UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
CÂMPUS DE ARAÇATUBA – FACULDADE DE ODONTOLOGIA

ANA MARIA VEIGA VASQUES

AVALIAÇÃO DA INFLUÊNCIA DO TABAGISMO NO DESENVOLVIMENTO DA PERIODONTITE APICAL EM RATOS WISTAR

ARAÇATUBA-SP
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Tese apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho"- UNESP, como parte dos requisitos para a obtenção do título de Doutora em Ciência, área de concentração: Endodontia.

Orientador: Prof.º Eloi Dezan Junior

Coorientador: Prof.º Luciano Tavares Angelo Cintra

Coorientador: Prof.º Carlos Roberto Emerenciano

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Resumo Geral

Vasques AMV. **Avaliação da influência do tabagismo no desenvolvimento da periodontite apical em ratos wistar.** 2023. 81f. [Tese]. Faculade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2023.

Resumo geral

A infecção endodôntica ocorre a após contaminação do tecido pulpar levando à uma colonização bacteriana dos canais radiculares resultando em uma resposta inflamatória dos tecidos periapicais e formação de uma lesão periapical, a periodontite apical (PA). O tabagismo, por sua vez, tem impactos prejudiciais à saúde oral e sistêmica sendo considerado um importante fator de risco para as doenças periodontais. Este trabalho tem por finalidade investigar a influência do tabagismo no desenvolvimento da periodontite apical em ratos. Trinta e dois ratos machos *Wistar* foram divididos em 4 grupos experimentais (n=8): Controle (sem periodontite apical induzida e sem inalação da fumaça do cigarro); FU (com inalação a fumaça do cigarro); PA (com periodontite apical induzida); FU+PA (com inalação a fumaça do cigarro e periodontite apical induzida). Para a inalar a fumaça do cigarro, grupo de cinco animais permaneceram em uma câmara de tabagismo inalando a fumaça de 10 cigarros por 8 minutos, três vezes ao dia, durante 50 dias. Decorridos 20 dias de inalação de fumaça do cigarro, foi realizada a cirurgia para indução da periodontite apical nos animais do grupo PA e FU+PA, no primeiro molar inferior direto, o qual permaneceu aberto na cavidade bucal por 30 dias. Nesses 30 dias subsequentes da indução da PA, os animais do grupo FU+PA e FU continuaram com a inalação a fumaça do cigarro. No 50º dia, os animais foram eutanasiados e coletados amostras de tecido hematológico para avalição dos níveis séricos de nicotina, cotinina, fosfatase alcalina, cálcio, fósforo, série vermelha e série branca. As hemimandibula removidas foram escaneadas em microtomógrafo para avaliar o volume da destruição óssea e processadas histologicamente para avaliação do perfil inflamatório e expressão das citocinas IL-6, IL-1β, TNF-α e dos marcadores do metabolismo ósseo RANKL e OPG. Os dados foram tabulados e aplicados os testes estatísticos de Mann-Whitney para os dados não paramétricos e teste *t* para os dados paramétricos, a um nível de significância de $P<0.05$. Com

relação a análise histológica o grupo FU+PA apresentou infiltrado inflamatório mais intenso em relação aos demais grupos (P<0,05). As citocinas pró-inflamatórias IL-6, IL-1β, TNF-α apresentaram alto padrão de imunomarcação para o grupo FU+PA (P<0,05). A análise histométrica mostrou maior área de reabsorção óssea no grupo FU+PA, assim como na análise microtomográfica (P<0,05). As citocinas RANKL esteve mais expressa no grupo FU+PA (P<0,05) e OPG teve maior expressão no grupo PA (P<0,05). Na análise hematológica houve um aumento na concentração de hemácias, hemoglobina e leucócitos para o FU+PA (P<0,05). Os níveis séricos de cálcio foram menores no FU+PA (P>0,05), a fosfatase alcalina e o cálcio se manteve constante em todos os grupos experimentais (P>0,05). A concentração sérica de nicotina e cotinina no grupo FU e FU+PA foram compatíveis com fumante humano.

Palavras-chave: endodontia, inflamação, periodontite periapical, reabsorção óssea, tabagismo.

Abstract

Vasques AMV. **Evaluation of smoking influence on the development of apical periodontitis in wistar rats**. 2023. 81f. [Thesis]. School of Dentistry, São Paulo State University, Araçatuba, 2023.

Abstract

Endodontic infection occurs mainly after pulp tissue contamination by a carious process, leading to bacterial colonization of root canal system and inflammatory response of periapical tissues, followed by formation of apical periodontitis (AP). It is widely known that smoking has harmful impacts on oral and systemic health and is considered a risk factor for periodontal diseases. The objective of this research was to investigate the influence of smoking on the development of AP in rats. Thirty-two male *Wistar* rats were divided into 4 experimental groups (n = 8): C - Control (no induced AP and no cigarette smoke inhalation); CSI (cigarette smoke inhalation); AP (induced apical periodontitis); CSI + AP (cigarette smoke inhalation and induced apical periodontitis). To inhale cigarette smoke, group with five animals remained in a smoking chamber inhaling smoke from 10 cigarettes for 8 minutes, three times daily, for 50 days. After 20 days of cigarette smoke inhalation, pulp chamber access was performed to induce apical periodontitis in the first mandibular right molar, which remained with pulp chamber exposed to oral cavity for 30 days in animals in the AP and CSI + AP group. During these 30 days after the AP induction, animals in the CSI + AP and CSI group continued to inhale cigarette smoke. On the 50th day, animals were euthanized and blood sample collected to assess serum levels of nicotine, cotinine, alkaline phosphatase, calcium, phosphorus, red series and white series. The hemimandibles were removed and scanned in microtomograph to assess bone volume and histologically processed to assess inflammatory profile and expression of cytokines IL-6, IL-1β, TNF-α and bone metabolism markers RANKL and OPG. Data were tabulated and statistically analyzed using Mann-Whitney tests for non-parametric data and analysis of variation test for parametric data, with a significance level of $P<0.05$. Regarding histological analysis, CSI+AP group showed more intense inflammatory infiltrate compared to other groups ($P < 0.05$). Histometric

analysis showed a larger area of bone resorption in the CSI + AP group, also observed in the microtomographic analysis ($P<0.05$). Group CSI+AP had elevated pro-inflammatory cytokines IL-6, IL-1β, TNF-α expression ($P<0.05$). The RANKL cytokines were also more expressed in the CSI+AP group ($P<0.05$), while OPG was more expressed in the AP group ($P<0.05$). The hematological analysis revealed an increase in the concentration of red blood cells, hemoglobin, and leukocytes for CSI+AP ($P<0.05$). Serum calcium levels were lower in CSI+AP ($P>0.05$), and alkaline phosphatase and calcium remained constant in all experimental groups ($P>0.05$). The serum concentrations of nicotine and cotinine in the CSI and CSI+AP group were compatible with human smokers.

Keywords: endodontics, inflammation, apical periodontitis, bone resorption smoking.

Sumário

Introdução Geral

Introdução geral

A periodontite apical (PA) é caracterizada pela reabsorção óssea em resposta a infecção bacteriana oriunda dos canais radiculares (Kawashima et al. 1996). Este processo inflamatório é uma resposta imune do hospedeiro contra a infecção bacteriana presente na polpa necrótica. Quando os mecanismos do sistema de defesa não conseguem erradicar a infecção, os tecidos periapicais sofrem reabsorção óssea, influenciada por fatores locais e sistêmicos (Xiong et al. 2007, Gomes - Filho et al. 2015).

A medicina endodôntica tem avançado nos estudos da periodontite apical sobre a doenças sistêmicas, e as consequências que podemos encontrar de uma combinação desses dois fatores. Cintra et al. (2018) realizaram um estudo correlacionando a PA com o diabetes e observaram que o diabetes pode influenciar a patogênese da PA, assim como a PA pode potencializar os efeitos sistêmicos que ocorrem como consequência do diabetes. Outros estudos avaliaram o comportamento da PA com as alterações hepáticas (Grønkjær et al. 2016), renais (Khalighinejad et al. 2017) e doenças cardiovasculares (Segura-Egea et al. 2010, Segura-Egea et al. 2011, Martins et al. 2016) confirmando que as essas alterações de ordem sistêmicas afetam a patogênese da PA.

O tabagismo apresenta impactos prejudiciais à saúde oral e sistêmica, considerado um importante fator de risco para várias doenças, dentre elas a doença periodontal (MacFarlane et al. 1992, Soder et al. 1999). Segundo dados da Organização Pan-Americana de Saúde (OPAS) e da Organização Mundial de Saúde (OMS) (Organização Mundial de Saúde 2019) estima-se que existam aproximadamente 1,1 bilhão de pessoas fumantes no mundo, sendo o tabaco responsável pela morte de 8 milhões de pessoas, dentre elas 7 milhões devido ao uso direto do tabaco (fumante ativo) e 1,2 milhões ao uso indireto do tabaco (fumante passivo).

Dos quase 4.000 componentes químicos presentes na fumaça do cigarro, a nicotina é o componente mais psicoativo (Nogueira-Filho et al. 2007, Talhout et al. 2011, Wu et al. 2013). Seu metabolismo hepático produz a cotinina, um metabólito importante, mais estável e com meia-vida mais longa que a nicotina, favorecendo

seu uso como marcador biológico para avaliação da exposição à fumaça do cigarro (Dempsey et al. 2002, Chou et al. 2008).

A fumaça do cigarro pode atingir o sistema respiratório através da inalação ativa ou passiva. Na inalação ativa a fumaça do cigarro é chamada de fumaça central ou convencional, resultado da queima do cigarro em altas temperaturas (acima de 950ºC) e da passagem da fumaça pela coluna e filtro do cigarro (Scherer et al. 1990). A inalação passiva ocorre quando a queima do cigarro é gerada em temperatura mais baixas (aproximadamente 350ºC), chamada de fumaça periférica ou de fluxo lateral, que ocorre durante a combustão lenta de um cigarro em um ambiente fechado. Esse tipo de fumaça (inalação passiva) contém quatro vezes mais compostos nocivos do que a fumaça principal (Schick et al. 2005). Nos dois tipos de exposição, a fumaça vai para os pulmões e, através da circulação sanguínea, atinge diferentes órgãos, alterando as rotas metabólicas e comprometendo a saúde e a resposta imune do indivíduo (Gao et al. 2015, Vasconcelos et al. 2016).

César Neto et al. (2003) avaliaram o impacto negativo que a inalação da fumaça passiva pode apresentar em relação a osseointegração de implantes de titânio inseridos em ratos, e concluíram que a inalação intermitente da fumaça do cigarro resulta em menor osseointegração devido a uma menor área óssea sendo parte desses defeitos ósseos causados principalmente pela nicotina. Rosa et al. (2017) estudaram a influência do fumo passivo em ossos de ratos imaturos, comprovando que a exposição passiva a fumaça do cigarro afeta a estrutura óssea, pois o osso longo dos ratos se apresentava mais curto, sugerindo sua interferência no crescimento ósseo.

Pesquisas clínicas e experimentais já demostraram que a fumaça do cigarro contém toxinas imunomoduladoras que são capazes de alterar a vascularização periférica, uma vez que a nicotina reduz a proliferação dos glóbulos vermelhos, altera o recrutamento e a função das células do sistema imunológico, e das citocinas pró-inflamatórias (Buduneli et al. 2018, Ferreira et al. 2018). A presença das citocinas no fluido gengival de pacientes fumantes com doença periodontal já foi detectada com uma redução significativa nesses pacientes das interleucinas IL-1β

(Petropoulos et al. 2004) e IL-6 (Tymkiw et al . 2011), e um aumento da expressão do TNF-α (Bostrom et al. 1999) . Em contraste, outros estudos clínicos mostraram níveis mais elevados de IL-1β e IL-6 em fumantes (Bostrom et al. 1998, Zhong et al. 2007).

As interleucinas IL-6, IL-1β e TNF-α também estão associadas com a patogênese da infecção endodôntica (Azuma et al. 2013, Cintra et al. 2016). A IL-6 é uma citocina presente em processos fisiológicos e patológicos e está relacionada ao desenvolvimento e progressão da inflamação (Nibali et al 2012). A IL-1β está relacionada com a reabsorção óssea periapical em complicações sistêmicas (Ng et al. 2008). Já o TNF-α atua como um importante mediador de lesões teciduais (Tansey et al. 2009), podendo induzir a atividade osteoclástica local (Cintra et al. 2016, Colić et al. 2009).

Além das alterações envolvendo as citocinas pro-inflamatórias, a nicotina afeta também o metabolismo ósseo modulando a proliferação e maturação de osteoblastos, e a produção de matriz extracelular (Walker et al. 2001, Akmal et al. 2004), podendo trazer um desequilíbrio para o sistema RANK/RANKL/OPG, que é uma das principais vias de ativação da reabsorção óssea (Suda et al. 1999, Walker et al. 2001). O ativador de receptores do ligante NF-κB (RANKL), também conhecido como fator de diferenciação de osteoclastos (Yasuda et al. 1998), é o fator mais potente para induzir a osteoclastogênese. O receptor natural do RANKL é a OPG (osteopretegerina) conhecida como fator inibidor da osteoclastogênese. Portanto a formação dos osteoclastos é regulada pelo equilíbrio entre OPG e RANKL.

Kawashima et al. (2007) encontraram níveis aumentados de RANKL em lesões periapicais duas semanas após a exposição pulpar em dentes de ratos. Buduneli et al (2008) observaram que fumantes com periodontite crônica exibiram níveis maiores de RANKL e menores de OPG na saliva em comparação aos pacientes não fumantes.

Outros marcadores relacionados a homeostase óssea são a fosfatase alcalina, o cálcio e o fósforo. A fosfatase alcalina é uma glicoproteína ligada à membrana liberada a partir de neutrófilos polimorfonucleares durante a inflamação, e osteoblastos durante a formação óssea (Perinetti et al. 2008). O cálcio e o fósforo

por sua vez são reguladores da mineralização e do crescimento ósseo (Scholz-Ahrens et al; 2007).

Considerando que as interleucinas IL-1β, IL-6 e TNF-α estão relacionadas com desenvolvimento da periodontite apical e com tabagistas, e a nicotina presente na fumaça do cigarro promove alterações sanguíneas nas séries vermelhas, brancas, e nas vias do metabolismo ósseo, este estudo propôs avaliar os efeitos do tabagismo no desenvolvimento da periodontite apical induzida em ratos. A hipótese nula testada é de que o tabagismo não tem influência no processo inflamatório, na marcação imunológica dos mediadores pró-inflamatórios e do metabolismo ósseo, e não tem capacidade de alterar as séries sanguíneas durante o desenvolvimento da PA.

Objetivo geral

Avaliar a influência do tabagismo no desenvolvimento da periodontite apical induzida em ratos.

Objetivos específicos

Artigo 1: Inflammatory profile of apical periodontitis exacerbated by cigarette smoke inhalation: histological and immunohistochemical analysis in rats. *Artigo publicado no *International Endodontic Journal**

- Avaliar os parâmetros hematológicos (série vermelha e série branca);
- Avaliar o perfil inflamatório na periodontite apical;
- Avalair a expressão das interleucinas IL-6, IL-1β e TNF-α como indicadores da inflamação local;

Artigo 2: Bone resorption in apical periodontitis enhanced by cigarette smoke inhalation: histometric, immunohistochemical and microtomographic analysis in rats

- Avaliar os níveis séricos dos marcadores do metabolismo ósseo: cálcio, fósforo e fosfatase alcalina;

- Avaliar a área de destruição dos tecidos periapicais pela análise histométrica;
- Avaliar a expressão de RANKL e OPG;
- Avaliar o volume da reabsorção óssea na periodontite apical através de microtomografia computadorizada.

Artigo 1

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ORIGINAL ARTICLE

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Inflammatory profile of apical periodontitis exacerbatedby cigarette smoke inhalation: Histological and immunohistochemical analysis in rats

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Abstract

Aim: The aim of this study was to evaluate the effects of cigarette smoke inhalation (CSI) on inflammation, pro-inflammatory mediators and haematological parameters in rats with induced apical periodontitis (AP).

Methodology: Thirty-two 3-month-old male Wistar rats were divided into four experimental groups (n = 8): C—Control; S—rats with CSI; AP—rats with AP; and SAP—rats with CSI + AP. Animals in groups S and SAP inhaled cigarette smoke by remaining inside a smoking chamber for 8 min, three times daily, for 50 days. After 20 days of smoke inhalation, animals in AP and SAP groups had the pulps of the lower right first molar exposed to oral environment for 30 days to induce AP. In these subsequent 30 days, animals in group S and SAP continued with CSI. On Day 50, animals were euthanized and mandibles were histologically processed to assess inflammatory infiltrate, immunohistochemical interleukins (IL-1β, IL-6 and TNF-α), and blood samples collected for laboratory analysis. The Mann–Whitney test was performed for non-parametric data and the pairwise analyses of Student's t-test for parametric data, with a significance level of $p < .050$.

Results: Inflammatory infiltrate was moderate in AP group and more severe in the SAP (p = .010). The interleukins IL-6, IL-1β and TNF-α were higher in SAP group ($p < .001$) when compared to the AP group. A greater number of red blood cells (p = .010), haemoglobin (p = .007) and neutrophils (p = .014) were observed in the SAP group in comparison with the AP group.

Conclusion: Cigarette smoke inhalation induced a more severe inflammatory infiltrate, with increased levels of pro-inflammatory cytokines and changes in haematological parameters in rats with induced AP. Thus, CSI aggravated AP, exacerbating the inflammatory response.

KEYWORDS
animal model, apical periodontitis, endodontics, inflammation, smokers, oral infections

INTRODUCTION

Smoking is a significant risk factor for oral and systemic health. According to data from the World Health Organization (WHO, 2019), it is estimated that approximately 1.1 billion smokers exist worldwide, with smoking being responsible for 8 million human deaths, including 7 million due to direct tobacco use (active smokers) and 1.2 million to indirect use (passive smokers).

Most adverse effects are from cigarette smoke. Nicotine, the main immunosuppressive compound, affects immunological and inflammatory responses by central and peripheral mechanisms (Mishra et al., 2008; Sopori, 2002; Sopori et al., 1998; Wang et al., 2003). The increase in cigarette smoke inhalation (CSI)-associated diseases was first recognized in the 1960s (Holt & Keast, 1977).

Smoking may be one of the risk factors for apical periodontitis (AP) since this practice induces a systemic inflammatory reaction and releases potentially destructive tissue substances such as collagenases and pro-inflammatory cytokines (Barbieri et al., 2011; Johannsen et al., 2014).

Apical periodontitis is characterized by a chronic inflammatory process with consequent bone destruction in response to bacterial infection from the root canals (Nair, 2004). The inflammatory infiltrate of this condition presents cells capable of producing pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α (Rôças et al., 2014), which act in the initiation and regulation of the inflammatory process by activation and proliferation of cells (Araujo-Pires et al., 2014).

Cytokines are proteins secreted by cells of injured tissues in response to microbial agents or other injuries, including smoking. IL-6 is a cytokine related to bone remodelling capable of activating and promoting the differentiation of immune cells and osteoclasts (Huang et al., 2001). IL-1β is a vital pro-inflammatory cytokine that plays a significant role in bone inflammation and resorption, making it an important parameter in periodontal research. TNF-α is involved in MMP secretion and osteoclastic activity (Cintra et al., 2016).

The association between smoking and AP is controversial in the literature. Whilst some authors showed a positive association between smoking and periapical lesions visualized on radiographs (Aleksejuniene et al., 2000; Cabanillas-Balsera et al., 2020a; Kirkevang & Wenzel, 2003; Krall et al., 2006; Segura-Egea et al., 2008, 2011). Other studies demonstrated no significant effect (Bergström et al., 2000; Frisk & Hakeberg, 2006; Rodriguez et al., 2013).

In a recent systematic review, smoking was shown to be a negative factor for the endodontic treatment prognosis, increasing in three times the possibility of extraction (Cabanillas-Balsera, Segura-Egea, Jiménez-Sánchez, et al., 2020). Because most studies involving smoking and periapical lesions are systematic reviews and radiographic correlations through imaging examinations and application of questionnaire to patients, the biological understanding of how changes may occur is limited. Due to the scarcity of *in vivo* reports, this study evaluated the effects of CSI on the development of induced AP in rats.

The animal model used was based upon a previous passive smoker protocol study, described by César-Neto et al. (2003), associated with an AP induction model (Cantiga-Silva et al., 2021; Dal-Fabbro et al., 2021). The null hypothesis tested was that smoking would not affect inflammatory, immunological or haematological parameters in rats with AP.

MATERIALS AND METHODS

The manuscript of this animal study has been written according to Preferred Reporting Items for Animal studies in Endodontology (PRIASE) 2021 guidelines (Nagendrababu et al., 2021). Experimental research approved by the Animal Ethics Committee (School of Dentistry, under protocol no. 00247-2020; Figure 1).

Sample size calculation

The sample size was estimated based on data from a previous study (Cantiga-Silva et al., 2021; Dal-Fabbro et al., 2019) considering the number of animals per group. Using an alpha error of 5% and 95% power, to recognize a significant difference of 1 in the median score, a minimum of seven animals per group was necessary. Considering eventual animal deaths during experimental period or laboratory processing, one more animal was added in each group, resulting in eight rats per group, totalling 32 animals.

Animals

Thirty-two male rats (*Rattus norvegicus albinus*, Wistar), with 3 months old, weighing 200–250 g each, were housed in a controlled temperature environment (22°C ± 1°C) with 12-h light cycle and access to food and water *ad libitum*. The animals were randomly assigned into four groups (n = 8): control (C)—rats without CSI and without AP; (S)—rats with CSI; (AP)—rats with AP; and (SAP)—rats with CSI and AP. The animals in the CSI groups were housed separately from the non-smokers.

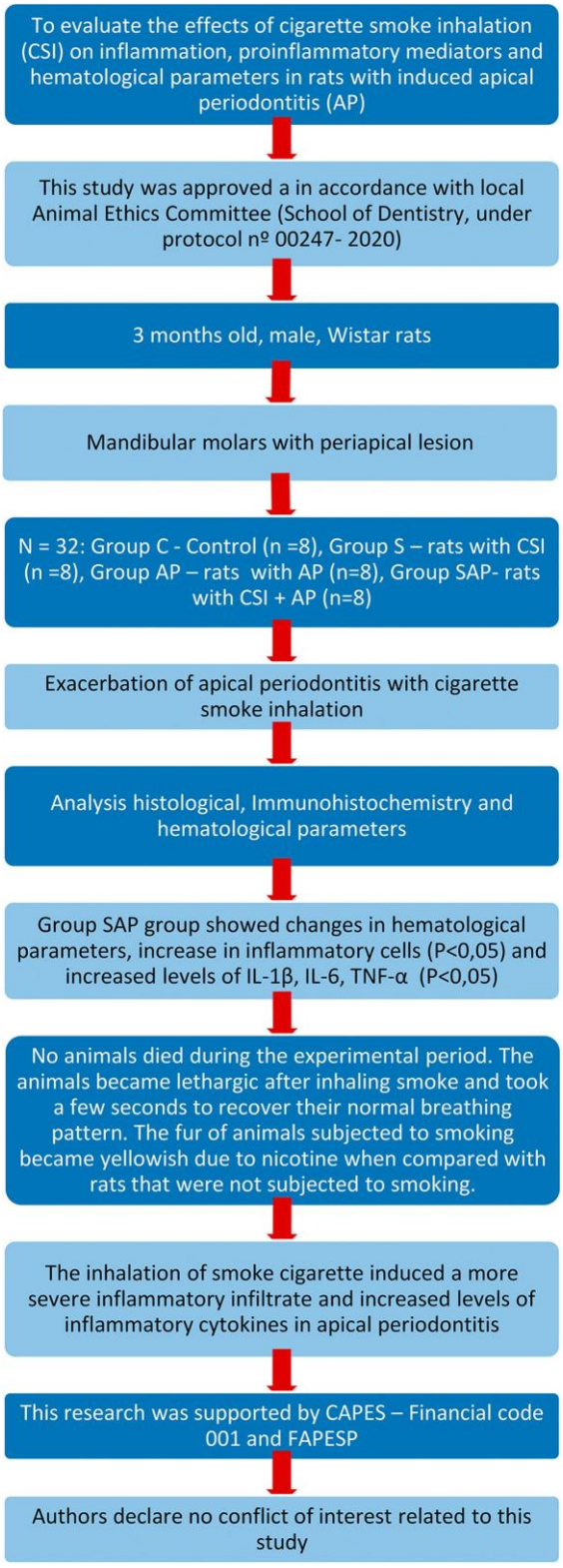

FIGURE 1 Study design flowchart: the Preferred Reporting Items for Animal Studies in Endodontology (PRIASE).

Cigarette smoke inhalation

Animals in the S and SAP group inhaled cigarette smoke via a full-body exposure chamber, simulating a passive smoker (César-Neto et al., 2003; Ercan et al., 2019; Nociti Júnior et al., 2002).

The smoking chamber was made of wood measuring 40 × 33 × 17 cm^3 with a glass lid for observing the animals and was divided into two compartments by a perforated screen. The largest compartment was used to maintain the animals, five at a time, and the smallest for burning cigarettes, which were attached to a wooden base. A fan was installed on the outer side of the main chamber. In the opposite wall of the fan, perforations were made for ventilation, generating a continuous flow of air inside the smoking chamber and keeping the cigarettes alight.

The animals were initially submitted to a period of gradual adaptation. On the first day, animals inhaled smoke of 10 cigarettes for 5 min once a day. Then, on the second day, animals inhaled cigarette smoke for 6 min and on the third day for 7 min (Carvalho et al., 2006). After adaptation period, the passive smoking protocol used was according to César-Neto et al. (2003), in which animals inhaled smoke of 10 cigarettes, for 8 min, three times a day, 7 days a week. The cigarette used was Marlboro (Phillips Morris; Rosa et al., 2017, Santiago et al., 2017), which contain 10 mg of tar, 10 mg of carbon monoxide and 0.8 mg of nicotine.

The animals in the smoking group (S and SAP) inhaled cigarette smoke 20 days before induction of apical periodontitis (SAP) and continued during the remaining experimental period of 30 days of pulp chamber exposed to oral environment, totalling 50 days of experiment (Figure S1).

Induction of apical periodontitis

The induction of periapical lesions was performed under general anaesthesia with the association of ketamine 75 mg/kg (Cetamin 10%, Syntec Brasil) and Xylazine 25 mg/kg (Xilazin 2%, Syntec Brasil), intramuscularly. The dental pulp of the lower right first molar was exposed to the oral environment with the aid of a carbon steel Ln Long Neck drill (Dentsply Sirona/Maillefer, Ballaigues, Switzerland) for a period of 30 days, to enable periapical lesion formation (Cantiga-Silva et al., 2021; Cintra et al., 2016).

Blood sample collection

At the end of the experimental period (Day 50), cardiac blood samples were collected from all groups, under general anaesthesia, and euthanized by exsanguination for evaluation of the red series: red blood cells (He), haemoglobin (Hb), globular volume (GV) and total plasma

protein (PPT); and white series: leukocytes, neutrophils, lymphocytes, monocytes and eosinophils. Blood samples were stored in tubes containing ethylenediaminetetraacetic acid and immediately transferred to a blind technician for processing.

Histological analysis

The mandibles were removed and sectioned in half, and the right hemimandibulae were stored in 10% formaldehyde solution for 24 h and decalcified in 10% buffered EDTA (Sigma-Aldrich), changed once a week for 3 months. After conventional laboratory processing, samples were embedded in paraffin. Then, semi-serial sections of 5 μm thick were cut and stained with haematoxylin and eosin. The distal root of the lower right first molar was standardized for all analyses (Cintra et al., 2016; Dal-Fabbro et al., 2019).

The intensity of the periapical inflammatory infiltrate was analysed in C, S, AP and SAP groups by a calibrated certified histologist (E.E.) blind to groups, using the Leica LAS X microscope (Leica Microsystems). At 10× magnification, the exit of the apical foramen was identified. At 400× magnification, cells were counted in an area encompassing the foramen exit region and the periapical tissue, assigning the following scores: no inflammation (score 1: 0 or few inflammatory cells), mild inflammation (score 2: <25 inflammatory cells), moderate inflammation (score 3: 25–125 inflammatory cells) and severe inflammation (score 4: >125 inflammatory cells; Cantiga-Silva et al., 2021).

Immunohistochemistry analysis

For immunohistochemistry analysis, three histological sections of each sample (5 μm thick), were used for each analysed interleukin. Positive immunoreactivity was defined as a brownish color in the cytoplasm of cells and in the extracellular matrix. A semi-quantitative analysis was performed to assess the intensity of the immunoreactive cells, and the extracellular matrix marking of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. A calibrated and blinded histologist (E.E.) evaluated the pattern of cytokine immunostaining and the following scores were assigned: Score 0: no immunolabelling (total absence of immunoreactive); Score 1: <20%—immunoreactive cells and low extracellular matrix labelling; Score 2: >20% and <40%—immunoreactive cells and low extracellular matrix labelling; Score 3: >40% and <60%—immunoreactive cells and moderate extracellular matrix labelling; Score 4: >60% and <80%—immunoreactive cells and high extracellular matrix labelling; and Score 5: >80%—immunoreactive cells and high extracellular matrix labelling (Cantiga-Silva et al., 2021).

Statistical analysis

The values were tabulated for each experimental group, and the data were analysed using Sigma Plot (V12.0 Systat Software Inc). After the Shapiro–Wilk test of normality, Mann–Whitney test was performed for non-parametric data and the pairwise analyses of Student's *t*-test for parametric data, with a significance level of $p < .050$.

RESULTS

Clinical observations

No animals died during the experimental period. The animals became lethargic after inhaling smoke and took a few seconds to recover their normal breathing pattern. The fur of animals subjected to smoking became yellowish due to nicotine when compared to rats that were not subjected to smoking. The concentration levels of nicotine and cotinine observed in the animals' blood after 20 days of CSI indicated that animals were classified as smokers at the moment of AP induction.

Histological and immunohistochemical analysis

Groups C and S did not present inflammatory response in the periapical region (score 1). The groups with apical periodontitis (AP and SAP) presented a chronic inflammatory infiltrate with necrotic areas. The SAP group presented an inflammatory infiltrate with more intense response (score 4) when compared to the AP group (score 3; $p = .010$; Figure 2; Table 1).

Immunohistochemical analysis showed increased levels of IL-1β, IL-6 and TNF-α cytokines in the AP and SAP groups; however, immunolabelling showed significant increase in all three cytokines in the SAP group, with high or extremely high scores when compared to the AP group (Figure 3; Table 1).

Haematological analysis

Table 2 shows the blood count values. The number of red blood cells (He), Hb and GV increased in the SAP group when compared to the AP group ($p < .050$). No statistical

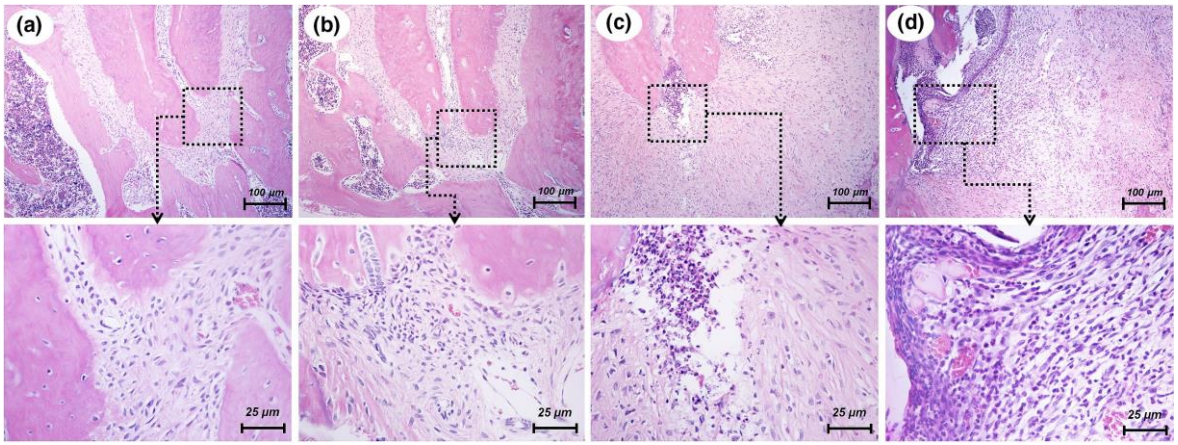

FIGURE 2 Histological sections of the periapical region (H&E). Group C (a); Group S (b); Group AP (c) and Group SAP (d). (a–d: 50× and 400× magnification).

TABLE 1 Median scores, range and *p*-values of histologic and immunohistochemical findings in rats from both groups.

Histologic criteria	Experimental groups				Statistical analysis
	C	S	AP	SAP	
Inflammatory infiltrate	1 (1-1)	1 (1-2)	3 (3-4)[a]	4 (4-4)[b]	Mann–Whitney $p = 0.010$
Immunoreactive cells					
IL-1β	1 (1-1)	1 (1-1)	2 (2-3)[a]	4 (3-4)[b]	$p = <0.001$
IL-6	1 (1-1)	1 (1-1)	3 (3-4)[a]	4,5 (4-5)[b]	$p = <0.002$
TNF-α	1 (1-1)	1 (1-1)	3 (3-4)[a]	4 (4-5)[b]	$p = <0.003$

Different superscript letters represent statistically significant differences.

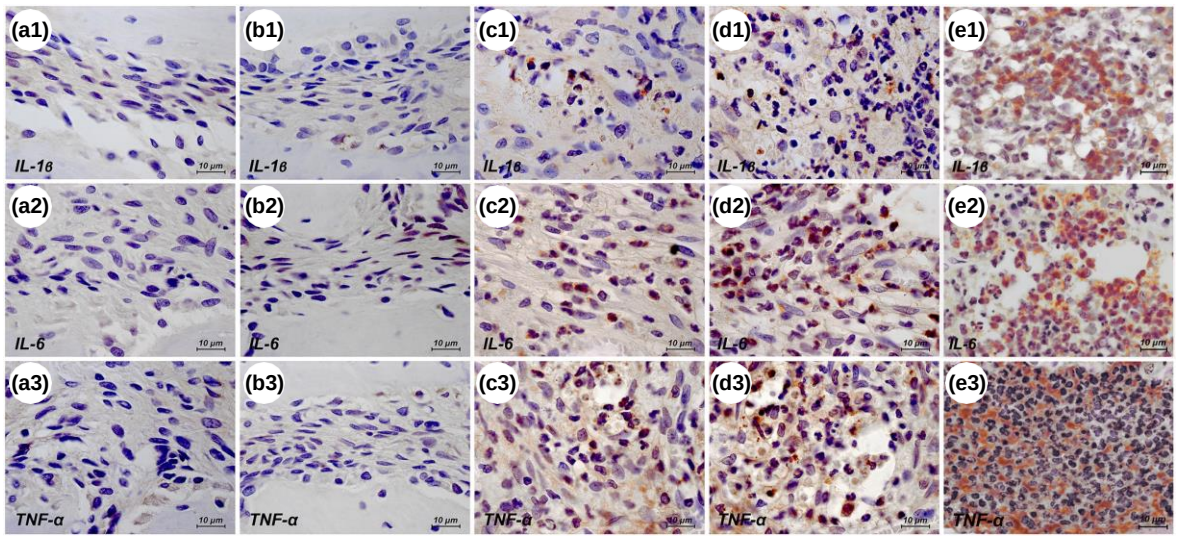

FIGURE 3 Representative images of immunohistochemical analysis of C (a1–a3), S (b1–b3), AP (c1–c3), SAP (d1–d3) and positive control (e1–e3) groups. Immunolabelling pattern for cytokines IL-1β (a1–e1), IL-6 (a2–e2) and TNF-α (a3–e3). (H&E staining, 1000× increase).

difference was shown for the concentration of total plastic proteins when comparing groups AP and SAP ($p = .072$).

Regarding inflammatory cells, the number of leukocytes was similar in AP and SAP groups ($p = .526$). When comparing groups with no apical lesion (C and S), the number of leukocytes was lower in S group ($p = .023$).

The SAP group presented a significant higher number of neutrophils in comparison with AP group ($p = .014$).

TABLE 2 Mean and standard deviation of blood parameters of the experimental groups.

	Experimental groups					
Blood parameters	**C**	**S**	**Statistical analysis**	**AP**	**SAP**	**Statistical analysis**
He ($\times 10^6$/μl)	7.96 ± 0.16[a]	7.60 ± 0.23[b]	0.005	8.00 ± 0.46[a]	8.65 ± 0.34[b]	0.010
Hb (g/dl)	16.51 ± 0.64[a]	16.27 ± 0.87[a]	0.565	16.25 ± 0.67[a]	17.41 ± 0.67[b]	0.007
VG	47.71 ± 1.38[a]	46.42 ± 1.98[a]	0.185	47.00 ± 2.44[a]	51.00 ± 2.16[b]	0.007
PPT (g/dl)	7.37 ± 0.31[a]	7.28 ± 0.12[a]	0.508	7.60 ± 0.38[a]	7.25 ± 0.25[a]	0.072
Leukocytes %	4.04 ± 0.47[a]	3.45 ± 0.36[b]	0.023	4.30 ± 0.50[a]	4.47 ± 0.48[a]	0.526
Neutrophils %	18.42 ± 8.84[a]	23.85 ± 8.57[a]	0.266	19.42 ± 3.50[a]	29.71 ± 8.88[b]	0.014
Lymphocytes %	76.00 ± 10.04[a]	78.28 ± 7.78[a]	0.642	77.71 ± 3.90[a]	82.42 ± 4.23[a]	0.051
Monocytes %	1.00 ± 0.00[a]	0.57 ± 0.53[a]	0.055	0.42 ± 0.53[a]	0.85± 0.70[a]	0.218
Eosinophils %	0.57 ± 0.78[a]	0.71 ± 0.48[a]	0.690	1.00 ± 0.81[a]	0.57 ± 0.53[a]	0.267

Different superscript letters represent statistically significant differences.

Lymphocytes were also higher in the SAP group, but there was no statistical difference between group AP ($p = .051$). The values of monocytes and eosinophils were similar between the groups analysed, showing no statistical difference.

DISCUSSION

The results from inflammatory, pro-inflammatory cytokine and blood profile analysis evidenced that CSI systemically affected the development of periapical lesion in rats, thus rejecting the null hypothesis.

Animal models were previously used to assess the mechanisms by which CSI can interfere with the body's response (Carvalho et al., 2006; César-Neto et al., 2003; Ercan et al., 2019). Also, a rat model for induced AP (Cantiga-Silva et al., 2021; Cintra et al., 2016; Dal-Fabbro et al., 2019, 2021) has been used, as rats have dental anatomy and oral microbiota similar to humans (Yoneda et al., 2017).

The methodology used allowed the simulation of passive smoking, as the animals were forced to breathe cigarette smoke in a closed environment. There were no deaths of animals during CSI. Disadvantages of this methodology may be related to possible eye irritation due to cigarette smoke, besides the hotter environment, caused by lit cigarettes, which may alter animal stress. The dosage of serum levels of nicotine and cotinine of animals submitted to this protocol (S and SAP) confirmed that the method produces serum levels of nicotine and cotinine compatible with human smokers.

Cigarette smoke contains more than 4000 toxic chemicals to the body such as carbon dioxide, carbon monoxide, tar and nicotine (Rennard, 2004; Sopori, 2002). After the cigarette is burned, these chemical compounds dissolve in the fluids of the oral epithelial linings and airways and spread throughout the body via the circulatory system (Lee et al., 2012). The consequences of chronic exposure to cigarette smoke lead to effects on systemic inflammation, promoting the activation, expansion and secretion of inflammatory mediators, which in turn lead to chronic recruitment of cells immune to inflammation.

The CSI protocol used in this study was based upon previous reports (César-Neto et al., 2003; Nociti Júnior et al., 2002), in which authors concluded that the volume of smoke from 10 cigarettes was the maximum amount animals could withstand for 8 min, three times a day, for 50 days. Serum assessment of cotinine levels, a nicotine metabolite, demonstrated that this intermittent CSI protocol produced serum levels correlated with active human smokers consuming 10 to 20 cigarettes per day (Gonzalez et al., 1996).

As observed in this study, the SAP group presented a higher number of inflammatory cells in the periapical region resulting not only from inflammation caused by AP, but also due to the inhalation of cigarette smoke. Neutrophils are one of the main components of inflammatory AP infiltrate in the acute phase, and they are known as the first in the line of defence that contributes to the progression of AP due to promoting chemotaxis and tissue damage (Braz-Silva et al., 2019). Segura-Egea et al. (2015) suggested that smoking has a negative effect on periradicular tissues by reducing blood supply and, consequently, restricting the distribution of nutrients and oxygen, in addition to promoting an intense inflammatory response in the periapical region. Furthermore, the study by Gomes et al. (2013) suggested that AP may contribute to a systemic immune response, potentially leading to increased systemic inflammation. When associated, these factors promote a more intense inflammatory response with consequent destruction of periapical tissues.

Changes in haematological index are used as physiological markers of organ and tissue damage. The pharmacological actions of nicotine led to changes in haematological parameters. The haemoglobin count was higher in the smoker group with apical periodontitis (SAP), and this fact is due to the combination of carbon monoxide (CO) with haemoglobin forming carboxyhaemoglobin (COHb). Haemoglobin affinity with CO is 200 times greater than its affinity with oxygen (Carallo et al., 1988). Thus, CO displaces oxygen from the haemoglobin, reducing the transportation of oxygen to tissues (Cronenberger et al., 2008), followed by bone marrow reducing its production of red blood cells in an attempt to increase the number of haemoglobin. It is believed that this increase in haemoglobin concentration is a human body's compensatory mechanism to supply the lack of oxygen (Aitchison & Russell, 1988).

In an experiment evaluating the effects of water pipe smoking on haematological parameters in rats, the authors showed significantly higher values in red blood cells and haemoglobin in the group of rats exposed to water pipe smoking in relation to the control (Miri-Moghaddam et al., 2014), results that corroborate the findings of our study. Malenica et al. (2017) also observed an increase in Hb in the blood of patients belonging to the smoker group in relation to non-smokers. The literature shows that haemoglobin and haematocrit levels increase significantly in individuals who smoke more than 10 cigarettes per day (Whitehead et al., 1995). Lakshmi et al. (2014) demonstrated that, in addition to the increase in haematocrit and Hb values in smokers, red cell count seems to increase as the intensity of smoking increases.

Leukocyte levels are also increased in smokers and these values depend on the frequency of smoking (Kurtoglu et al., 2013), as seen in our results. A group of researchers suggested that this increase in leukocyte count may occur due to the release of nicotine-induced catecholamines (Friedman et al., 1973). Furthermore, the irritation caused by the cigarette smoke on the bronchial tree and inflammation may be factors that contribute for a higher leukocyte count (Calapai et al., 2009; Malenica et al., 2017).

Cigarette smoke also stimulates the oxidative explosion of neutrophils, promoting the release of reactive oxygen species that are highly destructive to periapic tissues and altering the release of cytokines by inflammatory cells (Mirbod et al., 2001).

The interleukins IL-1β, IL-6 and TNF-α are pro-inflammatory cytokines involved in the AP development process and their levels seem to be altered by the influence of smoking. Abduljabbar et al. (2018) studied peri-sulcular fluid implant of smokers and non-smokers, observing an increase in these cytokines in the smokers' group.

Cytokines are greatly significant to evaluate the host response to infection, degradation and tissue repair. In the AP group, the values of the interleukins IL-1β, IL-6 and TNF-α were higher when compared to groups C and S ($p < .050$). The SAP group expressed a higher labelling of these interleukins when compared to the other groups ($p > .050$). On the one hand, this increase occurs because of the activation of inflammatory cells in response to bacterial lipopolysaccharide—releasing more cytokines in an attempt to fight infection (Johnson & Organ, 1997)—, and, on the other hand, by the harmful action of cigarette smoke (Mirbod et al., 2001).

In an *in vitro* study, nicotine was able to increase IL-6 release by osteoblasts (El-Ghorab et al., 1997), as well as increase TNF-α levels in gingival fluid in patients who had chronic periodontitis and used tobacco (BinShabaib et al., 2019; Boström et al., 1998).

In the evaluation of IL-1β mRNA expression in patients with chronic periodontitis, the expression increased twice as much when compared to the control group. Chronic periodontitis associated with smoking caused mRNA expression to increase eight times more (Meenawat et al., 2015), corroborating the findings of our study. Smokers with chronic periodontitis presented more periodontal destruction and systemic inflammatory markers than non-smokers with chronic periodontitis (Vijayakumar et al., 2020).

One of the explanations for this increase in cytokines in the SAP group may be due to the negative influence of smoking on periapical tissues, since nicotine can alter the immune response—including cytokine responses—by activating the cholinergic anti-inflammatory pathway (Pavlov & Tracey, 2005; Tracey, 2007).

Also, the fact that smoking would increase the formation and accumulation of advanced glycation end products (AGE) in oral tissues (Biswas et al., 2013; Katz et al., 2005, 2007; Xu et al., 2015) would be another factor involved in this process, modulating the inflammation of periodontal tissues by the nuclear factor kappa B (NF-κB) pathway (Xu et al., 2015). Moreover, nicotine can positively regulate the expression of AGE receptors by stimulating the formation of reactive oxygen species compromising oral tissues and increasing bone loss (Katz et al., 2005). Future studies should be carried out to assess bone metabolism in passive smokers with AP.

Health professionals should advise their patients about the harmful effects of smoking, warning about the damages that cigarette components may cause, jeopardizing oral and systemic health.

CONCLUSION

The smoke chamber methodology allowed the simulation of passive smokers in rats, as observed by elevated nicotine levels. CSI in rats induced a more intense periapical inflammatory reaction, higher levels of pro-inflammatory cytokines and haematological changes, aggravating AP.

AUTHOR CONTRIBUTIONS

Ana Maria Veiga Vasques contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, writing—original draft, writing—review and editing, visualization and funding acquisition. Ana Claudia Rodrigues da Silva contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, writing—original draft, writing—review and editing and visualization. Carlos Roberto Emerenciano Bueno and Marina Tolomei Sandoval Cury contributed to the data curation, validation, investigation, writing—review and editing and visualization. Edilson Ervolino contributed to the validation, formal analysis, resources, writing—review and editing and visualization. Luciano Tavares Angelo Cintra contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, writing—review and editing, visualization, supervision and project administration. Eloi Dezan Junior contributed to the conceptualization, methodology, validation, formal analysis, investigation, resources, writing—original draft, writing—review and editing, visualization, funding acquisition, supervision and project administration.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this study.

ETHICAL APPROVAL

Experimental research approved by the Animal Ethics Committee (School of Dentistry, under protocol no. 00247-2020).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Artigo 2

Bone resorption in apical periodontitis enhanced by cigarette smoke inhalation: histometric, immunohistochemical and microtomographic analysis in rats

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Running Title: Bone resorption exacerbated by smoking

Keywords: apical periodontitis, smokers, bone resorption, RANKL/OPG, micro-computed tomography

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Conflict of interest

Authors declare no conflict of interest related to this study.

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ABSTRACT

Objective

To evaluate the effects of cigarette smoke inhalation (CSI) associated with apical periodontitis (AP) induced in rats using markers of bone turnover and histometric, immunohistochemical, and microtomographic analysis.

Methodology

In total, 32 three-month-old male Wistar rats were divided into four experimental groups (n=8): C - Control; CSI - rats with CSI; AP - rats with AP; and CSI + AP - rats with CSI and AP. The rats of the CSI and CSI + AP groups inhaled cigarette smoke while remaining inside a smoke chamber for eight minutes, three times per day, for 50 days. After 20 days of smoke inhalation, the rats of the AP and CSI + AP groups had the pulps of their first right lower molar exposed to the oral cavity for 30 days to induce AP. In these subsequent 30 days, the rats of the CSI and CSI + AP group continued with CSI. On day 50, they were euthanized, their blood was collected to evaluate serum alkaline phosphatase, calcium, phosphorus, nicotine, and cotinine levels, and their mandibles were removed for histological processing to evaluate bone resorption via histometric, immunohistochemical (RANKL/OPG), and microtomographic analysis. The Mann-Whitney test was applied for nonparametric data and Student's *t*-test was applied for parametric data, with significance level of $P<0.05$.

Results

Serum calcium concentration was lower in the CSI + AP group compared to the AP group ($P=0.036$). Bone resorption occurred in the AP and CSI + AP groups. The histometric analysis showed a larger area of bone resorption for the CSI + AP group ($P=0.004$) and the microtomographic analysis showed greater volume of resorption ($P<0.001$) compared to the AP group. The CSI + AP group had a high immunostaining pattern for RANKL compared to the AP group ($P<0.001$), which had a higher immunostaining pattern for OPG ($P=0.015$) compared to the CSI + AP group.

Conclusion

Apical periodontitis associated with cigarette smoke inhalation resulted in a larger

area of resorption of alveolar bone tissue, shown in the histometric and microtomographic analyses, and higher immunostaining for RANKL, causing an imbalance for the RANK /RANKL/OPG pathway, with predominance of osteoclastic activity.
Keywords: apical periodontitis, smokers, bone resorption, RANKL/OPG, micro-computed tomography

Introduction

Apical periodontitis (AP) is a response to root canal infection characterized by the inflammation and the resorption of the periradicular tissues (Nair 2004). When defense mechanisms fail to eliminate infection, the periapical tissues suffer resorption influenced by local and systemic factors (Xiong et al. 2007, Gomes-Filho et al. 2015).

Tobacco has long been considered an important factor for public health worldwide due to the risks to the oral and systemic health. Besides active smokers, this concern also applies to passive smokers since exposure to cigarette smoke is related to an increased risk of cancer and heart and respiratory diseases (Vasconcelos et al. 2006).

Nicotine inhaled by passive smokers goes through the brain, occupying nicotinic acetylcholine receptors (Brody et al. 2011), which suggests that exposure to cigarette smoke can facilitate the development and maintenance of nicotine dependence among passive smokers, via a biological pathway similar to that of active smokers. Cotinin, a metabolite of absorbed nicotine, acts as a biomarker of exposure to tobacco smoke, regardless of whether the smoke source is the main current (inhaled and then exhaled smoke) or lateral flow (smoke produced by the tip of a burning cigarette), being then a strong indicator of nicotine dependence (Muscat et al. 2009).

Previous studies (Nociti-Junior et al. 2002, Cesar-Neto et al. 2003) reported that inhaling cigarette smoke passively affected the osseointegration of implants inserted in an animal model due to bone defects caused mainly by nicotine. A systematic review on the prevalence of AP in smoking patients showed that smokers have a higher rate of apical periodontitis, as well as endodontic treatments (Pinto et al. 2020a).

Nicotine is the main toxic component of cigarette smoke and believed to promote changes in bone metabolism by modulating the proliferation and maturation of osteoblasts, thus altering the production of extracellular matrix (Walker et al. 2001, Akmal et al. 2004).

The receptor activator of the NF-κB ligand (RANKL), also known as osteoclast

differentiation factor (Yasuda et al. 1998), is the most potent factor to induce osteoclastogenesis. The natural receptor of RANKL is OPG (osteoprotegerin), known as osteoclastogenesis inhibitory factor (Hsu et al. 1999) since it prevents the differentiation and activation of osteoclasts when bound to RANKL. Thus, bone homeostasis is regulated by the balance between RANKL and OPG (Tohme et al. 1991). Studies have suggested that nicotine can promote imbalance for the RANK/RANKL/OPG system, considered the main pathway of activation of bone resorption (Walker et al. 2001).

Alkaline phosphatase, calcium, and phosphorus are also involved in bone homeostasis. Alkaline phosphatase is a membrane-bound glycoprotein released from polymorphonuclear neutrophils during inflammation and by osteoblasts during bone formation (Perinetti et al. 2008), whereas calcium and phosphorus have their levels increased during bone resorption (Penido & Alon 2012).

Another tool to determine bone loss are imaging tests. Computed microtomography (micro-CT) is the current gold standard for the analyses to determinate bone loss in animal models since, besides the three-dimensional acquisition of the structures, the images have high resolution and the software used allows manipulation in different orientation planes to a better visualization (Kim et al. 2021).

Thus, this study aimed to evaluate bone resorption caused by apical periodontitis associated with passive smoking in rats using markers of bone turnover (alkaline phosphatase, calcium, and phosphorus) and histometric, microtomographic, and immunohistochemistry analyses of RANKL and OPG cytokines. The null hypothesis tested in this study is that passive smoking cannot change bone resorption of apical periodontitis in rats.

Materials and Methods

Sample estimation

Sample size was estimated based on data from a previous study (Cosme-Silva et al. 2020). Considering an alpha error of 0.05 and power (1-β) of 0.95 to recognize a significant difference, a minimum of six animals per group was indicated.

Considering the possible animal deaths during the experimental period or laboratory processing, two more rats were added to each group, resulting in eight rats per group, totaling 32 animals.

Animals

After approval by the local ethics committee (Araçatuba Dental School, no. 00247/2020), 32 three-month-old male rats (*Rattus norvegicus*, Wistar), weighing 200-250g each, were housed in a temperature-controlled environment (22°C ± 1°C), with a light cycle of 12 hours of brightness and 12 hours of darkness, access to food and water *ad libitum* throughout the experimental period. The animals were randomly distributed into four experimental groups (n=8) according to cigarette smoke inhalation (CSI) and induction of apical periodontitis (AP) into: Control group (C): animals without local and systemic alterations; CSI group: animals exposed to CSI; AP group: animals with only AP induction; CSI + AP group: animals exposed to CSI and AP induction. The animals of the CSI and CSI + AP groups were housed separately from the other groups (non-smokers).

Cigarette smoke inhalation protocol

The protocol followed was proposed by Cesar-Neto et al. (2003), simulating a passive smoker, in which the animals of the CSI and CSI + AP groups inhaled the smoke of 10 cigarettes (Marlboro, Phillips Morris, Santa Cruz do Sul, RS, Brazil), for eight minutes, three times per day, for 50 days.

Induction of apical periodontitis

After 20 days of cigarette smoke inhalation, the animals in the AP and CSI + AP groups were anesthetized intramuscularly with Ketamine 75 mg/kg-1 IM (Cetamin 10%; Syntec Brasil, São Paulo, Brazil) and Xylazine 25 mg/kg-1 IM (Xilazin 2%; Syntec Brasil), so that the pulp of the first lower right molar could be exposed to the oral cavity with the aid of a 0.5 mm diameter carbide bur (Jet Carbide 1/4; Kavo Kerr Group, Orange, CA, USA) and an operating microscope. The molars were exposed to the oral cavity for 30 days to induce apical periodontitis.

Blood sample collection for analysis of alkaline phosphatase, calcium, and phosphorus

At the end of the 50th day of cigarette smoke inhalation, the animals of all experimental groups were anesthetized, and cardiac puncture was performed to collect 5 ml of blood from each animal. The samples were conditioned in specific tubes and sent to a technician for laboratory processing.

Serum nicotine and cotinine levels

The serum obtained after the processing of blood samples was used for the analysis of nicotine and cotinine concentrations by high-performance liquid chromatography (HPLC).

Histometric analysis

After blood collection, the animals were euthanized by exsanguination. The right mandibles were removed and fixed in 10% formaldehyde, decalcified in EDTA solution, and histologically processed. The pieces obtained were cut in semiseries with 5 μm thickness and stained with hematoxylin-eosin (H&E). Histometric analysis was performed to verify any difference in bone structure loss in the periapical region due to smoking. The area of the periapical lesion was estimated considering the external surface of the cementum, periodontal ligament, and alveolar bone. A Leica LAS X microscope (Leica Microsystems, Nussloch - Germany) was used and the values were expressed in square micrometers (μm^2) as measurements of the apical lesion area (Cantiga-Silva et al. 2021).

Immunohistochemical Analysis

For the immunohistochemical analyses, three histological sections from each animal (5 μm thick) were obtained for staining with the RANKL and OPG cytokines. Semiquantitative analysis was performed to evaluate the intensity of immunoreactive cells and staining of the extracellular matrix. The brownish color of cells and extracellular matrix was considered as positive immunoreactivity, assigning the

following scores: score 1 (extremely low immunostaining), score 2 (low immunostaining), score 3 (moderate immunostaining), score 4 (high immunostaining), and score 5 (extremely high immunostaining) (Cantiga-Silva et al. 2021).

The receptor activator of the kappa-B ligand of the nuclear factor (RANKL) and osteoprotegerin (OPG) were analyzed in the periapical region, with a 1000× zoom under the microscope (Leica Microsystems, Wetzlar, Germany).

Microtomographic analysis

The right hemimandibles were digitized before histological processing using the μCT system (Bruker SkyScan 1174, Aartselaar, Belgium), with the following configurations: 50 kV, 800 μA, 0.5º rotation step, 7000 millisecond exposure, 3 frames averages, 14 a pixel image, and 16 camera pixel size, no filter.

The images were reconstructed using the NRecon software (v 1.6.9.8) with the following parameters: 3 of ring artefact correction, 86% of beam hardening correction, -24 of post alignment, and 7 of smoothing for all images. Then, the images were analyzed in the CTAn program (v 1.14.4.1) with sagittal view. The region of interest (ROI) was manually selected including the empty space of periradicular destruction and/or periodontal ligament space between the distal and mesial roots of the first right lower molar. The ROI was selected from the section where the roots were encapsulated by the alveolar bone and continued until the last section, when the alveolar bone appeared again. The relationship between tissue volume (TV) and alveolar bone volume (BV) was estimated to obtain the volume of bone destruction caused by AP.

Statistical analysis

The values were tabulated for each experimental group and the data were analyzed using Sigma Plot (V12.0 Systat Software Inc, San Jose, CA, USA). After the Shapiro-Wilk test of normality, the Mann-Whitney test was performed for nonparametric data and the pairwise analyses of Student's *t*-test for parametric data, with a significance level of $P<0.050$.

Results

Serum alkaline phosphatase, calcium, and phosphorus analysis

The activity of alkaline phosphatase was higher in the CSI + AP group (81.18 ± 13.39), with no statistical difference when compared with the AP group (P=0.434) (Table 1). Serum calcium levels were significantly lower in the CSI + AP group when compared with the AP group (P=0.036). The plasma concentration of phosphorus remained constant in all experimental groups (P>0.05).

Serum nicotine and cotinine levels

Serum nicotine and cotinine levels were below the detectable limit for the C and AP groups (nonsmokers). Table 1 shows that the CSI and CSI + AP groups (passive smokers) had detectable values of nicotine and cotinine. The mean nicotine values were 26.24 ng/ml ± 9.58 and 28.97 ng/ml ± 7.04 and the mean cotinine values were 294.06 ng/ml ± 64.86 and 332.71 ng/ml ± 35.34 for the CSI and CSI + AP groups, respectively.

Table 1. Mean and standard deviation of biochemical concentration of markers of bone turnover, nicotine, and cotinine.

Biochemical Analyses	C Group	CSI Group	P-value	AP Group	CSI + AP Group	P-value
Alkaline phosphatase (UL^{-1})	72.64 ± 14.85[a]	60.64 ± 13.14[a]	0.213	73.40 ± 16.34[a]	81.18 ± 13.39[a]	0.434
Calcium (mg dL^{-1})	10.52 ± 0.29[a]	10.74 ± 0.64[a]	0.508	10.30 ± 0.14[a]	10.02 ± 0.20[b]	0.036
Phosphorus (mg dL^{-1})	8.22 ± 1.82[a]	11.53 ± 4.02[a]	0.132	10.63 ± 3.25[a]	8.58 ± 1.65[a]	0.244
Nicotine (ng/ml)	Undetectable	26.24 ± 9.58	<0.001	Undetectable	28.97 ± 7.04	<0.001
Cotinine (ng/ml)	Undetectable	294.06 ± 64.86	<0.001	Undetectable	332.71 ± 35.34	<0.001

Different superscript letters represent statistically significant differences P<0.05.

Histometric analysis

The C and CSI groups showed a preserved periodontal ligament space and alveolar bone. The groups with periapical lesion, AP and CSI + AP, showed destruction of the periodontal ligament space and alveolar bone resorption. The area

of bone resorption was greater in the CSI + AP group when compared with the AP group (P=0.004) (Table 2).

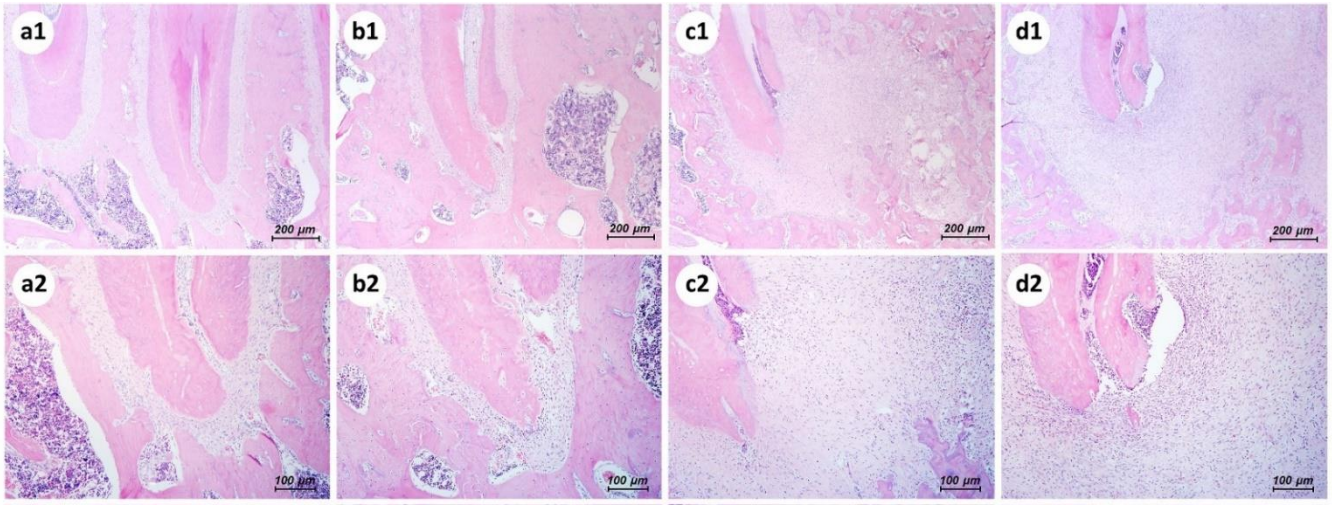

Figure 1. Histological sections (H&E) of the periapical region. **C** group: a1-a2: preserved periodontal ligament space and alveolar bone; **CSI** group: b1-b2: preserved periodontal ligament space and alveolar bone; **AP** group: c1-c2: destruction of the periodontal ligament and alveolar bone space. **CSI + AP** group: d1-d2: extensive area of bone resorption. (a1-d1: 50x; a2-d2: 100x).

Immunohistochemical Analysis

Figure 2 shows that the immunoreactivity for RANKL was higher in the AP and CSI + AP groups, being more intense (score 4) in the CSI + AP group (d2) (P<0.001). The groups without periapical lesion, C and CSI, had an extremely low immunostaining pattern (score 1). Immunoreactivity for OPG was higher in the AP and CSI + AP groups, having a high immunostaining pattern (score 4) in the AP group (figure 2.c1) (P=0.015).

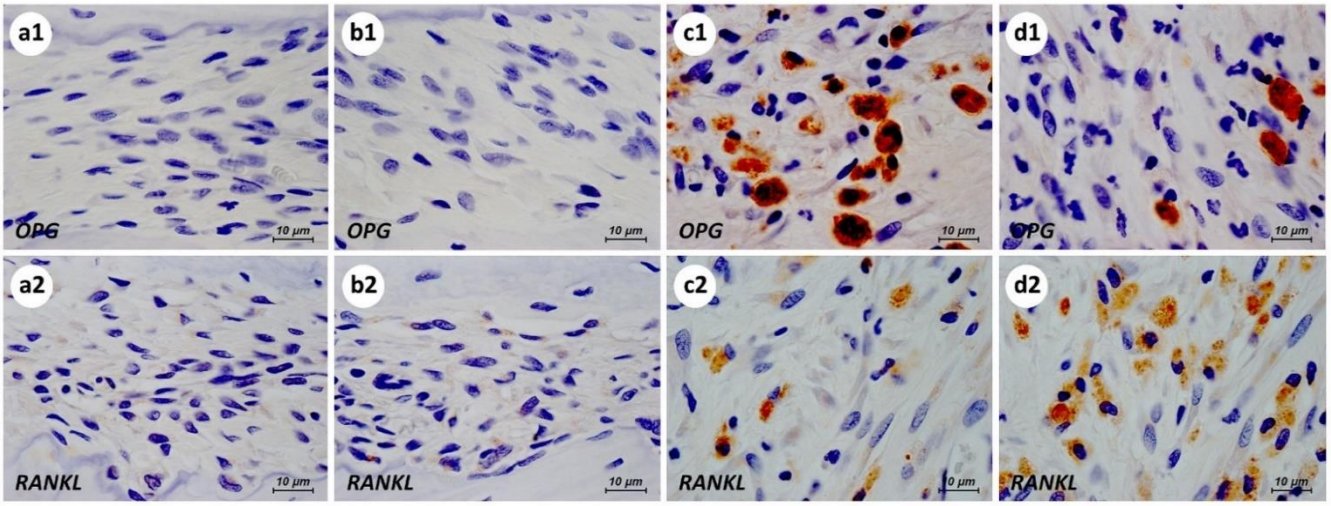

Figure 2. Representative images of the immunohistochemical analysis of the periapical region of groups **C**: (a1, a2); **CSI**: (b1, b2); **AP**: (c1, c2); **CSI + AP**: (d1, d2). Immunostaining for OPG (a1, b1, c1, d1) and RANKL (a2, b2, c2, d2) cytokines. (H&E staining, 1000x).

Micro-CT Analysis

The CSI + AP group showed a greater volume of bone resorption compared to the AP group (P<0.001) (table 2), characterized by the hypodense image between the distal and mesial roots of the first lower molar (figure 3.d) (yellow dotted line). In the groups without induction of apical periodontitis, C and CSI, the integrity of the periodontal ligament space and the preservation of the lamina dura and alveolar bone can be observed (figure 3.a.b).

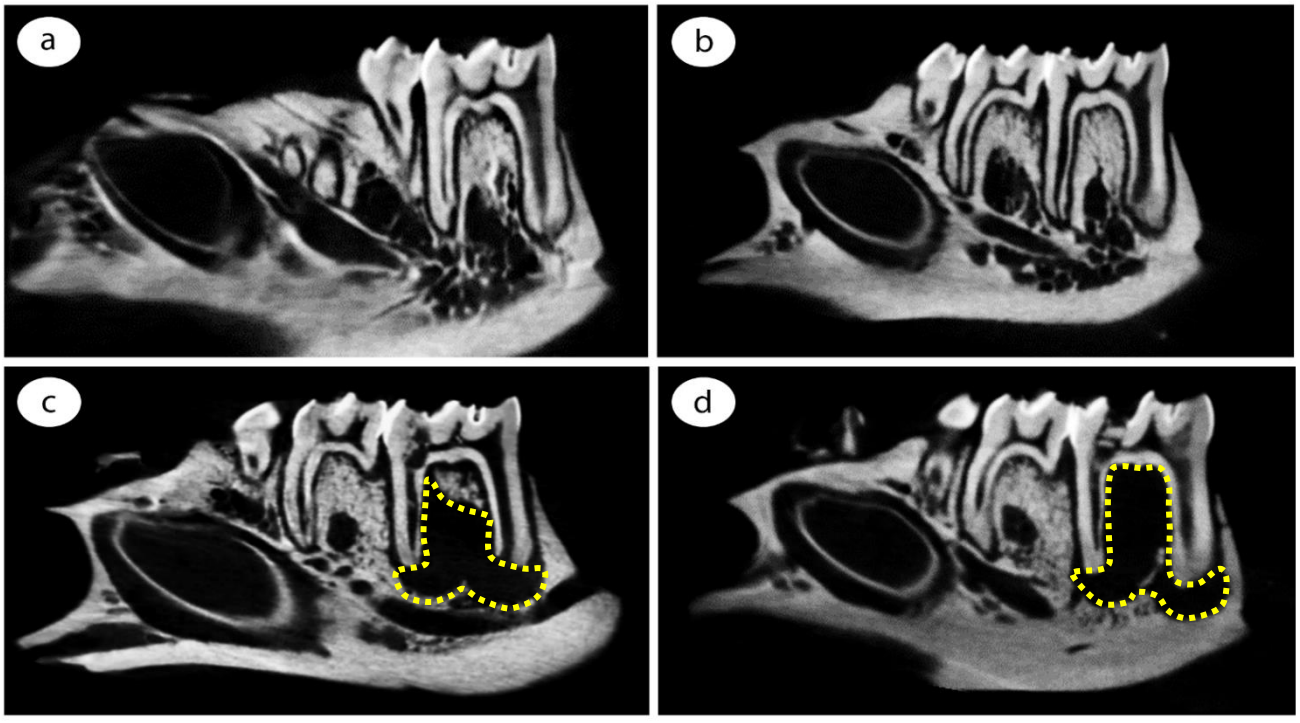

Figure 3. Images representing micro-CT, by sagittal view, of the first right lower molar of the **a**: Control group: preserved periodontal ligament space, lamina dura, and alveolar bone; **b**: CSI group: preserved periodontal ligament space, lamina dura, and alveolar bone; **b**: AP group: hypodense image (yellow dotted line) characteristic of bone resorption; **d**: CSI + AP group: hypodense image (yellow dotted line) between the mesial and distal roots of the first molar characterizing the volume of bone resorption.

Table 2. Median values, standard deviation, and mean histometric, immunohistochemical, and microtomographic findings.

Analyses	Experimental Groups				P-value
	C	**CSI**	**AP**	**CSI + AP**	
Histometric	3.42 ± 0.20	3.43 ± 0.23	6.18 ± 0.81[a]	7.32 ± 0.45[b]	***t*-test** P=0.004
Immunohistochemical					**Mann-Whitney**
OPG	1 (1-1)	1 (1-1)	4 (3-4)[a]	3 (3-2)[b]	P=0.015
RANKL	1 (1-1)	1 (1-1)	3 (3-4)[a]	4 (4-5)[b]	P=<0.001
Micro-CT volume	1.20 ± 0.40	1.72 ± 0.46	4.60 ± 1.38[a]	8.68 ± 0.70[b]	***t*-test** P=<0.001

Different superscript letters represent statistically significant differences (AP vs. SAP), P<0.05.

Discussion

The alveolar bone is a connective tissue supporting the teeth and is subject to the mechanical and pathological stress of oral infections, such as apical periodontitis, which induces an inflammatory response with consequent bone loss. Smoking had adverse effects on bone metabolism, facilitating the resorption of periapical bone tissue, thus rejecting the null hypothesis tested in this study.

The animal model adopted in this study has been used in research to induce apical periodontitis (Azuma et al. 2017, Dal Fabbro et al. 2019, Cantiga-Silva et al. 2021) and for exposure to cigarette smoke (Nociti-Júnior et al. 2002, Cesar-Neto et al. 2003).

New methodologies have been used for the early identification of diseases

that affect the periodontium, such as biochemical analyses. The biochemical method in periodontal diagnosis focuses on determining the constituents involved in local cellular metabolism that reflects the periodontal state (Champagne et al. 2003). Alkaline phosphatase is one of the constituents. It is a membrane-bound glycoprotein released from polymorphonuclear neutrophils during inflammation, osteoblasts during bone formation and fibroblasts of the periodontal ligament during periodontal regeneration (Perinetti et al. 2008). The potential value of alkaline phosphatase as a marker of periodontal disease activity has been identified by several researchers (Ishikawa & Cimasoni 1970, Nakashima et al. 1994, Chapple et al. 1999). The results of this research showed a higher serum alkaline phosphatase concentration in the CSI + AP group, but with no significant difference compared to the AP group.

Grover et al. (2016) analyzed the correlation of alkaline phosphatase activity in gingival crevicular fluid (GCF) with the clinical parameters of periodontal inflammation in smokers with chronic periodontitis and found that periodontal therapy reduced alkaline phosphatase levels. Chapple et al. (1999) observed that the total levels of alkaline phosphatase of the GCF may serve as a predictor of future or current activity of the disease.

Serum calcium levels decreased in the CSI + AP group when compared with the AP group. This can be explained by nicotine, as it reduces the body's calcium absorption, which affects osteogenesis and bone mineralization (Su 2004). Serum phosphorus levels remained constant in all experimental groups. Meral et al. (2015) observed similar results when compared the serum phosphorus levels in smokers and nonsmokers.

Serum nicotine and cotinine evaluation is also important. Cotinine is a nicotine metabolic, with a low rate of metabolism and renal excretion, thus its half-life is ten times greater than that of nicotine, being then a useful marker for evaluating tobacco dependence (Cuff et al. 1989). The evaluation of the serum cotinine level showed that this protocol of cigarette smoke inhalation produced serum levels correlated with human smokers who consume an average of 10 to 20 cigarettes per day (Gonzalez et al. 1996).

Serum nicotine and cotinine levels for non-smoking groups (C and AP) were lower than the detectable limit. In the group of passive smokers (CSI and CSI + AP) the values were 26.24 ± 9.58 ng/mL and 28.97 ± 7.04 ng/mL, respectively, for nicotine and 294.06 ± 64.86 ng/mL and 332.71 ± 35.34 ng/mL, respectively, for cotinine. Jain et al. (2021) evaluated serum cotinine concentration in different ethnic groups, with different types of cigarettes and tobacco, and found that cigarette users had an average 159.4 ng/mL serum cotinine concentration. In this study, people with serum cotinine levels above 3.3 ng/mL were classified as smokers.

During histometric analysis, the CSI + AP group had a larger area of bone resorption characterized by the destruction of periodontal ligament space, lamina dura, and alveolar bone. Osteoclasts are responsible for degrading the alveolar bone in apical periodontitis. For bone homeostasis, a balance between the processes of deposition and resorption of bone tissue is necessary, that is, a balance between the activity of osteoblasts and osteoclasts. A study by Laroche et al. (1994) showed that the components of cigarette smoke can decrease osteoblast activity. This may explain our findings since we observed a larger area of bone resorption in the CSI + AP group due to the periapical lesion with the components of cigarette smoke, which caused an imbalance of bone homeotasis with greater osteoclastic activity.

This becomes even more evident during the microtomographic analysis, considered the gold standard for the evaluation of periapical lesions in rats (Kang et al. 2013, Kalatzis-Sousa et al. 2017), in which the CSI + AP group presented greater volume of bone resorption due to cigarette smoke components and osteoclasts. This association enhanced bone resorption, corroborating the findings of Pinto et al. (2020b), who also observed alveolar bone destruction with the microtomography of nicotinic rats with apical periodontitis.

During the development of apical periodontitis, expressed pro-inflammatory cytokines are fundamental in the recruitment and activation of osteoclasts via the RANK/RANKL/OPG pathway (Hofbauer et al. 2004). The RANKL/OPG system is essential for regulating bone metabolism (Boyce & Xing 2008), since when RANKL binds to its NF-kB receptor (RANK), it stimulates the differentiation of osteoclasts and initiates bone resorption. However, OPG protects bone tissue from resorption by

binding to RANKL.

This study showed a significant increase in RANKL expression in the group with apical periodontitis associated with cigarette smoke inhalation. This can be explained by the local and systemic factor. Apical periodontitis is a local factor, in which many bacteria can be found (Hou et al. 2022), mainly gram-negative, which stimulate bone loss due to the lipopolysaccharide (LPS) constituent of its wall, stimulating then bone resorption by the activation of osteoclasts (Inada et al. 2006). Also, pro-inflammatory cytokines in apical periodontitis are expressed as IL-6, IL1-β, and TNF-α.

In a recent study (Vasques et al. 2022) on the expression of inflammatory cytokines in smoking rats with apical periodontitis, showed a higher expression of IL-6, IL1-β and TNF-α. Besides being related to the development of apical periodontitis, these cytokines are associated with the recruitment and differentiation of osteoclasts.

An in vitro study showed that IL-6 can stimulate RANKL expression in osteoclast precursors (Tamura et al. 1993). IL-1β can promote the differentiation of osteoclasts directly and indirectly (Kim et al. 2009). It can induce the proliferation of osteoclasts in the presence of M-CSF and RANKL (Cao et al. 2016), increase the production of RANKL in osteoblasts (Chen et al. 2020; Kitaura et al. 2020), raise the levels of M-CSF, necessary for the differentiation of osteoclasts, and decrease the levels of OPG (Tao et al. 2020; Roper et al. 2020). TNF-α, besides conducting inflammation, acts directly on osteoclasts in synergy with RANKL to promote bone resorption (Lam et al. 2000; Teitelbaum et al. 2006 ; Boyce et al. 2006).

Nicotine is the systemic factor, being the main cytotoxic component of cigarette smoke, which also acts in bone resorption. An *in vitro* study showed that nicotine associated with LPS can stimulate the formation of cells like osteoclasts (Tanaka et al. 2006) and increase bone resorption in smokers with periodontal diseases, besides increasing the levels of pro-inflammatory cytokines (Vasques et al. 2022), contributing directly and indirectly to an imbalance of the RANKL/OPG pathway, with predominance of osteoclastic activity.

Conclusion

Apical periodontitis associated with cigarette smoke inhalation resulted in higher osteoclastic activity evidenced by the area of bone resorption during histometric analysis and by the volume of bone resorption during microtomography. The higher expression of RANKL in the CSI + AP group created imbalance in the RANK/RANKL/OPG pathway, with the predominance of bone resorption. The methodology used allowed high levels of cotinine in animals, resembling human smokers. Therefore, cigarette smoke inhalation influenced the development of apical periodontitis, enhancing bone resorption.

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Conclusão geral

O tabagismo promoveu alterações no desenvolvimento da periodontite apical exacerbando a resposta inflamatória com consequente aumento das citocinas pro-inflamatórias IL-6, IL-1β e TNF-α, alterações em glóbulos vermelhos e brancos prejudicando a resposta imune do organismo. Com uma resposta inflamatória mais intensa, as citocinas pro-inflamatórias IL-6, IL-1β e TNF-α, juntamente com a nicotina, promoveram alterações no metabolismo ósseo através da ativação do sistema RANKL/OPG, e com isso o volume de destruição óssea foi maior quando a periodontite apical estava associada ao tabagismo.

Anexos

ANEXO A

Referências da introdução geral

of an estro-

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ANEXO B

Comitê de Ética em Pesquisa

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Influência do tabaco no desenvolvimento da periodontite apical em ratos"**, Processo FOA nº 00247-2020, sob responsabilidade de Eloi Dezan Júnior apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 01 de Dezembro de 2020.

VALIDADE DESTE CERTIFICADO: 01 de Novembro de 2022.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 01 de Dezembro de 2022.

CERTIFICATE

We certify that the study entitled **"Influence of tobaco in apical periodontis development"**, Protocol FOA nº 00247-2020, under the supervision of Eloi Dezan Júnior presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on December 01, 2020.

VALIDITY OF THIS CERTIFICATE: November 01, 2022.
DATE OF SUBMISSION OF THE FINAL REPORT: December 01, 2022.

Prof. Associado Guilherme de Paula Nogueira
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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ANEXO C

Author Guidelines International Endodontic Journal

1.GENERAL

The *International Endodontic Journal* publishes original scientific articles, systematic and narrative reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic health and well-being. Original scientific articles and reviews are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and the restoration of endodontically treated teeth. In addition, letters to the editor, book reviews, summaries and abstracts of scientific meetings, news items and significant obituaries and retirements are accepted subject to the approval of the Editor-in-Chief.

1.1 Preprints Policy

The *International Endodontic Journal* will consider for review articles previously available as preprints. Authors may also post the submitted version of a manuscript to a preprint server at any time. Authors are requested to update any pre-publication versions with a link to the final published article.

1.2. Data Sharing

The *International Endodontic Journal* encourages authors to share the data and other artefacts supporting the results in the paper by archiving it in an appropriate public repository. Authors should include a data accessibility statement, including a link to the repository they have used, in order that this statement can be published alongside their paper. All accepted manuscripts may elect to publish a data availability statement to confirm the presence or absence of shared data. If you have shared data, this statement will describe how the data can be accessed, and include a persistent identifier (e.g., a DOI for the data, or an accession number) from the repository where you shared the data. Sample statements are available **here**. If published, statements will be placed in the heading of your manuscript.

1.3 Open Access

The *International Endodontic Journal* is a subscription journal that offers an open access option. You'll have the option to choose to make your article open access after acceptance, which will be subject to an APC. You can **read more about APCs** and whether you may be eligible for waivers or discounts, through your institution, funder, or a country waiver.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in the *International Endodontic Journal*. Authors are encouraged to visit **Wiley Author Services** for further information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES

The *International Endodontic Journal* adheres to the below ethical guidelines for publication and research.

2.1 Authorship

The policy of the journal is that only ONE corresponding author is accepted.

Authors submitting a paper do so on the understanding that the manuscript has been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal.

The *International Endodontic Journal* adheres to the definition of authorship establsihed by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE, authorship criteria should be based on:

1. substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data
2. drafting the article or revising it critically for important intellectual content, and
3. final approval of the version to be published.

Authors should meet conditions 1, 2 and 3.

2.2 Ethical Approvals

Experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association **Declaration of Helsinki** (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board must also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been adopted.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain and discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study. The authors MUST upload a copy of the ethical approval letter when submitting their manuscript and a separate English translation. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

2.3 Clinical Trials

The *International Endodontic Journal* asks that authors submitting manuscripts reporting a clinical trial register the trial *a priori* in any of the following public clinical trials registries: **www.clinicaltrials.gov**, **https://www.clinicaltrialsregister.eu/**, **http://isrctn.org/** . Other primary registries if named in the WHO network will also be considered acceptable.

The clinical trial registration number and name of the trial register should be included in the Acknowledgements at the submission stage.

2.4 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

2.5 Acknowledgements

Authors should specify contributors to the article other than the authors accredited. Thus, the Acknowledgements should include the details (names and affiliations) of individuals who helped in conducting the research or statistical analysis or, translated, or edited the manuscript.

2.6 Conflict of Interest

Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. The *International Endodontic Journal* requires that both the corresponding author and co-authors must disclose any potential sources of conflict of interest. The *International Endodontic Journal* has adopted the recommendations provided by ICMJE (**http://www.icmje.org/**) related to conflicts of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company.

If authors are unsure whether a past or present affiliation or relationship should be disclosed in the manuscript, they are advised contact the Editor-in-Chief.

It is the responsibility of the corresponding author to have all authors of a manuscript fill out a conflict of interest disclosure form, and to upload all forms individually (do not combine the forms into one file) together while submitting the manuscript at **https://mc.manuscriptcentral.com/iej**.

The form can be accessed using the following link: **Conflict of Interest Disclosure Form**

2.7 Source of Funding

All sources of funding for a study must be disclosed. Authors must provide details about direct funding (e.g. consulting payments, fees) or ownership (shareholdings and patents) or indirect funding (e.g. donations of equipment, instruments) that was received. Authors must report the names of the sponsors or funding agency with funding/grant number. Funds and resources provided by academic institutions must also be disclosed to ensure transparency, e.g. academic staff, support staff, clinical facilities, purchase of consumables and equipment etc.

2.8 Appeal of Decision

The decision on a paper is final and cannot be appealed.

2.9 Permissions

If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

3. MANUSCRIPT SUBMISSION PROCEDURE

Manuscripts must be submitted electronically via the online submission site: **http://mc.manuscriptcentral.com/iej**.

The use of the online submission and peer review site enables immediate distribution of manuscripts and consequentially speeds up the review process. It also allows authors to track the status of their own manuscripts. Complete instructions for submitting a paper is available online and below. Further assistance can be obtained from **iejeditor@cardiff.ac.uk**.

3.1 ORCID

As part of the journal's commitment to supporting authors at every step of the publishing process, the *International Endodontic Journal* encourages authors to provide an ORCID iD when submitting a manuscript. Please see Wiley's resources on ORCID **here**

3.2. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission. However, only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the abstract, main text, references, tables, and figure legends, but no embedded figures or Title page. The Title page should be uploaded as a separate file. On a separate page after the references, please reference figures as 'Figure 1', 'Figure 2' etc. to match the tag name you choose for the individual figure files uploaded and provide a brief legend that described the image but does not repeat information that should be in the main body of the text. Manuscripts should be formatted as described in the Author Guidelines below.

3.3. Blinded Review

Manuscript that do not conform to the general aims and scope of the journal will be returned immediately without review. All other manuscripts will be reviewed by experts in the field, first by an associate editor and if appropriate by (generally) two or more referees. The *International Endodontic Journal* aims to forward the comments of the referees and to inform the corresponding author of the result of the review process. Manuscripts will be considered for fast-track publication under special circumstances after consultation with the Editor-in-Chief.

The *International Endodontic Journal* uses double blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper and the name(s) of the author(s) will not be disclosed to the reviewers.
To allow double blinded review, please submit (upload) your main manuscript and title page as separate files.
Please upload:

- Your manuscript without title page under the file designation 'main document'

- Figure files under the file designation 'figures'
- The title page and Acknowledgements where applicable, should be uploaded under the file designation 'title page'

All documents uploaded under the file designation 'title page' will not be viewable in the HTML and pdf format you are asked to review in the end of the submission process. The files viewable in the html and pdf format are the files available to the reviewer in the review process.

3.4. Suspension of Submission Mid-way in the Submission Process
You may suspend a submission at any phase before clicking the 'Submit' button and save it to submit later. The manuscript can then be located under 'Unsubmitted Manuscripts' and you can click on 'Continue Submission' to continue your submission when you choose to.

3.5. Submission of Revised Manuscripts
To submit a revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original Scientific Articles (includes Clinical Research (randomized control trials, cohort studies, case control studies, cross sectional studies, case series), Basic Research – Biological, Basic Research — Technical, and Education): These must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Scientific Articles must conform to the highest international standards in the field.

Review Articles (systematic and narrative): These are accepted for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy. Reviews should generally include a clearly defined search strategy and take a broad view of the field rather than merely summarizing the literature. Extensive or unbalanced citation of the authors´ own publications is not acceptable.

Clinical Techniques: These can describe significant improvements in clinical practice such as the report of a novel technique, a breakthrough in technology or practical approaches to recognised clinical challenges. They should conform to the highest scientific and clinical practice standards.

Case Reports: These can illustrate unusual and clinically relevant and new observations, but they must be of sufficiently high quality to be considered worthy of publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered. Illustrative material must be of the highest quality and healing

outcomes, if appropriate, should be demonstrated after an extended period – normally four years.

Supporting Information: The *International Endodontic Journal* encourages submission of adjuncts to printed papers via the supporting information website (see submission of supporting information below). Authors wishing to describe novel procedures or illustrate cases more fully with figures and/or video may are encouraged to utilise this facility.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited.

Presentation: Authors should pay special attention to the presentation of their research findings or clinical reports so that they may be communicated clearly. Technical jargon should be avoided as much as possible and clearly explained where its use is unavoidable. Abbreviations should also be kept to a minimum, particularly those that are not standard. The background and hypotheses underlying the study, as well as its main conclusions, should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

Article Preparation Support: Wiley Editing Services offers expert help with English Language Editing, as well as translation, manuscript formatting, figure illustration, figure formatting, and graphical abstract design – so you can submit your manuscript with confidence. Also, check out our resources for **Preparing Your Article** for general guidance about writing and preparing your manuscript.

Abbreviations: The *International Endodontic Journal* adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2. Reporting guidelines

5.2.1. Case reports/case series

Case reports should be written to comply with the Preferred Reporting Items for Case reports in Endodontics (PRICE) 2020 guidelines (Nagendrababu *et al.* 2020, doi:10.1111/iej.13285).

When submitting manuscripts that have been written using the PRICE 2020 guidelines, authors should include the following statement in the beginning of “Report” section: ‘‘This case report has been written according to Preferred Reporting Items for Case reports in Endodontics (PRICE) 2020 guidelines (Nagendrababu *et al.* 2020, doi: 10.1111/iej.13285).

A PRICE checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material.

The PRICE 2020 checklist and flowchart can be downloaded from: **http://pride-endodonticguidelines.org/price/.**

It is recommended that authors consult the following papers when writing case reports, which explains the rationale for the PRICE 2020 guidelines and their importance:

Nagendrababu V, Chong BS, McCabe P, Shah PK, Priya E, Jayaraman J, Pulikkotil SJ, Setzer FC, Sunde PT, Dummer PMH (2020) PRICE 2020 guidelines for reporting case reports in Endodontics: a consensus-based development. *International Endodontic Journal* **53**, 619-26. (**https://www.ncbi.nlm.nih.gov/pubmed/32090342**)

Nagendrababu V, Chong BS, McCabe P, Shah PK, Priya E, Jayaraman J, Pulikkotil SJ, Dummer PMH (2020) PRICE 2020 guidelines for reporting case reports in Endodontics: Explanation and elaboration. *International Endodontic Journal* **53**, 922-47. (**https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13300**)

5.2.2. Randomised clinical trials

Randomised clinical trials should be reported to comply with the Preferred Reporting Items for RAndomized Trials in Endodontics (PRIRATE) 2020 guidelines (Nagendrababu *et al.* 2020, doi: 10.1111/iej.13294).

When submitting manuscripts that have been written using the PRIRATE 2020 guidelines, authors should include the following statement in the beginning of “Materials and Methods” section: ‘‘This randomised clinical trial has been written according to Preferred Reporting Items for RAndomized Trials in Endodontics (PRIRATE) 2020 guidelines (Nagendrababu *et al.* 2020, doi: 10.1111/iej.13294).

A PRIRATE 2020 checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material. The PRIRATE 2020 checklist and flowchart can be downloaded from: **http://pride-endodonticguidelines.org/prirate/**

It is recommended that authors consult the following papers when writing manuscripts, which explains the rationale for the PRIRATE 2020 guidelines and their importance:

Nagendrababu V, Duncan HF, Bjørndal L, Kvist T, Priya E, Jayaraman J, Pulikkotil SJ, Pigg M, Rechenberg DK, Vaeth M, Dummer PMH (2020) PRIRATE 2020 guidelines for reporting randomized trials in Endodontics: a consensus-based development. *International Endodontic Journal* **53**, 764-73. (**https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13294**)

Nagendrababu V, Duncan HF, Bjørndal L, Kvist T, Priya E, Jayaraman J, Pulikkotil SJ, Dummer PMH (2020) PRIRATE 2020 guidelines for reporting randomized trials in Endodontics: Explanation and elaboration. *International Endodontic Journal* **53**, 774-03. (**https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13304**)

5.2.3. Epidemiological observational trials

Observational studies should be written using the STrengthening the Reporting of OBservational studies in Epidemiology' (STROBE) guidelines. Compliance with this should be detailed in the “Materials and Methods” section. (**www.strobe-statement.org**). A STROBE checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material.

It is recommended that authors consult the following papers when writing manuscripts, which explains the rationale for the STROBE guidelines and their importance:

Von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP, Strobe Initiative (2014) The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies. *International Journal of Surgery* **12**, 1495-9.

Vandenbroucke JP, von Elm E, Altman DG, Altman DG, Gøtzsche PC, Mulrow CD, Pocock SJ, Poole C, Schlesselman JJ, Egger M, STROBE Initiative. (2014) Strengthening the reporting of observational studies in epidemiology (STROBE): explanation and elaboration. *International Journal of Surgery* **12**, 1500–24

5.2.4. Diagnostic accuracy studies

Diagnostic accuracy studies should be written using the Standards for Reporting of Diagnostic Accuracy Studies (STARD) 2015 guidelines. Compliance with this should be detailed in the "Materials and Methods" section. A STRAD checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material. The STRAD checklist and flowchart can be downloaded from: **https://www.equator-network.org/reporting-guidelines/stard/**

It is recommended that authors consult the following papers when writing manuscripts, which explain the rationale for the STRAD guidelines and their importance:

Cohen JF, Korevaar DA, Altman DG, Bruns DE, Gatsonis CA, Hooft L, Irwig L, Levine D, Reitsma JB, de Vet HCW, Bossuyt PMM. (2016) STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. *BMJ Open* **6**, e012799

http://bmjopen.bmj.com/content/6/11/e012799.abstract

5.2.5. Animal studies

Animal studies should be written using the Preferred Reporting Items for Animal Studies in Endodontology (PRIASE) 2021 guidelines (Nagendrababu *et al.* 2021, doi: 10.1111/iej.13477).

When submitting manuscripts that have been written using the PRIASE 2021 guidelines, authors should include the following statement in the beginning of "Materials and Methods" section: ''The manuscript of this animal study has been written according to Preferred Reporting Items for Animal studies in Endodontology (PRIASE) 2021 guidelines (Nagendrababu *et al.* 2021, doi: 10.1111/iej.13477).

A PRIASE 2021 checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material. The PRIASE 2021 checklist and flowchart can be downloaded from: http://pride-endodonticguidelines.org/priase/

It is recommended that authors consult the following papers when writing manuscripts, which explain the rationale for the PRIASE 2021 guidelines and their importance:

Nagendrababu V, Kishen A, Murray PE, Nekoofar MH, de Figueiredo JA, Priya E, Jayaraman J, Pulikkotil SJ, Camilleri J, RM S, Dummer PMH (2021) PRIASE 2021 guidelines for reporting animal studies in Endodontology: a consensus-based development. *International Endodontic Journal* **54**, 848-57. (**https://onlinelibrary.wiley.com/doi/10.1111/iej.13477**)

Nagendrababu V, Kishen A, Murray PE, Nekoofar MH, de Figueiredo JA, Priya E, Jayaraman J, Pulikkotil SJ, Jakovljevic A, Dummer PMH (2021) PRIASE 2021 guidelines for

reporting animal studies in Endodontology: Explanation and Elaboration. *International Endodontic Journal* **54,** 858-86. (**https://onlinelibrary.wiley.com/doi/10.1111/iej.13481**)

5.2.6. Laboratory studies

Laboratory studies should be reported using the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu *et al.* 2021, doi: 10.1111/iej.13542).

When submitting manuscripts that have been written using the PRILE 2021 guidelines, authors should include the following statement in the beginning of "Materials and Methods" section: ''The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Nagendrababu *et al*. 2021, doi: 10.1111/iej.13542).

A PRILE checklist (for editors/referees) and flowchart (as a Figure to be included in the manuscript for readers) should also be completed and included in the submission material The PRILE 2021 checklist and flowchart can be downloaded from: **http://pride-endodonticguidelines.org/prile/**

It is recommended that authors consult the following papers when writing manuscripts, which explain the rationale for the PRILE 2021 guidelines and their importance:

Nagendrababu V, Murray PE, Ordinola-Zapata R, OA Peters, IN Rôças, JF Siqueira Jr, E Priya, J Jayaraman, SJ Pulikkotil, J Camilleri, C Boutsioukis, G Rossi-Fedele, PMH Dummer (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontics: a consensus-based development. *International Endodontic Journal* May 3. doi: 10.1111/iej.13542. (**https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13542**)

Nagendrababu V, Murray PE, Ordinola-Zapata R, OA Peters, IN Rôças, JF Siqueira Jr, E Priya, J Jayaraman, SJ Pulikkotil, N Suresh, PMH Dummer (2021) PRILE 2021 guidelines for reporting laboratory studies in Endodontics: Explanation and elaboration. *International Endodontic Journal* (**https://onlinelibrary.wiley.com/doi/abs/10.1111/iej.13565**)

5.2.7 Systematic reviews

The abstract and main body of the systematic review should be reported using the PRISMA for Abstract and PRISMA guidelines respectively (**http://www.prisma-statement.org/**). Authors submitting a systematic review must register the protocol in one of the readily-accessible sources/databases at the time of project inception and not retrospectively (e.g. PROSPERO database, OSF registries). The protocol registration number, name of the database or journal reference should be provided at the submission stage in the "Registration" section in the abstract and 'Methods' section in the main body of the text.

A PRISMA checklist and flow diagram as a Figure (to be included in the manuscript for readers) should also be included in the submission material. Source of funding (grant number, if available) should be added in the 'Acknowledgements' section.

It is recommended that authors consult the following papers, which help in the production of high quality reviews:

Moher D, Liberati A, Tetzlaff J, Altman DG, PRISMA Group (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLOS Medicine 6*, e1000097.

Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, Clarke M, Devereaux PJ, Kleijnen J, Moher D (2009)**The PRISMA statement for reporting**

systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *Journal of Clinical Epidemiology* **62**, e1-34.

Nagendrababu V, Duncan HF, Tsesis I, Sathorn C, Pulikkotil SJ, Dharmarajan L, Dummer PMH (2019) PRISMA for abstracts: best practice for reporting abstracts of systematic reviews in Endodontology. *International Endodontic Journal* **52,** 1096-1107. (**https://onlinelibrary.wiley.com/doi/10.1111/iej.13118**)

Nagendrababu V, Dilokthornsakul P, Jinatongthai P, Veettil SK, Pulikkotil SJ, Duncan HF, Dummer PMH (2020) Glossary for systematic reviews and meta-analyses. *International Endodontic Journal* **53,** 232-249.(**https://onlinelibrary.wiley.com/doi/full/10.1111/iej.13217**)

5.3. Structure

All manuscripts submitted to *International Endodontic Journal* should include Title Page, Abstract, Main Text, References and Acknowledgements, Tables, Figures and Figure Legends as appropriate

All manuscripts submitted to the *International Endodontic Journal* should include Title Page (separate file), Abstract, Main Text, References, Acknowledgements, Funding, Conflict of Interest, Tables, Figures and Figure Legends as appropriate

Title Page: The title page should bear: (i) Title, which should be concise as well as descriptive; (ii) Initial(s) and last (family) name of each author; (iii) Name and address of department, hospital or institution to which work should be attributed (excluding street names and postal codes); (iv) Running title (no more than 30 letters and spaces); (v) No more than six keywords (in alphabetical order); (vi) Name, full postal address, telephone, fax number and e-mail address of the author responsible for correspondence.

Abstract for Original Scientific Articles (includes Clinical Research (randomized control trials, cohort studies, case control studies, cross sectional studies, case series), Basic Research – Biological, Basic Research - Technical and Education) should be no more than 350 words giving details of what was done using the following structure:

- **Aim**: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.
- **Methodology**: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.
- **Results**: Give the main results of the study, including the outcome of any statistical analysis.
- **Conclusions**: State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.
- **Funding:**Provide the primary source of funding with grant number for the study (if no funding: say ‘none’).
- **Conflict of interest:** Specify potential conflicts of interest for all authors. If no conflict exists, the authors must explicitly state so.

Abstract for Systematic Review Articles should be no more than 350 words using the following structure where applicable:

- **Title:** Identify the report as a systematic review, meta-analysis, or both.
- **Background:** Provide a brief introduction of the subject and why it is important.
- **Objectives:** The research question including components such as participants, interventions, comparators, and outcomes. Use PICO format.
- **Methods:** Briefly describe i) the inclusion criteria, ii) provide databases searched and dates, iii) mention the method used to assess study quality (risk of bias) iv) meta-analysis methodology (if appropriate).
- **Results:** i) Number and type of included studies and participants ii) results for main outcomes (benefits and harms). If a meta-analysis was undertaken, include summary measures and confidence intervals. iii) direction of the effect in terms that are meaningful to clinicians and patients.
- **Discussion:** i) Strengths and ii) limitations of evidence.
- **Conclusions**: General interpretation of the results and important implications.
- **Funding:** Primary source of funding for the review (if no funding: say 'none').
- **Registration**: Registration number and name.

Abstract for Review Articles (narrative)
The Abstract should be unstructured and no more than 350 words.

Abstract for Clinical Techniques and Case Reports should be no more than 350 words using the following structure:

- **Aim**: Give a clear statement of the main aim of the report and the clinical problem which is addressed.
- **Summary**: Describe briefly the clinical technique(s) or the case report(s).
- **Key learning points**: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Main Text of Original Scientific Article should include Introduction, Materials and Methods, Results, Discussion and Conclusion:

- **Introduction**: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.
- **Material and Methods**: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association **Declaration of Helsinki** (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that

adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

Suppliers: Suppliers of materials should be named and their location (product: company, town/city, state, country) included.

• **Results**: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.

• **Discussion**: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a critical review of the methodology before discussing the results in light of previous work in the field. The Discussion should include the strengths and limitations of the work and should end with a brief summary and a comment on the potential clinical relevance of the findings, if any. Statements and interpretation of the data should be appropriately supported by original references.

• **Conclusion**: should contain a summary of the findings.

Main Text of Systematic Review Articles should be divided into Introduction, Methods, Results, Discussion, Conclusions, Funding and Conflict of Interest:

- **Introduction:**Should be focused to place the subject matter in context and to justify the need for the review.
- **Method:**Divide into logical sub-sections in order to improve readability and enhance understanding (e.g. details of protocol registration, literature search process, inclusion/exclusion criteria, data extraction, quality assessment, outcome(s) of interest, data synthesis and statistical analysis, quality of evidence).

- **Results:** Present in structured fashion (e.g. results of the search process, characteristics of the included studies, results of primary meta-analysis, additional analysis, publication bias, quality of evidence).

- **Discussion:** Should summarize the results, highlighting completeness and applicability of evidence, quality of evidence, agreements and disagreements with other studies or reviews, strength and limitations, implications for practice and research.

- **Conclusion(s):** Section should reach clear conclusions and/or recommendations on the basis of the evidence presented.
- **Funding:** Provide the primary source of funding with grant number for the study (if no funding: say ‘none’).
- **Conflict of Interest**: Need to specify potential conflicts of interest for all the authors. If no conflict exists, the authors must explicitly state so.

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