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Renata Oliveira Samuel

TESE DE DOUTORADO

PERFIL INFLAMATÓRIO LOCAL E SISTÊMICO DE RATOS WISTAR COM MÚLTIPLAS INFECÇÕES ENDODÔNTICAS

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Orientador: Prof. Dr. Luciano Tavares Angelo Cintra

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RENATA OLIVEIRA SAMUEL

Nascimento	14/01/1988 Uberaba – MG
Filiação	Aristides Samuel Júnior Vera Lúcia de Oliveira Samuel
2006-2009	Graduação em Odontologia na Universidade de Uberaba.
2011-2012	Especialização em Endodontia na Associação Brasileira de Odontologia (ABO/MG).
2011-2012	Mestrado em Ciência Odontológica, área de concentração em Endodontia na Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho", SP.
2013-2016	Doutorado em Ciência Odontológica, área de concentração em Endodontia na Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho", SP.
Associações	Associação Brasileira de Odontologia Associação Paulista de Cirurgiões Dentistas Sociedade Brasileira de Pesquisas Odontológicas Associação Internacional de Pesquisas Odontológicas

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SAMUEL, RO. **Perfil inflamatório local e sistêmico de ratos wistar com múltiplas infecções endodônticas**. 2016. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2016.

RESUMO

O objetivo deste trabalho foi colaborar com o entendimento dos mecanismos que envolvem a relação entre as infeções de origem endodôntica e o estado de homeostasia corpórea. Para isso investigamos o perfil inflamatório local da periodontite apical induzida e relacionamos com alterações no perfil sistêmico em ratos portadores de uma ou múltiplas infecções de origem endodôntica. Foram utilizados 30 ratos da linhagem Wistar distribuídos em 3 grupos de 10 animais: ratos saudáveis; 1AP – ratos com infecção endodôntica em um elemento dentário; 4AP ratos com infecções endodônticas em 4 elementos dentários. Após 30 dias, foi coletado sangue por punção cardíaca para a realização do hemograma (hemácias, volume globular, hemoglobina, VCM, CHCM, leucócitos, neutrófilos, linfócito, monócito, eosinófilo, basófilo e plaquetas); da quantificação sérica das citocinas pró-inflamatórias (TNF-α, IFN-γ, IL-6, IL-17, IL-23) por ELISA e da quantificação de óxido nítrico (NO). Após coleta sanguínea, os animais foram sacrificados, as maxilas e mandíbulas foram processadas para análise em microscopia de luz em coloração de H.E. e para marcação imunoistoquímica dos mediadores inflamatórios supracitados. Os resultados das diferentes análises e a relação entre os achados locais e sistêmicos foram analisados por testes estatísticos específicos para cada caso ($p<0,05$). Nas análises histológicas, foi confirmada a presença de periodontites apicais no 1AP e 4AP, com infiltrado inflamatório moderado. Além disso, foi observado aumento de todos os mediadores supracitados nas lesões quando comparado aos tecidos saudáveis ($p<0.05$). Nas análises séricas, foi observado aumento linfócitos, leucócitos, TNF-α, IL-6, IL-17 e IL-23 no 4AP quando comparado aos ratos saudáveis ($p<0.05$). Além disso, houve diminuição do NO no 1AP e 4AP quando comparados aos ratos saudáveis ($p<0.05$). O nível de IFN-γ não foi diferente entre os grupos ($p>0.05$). Conclui-se que a presença de periodontite apical em múltiplos elementos dentários pode levar a alterações séricas importantes decorrentes do aumento de linfócitos, leucócitos e das citocinas pró-inflamatórias TNF-α, IL-6, IL-17 e IL-23. Além disso, a periodontite apical em um ou em múltiplos elementos dentários leva a diminuição sérica dos metabólitos do NO.

Palavras chave: periodontite periapical, citocinas, óxido nítrico.

SAMUEL, RO. **Local and systemic inflammatory profile of Wistar rats with multiple endodontics infections**. 2016. Doctoral thesis - School of Dentistry, São Paulo State University. Araçatuba, 2016.

ABSTRACT

The aim of this work will contribute to understanding of the mechanisms that involve the relationship between infections of endodontic origin and state of bodily homeostasis. Thus, we propose to investigate the profile of local inflammatory induced apical periodontitis and correlate with changes in the profile systemic model with a onlyl or multiple endodontic infections. It were used 30 Wistar rats divided into three groups of 10 animals each: healthy rats; 1AP - rats with only endodontic infection; 4AP rats with multiple endodontic infections. After
30 days, blood was collected by cardiac puncture for complete blood count (RBCs, packed cell volume, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, leukocytes, neutrophil, lymphocyte, monocyte, eosinophil, basophil and platelets), serum quantification of proinflammatory cytokines (IL-17, IL-23, IL-6, TNF-α, IFN-γ) by ELISA and nitric oxide. After blood collection, the animals were killed, the jaws were processed for light microscopy in HE staining and immunohistochemical staining of inflammatory mediators above. The results of the different analyzes and the correlation between the local and systemic findings were analyzed by statistical tests specific to each case ($p<0,05$). Histological analysis confirmed the presence of apical periodontitis in 1AP and 4AP, with moderate inflammatory infiltrate. Furthermore, it was observed increase of all above mediators in lesions when compared to healthy tissue ($p <0.05$). In serum analysis, 4AP showed lymphocytes, leukocytes, TNF-α, IL-6, IL-17 and IL-23 increase when compared to healthy rats ($p <0.05$). Furthermore, there was a decrease of NO in 4AP and 1AP when compared to healthy rats ($p <0.05$). IFN-γ level was not different between the groups ($p>0.05$). It is concluded that the presence of apical periodontitis in multiple teeth can lead to significant alterations from increased serum lymphocytes, leukocytes and pro-inflammatory cytokines TNF-α, IL-6, IL-17 and IL-23. Furthermore, apical periodontitis in only or multiple teeth leads to decrease of serum levels of NO.

Keywords: periapical periodontitis, cytokines, nitric oxide

S U M Á R I O

Páginas

I. INTRODUÇÃO GERAL / REVISÃO DE LITERATURA

A inflamação é uma resposta natural do organismo contra uma infecção ou lesão do tecido com o objetivo de destruir agentes agressores. É um processo complexo que envolve a liberação de substâncias endógenas ou exógenas que podem atuar inibindo, mantendo, ativando ou amplificando uma resposta inflamatória (Del Prete 1992). Estas substâncias incluem moléculas de adesão celular (Giaever & Ward 1978), citocinas (como interleucinas e fator de necrose tumoral) (Le & Vilcek 1987), imunoglobulinas (Hektoen 1909), fatores de crescimento (Todaro et al., 1981), proteínas de fase aguda (como a proteína C-reativa) (Merriman et al., 1975) e até mesmo liberação de radicais livres (Bragt & Bonta 1980).

Embora estes mediadores sejam uma forma de defesa benéfica para o organismo, acontecem frequentemente desequilíbrios entre substâncias pró e anti-inflamatórias. Estes desequilíbrios podem levar tanto à inibição demasiada da inflamação (Hersh et al., 1983) quanto ao início de processos inflamatórios exacerbados que podem afetar todo o organismo pelo aumento de substâncias pró-inflamatórias de forma sistêmica (Bian et al., 2012).

O problema do excesso de substâncias pró-inflamatórias, como interleucinas, fatores de necrose tumoral e radicais livres é que uma vez liberadas, podem dar início ou potencializar a patogênese de diversas doenças também ativadas por esses mesmos mediadores, como por exemplo, diabetes, infarto, derrame, hipertensão, artrite reumatoide, doenças pulmonares, doença de Alzheimer, entre outras (Brod et al., 1991). Assim, um dos mecanismos que faz com que estes mediadores pró-inflamatórios sejam ativados e atuem de forma sistêmica, é a presença de infecções crônicas por longos períodos (Bian et al., 2012). Um exemplo clássico na literatura atual deste problema é a presença da doença periodontal e a potencialização de várias desordens autoimunes (El-Shinnawi & Soory 2013; Bansal et al., 2013).

Desta forma, a relação entre infecções orais e sistêmicas tem sido cada vez mais explorada nas pesquisas de área médica-odontológica (Segura-Egea et al., 2012; Cintra et al., 2013ab; Gomes et al., 2013; Cintra et al., 2014abc).

Estudos epidemiológicos realizados em todo o mundo mostram que a periodontite apical está presente em 22-65% dos dentes tratados endodonticamente (De Moor et al., 2000; Dugas et al., 2003; Siqueira et al., 2005). Entretanto, esta incidência é ainda maior em estudos utilizando tomografia computadorizada, mostrando a presença destas lesões em 78,1% dos dentes tratados (Paes et al., 2012). Relato recente da população brasileira mostrou presença de uma ou mais periodontites apicais em 62,7% dos pacientes que receberam tratamento endodôntico (Domínguez Sánchez et al., 2015). Assim, é comum um mesmo paciente apresentar mais de uma lesão periapical (Domínguez Sánchez et al., 2015).

Na presença da infecção endodôntica em um elemento dentário, nosso grupo de pesquisa têm observado em todos os trabalhos realizados (em ratos diabéticos e normoglicêmicos da linhagem Wistar) modificações no perfil sistêmico, como alterações em lipídios (Cintra et al., 2013a), creatinina (Cintra et al., 2013b), hemoglobina glicada (Cintra et al., 2014c), além de alterações em alguns parâmetros no hemograma (Cintra et al. 2014a) e em mediadores pró-inflamatórios como a IL-17 (Cintra et al., 2014b). No entanto, essas modificações na presença da infecção endodôntica levaram a um aumento tendencial, sem, contudo, apresentar diferenças do ponto de vista estatístico. Como estes estudos foram realizados com a presença da infeção endodôntica em apenas um elemento dentário, acreditamos que na ocasião de outros elementos portadores de infeção endodôntica poderíamos verificar esta influência de forma mais evidente.

Assim, a primeira hipótese do estudo é que a presença de vários focos de infecção de origem pulpar poderia acarretar no aumento dos níveis de alguns parâmetros sanguíneos que já classificamos como tendenciais na avaliação da infecção isolada em um dente.

Com relação à manutenção da infecção endodôntica observa-se, de forma semelhante à doença periodontal, a presença de um mecanismo imunológico complexo, com liberação de diversos mediadores inflamatórios e bioquímicos, capazes tanto de neutralizar as bactérias (em sua maioria gram negativas) e seus produtos (Rupf et al., 2000), como de promover efeitos deletérios, como a reabsorção óssea (Silva et al., 2007).

Depreende-se que as vias biológicas que a fazem a ligação entre a doença periodontal crônica e as desordens sistémicas são as mesmas que podem ligar a periodontite apical crônica à saúde geral (Silva et al., 2007). No entanto, diferente da doença periodontal, o assunto carece de informações com relação às infecções de origem endodôntica. Poucas são as evidências de que a periodontite apical cause alguma alteração diferente de abscessos esporádicos ou bacteremias transitórias (Mylona et al., 2012). Assim, o processo inflamatório sistêmico decorrente desta infecção é subestimado, mesmo diante da manutenção da lesão periapical por anos (Wu et al., 2006), além da forte evidência da presença de vários focos infecciosos em um mesmo indivíduo (Domínguez Sánchez et al., 2015).

Um exame básico e de excelência para verificação inicial de possíveis alterações sistêmicas decorrentes de infecções endodônticas é o hemograma, pois permite a avaliação da série vermelha, série branca e plaquetas, tanto quantitativamente quanto qualitativamente (Odagiri et al., 2011). Esse conjunto de parâmetros facilita o diagnóstico de doenças, possibilita a verificação da resposta do individuo à infecção e até mesmo a progressão de um tratamento (Gkrania-Klotsas et al., 2010).

Os leucócitos (representantes da série branca do hemograma) participam na defesa contra os patógenos, sendo os neutrófilos as células mais abundantes, constituindo entre 50-70% dos leucócitos, e seu número aumentado é comumente observado nas infecções bacterianas, como as infecções endodônticas (Ma et al., 2011; Carneiro et al., 2012). Além de atuar diretamente sobre o agente agressor, os leucócitos podem atuar ativando o processo inflamatório por meio de estímulos para produção de citocinas e quimiocinas (Bian et al., 2012).

Nosso grupo de pesquisa observou algumas alterações em hemograma (Cintra et al., 2014a). A presença da infecção endodôntica associada à infecção de origem periodontal mostrou aumento no volume corpuscular médio (VCM) em ratos normoglicêmicos de forma semelhante a ratos diabéticos. Parâmetros da série branca do hemograma também se mostraram alterados, tais como, aumento de leucócitos e neutrófilos em ratos com infecção endodôntica em um dente de forma semelhante a ratos diabéticos sem infecção (Cintra et al., 2014a). Além disso, dentre os diabéticos, houve aumento de neutrófilos em ratos com

infecções endodôntica e periodontal associadas quando comparados com ratos apenas hiperglicêmicos. O problema do aumento sérico desta célula é que ela pode ser a chave para uma sucessão de eventos pró-inflamatórios, devido à estimulação de vários mediadores (Cintra et al., 2014b). Isso pode influenciar uma série de efeitos deletérios ao organismo, como respostas inflamatórias exacerbadas (Bian et al., 2012).

Os neutrófilos atuam, por exemplo, estimulando a produção da interleucina 17 (IL-17) (Bian et al., 2012). A IL-17 é uma citocina pró-inflamatória secretada principalmente por células T helper, denominadas Th 17 (Gaffen 2011). É ativa em respostas inflamatórias, auto-imunes e antimicrobianas à patógenos em uma variedade de doenças infecciosas (Schenkein et al., 2010). Supõe-se que sua presença na periodontite apical esteja associada com exacerbação da resposta inflamatória, promovendo um aumento no número de neutrófilos e assim, aumentando a reabsorção óssea (Marçal et al., 2010).

Com relação à IL-17, nosso grupo de pesquisa observou um aumento sérico desta citocina quando a infecção endodôntica estava associada à infecção periodontal, tanto em ratos normoglicêmicos quanto em ratos diabéticos. Já com a presença apenas da infecção endodôntica ou periodontal isoladas, verificou-se que embora tenha existido um aumento sérico de IL-17, este resultado não foi estatisticamente significativo. Acredita-se que a presença de vários focos de infecção possa exacerbar o quadro sistêmico.

O reflexo do aumento sérico de IL-17 é potencializar processos inflamatórios em diversas doenças auto-imunes, ativando a produção de outros mediadores inflamatórios (Aggarwal et al., 2003). Para diferenciação das células Th17 e consequente produção de IL-17, se faz necessária a presença de outra citocina pró-inflamatória, a IL-23 (Aggarwal et al., 2003). A IL-23 ainda não foi explorada como a IL-17 quanto ao aumento sérico em infecções dentárias. Esta citocina geralmente é expressa por monócitos ativados, macrófagos, células dendríticas, células T, células B e células endoteliais (Oppmann et al., 2000). Desde a sua descoberta, a IL-23 tem sido associada com a patogênese de doenças autoimune, o que demonstra a importância de se estudar este mediador de forma sistêmica (Murphy et al., 2003; Wong et al., 2008; Nair et al., 2009). A relação da IL-23/IL-17 com a osteoclastogênese vem sendo descrita em algumas doenças como artrite reumatoide e doença periodontal (Murphy et al., 2003; Silva

et al., 2012). Existe apenas um estudo evidenciando a presença da IL-23 em periodontites apicais, em que foi demonstrado a participação ativa da IL-23 em fase aguda, até o 35º dia, concomitante com a fase de expansão da lesão (Hu et al., 2013).

Além de estimular a liberação de IL-17, a IL-23, uma vez ativada, também pode atuar diretamente sobre um subconjunto de macrófagos e células dendríticas, resultando na produção de outros mediadores inflamatórios, tais como IL-6 e TNF-α (Cua et al., 2003).

A interleucina 6 (IL-6) também é um dos mediadores liberados na periodontite apical (Prso, et al., 2007, Azuma et al., 2013). Afirma-se que esta citocina está envolvida desde a degradação da matriz extracelular que ocorre durante a inflamação da polpa (Wisithphrom & Windsor 2006), até a patogênese em si da lesão, estimulando também a ativação de células clásticas (Huang et al., 2001). Além disso, o nível desta citocina é mais expressivo em lesões sintomáticas (Prso, et al., 2007). Recentemente, foi mostrado aumento sérico da IL-6 em indivíduos portadores de periodontites apicais. Os pesquisadores notaram ainda que a presença aumentada da IL-6 teve uma correlação positiva com o desenvolvimento de doenças cardíacas (Cotti, et al., 2011).

Assim como ocorre com a IL-6, o fator de necrose tumoral alfa (TNF-α) e o interferon gama (IFN-γ), também são mediadores que atuam diretamente na patogênese da periodontite apical (Safavi & Rossomando 1991; Teixeira-Salum et al., 2010). Os efeitos biológicos destas citocinas compreendem a ativação de leucócitos como linfócitos T e B, ativação de macrófagos e células NK (Chen et al., 1999), atuando também na atividade osteoclástica local nestas lesões (Colić et al., 2009; Silva et al., 2011). Ambas as citocinas são produzidas por subclasses Th1 de células T CD4+ (Paul 1998) e são marcantes em fases agudas, no momento de expansão das lesões.

TNF-α e IFN-γ se relacionam com a produção de óxido nítrico (Cannon et al., 1998), um importante mediador biológico envolvido na fisiopatologia de diversas doenças atuando como mensageiro intra e intercelular liberado durante vários eventos, inclusive imunológicos (Moncada et al., 1991).

O óxido nítrico (NO), ou monóxido de nitrogênio permaneceu durante muitas décadas conhecido apenas como um gás poluente nocivo, sendo alvo de interesse apenas de químicos e ambientalistas (Fukuto 1995), até a sua

descoberta como uma molécula gerada de forma endógena por células de mamíferos (Palmer et al., 1988). O NO é um radical livre ubíquo, produzido por uma grande variedade de células, por meio de ações sintetizadoras de uma família de enzimas coletivamente denominadas de óxido nítrico sintases (NOS) (Hibbs et al., 1987). Há uma isoforma de NOS induzível (iNOS, NOS-2), que pode ser encontrada em quase todos os tipos celulares após estimulação (Forstermann et al., 1995).

O papel do NO em lesões periapicais é muito controverso. Por um lado, há evidencias de que a supressão de NO pela administração de aminoguanidina aceleraria o processo de cicatrização da periodontite apical (Farhad et al., 2011). No entanto, devido ao fato do IL-1 e TNF-α estimularem a produção de NO, acredita-se que baixas concentrações de NO aumente a produção destas citocinas pró-inflamatórias (que por sua vez atuam também na osteoclastogênese), estimulando assim a exacerbação do processo inflamatório (Silva et al., 2011). Ou seja, quanto menor a concentração de NO, maior a concentração de IFN-γ e TNF-α para ativação de mais NO (Silva et al., 2011). Assim, especula-se que o NO seja a chave da regulação da reabsorção óssea em lesões periapicais, já que ele pode atuar indiretamente regulando a diferenciação de osteoclastos pela inibição de algumas citocinas pró-inflamatórias cruciais neste processo (Silva et al., 2011).

O fato é que na presença de lesões periapicais existe NO (Fukada et al., 2008). Entretanto, o aumento sérico de NO estimula a patogênese de uma variedade de doenças, como doenças cardiovasculares, neuronais e diabetes (Rochette et al., 2013). Assim, tem sido mostrado que terapias anti NO podem ser promissoras para o controle da patogenia destas doenças (Rochette et al., 2013).

Diante do exposto, nota-se ainda deficiência de informações quanto à implicação destes mediadores pró-inflamatórios (TNF-α, IFN-γ, IL-6, IL-17, IL-23, NO) via sistêmica decorrente da infecção de origem endodôntica presente de forma isolada ou em diferentes regiões.

Assim, a segunda hipótese desta proposta abrange o entendimento do processo inflamatório sistêmico e local analisados por meio da quantificação por

ELISA em plasma sanguíneo e por meio da marcação imunoistoquímica nas áreas da lesão periapical.

II. OBJETIVOS

Objetivo Geral

Colaborar com o entendimento dos mecanismos que envolvem a relação das infecções orais com a saúde sistêmica.

Objetivos Específicos

1- Analisar as alterações em hemograma de ratos portadores de uma ou múltiplas periodontites apicais induzidas;

2- Analisar o perfil inflamatório sistêmico por meio da quantificação sérica do NO e das citocinas pró-inflamatórias TNF-α, IFN-γ, IL-6, IL-17 e IL-23 em ratos portadores de uma ou múltiplas periodontites apicais induzidas;

3- Analisar o perfil inflamatório local das lesões periapicais induzidas por meio da análise histológica (H.E), imunoistoquímica (TNF-α, IFN-γ, IL-6, IL-17, IL-23) e identificação do NO, pela quantificação do NOS.

III. Artigo 1

Apical periodontitis influences serum levels of cytokines and nitric oxide

Introduction: This study evaluated whether apical periodontitis (AP) in a single tooth or in multiple teeth affected serum levels of inflammatory mediators and influences blood homeostasis. **Methods:** Thirty male Wistar rats were divided into 3 groups of 10 rats each: control group, healthy rats; 1AP group, rats with AP in 1 tooth; and 4AP group, rats with AP in 4 teeth. After 30 days, the rats were anesthetized, and their blood was collected through cardiac puncture to quantify TNF-α, IFN-γ, IL-6, IL-17, IL-23, and nitric oxide (NO) levels. The rats were then sacrificed by administering an anesthetic overdose. Their maxillary and mandibular molars were collected and processed for histological analysis with hematoxylin-eosin and for immunohistochemical staining of the cytokines and NO-producing enzyme nitric oxide synthase (NOS). Results of these analyses were statistically analyzed; $P < 0.05$ was considered statistically significant. **Results:** Rats in the 1AP and 4AP groups showed increased IL-6, IL-17, IL-23, TNF-α, IFN-γ, and NOS expression; inflammatory cell infiltration, and moderate bone resorption in affected teeth. Serum TNF-α, IL-6, IL-17, and IL-23 levels were higher in rats in the 4AP group than in those in the control group ($P < 0.05$). Serum NO levels were significantly lower in rats in the 1AP and 4AP groups than in those in the control group ($P < 0.05$). Serum IFN-γ levels were not different among rats in the 3 groups ($P > 0.05$). **Conclusion:** These results suggested that AP affected blood homeostasis by altering the serum levels of inflammatory cytokines and NO.

Key Words: Apical periodontitis, cytokines, nitric oxide

Introduction

The theory of focal infection, which was proposed in the early 20th century, suggests that pathogenic microorganisms causing dental infections can disseminate systemically and trigger systemic changes (1). Thus, the prescribed treatment for infected teeth was extraction (2). This theory was rejected because of the lack of scientific evidence (3). However, results of several recent studies on this topic indicate that dental infections such as apical periodontitis (AP) may potentiate the pathogenesis of autoimmune diseases, mainly by increasing inflammatory cell infiltration and serum cytokine levels (4–8).

Pathogenesis of AP is complex and involves inflammatory mediators that activate as well as inhibit inflammation (9). T helper (Th) cells produce pro- and anti-inflammatory cytokines and seeking neutralize the pathogenic agents (10). Cells belonging to the Th1 lineage enhance the inflammatory process by secreting cytokines such as TNF-α and IFN-γ, which activate osteoclastogenesis and polymorphonuclear cells (11, 12). Cells belonging to the recently discovered Th17 lineage release cytokines such as IL-17 and IL-23 (13). In AP, Th17 cells are considered pro-inflammatory, as well as Th1 cells, which are also associated with increased bone resorption (14). IL-6 is produced by several cell types and is involved in acute-phase responses, B cell maturation, and macrophage differentiation. In addition, IL-6 plays a dual role in Th1/Th2 differentiation (15).

Nitric oxide (NO) is an important inflammatory mediator involved in AP (16). NO is a ubiquitous free radical produced in various cells by a family of enzymes collectively known as NO synthases (NOS) (17). Although the role of NO in AP is unknown, studies have shown that NO modulates the levels of proinflammatory cytokines such as IFN-γ and TNF-α during the pathogenesis of AP (18, 19).

Our previous research showed that periodontal diseases induce systemic alterations. Although periodontal diseases or AP in a single tooth does not induce significant systemic changes, periodontal diseases or AP in 2 teeth alters some systemic parameters (5–7). Therefore, the present study evaluated the serum levels of TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NO in rats with AP in a single tooth or in multiple teeth.

Material and Methods

Experimental Design

Three-month-old male Wistar rats (weight, 250–280 g) were housed in a temperature-controlled room (25°C) with a 12-/12-h dark/light cycle. The rats had *ad libitum* access to food and water. Experimental protocols of this study were approved by the institutional ethics committee and were conducted in accordance with relevant guidelines (Ethics Committee on Animal Use, Univ Estadual Paulista 2014-00108). The rats were divided into 3 groups of 10 rats each: control group: rats without AP, 1AP group: rats with AP in 1 tooth, and 4AP group: rats with AP in 4 teeth.

Induction of Oral Infections

Rats in the experimental groups were anesthetized by administering an intramuscular injection containing 13 mg/kg xylazine (Coopazine; Coopers Ltd Brazil, São Paulo, Brazil) and 87 mg/kg ketamine (Vetaset; Fort Dodge Animal Health Ltd, São Paulo, Brazil). AP was induced by exposing the pulp on the mesial surface of the first right maxillary molar of rats in the 1AP group and of the first and second right maxillary and mandibular molars of rats in the 4AP group by using surgical round burs (Broca LN Long Neck; Maillefer, Dentsply Ind. e Com. Ltda, Petrópolis, RJ, Brazil). AP was induced by exposing the pulps for 30 days (7,8).

Assessment of Serum Levels of TNF-α, lFN-γ, IL-6, IL-17, and IL-23

Blood was collected from the anesthetized rats (50 mg/kg sodium thiopental; Thiopentax, Cristália Itapira, SP, Brazil). After 30 days, venous blood samples (5 mL) were collected from the anesthetized rats through cardiac puncture after fasting the rats overnight for 8–12 h. The blood samples were centrifuged immediately at 1,800 × *g* and 4°C for 15 min to obtain plasma, which was immediately stored at -80°C. Serum cytokine levels were assessed by performing capture enzyme-linked immunosorbent assay (ELISA) by using commercial kits

(rat TNF ELISA set BD OptEIA™, cat #558535; BD Biosciences, San Diego, CA, USA; rat IFN-gamma DuoSet, cat #DY585; R&D Systems, Inc., Minneapolis, MN, USA; rat IL-6 ELISA set BD OptEIA™, cat #550319; BD Biosciences; rat IL-17A ELISA MAX™ Deluxe, cat #437904; Biolegend, San Diego, CA, USA; rat IL-23 ELISA kit, Uscn Life Science Inc., cat #SEA384Ra; Wuhan, Hubei, China), according to the manufacturers' instructions.

Assessment of Serum NO Levels

Serum NO levels were quantified using Griess reaction, as described by Guevara (1998) (20). For nitrate reduction, 100 µL serum samples were incubated with nitrate reductase (50 mU/100 µL of the sample), nicotinamide adenine dinucleotide phosphate, and reduced tetrasodium salt hydrate diluted in 20 µM/L Tris buffer for 30 min at room temperature. After reduction, the serum samples were incubated overnight with 900 µL methanol:diethyl ether mixture (3:1 v/v). The samples were then centrifuged (at 10000 rpm and 4°C for 10 min), and the supernatant was used for determining nitrite levels. For this, 50 µL 6.5 M/L HCl and 50 µL 37.5 µM/L sulfanilic acid were added to 200 µL deproteinized supernatant samples. After incubation at 4°C for 10 min, 50 µL 12.5 mM/L N-1-naphthylethylenediamine (NED) was added and the samples were incubated for 30 min at 4°C. The samples were then centrifuged (at 10000 rpm and 4°C for 10 min), and their absorbance was measured at 540 nm by using a microplate reader (Labsystems, Midland, ON, Canada). A standard curve was generated using sodium nitrite concentrations ranging from 1 to 100 µM/L.

Histopathological and Immunohistochemical Analyses

After obtaining blood samples, the rats were sacrificed by administering an overdose of sodium thiopental (150 mg/kg) intraperitoneally. Their maxillary and mandibular molars were immediately dissected, post-fixed in neutral-buffered formalin for 18 h (for fixing the samples and for preserving the antigenicity and tissues) (21), and decalcified in 17% buffered EDTA (pH 8; Sigma Chemical Co, St Louis, MO, USA). The first molar was sectioned semi-serially (thickness, 4 µm) along its longitudinal axis (21). The sections were stained with hematoxylin-eosin

for histological analysis or were used for performing immunohistochemical staining with an indirect immunoperoxidase technique by using primary antibodies against TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunohistochemical staining was performed according to a protocol described previously (21).

Intensity of inflammation was graded as follows: 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). Semiquantitative immunolabeling of TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NOS was performed by a certified histologist who was blinded to the treatments. Three histological sections were used for each animal, and positive immunoreactive (IR) was defined as a brownish color in the cytoplasm of the cells and extracellular matrix. Because immunolabeling of both the cells and the extracellular matrix is of great importance for our study, we performed semiquantitative analysis, which provides information on the numbers of immunoreactive cells and immunolabeling intensity of the extracellular matrix. The scores were assigned as follows (22): 0, complete absence of immunoreactive cells; 1 (low IR), few immunoreactive cells and weak labeling of the extracellular matrix (approximately one quarter of the immunoreactive cells); 2 (moderate IR), moderate number of immunoreactive cells and moderate labeling of the extracellular matrix (approximately one half of the immunoreactive cells); and 3 (high IR), large number of immunoreactive cells and strong labeling of the extracellular matrix (approximately three quarters of the immunoreactive cells).

Statistical Analysis

Statistical analyses and data tabulation were performed using SigmaPlot software (Systat Software Inc.; San Jose, CA, USA). Nonparametric data were analyzed by performing multiple comparisons with Kruskal–Wallis test followed by Dunn test. Parametric data were analyzed by performing analysis of variance followed by Tukey's test. Level of significance was set at 5%.

Results

Serum Levels of TNF-α, IFN-γ, IL-6, IL-17, and IL-23

Serum levels of TNF-α, IFN-γ, IL-6, IL-17, and IL-23 are shown in Table 1. Serum levels of TNF-α, IL-6, IL-17, and IL-23 were significantly higher in rats in the 4AP group than in rats in the control group ($P < 0.05$). Although rats in the 1AP group showed an increase in the serum levels of most cytokines compared with rats in the control group, the difference was not statistically significant ($P > 0.05$). Serum IFN-γ levels were not different among rats in the 3 groups ($P > 0.05$). Moreover, no difference was observed between rats in the 1AP and 4AP groups with respect to the levels of TNF-α, IL-6, IL-17, and IL-23 ($P > 0.05$; Table 1).

Serum Levels of NO

AP decreased serum NO levels in rats ($P < 0.05$; Table 1). Serum NO levels were significantly lower in rats in the 1AP and 4AP groups than in rats in the control group ($P < 0.05$). However, no significant difference was observed in serum NO levels between rats in the 1AP and 4AP groups ($P > 0.05$).

Histological and Immunohistochemical Analyses

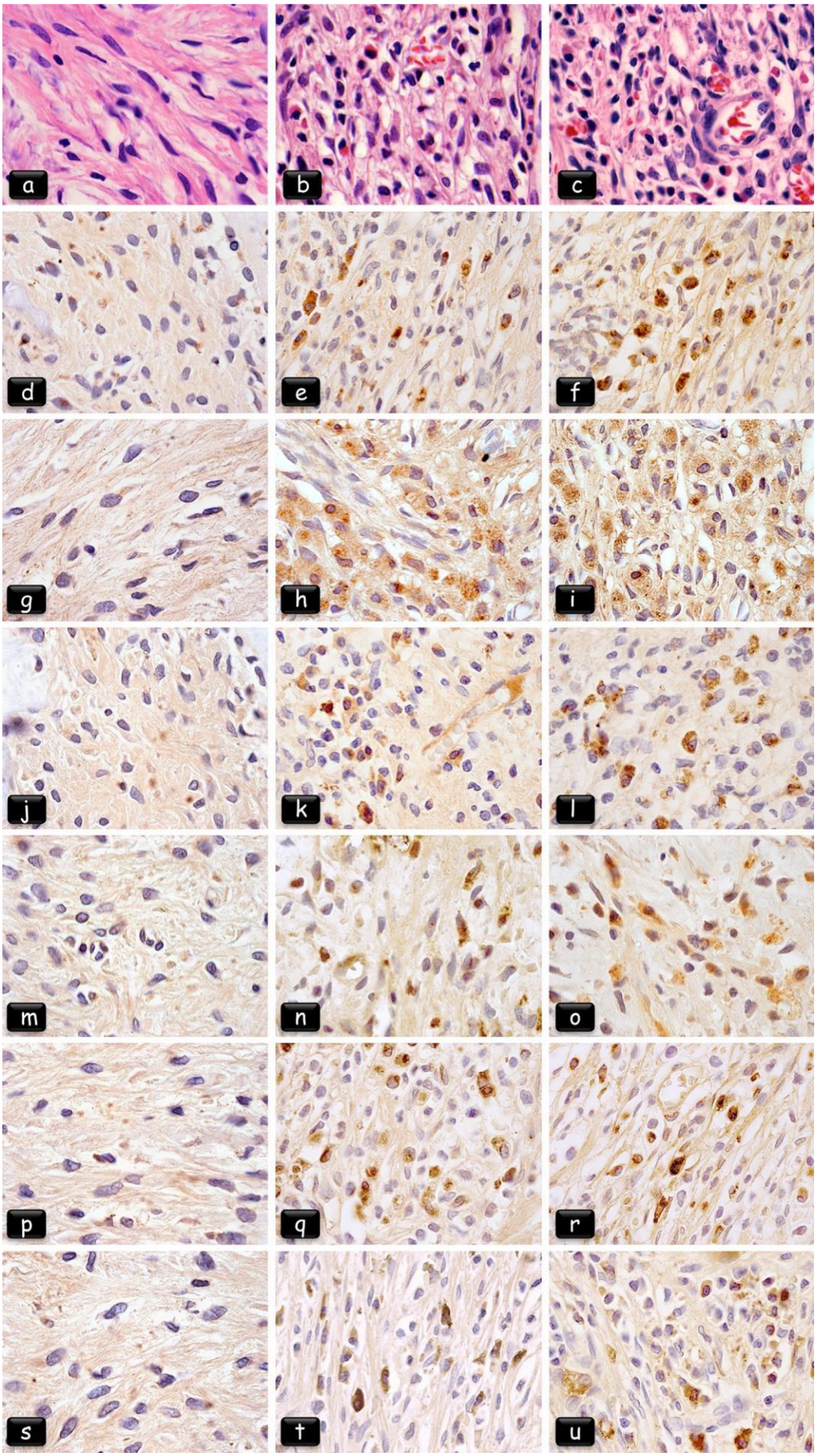
Histological analyses of tooth sections of rats in the experimental groups are shown in Figure 1. Tooth sections of rats in both 1AP and 4AP groups showed necrosis and AP at 30 days after pulp exposure. Moreover, tooth sections of rats in these groups predominantly showed moderate inflammatory infiltration of mononuclear cells (score 3). Tooth sections of rats in the control group showed no inflammation (scored 0). Evaluation of cytokines predominantly showed moderate immunolabeling for TNF-α, IFN-γ, IL-6, IL-17, IL-23, and NOS in the tooth sections of rats in the 1AP and 4AP groups, with no significant difference between the 2 groups. Sections of healthy teeth of rats in the control group showed complete absence of immunoreactive cells (score 0). Highest IR was observed in the tooth sections of rats in the 1AP and 4AP groups compared with those of rats in the control group ($P < 0.05$; Figure 1).

Table 1 - Serum levels of cytokines in pg/mL and NO in µmol/L.

	Inflammatory mediators	Groups		
		Healthy Rats	**1AP**	**4AP**
Kruskal Wallis (Mean, min - max values)	*TNF-α*	25.53 (15.3 - 26.9)[a]	29.21 (18.1 - 36.7)[a]	94.27 (43.4 - 158.0)[b]
	IFN-γ	40.72 (35.9 - 45.4)[a]	45.48 (35.9 - 78.7)[a]	40.72 (35.9 - 50.2)[a]
	IL-6	53.36 (32.4 - 113,4)[a]	53.24 (24.1 - 169.8)[a]	224.00 (67.4 - 463.8)[b]
	IL-23	13.25 (9.3 - 19.7)[a]	15.18 (10.5 - 20.7)[a]	37.46 (27.6 - 52.7)[b]
ANOVA (mean ± SD)	*IL-17*	96.44 ± 0.08[a]	96.45 ± 0.11[a]	96.6 ± 0.21[b]
	NO	11.09 ± 5.15[a]	6.56 ± 2.45[b]	5.58 ± 2.10[b]

*Different letters indicate significant statistical differences in lines ($p < .05$)

Figure 1 - Figure 1. Representative photographs of histological (a–c) and immunohistochemical analyses (d–o) of induced periapical lesions and healthy tooth tissues of rats. (a) Healthy tissue of rats in the control group without inflammation in the apical region (hematoxylin-eosin staining, ×1000). (b and c) Predominance of mononuclear cells in the inflammatory infiltrates of AP lesions (hematoxylin-eosin staining, ×1000) in (b) rats in the 1AP group and (c) rats in the 4AP groups. (d–f) *In situ* TNF-α expression in rats in the (d) control, (e) 1AP, and (f) in 4AP groups. (g–i) *In situ* IFN-γ expression in rats in the (g) control, (h) 1AP, and (i) 4AP groups. (j–l) *In situ* IL-6 expression in rats in the (j) control, (k) 1AP, and (l) 4AP groups. (m–o) *In situ* IL-17 expression in rats in the (m) control, (n) 1AP, and (o) 4AP groups. (p–r) *In situ* IL-23 expression in rats in the (p) control, (q) 1AP, and (r) 4AP groups. (s–u) *In situ* NOS expression in rats in the (s) control, (t) 1AP, and (u) 4AP groups. Magnification for a–o: ×1000.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Discussion

This study evaluated whether AP in a single tooth or in multiple teeth altered the serum levels of inflammatory mediators. Our results showed an increase in the serum levels of IL-6, IL-17, IL-23, and TNF-α in rats with AP lesions in multiple teeth. In contrast, serum NO levels decreased rats with AP lesions in a single tooth or in multiple teeth.

Increased serum levels of proinflammatory cytokines in rats with AP confirm the hypothesis that endodontic infections adversely affect systemic health similar to periodontal diseases, which is consistent with that reported by several studies (23–25). In our previous studies (5–7), we showed that periodontal diseases in a single tooth exerted an inflammatory response similar to AP in a single tooth, i.e., they did not significantly alter the serum levels of inflammatory cytokines (5–7). In contrast, AP associated with periodontal disease, affecting 2 teeth altered the numbers of inflammatory cells and levels of proinflammatory mediators in the blood (5–7).

AP-induced increase in the numbers of inflammatory cells and serum levels of cytokines can affect the balance of the bloodstream. Thus, against others pathogenic agent or pathological process, there are exacerbating inflammation in regions distant from the origin, leading to uncontrolled systemic inflammation (26). Although the function of inflammation is to protect host organisms from pathogenic agents, excessive inflammation leads to the loss of specificity and aggressively affects healthy tissues (26). For example, inflammation induced by periodontal diseases stimulates proinflammatory mediators, which in turn enhance the pathogenesis of autoimmune diseases such as diabetes, lupus, and rheumatoid arthritis (25).

TNF-α and IL-6 stimulate a proinflammatory response by recruiting more inflammatory cells and mediators to restrict a pathogenic agent or a pathological process (11). Once in the bloodstream, TNF-α and IL-6 can be attracted by chemotaxis to sites with advanced or incipient inflammatory processes, enabling inflammation and may cause exaggerated responses which damage healthy tissue (27). Similarly, a previous study showed that 20- to 40-year-old patients with AP were predisposed to cardiovascular diseases and showed a significant increase in the serum levels of IL-1, IL-2, and IL-6 (4).

Th17 cells function like Th1 cells in AP and systemic diseases and exacerbate AP-associated inflammation, including bone resorption, painful symptoms, and reacutization (28, 14). In a previous study, we showed that dental infections (AP and periodontal diseases) in 2 teeth increase serum levels of IL-17 and neutrophils (6). In the present study, serum levels of IL-17 and IL-23 increased in rats with AP in multiple teeth and were associated with bone resorption. Both IL-17 and IL-23 are involved in the pathogenesis of autoimmune diseases and act along with other inflammatory mediators to enhance pathological processes (28, 29).

In the present study, no difference was observed in serum IFN-γ levels among rats in the 3 groups. Biological effects of IFN-γ in AP include activation of leukocytes such as T and B cells, macrophages, and NK cells (30) and increase in local osteoclastic activity (12, 31). Our analysis was performed 30 days after AP induction when the disease was in the chronic phase; this may explain the lack of differences between rats in the groups. However, further studies are needed to determine whether AP temporarily increases the serum levels of IFN-γ because it is present in local tissues.

Another important observation of this study was the reduction in serum NO levels in rats with AP irrespective of the number of affected teeth. NO is synthesized by NOS from L-arginine. However, the role of NO in AP is unclear. Suppression of NO by aminoguanidine accelerates the treatment of AP (32). However, because IFN-γ and TNF-α stimulate NO production, low concentrations of NO may increase the production of these proinflammatory cytokines (which are also involved in osteoclastogenesis), thus exacerbating inflammation (18, 19).

In the present study, NO was detected in AP tissue despite its reduced levels in the blood. A recent study showed that serum levels of NO and its metabolites in patients with periodontal diseases increased locally at the site of infection and decreased in the blood (24).

Approximately 70%–90% of NO and its metabolites in the blood are derived from endothelial physiological activity (33). Given the importance of inter-relationships between different mediators involved in the pathogenesis of periapical lesion, perhaps AP may promotes imbalance in vascular homeostasis system, which can be stimulated by other inflammatory mediators (34).

Previous studies have shown that patients with periodontal diseases show impaired endothelium-dependent vasodilatation (34, 35) that may be associated with decreased NO production by the endothelium (34). Thus, an increase in NO production may occur locally in tissues with periodontal diseases and may not be reflected in the systemic levels of NO (24).

However, further studies are needed to elucidate mechanisms underlying the reduction of serum NO levels and its effect on systemic health.

Conclusion

Our results indicate that AP affects blood homeostasis by increasing the numbers of TNF-α-, IL-6-, IL-17-, and IL-23-secreting cells and by decreasing the levels of NO. Our results also indicate that an increase in the number of AP-affected teeth increases the changes in the serum levels of inflammatory mediators.

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The authors declare that they do not have any conflicts of interest related to this study.

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III. Artigo 2

Endodontic infections increase leukocyte and lymphocyte levels in the blood

Abstract

Purpose The aim of this study was to determine whether the presence of single or multiple apical periodontitis (AP) alters blood cell counts.

Material and Methods Thirty rats were divided into three groups: a control group comprising rats without AP; a group called 1AP comprising rats with AP in one tooth; and a group called 4AP comprising rats with AP in four teeth. Endodontic infections were induced by pulp exposure of the first right maxillary molar in the 1AP group, and by exposing the first and second right maxillary and mandibular molars in the 4AP group. A blood count was obtained 30 days after infection by collecting blood by cardiac puncture. The maxillae were dissected and stained with hematoxylin and eosin to evaluate the inflammatory infiltrates. The data were tabulated and subjected to statistical analysis ($P < 0.05$).

Results Histological analysis showed a predominance of mononuclear inflammatory cells. In blood, significant increase of leukocytes and lymphocytes in 4AP compared with the control and 1AP groups ($P < 0.05$).

Conclusions In the rat model, the presence of multiple AP can affect health by increasing lymphocyte levels in the blood.

Clinical Relevance The presence of endodontic infections can interfere with the blood profile, altering systemic health.

Keywords - Apical periodontitis, leukocytes, lymphocytes, blood count.

1. Introduction

Infections of endodontic origin are very common in the general population. They are primarily characterized by the presence of harmful agents and a complex immune system that functions by: (i) the neutralization of bacteria and their products; and (ii) the induction of tissue repair [1]. However, the equilibrium of this process depends on the presence of mediators, which activate and inhibit inflammation to avoid deleterious effects [2]. These effects, which are stimulated

by high bacterial load [1] or by diseases that affect the immune system, elevate levels of the mediators in the blood, and activate inflammation [3, 4].

The increase of inflammatory cells and mediators in the blood potentiates local inflammatory processes, such as apical periodontitis (AP) [5], and may accelerate the pathogenesis of autoimmune diseases such as rheumatoid arthritis and psoriasis [6]. Therefore, it has been suggested that a bidirectional relationship exists between dental infections and systemic health [7, 8].

Previous studies have shown that infections of dental origin, especially periodontal diseases, have a direct relationship with autoimmune diseases [3, 4] and potentiate metabolic upsets by increasing glycosylated hemoglobin [4, 9], triglycerides [10, 11], inflammatory cells, mediators [4], and oxidative stress [12]. However, few studies have been conducted with respect to infections of endodontic origin. We have previously reported that the presence of AP in one tooth led to slight alterations in the blood, as was also shown in periodontal disease localized to one tooth [3, 4, 13]. However, in periodontal disease that was concomitant with AP, significant blood alterations were observed [13]. To date, there is no consistent evidence that endodontic infection alone can lead to alterations, and it is possible that the presence of more than one tooth with AP may lead to a significant increase in inflammatory cells, among other alterations in the blood.

The blood count is a highly reliable test and has been used for blood evaluation in several studies [13-15]. It is possible to evaluate several blood parameters such as the levels of erythrocytes, leukocytes, neutrophils, and lymphocytes. Those cells are directly related to the presence of infections such as AP [13]. Thus, the aim of this study was to assess blood counts from rats with single or multiple AP to determine whether AP produces alterations in the blood profile.

2. Material and Methods

2.1. Experimental Animals

The experimental protocol was approved by the institutional ethics committee and was conducted in accordance with the relevant guidelines of the Ethics Committee on Animal Use (2014-00108). Three-month-old male Wistar rats weighing 250–280 g were housed in a temperature-controlled room (25°C) with a 12-h dark/light cycle. Food and water were provided *ad libitum*.

2.2. Induction of AP

The rats were divided into three groups of ten rats each: a control group– rats without AP; a group called 1AP– rats with AP on the first molar of the right maxillae; and a group called 4AP– rats with AP on the first and second right maxillary, and on the mandibular molars.

Anesthesia was administered to each rat in the experimental groups by an intraperitoneal injection of xylazine (13 mg/kg; Coopazine; Coopers Ltd Brazil, São Paulo, Brazil) combined with ketamine (87 mg/kg; Vetaset; Fort Dodge Animal Health Ltd, São Paulo, Brazil). For the induction of AP, the pulps of the molars were exposed on the mesial surface by using a surgical round bur of 0.1 mm diameter (Broca LN Long Neck, Dentsply Maillefer Ind. e Com. Ltda, Petrópolis, Brazil).

2.3. Blood Sample Collection and Determination of Hematologic Parameters

After 30 days, the rats were again anesthetized as previously described and a cardiac puncture was performed to collect 5 mL blood from each subject. The samples were collected in ethylenediaminetetraacetic acid (EDTA), mixed, and immediately transferred to a technician, who was blinded to the case status, for processing. The following parameters were determined using an automated analyzer (ABX Micros ABC Vet; Horiba ABX Diagnostics, Montpellier, France): red blood cell concentration; packed cell volume (also known as hematocrit);

mean corpuscular hemoglobin level; mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC); and leukocyte, neutrophil, lymphocyte, monocyte, basophil, and eosinophil counts. Next, the values for each of these hematologic parameters were subjected to analysis of variance followed by the Tukey's test ($P < 0.05$).

2.4. Tissue Processing and Morphometric Evaluation

After blood sample collection, the rats were sacrificed using an overdose of anesthetic solution. Their maxillae were removed and subjected to conventional histological processing, as described in a previous study [13].

The extent and severity of the inflammation were evaluated by examining the inflammatory infiltrate. The average number of cells per field in the inflammatory infiltrate and the extent of the inflammation beyond either apical foramen were considered. For each experimental group, the number of cells in the infiltrate was calculated as the average of 10 separate fields (400× magnification), according to the method described in a previous study [4]. The severity of the inflammation was evaluated by assigning one of the following grades to the infiltrate: absent (0 to few inflammatory cells: score 1), mild (< 25 cells: score 2), moderate (25–125 cells: score 3), and severe (> 125 cells: score 4). Analyses were performed blindly by a single calibrated operator. Inflammation scores were statistically analyzed (Kruskal–Wallis test, $P < 0.05$) [4].

3. Results

3.1. Blood count

The results of the blood tests are shown in Table 1. Among the inflammatory cells, a significant increase in leukocytes and lymphocytes was observed in 4AP compared with the control ($P < 0.05$). Lymphocytes and leukocytes increased to 43.9% and 36% in 4AP, respectively. Monocytes and neutrophils also increased to 23% and 13%, respectively, in 4AP compared with the control; however, this difference was not significant. Mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), eosinophils, erythrocytes, and

packed cell volume (also known as hematocrit) showed no significant difference ($P > 0.05$) (Table 1).

3.2. Histologic findings

Representative hematoxylin–eosin stained sections are shown in Figure 1. Thirty days after AP induction, the pulp showed total necrosis and AP was established in the 1AP and 4AP groups. The AP was exclusively restricted to the periapex region. Moreover, the AP was composed primarily of neutrophils (polymorphonuclears) and mononuclear cells, and comprised moderate inflammatory infiltrate in most samples (Table 2). There was no difference between the 1AP and 4AP groups. In the control group, none of the samples showed inflammatory infiltrates, and all the samples received a score of 0 (Table 2).

Table 1 - Mean and Standard Deviation of the Blood Cell Parameters.

Hematologic Parameters	**Groups (Mean** ± SD)*		
	Healthy Rats	**1AP**	**4AP**
Leukocytes	5.86±0.83[a]	6.56±1.53[a]	7.96±0.81[b]
Lymphocytes	39.39±7.65[a]	43.50±8.39[a]	56.70±9.56[b]
Neutrophils	18.42±4.34[a]	19.35±3.86[a]	20.86±5.31[a]
Hematocrit	45.33±2.92[a]	44.85±2.11[a]	43.30±1.70[a]
Hemoglobin	15.45±0.91[a]	14.95±0.52[a]	14.50±0.27[a]
MCV (fL)	54.49±0.74[a]	53.50±1.76[a]	52.79±1.11[a]

*Different letters indicate significant statistical differences in lines ($p < 0.05$)

Table 2 - Scores and median of the histological findings.

	Groups		
Intensity of inflammatory Infiltrade	**Healthy Rats**	**1AP**	**4AP**
1 - Absent	10/10	0/10	0/10
2 - Mild	0/10	1/10	0/10
3 - Moderate	0/10	8/10	8/10
4 - Severe	0/10	1/10	2/10
Median	**1[a]**	**3[b]**	**3[b]**

*Different letters indicate significant statistical differences in lines ($p < 0.05$)

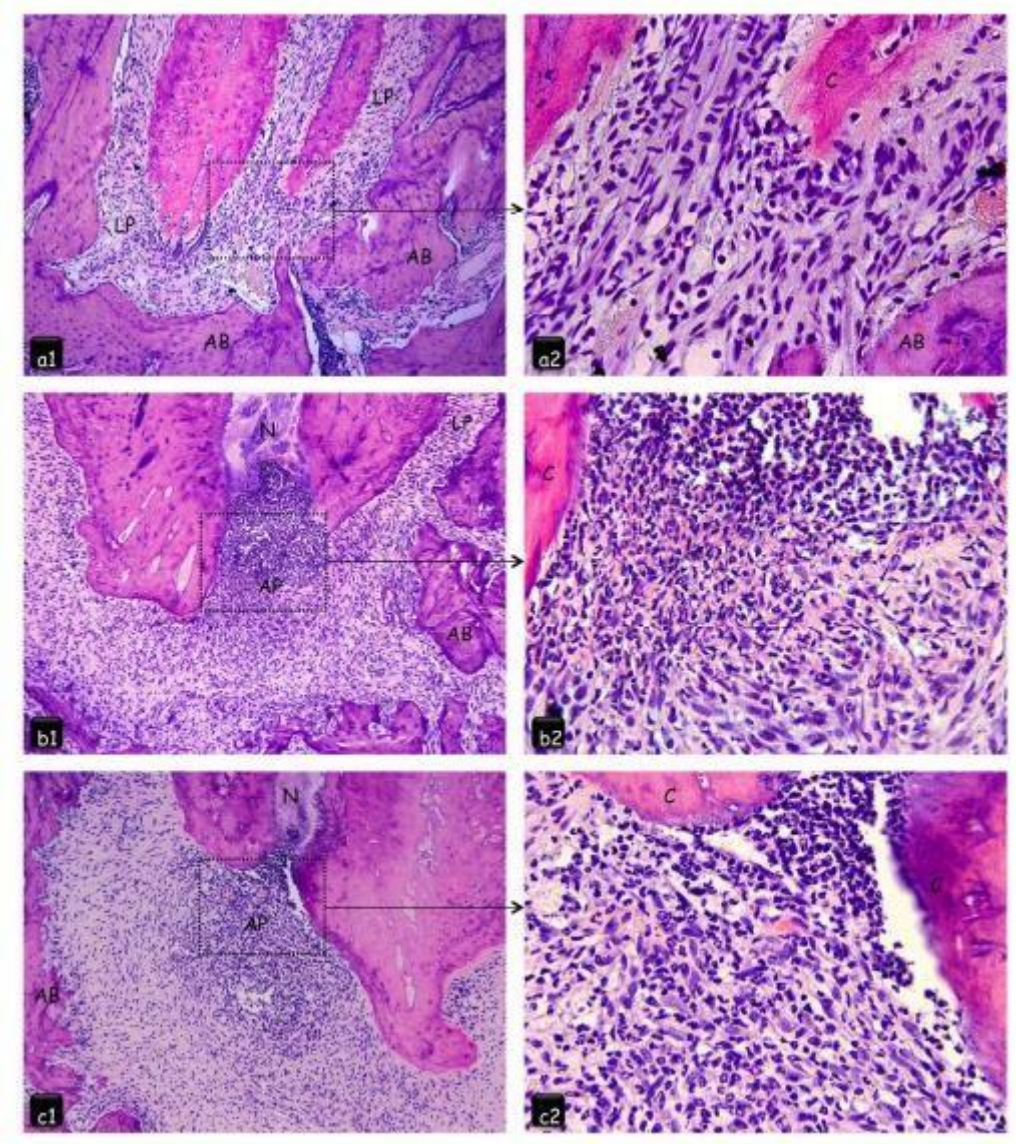

Figure 1- Histological findings after 30 days of AP induction. A1 and A2) Control group (healthy rats), increase of 100x and 400x, respectively. Absent of inflammatory infiltration on apical and periapical regions. B1 and B2) 1AP, increase of 100x and 400x, respectively: moderate inflammatory cell infiltration in the periodontal ligament region and large area of bone resorption is visible with predominance of mononuclear cells. C1 and C2) 4AP, increase of 100x and 400x, respectively: a moderate inflammatory cell infiltration in the periodontal ligament region and large area of bone resorption is visible with predominance of mononuclear cells.

4. Discussion

The present study determined whether single or multiple AP leads to blood alterations. Our results confirmed that endodontic infections in multiple teeth can lead to an increase in blood lymphocyte levels. The histologic features also confirmed this relationship: in addition to increased levels of lymphocytes in the blood, there were increased levels of lymphocytes in response to bacterial infection in AP in the 1AP and 4AP groups.

The prevalence of lymphocytes in AP is explained by the experimental period of 30 days, since at this stage the lesion has the characteristics of chronic inflammation [16]. In a previous study, we observed similar results in rats with periodontal disease associated with AP [13]. Although there was no significant increase in the presence of dental infection in two teeth, there was an increase of 61% in the average number of leukocytes in the group with AP concomitant with periodontal disease compared with those without dental infections [13].

The increased numbers of inflammatory cells resulting from AP may activate and enhance the deleterious effects of autoimmune diseases. They may also activate inflammatory mediators and increase oxidative stress, exacerbating inflammation in different foci throughout the body [17]. Because the biological pathways that activate inflammation and autoimmunity are the same, the presence of AP can increase the deleterious effects of autoimmune diseases, and the pathogenesis of autoimmune disease can also potentiate the effects of AP in a bidirectional relationship [18, 19].

Confirming this bidirectional relationship, models with diabetes and oral infections, for example, have shown that various parameters are altered in the presence of systemic diseases, and periodontal and periapical lesions. These alterations include increases in blood glucose [4], glycosylated hemoglobin [4], triglycerides [11], inflammatory mediators such as IL-17 and neutrophils [3], and insulin resistance [20]. Cardiovascular diseases have also been reported to be associated with AP [21, 22]. Moreover, histological, and radiographic analysis of the lesions has revealed a significant increase in bone loss in diabetic rats compared with non-diabetic rats with the same dental infections [3].

Our results showed that blood neutrophil levels were not significantly different between the groups. This result is consistent with a previous report, which also showed that neutrophil levels in the presence of concomitant periodontal disease and AP were not significantly different [13]. In diabetic rats with AP and concomitant periodontal disease, the lesions showed acute inflammation even at 30 days, which also led to increased levels of polymorphonuclear cells [13]. This indicates that diabetes enhances the inflammatory process. In the present study, the rats with AP were healthy. Thus, after 30 days, AP showed chronic inflammation and lower levels of neutrophils between groups were expected, since these cells are the first line of defense [16].

In the present study, there was no significant difference in MCV, hematocrit, and hemoglobin in the presence of AP. However, it was noticeable that all these parameters were slightly reduced in the presence of multiple AP compared with the control group. In the presence of aggressive periodontal disease, some studies have also reported a reduction of red blood cells and hemoglobin levels, suggesting that periodontal disease may be associated with the pathogenesis of mild anemia [23, 24]. Since in the present study AP was induced in only four teeth, the difference between the groups was not significant, as observed in generalized periodontitis.

In the present study, the presence of AP in one tooth did not result in significant differences compared with uninfected rats. These data are consistent with those of a previous study [13], and were therefore expected. However, a slight increase in some parameters suggested the need for induction of infection in multiple teeth to observe significant differences, as observed in some patients.

It is common for a single patient to show foci of endodontic infections in multiple teeth [25].

Experimental induction of AP in rats is a consistent method and an accepted model for studying AP [26]. The use of experimental animals in this model allowed the study to be performed under standardized conditions where animals of the same strain, sex, and weight received the same food, experienced the same stress, and shared the same accommodation [26]. Such a study in humans would not be ideal owing to the variability of samples, which could put the results into question. In addition, blood count is an extremely reliable test and has been used in several medical evaluations and scientific studies [13-15]. For these reasons, we found it prudent to use blood count in rats to confirm the hypothesis that infections of endodontic origin may influence systemic health.

The scientific community has not extensively explored the relationship between AP and systemic health. However, the existence of this relationship in periodontal disease has been well established by several *in vivo* and *in vitro* studies [27-29]. Although both infections have a similar pathogenesis with the same bacterial profile, and a similar inflammatory process with subsequent bone resorption, the treatment of endodontic infection is still not seen as a form of health promotion.

Given the results of this study, it should be noted that AP can activate or enhance the deleterious effects of autoimmune diseases, owing to the significant systemic alterations that occur in the blood, such as increased lymphocyte counts.

5. Conclusion

In conclusion, the presence of multiple endodontic lesions can affect health by increasing lymphocyte levels in the blood.

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

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VI. ANEXOS

VI.I. Material e Métodos utilizados

A) Animais

Foram utilizados 30 ratos machos (Rattus albinus, Wistar), pesando aproximadamente 180g, provenientes do biotério da Faculdade de Odontologia de Araçatuba - UNESP. Os animais foram mantidos em ambiente com temperatura entre 22 e 24ºC com ciclo de luz controlada (12 horas claro e 12 horas escuro) e em gaiolas coletivas, quatro ratos por gaiola, alimentados durante todo o período experimental com dieta sólida e água “ad libitum”, exceto nas primeiras 24 horas após a intervenção. Os procedimentos experimentais propostos neste estudo foram aprovados pelo comitê de conduta ética no uso de animais em experimentação (CEUA 2014-00108 Unesp).

B) Drogas empregadas

Para anestesia dos animais foi utilizado via intramuscular um sedativo à base de xilazina (Dopaser, Calier S.A. - Barcelona, Espanha – 10mg/Kg) e um anestésico à base de Cloridrato de Ketamina a 5% (Vetanarcol, König S. A. – Avellaneda, Argentina – 25mg/Kg).

C) Divisão em grupos

Os ratos foram distribuídos em 3 grupos de acordo com os procedimentos locais de indução das doenças pulpares:

- Ratos saudáveis: ratos sem infecções endodôntica.

- 1AP: ratos com uma infecção endodôntica induzida.

- 4AP: ratos com quatro infecções endodônticas induzidas.

D) Indução da infecção pulpar:

Para o desenvolvimento da infecção pulpar, 20 ratos (1AP e 4AP) foram anestesiados. Para o grupo 1AP foi exposta a polpa do primeiro molar superior direito de cada animal (Cintra et al., 2012; Cintra et al., 2013). Para o grupo 4AP, foram expostas as polpas dos primeiros e segundos molares superiores e inferiores direitos. Para isso foi empregada uma broca de aço carbono dotada de uma esfera na extremidade com 0,1mm de diâmetro (Broca Ln Long Neck - Maillefer, Dentsply, Tulsa, OK). Assim, todas as exposições pulpares foram padronizadas com 0,1mm de diâmetro. Todas as exposições foram sondadas, confirmando a exposição do tecido pulpar pela observação do com sangramento (Haug & Heyeraas, 2003) (Figura 1).

E) Coleta do sangue

Aos 30 dias pós-operatórios os animais foram novamente anestesiados e foi realizada a coleta de 5ml de sangue por meio de punção cardíaca. Posteriormente, os animais foram mortos por uma sobredose anestésica (Figura 1H).

Com as amostras sanguíneas foram analisado os parâmetros: hemácias, volume globular (hematócrito), hemoglobina, VCM, CHCM, leucócitos, neutrófilos, linfócitos, monócitos, eosinófilos, basófilos e plaquetas.

As amostras sanguíneas para NO, TNF-α, IFN-γ, IL-6, IL-17 e IL-23 foram colocadas em heparina (10U/ml), na sequencia centrifugadas e armazenadas imediatamente a -80°C.

F) Coleta das maxilas e mandíbulas

As peças foram coletadas e fixadas em formaldeído a 4% por 24 horas. Foram então lavadas por 12 horas em água corrente e desmineralizadas em ácido etilenodiamino tetracético (EDTA) a 10% e, finalmente lavadas por 24 horas em água corrente. Na sequência, as peças foram desidratadas em álcool,

diafanizadas em xilol e incluídas em parafina. Uma vez incluídas, as peças foram cortadas em micrótomo Leica RM 2045 (Leica Microsystems, Nussloch – Germany) com espessura de 5 µm e colocados em laminas especiais (Star-frost ®).

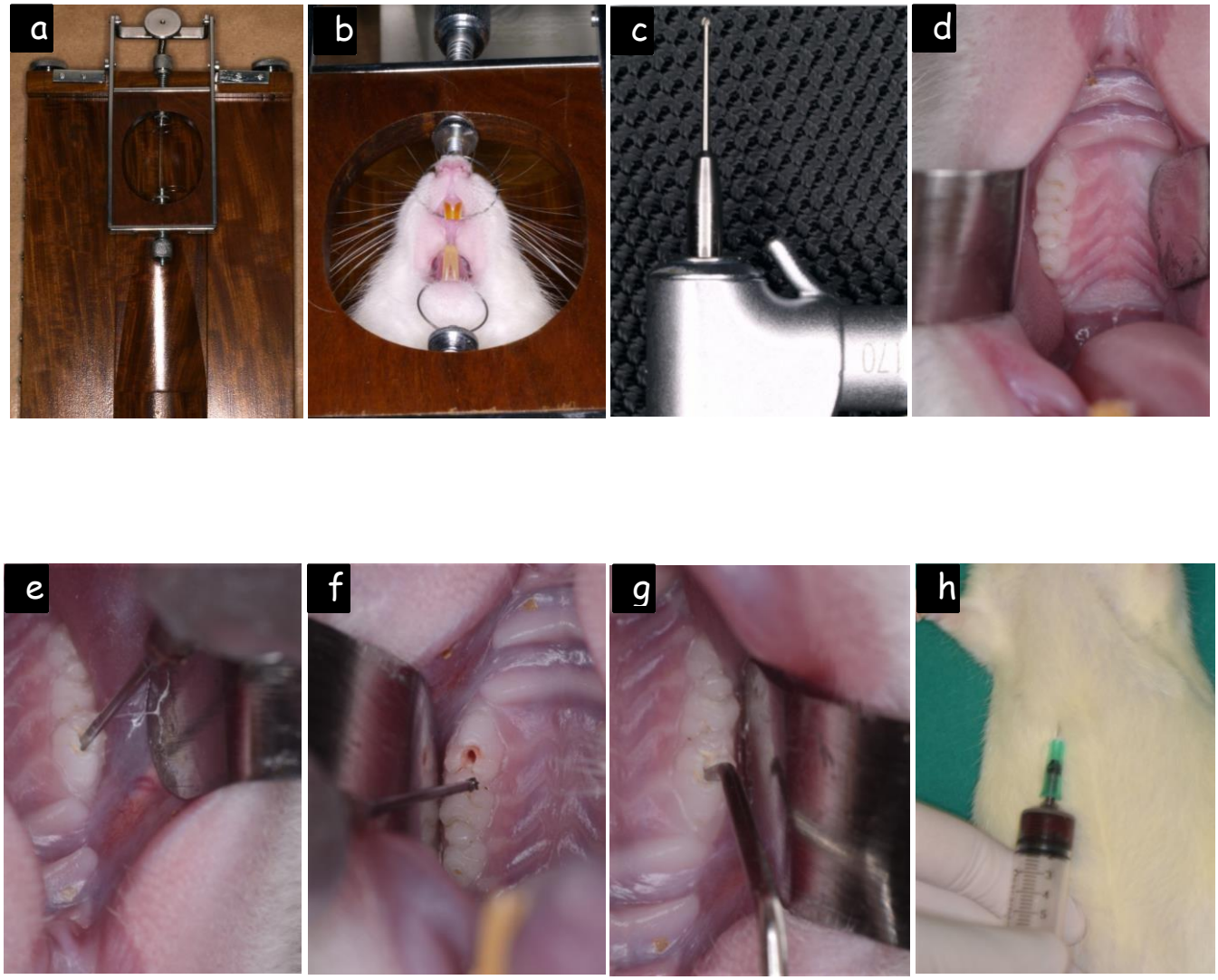

Figura 1. a) Mesa utilizada para proporcionar abertura bucal nos ratos. b) Rato posicionado para experimento. c) Broca Ln utilizada para realização da abertura coronária. d) Imagem do campo operatório. e) Abertura coronária, caída na câmara pulpar (padronização da abertura: até que entre totalmente a parte ativa da broca). f) Visualização da exposição pulpar após abertura. g) Desorganização do tecido pulpar com auxílio da sonda exploradora. h) Coleta do sangue via punção cardíaca após 30 dias de exposição pulpar.

VI.II. Forma de análise dos resultados

A) Análise sistêmica

A1) Hemograma

Para a realização do hemograma, foi utilizado o analisador automático ABX (ABX Micros ABC Vet - Horiba ABX Diagnostics, Montpellier, France). Foram observados os seguintes parâmetros: Hemáceas (106/µL), Volume Globular (%), Hemoglobina (g/dL), VCM (fL), CHCM (%), Leucócitos (10^3/µL), Neutrófilos (%), Linfócito (%), Monócito (%), Eosinófilo (%) e Basófilo (%).

Foi empregado o kit ABX VetPack que é composto por 3 reagentes:

O ABX VET Pack, R3 é uma solução salina e tampão eletrolítica que permite a diluição e a preparação da amostra de sangue para análise. O fluxo dos sistemas hidráulicos do aparelho é garantido pela presença de surfactante não iônico. A ação eletrolítica aceita a contagem das células por impedância. Este reagente diferencia populações morfológicas de leucócitos e é utilizado nos ciclos de enxágue e limpeza dos sistemas hidráulicos do instrumento.

O ABX VET Pack, R2 decompõe a membrana celular das hemáceas. Pela adição do agente surfactante, a hemoglobina é libertada. Todo o ferro heme é oxidado e os complexos resultantes são quantificados por espectrofotometria a um comprimento de onda de 530 nm. O detergente presente na solução também diferencia populações morfológicas de leucócitos.

ABX VET Pack, R1 utiliza a ação combinada de uma enzima proteolítica com um detergente para eliminar os resíduos de proteína e evitar que os tubos hidráulicos fiquem obstruídos e/ou bloqueiem o fluxo. Também é usado para decompor os acúmulos de proteína nas aberturas e câmaras de contagem.

A2) Quantificação das citocinas pró-inflamatórias TNF-α, IFN-γ, IL-6, IL-17 e IL-23 no plasma

Inicialmente foram pipetados os anticorpos de captura (1:1000; BD Biosciences, CA, USA) de cada uma das citocinas em suas respectivas placas. As placas permaneceram em overnight na geladeira por 16 horas. No dia seguinte, todos os reagentes foram colocados à temperatura ambiente antes de procederem os experimentos. As placas foram então lavadas e aspiradas por 4 vezes com solução tampão (PBS) associada a Tween 20 (Sigma, MO, USA). Foram adicionados então 200ul de solução tampão associada à albumina por poço. A placa permaneceu incubada e selada por 1 hora à temperatura ambiente. Em seguida, ela foi lavada novamente com PBS+Tween 20 (Sigma, MO, USA) e aspirada por 4 vezes.

Foram pipetados então 100µl do recombinante, e foi realizada a diluição seriada para fazer a curva padrão. Foram pipetadas 50µl das amostras nos poços apropriados. Novamente foram realizados os selamentos das placas e a incubação das amostras à temperatura ambiente por 2 horas. A aspiração e lavagem foram realizadas novamente da mesma forma já descrita. Foram adicionados 100µl de anticorpo secundário (1:1000; BD Biosciences, CA, USA) em cada poço, as placas de vedação foram colocada para incubação da amostra por 1 hora à temperatura ambiente. Novamente foram realizadas a aspiração e lavagem. Então foram adicionados 100µl de reagente enzimático diluído (ExtrAvidin®−Peroxidase, Sigma, MO, USA) em cada poço. Foi feito o selamento da placa e a incubação por 30 minutos à temperatura ambiente. A aspiração e lavagem nesta etapa foram feitas 7 vezes os poços foram mergulhados por um minuto em tampão de lavagem. Posteriormente foram adicionados 100µl de solução de substrato TMB (BD Biosciences, CA, USA) em cada poço. A incubação foi realizada sem o selador de placa por trinta minutos à temperatura ambiente no escuro. Por fim, foram adicionados 50µl de solução de parada (H_2SO_4) em cada poço. A leitura foi realizada em espectofotômetro ajustado para o comprimento de onda de 450nm.

A3) Quantificação de metabólitos do NO no plasma

Os níveis séricos de NO foram quantificados utilizando a reacção de Griess, conforme descrito por Guevara (1998). Para a redução de nitrato, 100μL de soro foram incubadas com nitrato redutase (50 mU/100 mL da amostra), fosfato de dinucleótido de nicotinamida e adenina, e reduzida com sal tetrassódico hidraado diluído em 20 μM/L de tampão Tris durante 30 min à temperatura ambiente. Após a redução, as amostras de soro foram incubadas durante a noite com 900 uL de metanol éter dietílico (3:1 v/v). As amostras foram então centrifugadas (a 10000 rpm e 4°C durante 10 min), e o sobrenadante foi utilizado para determinar os níveis de nitritos. Posteriormente, 50 uL de 6,5 M/L de HCl e 50 ul de 37,5 uM/L de ácido sulfanílico foram adicionados a 200 ul de sobrenadante desproteinizado amostras. Depois as amostras foram incubadas a 4°C durante 10 min, foi adicionado 50 uL de 12,5 mM/L de N-1-naftiletilenodiamina (NED) e as amostras foram incubadas durante 30 min a 4 ° C. As amostras foram então centrifugadas (a 10000 rpm e 4 ° C durante 10 min), e a sua absorvância foi medida a 540 nm usando um leitor de microplacas (Labsystems, Midland, ON, Canada). Uma curva padrão foi gerada usando concentrações de nitrito de sódio na gama de 1 a 100 μM/L.

B) Análise local

B1) Análise histológica das lesões periapicais

Para a análise histológica foram empregadas 5 lâminas com 3 cortes teciduais cada, preparadas e coradas com hematoxilina e eosina (Lillie, 1954). A análise histológica foi utilizada para caracterização do perfil inflamatório das lesões periapicais (Cintra et al. 2013c). Os resultados foram expostos por meio de duas análises, sendo uma descritiva e outra quantitativa. As lâminas contendo os cortes representativos de cada espécime foram avaliadas sob a microscopia óptica e utilizadas na análise descritiva. A análise descritiva consistiu na

descrição dos fenômenos histopatológicos, procurando caracterizá-los globalmente em função das variáveis experimentais (Cintra et al. 2013c).

A análise quantitativa foi realizada por meio da atribuição de escores, graduando as magnitudes dos fenômenos histopatológicos de forma dissociada e da análise histométrica das estruturas do periodonto apical (Cintra et al. 2013c). As variáveis estudas foram: infiltrado inflamatório quando à sua intensidade e extensão; presença de reabsorções radiculares e perda de estrutura óssea periapical. Foram atribuídos escores para os critérios intensidade e extensão do infiltrado inflamatório e para a presença de reabsorções radiculares. Para a perda de estrutura óssea foi realizada a mensuração linear e por área, respectivamente, empregando programa de imagens específico (Leica QWin Plus - Leica Microsystems, Nussloch – Germany). Os critérios considerados para cada análise foram:

1 – Intensidade do infiltrado inflamatório

A intensidade do processo inflamatório foi analisada em conformidade com o número médio aproximado de células inflamatórias presentes em diferentes campos de um mesmo espécime, examinados em aumento de 400x junto ao periápice dentário (Quadro 1).

Quadro 1 – Escores para o critério intensidade do infiltrado inflamatório

Escore 1	Células inflamatórias ausentes ou em número desprezível;
Escore 2	Infiltrado inflamatório discreto (menos de 10 células por campo);
Escore 3	Infiltrado inflamatório moderado (entre 10 e 25 células por campo);
Escore 4	Infiltrado inflamatório intenso (mais que 25 células por campo).

2 – Extensão do infiltrado inflamatório

A extensão do infiltrado inflamatório foi estabelecida em função da alteração do tecido periapical o (Quadro 2).

Quadro 2 – Escores para o critério extensão do infiltrado inflamatório

Escore 1	Células inflamatórias ausentes ou em número desprezível;
Escore 2	Células inflamatórias ocupando extensão de até 300µm do ápice radicular;
Escore 3	Células inflamatórias ocupando extensão de até 600µm do ápice radicular;
Escore 4	Células inflamatórias ocupando extensão maior que 600µm do ápice radicular.

3 – Presença de reabsorções radiculares

A presença de reabsorções radiculares foi analisada em conformidade com o número médio de lacunas de reabsorção presentes em diferentes campos, de um mesmo espécime, examinados em aumento de 400x junto ao periápice dentário (Quadro 3).

Quadro 3 – Escores para o critério presença de reabsorções radiculares

Escore 1	Ausência de Lacunas de reabsorção;
Escore 2	Presença discreta de lacunas de reabsorção cementária (menos de 3 lacunas por campo);
Escore 3	Presença moderada de lacunas de reabsorção cementária e reabsorção dentinária ao acaso (mais de 3 e menos que 6 lacunas por campo);
Escore 4	Presença intensa de lacunas de reabsorção cementária e reabsorção dentinária (mais que 6 lacunas por campo).

4 – Perda de estrutura óssa

Foram selecionados cortes representativos de cada espécime dos grupos com doença pulpar com e sem diabetes (Grupos 2 e 4) para a verificação da perda de estrutura óssea periapical proveniente da infeção pulpar. Foi empregado o software Leica QWin Plus (Leica Microsystems, Nussloch – Germany) e os valores foram expressos em micrometro quadrado (µm2) obtidos em medidas de área.

B2) Imunoistoquímica para determinação do NOS e das citocinas pró-inflamatórias TNF-α, IFN-γ, IL-6, IL-17 e IL-23 nas lesões periapicais

Os cortes histológicos foram desparafinizados em xilol e hidratados em série decrescente de etanol. A recuperação antigênica foi realizada através da imersão das lâminas histológicas em tampão Diva Decloaker® (Biocare Medical, CA, USA), em câmara pressurizada Decloaking Chamber® (Biocare Medical, CA, USA), a 95°C, por 10 minutos. Ao término de cada uma das etapas da reação imunoistoquímica foram efetuadas lavagens em tampão fosfato salino (PBS) 0,1M, pH 7,4. Os cortes histológicos foram imersos em 3% de peróxido de hidrogênio por 1 hora e em 1% de soro albumina bovino por 12 horas, para o bloqueio da peroxidase endógena e bloqueio dos sítios inespecíficos, respectivamente. As lâminas histológicas contendo amostras de todos os grupos experimentais foram divididas em seis lotes, e cada lote foi submetido à incubação com seus respectivos anticorpos primários. Os anticorpos primários foram diluídos em PBS acrescido de 0,1% Triton X-100 (PBS-TX), durante 24 horas, em câmara úmida. Nas etapas subseqüentes foi empregado o Universal Dako Labeled (HRP) Streptavidin-Biotin Kit® (Dako Laboratories, CA, USA). As secções histológicas foram incubadas no anticorpo secundário biotinilado, durante 2 horas, e posteriormente tratadas com estreptavidina conjugada com a peroxidase da raiz forte (HRP), por 1 hora. Na revelação foi empregado como cromógeno o 3,3'- tetracloridrato de diaminobenzidina (DAB chromogen Kit®, Dako Laboratories, CA, USA) e em seguida a contracoloração foi realizada com hematoxilina de Harris. Como controle negativo, os espécimes foram submetidos aos procedimentos descritos anteriormente suprimindo-se a utilização dos anticorpos primários.

C) ANÁLISE ESTATÍSTICA

A análise estatística foi realizada por meio do programa SigmaPlot 12.0™ (Chicago, IL, USA). A verificação da distribuição normal das variáveis contínuas quantitativas foi feita pelos testes de Kolmogorov-Smirnov e Shapiro-Wilk. As variáveis que apresentarem distribuição normal foram expressas em média e ± desvio padrão e aquelas que apresentaram distribuição não normal foram expressas em mediana com valor mínimo e máximo e percentis.

Os valores quantitativos das análises que seguiram uma distribuição normal foram aplicados o teste ANOVA, enquanto que para comparações múltiplas dos resultados entre os grupos estudados, foi utilizado o teste Tukey.

Para as análises histológicas foram atribuídos escores e para os valores quantitativos das análises que não seguiram uma distribuição normal, foi aplicado o teste de Kruskal-Wallis, e quando observada alguma diferença significativa, foi realizado o cruzamento entre os grupos, pelo teste de comparações múltiplas de Dunn (Dunn, 1958). Também foi empregado o teste de Mann Whitney (Siegel et al., 1956) para comparação entre dois grupos isoladamente.

Os resultados foram considerados estatisticamente significativos quando a probabilidade for menor 5% ($p<0,05$).

VI.III. Normas para publicação dos periódicos

VI.III.I. Journal of Endodontics

Guidelines for Publishing Papers in the JOE

1. General Points on Composition

1. Authors are strongly encouraged to analyze their final draft with both software (*e.g.*, spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery since their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision, or have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the*JOE*:

2. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section “c” below), or sentences with little to no transition within a paragraph.
3. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors’ names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland et al., reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al., 1983)." In this example, the paragraph’s subject is sodium hypochlorite and sentences should focus on this subject.
4. Sentences are stronger when written in the active voice, *i.e.*, the subject performsthe action. Passive sentences are identified by the use of passive verbs such as “was,” “were,” “could,” etc. For example: “Dexamethasone was found in this study to be a factor that was associated with reduced inflammation,” can be edited to: “Our results demonstrated that dexamethasone reduced inflammation.” Sentences written in a

direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

5. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.)
6. Use parallel construction to express related ideas. For example, the sentence, "Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method," can be edited to "Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation." The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.
7. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used," can be edited to "Caution must be used when conclusions are drawn from the results of this study."
8. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.
9. Authors should be aware that the *JOE* uses iThenticate, plagiarism detection software, to assure originality and integrity of material published in the *Journal*. The use of copied sentences, even when present within quotation marks, is highly discouraged. Instead, the information of the original research should be expressed by new manuscript author's own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected, or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

2. Organization of Original Research Manuscripts

Please Note: *All abstracts should be organized into sections that start with a one-word title (in bold),* i.e.*, Introduction, Methods, Results, Conclusions, etc., and should not exceed more than 250 words in length.*

1. **Title Page:** The title should describe the major emphasis of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard—it represents your major opportunity to solicit readers to spend the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (*e.g.*, use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts

Submitted to Biomedical Journals at *www.icmje.org*). The manuscript title, name and address (including email) of one author designated as the corresponding author. This author will be responsible for editing proofs and ordering reprints when applicable. The contribution of each author should also be highlighted in the cover letter.

2. **Abstract:** The abstract should concisely describe the purpose of the study, the hypothesis, methods, major findings and conclusions. The abstract should describe the new contributions made by this study. The word limitations (250 words) and the wide distribution of the abstract (*e.g.*, PubMed) make this section challenging to write clearly. This section often is written last by many authors since they can draw on the rest of the manuscript. Write the abstract in past tense since the study has been completed. Three to ten keywords should be listed below the abstract.

3. **Introduction:** The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. The purpose of the study, the tested hypothesis and its scope should be clearly described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the *JOE*. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to succinctly summarize the gap in knowledge that the study addresses. It is important to note that many successful manuscripts require no more than a few paragraphs to accomplish these goals. Therefore, authors should refrain from performing extensive review or the literature, and discussing the results of the study in this section.

4. **Materials and Methods:** The objective of the materials and methods section is to permit other investigators to repeat your experiments. The four components to this section are the detailed description of the materials used and their components, the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the essential aspects used in the present study. Thus, the reader should still be able to understand the method used in the experimental approach and concentration of the main reagents (*e.g.*, antibodies, drugs, etc.) even when citing a previously published method. The inclusion of a "methods figure" will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a **commercial product**, the manuscript must state that they either followed manufacturer's protocol *or*specify any changes made to the protocol. If the study used an ***in vitro*** **model** to simulate a clinical outcome, the authors must describe experiments made to validate the model, or previous literature that proved the clinical relevance of the model. Studies on **humans** must conform to the Helsinki Declaration of 1975 and state that the institutional IRB/equivalent committee(s) approved the protocol and that informed consent was obtained after the risks and benefits of participation were described to the subjects or patients recruited. Studies involving **animals** must state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests

were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, drop-outs from clinical trials, the effects of important confounding variables, and bivariate versus multivariate analysis.

5. **Results:** Only experimental results are appropriate in this section (*i.e.*, neither methods, discussion, nor conclusions should be in this section). Include only those data that are critical for the study, as defined by the aim(s). Do not include all available data without justification; any repetitive findings will be rejected from publication. All Figures, Charts and Tables should be described in their order of numbering with a brief description of the major findings. Author may consider the use of supplemental figures, tables or video clips that will be published online. Supplemental material is often used to provide additional information or control experiments that support the results section (*e.g.*, microarray data).

6. **Figures:** There are two general types of figures. The first type of figures includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (*i.e.*, about 175 mm wide X 125 mm high when published in the *JOE*), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing to emphasize the most important feature of each photomicrograph, but it greatly increases the total number of illustrations that you can present in your paper. Remember that each panel must be clearly identified with a letter (*e.g.*, “A,” “B,” etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Jeger et al (J Endod 2012;38:884–888); Olivieri et al., (J Endod 2012;38:1007 1011); Tsai et al (J Endod 2012;38:965–970). Please note that color figures may be published at no cost to the authors and authors are encouraged to use color to enhance the value of the illustration. Please note that a multipanel, composite figure only counts as one figure when considering the total number of figures in a manuscript (see section 3, below, for maximum number of allowable figures).

   The second type of figures are graphs (*i.e.*, line drawings including bar graphs) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). Examples include a graph depicting pain scores over time, etc. Graphs should be used when the overall trend of the results are more important than the exact numerical values of the results. For example, a graph is a convenient way of reporting that an ibuprofen-treated group reported less pain than a placebo group over the first 24 hours, but was the same as the placebo group for the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

7. **Tables:** Tables are appropriate when it is critical to present exact numerical values. However, not all results need be placed in either a table or figure. For example, the following table may not be necessary:

% NaOCl	N/Group	% Inhibition of Growth
0.001	5	0
0.003	5	0
0.01	5	0
0.03	5	0
0.1	5	100
0.3	5	100
1	5	100
3	5	100

8. Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl (N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).

9. **Discussion:** This section should be used to interpret and explain the results. Both the strengths and weaknesses of the observations should be discussed. How do these findings compare to the published literature? What are the clinical implications? Although this last section might be tentative given the nature of a particular study, the authors should realize that even preliminary clinical implications might have value for the clinical readership. Ideally, a review of the potential clinical significance is the last section of the discussion. What are the major conclusions of the study? How does the data support these conclusions

10. **Acknowledgments:** All authors must affirm that they have no financial affiliation (*e.g.*, employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest should be disclosed. Any author for whom this statement is not true must append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Likewise the sources and correct attributions of all other grants, contracts or donations that funded the study must be disclosed

11. **References:** The reference style follows Index Medicus and can be easily learned from reading past issues of the *JOE*. The *JOE* uses the Vancouver reference style, which can be found in most citation management software products. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

3. Manuscripts Category Classifications and Requirements

Manuscripts submitted to the *JOE* must fall into one of the following categories. The abstracts for all these categories would have a maximum word count of 250 words:

1. CONSORT Randomized Clinical Trial-Manuscripts in this category must strictly adhere to the Consolidated Standards of Reporting Trials-CONSORT- minimum guidelines for the publication of randomized clinical trials. These guidelines can be found at *www.consort-statement.org/*. These manuscripts have a limit of 3,500 words, [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

2. Review Article-Manuscripts in this category are either narrative articles, or systematic reviews/meta-analyses. Case report/Clinical Technique articles even when followed by extensive review of the literature will should be categorized as "Case Report/Clinical Technique". These manuscripts have a limit of 3,500 words, [including abstract, introduction, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
3. Clinical Research (*e.g.*, prospective or retrospective studies on patients or patient records, or research on biopsies, excluding the use of human teeth for technique studies). These manuscripts have a limit of 3,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

4. Basic Research Biology (animal or culture studies on biological research on physiology, development, stem cell differentiation, inflammation or pathology). Manuscripts that have a primary focus on biology should be submitted in this category while manuscripts that have a primary focus on materials should be submitted in the Basic Research Technology category. For example, a study on cytotoxicity of a material should be submitted in the Basic Research Technology category, even if it was performed in animals with histological analyses. These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or 4 tables*.
5. Basic Research Technology (Manuscripts submitted in this category focus primarily on research related to techniques and materials used, or with potential clinical use, in endodontics). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 3 figures and tables *.
6. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.

* Figures, if submitted as multipanel figures must not exceed 1 page length. Manuscripts submitted with more than the allowed number of figures or tables will require approval of the *JOE* Editor or associate editors. If you are not sure whether your manuscript falls

within one of the categories above, or would like to request preapproval for submission of additional figures please contact the Editor by email at *jendodontics@uthscsa.edu*.

Importantly, adhering to the general writing methods described in these guidelines (and in the resources listed below) will help to reduce the size of the manuscript while maintaining its focus and significance. Authors are encouraged to focus on only the essential aspects of the study and to avoid inclusion of extraneous text and figures. The Editor may reject manuscripts that exceed these limitations.

Available Resources:

Strunk W, White EB. The Elements of Style. Allyn & Bacon, 4th ed, 2000, ISBN 020530902X.
Day R. How to Write and Publish a Scientific Paper. Oryx Press, 5th ed. 1998. ISBN 1-57356-164-9.
Woods G. English Grammar for Dummies. Hungry Minds:NY, 2001 (an entertaining review of grammar).
Alley M. The Craft of Scientific Writing. Springer, 3rd edition 1996 SBN 0-387-94766-3.
Alley M. The Craft of Editing. Springer, 2000 SBN 0-387-98964-1.

VI.III.II. Clinical Oral Investigations

Guidelines for Publishing Papers in the CLOI

Types of papers

Papers may be submitted for the following sections:

Original articles

Invited reviews

Short communications

Letters to the editor

It is the general policy of this journal not to accept case reports and pilot studies.

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

The Springer Author Academy is a set of comprehensive online training pages mainly geared towards first-time authors. At this point, more than 50 pages offer advice to authors on how to write and publish a journal article.

Text Formatting

Manuscripts should be submitted in Word.

Use a normal, plain font (e.g., 10-point Times Roman) for text.

Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

Use tab stops or other commands for indents, not the space bar.

Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

LaTeX macro package (zip, 182 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].

2. This result was later contradicted by Becker and Seligman [5].

3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of *California*

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

ISSN.org LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

EndNote style (zip, 2 kB)

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

Tables

All tables are to be numbered using Arabic numerals.
Tables should always be cited in text in consecutive numerical order.
For each table, please supply a table caption (title) explaining the components of the table.
Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and illustrations guidelines

Electronic Figure Submission

Supply all figures electronically.

Indicate what graphics program was used to create the artwork.

For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable.

Vector graphics containing fonts must have the fonts embedded in the files.

Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

Definition: Black and white graphic with no shading.

Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

All lines should be at least 0.1 mm (0.3 pt) wide.

Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi.

Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art

Definition: Photographs, drawings, or paintings with fine shading, etc.

If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves.

Halftones should have a minimum resolution of 300 dpi.

Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc.

Combination artwork should have a minimum resolution of 600 dpi.

Color Art

Color art is free of charge for online publication.

If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent.

If the figures will be printed in black and white, do not refer to color in the captions.

Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

To add lettering, it is best to use Helvetica or Arial (sans serif fonts).

Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt).

Variance of type size within an illustration should be minimal, e.g., do not use 8-pt type on an axis and 20-pt type for the axis label.

Avoid effects such as shading, outline letters, etc.

Do not include titles or captions within your illustrations.

Figure Numbering

All figures are to be numbered using Arabic numerals.

Figures should always be cited in text in consecutive numerical order.

Figure parts should be denoted by lowercase letters (a, b, c, etc.).

If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures,

"A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions

Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file.

Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type.

No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption.

Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs.

Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

Figures should be submitted separately from the text, if possible.

When preparing your figures, size figures to fit in the column width.

For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm.

For books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that

All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware)

Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements)

Any figure lettering has a contrast ratio of at least 4.5:1

Integrity of research and reporting

Ethical standards

Manuscripts submitted for publication must contain a statement to the effect that all human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

It should also be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted.

These statements should be added in a separate section before the reference list. If these statements are not applicable, authors should state: The manuscript does not contain clinical studies or patient data.

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Conflict of interest

Authors must indicate whether or not they have a financial relationship with the organization that sponsored the research. They should also state that they have full control of all primary data and that they agree to allow the journal to review their data if requested.

Therefore the manuscript must be accompanied by the “Conflict of Interest Disclosure Form”. To download this form, please follow the hyperlink on the right.

The manuscript must also be accompanied by the “Authorship & Disclosure Form”. To download this form, please follow the hyperlink on the right.

IV. Comitê de ética

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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 **"Perfil inflamatório local e sistêmico de ratos wistar com múltiplas infecções endodônticas"**, Processo FOA nº 2014-00108, sob responsabilidade Luciano Tavares Angelo Cintra apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 12 de março de 2014.

VALIDADE DESTE CERTIFICADO: 28 de Julho de 2016.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 28 de Agosto de 2016.

CERTIFICATE

We certify that the study entitled **"Local and systemic inflammatory profile of rats wistar with multiple endodontics infections"**, Protocol FOA nº 2014-00108, under the supervision of Luciano Tavares Angelo Cintra presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on March 12, 2014.

VALIDITY OF THIS CERTIFICATE: July 28, 2016.
DATE OF SUBMISSION OF THE FINAL REPORT: August 28, 2016.

Prof. Dr. Edilson Ervolino
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br