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DESENVOLVIMENTO DA PERIODONTITE APICAL EM RATOS SADIOS E COM DIABETES TIPO 2: EFEITO DO TRATAMENTO COM METFORMINA

DISSERTAÇÃO DE MESTRADO

Camilla dos Santos Tibúrcio Machado

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Camilla dos Santos Tibúrcio Machado

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Odontológicas, Área de Concentração em Odontologia, Ênfase em Endodontia, da Universidade Federal de Santa Maria (UFSM - RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Odontológicas**

Orientador: Prof. Dr. Carlos Alexandre Souza Bier

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DESENVOLVIMENTO DA PERIODONTITE APICAL EM RATOS SADIOS E COM DIABETES TIPO 2: EFEITO DO TRATAMENTO COM METFORMINA

elaborada por
Camilla dos Santos Tibúrcio Machado

como requisito parcial para obtenção do grau de
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Santa Maria, 25 de agosto de 2014.

DEDICATÓRIA

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Odontológicas
Universidade Federal de Santa Maria

DESENVOLVIMENTO DA PERIODONTITE APICAL EM RATOS SADIOS E COM DIABETES TIPO 2: EFEITO DO TRATAMENTO COM METFORMINA

AUTORA: CAMILLA DOS SANTOS TIBÚRCIO MACHADO
ORIENTADOR: CARLOS ALEXANDRE SOUZA BIER
Data e Local da Defesa: Santa Maria, 25 de agosto de 2014.

O diabetes é reconhecido por ser uma desordem metabólica, pois seu estabelecimento promove uma série de alterações no funcionamento do organismo como um todo, repercutindo inclusive, na cavidade oral. Uma das principais causas destas alterações é a quantidade excessiva de espécies reativas de oxigênio (ROS) que está presente no organismo do paciente com diabetes, levando a um aumento do estresse oxidativo. A metformina, o anti-hiperglicemiante mais utilizado para o tratamento do diabetes tipo 2, também possui a propriedade sequestradora de ROS. Adicionalmente, estudos recentes têm mostrado um maior reparo ósseo associado ao tratamento com o fármaco. No entanto, não existem dados disponíveis na literatura informando se a metformina é capaz de alterar o desenvolvimento das periodontites apicais (PA) em ratos com diabetes tipo 2. Portanto, o objetivo principal deste estudo foi determinar se a metformina tem a capacidade de prevenir ou interferir no desenvolvimento das PA em ratos sadios e com diabetes tipo 2. Além disso, parâmetros bioquímicos relacionados com desordem metabólica foram avaliados a fim de complementar o estudo farmacológico. Para tal, quarenta e oito ratos *Wistar* foram utilizados. O modelo de diabetes tipo 2 foi alcançado submetendo vinte quatro animais à ingestão de uma dieta especial [*High Fat, Low Protein, Moderate Carbohydrate* (HFLPMC)] por 70 dias. Os animais sadios receberam uma dieta padrão [standard diet (SD)] durante todo o período experimental. Dentro de cada dieta, os ratos foram divididos conforme os protocolos de tratamento medicamentoso a seguir: i) solução salina (diariamente/quatro semanas); ii) metformina (500mg/kg, diariamente/duas semanas); iii) metformina (500mg/kg, diariamente/quatro semanas). A abertura da câmara pulpar para propiciar o desenvolvimento das PA foi realizada na sexta semana do experimento e uma semana antes da eutanásia os animais foram submetidos ao teste oral de tolerância à glicose. Após dez semanas do início do experimento, os animais foram eutanasiados e as seguintes estruturas foram coletadas e pesadas: fígado, tecido adiposo abdominal e marrom. A mandíbula foi removida e dissecada e radiografias periapicais foram obtidas para mensurar o tamanho da PA. Adicionalmente, amostras de sangue foram retiradas para posterior determinação do colesterol total e HDL e insulina plasmática. Os níveis de catalase e glutationa reduzida (GSH) foram mensurados a fim de determinar o estresse oxidativo no fígado. Os níveis de glicose sanguínea após 30 minutos de administração de sacarose foram maiores no grupo HFLPMC+Salina que no grupo SD+Salina, porém a metformina não teve efeito sobre este parâmetro. Os animais submetidos à ingestão da dieta HFLPMC e salina apresentaram concentrações superiores de insulina plasmática e níveis significativamente inferiores de catalase (p = 0,0267) que os animais do grupo SD+Salina. Um comportamento similar à catalase foi observado nos níveis de GSH e a metformina aumentou a concentração de ambos nos animais alimentados com a dieta HFLPMC. O tratamento com metformina não afetou o tamanho da PA. Portanto, com base nos nossos resultados, sugere-se que a droga não tem efeito positivo sobre o metabolismo ósseo.

Palavras-chave: Endodontia. Lesão periapical. Hipoglicemiantes.

ABSTRACT

Master Thesis
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APICAL PERIODONTITIS DEVELOPMENT IN HEALTHY AND TYPE-2 DIABETES RATS: EFFECT OF METFORMIN TREATMENT

AUTHOR: CAMILLA DOS SANTOS TIBÚRCIO MACHADO
ADVISOR: CARLOS ALEXANDRE SOUZA BIER
Place and Date of Defense: Santa Maria, August 25, 2014.

Diabetes is classified as a metabolic disorder, since its establishment promotes several systemic and oral changes. One of the causes of these alterations is the excess of reactive oxygen species (ROS) presents in the diabetic patient tissues, leading to oxidative stress increase. Metformin, the anti-hyperglycemic more used in the type-2 diabetics treatment, is a ROS scavenger. Furthermore, recent studies have shown a bone repair increase attributed to the drug. However, there are not reports searching the relationship between metformin and apical bone repair in the type-2 diabetes individuals. Therefore, the aim of this study was evaluate the treatment effect with metformin on the development of apical periodontitis (AP) in healthy and type-2 diabetes rats. Addicionally, biochemical parameters related to metabolic disorder were evaluated in order to complement the pharmacological study. For this purpose, forty eight *Wistar* rats were fed either with a standard diet (SD) or high fat, low protein, moderate carbohydrate diet (HFLPMC). Rats in each diet were divided into three subgroups: i) saline (daily/four wk); ii) metformin (500mg/kg, daily/two wk); iii) metformin (500mg/kg, daily/four wk). At the sixth experimental week, the pulp chambers of first mandibular molars were exposed in order to permit the AP development. Glucose tolerance test was performed in the week before euthanasia. At the tenth experimental week, the animals were euthanized and it was collected the fallowing structures: liver, abdominal adipose tissue, interscapular brown adipose tissue and mandible. Also, it was collected blood to determine levels of plasma insulin, total and HDL cholesterol. Catalase and reduced glutathione (GSH) were measured and the size of apical periodontitis was estimated from radiographs of the mandible. After 30 minutes of sucrose administration, the rats fed with HFLPMC diet presented greater blood glucose levels than the ones fed with SD. However, the metformin administration did not affect this concentration. Moreover, plasma insulin levels were higher in the HFLPMC+Saline group than in the SD+Saline group. Catalase levels in the HFLPMC+Saline group were significantly lower than SD+Saline group (p = 0,0267) and metformin increased these levels in the animals fed with HFLPMC diet. It was observed a similar behavior with GSH levels. In the radiographic analysis, treatment with metformin did not affect the AP size. Therefore, based on the results, we suggest that metformin has not a positive effect over the bone metabolism.

Keywords: Endodontics. Periapical lesion. Hypoglycemics.

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LISTA DE ABREVIATURAS E SIGLAS

HFLPMC	*High Fat, Low Protein, Moderate Carbohydrate* (Alto Conteúdo de Gordura, Baixo Conteúdo de Proteína e Moderada Concentração de Carboidrato)
SD	*Standard Diet* (Dieta Padrão)
PA	Periodontite(s) Apical(ais)
AP	*Apical Periodontitis*
ROS	*Reactive Oxygen Species* (Espécies Reativas de Oxigênio)
AGEs	*Advanced Glycation End Products* (Produtos Finais de Glicação Avançada)
HDL	*High-density Lipoprotein* (Lipoproteína de Alta Densidade)
GSH	*Reduced Glutathione* (Glutationa Reduzida)
TRX	*Thioredoxin Reductase* (Tiorredoxina Redutase)
SOD	*Superoxide Dismutase* (Superóxido Dismutase)
RANKL/OPG	*Receptor Activator of Nuclear Factor Kappa-β Ligand and Osteoprotegerin Ratio* (Relação entre o Ligante do Receptor do Fator Nuclear Kapa β e Osteoprotegerina)
T-RFLP	*Terminal Restriction Fragment Lenght Polymorphism* (Técnica de Análise do Polimorfismo dos Fragmentos Terminais de Restrição)
wk	*Weeks* (Semanas)
micro-CT	*Micro Computed Tomography* (Micro Tomografia Computadorizada)
% BW	*Percentage of Body Weight* (Porcentagem de Peso Corporal)
b.w	*Body Weight* (Peso Corporal)

LISTA DE SÍMBOLOS

s	*Second(s)* (Segundo(s))
µl	*Microliter* (Microlitro(s))
µM	*Micromolar* (Micromolar)
g/kg	*Gram(s) per kilogram* (Grama(s) por Quilograma)
mg/kg	*Milligram(s) per kilogram* (Miligrama(s) por Quilograma)
ng/ml	*Nanogram(s) per Milliliter* (Nanograma(s) por Mililitro)
mg/dl	*Milligram(s) per deciliter* (Miligrama(s) por Decilitro)
mmol/min/mg	*Milimole per minute per miligrama* (Milimol por Minuto por Miligrama)
σ	*Standard Deviation* (Desvio Padrão)
$\bar{M}$	*Mean* (Média)

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INTRODUÇÃO

Na sociedade atual, homens e mulheres trabalham em ritmo acelerado, vivem sob constante sobrecarga emocional, alimentam-se de forma inadequada e não possuem tempo para a prática de atividade física. Como consequência deste estilo de vida, sobrepeso e doenças sistêmicas são características cada vez mais frequentes na ficha clínica dos pacientes que procuram atendimento odontológico.

Neste cenário, uma doença que merece atenção especial é o diabetes, pois sua alta prevalência e o aumento da sua incidência a tornaram um verdadeiro problema de saúde pública. De acordo com dados publicados pela Organização Mundial da Saúde, cerca de 347 milhões de pessoas em todo mundo têm diabetes (WORLD HEALTH ORGANIZATION, 2013). No Brasil, baseado nos números do Censo-IBGE de 2010, estima-se que 12 milhões de pessoas sejam portadoras da doença (SOCIEDADE BRASILEIRA DE DIABETES, 2012).

Uma característica comum aos indivíduos diabéticos é o aumento da concentração da glicose sanguínea decorrente da deficiência na secreção ou na ação da insulina (AMERICAN DIABETES ASSOCIATION, 2005; KIDAMBI e PATEL, 2008). A insulina é um hormônio sintetizado nas células β que compõem as ilhotas de Langerhans do pâncreas e possui um papel fundamental para o metabolismo dos carboidratos, além de agir diretamente no metabolismo de gorduras e proteínas (CONSTANZO, 2007; GUYTON, 2012).

As duas formas mais predominantes da doença são o diabetes tipo 1 e o tipo 2. Quando o indivíduo apresenta anticorpos específicos, ocorre a destruição autoimune das células β do pâncreas e o paciente desenvolve o diabetes tipo 1. A presença destes anticorpos está relacionada à herança genética e especula-se a sua relação com fatores ambientais (AMERICAN DIABETES ASSOCIATION, 2005). Como as células produtoras de insulina são destruídas, o indivíduo necessita da administração exógena do hormônio para sobreviver. Este tipo da doença é responsável por 5 a 10% dos casos de diabetes e acomete principalmente crianças e adolescentes (AMERICAN DIABETES ASSOCIATION, 2005; MILEY e TEREZHALMY, 2005).

Ao contrário do que ocorre no diabetes tipo 1, o desenvolvimento do diabetes tipo 2 não se dá em função da destruição das células β , mas sim, devido a uma resistência dos tecidos ao hormônio insulina, que pode também se apresentar de forma relativamente deficiente (AMERICAN DIABETES ASSOCIATION, 2005; KIDAMBI e PATEL, 2008). Esta é a

forma mais comum da doença, compreendendo entre 90 e 95% de todos os casos e está intimamente ligada à predisposição genética e a determinados fatores de risco, tais como sobrepeso, excesso de gordura abdominal, idade avançada e sedentarismo (AMERICAN DIABETES ASSOCIATION, 2005).

Várias complicações orais e sistêmicas estão envolvidas com a presença do diabetes o que o leva a ser classificado como uma desordem metabólica (KIDAMBI e PATEL, 2008). Dentre as manifestações bucais de maior prevalência estão doença periodontal (LÖE, 1993; MILEY e TEREZHALMY, 2005), xerostomia, cárie dental e candidíase (MILEY e TEREZHALMY, 2005). Além disso, alguns estudos têm buscado correlacionar o aumento na prevalência de lesões periapicais de origem endodôntica com a doença (LOPEZ-LOPEZ et al., 2011). Com relação às manifestações sistêmicas, o indivíduo diabético pode apresentar desde problemas na cicatrização de feridas até um aumento do risco de infarto do miocárdio. Tais complicações têm sido relacionadas à formação de produtos finais de glicação avançada (AGEs, do inglês: *advanced glycation end products*) (FENG et al., 2005). A falta de insulina aumenta a formação dos AGEs através da quebra dos lipídeos e das proteínas. Os AGEs, em maiores quantidades nos tecidos dos pacientes diabéticos, aumentam a expressão de citocinas pró-inflamatórias produzindo inflamação vascular crônica e auxiliando na formação dos ateromas (FENG et al., 2005). Estas alterações teciduais produzidas pelo acúmulo de AGEs favorecem, entre outros, o desenvolvimento de infarto do miocárdio, acidente vascular cerebral e doença vascular periférica (KIDAMBI e PATEL, 2008). A formação de AGEs também tem uma contribuição importante no processo de cicatrização das feridas, pois a sua interação com o seu receptor promove a liberação de moléculas pró-inflamatórias que impedem o fechamento da lesão (GOOVA et al., 2001).

Além disso, vários trabalhos têm evidenciado a relação do diabetes com o aumento do estresse oxidativo, sendo este um dos responsáveis pelas complicações advindas da desordem. O aumento do estresse oxidativo pode ser devido à redução das defesas antioxidantes do organismo ou à elevação na produção de radicais livres (SINGH e JIALAL, 2008), também conhecidos por espécies reativas de oxigênio (ROS, do inglês: *reactive oxygen species*). O desequilíbrio entre a quantidade de antioxidantes intracelulares e de ROS faz com que os ROS, em excesso, ataquem e danifiquem proteínas, lipídios e ácidos nucleicos (VINCENT et al., 2004).

Hipóteses têm sido levantadas envolvendo mecanismos moleculares pelos quais a hiperglicemia provoca complicações no organismo como um todo. Entre elas estão o aumento do fluxo da via do poliol, aumento da formação de AGEs, ativação da proteína quinase C e

fluxo aumentado através da via da hexosamina (BROWNLEE, 2001). Todos estes eventos têm em comum a formação de ROS de forma excessiva (BROWNLEE, 2001; DU et al., 2000; NISHIKAWA et al., 2000; VINCENT et al., 2004). Outro local de formação de ROS é na mitocôndria, onde sua produção é dada de forma normal como subproduto dos processos metabólicos (VINCENT et al., 2004).

Para combater os ROS que são formadas fisiologicamente ou patologicamente no organismo, o sistema imunológico lança mão de antioxidantes endógenos, que são moléculas conhecidas como *scavengers* (sequestradores). Entre elas estão a glutationa (GSH), a tiorredoxina redutase (TRX) e enzimas antioxidantes como superóxido dismutase (SOD), catalase entre outras. Fontes suplementares de antioxidantes estão disponíveis na dieta e são representados pela vitamina C e E (VINCENT et al., 2004).

Vários estudos têm utilizado substâncias com potencial antioxidante, entre elas ácido lipólico (SINGH e JIALAL, 2008; VINCENT et al., 2004), coenzima Q10, flavonoides, zinco (VINCENT et al., 2004) e tempol (BRILHANTE WOLLE et al., 2012; RAFIKOVA, SALAH e TOFOVIC, 2008; RITCHIE et al., 2007; WOLLE et al., 2013; ZHANG et al., 2003). Os antioxidantes endógenos normalmente não são utilizados em estudos, pois possuem alto peso molecular e tem reduzida disponibilidade após filtração glomerular, conferindo-lhes importante limitação para sua administração (RAFIKOVA, SALAH e TOFOVIC, 2008).

Outra droga que tem demonstrado efeito inibidor sobre ROS é a metformina (ZHEN, CHEN e TANG, 2010), o agente anti-hiperglicemiante mais usado para o tratamento de diabetes tipo 2 (ALEXANDER et al., 2008). Adicionalmente, pesquisas na área médica e odontológica têm encontrado novas funções terapêuticas para a droga, atribuindo o seu uso a um maior reparo ósseo (LIU et al., 2012; MAI et al., 2011).

Zhen, Chen e Tang (2010) avaliaram, num estudo *in vitro*, a ação da droga no crescimento e diferenciação de culturas de osteoblastos. Os autores observaram maior proliferação celular e, em três semanas, houve um aumento marcante na formação de nódulos de mineralização nas culturas tratadas com o medicamento. Além disso, foi observado um aumento na capacidade de diferenciação celular e uma redução significativa de ROS intracelulares.

Pradeep et al. (2013) avaliaram, através de um ensaio clínico randomizado, o efeito da metformina em gel aplicada localmente e associada à raspagem e alisamento radicular em defeitos ósseos de paciente com doença periodontal crônica. Após 3 e 6 meses de acompanhamento, os grupos tratados com gel de metformina e raspagem radicular, em ambas concentrações, tiveram significativa diminuição na profundidade de sondagem e ganho no

nível de inserção clínica quando comparados ao grupo que recebeu um gel placebo e raspagem e alisamento radicular.

Liu et al. (2012) avaliaram o efeito da administração intramuscular de metformina em ratos saudáveis com lesão periapical induzida. Após 28 dias de tratamento medicamentoso, os tamanhos das lesões do grupo experimental foram significativamente menores que as do grupo controle. Também houve redução significativa na razão RANKL/OPG nos animais que tiverem a administração do medicamento, indicando menor reabsorção óssea. Além disso, os autores sugerem que o efeito anti-inflamatório da droga também pode ter contribuído para o bom resultado do estudo.

Embora sejam crescentes os números de trabalhos publicados em relação ao papel da metformina sobre o metabolismo ósseo, ainda há um longo caminho a ser percorrido a fim de se obter conclusões concretas. Na área da odontologia, especialmente da endodontia, os trabalhos utilizando o medicamento com tais finalidades são bastante escassos. Adicionalmente, até o presente momento, não há nenhuma informação disponível na literatura científica a respeito do resultado da terapia sistêmica com a metformina sobre o desenvolvimento das lesões periapicais em indivíduos com diabetes tipo 2. Baseado nestas observações, o objetivo principal do presente estudo foi avaliar através de análise radiográfica em ratos sadios e com diabetes tipo 2, se a metformina tem a capacidade de prevenir ou interferir no desenvolvimento das periodontites apicais. Além disso, parâmetros bioquímicos relacionados com desordem metabólica foram avaliados a fim de complementar o estudo farmacológico. Com o desenvolvimento deste trabalho buscou-se compreeender o processo de desenvolvimento da periodontite apical num indivíduo portador da desordem e que esteja sob tratamento com metformina antes da infecção endodôntica se instalar, bem como, naqueles indivíduos que iniciaram o tratamento para o diabetes concomintantemente à invasão bacteriana ao sistema de canais radiculares.

ARTIGO - APICAL PERIODONTITIS DEVELOPMENT IN TYPE-2 DIABETES RATS: EFFECT OF METFORMIN TREATMENT

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Title Page

Title: Apical periodontitis development in type-2 diabetes rats: effect of metformin treatment.

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

Abstract

Objectives: To evaluate the effects of treatment with metformin on the development of apical periodontitis in healthy and type-2 diabetes rats. Our hypothesis is that metformin may prevent the apical periodontitis progression.

Methods: Forty eight male *Wistar* rats were fed with a standard diet (SD) or high fat, low protein, moderate carbohydrate diet (HFLPMC). Rats in each diet were divided into three subgroups: i) saline (daily/four weeks); ii) metformin (500mg/kg, daily/two weeks); iii) metformin (500mg/kg, daily/four weeks). At the sixth experimental week, the pulp chambers of first mandibular molars were exposed in order to permit the apical periodontitis development. Glucose tolerance test was performed in the week before euthanasia. At the tenth experimental week, the animals were euthanized and it was collected the liver, abdominal and interscapular brown adipose tissue and mandible. Also, blood was collected to determine levels of plasma insulin, total and HDL cholesterol. Catalase and reduced glutathione (GSH) were measured and the size of apical periodontitis was estimated from radiographs of the mandible.

Results: After 30 min of sucrose administration, the rats fed with HFLPMC diet had greater blood glucose levels than the rats fed with SD. However, the metformin administration did not affect this parameter. Moreover, plasma insulin levels were higher in the HFLPMC+Saline group than in the SD+Saline group. Catalase levels in the HFLPMC+saline group were significantly lower than SD+Saline group ($p = 0.0267$) and metformin increased these levels in the animals fed with HFLPMC diet. A similar profile was observed with GSH levels. In the radiographic analysis, the treatment with metformin did not affect the apical periodontitis size.

Conclusions: The data demonstrated that metformin did not alter the apical periodontitis size by radiographic measurement.

Keywords: Periapical Periodontitis; Hypoglycemics; Diabetic; High-fat diet

Introduction

Modern lifestyle, which people are submitted nowadays generate several health problems, such as hypertension, heart disease and diabetes. Consequently, presence of systemic diseases is an increasingly common feature in the clinical record of patients that are seeking for dental care.

Diabetes is a disease that should be highlighted since its presence involves systemic and oral alterations (1, 2). One of the reasons to the onset of these disorders is the increase of the oxidative stress caused by excessive production of reactive oxygen species (3). Several drugs have ability to eliminate the excessive ROS in the tissues and they are widely used in the scientific studies in the present days (4-6). One of them is the metformin which is recognized to its anti-hyperglycemic effect. Additionally, studies have being shown new therapeutic functions to the drug, attributing its use to a larger bone repair (7, 8).

Since that World Health Organization estimates that in 2030 the diabetes will be the seventh death cause in the world (9), it is very important that research about this disease continue being realized. Additionally, the lack of information on new therapeutic functions of metformin in the odontological setting gives new opportunities for investigations in this area. Recent results showed that the metformin administration decreased the apical periodontitis size in healthy rats compared to rats treated with saline (7).

Therefore, the aim of this study is to evaluate by radiographic analysis in a rat model of type-2 diabetes and healthy rats if metformin has ability to prevent the apical periodontitis development, as well as interfere in this process. Hypothesis defined for this study is that the metformin administration will hamper the apical periodontitis progression.

Materials and Methods

Animals

Experimental procedures were performed accordingly with Federal Brazilian Law number 11.794, which determines the rules about the animal handling in scientific studies. The study was approved by the local animal ethics committee (CEUA – protocol number 13/00360) of Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-PUCRS, Brazil). The animals used in the experimental were forty eight male *Wistar* rats with about 30

days old and they were housed under controlled conditions (temperature: 22 ± 1 °C, 70% humidity, and a 12h/12h light-dark cycle). They had free access to food and filtered water.

Diets and Induction of type-2 diabetes

The animals initially were divided into two groups, according to the diet: standard diet (SD) and high fat, low protein, moderate carbohydrate diet (HFLPMC). They received these diets during all experimental period of seventy days. The prepared chow was based on the protocol used in a previous study (10). The diets nutritional composition is described in Table 1. In order to induce a model of type-2 diabetes, we fed twenty four rats with HFLPMC diet during seventy days and additionally we added 20% sucrose in the drink water during the first thirty days.

Sample distribution and Protocol of treatment with metformin

The animals were divided into six groups: SD Saline, SD+Metformin 2 weeks, SD+Metformin 4 weeks; HFLPMC+Saline, HFLPMC+Metformin 2 weeks and HFLPMC+Metformin 4 weeks.

The treatment with metformin (Abhilash Chemicals, Tamil Nadu, India) was initiated at two different moments: the SD+Metformin 2 weeks and HFLPMC+Metformin 2 weeks groups received the drug from the eighth to tenth week, while the SD+Metformin 4 weeks and HFLPMC+Metformin 4 weeks groups received metformin from the sixth to the tenth week, since the day in which apical periodontitis was induced. The drug concentration (500 mg/kg) was determined according to previous studies (11, 12) and the route of administration was gavage. The groups SD+Saline and HFLPMC+Saline received 20 ml/kg of saline solution orally.

Apical periodontitis induction

The protocol to develop the apical periodontitis was performed as previously described (5, 6), with minor adaptations. Briefly, the rats were anesthetized by i.p. injection of an association of xylazine (10 mg/kg) and ketamine (100 mg/kg). A metal device was confectioned (13) aiming to permit an optimal opening mouth for a free access to the right mandibular first molar. The teeth opening was performed at the sixth experimental week with

a round bur long shank number 1011 (KG Sorensen, São Paulo, SP, Brazil) using high-speed rotation, under constant irrigation. Pulp chambers were left exposed to the oral cavity for four weeks, in order to permit apical periodontitis development.

Glucose tolerance test

One week before the euthanasia, the glycaemia was taken in four moments: after 16 h overnight food deprivation and 30 min, 60 min and 120 min after via oral administration of sucrose solution (2g/kg b.w.). The glucose was measured by using a glucosimeter (Onetouch® Select Simple™, Johnson & Johnson Medical Devices & Diagnostics Group - Latin America, L.L.C.) with the blood sample collected through a small puncture on the tip of the tail.

Euthanasia and Biochemical Analyzes

In the seventieth day of the experiment, the animals were euthanized by deep anesthesia with isofluorane. The blood was rapidly acquired by heart puncture with vacutainer disposal (BD Vacutainer® Eclipse™ Blood Collection Needle) and it was centrifuged to separate the plasma and serum, which were maintained refrigerated for later measurements of plasma insulin, total and HDL cholesterol.

Abdominal adipose tissue, interscapular brown adipose tissue and liver were collected and weighted. Liver samples were frozen for posterior determination of oxidative stress by catalase and reduced glutathione (GSH) levels, according to methodology described previously (14). The insulin levels were measured by radioimmunoassay (Immunotech - Beckman Coulter Company, Marseille, France) using 100 µl of plasma (15). Total and HDL cholesterol were determined spectrophotometrically by enzymatic colorimetric tests (Bioclin, Belo Horizonte, MG, Brazil) according to the recommendations of the manufacturer.

Radiographic analysis

After euthanasia, the right side of mandibles were removed and dissected. Digital periapical radiographs were conducted in a standardized manner using a Soredex digital sensor (Tuusula, Finland) and a x-ray unit Gnattus, Ribeirão Preto, SP, Brazil), with an

exposure time of 0.2 s. The x-ray beam was perpendicularly positioned to 30 cm from the mandible-sensor set.

The apical periodontitis sizes (in pixels) were determined by an endodontist blinded to the experimental groups, using the software Adobe Photoshop CS6 (Adobe Systems Incorporated®, SanJose, CA) (5, 6).

Statistical analysis

The sample size was established in 8 animals per group based on previous literature studies (5-7, 16). The normality test used was Shapiro-Wilk test and data were analyzed by one-way ANOVA and *post hoc* Bonferroni test and Kruskal-Wallis test with Dunn post hoc to the catalase levels. Results are expressed as the mean ± the standard error of the mean (SEM) and α was established in 0.05 to determine the statistical significance level ($p < 0.05$).

Results

Glucose tolerance test

After thirty minutes of sucrose administration, the HFLPMC+Saline group revealed an increase in glucose concentration greater than the SD+Saline group, although this effect was not statistically significant (confidence intervals overlap), as shown in Table 2. The metformin treatment did not reduce the glucose concentration at the time of thirty minutes.

Plasma insulin, total cholesterol, HDL cholesterol, abdominal adipose tissue, interscapular brown adipose tissue and liver weight

The data related to the levels of plasma insulin, total cholesterol, HDL cholesterol, abdominal adipose tissue, interscapular brown adipose tissue and liver weight are shown in Table 3.

There was not statistical difference in the plasma insulin levels ($p = 0.6284$) between the groups. However, the HFLPMC+Saline group presented a higher concentration at these levels than SD+Saline group.

Concentration of total and HDL cholesterol were similar between the groups. Additionally, there was not difference between abdominal adipose tissue and interscapular

brown adipose tissue percentage. On the other hand, metformin treatment increased the percentage of liver weight compared to the saline groups.

Catalase and GSH levels

In order to determine oxidative stress, catalase and GSH levels were obtained. As it can be observed in the Figure 1A, there was significant reduction in the catalase levels in the HFLPMC+Saline compared to SD+Saline group (p = 0.0267). Furthermore, the metformin treatment in the animals fed with HFLPMC diet increased the liver catalase levels. Nonetheless, this was not noticed for the animals fed with standard diet.

Similarly, the GSH levels were lower in the HFLPMC+Saline compared to SD+Saline group, although it does not have statistical significance. Also, the metformin treatment increased the GHS levels in the HFLPMC+Metformin 2 weeks and HFLPMC+Metformin 4 weeks groups (Figure 1B).

Radiographic analysis

Aiming to reduce possible bias, each radiographic image was analyzed ten times for the same examiner blinded to the experimental groups. Afterwards, data were tabbed and mean ($\bar{M}$) and standard deviation (σ) were obtained. Data have normal distribution. We have observed that the results obtained from the first measure were in its majority out of the interval in between $\bar{M} - 2\sigma$ and $\bar{M} + 2\sigma$. Thus, the first day data were excluded and a new approach was done, where the statistics were computed by using this new set of data, i.e., the nine times data remaining. Again, the interval in between $\bar{M} - 2\sigma$ and $\bar{M} + 2\sigma$ was evaluated and the values out of this range were excluded. After the two rounds of data evaluation, he bias was significantly minimized.

There was not significant statistical difference in the apical periodontitis size between animals treated with saline and metformin. Furthermore, the HFLPMC diet did not have influence in the process (Figure 2).

Discussion

Our findings show that the apical periodontitis size was statistically similar among the six groups. Therefore, they suggest that the metformin does not prevent neither interfere in the apical periodontitis progression. Metformin is the anti-hyperglycemic agent most commonly used to treat the type-2 diabetes (17) and it controls the glycemic levels by the gluconeogenesis inhibition and insulin sensitivity increase (18). In contrast to our results, other previous study showed that healthy rats treated with intramuscular injections of metformin during twenty eight days obtained a decrease in the apical periodontitis size compared to the saline group (7). However, divergences between our data and those of Liu et al. 2012, may be due to methodological particularities, such as metformin administration route and method of lesion size measurement were different.

Although most of researches indicate beneficial effects of metformin on bone metabolism (19-21), a recent report has demonstrated that metformin did not improve osteoporosis. Accordingly, bone trabecular and cortical parameters measured by micro-CT in tibia of ovariectomised mice was not improved by treatment with metformin (22). Likewise, the treatment with 100 μM metformin did not affect the bone formation and bone reabsorption in osteoblast cell culture from rats calvarial bone and osteoclasts cells culture derived from the bone marrow of juvenile mice (23).

Our study employed a type-2 diabetes model induced by HFLPMC diet intake. Impaired glucose tolerance and higher insulin levels were noticed in the rats fed with this diet, although the difference was not significant between this group and the one that was fed with standard chow. Moreover, remarkable reduction in the catalase and GSH levels promoted by HFLPMC diet intake confirms the production of oxidative stress in the liver samples. Similar results were obtained in other reports which reproduced the type-2 diabetes model in rats by glucose solution intake (6, 15). In an earlier study which used the same diet of ours, the data obtained for the group fed with this diet were similar, where it was reported a significant increase in the oxidative stress and in the levels of blood glucose during the glucose tolerance test (10). However, in our study, the difference in the blood glucose concentration was not statistically significant probably owing to the experimental period that was smaller than that adopted by Souza et al. (2007).

Other report performed in rats submitted to similar diet intake, during four months, did not find differences in the total and HDL cholesterol between animals fed with standard diet and high fat diet, similarly to our results (24). Notwithstanding, they showed that animals fed

with high fat diet had adipose tissue depots heavier than standard diet group. In contrast, our data did not show any difference, probably because the smaller experimental time. In opposition to what has been expected, metformin treatment did not affect these parameters.

Another change observed in the HFLPMC group was the liver weight. Animals submitted to high fat diet intake had a slight decrease in the liver weight and the metformin had positive effect on this point. The liver weight reduction might be accompanied for an increase of lipid concentration. These changes can be explained by the inferior protein intake in the HFLPMC group. Since there is a little input of amino acids in the organism, the lipoprotein synthesis in the liver is impaired and the fat acids transport to the peripheral tissues is harmed (25). As a result, there is an increase on the liver lipid content which make it lighter.

We also investigated the role of metformin on the plasma insulin levels and catalase and GSH content. The results show that metformin did not have the expected effect on the plasma insulin in the rats fed with standard diet, i.e., it increased the insulin levels. Nonetheless, among the rats submitted to HFLPMC diet intake, the treatment with metformin, during two weeks, caused a small decrease on the insulin levels, whereas these levels were slightly increased after four weeks of drug administration. Regarding oxidative stress indicators, metformin increased the catalase and GSH levels in the rats fed with HFLPMC diet, even though this was not statistically significant. Likewise, previous reports stated that 500 mg/kg/daily and 500 mg/kg/twice daily of metformin administration during eight and four weeks, respectively, improved significantly the tissue catalase and GSH concentrations in diabetic animals (11, 26). Our study did not achieve a level of statistical significance most likely due to posology and administration time adopted, which was smaller than the aforementioned.

Our data indicate a negative correlation between plasma insulin, and catalase and GSH levels, i.e, the higher the plasma insulin levels are, the lower are the catalase and GSH levels. These results are coherent with previous data, demonstrating that increase of plasma insulin levels was followed by an increase of the oxidative stress, in a model of metabolic syndrome induced by fructose administration in rats (27).

Some considerations can be taken into account regarding our experimental procedure. We believe that the experimental period could be longer in order to have statistical significance levels for all data considered in this study. Another important issue is the method used to obtain the apical periodontitis size. We used a radiographic method, which provides a two-dimensional image of a three-dimensional structure. Therefore, the information on the

apical periodontitis size is not complete since the apical periodontitis depth cannot be obtained.

We demonstrated in our study that metformin administration have not the expected effect on the apical periodontitis development. However, the evidence found in this work cannot provide definitive conclusions. Based on our experimental model, we suggest that more studies using similar protocols with longer treatment periods and micro-CT, e.g., such as measurement method of apical periodontitis should be conducted. Finally, our data, allied to previous literature evidence can provide useful information for delineating future clinical studies.

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TABLE 1. Nutritional composition of Standard Diet and HFLPMC Diet in %

Nutritional Composition	Standard Diet[a]	HFLPMC Diet
Energy Value	331.5 kcal	389.19 kcal
Carbohydrate	56	60.25[b]
Protein	19	6.83[c]
Fat	3.5	13.43[d]
Cellulose	4.6	1.15[e]
Vitamins and Minerals	5	1.25[e]

[a] Standart Diet = Chow Nuvilab®
[b] HFLPMC ingredients considered as carbohydrate are corn starch, white sugar and part of condensed milk
[c] HFLPMC ingredients considered as protein are part of crushed chow and condensed milk
[d] HFLPMC ingredient considered as fat is soy oil
[e] HFLPMC ingredient considered as Cellulose and Vitamins and Mineral is crushed chow

TABLE 2. Glucose blood of rats fed with Standard Diet (Chow Nuvilab®) and HFLPMC Diet submitted to treatment with Metformin (500 mg/kg) during two and four weeks or vehicle (saline). Sample blood were collected after 16 fasting hours, 30, 60 and 120 minutes after sucrose solution (2 g/kg body weight) administration by gavage

	Fasting (IC 95%)	30 min (IC 95%)	60 min (IC 95%)	120 min (IC 95%)
SD+Saline	90.5 (87.1 – 93.9)	120 (115 – 125)	104 (91.8 – 116)	89.5 (83.2 – 95.8)
SD+Metformin 2 Weeks	82.1 (75.7 – 88.5)	122 (110 – 134)	101 (89.7 – 111)	86.1 (80.4 – 91.8)
SD+Meformin 4 Weeks	91.8 (82.8 – 101)	129 (108 – 150)	106 (98.1– 114)	91.9 (85.5– 98.3)
HFLPMC+Saline	94 (75.0 – 113)	136 (112 – 159)	107 (98.2 – 117)	89.4 (78.2 – 101)
HFLPMC+Metformin 2 Weeks	80.7 (71.5 – 89.9)	137 (125 – 149)	114 (108 – 119)	90.7 (79.6 – 102)
HFLPMC+Metformin 4 Weeks	95.3 (66.2 – 124)	140 (125 – 155)	130 (104 – 156)	98.7 (84.7 – 113)

TABLE 3. Data related of systemic parameters such as plasma insulin levels, total cholesterol, HDL cholesterol, liver weight, abdominal fat weight and brown fat weight (in % of body weight) in rats fed with Standard Diet (Chow Nuvilab®) and HFLPMC Diet submitted to treatment with Metformin (500mg/kg) during two and four weeks or vehicle (saline). Values are expressed as mean and standard error of the mean

	Standard Diet			HFLPMC Diet		
	Saline	Metfomin 2 weeks	Metfomin 4 weeks	Saline	Metfomin 2 weeks	Metfomin 4 weeks
Plasma Insulin (ng/ml)	0.27 ±0.12	0.37 ±0.20	0.44 ±0.22	0.38 ±0.21	0.34 ±0.06	0.41 ±0.32
Total Cholesterol (mg/dL)	63.48 ±14.27	67.06 ±13.03	67.88 ±14	62.87 ±8.14	66 ±7.46	69.79 ±10.29
HDL Cholesterol (mg/dL)	51.13 ±8	50.66 ±13.04	53.51 ±8.12	50.54 ±6.22	52.86 ±5.89	54.69 ±7.58
Liver Weight (% BW)	3.3 ±0.19[a]	3.56 ±0.22[b]	3.63 ±0.27[c]	3.14 ±0.22[b,c,d]	3.71 ±0.36[a,b,d]	3.48 ±0.1
Abdominal Adipose Tissue (% BW)	1.67±0.12	1.91 ±0.59	1.55 ±0.2	1.67 ±0.44	1.64 ±0.29	1.64 ±0.46
Interscapular Brown Adipose Tissue (% BW)	0.16 ±0.03	0.18 ±0.03	0.14 ±0.03	0.16 ±0.04	0.15 ±0.05	0.17 ±0.06

Same superscript letters in the same line represent statistical differences ($P<0.05$) using one-way ANOVA and Bonferroni *post hoc*

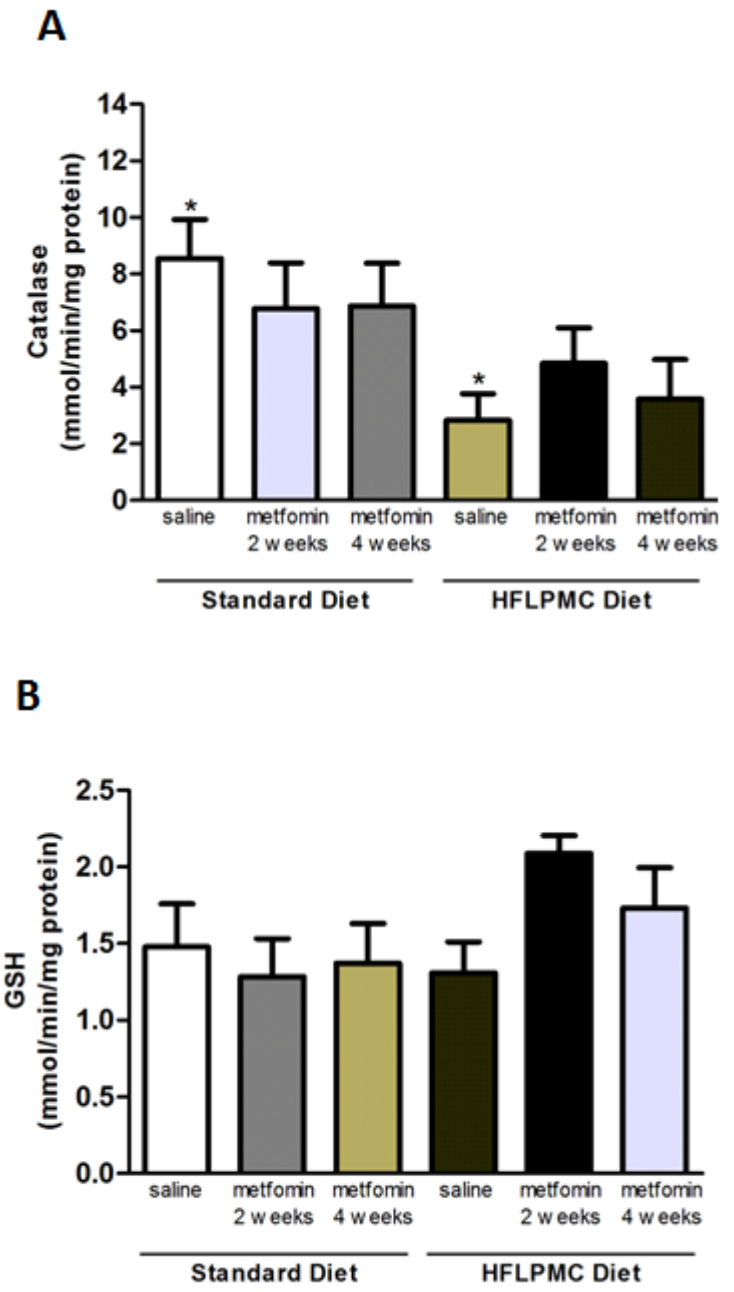

Figure 1 – Catalase (A) and GSH (B) levels in liver samples of rats fed with standard and HFLPMC diet during ten weeks. Columns represent the mean and vertical lines show the standard error of mean (n=8) (*p<0.05).

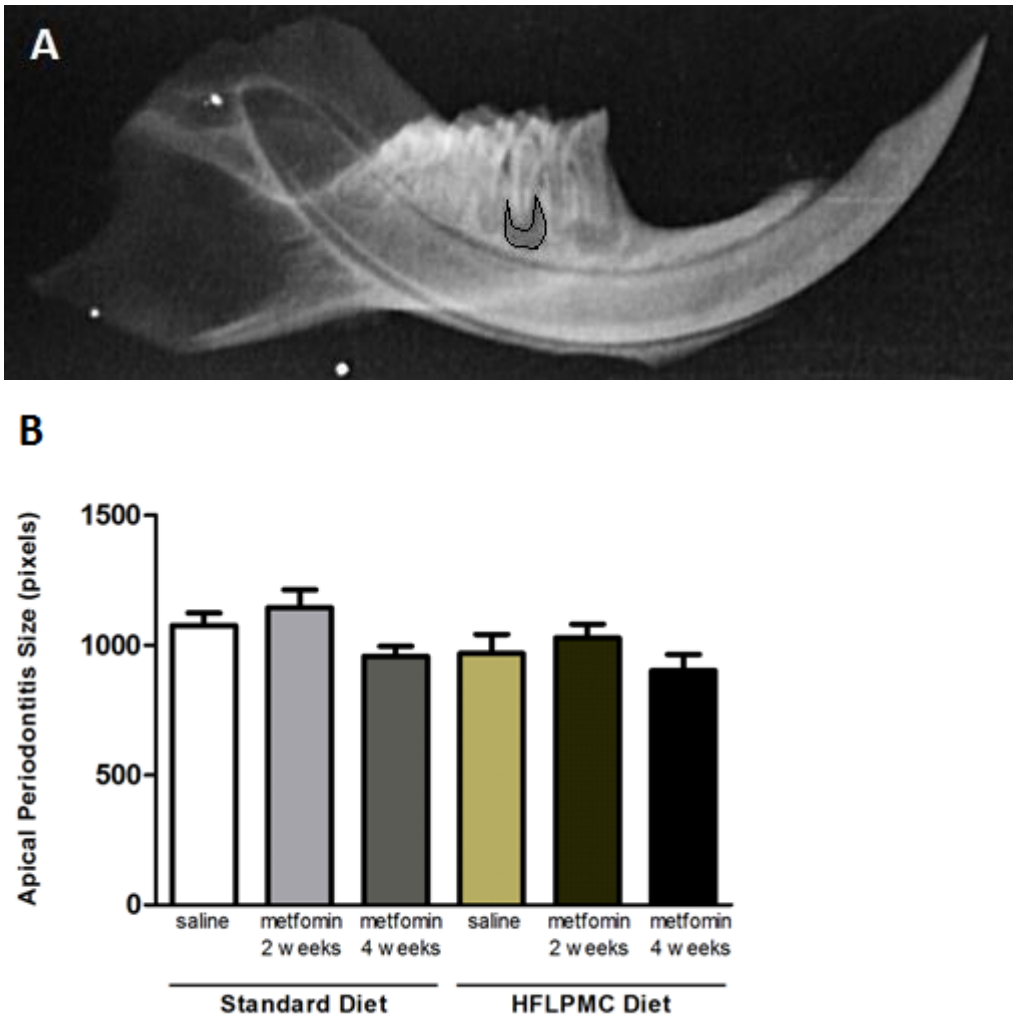

Figure 2 – (A) Periapical radiography of mandible sample. Circumscribed area represents the apical periodontitis extension. (B) Apical periodontitis size of rats fed with standard and HFLPMC diet during ten weeks. Columns represent the mean and vertical lines show the standard error of mean (n=8) (*$p<0.05$).

CONCLUSÃO

Os resultados obtidos no presente estudo sugerem que a metformina não tem efeito positivo sobre o metabolismo ósseo, uma vez que este medicamento não foi capaz de prevenir ou interferir no processo de reabsorção óssea periapical. No entanto, nenhuma conclusão definitiva deve ser obtida acerca do assunto tomando como base apenas o tamanho das lesões obtidas por radiografias periapicais. Portanto, a fim de fornecer mais dados que irão complementar os resultados deste estudo, análises adicionais estão sendo realizadas. Estas análises compreendem a histologia dos tecidos periapicais envolvidos no processo de reabsorção e a caracterização das comunidades microbianas presentes nos canais radiculares infectados, bem como na saliva e placa bacteriana através da técnica de T-RFLP (*Terminal Restriction Fragment Lenght Polymorphism*).

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Anexo A – Carta de aprovação da Comissão de Ética no Uso de Animais

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 112/13 - CEUA

Porto Alegre, 03 de dezembro de 2013.

Prezado Sr(a). Pesquisador(a),

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 13/00360, intitulado **"Diferenças entre a microbiota dos canais radiculares infectados de ratos normais diabéticos tipo 2: efeito do tratamento com metformina".**

Sua investigação, respeitando com detalhe as descrições contidas no projeto e formulários avaliados pela CEUA, está **autorizada** a partir da presente data.

Informamos que é necessário o encaminhamento de relatório final quando finalizar esta investigação. Adicionalmente, ressaltamos que conforme previsto na Lei no. 11.794, de 08 de outubro de 2008 (Lei Arouca), que regulamenta os procedimentos para o uso científico de animais, é função da CEUA zelar pelo cumprimento dos procedimentos informados, realizando inspeções periódicas nos locais de pesquisa.

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

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Anexo B – Guidelines for Publishing Papers in the *Journal of Endodontics*

Guidelines for Publishing Papers in the JOE

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

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

b. 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.

c. 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.

d. 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.

e. 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.)

f. 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.

g. 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."

h. 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.

i. Authors should be aware that the *JOE* uses iThenticate, plagiarism detection software, to assure originality and integrity of material published in the *Journal*. The use of copied sentences, even when present within quotation marks, is highly discouraged. Instead, the information of the original research should be expressed by new manuscript author's own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected, or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

2. **Organization of Original Research Manuscripts**

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

a. **Title Page:** The title should describe the major emphasis of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard—it represents your major opportunity to solicit readers to spend the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (*e.g.*, use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at *www.icmje.org*). The manuscript title, name and address (including email) of one author designated as the corresponding author. This author will be responsible for editing proofs and ordering reprints when applicable. The contribution of each author should also be highlighted in the cover letter.

b. **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.

c. **Introduction:** The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. The purpose of the study, the tested hypothesis and its scope

should be clearly described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the *JOE*. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to succinctly summarize the gap in knowledge that the study addresses. It is important to note that many successful manuscripts require no more than a few paragraphs to accomplish these goals. Therefore, authors should refrain from performing extensive review or the literature, and discussing the results of the study in this section.

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

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

f. **Figures:** There are two general types of figures. The first type of figures includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (*i.e.*, about 175 mm wide X 125 mm high when published in the *JOE*), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing to emphasize the most important feature of each photomicrograph, but it greatly increases the total number of illustrations that you can present in your paper. Remember that each panel must be clearly identified with a letter (*e.g.*, "A","B", etc.), in order for the reader to understand each

individual panel. Several nice examples of composite figures are seen in recent articles by Jeger et al (J Endod 2012;38:884–888); Olivieri et al., (J Endod 2012;38:1007 1011); Tsai et al (J Endod 2012;38:965–970). Please note that color figures may be published at no cost to the authors and authors are encouraged to use color to enhance the value of the illustration. Please note that a multipanel, composite figure only counts as one figure when considering the total number of figures in a manuscript (see section 3, below, for maximum number of allowable figures).

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

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

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

h. 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).

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

j. **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

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

3. **Manuscripts Category Classifications and Requirements**

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

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

B. Review Article-Manuscripts in this category are either narrative articles, or systematic reviews/meta-analyses. Case report/Clinical Technique articles even when followed by extensive review of the literature will should be categorized as "Case Report/Clinical Technique". These manuscripts have a limit of 3,500 words, [including abstract, introduction, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

C. Clinical Research (*e.g.*, prospective or retrospective studies on patients or patient records, or research on biopsies, excluding the use of human teeth for technique studies). These manuscripts have a limit of 3,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

D. Basic Research Biology (animal or culture studies on biological research on physiology, development, stem cell differentiation, inflammation or pathology). Manuscripts that have a primary focus on biology should be submitted in this category while manuscripts that have a primary focus on materials should be submitted in the Basic Research Technology category. For example, a study on cytotoxicity of a material should be submitted in the Basic Research Technology category, even if it was performed in animals with histological analyses. These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or 4 tables*.

E. Basic Research Technology (Manuscripts submitted in this category focus primarily on research related to techniques and materials used, or with potential clinical use, in endodontics). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 3 figures and tables *.

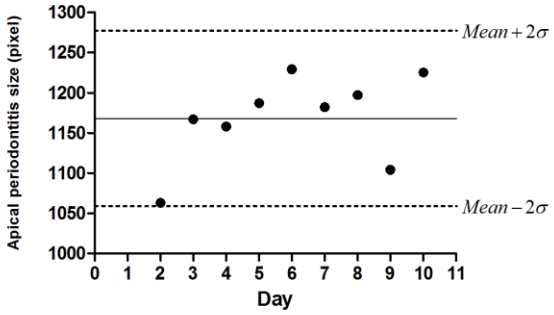
F. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and

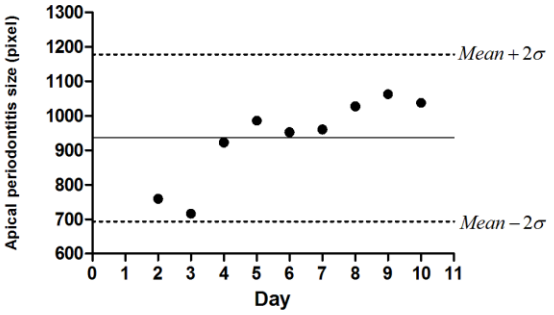
acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.

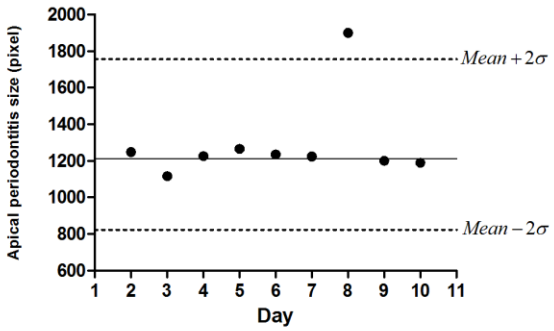
* Figures, if submitted as multipanel figures must not exceed 1 page length. Manuscripts submitted with more than the allowed number of figures or tables will require approval of the *JOE* Editor or associate editors. If you are not sure whether your manuscript falls within one of the categories above, or would like to request preapproval for submission of additional figures please contact the Editor by email at *jendodontics@uthscsa.edu*.
Importantly, adhering to the general writing methods described in these guidelines (and in the resources listed below) will help to reduce the size of the manuscript while maintaining its focus and significance. Authors are encouraged to focus on only the essential aspects of the study and to avoid inclusion of extraneous text and figures. The Editor may reject manuscripts that exceed these limitations.

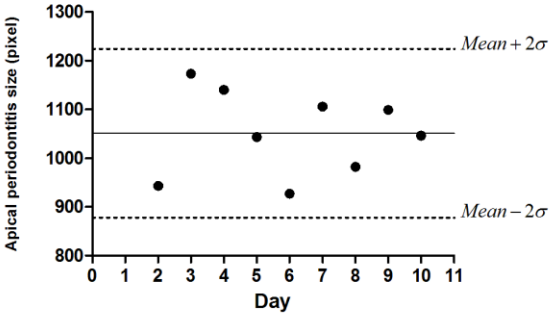
Available Resources: Strunk W, White EB. The Elements of Style. Allyn & Bacon, 4th ed, 2000, ISBN 020530902X. Day R. How to Write and Publish a Scientific Paper. Oryx Press, 5th ed. 1998. ISBN 1-57356-164-9. Woods G. English Grammar for Dummies. Hungry Minds: NY, 2001 (an entertaining review of grammar).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

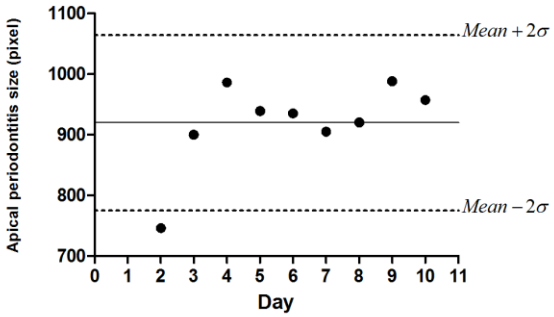
Apêndice A – Gráficos representativos das nove mensurações das lesões periapicais utilizadas para cada amostra. Valores fora do intervalo entre $\bar{M} - 2\sigma$ e $\bar{M} + 2\sigma$ não foram considerados no cálculo do tamanho das lesões periapicais.

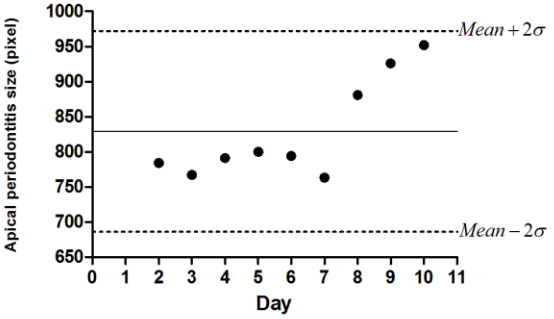












Apêndice B – Eixo temporal referente ao período experimental.