto na resposta imune inflamatória para que possamos buscar novas terapias que auxiliem no tratamento da lesão periapical.

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ANEXO A – Comprovante de artigo publicado

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Dear Dr. Rayana Khoury,

You have been listed as a Co-Author of the following submission:

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Corresponding Author: Marcia Valera
Co-Authors: Rayana D Khoury, MS Student; Felipe S Matos, PhD student; Renata F Prado, PhD; Claudio Antonio T Carvalho, PhD; Flávia Goulart R Cardoso, PhD;
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Journal of Endodontics

ANEXO B – Certificado do Comitê de Ética em Pesquisa

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José dos Campos
Instituto de Ciência e Tecnologia

CERTIFICADO
CEUA – Comissão de Ética no Uso de Animais

CERTIFICAMOS, que a proposta intitulada "**O Estresse crônico e a via de sinalização adrenérgica na progressão da periapicopatia e na reintegração pós-reimplante dentário: Um alvo terapêutico potencial?** " registrada com o nº **001 /2016** , sob a responsabilidade de **RENATA FALCHETE DO PRADO** e que envolve a utilização de animais pertencentes ao filo Chordata subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009 e com as Normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS** (CEUA – ICT – CAMPUS DE SÃO JOSÉ DOS CAMPOS-UNESP), em reunião de 11/03/2016.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da Autorirzação	01/04/2016 a 30/11/2017
Espécie/linhagem/raça	Rato/heterogênico Wistar
Nº de Animais	112
Peso/Idade	400 grs / 90 dias
Sexo	MACHO
Origem	Biotério Central – Campus de Botucatu-UNESP

São José dos Campos, 11 de março de 2016

Profa.Dra. **CRISTIANE YUMI KOGA ITO**
Vice- Coordenadora

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