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RENAN DAL FABBRO

Efeito do consumo crônico de álcool na lesão periapical induzida em ratos

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Dissertação apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" UNESP como parte dos requisitos para obtenção do título de Mestre em Endodontia.

Orientador: Prof. Tit. João Eduardo Gomes Filho

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Dedicatória

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“Your work is going to fill a large part of your life, and the only way to be truly satisfied is to do what you believe is great work. And the only way to do great work is to love what you do. If you haven't found it yet, keep looking. Don't settle. As with all matters of the heart, you'll know when you find it”

Steve Jobs

Resumo

Fabbro, RD. **Efeito do consumo crônico de álcool na lesão periapical induzida em ratos**, 2017. 63p. Dissertação (Mestrado em Endodontia) – Universidade Estadual Paulista (Unesp), Faculdade de Odontologia, Araçatuba.

Objetivo: analisar o efeito do consumo crônico de álcool no desenvolvimento da lesão periapical. **Material e métodos:** Trinta e dois ratos foram divididos em 4 grupos: Controle (C): sem periodontite apical (PA) e dieta não alcoólica (DNA); (AL): sem PA e dieta alcoólica (DA); (AP): com PA e DNA; (AP+AL): com PA e DA. Solução alcoólica à 20% foi fornecida aos grupos AL e AP+AL como única fonte de hidratação por todo experimento. A PA foi induzida nos primeiros molares inferiores esquerdos ao final da 4ª semana de administração da dieta alcoólica. Alterações de peso, e quantidade de alimentos sólidos e líquidos foram tabulados ao longo das 8 semanas. Ao final deste período, os animais foram eutanaziados e as mandíbulas removidas para análise da densidade óssea seguida do processamento histológico para histomorfometria, bem como análise por imunohistoquímica da expressão das proteínas RANKL, OPG, TRAP, HIF-1α e ALP. As comparações múltiplas dos resultados foram realizadas por análise de variância seguida pelo teste de Tukey. Para dados não paramétricos foi utilizado o teste de Mann-Whitney nas comparações CvsAL e APvsAP+AL. O nível de significância utilizado foi de $p<0,05$. **Resultados:** animais que receberam a dieta alcoólica tiveram um ganho de peso inferior aos dos outros grupos $p<0,05$. A área da região periapical não foi influenciada pela administração da solução alcoólica, entretanto, o infiltrado inflamatório foi mais intenso em AP+AL comparado à AP $p<0,05$. Análise radiográfica mostrou diferença apenas entre os grupos com e sem PA. O grupo AP+AL mostrou os maiores valores para indicadores de osteoclastogênese TRAP, HIF-1α e RANKL $p<0,05$. **Conclusão:** A dieta alcoólica exerceu efeito significativo na severidade da periodontite apical, exacerbando a resposta inflamatória e a osteoclastogênese.

Palavras chave: Periodontite periapical, alcoolismo, endodontia

Abstract

Fabbro, RD. **Chronic alcohol consumption effect on induced apical periodontitis in rats**, 2017. 62p. Dissertation (Master's Degree in Endodontics) – São Paulo State University (Unesp), School of Dentistry, Araçatuba.

Aim: evaluate the effect of chronic alcohol consumption on the periapical lesion. **Material and methods:** Thirty-two rats were divided into 4 groups: Control (C): without apical periodontitis (AP) and non-alcoholic diet (NDA); (AL): without AP and alcoholic diet (AD); (AP): with AP and NDA; (AP + AL): with AP and AD. Alcoholic solution at 20% was given to the AL and AP+AL groups as the sole source of hydration throughout the experiment. AP was induced in the lower left first molars at the end of the 4th week. Changes in weight, and amount of solid and liquid foods were recorded over 8 weeks. At the end of this period, the animals were euthanized and the jaws removed for of x-ray bone density analysis followed by histological processing for histomorphometry, as well as immunohistochemical analysis for RANKL, OPG, TRAP, HIF-1α and alkaline phosphatase. Multiple comparisons of results were performed by analysis of variance followed by the Tukey test. For non-parametric data the Mann-Whitney test was used in the comparisons CvsAL and APvsAP+AL. The level of significance was set at $p < 0.05$. **Results:** animals that received alcoholic diet had a weight gain lower than the other groups $p < 0.05$. The periapical region area was not influenced by the administration of the alcohol solution, however, the inflammatory infiltrate was higher in AP+AL compared to the AP $p < 0.05$. Radiographic analysis showed difference only in the comparisons between the groups with and without apical periodontitis. The AP+AL group showed the highest values for osteoclastogenesis markers TRAP, HIF-1α and RANKL $p < 0.05$. **Conclusion:** Alcoholic diet had a significant effect on the severity of apical periodontitis, exacerbating the inflammatory response and osteoclastogenesis.

Keywords: Apical periodontitis, alcoholism, endodontics

Sumário

Introdução Geral

INTRODUÇÃO GERAL

O álcool é uma substância não essencial para a vida, consumida mundialmente em ambos os contextos sociais e culturais (1), e três tipos de consumo de álcool já foram reconhecidos: o consumo agudo, o elevado-crônico e o consumo leve (2). Estima-se que 2 bilhões de pessoas no mundo consomem bebidas alcoólicas e o consumo excessivo afeta a economia de toda a sociedade e a qualidade de vida, uma vez que traz prejuízo à saúde (1).

O consumo agudo de álcool prejudica uma variedade de tarefas cognitivas e de desempenho, incluindo a memória e a aprendizagem (3, 4). Além disso, o consumo crônico de álcool leva a déficits de memória que persistem mesmo após a abstinência a longo prazo (5-8). Também traz efeitos prejudiciais ao cérebro, fígado, músculos e esqueleto, sendo responsável por um número elevado de mortes (75.000 americanos a cada ano) (9). Um dos órgãos que mais sofre influência da droga é o fígado, causando aumento da atividade específica do citocromo P4502E1, do conteúdo de triglicérides e do peso do fígado (10). O consumo crônico de álcool também pode levar à doença hepática diretamente, bem como contribuir para a progressão de doenças que possuem outras etiologias, como a hepatite viral (11). O consumo excessivo de etanol também pode resultar na reparação óssea deficiente (10), sendo conhecido por induzir a osteoporose secundária (2).

Os efeitos do consumo de álcool no tecido ósseo estão ligados à dose ingerida e à duração de consumo, uma vez que o baixo consumo de álcool não é prejudicial para o tecido ósseo mas o consumo moderado já pode ter efeitos negativos, dependendo do sexo, idade, estado hormonal, e tipo de bebida consumida (12).

Em um estudo foi relatado que consumo leve de álcool,

preferencialmente vinho, resultou num aumento da densidade mineral óssea da coluna e de todo o corpo em mulheres pós-menopausa (13). Entretanto, em casos onde o consumo foi elevado e crônico, houveram reduções na densidade mineral óssea, além da massa e resistência óssea (12, 14-16).

Recentemente foi relatada a redução da espessura cortical óssea, diminuição da espessura e do volume trabecular ósseo, bem como diminuição da taxa de formação óssea, da taxa de aposição mineral e aumento da superfície corroída, em pacientes negros do sexo masculino sofrendo de doença óssea induzida por álcool e pancreatite (17, 18).

O consumo de álcool também constitui um importante fator de risco para fraturas (19), sendo quatro vezes maior em alcoólatras crônicos em comparação à pacientes não alcoólotras com as mesmas características (12, 20). A baixa massa óssea frequentemente encontrada em alcoólatras é o principal fator predispontente ao aumento da incidência dessas lesões (12, 21). Além disso, o uso do álcool cria condições que favorecem quedas acidentais, independentemente de alterações ósseas (22).

A osteoporose, caracterizada por uma redução generalizada na densidade mineral óssea e deterioração da microarquitetura do tecido ósseo, é uma doença multifatorial (23). Evidências indicam que o etanol promove a osteoporose por meio da alteração da produção e da reabsorção no processo de remodelação óssea (9), resultando na fragilidade e porosidade do osso, aumentando sua susceptibilidade a fraturas.

Vários estudos têm mostrado que o álcool pode inibir a formação de novo osso em um local da fratura quando a formação óssea é necessária para a reparação (10, 24, 25). Os efeitos do consumo de álcool na cura da fratura também parecem ser dose-dependente (2). Os efeitos diretos do álcool sobre o tecido ósseo se relacionam com uma diminuição nos índices de atividade dos osteoblastos e diminuição da diferenciação em osteoblastos humanos (26), assim como um aumento da reabsorção óssea

realizada por osteoclastos (27). Foi observada também mudança na morfologia dos osteócitos e o aumento da apoptose de osteócitos (2).

Também foi demonstrado que o tratamento com álcool em células tronco da medula óssea diminui a osteogênese e aumenta a adipogênese (28, 29)**.** O aumento da osteoclastogênese pelo álcool pode ser mediado também por citocinas, como mostrado pelo aumento da expressão de Interleucina 6 (IL-6) e do Receptor Ativador do Fator Nuclear -κB Ligante (RANKL) em ratos tratados com solução alcoólica (30).

A descoberta da via RANKL/OPG (osteoprotegerina) contribuiu para a compreensão de como a formação e a reabsorção óssea são reguladas. RANKL e OPG são membros das superfamílias do fator de necrose tumoral (TNF) e do receptor de TNF (TNFr), respectivamente, e ao se ligarem ao Receptor Ativador do Fator Nuclear kB (RANK) não só regulam a formação, ativação e sobrevivência dos osteoclastos na modelagem e remodelamento ósseo normal, mas também em várias outras condições patológicas caracterizadas por aumento do turnover ósseo, como por exemplo no desenvolvimento da periodontite apical. Basicamente, o RANKL é sintetizado pelas células da linhagem osteoblástica, células imunes e algumas células cancerígenas. Este fator liga-se ao receptor de superfície de osteoclastos, RANK e estimula a reabsorção óssea através da osteoclastogênese e da ativação de osteoclastos maduros multinucleados. A OPG que é secretada por osteoblastos como um receptor de armadilha para RANKL, evita que o RANKL se ligue ao RANK, prevenindo a reabsorção óssea (31).

Estes efeitos podem ser exercidos diretamente ou indiretamente através dos muitos tipos de células, hormônios, e fatores de crescimento que regulam o metabolismo ósseo. Além de fornecer apoio estrutural, o osso é um grande armazém de cálcio e outros minerais (32). O intestino delgado absorve o cálcio dos alimentos ingeridos, e os rins excretam o excesso de cálcio (33). Uma concentração adequada de cálcio na corrente

sanguínea é necessária para o bom funcionamento dos nervos e músculos (33). O corpo monitora a concentração de cálcio e responde através da ação dos hormônios, vitaminas e fatores de crescimento locais para regular a distribuição de cálcio entre o sangue e ossos (34). O álcool pode perturbar este equilíbrio por afetar os hormônios que regulam o metabolismo do cálcio, bem como os hormônios que influenciam o metabolismo do cálcio indiretamente (por exemplo, hormônios esteroides reprodutivos e hormônio do crescimento) (35). Um exemplo é a diminuição dos níveis séricos de vitamina D, resultando em má absorção de cálcio através do intestino, levando a baixos níveis séricos de cálcio (2).

Os ossos são constituídos por tecido mineralizado que consiste principalmente em cálcio (Ca) e fósforo (P) (36). Estes elementos são organizados formando cristais de hidroxiapatita e algumas condições ou patologias que afetam este tecido podem alterar a distribuição quantitativa desses elementos e, consequentemente, a composição estequiométrica da hidroxiapatita (23). A deficiência de estrogênio induzida pela realização de ovariectomia em ratos, associada ao consumo de álcool pode influenciar negativamente a qualidade do osso alveolar, e alterar a sua composição mineral, mostrando uma mudança significativa na composição estequiométrica da hidroxiapatita na crista óssea alveolar, levando a uma redução nas relações Ca/P (37). O estrogênio diminui a reabsorção óssea e aumenta a formação de osso através de regulação positiva de OPG (um regulador negativo da atividade osteoclática e osteoclastogênese) e regulação negativa de RANKL (um regulador positivo da atividade osteoclástica e osteoclastogênese) (38).

Uma diminuição do nível sérico de estradiol foi relatado em mulheres alcoólatras (39) e em ratas tratadas com álcool (40). O estradiol sérico baixo mostrou ser um fator importante para a perda óssea induzida pelo etanol, uma vez que provoca um aumento do receptor ativador NF-κB ligante (RANKL).

A Fosfatase Ácida Resistente ao Tartrato (TRAP), considerada um marcador imunohistoquímico de osteoclastos, é expressa por osteoclastos, macrófagos, células dendríticas e uma série de outros tipos de células. Tem um papel crítico em muitos processos biológicos, incluindo desenvolvimento esquelético, síntese e degradação de colágeno, mineralização óssea, produção de citocinas por macrófagos e células dendríticas e recrutamento de macrófagos (41). Por sua vez, o Fator de Transcrição Induzível de Hipóxia (HIF-1α) regula o acoplamento angiogênico-osteogênico e a reabsorção óssea mediada por osteoclastos. O HIF-1α é conhecido por melhorar a diferenciação de osteoclastos, sendo identificado como parte de essencial via para a osteoclastogênese aumentada in vivo (42). Ambos marcadores, TRAP e HIF-1α, são frequentemente utilizados para monitorar o desenvolvimento de patologias ósseas.

Baixos níveis de osteocalcina, um marcador bioquímico de formação óssea sintetizado principalmente por osteoblastos, foram encontrados em pacientes com alcoolismo elevedo crônico, provavelmente devido ao fato do álcool causar uma diminuição da produção/secreção pelos osteoblastos ou à um aumento na taxa de depuração (43).

O álcool também tem implicações no osso alveolar. A ingestão da *Cachaça*, uma bebida alcoólica tipicamente brasileira, pode afetar adversamente o osso alveolar, tanto na presença como na ausência de formação de biofilme, aumentando a perda óssea e reduzindo a densidade óssea na periodontite induzida (44). Estes resultados suportam os dados clínicos que apontam o consumo de álcool como um indicador de risco para as doenças periodontais (45).

A periodontite apical é uma doença inflamatória crônica de tecidos perirradiculares causada por agentes etiológicos de origem endodôntica, levando à destruição óssea (46). A maioria das células e moléculas de sinalização envolvidas na patogênese das lesões periodontais também participam nas lesões periapicais (47). Assim uma vez que o osso alveolar

sofre com o consumo excessivo de álcool, é provável que haja alterações na formação da lesão periapical em animais sob o consumo de álcool, devido às semelhanças dos mecanismos celulares e moleculares, podendo exercer um efeito potencializador sobre a progressão da lesão periapical.

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Artigo 1

Chronic alcohol consumption effect on induced apical periodontitis in rats

Introduction

Alcohol consumption is widely spread worldwide in both social and cultural contexts (1). According to the World Health Organization, it is estimated that 2 billion people consume alcohol, leading to about 3.3 million deaths each year, or 5-9% of all global deaths, then its excessive intake affects the economy and the quality of life of the whole society (1).

Excessive chronic alcohol consumption causes harmful effects to the brain, liver, muscles and skeleton (2-6). The effects on bone tissue are dose-dependent, where an excessive and long-term consumption leads to deleterious effects, decreasing bone mass, bone mineral density, and poor bone repair (5, 7).

The direct effects of alcohol in bone tissue are related to a decreasing in osteoblast differentiation and activity, as well as an increasing on bone resorption performed by osteoclasts (8). Such local effects are due to a up regulation of Receptor Activator of NF-κB ligand (RANKL), a positive regulator of osteoclastic activity and osteoclastogenesis, and down regulation of osteoprotegerin (OPG), a negative regulator of osteoclast activity and osteoclastogenesis (9). Studies using animal models have suggested that alcohol consumption can adversely affect the alveolar bone, increasing bone loss and reducing bone density (10, 11).

Periodontal disease and apical periodontitis are very common oral infections (12). Once excessive alcohol consumption leads to alveolar bone loss (10), it is possible that the apical periodontitis (AP) can also be affected by chronic alcohol consumption due to the similarities in the biological processes.

There is no information available concerning the effect of alcohol on AP in animals, therefore the aim of this study was to evaluate the AP development in rats under or not chronic alcohol diet. Thus, the hypothesis tested in this study was if an excessive chronic alcohol administration

enhances the size and severity of AP due the imbalance in RANKL/OPG ratio.

Material and Methods

Animals

The study was submitted and approved by the ethics committee (Ethics Committee on Animal Use - Araçatuba Dental School, FOA nº00502-2016). Thirty-two male rats (*Rattus norvegicus*, Wistar) aged four months, weighing 250-300g each were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity) with a 12h light-dark cycle, were used. The animals were randomly assigned into four groups (n = 8): control group (C) - rats without apical periodontitis and regular diet; apical periodontitis and regular diet (AP); alcoholic diet without apical periodontitis (AL); apical periodontitis and alcoholic diet (AP+AL).

Alcohol consumption

The alcohol consumption was conducted as described by De Deco et al (13) with minor changes regarding the solution concentration. Starting on day one (experiment beginning) at 5% (v/v) until reaching 20% (v/v) (14^{th} day), increasing 1% per day, in order to avoid rejection. Subsequently, the animals ingested 20% (v/v) of alcohol solution until the last day (56^{th} day). The alcohol solution was prepared daily by dilution of absolute ethyl alcohol in water. It was administrated to the AL groups in drinking fountains, as the sole source of water for eight weeks. For C and AP groups, water was freely available throughout the experiment (from 1^{st} to 56^{th} day). The solid diet consisted of a commercial feed (Labina-Purina®, Paulinia, Brazil) and was available freely throughout the experiment for all groups. The animals, as well as the amount of solid feed consumed by each group were weighed daily. The amount of alcohol consumption was quantified daily taking into account the decreasing on the original volume present in the bottles and defined in ml/day.

Apical Periodontitis Induction

Four weeks before euthanasia (28th day), under general anesthesia with ketamine 75 mg/kg IM (Cetamin 10%, Syntec do Brasil, São Paulo, Brazil) and xylazine 25 mg/kg IM (Xilazin 2%, Syntec do Brasil, São Paulo, Brazil), the animals were placed on specific surgical tables, had the coronary pulp tissue exposed and disorganized through the coronary opening of the lower first left molar using a spherical carbide burr with 0.5mm diameter (Jet Carbide ¼, Kavo Kerr Group, Orange California, USA) (14).

Radiographic density (RD)

Digital radiographic images (storage phosphorplate, Digora, Soredex, Orion Corporation, Helsinki, Finland) were obtained with a GE-100 x-ray unit (70 kVp, 10 mA,12 pulses, General Electric, Waukesha, WI, USA). The target-to-film distance was set to 40cm. The incidence of the radiation focus was perpendicular to the film-object plane. Phosphorus-activated optical plate and a nine-step aluminum step wedge (6063 alloy, ABNT) were used concomitantly with the specimens to capture images in the TIFF format (tagged image file format) 24 bits. The phosphor plate was scanned in the dedicated Digora scanner (Orion Corporation, Helsinki, Finland), and image was displayed in Digora sotware for Windowns, version 1.51

Radiographic density (RD) analysis was performed on the periapical area and on the aluminum penetrometer. A standardized area (in pixels) was established for each item analyzed: periapical region of distal root of the first lower molar (9x9 pixels) and each step of aluminum step-wedge (40x40 pixels). Three repetitions of measurements were made after the area had being selected, with the mean values of gray shades in the areas, automatically calculated by the Digora software, corresponding to the DR of bone and penetrometer steps respectively.

The values expressed in grayscale are converted to millimeters of aluminum (mmAl) by equations obtained through the scatter plot, relating the values of DR of each aluminum step and its corresponding thickness (18). The equation of each image is obtained from three values of the graph (linear regression): the value of radiopacity for the aluminum step closest to that of radiopaque periapical area, the step above and step below. A scatter plot was constructed for each radiographic image due to the variation of aluminum steps amongst images.

Histometric Analyses

All animals were euthanized after eight weeks (56^{th} day) from the experiment beginning. Immediately after, the jaws were removed, sectioned in hemi-jaws and fixed in 10% neutral formalin for 24 hours. Then, washed in running water for 24 hours and decalcified in 10% EDTA solution for approximately 45 days. After this period, the specimens were washed in running water (24 hours), dehydrated, diaphanized and embedded in paraffin by the routine method. The obtained blocks were 06 micrometers semi-serial cut and stained with hematoxylin and eosin (HE). Slides without staining were reserved for immunohistochemistry.

Histologic analysis was conducted by using the following guidelines: quality and extension of inflammation, cellularity pattern of dental and periodontal tissues to score the inflammatory infiltrate as follows: score 0 (absent to few inflammatory cells), score 1 (less than 25 cells and mild reaction), score 2 (between 25 and 125 inflammatory cells and moderate reaction), and score 3 (125 or more cells and severe reaction). Presence or absence of necrosis was also recorded (15).

For histometric analysis of periapical lesion size, the periapical area associated to the distal root of the lower first molar was measured using Leica Microsystems Software (Leica, Wetzlar, Germany). The area was calculated by rounding up the lesion borderline, considering the outer

external surface of alveolar bone, and it was expressed in square millimeters, using an image processing system that consisted of light microscope (DM 4000 B; Leica), color camera (DFC 500; Leica, Wetzlar, Germany), color image processor (Leica Qwin V3 software; Leica), and a personal computer (Intel Core I5, Microsoft Windows 10) (16).

Immunohistochemical Analyses

Immunolabeling in the histological sections were performed by using an indirect immunoperoxidase technique (15): primary antibodies (1:100) against TRAP (Goat anti-TRAP-SC30832, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ALP (Goat-SC15065, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF-1alpha (Rabbit-SC10790, Santa Cruz Biotechnology, Santa Cruz, CA, USA), RANKL (Goat anti-RANKL-SC7627, Santa Cruz Biotechnology, Santa Cruz, CA, USA), OPG (Rabbit anti-OPG-SC11383, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Universal secondary antibody (Universal LSAB + Kit / HRP, Rb / Mo / Goat) was used. The reaction was amplified with the avidin-biotin system (ABC-Vectastain Elite ABC Kit - Peroxidase Standard, reagent A and B only - PK6100 - Vector Laboratories) and disclosed using diaminobenzidine (DAB) as a chromogen. The specificity of the labeling was confirmed through a negative control.

Alkaline Phosphatase (ALP), Hypoxia-Inducible Factor-1 alpha (HIF-1alpha), Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) and Osteoprogeterin (OPG) were analyzed in the vicinity of the periapical region at a 400x magnification (Leica Microsystems, Wetzlar, Germany). A semi-quantitative analysis of the immunostaining was carried out using three histological sections of each block. The adopted immunolabeling pattern (IP) were assigned as follows (17): score 0 - null immunostaining pattern: total absence of immunoreactive cells (IR); score 1 - Low IP: about 1/4 of the IR cells per area; score 2 - Moderate IP: about 1/2 of the IR cells per area; score 3 - High standard of immunostaining: about 3/4 of the IR cells per area.

The TRAP analysis was performed to quantify bone resorption associated cells following the previously described protocol (16). Initially the perimeter of the lesion at the bone level was determined by contouring the borderline of periapical lesion with the aid of Leica Microsystems Software, then quantifying mature osteoclasts or multinucleated TRAP-positive cells at the perimeter, being expressed in multinucleated TRAP-positive cells per square millimeters.

Statistical Analysis

The data were analyzed using GraphPad Prism 7 software (La Jolla, CA). After the Shapiro–Wilk test of normality, the Mann-Whitney test was performed for nonparametric data, and analysis of variance followed by the Tukey test was performed for parametric data. The level of significance was 5%.

Results

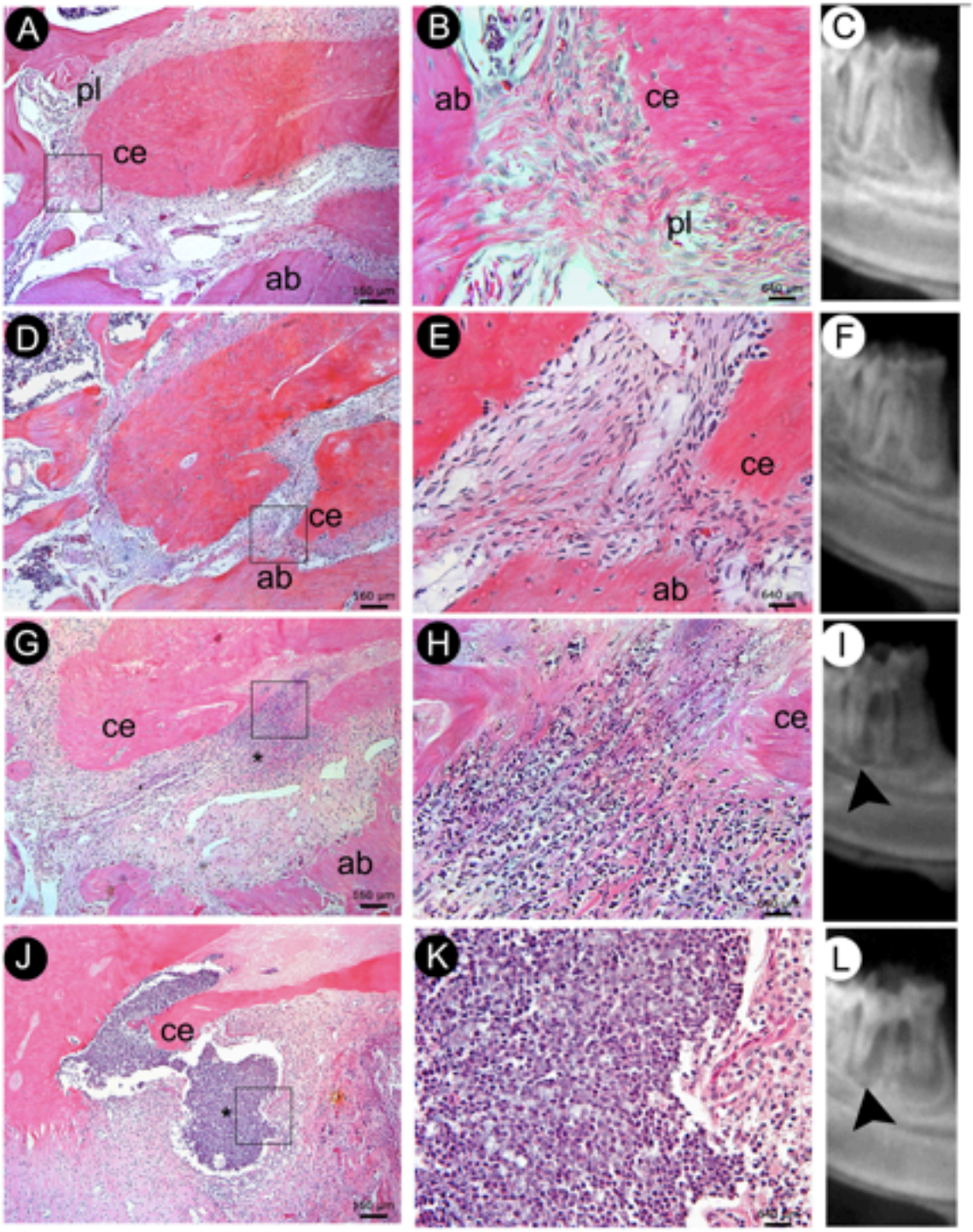
Body weight and Alcohol/Solid diet intake

In all groups, there was rat weight gain during the experiment. The AP had more percentage weight gain (48,3%). All groups that did not receive the alcohol diet gained more weight compared to alcoholic groups, once the solid diet consumption was 29,47±6,09 (g/animal/day) for C and AP groups, and 15,92±3,80 for AL and AP+AL groups, being statistically significant ($p<0.05$) (Table 1).

Histopathologic and Histometric Analysis

Groups without apical periodontitis induction did not present inflammatory response. In contrast, for the groups with apical periodontitis, AP and AP+AL, an inflammatory infiltrate with necrotic areas was observed. The AP+AL group presented more intense chronic inflammatory infiltrate and more areas of bone resorption compared to the AP group ($p = 0.186$) (Table 1, Figure1).

Figure 1: Histologic and radiographic aspects of periapical regions (A,B,D,E). Histologic images showing normal condition in C and AL groups. Apical periodontitis with greater severity in AP+AL rats (J,K). Periapical radiographs of mandibular first molar in C group (C), AL (F), AP (I), AP+AL (L). Arrowheads indicate apical periodontitis (I-L). ab - alveolar bone; ce - cementum; pl – periodontal ligament. *Inflammatory infiltrate. Hematoxylin-eosin staining. Rectangle shows area elected for 400X magnification. Original magnification: A, D, G, J 100X; B, E, H, K 400X.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Immunohistochemistry

The immunoreactivity pattern for TRAP, ALP, HIF-1α, RANKL, OPG are described below and in Figure 2.

TRAP: The immunolabeling technique for TRAP was remarkably specific to osteoclasts. The AP+AL group showed the highest load of TRAP-positive multi-nucleated cells per mm in the periapical region than the other groups ($p<0.05$).

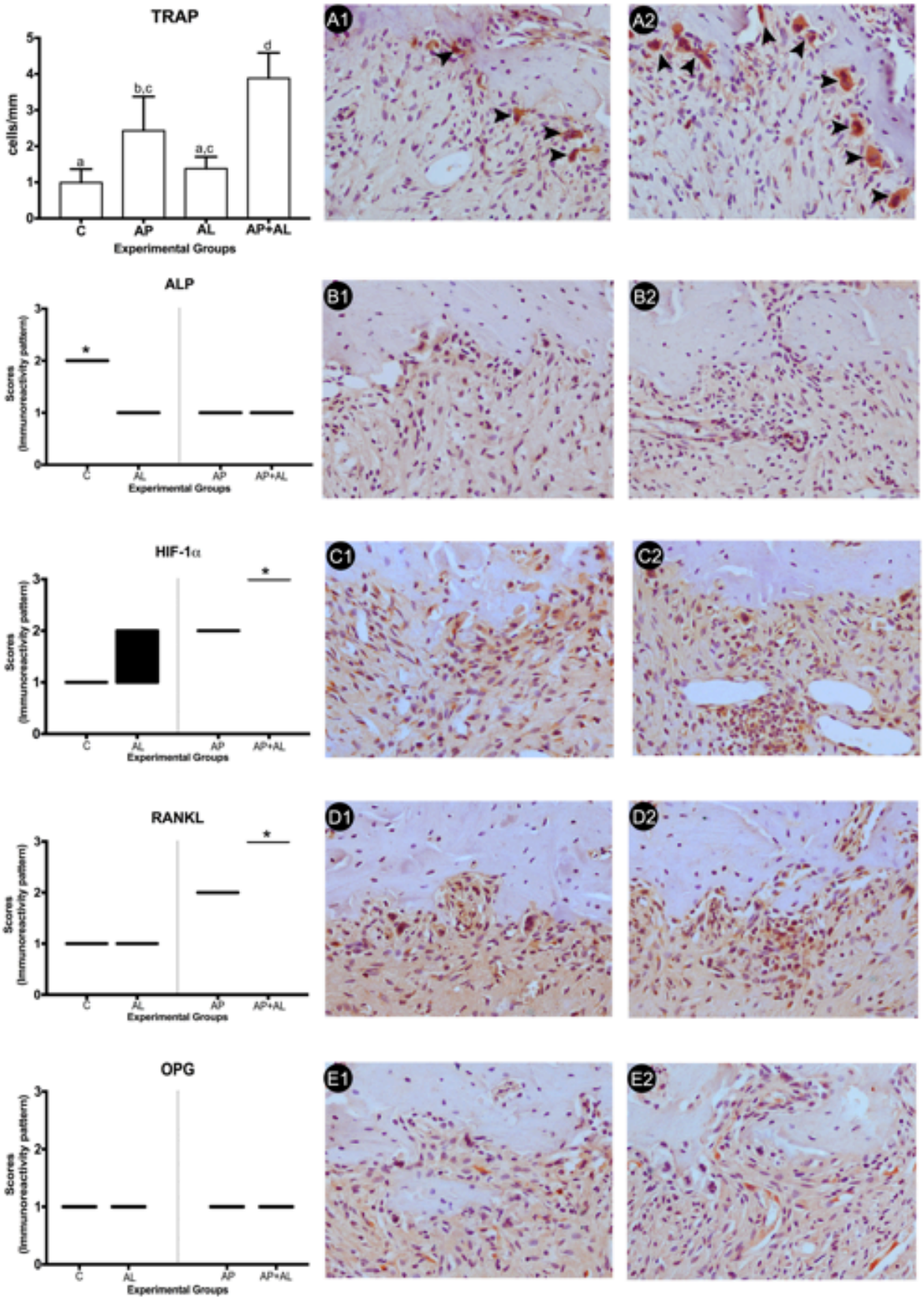
ALP: For control group, the score 2 predominated, being higher than AL group ($p<0.05$). For all groups with apical periodontitis induction, score 1 prevailed regardless the liquid diet imposed ($p>0.05$).

HIF-1α: The score 1 was predominant for control group, showing no statistical difference to AL group, which presented a mixed 1 and 2 score. The AP group showed totality in marking 2, and AP+AL an absolute marking for score 3 ($p = 0.0002$).

RANKL: For groups without apical periodontitis induction the score 1 predominated. In the AP+AL group score 3 prevailed, with significant difference when compared to the AL group ($p = 0.0013$).

OPG: In all groups prevailed scored 1. There were no significant differences between any experimental groups.

Figure 2: Number of TRAP-positive cells per mm in the perimeter of apical periodontitis of distal root of mandibular first molar in different treatment groups (A1-A2). Photomicrographs showing TRAP-positive cells (arrowheads) in AP and AP+AL groups, respectively. Charts showing scores for ALP, HIF-1α, RANKL and OPG. Photomicrographs showing the histological appearance of immunolabeling for ALP (B1-B2), HIF-1α (C1-C2), RANKL (D1-D2) and OPG (E1-E2), in AP (B1, C1, D1 and E1) and AP+AL (B2, C2, D2, and E2) groups.


TRAP
cells/mm
a
b,c
a,c
d
C
AP
AL
AP+AL
Experimental Groups
A1
A2
ALP
Scores (Immunoreactivity pattern)
*
C
AL
AP
AP+AL
Experimental Groups
B1
B2
HIF-1α
Scores (Immunoreactivity pattern)
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C
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Experimental Groups
C1
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RANKL
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AP+AL
Experimental Groups
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Scores (Immunoreactivity pattern)
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Radiographic Analysis

The RD of periapical region in groups with apical periodontitis was lower than that observed in the groups without induction ($p<0.05$). Moreover, the administration of alcohol did not alter RD in AP+AL group when compared to AP only (Table 1).

Table1: Scores assigned to the criteria of intensity and extent of inflammatory infiltrate, Presence or absence of necrosis, Periapical Lesion Areas, Average Weight Gain, and radiography density of periapical area.

Group	Score					Necrosis	Periapical Area (mm^2)	Average Weight Gain %	Rx (mm/Al)
	0	1	2	3	Med				
C	7/7	0/7	0/7	0/7	0^a	0%	$0,15 \pm 0,05^a$	$31,06^a$	$2,21 \pm 0,17^a$
AL	6/6	0/6	0/6	0/6	0^a	0%	$0,17 \pm 0,04^a$	$14,39^b$	$2,12 \pm 0,14^a$
AP	0/8	4/8	4/8	0/8	$1,5^b$	100%	$0,94 \pm 0,36^b$	$48,3^a$	$1,62 \pm 0,33^b$
AP+AL	0/6	0/6	3/6	3/6	$2,5^c$	100%	$1,03 \pm 0,23^b$	$9,83^b$	$1,76 \pm 0,24^b$

C, control group; AL, alcoholic diet; AP; apical periodontitis; AP+AL, alcoholic diet and apical periodontitis. In the same column, different letters indicate significant differences ($p<0.05$).

Discussion

In the present study, the effects of chronic alcoholism in the periapical region of rats' tooth were evaluated through the animal exposure to a 20% alcohol solution via drinking water as the sole source of hydration that was used for 8 weeks to characterize the pattern of excessive chronic alcoholism (19). This model was chosen due to be more natural, mimicking human consumption more than gavage and intraperitoneal injections, which causes stress to the animal and provides large amounts of alcohol at once (20). Another commonly used model is the Lieber DeCarli diet that contains alcohol, protein and caloric needs, however besides it is widely used it restricts the access to solid foods and, therefore, does not exactly simulate regular ethanol consumption in humans (19, 21, 22). This way, the spontaneous consumption chosen was considered offering better characteristics for the present purpose.

In the present study, the animals that received alcoholic diet had a daily consumption of the solid diet inferior to those that did not receive it, showing almost no changes on corporal weight along the experiment different from that observed with the rats under regular diet. Whereas alcohol is highly caloric the rats under alcohol intake felt satiety (19).

During the radiographic density analysis, it was not possible to detect influence of chronic alcohol consumption on the bone density in periapical region, being clear only the difference between groups with lesion and without lesion, matching with the histometric findings. It is important to record that radiographic analysis is the most routinely diagnosis method used in dentistry but it may not be enough to scientifically evaluate a therapeutic strategy or propose a scientific fundament like the present purpose.

The present results showed a significant larger inflammatory infiltrate in apical lesions under chronic alcohol intake, characterized by an enlarged periapical space between bone and cementum, indicating that bone tissue was pathogenically resorbed due to inflammatory response. These results matches to previous ones that revealed the ability of chronic alcohol

consumption to alter the expression of inflammatory cells and their chemical mediators (7). In rats, studies have suggested that alcohol consumption may increase the alveolar bone loss (10, 23). In fact, chronic alcohol consumption has been associated with a lower bone mineral content and bone density in trabecular and cortical long bones, increasing the risk of osteoporotic fracture (24-26). Some periodontal bone hazard has also been demonstrated after alcohol intake such as a lower percentage of periodontal bone support (10, 23) and a lower alveolar bone volume (27). On the other hand, other investigations did not demonstrate any effect of alcohol consumption upon alveolar bone (28). Moreover, Liberman et al. (29) concluded that the intake of low concentrations of alcohol did not affect the alveolar bone loss. More than that, there are still conditions in which lower alcohol consumption resulted on bone and mineral homeostasis (30). The different animal study results differ each other due to the type of alcoholic beverage, dose of ethanol, time and via of alcohol administration and animal age.

However, to date, there is no study regarding the effects of alcohol ingestion on apical periodontitis. The periapical inflammation is a complex process that occurs as a consequence of a bacterial infection or trauma and can result in bone resorption (31). During this process, pro-inflammatory cytokines are expressed, playing a key role in the recruitment and activation of osteoclasts through the OPG/RANKL/RANK system (9).

In the present study, it was observed change on OPG/RANKL system, which is fundamental for the regulation of bone metabolism (32). Receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) are members of TNF superfamily (32). In bone, RANKL stimulates osteoclastic differentiation and enhances the activity of mature osteoclasts and inhibits their apoptosis (32). RANKL exerts its function by binding to receptor activator of NF-κB (RANK) present on the membrane of mononuclear osteoclast precursors (33). The present study revealed significantly more expression of RANKL in apical periodontitis under alcohol intake corroborating the alcohol's ability to potentiate RANKL release (34). OPG

protects the bone tissue from excessive reabsorption by binding to RANKL, thus preventing it from binding to RANK (32). In the present study, there was no observed difference in the levels of OPG amongst all groups. However, even though OPG levels are constants, RANKL/OPG ratio changes in favor of osteoclastogenesis, due increased RANKL expression.

In the present investigation, the highest values of HIF-1α were found in AP under alcohol intake showing a positive effect of the alcoholic diet on its increase, as reported in previous study (35). HIF-1α is a hypoxia-inducible factor, induced by hypoxia and RANKL via activation of NF-kB. HIF-1α activated results in recruitment and activation of osteoclasts, leading to enhanced bone resorption on site (36). Moreover, immunolabeling to TRAP (tartrate-resistant acid phosphatase) that reveals osteoclast activity (37) demonstrated more TRAP-positive cells in animals that consumed alcoholic diet than those who consumed potable water, which is consistent with previous study that show a direct effect of alcohol on the amount of TRAP-positive cells, since it is evaluated by the ratio of RANKL to its decoy receptor, OPG (11).

The relationship between chronic alcohol consumption and periapical lesion is complex and more studies are needed to elucidate the doubts and variations of the presented method.

Conclusion

The excessive chronic alcohol consumption increased the severity of apical periodontitis via exacerbation of local inflammatory response, potentiating osteoclastogenesis, recruitment and activity of osteoclasts.

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Anexo A – Comitê de Ética

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

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

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Efeito do consumo crônico de álcool na lesão periapical induzida em ratos"**, Processo FOA nº 00502-2016, sob responsabilidade de João Eduardo Gomes Filho apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 04 de Outubro de 2016.

VALIDADE DESTE CERTIFICADO: 04 de Dezembro de 2017.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 04 de Janeiro de 2018.

CERTIFICATE

We certify that the study entitled **"Chronic alcohol consumption effect on apical lesion induced in rats"**, Protocol FOA nº 00502-2016, under the supervision of João Eduardo Gomes Filho presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on October 04, 2016.

VALIDITY OF THIS CERTIFICATE: December 04, 2017.
DATE OF SUBMISSION OF THE FINAL REPORT: January 04, 2018.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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Anexo B: Protocolo Imunohistoquímico

Técnica Da Imunoperoxidase Indireta

1º Dia

1. Desparafinização

   1º Ciclo: Colocar lâminas na estufa por 30 minutos para derreter a parafina (Estufa a 56oC – 60oC).

   2º Ciclo: Bateria:

   a) Xilol I – 5 minutos

   b) Xilol II – 5 minutos

   c) Xilol III – 10 minutos

   d) Álcool 100 I – 2 minutos

   e) Álcool 100 II – 2 minutos

   f) Álcool 100 III – 2 minutos

   g) Álcool 95 – 2 minutos

   h) Álcool 70 – 2 minutos

2. Lavagens

   a) PBS – 5 minutos

   b) PBS–Tritonx100 – 5minutos

   c) PBS – 5minutos

3. Recuperação Antigênica:

   a) 198 ml de água destilada + 2 ml de tampão citrato 100x

   b) 5 minutos – 95ºC

4. Lavagens

   a) PBS – 5 minutos

   b) PBS–Tritonx100 – 5minutos

   c) PBS– 5minutos

5. Bloqueio da Peroxidase Endógena

    a) 180 mL de PBS + 20 mL de H_2O_2 / 1 hora

6. Lavagens

    a) PBS – 5 minutos

    b) PBS–Tritonx100 – 5minutos

    c) PBS– 5minutos

7. Bloqueio da Biotina Endógena

    a) 200 mL de PBS + 9 g de leite em pó (Molico ®) / 1 hora

8. Lavagem

    a) PBS – 5 minutos

9. Bloqueio dos sítios inespecíficos

    a) 200 mL de PBS – Triton x 100 + 3g de soro albumina bovina (BSA) / Overnight

2º Dia

1. Incubação do anticorpo primário

    a) 200µL de solução contendo anticorpo primário / 24 horas

3º Dia

1. Lavagens

    a) PBS – 5 minutos

    b) PBS–Tritonx100 – 5minutos

    c) PBS – 5minutos

2. Incubação do anticorpo secundário biotinilado

    a) 200µL solução contendo anticorpo secundário biotinilado / 1 hora

3. Lavagens

    a) PBS – 5 minutos

b) PBS–Tritonx100 – 5minutos

c) PBS – 5minutos

4. Incubação na Estreptavidina Conjugada

a) 200µL de estreptavidina conjugada / 1 hora

5. Lavagens

a) PBS – 5 minutos

b) PBS–Tritonx100 – 5minutos

c) PBS – 5minutos

6. Revelação com Cromógeno – DAB

a) 200µL de DAB substrato (2,5 mL) + 2 gotas de DAB cromógeno / 200µl por lâmina (tempo variável)

7. Lavagens

a) PBS – 5 minutos

b) Água destilada – 5 minutos

8. Contra-coloração: O corante muda dependendo domarcador. Para hematoxilina

a) Hematoxilina de Harris (tempo variável)

b) Água destilada – 1 minuto

c) Álcool 70% – 2 minutos

d) Álcool 95% – 2 minutos

e) Álcool 100% I– 2 minutos

f) Álcool 100% II – 2 minutos

g) Xilol – 2 minutos

h) Xilol I – 5 minutos

i) Xilol II – 5 minutos

j) Xilol III – 10 minutos

10. Deixar secar na estufa, montar com Permount e lamínula

Anexo C – Guidelines for Publishing Papers in the Journal of Endodontics

Writing an effective article is a challenging assignment. The following guidelines are provided to assist authors in submitting manuscripts.

The JOE publishes original and review articles related to the scientific and applied aspects of endodontics. Moreover, the JOE has a diverse readership that includes full-time clinicians, full-time academicians, residents, students and scientists. Effective communication with this diverse readership requires careful attention to writing style.

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. 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 developthe 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 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.
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. General Points on the Organization of Original Research Manuscripts

1. **Title Page:** The title should describe the major conclusion 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).

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. The purpose of the study, the tested hypothesis and its scope should be 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 provide sufficient detail. However, many successful manuscripts require no more than a few paragraphs to accomplish these goals.
4. **Material and Methods:** The objective of the methods section is to permit other investigators to repeat your experiments. The three components to this section are 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 particular aspects used in the present study. 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 should state that they either followed manufacturer's protocol or specify any changes made to the protocol. Studies on humans should conform to the Helsinki Declaration of 1975 and state that the institutional IRB approved the protocol and that informed consent was obtained. Studies involving animals should 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, drop-outs from clinical trials, etc.

5. **Results:** Only experimental results are appropriate in this section (i.e., neither methods nor conclusions should be in this section). Include only those data that are critical for the study. Do not include all available data without justification, any repetitive findings will be rejected from publication. All Figs./Charts/Tables should be described in their order of numbering with a brief description of the major findings.
6. **Figures:** There are two general types of figures. The first type of figure 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 on your part given the small size of each panel, you will only be able to illustrate the most important feature of each photomicrograph. 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 Chang, et al, (JOE 28:90, 2002), Hayashi, et al, (JOE 28:120, 2002) and by Davis, et al (JOE 28:464, 2002). At the Editor's discretion, color figures may be published at no cost to the authors. However, the Editor is limited by a yearly allowance and this offer does not include printing of reprints.

The second type of figure are graphs (i.e., line drawings) 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 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
0.001	5	0
0.003	5	0

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).

8. **Discussion:** The conclusion section should describe the major findings of the study. Both the strength and weaknesses of the observations should be discussed. What are the major conclusions of the study? How does the data support these conclusions? 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?
9. **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

10.References: The reference style follows Index Medicus and can be efficiently learned from reading past issues of the JOE. 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.