UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

Leticia Citelli Conti

DISSERTAÇÃO

INTER-RELAÇÃO ENTRE A PERIODONTITE APICAL E A ATEROSCLEROSE. ANÁLISE METABÓLICA, HISTOLÓGICA E DO PERFIL LIPÍDICO

ARAÇATUBA- SP

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP como parte dos requisitos para obtenção do título de Mestre em Ciência Odontológica, área de concentração em Endodontia.

Orientador: Prof. Adj. Luciano Tavares Angelo Cintra
Coorientadora: Profª. Dra. Suely Regina Mogami Bomfim

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Dedicatória

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meus pais: ***Isabel de Lurdes Citelli Conti*** e ***Marcio Forlan Conti***.

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"Carreguem a minha carga e aprendam de mim, porque sou manso e humilde de coração, e vocês encontrarão descanso para suas vidas. Por que a minha carga é suave e meu fardo é leve." (Mt 11:29)

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"Se eu orar, se eu clamar, as muralhas não resistiram ao Poder de Meu Deus"
Pedi e Recebereis (Vagner Bittencourt)

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Tua família (Anjos de Resgate)

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Eduque o jovem no caminho que deve andar, e até a velhice ele não desviará.
Provérbio (22:6)

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Amigos pela fé (Anjos de Resgate)

Epígrafe

“Nada é igual ao Seu redor

Tudo se faz no Seu olhar

Todo o universo se formou no Seu falar

Teologia pra explicar

Ou Big Bang pra disfarçar

Pode alguém até duvidar

Sei que há um Deus a me guardar

E eu, tão pequeno e frágil, querendo Sua atenção

No silêncio encontro resposta certa, então

Dono de toda **CIÊNCIA, SABEDORIA e PODER**

Ó, dá-me de beber da água da fonte da vida

Antes que o haja houvesse

Ele já era Deus

Se revelou ao seus

Do crente ao ateu

Ninguém explica Deus

E se duvida ou se acredita

Ninguém explica

Ninguém explica Deus

Ninguém explica

Ninguém explica Deus”

Ninguém explica Deus – Preto no Branco (Clóvis Pinho)

Conti LC. Inter-relação entre a periodontite apical e a aterosclerose. Análise metabólica, histológica e do perfil lipídico, 2018. Dissertação (Mestrado em Endodontia) – Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araçatuba.

Resumo

A aterosclerose é uma doença cardiovascular inflamatória crônica, caracterizada pelo acúmulo de placas de gordura nos vasos sanguíneos em consequência de danos no endotélio provocada por diversos fatores de risco, dentre eles o acúmulo de lipídeos. As infecções dentárias também são doenças de caráter inflamatório, dentre elas, está a infecção endodôntica que resulta no desenvolvimento da periodontite apical. Diante dos estudos apresentados na literatura especializada, dos quais os resultados ainda carecem de comprovação científica sobre possíveis correlações entre a periodontite apical com alterações sistêmicas, o objetivo deste trabalho foi avaliar a inter-relação entre a periodontite apical e a aterosclerose. Foram utilizados 40 ratos Wistar distribuídos em 4 grupos de 10 animais: ratos controle (C); ratos com periodontite apical (AP); ratos com aterosclerose (AT); ratos com periodontite apical e com aterosclerose (AP+AT). A aterosclerose foi induzida com a administração de uma dieta com alto teor lipídico, juntamente com um procedimento cirúrgico realizado na carótida comum direita associada a uma super dosagem de vitamina D_3. A periodontite apical foi induzida 30 dias após a indução cirúrgica da aterosclerose, pela exposição da polpa dentária coronária ao meio bucal, dos primeiros e segundos molares superiores e inferiores direito. O peso corporal foi obtido aos 0, 15, 45 e 75 dias e o consumo de ração foi mensurado nos períodos de 7, 15, 45 e 75 dias. Aos 45 e 75 dias, foi realizada punção cardíaca para remoção do tecido hematológico e as concentrações séricas do perfil lipídico foram mensurados. Aos 75 dias, os ratos foram eutanasiados e os órgãos: cérebro, coração, pulmão, fígado, baço, rim e gônadas foram pesados individualmente. A maxila, a mandíbula e a carótida foram processadas para análise histológica (hematoxilina e eosina). Os resultados das diferentes análises e a relação entre os achados locais e sistêmicos foram tabulados e analisados por testes estatísticos específicos para cada caso ($p<0,05$). A aterosclerose foi capaz de induzir a perda de peso dos animais no período inicial do experimento, no

entanto, ao final do experimento apenas o grupo AP+AT apresentou peso inferior quando comparado ao grupo controle. O consumo de ração foi semelhante entre todos os grupos. As concentrações séricas do colesterol total e do LDL-C foram, estatisticamente mais elevados nos grupos portadores de aterosclerose (AT e AP+AT) quando comparados aos outros grupos ($p < 0,05$). Nos grupos AP e AT, a presença das doenças foram capazes de aumentar os níveis de triglicérides, comparados ao controle, sem diferença estatística entre si. ($p < 0,05$). Já no grupo AP+AT os níveis de triglicérides foram superiores quando comparados aos grupos AP e AT. Houve aumento no peso do cérebro e redução no peso do pulmão nos grupos AP, AT, AP+AT quando comparados ao grupo C. O peso do baço no grupo AP apresentou-se elevado quando comparado aos demais grupos. Nas análises histológicas, o grupo AP+AT apresentou infiltrado inflamatório mais intenso e reabsorção óssea mais exacerbada quando comparada ao grupo AP. Foi possível detectar alterações morfológicas nas artérias carótidas dos animais portadores de aterosclerose. Conclui-se que a periodontite apical e a aterosclerose, isoladas ou associadas, influenciam no peso corporal, no metabolismo, no perfil lipídico, nas lesões periapicais e nas carótidas de ratos Wistar.

Palavras-Chave: Periodontite apical; aterosclerose; peso corporal; perfil lipídico; metabolismo; histologia; alterações sistêmicas.

Conti LC. Interrelationship between apical periodontitis and atherosclerosis. Metabolic, histological and lipid profile analysis, 2017. Dissertation (Master's Degree in Endodontics) – São Paulo State University (Unesp), School of Dentistry, Araçatuba.

Abstract

Atherosclerosis is a chronic inflammatory cardiovascular disease, characterized by the accumulation of lipid in the blood vessels, due to risk factors capable of causing injuries to the endothelium. Dental infections are also pathologies of inflammatory origin, among them is the endodontic infection that results in the development of apical periodontitis. In view of the studies presented in the specialized literature, of which the results still lack scientific evidence on possible correlations between apical periodontitis with systemic alterations, the objective of this study was to evaluate the possible interrelationship between apical periodontitis and atherosclerosis. Forty male Wistar rats were distributed in 4 groups of 10 animals: control rats (C); rats with apical periodontitis (AP); rats with atherosclerosis (AT); rats with AP and atherosclerosis (AP+AT). Atherosclerosis was induced by using a high-lipid diet together with right common carotid surgery and a super dosage of vitamin D_3. Apical periodontitis was induced 30 days after the surgical induction of atherosclerosis, by exposure of the coronary tooth pulp to the oral environment, of the first and second molars, upper and lower right. Body weight was obtained at 0, 15, 45 and 75 days and food consumption was measured on 7, 15, 45, and 75 days. At 45 and 75 days, cardiac puncture was performed to remove hematological tissue and serum levels of the lipid profile were measured. At 75 days the rats were euthanized and the organs: brain, heart, lung, liver, spleen, kidney and gonads were weighed individually. The upper and lower jaws and carotid were processed for histological analysis (hematoxylin and eosin). The results of the different analyzes and the relation between the local and systemic findings were tabulated and analyzed by specific statistical tests for each case ($p < 0.05$). Atherosclerosis induced the weight loss of the animals in the initial period of the experiment, however, at the end of the experiment only the AT+AP group presented lower weight when compared to group C. Feed consumption was similar among all groups. The levels of total cholesterol and low-density lipoprotein cholesterol (LDL-C) were statistically higher in the groups

with atherosclerosis (AT and AP+AT) when compared to the other groups ($p < 0.05$). In the AP and AT groups, the presence of infections were able to increase triglyceride levels, compared to control, with no statistical difference between them. ($p < 0.05$). In the AT+AP group, the triglyceride levels were higher when compared to the AP and AT groups. There was an increase in brain weight and a reduction in lung weight in the AP, AT, AP+AT groups when compared to group C. The spleen weight in the AP group was elevated when compared to the other groups. In the histological analyzes, the AP+AT group presented more severe periapical inflammatory infiltrates and more exacerbated bone resorption when compared to the AP group. It was possible to detect morphological changes in the carotid arteries of animals with atherosclerosis. It is concluded that apical periodontitis and atherosclerosis, whether isolated or associated, influence body weight, metabolism, lipid profile, periapical lesions and in the carotids of Wistar rats.

Keywords: Apical periodontitis; atherosclerosis; body weight; lipid profile; metabolism; histology; systemic disorders.

S U M Á R I O

Páginas

I. Introdução

I. INTRODUÇÃO

A aterosclerose é uma doença cardiovascular inflamatória caracterizada pela alteração no endotélio vascular (Ross, 1999; Verma et al., 2002; Libby, 2002) e causa enrijecimento das paredes arteriais (Lönn et al., 2012). Acredita-se que a presença de lipídeos oxidados, agentes infecciosos (Lou-Bonafonte et al., 2012) e fatores de risco, como a diabetes mellitus, hipertensão arterial, hipercolesterolemia, obesidade, distúrbios metabólicos (Hussain et al., 2015), tabagismo e predisposição genética, podem provocar lesões iniciais no endotélio dando início ao processo aterogênico (Eaton, 2005).

Na patogenia da doença aterosclerótica, essas lesões iniciais que ocorrem no endotélio, geram uma condição inflamatória subclínica crônica, na qual há constante liberação de citocinas pró-inflamatórias, infiltração de monócitos, produção de espécies reativas de oxigênio e oxidação de moléculas de lipídios e proteínas (Iwata & Nagai, 2012). Essas lesões endoteliais geram um aumento da permeabilidade vascular e facilita a penetração no espaço subendotelial, principalmente das lipoproteínas de baixa densidade (LDL) (Homma, 2004). Em resposta a essa injúria, formam-se placas ou estrias de gorduras, chamadas de ateromas. Essas placas são compostas principalmente por células sanguíneas, células espumosas, lipídeos e cálcio (Ravnskov, 1998). Os ateromas geram expansão vascular, podem restringir o fluxo de sangue e em caso de ruptura da parede vascular, originam coágulos com consequente isquemia (Lönn et al., 2012). A obstrução e a ruptura das artérias ateroscleróticas, geralmente causam infarto do miocárdio, enquanto que o bloqueio do fluxo sanguíneo das artérias carótidas pode causar acidente vascular cerebral (Davies, 2000).

Segundo a Organização Mundial da Saúde em 2012, as doenças cardiovasculares foram responsáveis por 17,5 milhões de mortes, representando 31% de todas as mortes em nível global e são consideradas as principais causas de mortalidade mundial (Paho/Who, 2016). Dentre essas doenças, está previsto que em 2020, a aterosclerose será a principal causa de morte no mundo (Scott, 2002).

Além da aterosclerose, as infecções dentárias também são doenças inflamatórias. Dentre elas, está a infecção endodôntica que resulta no

desenvolvimento da periodontite apical (Colic et al., 2010). O processo inflamatório ocorre como uma resposta do organismo ao se deparar com uma infecção ou com uma lesão do tecido. Esta atua objetivando a destruição de agentes agressores. Esse processo, envolve a liberação de substâncias endógenas ou exógenas, elas podem atuar amplificando, inibindo, mantendo ou ativando uma resposta inflamatória (Del Prete, 1992). Estas substâncias são compostas por diferentes moléculas, como por exemplo: citocinas (como interleucinas e fator de necrose tumoral) (Le & Vilcek, 1987), fatores de crescimento (Todaro et al., 1981), imunoglobulinas (Hektoen, 1909), proteínas de fase aguda (como a proteína C-reativa) (Merriman et al., 1975), liberação de radicais livres (Bragt & Bonta 1980) e molécula de adesão celular (Giaever & Ward 1978).

Apesar destes mediadores proporcionarem uma defesa favorável para o organismo, podem acontecer desequilíbrios entre substâncias pró e anti-inflamatórias, sendo que, estes desequilíbrios, podem resultar no início de processos inflamatórios exacerbados, vindo a afetar todo o organismo por meio do aumento de substâncias pró-inflamatórias de forma sistêmica (Bian et al., 2012) ou também, pela inibição excessiva da inflamação (Hersh et al., 1983).

A periodontite apical é caracterizada, histologicamente, por um tecido granulomatoso, infiltrado por diversos tipos de células inflamatórias (Lukic et al., 2008). Ela é formada devido à resposta do hospedeiro ao estímulo antigênico contínuo procedente dos canais radiculares contaminados (Zhang & Peng, 2005; Xiong et al., 2009). A resposta do hospedeiro envolve o recrutamento de diferentes células inflamatórias e a participação de uma extensa rede de mecanismos imunológicos (Xiong et al., 2009; Colic et al., 2010). Estudos comprovam que os mediadores inflamatórios são formados em resposta à presença de bactérias e seus produtos, os quais induzem e mantêm a resposta inflamatória (Xiong et al., 2009; Colic et al., 2010; Fan et al., 2011), que se mostram associadas à deterioração da matriz extracelular e posteriormente à reabsorção óssea (Nair, 2004; Graves et al., 2011). Contudo, a periodontite apical na maioria das vezes trata-se de uma infecção crônica que geralmente permanece assintomática (Cotti et al., 2011).

O excesso de substâncias pró-inflamatórias, como interleucinas, fatores de necrose tumoral e radicais livres podem acarretar resultados desfavoráveis,

pois quando liberadas, podem dar início ou potencializar a patogênese de diversas doenças, que também são ativadas pelos mesmos mediadores (Brod et al., 1991). 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 períodos longos (Bian et al., 2012).

Estudos epidemiológicos mostraram que a população brasileira possui uma média de 2,7 periodontites apicais presentes por indivíduo (Marotta et al., 2012), denotando ser comum a presença de mais de um foco residual de infecção em um mesmo paciente. Mundialmente, estudos mostraram que a periodontite apical está presente em 22 - 65% dos dentes com tratamento endodôntico (De Moor et al., 2000; Dugas et al., 2003; Siqueira et al., 2005).

Desta forma, a relação entre infecções orais e sistêmicas tem sido cada vez mais explorada nas pesquisas da área médica-odontológica (Segura-Egea et al., 2012; Cintra et al., 2013ab; Gomes et al., 2013; Cintra et al., 2014abc). Com relação às doenças cardiovasculares, estudos odontológicos existentes vêm suportando a hipótese de que a inflamação e a infecção podem estar ativamente envolvidas na aterogênese (Epstein et al., 1999). No entanto, diferente da doença periodontal, são escassos os estudos e carecem de informações, com relação às infecções de origem endodôntica (Mylona et al., 2012).

Estudos mostram que a presença da periodontite apical, após o tratamento endodôntico, está positivamente correlacionada com o risco e incidência de doenças cardiovasculares (Gomes et al., 2015). Neste contexto, a aterosclerose está sendo alvo de intensos estudos (Bartova et al., 2014; Tapashetti et al., 2014; Kudo et al., 2015), entretanto, a maioria destes estudos envolvem apenas a doença periodontal, onde foi observado aumento de marcadores que podem ser utilizados para prever a ocorrência de doenças cardiovasculares (Tapashetti et al., 2014; Etemadifar et al., 2015). Além disso, estudos realizados em humanos mostraram que a presença da doença periodontal está correlacionada com o risco de pacientes desenvolverem aterosclerose (Bartova et al., 2014; López, 2014; Kudo et al., 2015). Diante desta hipótese já confirmada com relação à doença periodontal, acredita-se ser necessário a realização de estudos que envolvam a periodontite apical em busca da compreensão dos mecanismos responsáveis pelo aumento do risco de se

desenvolver doenças cardiovasculares em pacientes com infecções bucais.

A influência da infecção endodôntica na saúde sistêmica tem sido alvo de intensos estudos em nosso grupo de pesquisa (Cintra et al., 2013a; Cintra et al., 2013b; Cintra et al., 2014a; Cintra et al., 2014b; Cintra et al., 2014c; Azuma et al., 2017; Prieto et al., 2017). Em estudos em ratos normoglicêmicos e ratos diabéticos, foi visto que a infecção endodôntica quando associada com a diabetes e com a doença periodontal pode influenciar os níveis de triglicérides (Cintra et al., 2013a), creatinina (Cintra et al., 2013b), hemoglobina glicada (Cintra et al., 2014c), mediadores pró-inflamatórios como a IL-17 (Cintra et al., 2014a), além de alterar alguns parâmetros do hemograma, destacando-se a série branca (Cintra et al., 2014b), bem como é capaz de alterar o sinal insulínico do sangue e do tecido muscular (Astolphi et al., 2013; Astolphi et al., 2015).

Tendo em vista que a aterosclerose é uma doença inflamatória que conduz a um aumento sistêmico de mediadores inflamatórios (Tedgui & Mallat, 2006; Weber et al., 2008; Ait-Oufella et al., 2011), seria oportuno verificar se a presença desta doença pode atuar na patogênese da periodontite apical.

Uma avaliação interessante a ser explorada é a análise do perfil lipídico, pois um dos principais fatores envolvidos no desenvolvimento das doenças cardiovasculares é o consumo de uma dieta rica em gorduras, proteínas animais, açúcares e sal (Daniels & Greer, 2008). A elevada concentração de colesterol total, triglicerídeos e de LDL, são fatores de risco importantes para doenças coronárias, uma vez que a deposição de LDL na camada subendotelial consiste em uma etapa importante para o início e progressão da aterosclerose (Stocker & Keaney, 2004; Houston et al., 2009). Desta forma, os níveis de colesterol total, triglicérides, lipoproteína de alta e de baixa densidade (HDL e LDL) de ratos com infecção endodôntica e aterosclerose foram mensurados em nosso estudo para detectar a possível relação bidirecional entre essas duas patologias.

Neste contexto, procuramos desenvolver um estudo com o intuito de preencher parte das lacunas existentes na literatura atual, buscando investigar as alterações locais e sistêmicas, por meio de análises metabólicas, sanguíneas, histológicas em lesões periapicais e em artérias carótida, para observar se há uma possível inter-relação entre a periodontite apical e a aterosclerose.

II. Artigo 1

Archives of Oral Biology

Oral health, atherosclerosis, and body weight

Abstract

Objective: To evaluate the effects of apical periodontitis (AP) on organ weight in Wistar rats with atherosclerosis.

Design: Forty male Wistar rats (*Rattus norvegicus albinus*) were divided into four groups of ten animals each: control rats, rats with AP, rats with atherosclerosis (AT), and rats with AP and atherosclerosis (AP+AT). Atherosclerosis was induced by using a high-lipid diet together with right common carotid surgery and a super dosage of vitamin D_3. AP was induced via dental pulp exposure to the oral environment. After 30 days, the rats were euthanized, and their brain, gonads, heart, kidney, liver, lungs, and spleen were individually weighed. Lipid profile (total cholesterol and triglyceride levels), feed intake, organ weight, and body weight were statistically analyzed ($P<0.05$).

Results: Total cholesterol levels were higher in all rats with atherosclerosis regardless of the presence of AP ($P<0.05$). However, AP influenced the triglyceride levels in the AP and AP+AT groups ($P<0.05$). Feed intake was similar among all groups. Weight loss was observed in the AT rats until the induction of AP ($P<0.05$). At the end of the experiment, only the AP+AT group presented weight loss in relation to the control ($P<0.05$). Compared to the control group, the AP+AT group showed an increase in brain weight, and the AP group showed an increase in liver and spleen weights and a decrease in lung weight ($P<0.05$).

Conclusion: AP and atherosclerosis, whether isolated or in combination, influence the body weight and metabolism in Wistar rats.

Keywords: Apical periodontitis; Atherosclerosis; Body weight; Lipid profile

1. Introduction

Cardiovascular diseases are the main causes of mortality worldwide (Gomes et al., 2016), accounting for three out of ten deaths (Berlin-Broner, Febbraio, & Levin, 2017). Atherosclerosis is considered the main cause of heart disease (Chen, Li, Zhao, Chen, & Wang, 2014), and it can be defined as a chronic progressive disease characterized by the accumulation of calcium, fibrous elements, and lipids (including cholesterol and triglycerides) that cause thickening of arterial walls (Lu & Daugherty, 2014). This change occurs in the walls of medium- and large-caliber arteries and results in their stiffening (Lönn, Dennis, & Stocker, 2012). Atherosclerosis is considered a multifactorial disease, whose risk factors include circulating lipoproteins (hypercholesterolemia), hypertension, genetic predisposition, obesity, smoking, and diabetes (Bartova et al., 2014).

Oral infections such as periodontal disease and apical periodontitis (AP) are related to the pathogenesis of heart disease (Mattila et al., 1989; Cotti & Mercuro, 2015; Segura-Egea, Martín-González, & Castellanos-Cosano, 2015), especially in association with atherosclerosis (Krell, McMurtrey, & Walton, 1994; Beck et al., 2001; Niedzielska, Janic, Cierpka, & Swietochowska, 2008; Patil & Sinha, 2013; Petersen et al., 2014; Velsko et al., 2014; Kudo et al., 2014; Etemadifar, Konarizadeh, Zarei, Farshidi, & Sobhani, 2015). Although the etiology and pathogenesis of both oral infections are different, they present a common microbiota, such as the presence of anaerobic bacteria including Gram-negative bacteria (Noiri, Li, & Ebisu, 2001), which increase and propagate inflammatory mediators (Sun, Chen, Zhang, Ren, & Qin, 2010; Takano et al., 2010; Cintra et al., 2014a). Endodontic infection causes a local periapical tissue response (AP), which is evidence that it may also contribute to systemic inflammation (Caplan et al., 2006; Doyle, Hodges, Pesun, Baisden, & Bowles, 2007; Cintra et al., 2016; Samuel et al., 2017).

With scientific advancement, interdisciplinary research has become of paramount importance in supporting treatments in which health and disease mechanisms need to be widely understood (Cintra et al., 2017), so that future risks to the patient are avoided (Rautemaa, Lauhio, Cullinan, & Seymour, 2007). The correlation between periodontal disease and heart disease is well

established (Lockhart et al., 2012). However, studies in the current literature do not provide sufficient evidence to support the interrelationship between AP and atherogenesis (Berlin-Broner et al., 2017), especially in relation to organ weight and body weight.

Therefore, this study investigated the possible effects of AP on the body weight, organ weight, and lipid profile of Wistar rats with atherosclerosis. We hypothesized that AP may influence the weight and metabolism in rats with atherosclerosis.

2. Material and Methods

2.1. Experimental design

Forty male Wistar rats (*Rattus norvegicus albinus*), with an initial mean weight of 120 g, were used in this study. The rats were housed in mini-isolators for rats (Alesco, São Paulo, SP, Brazil), kept in temperature-controlled rooms (22-24°C), and given ad libitum access to water. Different diets were offered depending on the study group. The experimental procedures were approved by and conducted in accordance with the guidelines of the institutional ethics committee and in compliance with the U.K. Animals (Scientific Procedures) Act 1986 (00358-2016).

2.2. Induction of atherosclerosis

Atherosclerosis was induced by using a high-lipid diet together with a surgical procedure of the right common carotid artery and a super dosage of vitamin D_3.

Initially, the rats were fed for 15 days (from 1st day) with a high-lipid diet composed of a combination of 75% commercial food, 20% lard, and 5% sugar. The sugar was dissolved in the liquid (melted) lard, and then, the ration was immersed in the compound liquid to absorb it. The high-lipid diet protocol was adapted from previous studies (Zhou, Pan, & Zhai, 2011; Chen et al., 2014). The compositions of the commercial food (Labina® - Purina Agribrands do Brasil Ltda, Paulínia, SP, Brazil) and high-lipid diet are described in Table 1.

Table 1. Composition of the commercial food and high-lipid diet fed to rats

	Food composition Amount (g/100 g)	
Component	**Commercial food**	**High lipid diet**
Humidity (Max)	13.00	09.75
Crude Protein (Min)	23.00	17,25
Ethereal Extract (Min)	04.00	03.00
Fibrous Matter (Max)	05.00	03.75
Mineral Matter (Max)	10.00	07.50
Calcium (Max)	01.30	00.98
Phosphorus (Min)	00.85	00.64
Lard	-	20.00
Sugar	-	05.00

On the 15th day, the rats were subjected to the surgical procedure. The rats were fasted for 8 h before the procedure. Half of the rats (20) were anesthetized using intramuscular administration of ketamine (87 mg/kg) and xylazine (13 mg/kg) (Rompum - Bayer SA, São Paulo, SP, Brazil). The trichotomy was performed in the frontal portion of the midline of the neck, and then Iodopovidone asepsiswas performed in the region to be operated. The rats were operated on surgical tables for rats in the supine position, and an incision was made in the midline of the neck at the level of the carotid plexus. The right carotid artery was isolated using a retractor, to avoid damage to the adjacent anatomical structures, and a non-absorbable suture was placed (Seda, 5-0 - Ethicon Johnson and Johnson, São Paulo, Brazil). A sterile insulin needle, with an external diameter of 0.3 mm, was placed over and parallel to the isolated artery. Artificial ligation was made at a distance of 1.5 cm from the aortic arch, causing strangulation to promote carotid stenosis. Subsequently, the needle was removed but the suture was retained around the vessel to ensure it had an internal diameter of approximately 0.3 mm for facilitating the formation of atheromas (Wang et al., 2015; Nam et al., 2009; Hua et al., 2006). The tissues were then approximated and suturing was performed using interrupted stitches with nonabsorbable wire (Nylon, 5-0 - Ethicon Johnson and Johnson, São Paulo SP, Brazil).

One day after the surgical procedure (16th day), a super oral dose of

vitamin D_3 equivalent to 90,000 IU (Manipulated and equivalent to 45,000 IU per drop or 1,125,000 IU/ml – Apothicario®, Araçatuba, SP, Brazil) was given for 2 days after the artificial ligation was performed (Wang et al., 2015).

2.3. Induction of oral infection

Thirty days after the surgical procedure, i.e., the 45th day after atherosclerosis induction, half of the rats that underwent carotid artery ligation (10 rats) and half of the rats that did not receive the ligation (10 rats) were anesthetized according to the previously described protocol.

For inducing AP, the dental pulps of the first and second upper and lower right molars of each rat were exposed using surgical round burs LN (Burs Long Neck - Maillefer, Dentsply Ind. e Com. Ltda, Petrópolis, RJ, Brazil) (Cintra et al., 2013; Cintra et al., 2014a; Cintra et al., 2014b; Cintra et al., 2016). The dental pulps were exposed in the oral cavity for 30 days, until the end of the experiment.

2.4. Experimental design

After the respective inductions, the rats were assigned into the following groups: control group (C), AP group, atherosclerosis (AT) group, and AP plus atherosclerosis (AP+AT) group (Table 2).

Table 2. Distribution of the experimental groups according to local procedures and systemic conditions of the rats

LOCAL AND SYSTEMIC CONDITIONS		APICAL PERIODONTITIS	
		NO	**YES**
ATHEROSCLEROSIS	**NO**	Group C	Group AP
	YES	Group AT	Group AP+AT

2.5. Rats euthanasia and confirmation of AP and AT induction

The rats were euthanized 75 days after starting the high-lipid diet, 60 days after the carotid artery ligation, and 30 days after AP induction (Figure 1) by using an anesthetic overdose of thiopental sodium (Thiopentax; Cristália, Itapira, SP, Brazil).

Timeline experimental

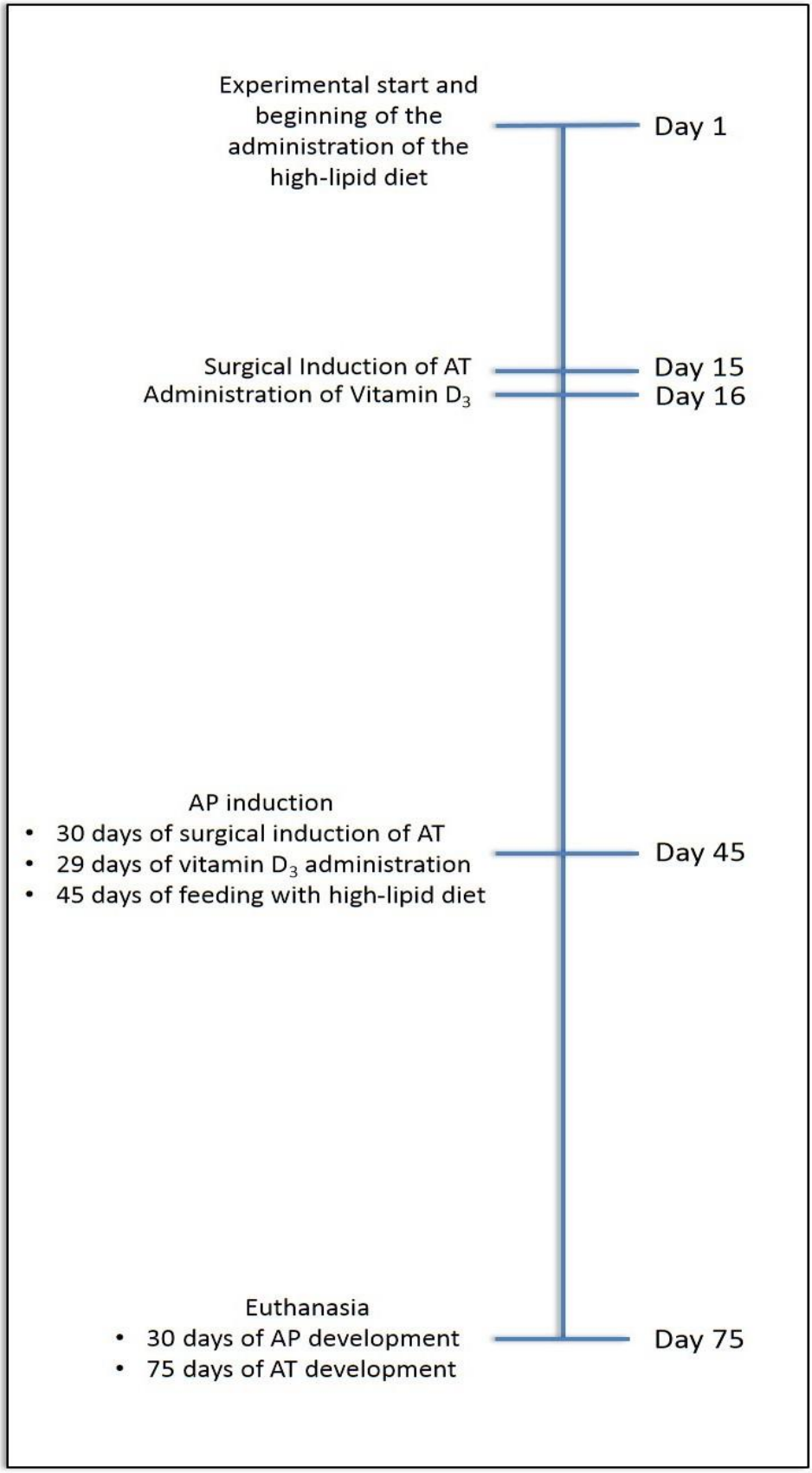

Fig. 1. Timeline of the experiment

To confirm the induction of AT, carotid ultrasound was performed and the lipid profile was checked by quantifying the total cholesterol and triglyceride levels. Ultrasound was performed 60 days after carotid artery ligation. Anesthetized rats were placed in the dorsal decubitus position; trichotomy of the neck region to be analyzed was performed; and the ultrasound gel was used as an acoustic coupling medium. The right common carotid artery was visualized in the anterolateral longitudinal view by using the ultrasound system and a megahertz transducer (Veterinary ultrasound, Mindray DP 2200 VET).

For quantifying the lipid profile, venous blood samples were collected by means of cardiac puncture. Samples were centrifuged immediately after collection at 1800×g for 15 min at 4°C to obtain the serum. The levels of total cholesterol and triglycerides were measured enzymatically by using a commercial kit (Triglycerides Liquiform Labtest® and Cholesterol Liquiform Labtest®, respectively - Labtest Diagnostica Ind. E Com. Ltda, Lagoa Santa, MG, Brazil) as described previously (Trinder, 1969).

After blood collection and sacrificing the rats, the upper and lower jaws were dissected, processed, and subjected to radiographic analysis.

2.6. Measurement of food consumption

Rats in the AT and AP+AT groups received the high-lipid diet, and rats in the others groups received the commercial food Labina® (Purina Agribrands do Brasil Ltda) and water ad libitum. The energy content of Labina® was 3.3 Kcal/g (Table 1). Food consumption was measured on 7, 15, 45, and 75 days during the experiment.

2.7. Measurement of body weight and organ weight

The rats were weighed individually on a digital scale (model 707; Seca, Hamburg, Germany) on 0, 15, 45, and 75 days after calibration of the initial weight of the rats. After sacrificing the rats, their brain, gonads, heart, kidney, liver, lungs, and spleen were dissected by a trained researcher and weighed on a digital scale (model 707; Seca, Hamburg, Germany). The relative weight of each organ was obtained while weighing and was evaluated according to the calculated index between the organ weight and body weight of each animal multiplied by 100 (i.e., organ weight/body weight × 100) (Kumar, Sharmila Banu, & Murugesan, 2009;

Eleazu, Iroaganachi, Okafor, Ijeh, & Eleazu, 2013; Cintra et al., 2017).

2.8. Statistical analysis

The total values were tabulated for each experimental group, and the data were analyzed by a single blinded, but calibrated, operator. The results were submitted to one-way ANOVA statistical tests, followed by Tukey multiple comparisons tests. $P<0.05$ was considered significant.

3. Results

3.1. AP and AT inductions

AP induction was confirmed by radiographic examination of the jaws. Radiographs acquired after sacrifice showed radiolucent areas in the periapical region in the AP and AP+AT groups (Figure 2), indicating AP formation.

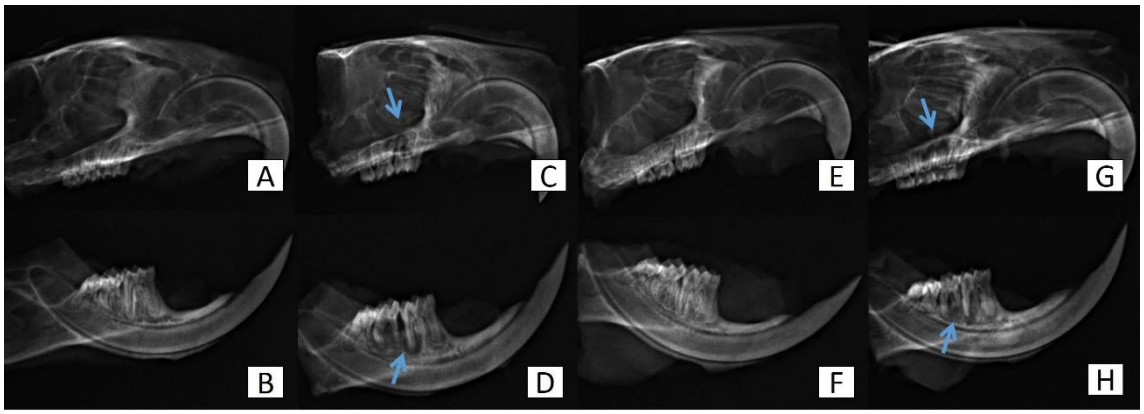

Fig. 2. Radiographic appearance after apical periodontitis (AP) induction. A and B: Upper and lower jaws of the control (C) group; C and D: Upper and lower jaws of the AP group, showing the periapical lesions (blue arrow); E and F: Upper and lower jaws of the atherosclerosis (AT) group; G and H: Upper and lower jaws of the AP+AT group, showing the periapical lesions (blue arrow).

AT induction was confirmed by ultrasound examination and by measuring the total cholesterol and triglyceride levels. On ultrasound examination, the anatomical aspect of normality in the carotid artery could be observed in the control and AP groups. In the images of the AT and AP+AT groups, the presence of constriction by artificial ligation and expansive arterial remodeling in the proximal region of the artificial ligature were observed (Figure 3).

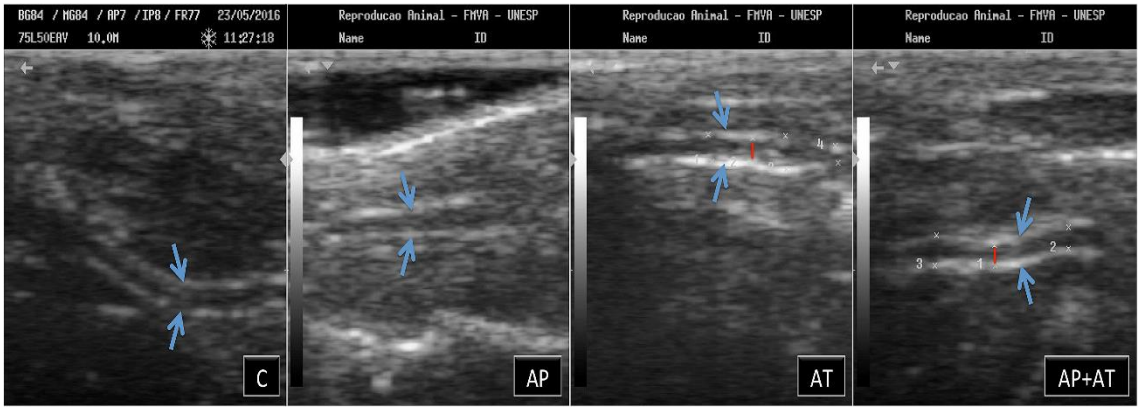

Fig. 3. Ultrasound images corresponding to 60 days after the surgical procedure performed on the right carotid artery of the rats. (C and AP) Control and apical periodontitis groups. Images showing the anatomical aspect of normality in the carotid (blue arrow). (AT and AP+AT) Atherosclerosis and AP+AT groups. Images showing arterial constriction (red dash) after artificial ligation placement and expansive arterial remodeling in the proximal region of the artificial ligature in the interventional groups.

Regarding the lipid profile, blood analysis was performed in all rats on the 75th day. In the rats with atherosclerosis (AT and AP+AT groups), cholesterol levels were statistically higher than those in the other two groups (control and AP groups) ($P<0.05$). The presence of endodontic infection (AP) and atherosclerosis (AT) in isolation increased the triglyceride levels more than those seen in the control group. However, both groups did not present significant differences between them ($P>0.05$). The AP+AT group had higher triglyceride levels than did the other groups (control, AP, and AT) (Table 3).

Table 3. Mean and standard deviation (mean ± SD) values of the lipid profile of the rats from the four groups

Groups	Lipid profile			
	Cholesterol levels (mg/dL)		Triglyceride levels (mg/dL)	
	Mean	SD	Mean	SD
C	68.90[a]	10.51	63.05[a]	15.56
AP	68.33[a]	9.58	85.18[b]	9.24
AT	92.95[b]	23.06	91.20[b]	19.34
AP+AT	108.45[b]	26.18	120.49[c]	18.91

* Same letters indicate the absence of statistical difference among the groups ($p > 0.05$)

3.2. Feed consumption

The feed intake of control and AT rats was not significantly different (P>0.05). However, after the surgical induction of atherosclerosis and oral infections, feed consumption decreased in both groups but returned to normal after 7 days. After these procedures, consumption was similar across all groups throughout the study duration (P>0.05) (Figure 4).

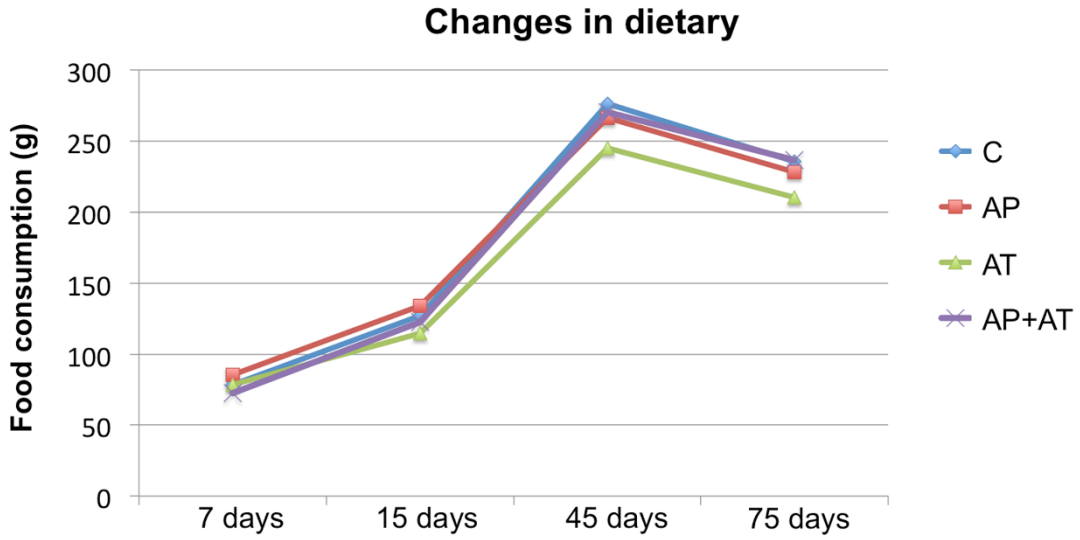

Fig. 4. Changes in dietary intake of the control and atherosclerotic rats. C, control rats; AP, apical periodontitis; AT, atherosclerosis; AP+AT, with both AP and AT.

3.3. Body weight

In the initial period (day 0), the rats weighed approximately 120 g. The weights were similar until the surgical induction of isolated atherosclerosis (day 15). Thereafter, the rats with atherosclerosis (AT and AP+AT groups) presented a significant reduction in body weight than did the rats in the other groups (P<0.05). After the induction of AP (day 45), the weights of the rats in the AT and AP+AT groups remained lower than those of rats in the other groups (P>0.05). However, after that period, the rats in the AT group regained body weight, which was similar to that of rats in the other groups. However, rats in the AP+AT group presented reduced body weight when compared to that of rats in the control group until the end of the experiment (Table 4).

Table 4.
Changes in body weight in all groups

Groups	Evaluation Periods							
	Dia 0		Dia 15		Dia 45		Dia 75	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C	117,3[a]	10,812	235,5[a]	21,041	392,9[a]	29,816	455,4[a]	32,827
AP	129,3[a]	13,557	237,5[a]	17,444	388[a]	17,224	444,5 [ab]	15,869
AT	125,9[a]	5,405	231,9[a]	21,728	337,9[b]	29,823	434,7[ab]	39,156
AP+AT	123,7[a]	12,798	245,7[a]	32,606	323,4[b]	60,833	409,8[b]	65,499

* Same letters indicate the absence of statistical difference among the groups (p > 0.05).

3.4. Organ weight

The variation in the relative weight of the organs of the rats according to the groups is shown in Figure 5.

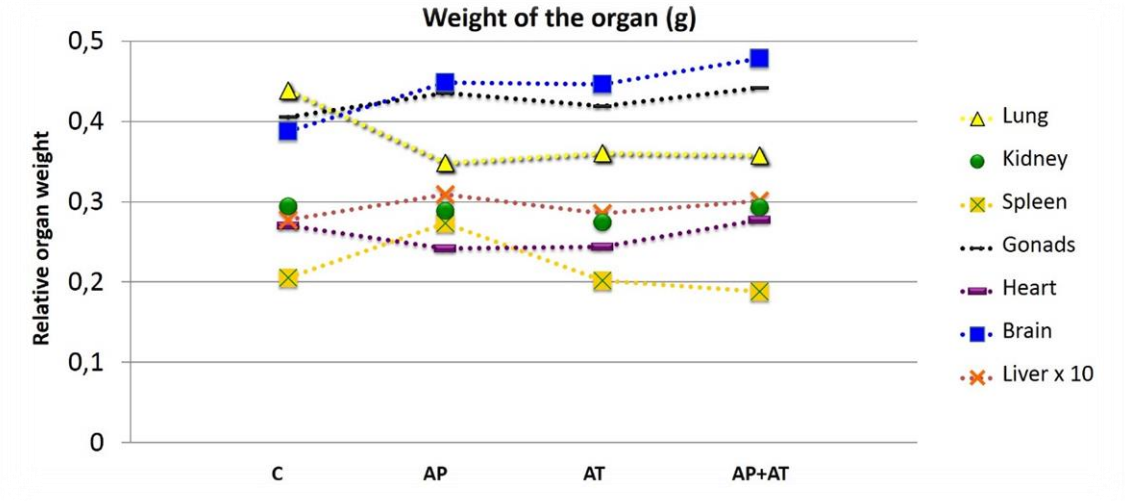

Fig. 5. Variation in the relative weight of the organs of the rats in the four groups; C, control rats; AP, apical periodontitis; AT, atherosclerosis; AP+AT, with both AP and AT.

3.4.1. Liver

The AP group showed an increase in liver weight when compared to that of rats in the other groups; however, this increase was statistically significant only when compared to that of the control group (P<0.05).

3.4.2. Kidney

The AP, AT, and AP+AT groups showed a reduction in the weight of the kidney (in decreasing order) when compared to that of the control group; however, they did not present a statistical difference between them ($P<0.05$).

3.4.3. Spleen

Endodontic infection promoted a more significant increase in spleen weight in the AP group than in the other groups. The spleen of the rats in the AP+AT group presented the lowest weight, but without statistical difference.

3.4.4. Brain

The brain weight was higher in the AP+AT group; however, this increase was statistically significant only when compared to that of the control group.

3.4.5. Heart

The weight of the heart was higher in the AP+AT group than in the other groups, but without considerable statistical difference.

3.4.6. Lungs

The weight of the lungs was lower in the rats that presented some type of alteration (AP, AT, and AP+AT groups), with the AP group showing the lowest lung weights. However, this difference was statistically significant only when the AP, AT, and AP+AT groups were compared to the control group ($P<0.05$).

3.4.7. Gonads

The AP+AT group presented greater gonadal weight than did the other groups, but without considerable statistical difference.

4. Discussion

This study investigated the effects of AP on the body weight, organ weight, and lipid profile of Wistar rats with atherosclerosis. The results showed that AP influenced the weight and metabolism in rats with atherosclerosis, thus proving our hypothesis. These results are also consistent with those of other studies that demonstrated a positive relationship between AP and atherosclerosis (Caplan,

Pankow, Cai, Offenbacher, & Beck, 2009; Petersen et al., 2014).

For the experiments, we used a rat model of AP that has been used by our research group to study the interrelationships of AP with systemic alterations and diseases (Cintra et al., 2014a; Cintra et al., 2014b; Prieto et al., 2017; Azuma et al., 2017). The presence of periapical lesions was confirmed radiographically and it indicated the successful induction of AP.

To induce atherosclerosis, we combined three methods used in previous studies to ensure successful model development and implementation. The first method was the administration of a high-lipid diet to the rats (Ding et al., 2010; Zhou et al., 2011; Bond, Hultgårdh-Nilsson, Knutsson, Jackson, & Rauch, 2014; Chen et al., 2014; He et al., 2014) throughout the experimental period. The continuous administration of fatty diets induces changes in vessel walls, such as the recruitment of immune cells and increased production of cytokines, which may lead to structural rupture of the carotid wall (Zhou et al., 2011; Fotis et al., 2012). After 15 days of the high-lipid diet, a constrictive artificial ligation (Hua et al., 2006) was made in the right carotid artery (Bond et al., 2014). This surgical procedure causes lumen stenosis, which promotes changes in the hemodynamic state within the blood vessel (Stroud, Berger, & Saloner, 2000) and increases the residence time of circulating macromolecules, thereby promoting the absorption of lipoproteins (Himburg et al., 2004) and resulting in endothelial dysfunction. All of these physiological events are capable of inducing atherosclerosis in an accelerated manner (Nam et al., 2009). Finally, 1 day after the surgical procedure, the rats received a high dose of vitamin D_3 for 2 days (Chen et al., 2014; Wang et al., 2015). The main function of vitamin D is to maintain calcium concentrations at a sufficient level for homeostasis and to increase the efficiency of the small intestine in absorbing calcium (Holikm, 2006); the purpose of this vitamin overdose was to cause hypercalcemia. In this way, calcium absorption would be favored by the organism, thereby causing calcium deposition in the carotid wall and possibly causing calcification of the atheromas. The presence of calcification in the carotid wall was confirmed using ultrasound images.

According to our results, the presence of four local oral infections in rats with atherosclerosis not only altered the triglyceride levels but also caused significant changes in the relative weight of the brain, lungs, liver, and spleen. These results corroborate with those of other studies that demonstrated systemic

alterations in the lipid profile (Cintra et al., 2013; Chen et al., 2014), body weight (Cintra et al., 2017), blood glucose levels (Cintra et al., 2014b), glycated hemoglobin (Cintra et al., 2014c), hemogram (Cintra et al., 2014b), and inflammatory mediators (Cintra et al., 2014a) with simultaneous oral infections (AP) in animal models with systemic alterations.

Hyperlipidemia is a major cause of atherosclerosis and changes induced by atherosclerosis, such as cardiovascular diseases (Gobalakrishnan, Asirvatham & Janarthanam, 2016). In our study, the rats with atherosclerosis (AT and AP+AT groups) had higher cholesterol levels than did rats without atherosclerosis. These results are consistent with strong medical scientific evidence suggesting elevated cholesterol levels in patients with atherosclerosis (Keys, 1980; Saller, Meier, & Brignoli, 2001; Bersot, 2011). Although the AP+AT group did not present statistical difference when compared to the AT group, the cholesterol levels were higher in the AP+AT group. This result corroborates with that of another study, which demonstrated that the presence of oral infections caused changes in the lipid profile of diabetic rats (Cintra et al., 2013).

Our results showed an increase in serum triglyceride levels in the groups with atherosclerosis (AT and AP+AT) than in the other groups analyzed, which was consistent with the finding of another study that showed increased levels of triglycerides in animals prone to atherosclerosis (Gobalakrishnan et al., 2016). In addition, we observed that AP increased the triglyceride levels in rats with atherosclerosis. This scientific finding corroborates with that of studies showing the prevalence of high levels of triglycerides in patients with periodontal diseases (Losche, Karapetow, Pohl, Pohl, & Kocher, 2000; Golpasand Hagh, Zakavi, Hajizadeh, & Saleki, 2014). Another study showed a higher incidence of periapical lesions in patients with high triglyceride levels (Kimak, Strycharz-Dudziak, Bachanek, & Kimak, 2015).

The results of a study on rats with diabetes and endodontic infection also showed changes in the lipid profile and concluded that both alterations are capable of altering triglyceride levels (Cintra et al., 2013). The high values of the lipid profile in our study on rats with AP combined with AT support the hypothesis that endodontic infections are capable of adversely affecting systemic health. In addition, some studies have shown the worsening of a systemic disorder in the presence of endodontic infection (Kodama et al., 2011, Wang et al., 2011, Cintra

et al., 2017).

In addition to the changes observed in the lipid profile, the assessment of body weight at 45 days revealed a reduced weight in rats with atherosclerosis than in the other rats. At that time, AP had not been induced, suggesting that the presence of atherosclerosis could induce weight loss in these rats. This result is consistent with that of another study that also observed a weight reduction in animals fed a high-fat diet that received vitamin D_3 (Chen et al., 2014). This fact can be attributed to the composition of the diet, which resulted in reduced food intake because it contained ingredients rich in lipids. The administration of vitamin D_3 also induced excess calcium levels in these rats and, consequently, resulted in a lower food intake. At the end of the experiment, the rats with both alterations (AP+AT) had the lowest body weights when compared to the rats in the other groups. This weight loss can be attributed to all the aspects discussed above and to the presence of two foci of inflammation that were able to generate an exacerbated metabolic disorder (Sánchez-Domínguez et al., 2015). Thus, the study confirms the hypothesis that endodontic infections are capable of aggravating the effects of atherosclerosis (Petersen et al., 2014).

Regarding organ weight, an increase in brain weight was observed in the AP+AT group. Corroborating this result, another study showed that the ingestion of pro-inflammatory drugs and proteins could cause a local pro-inflammatory state in the hypothalamus (De Souza et al., 2005). Another study on an animal model and using a diet with a similar lard content as in our study showed the occurrence of inflammation in the brain, specifically in the hypothalamus (Dorfman & Thaler, 2015). We think this inflammation was supposedly responsible for the increase in brain weight. Regarding the association of AP in this group, brain inflammation may have been exacerbated by the inflammatory process from AP, since data from another study showed that the presence of AP in pregnant rats could increase the levels of IL-6, IL-1β, and TNF-α in fetal brain tissue (Bain, 2013).

Using this same measurement parameter, the liver and spleen of the rats in the AP group presented greater weight than did those of rats in the other groups. These results are consistent with those of other studies that demonstrated that the presence of AP in rats elevated the levels of proinflammatory cytokines (IL-2 and IL-6) and caused pathological changes in

the liver and spleen (Zhang, Huang, Lu, Zhang, & Cai, 2016).

A reduction in lung weight was also observed in the AP group. Although the association of lung diseases with periodontal diseases has been extensively studied (Holtfreter et al., 2013; Fernández-Plata et al., 2015; Zeng et al., 2016), further studies are needed to determine if the presence of AP is capable of altering respiratory system health, as observed in our study with the changes in lung weight.

These significant changes in organ weight show that the presence of AP and AT, either alone or in combination, can lead to metabolic alteration, resulting in deleterious effects on the body. These results suggest that the combination of a local and a systemic alteration could generate or aggravate a metabolic disorder, as shown in another study on rats in which AP and periodontal disease could potentiate the harmful effects of diabetes (Cintra et al., 2017).

The results of the present study provide evidence that endodontic infections not only cause local responses to the periapical tissues but are also capable of generating systemic alterations. The findings of our study contribute to demystifying the hypothesis that AP is exclusively a local phenomenon. However, more studies are needed to strengthen the hypothesis that there are possible relationships between endodontic infections and systemic diseases, which in the future will have considerable implications for the treatment of patients who present both changes.

5. Conclusion

We conclude that the apical periodontitis and atherosclerosis, whether isolated or in combination, can influence the body weight and metabolism in Wistar rats. In addition, the presence of AP exacerbates the deleterious effects of atherosclerosis in a Wistar rat model.

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III. Artigo 2

Journal of Endodontics

Relationship between apical periodontitis and atherosclerosis: Lipid profile and histological study

Abstract:

Introduction: We aimed to investigate the relationship between apical periodontitis and atherosclerosis by lipid profile measurement, and histological and histometric evaluation. **Methods:** Forty male Wistar rats were assigned to the following 4 groups: control group (C), group with apical periodontitis (AP), group with atherosclerosis (AT) and group with AP and AT (AP+AT). Atherosclerosis was induced by using a high-lipid diet together with an artificial ligature in the carotid artery and a super dosage of vitamin D_3. AP was induced via pulp exposure to the oral environment. Serum levels of total cholesterol (TC), triglycerides levels (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured at 45 and 75 days with blood samples. The upper and lower jaws and carotid artery were collected and processed for histologic analysis. The data were tabulated and submitted to statistical analysis ($P < .05$). **Results:** At 45th day, the animals with atherosclerosis induced presented high TC, TG and LDL-C levels when compared to the other animals ($P < .05$). At 75th day, the AT and AP+AT groups presented higher levels of TC and LDL-C compared to the groups without AT ($P < .05$). The TG level was higher in the AP+AT group, compared to the all other groups ($P < .05$). The AP and AT groups present higher TG levels as compare to C groups ($P < .05$). Alterations were detected in the carotid artery of the groups with atherosclerosis (AT and AP+AT). The intensity of the inflammatory infiltrate and the bone resorption lesion was significantly larger in the AP+AT group compared with AP group ($P < .05$). **Conclusion:** Apical periodontitis influence triglyceride levels regardless of the presence of atherosclerosis, and atherosclerosis intensified inflammatory infiltrate and increased bone resorption in the apical periodontitis area.

Key words: Apical periodontitis; atherosclerosis; histology, lipid profile, systemic disorders.

Introduction

When associating cardiovascular diseases with chronic inflammatory processes, recent studies have shown that infectious inflammatory diseases of the oral cavity can be considered risk factors for metabolic disorders and systemic pathological alterations (1, 2). Among oral infections, periodontal disease and apical periodontitis may be causative agents of systemic diseases (3) and therefore have been studied because they present a favorable context for the pathogenesis of heart diseases (4, 5). Apical periodontitis is an inflammatory disease, characterized by the destruction of periapical tissues. Inflammatory mediators in reaction to bacterial infection trigger this periapical alteration (6). Evidence show that the prevalence of AP is 34-61% (7) and the medical and dental scientific community has been investigating the probability of AP being associated with systemic alterations (5).

Among the systemic alterations, atherosclerosis is a pathological process that occurs due to lipoproteins retention in the subendothelial layer of large and medium arteries, and the accumulation of fat plaques triggers innate immune responses. T cells perform an important role towards these responses, in which they react against lipid proteins that consequently the disease progress slowly by means of a chronic inflammatory process (8). This vascular modification is capable of initiate cardiovascular diseases, which remain the leading causes of global morbidity and mortality (9).

Hypercholesterolemia causing various endothelial dysfunctions, and it is considered a risk factor for atherosclerosis (10). Among the cholesterol fractions, low-density lipoprotein (LDL-C) at high levels influences in atherogenesis (11). In human studies, previous reports have found that total cholesterol and triglyceride levels are higher in patients with periodontal disease compared to those with good oral health (12, 13). In animal study, the association of endodontic infection with periodontal disease was capable to increase the triglyceride levels in diabetic rats (1).

Several studies have indicated the possible correlation between periodontal disease and cardiovascular diseases (14). The first prospective cohort study followed for 14 years 1000 individuals and detected a 25% increase in cardiovascular disease in patients diagnosed clinically with periodontal disease

(14). Another study observed that the dental health of 100 individuals who had myocardial infarction was significantly lower when compared to healthy subjects (4). When analyzing aorta of patients who underwent heart surgery, it was possible to detect bacterial DNA in 23 of 26 samples (88.5%), among the pathogens, *Porphyromonas gingivalis (*15). Taking into consideration the same periodontal pathogen, it was possible to demonstrate in another study that *Porphyromonas gingivalis* presents several properties that may play an important role in cardiovascular diseases, such as mediators of LDL oxidation, foam cells formation and rupture of the atherosclerotic plaque (16). Research has shown both diseases are independently associated with classic cardiovascular risk factors (14). On the other hand, studies that address the association of apical periodontitis with cardiovascular diseases are scarce (17) and the results presented at literature to date are not conclusive, but suggest an association between endodontic infections with systemic alterations (5).

Thus, it would be important to evaluate the standardized animal model, the relationship between apical periodontitis and atherosclerosis. Therefore, this study aimed to evaluate, locally by means of histological and histometric analysis the influence of the atherosclerosis in the apical periodontitis development and, the influence of apical periodontitis in the lipid profile of rats with atherosclerosis.

Material and Methods

Experimental design

The experimental procedures of this study were approved by the Institutional Ethics Committee of the Universidade Estadual Paulista, Sao Paulo, Brazil and were conducted according to the relevant guidelines of the Ethical Conduct Committee on Animal Experimentation (00358-2016).

Forty male Wistar rats *(Rattus norvegicus albinus, Wistar)*, weighing on average 120g, were housed in mini-isolators for rats (Alesco, São Paulo, Brazil). The rats were kept in a temperature-controlled environment and given *ad libitum* access to water. Each study group received a different diet type.

Induction of atherosclerosis

During the whole experimental period, half of the animals (N = 20) received a high-lipid diet, composed of 75% of commercial food, 20% lard and 5% sugar

(18, 19). At 15th day, the rats were anesthetized with intramuscular administration of ketamine (87 mg / kg) (Francotar - Virbac do Brasil Ind e Com Ltda, Roseira, Brazil) and xylazine (13 mg / kg) (Rompum - Bayer SA, São Paulo, Brazil) for the surgical induction of atherosclerosis. The trichotomy and disinfection with Iodopovidone were performed in the region to be operated. Next, the animals were placed in supine position at surgical tables for rats and an incision was made in the midline of the neck, at carotid plexus position. The right carotid artery was isolated in order to avoid damage to the blood vessels and nerves of this region. A sterile needle with an outer diameter of 0.3 mm was placed parallel and over to the artery. A ligature was made around the carotid artery at a distance of 1.5 cm from its bifurcation, using a suture segment (20). Then, the needle was removed and the ligature remained around the carotid artery. Thus, the inner diameter of the stenosis remained approximately 0.3 mm in diameter.

One day after the surgical procedure (16th days), an oral dose of vitamin D_3 equivalent to 90.000 IU (Apothicaria Manipulated and equivalent to 45.000 IU per drop or 1.125.000 IU / ml), was given for two days, to the animals with the artificial ligation performed (21). The other animals were fed with commercial food (Labina® - Purina Agribrands do Brasil Ltda, Paulínia, São Paulo, Brazil), underwent the same surgical procedure, however, without the artificial ligature and instead of vitamin D_3, physiological saline was used.

Induction of apical periodontitis

At 45th day, apical periodontitis was induced in AP and AP+AT groups using surgical round burs 0.1 mm diameter (Broca Ln Long Neck, Maillefer, Dentsply Ind and Com Ltda, Petrópolis, Brazil). The animals of these groups were anesthetized according to the previously described protocol and the pulps of the first and second upper and lower right molars of each animal were exposed to the oral cavity and they remained until the end of the experimental period.

After the inductions protocols, the rats were divided into 4 groups (10 rats / group):

Group C: control rats;

Group AP: rats with apical periodontitis;

Group AT: rats with atherosclerosis;

Group AP+AT: rats with apical periodontitis and atherosclerosis.

Collection of blood samples for determination of serum lipid profile

At 45th and 75th days, after a fasting period for 8 - 12 h, the animals were anesthetized according to the protocol described previously and by a cardiac puncture blood samples were collected from each animal to determine the lipid profile. The blood samples were placed in vacuum tubes for collection, containing clot activator spread on the wall of the tube, to accelerate the coagulation process, and with separator gel to obtain a better quality serum. The samples were then centrifuged immediately after collection at 1800 x g for 15 minutes at 4 ° C to obtain the serum. Plasma total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) levels were measured enzymatically by using a commercial kit (Cholesterol Liquiform Labtest®, Triglycerides Liquiform Labtest®, HDL Labtest® Cholesterol, Labtest Diagnostica Ind. E Com. Ltda, Lagoa Santa, MG). The levels of low-density lipoprotein cholesterol (LDL-c) were obtained by the Friedewald Formula: LDL-C = (CT – HDL-C) - (TG / 5) (22).

Collection and processing of tissues for histologic evaluation

At 75th day, after the second blood collection, the animals were euthanized with an overdose of the anesthetic solution. Their upper and lower jaws and carotid of each animal were dissected and fixed in 10% formaldehyde at neutral pH for 24 hours, then were washed in running water for a period of 12 hours to remove the entire fixative solution. The upper and lower jaws were demineralized with 17% buffered EDTA (Sigma Chemical Co., St. Louis, MO). Later, both the maxillae and the carotids were dehydrated with ethanol, diaphanized with xylol and embedded in paraffin. Serial paraffin sections (5 μm) were obtained in the mesial-distal aspects of the right upper and lower first molars and of the carotid in the transverse and longitudinal directions. The tissue sections were stained with hematoxylin-eosin and evaluated under optical microscopy (DM 4000 B, Leica, Wetzlar, Germany).

Histological analysis of the periapical region was performed using the following parameters: the intensity of the inflammatory infiltrate and the bone resorption of the periapical lesion. The intensity of inflammation was graded in the apical third of the mesial root of the upper and lower molars by scores attributed: absent (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).

For the AP and AP + AT groups, the periapical lesion area was calculated by rounding the bone resorption present in the apical third of the root, considering the outer external surface of alveolar bone, with a magnification of 50x. For each rat subject, 5 serial histologic sections were measured histometrically using an image processing system, which consisted of a light microscope (DM 4000 B, Leica), a color image processor (Leica Qwin V3 software, Leica), a color camera (DFC 500, Leica) (Cintra, 2014) and a personal computer (Intel Core I5, Intel Corp, Santa Clara, CA; Windows 10, Microsoft Corp, Redmond, WA). The analyzes were performed blindly by a single operator calibrated.

Statistical analysis

The total values of the lipid profile (CT, TG, HDL-C and LDL-C) were submitted to One-way ANOVA test, followed by Tukey multiple comparisons tests, considering $p < 0.05$.

The total values were tabulated for each experimental group and the data were analyzed by a single operator blindly calibrated. The results of the scores attributed to inflammation were statistically analyzed using the Mann Whitney test, $p < 0.05$, and the measurements of periapical lesions using the Student's T-test, $p < 0.05$.

Results

Lipid profile Serum

The effect of atherosclerosis was investigated on the lipid profile of animals with endodontic infections. The results of the dosed fat fractions of lipid profile (TC, TG, HDL-C and LDL-C) are shown in table 1 and 2. At 45th day, the animals with atherosclerosis induced presented high TC, TG and LDL-C levels when compared to the other animals ($p < 0.05$). The HDL-C levels were similar among all animals ($p < 0.05$) (table 1).

Table 1. Mean and standard deviation (SD) values of the lipid profile at 45 days of the rats

Animal condition	Lipid profile at 45 days (mg/dL)							
	TC		TG		HDL-C levels		LDL-C levels	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	66.35[a]	11.43	80.43[a]	12.55	43.92[a]	7.55	8.27[a]	12.15
Induced atherosclerosis	93.70[b]	25.03	90.68[b]	25.56	42.78[a]	6.20	33.36[b]	17.06

* Same letters indicate the absence of statistical difference among the groups (p > 0.05); *Legends:* total cholesterol (TC), triglycerides levels (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

At 75th day (30 days after AP induction), the AT and AP+AT groups presented higher levels of TC and LDL-C compared to the groups without AT, regardless of the endodontic infection presence (p <0.05). The TG level was higher in the AP+AT group, compared to the all other groups (p <0.05). The AP and AT groups present higher TG levels as compare to C groups (p <0.05). The AP and AT group present similar TG levels (p >0.05). The HDL-C levels were similar among of all groups (p >0.05) (table 2).

Table 2. Mean and standard deviation (SD) of the lipid profile at 75 days of the all groups

Groups	Lipid profile at 75 days (mg/dL)							
	TC		TG		HDL-C levels		LDL-C levels	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C	69.40[a]	9.47	63.05[a]	11.96	31.13[a]	5.35	25.72[a]	8.57
AP	68.88[a]	9.05	88.18[b]	8.32	28.61[a]	5.79	22.69[a]	6.48
AT	94.05[b]	21.88	93.20[b]	18.97	28.83[a]	4.38	46.55[b]	19.22
AP+AT	111.40[b]	23.38	121.84[c]	15.24	32.29[a]	4.07	54.60[b]	18.49

* Same letters indicate the absence of statistical difference among the groups (p > 0.05)

Legends: total cholesterol (TC), triglycerides levels (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), control group (C), group with apical periodontitis (AP), group with atherosclerosis (AT) and group with AP and AT (AP+AT).

Histological and histometric analysis of the periapical lesions

To evaluate the intensity of the inflammatory infiltrate and bone resorption in apical periodontitis, histologic images of the periapical region H&E-stained from the different experimental groups were analyzed and are shown in figure 1.

In groups C and AT (without endodontic infection), no inflammation was observed in the periapical regions. However, in the AP and AP+AT groups, the pulp showed signs of total necrosis 30 days after exposure. The periodontal ligament region, near the apical apex, was disorganized, with the presence of necrotic tissue and underlying inflammation in all teeth. Near the bone tissue, active resorption gaps were found throughout the periphery of the granuloma (Figure 1).

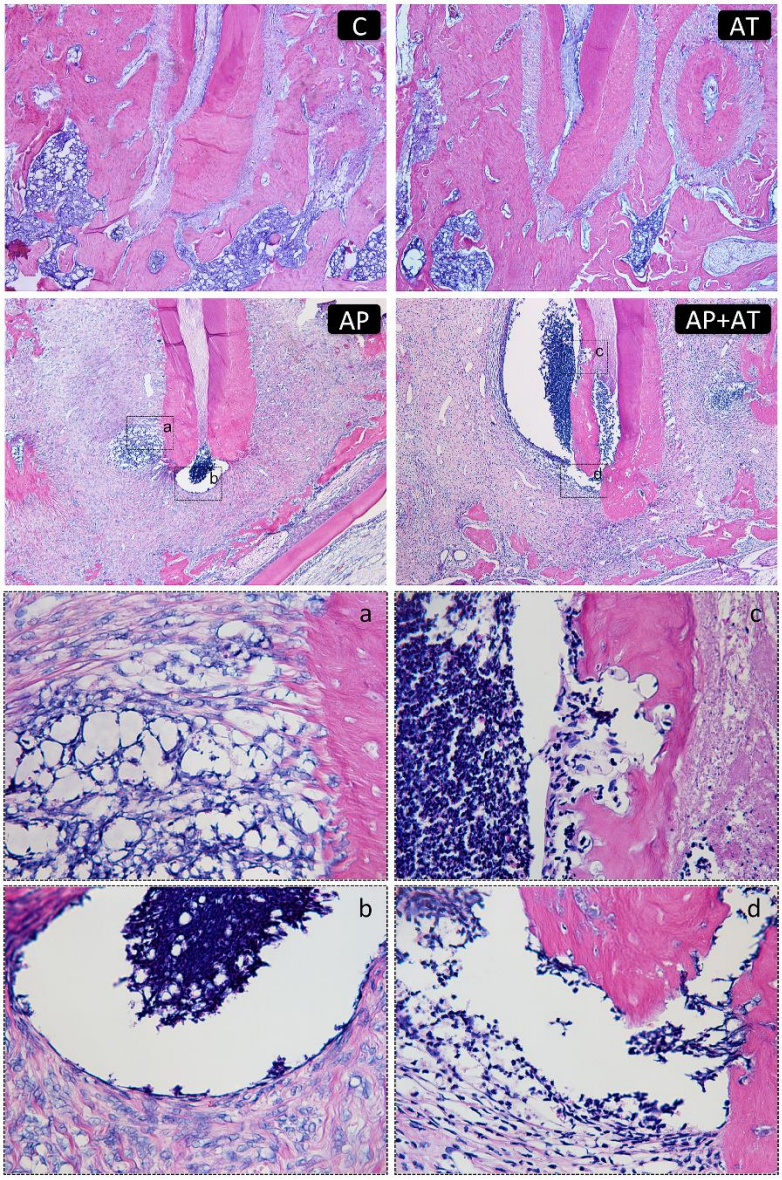

Figure 1. Histologic images of periapical lesions at 30 days after pulp exposure to the oral environment (H&E staining). (C) Group C. The apical and periapical regions are free of inflammatory infiltrates (hematoxylin, x100). (AT) Group AT. Similar to C, the pulp and periodontal tissues are shown with normal aspect (hematoxylin, x100). (AP, a and b) Group AP. (AP) A moderate inflammatory cell infiltration the area surrounding tooth apex can be observed and large area of bone resorption is visible (hematoxylin, x100); (a) Infiltration of inflammatory cells near the dentin region can be observed (hematoxylin, x1000); (b) Presence of acute inflammatory cell concentrations near the tooth apex region, areas of bone resorption are visible and severe disorganization of the periodontal ligament. (hematoxylin, x1000). (AP+AT, c and d) Group AP+AT. (AP+AT) Severe acute, inflammatory cell concentrations near the tooth apex region can be observed and large bone resorption area are visible (hematoxylin; x100); (c) Intense inflammatory cells concentrations and presence of intense dentin resorption (hematoxylin, x1000); (d) Intense acute inflammatory cell concentrations near the tooth apex region, and large bone resorption (hematoxylin, x1000). *Arrowheads* indicate margins of apical periodontitis.

Histologically, AP+AT animals presented more severe periapical inflammatory infiltrates than those in the AP group (P = 0.038067). In these groups, regions of the cementum surface of most specimens exhibited areas of resorption. All the lesions found were of characteristics compatible with periapical granuloma with origin due to pulp necrosis. Histologically, the AP+AT group exhibited the most significant alveolar bone loss (P = 0.031742), when compared to the AP group (Table 3).

Table 3. Scores, median and mean of periapical lesion size of the all groups

Intensity of inflammatory Infiltrate	Groups				Statistical analysis
	C	AP	AT	AP+AT	
1 – Absent	20/20	0/20	20/20	0/20	*Mann Whitney Test (AP x AP+AT) P= 0.038067*
2 – Mild	0/20	6/20	0/20	3/20	
3 - Moderate	0/20	9/20	0/20	6/20	
4 – Severe	0/20	5/20	0/20	11/20	
Median*	1	3[a]	1	4[b]	
Lesion size ($x10^4$ μm^2 ± SD*)	-	151.41±49.11[a]	-	206.41±56.28[b]	*T test Student P= 0.031742*

*Different letters indicate significant statistical differences in rows (P < .05)

Legends: control group (C), group with apical periodontitis (AP), group with atherosclerosis (AT) and group with AP and AT (AP+AT), standard deviation (SD).

Descriptive histological analysis of carotid artery

To evaluate the morphology of carotid artery, histological images H&E-stained from the different experimental groups were analyzed and are shown in figure 2. The histological analysis of C and AP groups sections showed that the carotid intima tunic was normal and intact. Endothelial cells were orderly and continuous organized, without morphological alterations. However, in the AT and AP+AT groups, there was a notable thickening of the carotid intima tunic, with exacerbated proliferation of the endothelial cells. Disorganization of the cell layers and presence of the foam cell layer was also detected. In addition,

calcification areas and necrosis were found in the carotid intima tunic of some specimens. Vacuoles were observed in regions where adipocyte cells were probably present (Figure 2).

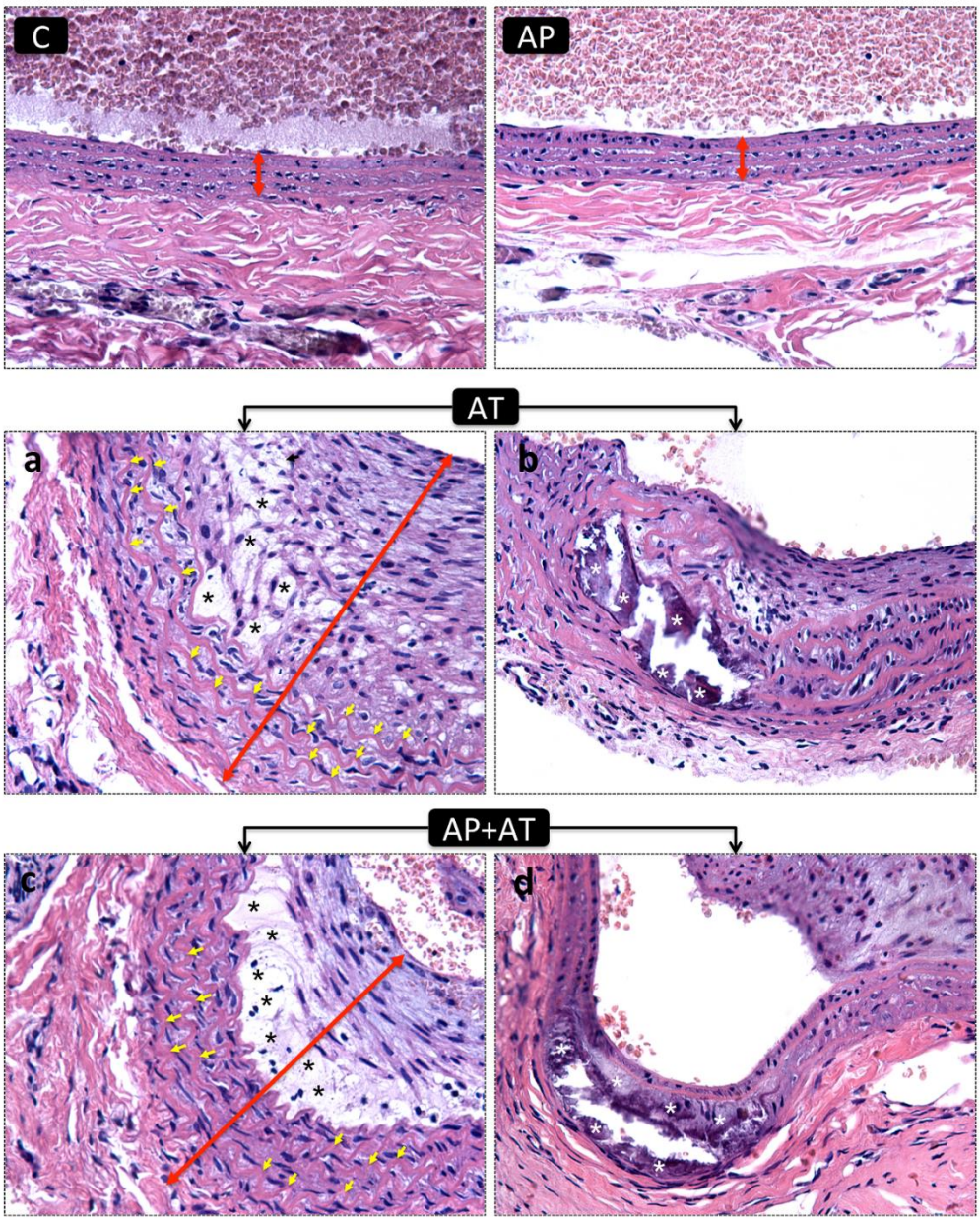

Figure 2. Histological images corresponding to 60 days after the surgical procedure performed on the right carotid artery of the rats of different experimental groups (H&E staining). (C) Group C and (AP) Group AP. Normal and intact appearances of carotid artery. Carotid intima tunic (red arrow) was smooth and thin. Endothelial cells were arranged orderly and continuous organized, without morphological alterations (original magnification, x100). (a and b) Group AT and (c and d) Group AP+AT. In these two groups there was a notable thickening of the carotid intima tunic (red arrow), with exacerbated proliferation of the endothelial cells. Calcification areas and necrosis (white asterisk) were found in the carotid intima tunic of some specimens. Vacuoles (black asterisk) were observed in regions where adipocyte cells were probably present (original magnification, x100 and x400, respectively).

Discussion

In the present study, the association of endodontic infection with atherosclerosis in Wistar rats not only exacerbated bone resorption in periapical lesions, but also increased the intensity of the inflammatory infiltrate, as well as promoted an increase in triglyceride levels, suggesting an interrelation of endodontic infection with atherosclerosis capable of causing metabolic changes.

The animals that underwent atherosclerosis induction with a high-lipid diet (9) associated with the surgical procedure performed on the right carotid artery (20) and with a dose in high concentration of vitamin D_3 (21) presented significant alterations in the morphology of the carotid, resulting in modification of the carotid intima tunic and the formation of the foam cell layer. These induction methods performed in the AT and AP+AT groups have been used in several animal studies and have a high success rate reported in the literature (18), since they are able to repeat several aspects of atherosclerosis that occur in humans. Changes in the carotid artery are compatible with those of other animal studies that also presented atherosclerosis as a systemic alteration (9, 21). However, none of these studies has associated the induction of atherosclerosis with endodontic infection in rats to evaluate whether there is a correlation between these two pathologies. Additional studies to elucidate the mechanisms that occur in this interrelationship are necessary to verify this hypothesis.

Oral infection was induced by a consolidated method used in previous studies (23). The molars pulps of the rats were exposed in the oral cavity for 30 days, sufficient period for the formation of apical periodontitis, as well as previous studies (24). The induction model was confirmed by histological images of the pulp that showed necrosis and of the apical third of the root that contained periapical lesions, with inflammatory infiltrate.

Hyperlipidemia is considered a risk factor for atherosclerosis (25). The results of CT, TG and LDL-C measurements performed in both periods (45 and 75 days), showed that the AT-induced animals had the highest values when compared to the others. These results, along with the morphological changes observed in the carotid arteries of these animals, are in agreement with evidence that hypercholesterolemia is capable of causing pathologies such as endothelial damage and changes in the myocardium (26). In addition, at 75th

day, the AP+AT group presented the highest values of TC, TG and LDL-C, however only the TG presented statistical difference when compared to the other animals. Considering the similarity between the inflammatory state generated by periodontal disease and that of endodontic disease, these elevated TG data corroborate with studies of periodontics performed in rat models and in patients with diabetes as a systemic alteration associated with periodontal disease, which presented levels elevated triglycerides (12, 27). We suggest that the increase in levels of lipid profile, especially TG, observed in atherosclerotic rats with apical periodontitis, may have occurred due to the association of the two induced pathologies that are of inflammatory origin.

It is known that HDL-C acts to protect against the formation of atherosclerotic plaque and its high indexes are associated with a lower occurrence of cardiovascular diseases. However high LDL values are the main causes and conditions induced by atherosclerosis, such as cerebrovascular disease, coronary disease and peripheral vascular disease (25).

The results showed a reduction of HDL-C and an increase of LDL-C, when compared the data obtained in the period of 45 days with the 75 days data. These results are consistent with another study that induced atherosclerosis in rats by the administration of high lipid diet (25), because rats fed with a high lipid diet for thirty days had a significant increase in TC, LDL-C, VLDL-C, TG levels and a decrease in the HDL-C level (28).

In all animals without AP, no inflammation and no bone resorption was observed in the periapical area. However, the periapical lesion induced by exposure of the coronary pulp to the oral environment (24) was significantly higher in the AP+AT group when compared to the AP group. This result corroborates previous studies in diabetic rats with apical periodontitis (29, 23). A human study found that bone loss over 40% from periodontal disease was associated with a threefold increase in mortality from cardiovascular diseases (30). Another study found a positive correlation between alveolar bone loss in humans, assessed by panoramic radiographs, with increased calcification in the internal wall of the carotid artery (31).

Regarding inflammatory infiltrate, microscopic examination revealed no inflammation in C and AT groups. However, histologically the periapical inflammatory infiltrates in the AP+AT group were more intense presenting more

inflammatory cells when compared to the AP group. These results are consistent with studies that also presented more severe inflammation in rats with oral infection associated with others systemic pathology (23). In periodontics, it is believed that there are several mechanisms that can trigger or aggravate atherosclerotic processes, such as activation of the innate immune system, bacteremia triggered by dental intervention, involvement of inflammatory mediators activated by antigens of dental infections in atheromas and common predisposing factors that influence both pathologies (32). Studies with humans indicate that periodontitis may influence atherogenesis (2, 33) as well as cardiovascular events (14, 30, 34).

Although the etiology and pathogenesis of periodontal disease and apical periodontitis are different, it is well established in the literature that both have a common microbiota (35). Moreover the mechanisms that relate endodontic disease as a risk for coronary heart disease may be similar to the hypothetical association between periodontal disease and coronary heart disease, because in both cases the presence of the bacterial infection generates a localized inflammatory response that is able to release systemic cytokines, which may lead to subsequent deleterious vascular effects (17).

It is important that this comparison be made so that more studies are carried out in order to clarify if there is this influence of AP in general health with the presence or absence of systemic diseases, with greater emphasis in the case of atherosclerosis. In summary, this study may serve as a parameter for further investigations to be carried out in order to uncover the possible association of atherosclerosis with apical periodontitis.

Conclusion

The results of this study indicate that apical periodontitis influence triglyceride levels regardless of the presence of atherosclerosis, and Atherosclerosis was able to intensified inflammatory reaction and increased bone resorption in the apical periodontitis area in addition to influencing the lipid profile.

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a. 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.

b. 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.

c. Sentences are stronger when written in the active voice, that is, the subject performs the 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.

d. 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.)

e. 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.

f. 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."

g. 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.

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