Universidade Estadual Paulista "Julio de Mesquita Filho" – Faculdade de Odontologia de Araçatuba

Programa de Pós Graduação em Ciência Odontológica, Endodontia

Christine Men Martins

Tese Doutorado

INFLUÊNCIA DA HIPERTENSÃO ARTERIAL NO PADRÃO FENOTÍPICO DA LESÃO PERIAPICAL, NA DIFERENCIAÇÃO DOS OSTEOCLASTOS, NA RESPOSTA TECIDUAL E NA CAPACIDADE DE MINERALIZAÇÃO DO CIMENTO REPARADOR MTA

Orientador: Prof. Titular João Eduardo Gomes Filho

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Epígrafe

Se você abre uma porta, você pode ou não entrar em uma nova sala.

Você pode não entrar e ficar observando a vida.

Mas se você vence a dúvida, o temor, e entra, dá um grande passo: nesta sala vive-se!

Mas, também, tem um preço...

São inúmeras outras portas que você descobre. Às vezes curte-se mil e uma.

O grande segredo é saber quando e qual porta deve ser aberta.

A vida não é rigorosa, ela propicia erros e acertos.

Os erros podem ser transformados em acertos quando com eles se aprende.

Não existe a segurança do acerto eterno.

A vida é generosa, a cada sala que se vive, descobre-se tantas outras portas.

E a vida enriquece quem se arrisca a abrir novas portas.

Ela privilegia quem descobre seus segredos e generosamente oferece afortunadas portas.

Mas a vida também pode ser dura e severa.

Se você não ultrapassar a porta, terá sempre a mesma porta pela frente.

É a repetição perante a criação, é a monotonia monocromática perante a multiplicidade das cores, é a estagnação da vida...

Para a vida, as portas não são obstáculos, mas diferentes passagens!

Içami Tiba

Resumo

MARTINS, CM. **Influência da hipertensão arterial no padrão fenotípico da lesão periapical, na diferenciação dos osteoclastos, na resposta tecidual e na capacidade de mineralização do cimento reparador MTA.** Tese [Doutorado]. Araçatuba: Universidade Estadual Paulista; 2016.

Resumo

Entre as consequências da forma de ação do MTA e seus produtos encontra-se a participação na indução da mineralização nos tecidos onde é aplicado e a redução da inflamação ali presente. Sendo a hipertensão arterial uma desordem crônica de cunho inflamatório que parece agir negativamente na mobilização do cálcio e nas estruturas ósseas do organismo, pode-se inferir que o desenvolvimento da lesão periapical e o seu tratamento por meio do uso do MTA podem ser alterados pela presença do estado hipertensivo. Dessa forma, o objetivo do presente trabalho foi de estudar a influência da hipertensão arterial no padrão fenotípico da lesão periapical, na diferenciação dos osteoclastos, na resposta inflamatória tecidual e na capacidade de mineralização dos cimentos reparadores à base de MTA. Para isso o trabalho foi dividido em três artigos. O artigo 1 comparou aspectos potenciais da formação da lesão periapical nas condições de hipertensão e normotensão, tendo como hipóteses nulas que a hipertensão não altera a quantidade de osteoclastos diferenciados, o tamanho da lesão periapical e a expressão das citocinas inflamatórias IL1α, IL1β e TNFα da lesão periapical. Esse artigo teve como resposta que, apesar de não haver diferenças estatisticamente significantes entre o tamanho da lesão periapical e a expressão de citocinas inflamatórias, ratos hipertensos apresentaram um elevado número de osteoclastos diferenciados. Já o artigo 2 investigou se a hipertensão afeta a resposta tecidual do MTA branco e cinza implantados subcutaneamente em ratos, bem como a capacidade dessas substâncias para induzir a mineralização, sendo a hipótese nula testada que a hipertensão não altera a resposta tecidual e

capacidade de mineralização do MTA. Por meio dos resultados para as análises histológicas com as colorações Hematoxilina e Eosina e Von Kossa e sob luz polarizada, observou-se que a hipertensão exacerba a resposta inflamatória e diminui a capacidade de mineralização, prejudicando, dessa forma, tanto o reparo tecidual quanto a mineralização. Por sua vez, o artigo 3 investigou se hipertensão afeta a resposta de mineralização do MTA branco e cinza implantados subcutaneamente em ratos, através dos biomarcadores osteoblásticos RUNX-2, OPN e OCN em ratos, sendo a hipótese nula que a habilidade de mineralização do MTA não é afetada pela hipertensão. Os resultados apontaram para o prejuízo da capacidade de mineralização para o MTA frente à hipertensão. Então, de forma geral, pode-se concluir que há a associação da hipertensão com periapicopatias de origem endodôntica e seu tratamento, sendo que a hipertensão parece interferir negativamente na quantidade de osteoclastos e na ação do MTA quanto a resposta inflamatória e a capacidade de mineralização. Isso pode colocar a hipertensão como um fator prejudicial para o sucesso do tratamento/retratamento endodôntico.

Palavras-chave: hipertensão, doenças periapicais, inflamação, calcificação, MTA.

Abstract

MARTINS, CM. **Influence of hypertension in periapical lesion phenotype pattern, in osteoclastic differentiation, in tissue response and mineralization capacity of MTA repairing cement.** Thesis [PhD]. Araçatuba: Sao Paulo State University; 2016.

Abstract

Among MTA and its products consequences, it is found the participation in mineralization induction in tissue where it is applied and reduction of inflammation maybe present. High blood pressure is a chronic inflammatory disorder that seems acting negatively on calcium mobilization and bone structures of the body. So it can be inferred that periapical lesion development and its treatment using MTA can be altered by the presence of a hypertensive state. Thus, the objective of this research was to study the influence of hypertension in periapical lesion phenotypic, in osteoclast differentiation, in tissue inflammatory response and mineralization ability of MTA repair cements. For this, work was divided into three articles. Article 1 compared potentials aspects of periapical lesion formation in hypertensive and normotensive conditions. Null hypothesis was high blood pressure does not change the number of differentiated osteoclasts, periapical lesion size and expression of IL1α, IL1β and TNFα inflammatory cytokines in apical periodontitis. Among results, although there was no statistically significant difference between periapical lesion size and inflammatory cytokines expression, hypertensive mice showed a large number of differentiated osteoclasts. Article 2 investigated whether hypertension affect tissue response and mineralization ability of white and grey MTA implanted subcutaneously in rats. Null hypotheses were that high blood pressure did not alter tissue response and mineralization capacity against MTA. Through histological analyzes with Hematoxylin and Eosin and Von Kossa stains and under Polarized Light was observed hypertension exacerbates inflammatory response and decrease mineralization

capacity, damaging both tissue repair and mineralization. In turn, article 3 investigated whether hypertension affects white and gray MTA mineralization response when they were implanted subcutaneously in rats through osteoblast biomarkers RUNX-2, OPN and OCN. Null hypothesis was that MTA mineralization ability it is not affected by hypertension. The results pointed to the prejudice of MTA mineralization capacity against hypertension. So, in general, it can be concluded that there is an association between periapical problems and its treatment, wherein hypertension impairs number of osteoclasts and MTA action in inflammatory response and mineralization, thus it may be a detrimental factor for successful of endodontic treatment or retreatment.

Key-Words: Hypertension, periapical disease, inflammation, calcification, MTA.

Lista de Abreviaturas

BMC – células da medula óssea

BMD – do inglês, boné mineral density

BMM – do inglês bone marrow-derived macrophages

BPH/2J – camundongos hipertensos

BPN/3J – camundongos normotensos

CO_2 – gás carbônico

ELISA – do inglês, Enzyme-Linked Immunosorbent Assay

FBS – soro fetal bovino

Fig. – Figura

G – gramas

HCl – ácido clorídrico

HE – Hematoxilina Eosina

Hg – mercúrio

HRP – do inglês, horseradish peroxidase

IL10 – interleucina 10

IL1α – interleucina 1 alfa

IL1β – interleucina 1 beta

IL6 – interleucina 6

IP – intraperitoneal

IRM - material restaurador intermediário

Kg – quilogramas

M – molar

M-CSF – do inglês macrophage colony-stimulating fator

Mg – miligramas

microCT – micro tomografia computadorizada

mL – mililitros

Mm – milímetros

MTA – Mineral Trióxido Agregado

OPC – osteocalcina

OPG – do inglês osteoprotegerin

OPG – osteopontina

pH – potencial Hidrogeniônico

RANKL – do inglês Receptor activator of nuclear factor kappa-beta ligand

RUNX-2 – do inglês Runt-related transcription factor 2

SD – desvio padrão

SHR – ratos hipertensos

TLR4 – do inglês toll like receptor 4

TNF – Fator de Necrose Tumoral

TNFα – fator de necrose tumoral alfa

TRAP - tartrateresistant acid phosphatase

VK – Von Kossa

Vs – versus

αMEM – meio essencial mínimo alfa

µCT – Micro Tomografia Computadorizada

µg – microgramas

μm^2 – micrometros quadrados

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Table 1: Inflammatory cells and mineralization area average in hypertension and normotension conditions

Lista de Figuras

Artigo 1

Figure 1: Average of osteoclast number per well is described on figure 7. The value on x axis is number of osteoclasts differentiated. N=5 each group. Difference statistically significant between hypertensive group (BPH/2J) and normotensive group (BPN/3J).

Figure 2: Average of periapical lesion size in pixels to hypertensive (BPH/2J) and normotensive (BPN/3J) condition. The value is in mm^3. N=5 each group. No difference statistically significant.

Figure 3: Average of IL1α, IL1β and TNFα expression. The value is in picograms cytokine per milligram periapical tissue. N=5 each group for each cytokine. No difference statistically significant.

Artigo 2

Figure 1. Inflammatory response to white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by H&E staining (n = 6 per group). A) Gray MTA; B) white MTA; C) IRM; and D) control. 1) Normotensive day 7; 2) hypertensive day 7; 3) normotensive day 30; and 4) hypertensive day 30. H&E staining (100×) shows fibrous capsule formation with infiltration of macrophages and lymphocytes. The inflammatory response was increased by hypertension and in the presence of MTA and IRM.

Figure 2. Mineralization in response to white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by von Kossa staining (100×). A) Gray MTA; B) white MTA; C) IRM; and D) control. 1) Normotensive day 7; 2) hypertensive day 7; 3) normotensive day 30; and 4) hypertensive day 30. Black areas represent mineralization. Both types of MTA induced mineralization, which was not observed for IRM or the control group;

dark areas of tissue associated with IRM were necrotic. More consistent mineralization was observed in normotensive rats, especially at 30 days.

Figure 3. Mineralization in response to white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by polarized light microscopy (100×). A) Gray MTA; B) white MTA; C) IRM; and D) control. 1) Normotensive day 7; 2) hypertensive day 7; 3) normotensive day 30; and 4) hypertensive day 30. Birefringent structures in the tissue represent calcite crystals, which were observed for different types of MTA but not for IRM or the control group. More consistent birefringence was observed in normotensive rats, especially at 30 days.

Artigo 3

Figure 1. RUNX-2 immunostaining pattern. (A) Graph about the pattern of immunelabeling in differents experimental groups. **(B)** Photomicrograph showing RUNX-2 cells - immunoreactive (RUNX2-IR) (red arrows). **(C-J)** Photomicrographs showing immunostaining for RUNX-2 at 7 days post-implantation of the empty tube or cement content in NT-CONTROL (C), SHR-CONTROL (D), NT-IRM (E), SHR-IRM (F), NT-WHITE MTA (G), SHR-WHITE MTA (M), NT-GREY MTA (I) and SHR-GREY MTA (J). **Original magnification:** B: 5000x; C-J: 1000x. Scale bars: B: 10 micrometres; C-J: 25 micrometers. **Counter-staining:** *Fast Green*.

Figure 2. OPN immunostaining pattern. (A) Graph about the pattern of immunelabeling in differents experimental groups for RUNX-2 protein. **(B)** Photomicrograph showing osteopontin cells - immunoreactive (OPN-IR) (red arrows). **(C-J)** Photomicrographs showing immunostaining for OPN at 30 days post-implantation of the empty tube or cement content in NT-CONTROL (C), SHR-CONTROL (D), NT-IRM (E), SHR-IRM (F), NT-WHITE MTA (G), SHR-WHITE MTA

(M), NT-GREY MTA (I) and SHR-GREY MTA (J). **bv:** blood vessels. **Original magnification:** B: 5000x; C-J: 1000x. Scale bars: B: 10 micrometres; C-J: 25 micrometers. **Counter-staining:** *Harris hematoxylin*.

Figure 3. OCN immunostaining pattern. (A) Graph about the pattern of immunelabeling in differents experimental groups for OCN protein. **(B)** Photomicrograph showing osteocalcin cells - immunoreactive (OCN-IR) (red arrows). **(C-J)** Photomicrographs showing immunostaining for OCN at 30 days post-implantation of the empty tube or cement content in NT-CONTROL (C), SHR-CONTROL (D), NT-IRM (E), SHR-IRM (F), NT-WHITE MTA (G), SHR-WHITE MTA (M), NT-GREY MTA (I) and SHR-GREY MTA (J). **bv**: blood vessels. **Original magnification:** B: 5000x; C-J: 1000x. Scale bars: B: 10 micrometres; C-J: 25 micrometers. **Counter-staining:** *Harris hematoxylin*.

Sumário

Sumário

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Introdução Geral

Introdução

As dores orofaciais de origem dental acometem grande parte da população mundial, sendo um problema significante em saúde pública e o provável tratamento para essas dores refere-se à endodontia. Entre as pessoas acometidas por essas dores podem estar presentes as hipertensas, uma vez que a hipertensão arterial é uma desordem crônica de etiologia multifatorial e de grande expressividade na população brasileira e mundial (1), sendo que estudos apontam que cerca de 25% da população apresenta essa patologia (2). Nos últimos anos vem se destacando um aumento dessa morbidade em decorrência da mudança dos padrões alimentares e estilo de vida da população, além do crescimento da população idosa e do aumento da longevidade (3).

O diagnóstico da hipertensão arterial se dá pela presença da pressão sistólica ser maior que 140 mm de mercúrio e/ou a diastólica ser maior que 90 mm de mercúrio (1). A hipertensão arterial é caracterizada pelo aumento da resistência vascular periférica ao fluxo sanguíneo, em grande parte devido à remodelação vascular, fazendo com que a pressão do sangue nas artérias seja maior que o normal (4-6). Assim, essa desordem crônica se torna um dos principais fatores de risco para a ocorrência de doenças cardiovasculares e renais, colocando a hipertensão arterial em uma das primeiras posições no ranking de problemas mundial de saúde pública (1,4,7).

Na literatura estão descritos diversos estudos acerca da inter-relação hipertensão e problemas bucais. Segura-Egea e colaboradores realizaram estudos retrospectivos e observaram, em 2010, maior prevalência de periodontite apical crônica em pacientes hipertensos do que em normotensos (8) e em 2011 que a associação dessa morbidade com o hábito do fumo pode aumentar a prevalência de periodontite apical crônica (9).

A hipertensão parece atuar de forma dupla relacionada com problemas bucais. Ou seja, ela parece favorecer para o aparecimento de problemas bucais assim como pessoas hipertensas parecem ter mais susceptibilidade para a ocorrência de tais problemas. A hipertensão arterial pode levar ao aumento de pressão nas arteríolas do osso alveolar e consequentemente levar à uma pequena hemorragia (10-12). Além disso, pacientes com doenças sistêmicas, como a hipertensão, tem uma baixa resistência à infecção bacteriana e capacidade de reparo tecidual depois do tratamento endodôntico (13,14). A infecção periodontal apresenta uma grande quantidade de espécies patógenas e mediadores inflamatórios que podem criar uma carga inflamatória sistêmica e aumentar o risco de desenvolvimento da hipertensão ou outras doenças cardiovasculares (15-21). Bonato e colaboradores em seu estudo, induziram a periodontite e observaram um recrutamento adicional de neutrófilos, devido ao aumento da presença de TNF e outras citocinas envolvidas na emissão do sinal para respostas imunológicas (15). Da mesma forma, os autores descobriram que o número de neutrófilos presentes é maior em ratos hipertensos do que nos normotensos, sendo assim, a hipertensão parece promover um processo inflamatório e, na presença de pequenos focos inflamatórios, essa é potencializada (15).

A hipertensão pode aumentar a susceptibilidade de desenvolvimento de patologias que afetam a saúde bucal, especialmente pelo fato da hipertensão influenciar negativamente na saliva. Elias e colaboradores em 2006, conduziu um estudo no qual avaliaram a saliva e observaram que o fluxo salivar e a concentração média de proteínas na saliva eram reduzidos, embora não observaram mudanças na atividade da amilase salivar (22).

Os mesmos autores, por meio da análise de dureza, observaram que ratos hipertensos apresentam uma menor resistência do esmalte e da dentina (22). Na mesma linha de raciocínio,

Inoue e colaboradores em 2007 sugeriram que o mecanismo de mineralização de ratos hipertensos é anormal, devido ao osso trabecular apresentar uma quantidade menor de mineral (23).

Além desses fatores, a hipertensão pode causar mudanças histométricas e moleculares no osso alveolar, mesmo na ausência de um processo inflamatório. Bastos e colaboradores em 2010, encontraram aumento da expressão das proteínas RANKL, consequentemente uma maior proporção das proteínas RANKL/OPG e isso, combinado com outros fatores, causam uma redução da densidade óssea. Isso se deve ao fato da proteína RANKL ativar os osteoclastos, que são os responsáveis pelo processo de reabsorção, e a proteína OPG ser um fator inibitório da osteoclastogênese (24). Esses dados sugerem que a hipertensão pode afetar diretamente o osso alveolar. Zhang e colaboradores em 2009, também descobriram que a densidade mineral óssea é menor em ratos hipertensos, o que pode confirmar o aumento da perda óssea na presença da pressão alta (25).

Bastos e colaboradores em 2010 estudaram que não só a densidade óssea do osso pré-existente é afetada pela hipertensão, mas também o osso neoformado. Nesse estudo, a área do osso trabecular em ratos normotensos com 150 dias de idade era significantemente maior do que na condição sistêmica de pressão alta. Somando-se a isso, oito dias depois da realização do defeito ósseo, a densidade óssea do osso neoformado era significantemente menor (24).

Foi sugerido que a hipertensão pode contribuir para a diminuição da retenção de dentes tratados endodonticamente. Mindiola e colaboradores em 2006 descobriram que 7,8% dos dentes tratados endodonticamente em pacientes com hipertensão o processo de reparo foi considerado insatisfatório. Quando se agrega à essa situação sistêmica a diabetes, a insatisfatoriedade era ainda maior (26), o que pode justificar a hipótese que doenças sistêmicas como Diabetes Melitus,

doenças arteriais e hipertensão aumentam o risco de extração dentária depois da realização do tratamento endodôntico ou retratamento (27).

O tratamento endodôntico objetiva o restabelecimento da normalidade dos tecidos apicais e periapicais perdidos (28-31) por meio de uma limpeza profunda e desinfecção do sistema de canais radiculares para controlar os microorganismos patogênicos e do completo selamento tridimensional do canal radicular com materiais obturadores que apresentem propriedades físicas e biológicas apropriadas para o reparo tecidual, por meio da indução da mineralização nos tecidos onde é aplicado (32-38).

No entanto, falhas no tratamento endodôntico podem ocorrer e, quando bem indicada, a cirurgia paraendodôntica com obturação retrógrada é uma ótima alternativa. Vários materiais são utilizados para esse fim e para ser considerado ideal eles devem ter características como a biocompatibilidade, adesividade, boa vedação e estabilidade dimensional especialmente perante as cargas mecânicas da oclusão, capacidade de induzir osteogênese e cementogênese, facilidade de preparo, esterilidade, radiopacidade, não capacidade de expansão e não ser irritante aos tecidos periapicais.

Atualmente, existem no mercado diversas pastas e cimentos com as mais variadas bases: óxido de zinco e eugenol, resina epóxica, ionômero de vidro, hidróxido de cálcio e, mais recentemente, o agregado trióxido mineral (MTA) (32-35,37,39,40).

Com o objetivo inicial de proporcionar o selamento de comunicações patológicas ou iatrogênicas entre o sistema de canais radiculares e a superfície externa do dente, o MTA foi desenvolvido. Além disso, em decorrência das suas excelentes propriedades físico-químicas e biológicas, ele passou a ser utilizado também em pulpotomias, capeamentos pulpares diretos,

apicogêneses e apicificações (34,39,41-51). Foi demonstrado que o MTA tem comportamento semelhante ao hidróxido de cálcio quando aplicado nos diferentes tecidos (52).

Após estudos realizados com o MTA, demonstrou ser este um cimento biocompatível, hidrofílico, radiopaco, com ação antimicrobiana, apresenta boa capacidade de selamento marginal, é indutor de dentinogênese, cementogênese e osteogênese, não é tóxico e/ou carcinogênico. Encontra-se disponível no mercado sob os nomes comerciais de MTA ProRoot® (Dentsply) e Ângelus MTA®, ambos com características físico-químicas e biológicas semelhantes (34,39,41-46,48-51,53-55).

Entre esses MTA, existem as variações de MTA Branco e Cinza. Embora sejam bastante semelhantes, eles apresentam algumas diferenças. Estudos sobre a biocompatibilidade tecidual e celular por meio dos osteoblastos, a cicatrização de injúrias pulpares e a formação de ponte de tecido duro mostraram resultados semelhantes para branco e cinza MTA (56,57). Em contrapartida, alguns estudos descobriram que o MTA Cinza promove melhor crescimento, adesão e diferenciação de células osteoblásticas (58). MTA Branco tem um tempo de presa mais baixo do que o MTA Cinza, o que explica sua maior solubilidade e liberação iônica (56-59). No entanto, o MTA cinza tem altos níveis de arsênio, aluminoferrite tetracalcico, ferro, cádmio e cromo, que podem levar à rejeição pelo tecido, inflamação ou reações alérgicas (59).

O IRM® (Dentsply DeTrey, Germany) é um cimento que possui sua composição a base de Óxido de Zinco e Eugenol reforçado por polímeros. É indicado para restaurações temporárias de longa espera, para forramento de cavidades sob restaurações de amálgama e para o uso em odontopediatria e odontogeriatria. Possui alto vedamento marginal, rápida presa, alta resistência a compressão, além de propriedades sedativas, o que proporciona menores índices e infiltração marginal, agilidade e facilidade de preparo e uso e menor índice de dor pós-operatória. Devido à

essas características, ele também é utilizado como cimento obturador retrógrado, no entanto em função da presença do Eugenol, o IRM apresenta certa toxicidade aos tecidos periodontais. Além disso, apresenta certa solubilidade ao longo do tempo (60,61).

Considerando que uma das finalidades do MTA e seus produtos é participar na indução da mineralização nos tecidos onde é aplicado e reduzir a inflamação ali presente e que a hipertensão arterial é uma desordem crônica de cunho inflamatório que parece induzir uma mobilização do cálcio do osso a ser excretado pelos rins, justifica-se o estudo da resposta tecidual de animais hipertensos a materiais endodônticos indutores de mineralização.

Proposição Geral

Proposição

Objetiva-se no presente trabalho a análise da influência da hipertensão arterial no padrão fenotípico da lesão periapical, na diferenciação dos osteoclastos, na resposta inflamatória tecidual e na capacidade de mineralização dos cimentos reparadores à base de MTA. As hipóteses nulas testadas são:

- O estado hipertensivo não altera o tamanho da lesão periapical analisada pela Micro Tomografia Computadorizada;

- A expressão de citocinas inflamatórias IL1α, IL1β e TNFα da lesão periapical em ratos hipertensos não se diferencia da expressão das mesmas em condição sistêmica normal;

- A quantidade de diferenciação de células da medula óssea em osteoclastos não altera devido à presença de um estado hipertensivo;

- O estado hipertensivo não altera a resposta inflamatória tecidual avaliada pela coloração de Hematoxilina e Eosina frente ao cimento reparador MTA;

- A mineralização avaliada pela coloração de Von Kossa e sob Luz Polarizada não se diferencia de acordo com os dois estados sistêmicos;

- A hipertensão não altera o processo de diferenciação osteogênica por meio da análise de imunomarcação dos biomarcadores RUNX-2, OCN e OPN.

Artigo 1

RELATIONSHIP BETWEEN HYPERTENSION AND PERIAPICAL LESION: AN *IN VIVO* AND *IN VITRO* STUDY

ABSTRACT

The aim of this study was to compare potential aspects of periapical lesion formation in hypertensive and normotensive condition using hypertensive BPH/2J and wild type control BPN/3J mice. Bone marrow stem cells were isolated from adult mice femur in 2 strains and osteoclast differentiation was evaluated by Tartrate-resistant acid phosphatase (TRAP) *in vitro.* The mandibular first molars of both strains had their dental pulp exposed. At day 21 the mice were euthanized and right mandibular molars were used to evaluate the size and phenotype of periapical lesion by microCT. Proteins were extracted from periapical lesion on left side and the expression of IL1α, IL1β and TNFα was analyzed by ELISA. The amount of differentiated osteoclastic cells was nearly double in hypertensive mice when compared to the normotensive strain ($p<0.03$). Periapical lesion size did not differ between hypertensive and normotensive strains ($p>0.7$). IL1α, IL1β and TNFα cytokines expression were similar for both systemic conditions ($p>0.05$). Despite the fact that no differences could be observed in periapical lesion size and cytokines expression on the systemic conditions tested, hypertension showed elevated number of osteoclast differentiation.

Key-words: Hypertension, Periapical diseases, Inflammation.

INTRODUCTION

The association between periapical inflammatory and infectious process and systemic disease is raising researches interest lately. In the past, the focal infection theory was applied as a causal relationship between oral infections and heart disease, like infective endocarditis (1). However, in 2012 the American Association of Endodontists stated that decades of research opposes the beliefs of focal infection theory proponents. To date, there is no valid, scientific evidence linking endodontically treated teeth and systemic diseases (2).

Oral health will certainly improve the overall good health (3), and although there is no causal relationship (2), an association between oral infections and certain systemic conditions, such as hypertension, can be inferred. This association may depend on common risk factors such as dysregulation of biological functions including immune response (4-7).

The diagnosis of hypertension can be given by a systolic blood pressure higher than 140 mm Hg, a diastolic blood pressure higher than 90 mm Hg, or both (8). Essential hypertension is due to genetic factors associated with unhealthy lifestyle and renal disorder is the cause of secondary hypertension (9).

It is known that hypertension can be considered an inflammatory disease (10). TLR4 signal links hypertension and periapical inflammation (11) and lymphocytes T cells are responsible to the hypertension development (12) mediated by angiotensin II (13). Pro-inflammatory cytokines like TNFα and IL-6 are larger presented in hypertensive condition (14) and the opposite is true for IL-10, an anti-inflammatory cytokine (15).

Although the relationship between this systemic disorder and periapical lesion has been indicated, it is paramount that such connection be scientifically reinforced. Studies have shown correlation between hypertension and periodontal disease (16,17) as well as amount of salivary

flow and its protein concentration (18). Changes in hard tissue structures like enamel, dentin and bone are also affected by hypertension (18-22). Few studies can be found correlating endodontic disease and hypertension. Allareddy et al., 2010 conducted a retrospective study where he found that 24,6% patients hospitalized for periapical abscess were hypertensive (23). Likewise, another study found that almost 8% of endodontically treated teeth in hypertensive patients were not considered satisfactory (24).

Thus, the hypothesis tested in this study was that osteoclast differentiation from bone marrow cells, periapical lesion size and inflammatory cytokine expression in hypertensive mice are higher than in normotensive ones. Therefore, this study aimed to compare potential aspects of periapical lesion formation on hypertensive and normotensive conditions.

METHODS

Animals

This experimental animal study was submitted to the approval of the Animal Experiment Committee of Forsyth Institute, nº 14/004. BPH/2j and its normal control BPN/3J mouse strains were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice were maintained in accordance with the guidelines of the Animal Experiment Committee of Forsyth Institute.

Osteoclast differentiation from bone marrow cells

Bone marrow cells (BMCs) were isolated from femora of BPH/2J and BPN/3J strain mice. The sample size was 5 mice each divided in 40 wells. The initial density of cells was $1{,}5x10^6$ cells/μL in a 96 well plate. The cells were incubated for 5 days in α-MEM containing 10% inactivated FBS with M-CSF (50 ng/mL). After the incubation, the adherent cells were collected

as bone marrow-derived macrophages (BMMs). The BMMs were cultured in the presence of M-CSF (25 ng/ml) and RANKL (100 ng/ml) for 8 days. Osteoclast formation was evaluated by measuring the tartrateresistant acid phosphatase (TRAP) activity as an early differentiation marker. After TRAP staining the cells with more than three nuclei were counted as TRAP-positive multinucleated cells.

Periapical lesion stimulation

It was used female and male mice with 7 weeks old. The sample size was 5 mice for hypertensive group and 5 mice for normotensive group. The mice were anesthetized via intra peritoneal (IP) injection with ketamine HCl (80 mg/kg) and xylazine (10 mg/kg) and were placed on a jaw retraction board. The dental pulps of both mandibular first molars were exposed using an electric dental hand piece with a no. 1/4 round bur under a surgical microscope. The pulp chambers were open until the entrance of the canals could be visualized and probed with a size 6 endodontic file. On day 21 after pulp exposure, mice were sacrificed using the CO2 gas chamber and mandibles were isolated and dissected free of soft tissue. Right hemimandibles were fixed in fresh 4% paraformaldehyde in PBS and scanned by Micro Tomography Computed to analyze the periapical lesion size. Left hemimandibles were immediately frozen for protein extraction and analysis of the pro-inflammatory cytokine expression.

General bone and periapical lesion phenotype

After fixation the paraformaldehyde in the samples was reduced by Distilled Water and the samples were scanned in Micro Tomography Computed. The angles of the image were adjusted

using the ImageJ program and the periapical lesion size was measured by Adobe Photoshop56 program. The periapical lesion size was recorded in square micrometer.

Pro-inflammatory cytokine expression from periapical lesion proteins

It was used 5 samples each group, hypertensive or normotensive group. For protein extraction, frozen periapical tissue samples were disrupted in a cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with 50 μg/ml gentamicin (Sigma-Aldrich, St. Louis, MO) using FastPrep-24 with matrix A (both MP Biomedicals, Solon, OH). The supernatant was collected after centrifugation and assays for cytokines in extracts employed commercially available ELISA kits obtained from R&D Systems (DuoSets) and were used according to the manufacturer's instructions to evaluate periapical tissue levels of IL-1α, IL-1β and TNF-α. The concentration of each cytokine was calculated with reference to a standard curve constructed using recombinant cytokines provided with each kit. Results were expressed as picograms cytokine per milligram periapical tissue.

Statistical analysis

Normality was defined by Shapiro-Wilk test. Test T Tukey was performed to assess the difference between the groups. Values of $p<0.05$ was considered statistically significant.

RESULTS

Osteoclast differentiation from bone marrow cells

The average of differentiated osteoclast number per well is described on figure 1. The hypertensive group presented almost twice the number of differentiated osteoclast when compared

with normotensive group, 97 and 45, respectively. There is a statistically significant difference between the two groups ($p<0.03$).

General bone and periapical lesion phenotype

The average of periapical lesion size in pixels on hypertensive and normotensive condition is on figure 2. The average of lesion size on the hypertensive group was 2,6mm^3 compared to 2,7 mm^3 in normotensive group. No statistically significant difference between the groups was observed ($p>0.07$). There was no visible difference between the groups regarding to the bone structure or phenotype pattern.

Pro-inflammatory cytokine expression from periapical lesion proteins

The average of IL1α, IL1β and TNFα expression is described on figure 3. For all cytokines the tendency is higher expression on periapical lesion group than in control (no exposed) group. Although there is no difference statically significant for all cytokines ($p>0.05$), hypertensive condition presented higher expression of cytokines IL1α and TNFα than the normotensive condition.

DISCUSSION

Although there is a high prevalence of hypertension in patients hospitalized for periapical abscess (23) and the indications of the negative influence of hypertension in the periapical lesion, our results did not show a clear relationship between this health condition and periapical lesion phenotype. The periapical lesion size and cytokines expression from periapical lesion were similar independently the systemic condition.

The hypertensive group presented almost twice the number of differentiated osteoclast compared with normotensive group. Since the final outcome of periapical lesion and periodontitis is bone destruction, the effect of metabolic disorders on bone needs to be considered. The osteoclasts are responsible for bone resorption and angiotensin II may be the link. Hypertension is mediate by angiotensin II, molecule responsible to activate osteoclasts due to up-regulated RANKL expression in osteoblasts (25). In other words, angiotensin II induce the expression of RANKL thru receptor activator of NF-κB ligand in osteoblasts, leading to the activation of osteoclasts (25), which will be responsible to bone fracture, osteoporosis and also bone destruction in endodontics. In a ligature-induced periodontitis model, increased RANKL/OPG ratio toward more osteoclastic condition was observed in the SHR rat vs. normotensive controls (20,21). Hypertension also may negatively affect bone mineral density (BMD) due to abnormal metabolism of 1,25-dihydroxyvitamin D, a key regulator of calcium homeostasis and bone metabolism, intestinal calcium transport, and angiotensin II-mediated osteoclast activation (25,26).

Pulp inflammation can soon spread in apical direction and so an immune-inflammatory response occurs thru the action of immune cells resulting in abscess formation which is an acute phase (27). After that, a lymphocytic infiltration will happen as a defense mechanism against systemic spread of bacteria and/or bacterial by-products to other sites in the body and this is the granuloma phase (27).

In our study, the protocol used was doing pulp exposure and sacrifice the mice after 21 days. Some studies reported that in this period we have granuloma phase (28) and also the immune/inflammatory response and the systemic and local bone metabolism can be responsible for the higher prevalence of chronic apical periodontitis in hypertensive patients when compared to normotensive ones (16).

The immune system is not a primary cause of hypertension, but it is a secondary factor following initiation of pre-hypertension, which is mainly caused by genetics and lifestyle exhibiting a modest elevation of blood pressure about 135 to 140 mmHg. Pre-hypertension and the following vascular injuries lead to production of damage-associated molecular patterns (DAMPs), neoantigens, and immune regulatory mediators promoting immune and inflammatory response. The Toll-like receptor 4 (TLR4) plays a fundamental role in pathogen recognition and activation of innate immunity, being the key proinflammatory signaling in induction of hypertension target organ damages and periapical lesions (5,29-30). So, it can be state that hypertension can be considered an inflammatory vascular process (10) and this is why studies suggest a greater tendency to a chronic nature of lesions in hypertensive patients (10).

In our study it was observed the expression of IL1α, IL1β and TNFα from the periapical lesion, which shows the inflammatory response. In our study, we did not found statistically significant difference between expression on pulp exposed group compared to non-exposed one. Nevertheless, it was expected that as the pulp exposure induces periapical lesion, that present together inflammation and bone resorption.

The results of the present study must be checked in different models once BPH/2J mice are genetically modified and the signs, symptoms and consequences of hypertension start to appear in older mice. However, to have a standardized periapical lesion the researches usually use young adult mice. In our research we used 7 weeks old mice, which is an adult mice, being not so young and not as old as necessary. So, we had a wide standard variation intragroup, as maybe some mice presented sequelae of hypertension while others did not.

Despite the periapical lesion size and cytokines expression being similar for the different systemic conditions, hypertension condition lead to a higher osteoclasts differentiation, which may influence the endodontic treatment outcome in such systemic condition.

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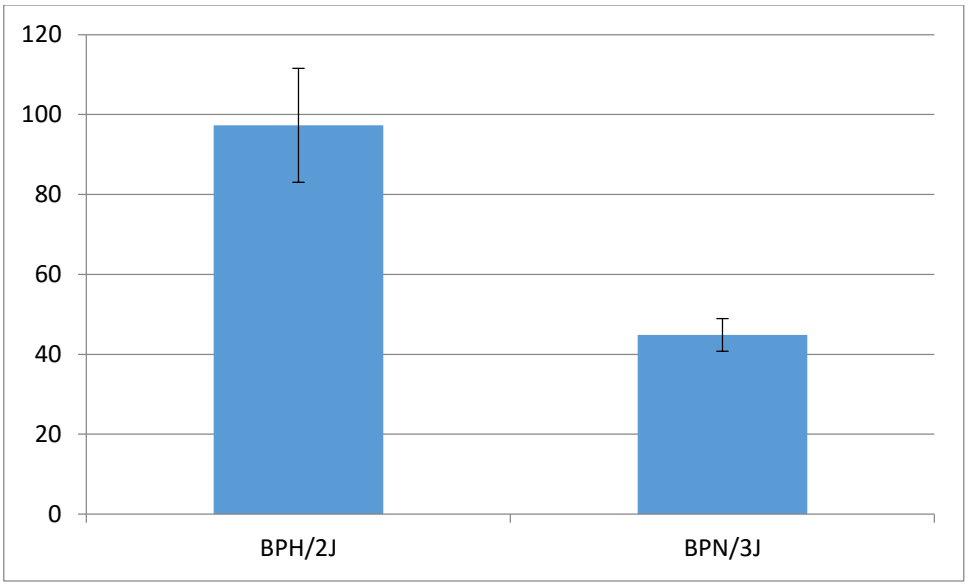


Figure 1: Average of osteoclast number per well is described on figure 7. The value on x axis is number of osteoclasts differentiated. N=5 each group. Difference statistically significant between hypertensive group (BPH/2J) and normotensive group (BPN/3J).

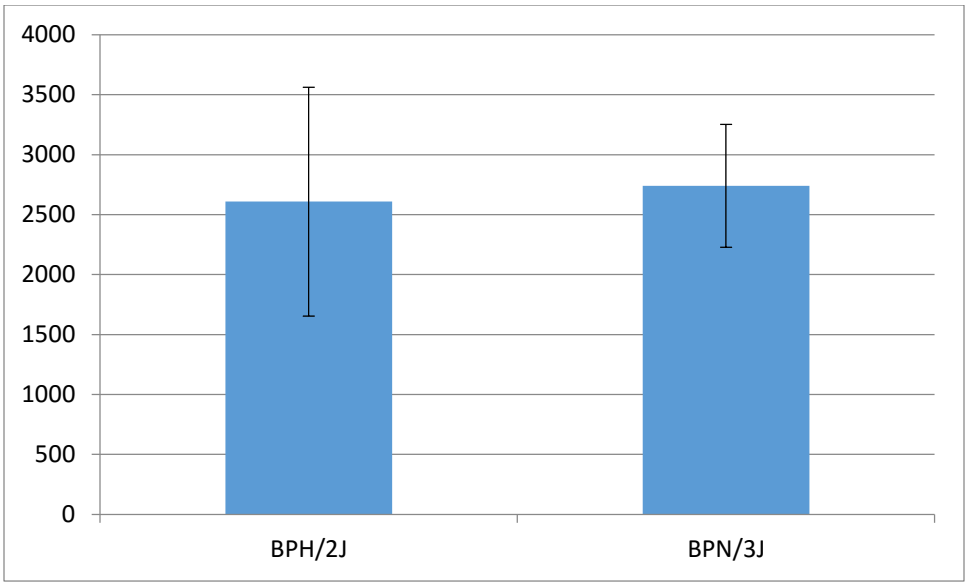
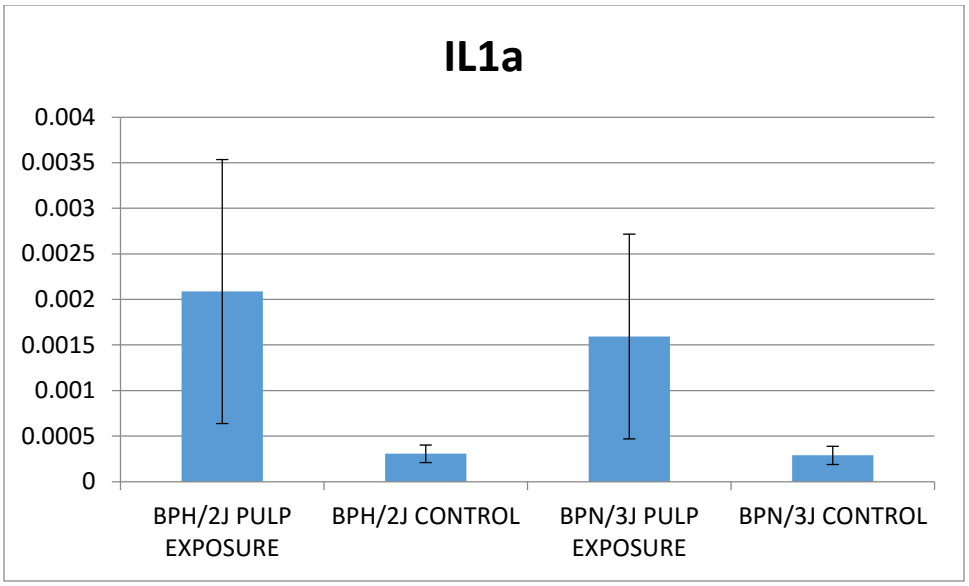
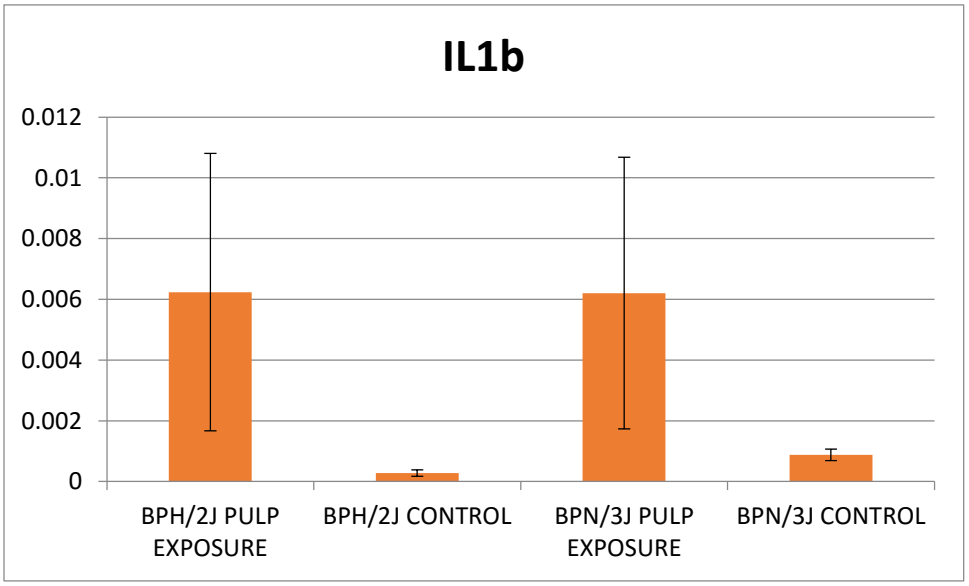


Figure 2: Average of periapical lesion size in pixels to hypertensive (BPH/2J) and normotensive (BPN/3J) condition. The value is in mm^3. N=5 each group. No difference statistically significant.


IL1a
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
BPH/2J PULP EXPOSURE
BPH/2J CONTROL
BPN/3J PULP EXPOSURE
BPN/3J CONTROL


IL1b
0.012
0.01
0.008
0.006
0.004
0.002
0
BPH/2J PULP EXPOSURE
BPH/2J CONTROL
BPN/3J PULP EXPOSURE
BPN/3J CONTROL

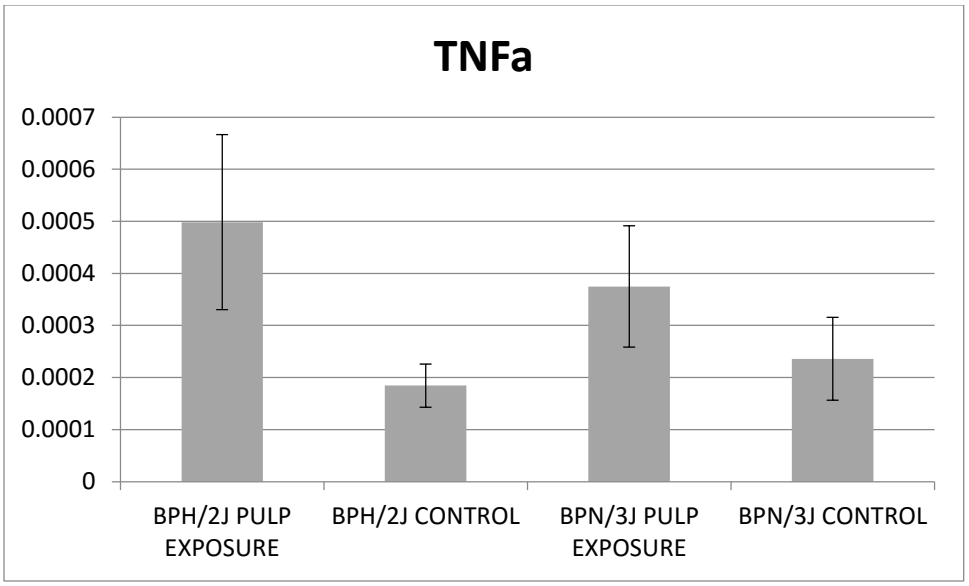


Figure 3: Average of IL1α, IL1β and TNFα expression. The value is in picograms cytokine per milligram periapical tissue. N=5 each group for each cytokine. No difference statistically significant.

Artigo 2

HYPERTENSION UNDERMINES MINERALIZATION-INDUCING CAPACITY OF AND TISSUE RESPONSE TO MINERAL TRIOXIDE AGGREGATE ENDODONTIC CEMENT

ABSTRACT

Introduction: This study evaluated the effect of hypertension on tissue response to and mineralization capacity of white and gray mineral trioxide aggregate (MTA)-Angelus, an endodontic reparative cement.

Methods: Polyethylene tubes containing gray MTA, white MTA, or intermediate restorative material (IRM; positive control) or an empty tube (negative control) were implanted into the dorsal connective tissue of spontaneous hypertensive and Wistar rats (n = 12 each). Six rats in each group were sacrificed after 7 days, and the remainder after 30 days. Tubes with surrounding tissue were removed and a histological analysis was carried out by hematoxylin and eosin and von Kossa staining and examination by polarized light microscopy.

Results: The inflammatory response to all materials was greater in hypertensive as compared to normotensive rats ($P < 0.05$). Positive von Kossa staining and birefringent structures in polarized light were observed for both gray and white MTA ($P > 0.05$), but these were more pronounced in normotensive rats ($P < 0.05$). Necrotic areas with positive von Kossa staining were observed for IRM.

Conclusion: Hypertension undermines tissue repair and mineralization, which can negatively affect treatment outcome. Nonetheless, mineralization in response to MTA was observed even under hypertensive conditions.

Key words: Hypertension, Periapical diseases, Calcification.

Acknowledgement: The authors deny any conflicts of interest.

INTRODUCTION

Hypertension is a disease that affects around 25% of the general population and 50% of individuals over 60 years old (1). It is defined as high blood pressure, i.e., systolic blood pressure > 140 mm Hg, diastolic blood pressure > 90 mm Hg, or both (1).

Hypertension is considered as an inflammatory disease that has systemic effects, including calcium loss from the body (2,3). It has been suggested that hypertension is an inflammatory vascular process that increases T lymphocyte and natural killer cell infiltration and proliferation, with deposition of perivascular macrophages and increased expression of collagen type I and III, interleukin 16, lymphocyte chemotactic factor, and cytotoxic cytotoxic T lymphocyte-associated protein 4 (2-5). Moreover, it is associated with an increase in C-reactive protein, which is considered as an inflammatory marker (6).

Epidemiological studies have found an association between periodontal disease and hypertension that implicates oral infection and the immune/inflammatory response (7). It was observed that 24.6% of patients hospitalized for periapical abscesses had hypertension (8). Periapical lesions occur as an inflammatory response to infection; along with hypertension, it can lead to vascular injury and inflammation. However, there are no study linking hypertension and periapical lesion healing in the literature.

Hypertension can also lead to abnormalities in mineralization due to increased serum levels of parathyroid hormone, whose main function is to stimulate the breakdown of bone by activation and proliferation of osteoclasts and thereby increase blood calcium levels. Abnormal vitamin D metabolism causes reduced serum ionized calcium, and decreases calcium absorption (3). In addition, trabecular bone mineral content is decreased in both children and adults (9,10), which is observed not only in pre-existing but also in newly formed medullar bone tissue (11). This

morbidity leads defects in bone healing (9,11,12) and to a high rate of implant loss due to defects in osseointegration (12,13). Hypertension may also increase the receptor activator of nuclear factor κB ligand/osteoprotegerin ratio, which would tend to favor a more osteoclastic state (9). Thus, hypertension can affect bone metabolism and may be a risk factor for bone diseases including periodontitis, periapical lesions, and rheumatoid arthritis (14).

Endodontic treatment aims to control infection and restore apical and periapical tissues (15). Apical periodontitis may persist due to the presence of bacteria in the apical root canal space (16), which can be treated by endodontic surgery with retrofilling (16). The endodontic reparative cement mineral trioxide aggregate (MTA) is used in this process owing to its excellent physical, chemical, and biological properties, including its ability to induce mineralization in tissues at the site of application (17-19).

Few studies have investigated the difference between the white and gray types of MTA with respect to their potential for inducing tissue regeneration, biocompatibility, and sealing ability (20-22). In the present study, we investigated whether hypertension affects the tissue response to white and gray MTA implanted subcutaneously into rats as well as the ability of these substances to induce mineralization.

METHODS

The study was approved by the Ethical Committee of Aracatuba Dental School (protocol no. 2013-00961).

Animals

Male spontaneously hypertensive and Wistar rats (n = 12 each; age: 2–3 months; 250–280 g) were used as hypertensive (experimental) and normotensive (control) animals, respectively.

Surgical procedures

White and gray MTA (Angelus Industry Ontological Products, Londrina, Brazil) and intermediate restorative material (IRM) (Dentsply Caulk, Milford, DE, USA) were prepared according to the manufacturers' recommendations and placed in 72 sterile polyethylene tubes (internal diameter: 1.0 mm; external diameter: 1.6 mm; length: 10 mm) (Abbott Labs of Brazil, São Paulo, Brazil) with a lentulo spiral (Maillefer Dentsply, Tulsa, OK, USA); 24 empty tubes were used as the control. Each tube was completely filled with the material.

Animals were anesthetized with xylazine (10 mg/kg) (Anasedan/Vetbrands Division Animal Health, Sao Paulo, Brazil) and ketamine (25 mg/kg) (Cetamin Syntec do Brazil, São Paulo, Brazil). Their backs were shaved and the skin disinfected with 5% iodine solution; a 2-cm incision was made from head to tail with a #15 Bard-Parker blade (Becton-Dickinson, Franklin Lakes, NJ, USA). Four pockets 6 cm apart were created on each side of the incision into which three tubes (filled with white or gray MTA or IRM) and an empty tube were implanted. The skin was closed using Ethicon 4.0 silk sutures (Johnson & Johnson, Sao Paulo, Brazil).

Histological analysis

At 7 and 30 days after tube implantation, animals were euthanized by an overdose of anesthetic and the tubes with surrounding tissue were removed and fixed in 10% formalin solution (pH 7.0), cut transversely into two halves that were each cut longitudinally using a sharp blade to allow contact between the surfaces and processing solutions. Specimens were embedded in glycol

methacrylate with historesin. Serial sections 3 μm thick were stained with hematoxylin and eosin (H&E), while 10-μm sections were processed by von Kossa staining or directly examined under polarized light (23).

Tissue in contact with the material at the tube opening was assessed by counting the average number of inflammatory cells stained by H&E in 10 randomly selected microscopic fields at 400× magnification by one evaluator blinded to the experimental condition. Mineralization per μm^2 was determined using Qwin software (Leica Microsystems, Wetzlar, Germany). An average value for each material was determined as the total number of cells counted in 10 microscopic fields at 400× magnification. Results were evaluated by three-way analysis of variance and P values < 0.05 were considered significant.

RESULTS

Hypertension exacerbates the inflammatory response to endodontic reparative cement

The average number of inflammatory cells at the mouth of polyethylene tubes was determined on days 7 and 30 (Table 1). The inflammatory response to gray MTA decreased over time and was similar to the response to white MTA under normotensive conditions. However, the inflammatory response was increased in hypertensive rats ($P < 0.05$) and also in the presence of IRM relative to the control group. All materials induced greater inflammation in hypertensive as compared to normotensive rats ($P < 0.05$).

The inflammatory response to all materials as well as to the empty tube was exacerbated by hypertension (Fig. 1). Macrophages and lymphocytes were the predominant cell types detected in the tissue. Fibrous capsules were observed surrounding the tubes under both hyper- and normotensive conditions that were thinner at 30 vs. 7 days.

Hypertension inhibits mineralization

An analysis of birefringence under polarized light revealed the presence of calcium carbonate particles for white and gray MTA but not for the other two groups (Table 1). Under normotensive conditions, the birefringent area (μm^2) was greater on day 30 than on day 7 ($P < 0.05$); however, in hypertensive rats, the areas were similar at both time points. The mineralized area in contact with white MTA vs. other substances was greater in both hyper- and normotensive rats; there were no birefringent areas observed for IRM or the control group ($P < 0.05$).

Calcium particles were observed by von Kossa staining, with dark areas representing tissue necrosis. Under hypertensive conditions, mineralization was similar on day 30 for both types of MTA and higher than in the IRM group. A decrease in mineralization was observed over time for gray MTA ($P < 0.05$); however, for white MTA, this trend was seen only in normotensive rats, whereas under hypertensive conditions, mineralization was increased at 30 as compared to 7 days ($P < 0.05$). The area of positive staining was lower overall in the IRM group, in which hypertension exacerbated this response ($P < 0.05$). There were no areas positive for von Kossa staining in the control group ($P < 0.05$).

Based on von Kossa staining (Fig. 2) and polarized light microscopy (Fig. 3) analyses, the two types of MTA had more mineralization than the other groups. In general, mineralization was more consistently observed in the absence of hypertension.

DISCUSSION

This is the first study evaluating the effect of hypertension on tissue response to MTA, including mineralization. The results demonstrate that hypertension enhances the inflammatory response and decreases the capacity of MTA to induce mineralization.

Hypertension is an inflammatory vascular process (2) in which high levels of angiotensin II stimulate the infiltration and proliferation of T lymphocytes and natural killer cells (24). In addition, increases in aldosterone and sodium production enhance the accumulation of perivascular macrophages in the heart (4) and induce the expression of collagen type I and III, interleukin 16, lymphocyte chemotactic factor, and cytotoxic T lymphocyte-associated protein 4, which are associated with arterial inflammatory lesions, fibrosis, and vascular calcification (5). Hypertension is also associated with the upregulation of C-reactive protein, an inflammatory marker that also promotes inflammation by stimulating the release of proinflammatory cytokines such as interleukin-6, interleukin-1β, and tumor necrosis factor α by monocytes and induce the expression of intracellular and vascular cell adhesion molecules by endothelial cells (6).

In the present study, we observed that hypertension increased inflammation in response to a foreign substance. The combined effect of hypertension and endodontic cement increased inflammation, as evidenced by the higher number of neutrophils in hypertensive as compared to normotensive rats. This is consistent with another report demonstrating that the effects of foreign materials on inflammation is exacerbated when there is co-existing hypertension (25).

Mineralization was evaluated by von Kossa staining and birefringence under polarized light. The former is used to detect calcium deposits and hence, mineralization area (26). On the other hand, polarized light reveals birefringent calcium carbonate particles in the tissue and as such, provides insight into the mechanism by which mineralization occurs since calcium carbonate nuclei initiate mineralization (27).

Hypertension caused less consistent mineralization, possibly due to increased mobilization of calcium from bone and calcium excretion by the kidneys under these conditions (3). Secondary activation of parathyroid hormone leads to activation of vitamin D, which stimulates the

breakdown of bone by activation and proliferation of osteoclasts; calcium release is thereby increased and is absorbed by the intestine and resorbed by the kidneys (3) owing to the high levels of angiotensin II (28-30).

Under normotensive conditions, gray MTA was associated with a smaller birefringent area at 30 as compared to 7 days. This was not surprising, since in the days after surgery, tissue is regenerating and has a larger birefringent area, which is more restricted during the healing period (31). Under hypertensive conditions, birefringence was maintained over time; this may be explained by the presence of exudate from the persistent inflammatory response, which caused ion solubilization and diffusion (32).

Greater birefringence was observed in response to white as compared to gray MTA. This can be ascribed to the different properties of the two MTAs. Studies on leakage, tissue and osteoblasts biocompatibility, pulp wound healing, and hard tissue bridge formation showed similar results for white and gray MTA (22,33). In contrast, some studies have found that gray MTA promotes the growth, adherence, and differentiation of osteoblastic cells while showing poor leakage results (34). White MTA has a lower setting time than gray MTA, which explains its higher solubility and ionic release (20,22,23,34). However, gray MTA has high levels of arsenic, tetracalcium aluminoferrite, iron, cadmium, and chromium, which can lead to rejection by tissue, inflammation, or allergic reactions and thereby compromise mineralization (20). Although these properties can induce greater calcium carbonate formation, we did not detect increased mineralization with this substance by von Kossa staining.

Mineralization in response to MTA was observed even under hypertensive conditions. MTA contains calcium oxide and calcium phosphate; the former reacts with water from tissue to form calcium hydroxide, which dissociates into calcium and hydroxyl ions. Calcium ions react in

turn with carbon dioxide in tissue to form crystals of calcite—a polymorph of calcium carbonate—that are birefringent under polarized light and are deposited in hard tissue (34). Necrotic areas may also be positive for von Kossa staining; this is consistent with the absence of polarization observed with IRM, which is toxic and causes necrosis upon direct contact with tissue but does not release calcium to form calcium carbonate.

MTA is biocompatible and exhibits good marginal sealing; in addition, it induces dentinogenesis, cementogenesis, and osteogenesis (18,19). The hydration chemistry of MTA is important dictates its chemical stability, physical durability, biocompatibility, and activities in tissue. When MTA powder is mixed with water, its components alite and belite react to form a poorly crystalline calcium silicate hydrate gel and portlandite, whereas the aluminate, ferrite, and gypsum react with water to generate ettringite, which is converted into thermodynamically stable monosulphate (35).

In conclusion, although hypertension can jeopardize tissue repair—including mineralization—and thus negatively impact endodontic treatment outcome, MTA still has satisfactory mineralization ability.

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Table 1: Inflammatory cells and mineralization area average in hypertension and normotension conditions

	Inflammatory – Hematoxylin Eosin*			
	7 days		30 days	
Material	Normotensive Mean±SD	Hypertensive Mean±SD	Normotensive Mean±SD	Hypertensive Mean±SD
MTA Grey	235.00±96.29	303.55±50.36[1]	194.18±25.41	206.22±47.82[1]
MTA White	215.38±30.69	334.25±117.61[1]	179.08±66.16	371.42±122.39[1]
IRM	177.60±58.61	213.20±39.00[1]	326.78±96.77	374.00±56.15[1]
Control	376.37±92.27	301.34±95.27	303.62±58.37	297.63±63.23
	Mineralization – Von Kossa**			
	7 days		30 days	
Material	Normotensive Mean±SD	Hypertensive Mean±SD	Normotensive Mean±SD	Hypertensive Mean±SD
MTA Grey	395447,41±121643.92	468748,73±74096.83[2]	307126,92±40447.09	315192,98±43582.08[2]
MTA White	477389,78±113190.85	288507,07±44017.33[2]	570670,05±8955.82	336474,21±29138.79[2]
IRM	155233,13±52021.17	291139,31±51986.91[2]	63600,92±25962.82	206223,83±57863.62[2]
Control	0,00±0[3]	0,00±0[3]	0,00±0[3]	0,00±0[3]
	Mineralization – Polarized Light**			
	7 days		30 days	
Material	Normotensive Mean±SD	Hypertensive Mean±SD	Normotensive Mean±SD	Hypertensive Mean±SD
MTA Grey	94564,65±19793.50	69745,84±12573.40[2]	22018,27±5074.59	65392,96±6573.77[2]
MTA White	319889,79±96245.49	216619,38±37825.46[2]	48069,21±11328.04	235522,79±35704.59[2]
IRM	0,00[4]±0	0,00±0[4]	0,00±0[4]	0,00±0[4]
Control	0,00±0[3]	0,00±0[3]	0,00±0[3]	0,00±0[3]

*Mean number of inflammatory cells (n = 6 per group). [1]Significant difference between hypertensive and normotensive groups for white MTA, gray MTA, and IRM ($P < 0.05$). **Mean area of mineralization associated with white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by von Kossa staining and polarized light microscopy (n = 6 per group). [2]Significant difference between day 7 and 30 for gray and white MTA and IRM in the hypertensive vs. normotensive group. [3]Significant difference between control and other materials. [4]Significant difference between IRM and both types of MTA.

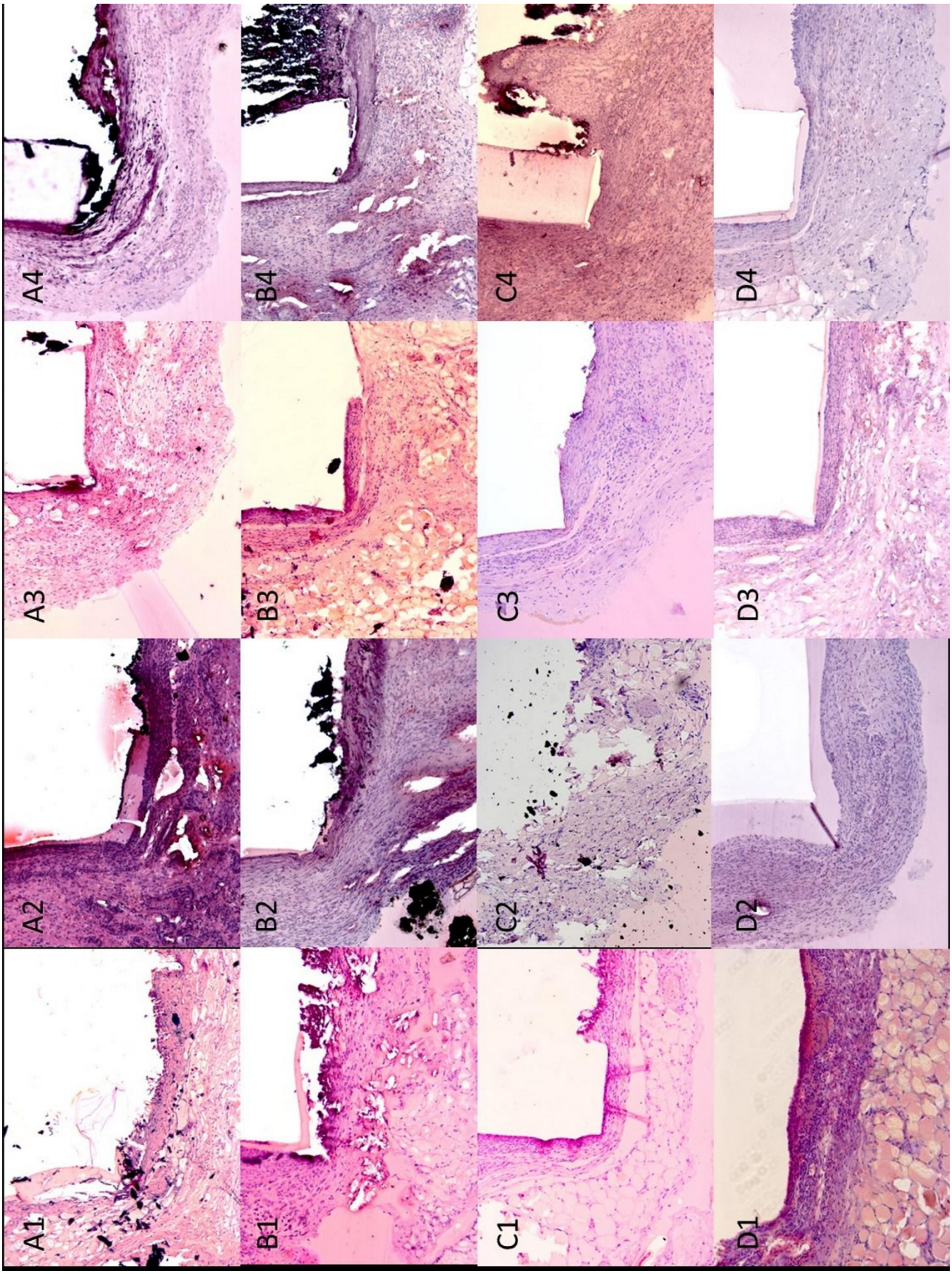
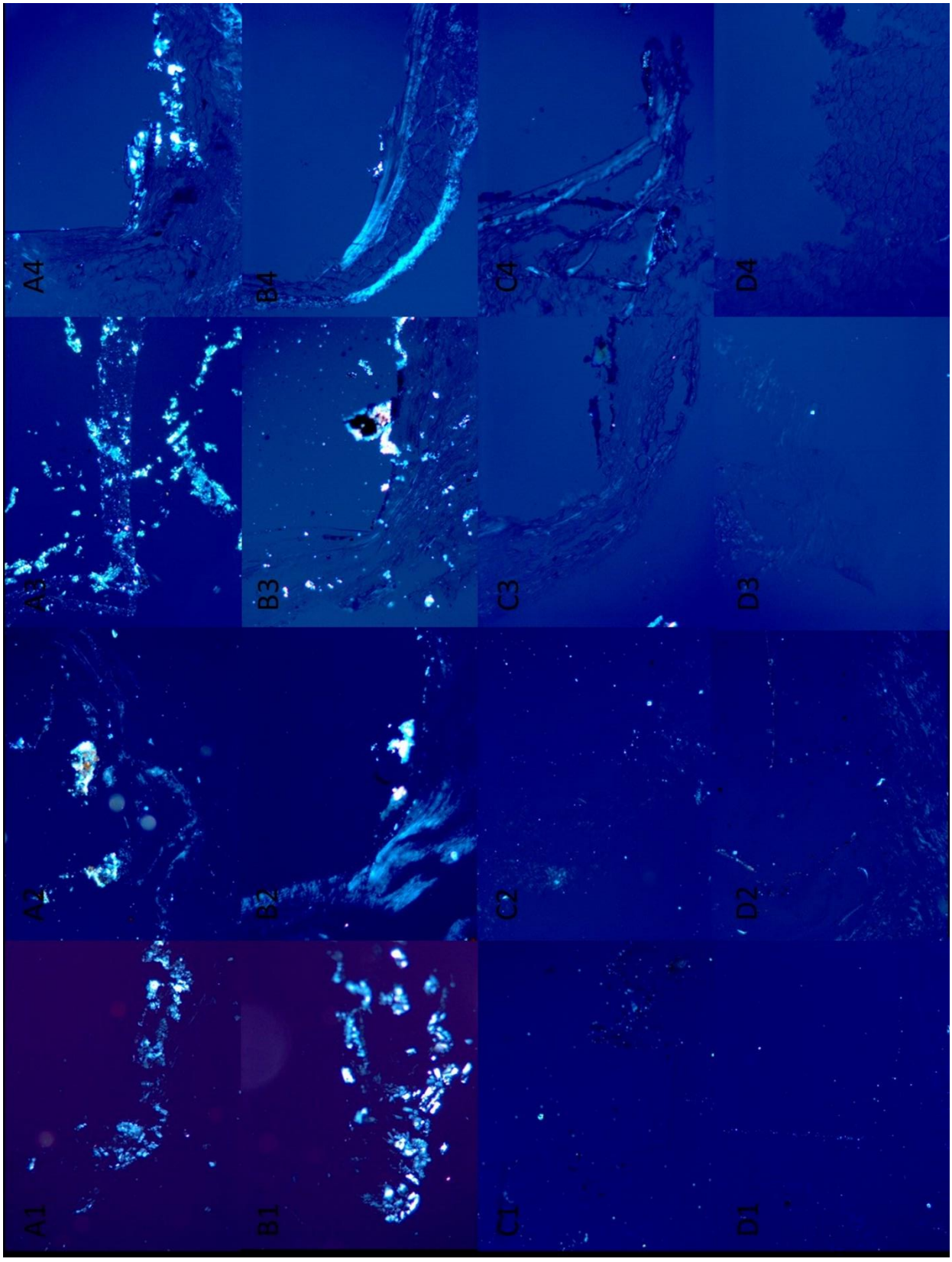

A1
A2
A3
A4
B1
B2
B3
B4
C1
C2
C3
C4
D1
D2
D3
D4

Figure 1. Inflammatory response to white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by H&E staining (n = 6 per group). A) Gray MTA; B) white MTA; C) IRM; and D) control. 1) Normotensive day 7; 2) hypertensive day 7; 3) normotensive day 30; and 4) hypertensive day 30. H&E staining (100×) shows fibrous capsule formation with infiltration of macrophages and lymphocytes. The inflammatory response was increased by hypertension and in the presence of MTA and IRM.


A4
B4
C4
D4
A3
B3
C3
D3
A2
B2
C2
D2
A1
B1
C1
D1

Figure 2. Mineralization in response to white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by von Kossa staining (100×). A) Gray MTA; B) white MTA; C) IRM; and D) control. 1) Normotensive day 7; 2) hypertensive day 7; 3) normotensive day 30; and 4) hypertensive day 30. Black areas represent mineralization. Both types of MTA induced mineralization, which was not observed for IRM or the control group; dark areas of tissue associated with IRM were necrotic. More consistent mineralization was observed in normotensive rats, especially at 30 days.


A4
B4
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A3
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A1
B1
C1
D1

Figure 3. Mineralization in response to white or gray MTA, IRM, or empty tubes at 7 and 30 days in hyper- and normotensive rats, as determined by polarized light microscopy (100×). A) Gray MTA; B) white MTA; C) IRM; and D) control. 1) Normotensive day 7; 2) hypertensive day 7; 3) normotensive day 30; and 4) hypertensive day 30. Birefringent structures in the tissue represent calcite crystals, which were observed for different types of MTA but not for IRM or the control group. More consistent birefringence was observed in normotensive rats, especially at 30 days.

Artigo 3

HYPERTENSION DECREASES IMMUNOSTAINING PATTERN OF RUNX-2, OPN AND OCN BIOMARKERS TO MINERAL TRIOXIDE AGREGATE ENDODONTIC CEMENT

ABSTRACT

Aim: This study investigated whether hypertension affects the white and gray MTA mineralization thru implanted subcutaneously into rats by bone osteoblastic biomarkers.

Methodology: Polyethylene tubes containing gray MTA, white MTA, intermediate restorative material (IRM; positive control) and an empty tube (negative control) were implanted into the dorsal connective tissue of spontaneous hypertensive (n=12) and Wistar rats (n=10). Half rats in each group were sacrificed after 7 days, and the remainder after 30 days. Tubes with surrounding tissue were removed and immunostaining was performed to detect RUNX-2, OPN and OCN proteins.

Results: Under hypertensive condition in 30 days, both MTA presented pattern of immunostaining for RUNX-2 from low to moderate, lower result compared to normal blood pressure and to 7 days ($p<0.05$). OPN and OCN protein expression to both MTA was considered low under 7 or 30 days for hypertensive condition, also lower result than normal blood pressure in 30 days ($p<0.05$). No immunostaining for all biomarkers in CONTROL and IRM groups ($p<0.05$).

Conclusion: Hypertension decreases immunostaining pattern of RUNX-2, OPN and OCN biomarkers to MTA endodontic cement. Thus, hypertension can jeopardize MTA mineralization ability and having negative impact endodontic treatment outcome.

Key words: Hypertension, Mineral Trioxide Aggregate, Periapical diseases, Calcification.

INTRODUCTION

Controlling infection and restoring apical and periapical tissues is the main goal of endodontic treatment (1). However, in some cases failure is observed, so we can resort to paraendodontic surgery for retrograde treatment with retrofilling material (2). Mineral trioxide aggregate (MTA) is an endodontic reparative cement and is the first choice in this cases (3).

Commercially we have 2 types of MTA, white and grey, and studies have shown similar results for both (4), even though it is described that gray MTA is better regarding to growth, adherence and differentiation of osteoblastic cells (5) and white MTA presents lower setting time and higher solubility and ionic release (6). In general, MTA is biocompatible and exhibits good marginal sealing and also contains calcium oxide and calcium phosphate (4,7). These substances react to tissue fluids inducing mineralization, such as dentinogenesis, cementogenesis, and osteogenesis (7-10).

Hypertension is considered an inflammatory disease that has systemic effects (11-13), including in bone metabolism, thru calcium loss, decrease trabecular bone mineral content, increase osteoclastic function (14-18). Therefore, it can be suggested that hypertension may exacerbate bone destruction in periapical lesion caused by pulp infection and decrease MTA mineralization activity.

Systolic blood pressure higher than 140 mm Hg and/or diastolic blood pressure higher than 90 mm Hg characterizes hypertension (19). It is well known that this systemic condition leads to activation and proliferation of osteoclasts, due to increased RANKL/OPG ratio mediated by angiotensin II. Also, these parathyroid hormone leads to an abnormal metabolism of Vitamin D which will increase blood calcium levels, reduce serum ionized calcium, and decrease calcium absorption (15,17,20,21).

Although this studies related in literature, there is a gap information regarding to the influence of hypertension in MTA mineralization ability. The null hypothesis is that MTA mineralization ability is not affected by hypertension. Thus, in the present study, we investigated whether hypertension affects the white and gray MTA mineralization thru implanted subcutaneously into rats by bone osteoblastic biomarkers.

MATERIAL AND METHODS

The study was approved by the Ethical Committee of Aracatuba Dental School (protocol no. 2013-00961).

Animals

Male spontaneously hypertensive (n = 12; age: 2–3 months; 250–280 g) and Wistar rats (n = 10; age: 2–3 months; 250–280 g) were used as hypertensive (experimental) and normotensive (control) animals, respectively.

Experimental material

White and gray MTA (Angelus Industry Ontological Products, Londrina, Brazil) and intermediate restorative material (IRM) (Dentsply Caulk, Milford, DE, USA) were prepared according to the manufacturers' recommendations and placed in 66 sterile polyethylene tubes (internal diameter: 1.0 mm; external diameter: 1.6 mm; length: 10 mm) (Abbott Labs of Brazil, São Paulo, Brazil) with a lentulo spiral (Maillefer Dentsply, Tulsa, OK, USA); 22 empty tubes were used as the control. Each tube was completely filled with the material.

All of this experimental material was implanted in each rat, normotensive or hypertensive. Thus, 8 groups were formed: NT-Control, an empty tube implanted in normotensive rat; NT-IRM, a polyethylene tube completely filled with IRM implanted in normotensive rat; NT-WHITE MTA, a polyethylene tube completely filled with White MTA implanted in normotensive rat; NT-GREY MTA, a polyethylene tube completely filled with Grey MTA implanted in normotensive rat; HT-Control, an empty tube implanted in hypertensive rat; HT-IRM, a polyethylene tube completely filled with IRM implanted in hypertensive rat; HT-WHITE MTA, a polyethylene tube completely filled with White MTA implanted in hypertensive rat; HT-GREY MTA, a polyethylene tube completely filled with Grey MTA implanted in hypertensive rat.

Surgical procedures

Animals were anesthetized with xylazine (10 mg/kg) (Anasedan/Vetbrands Division Animal Health, Sao Paulo, Brazil) and ketamine (25 mg/kg) (Cetamin Syntec do Brazil, São Paulo, Brazil). Their backs were shaved and the skin disinfected with 5% iodine solution; a 2-cm incision was made from head to tail with a #15 Bard-Parker blade (Becton-Dickinson, Franklin Lakes, NJ, USA). Four pockets were created on each side of the incision into which three tubes (filled with white or gray MTA or IRM) and an empty tube were implanted. The skin was closed using Ethicon 4.0 silk sutures (Johnson & Johnson, Sao Paulo, Brazil) (22).

Immunohistochemical analysis

At 7 and 30 days after tube implantation, animals were euthanized by an overdose of anesthetic and the tubes with surrounding tissue were removed and fixed in 10% formalin solution (pH 7.0), cut transversely into two halves that were each cut longitudinally using a sharp blade to allow

contact between the surfaces and processing solutions. Specimens were embedded paraffin. Serial sections 5µm thick were deparaffinized in xylene and hydrated in decreasing ethanol series (100º - 100º - 100º - 90º - 70 ° GL). Antigen retrieval was performed by immersion of histological slides in citrate buffer (Diva decloaker®, Biocare Medical, Concord, CA, EUA), in pressurized chamber (Decloaking chamber®, Biocare Medical, Concord, CA, EUA) at 95°C for 20 minutes. At each immunohistochemistry stage, histological sections were washed in 0.1M PBS, pH 7.4. Thereafter, sections were immersed in 3% hydrogen peroxide for 1 hour and 1% bovine serum albumin for 12 hours to block endogenous peroxidase activity and to block nonspecific sites, respectively. Sections containing samples from each experimental group were divided into three batches and each batch was incubated with the following primary antibodies: anti-RUNX-2 mouse generated in rabbit (SC-10758, Santa Cruz Biotechnology, Santa Cruz, CA, EUA), anti-OCN mouse generated in goat (SC-18319, Santa Cruz Biotechnology, Santa Cruz, CA, EUA) and anti-OPN mouse generated in goat (SC-18319, Santa Cruz Biotechnology, Santa Cruz, CA, EUA). The sections were incubated with biotinylated secondary antibody for 2 hours and subsequently treated with streptavidin conjugated with horseradish peroxidase - HRP for 1 hour (Universal Dako Labeled HRP Streptavidin-Biotin Kit®, Dako Laboratories, CA, EUA). The revelation was performed using as the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB chromogen Kit®, Dako Laboratories, CA, EUA). Staining was performed with Fast Green for RUNX-2, and Harris hematoxylin for OPN and OCN, and then dehydration in ethanol, diaphanization in xylene and coating with mounting means (Permount, Fisher Scientific, San Diego, CA, USA) and glass coverslip. As negative control, were performed all procedures previously described suppressing the use of primary antibody in specimens.

A certified histologist (EE), blinded to the treatments, performed the immunohistochemical analyses. A semiquantitative analysis of RUNX-2, OPN and OCN immunostaining was performed at ×400 magnification in optic microscope (Axiolab, Carl Zeiss). Immunostaining was defined by brown color present in nucleus for RUNX-2, and in cytosolic and extracellular compartment in the case of OPN and OCN. Three histologic sections from each animal were used. A semi-quantitative analysis of immunostaining for three areas 600µm x 800µm each histological section was made. The criteria for immunostaining pattern was based as previously described (23): Score 0 - no immunostaining (total absence of immunoreactive cells), Score 1 - low standard immunostaining, Score 2 - moderate standard immunostaining and Score 3 - high standard immunostaining.

Statistical analysis

Data were analyzed using the BioEstat software (5.0, Manaus, AM, Brazil). The normality of data was analyzed using the Shapiro-Wilk test. When a significant difference was detected by Mann-Whitney, multiple comparisons were performed using Tukey's test ($p<0.05$).

RESULTS

Decreased immunostaining pattern of RUNX-2, OPN and OCN biomarkers against hypertension

Immunoreactive cells to RUNX-2 presented brown color in cell nucleus. Immunoreactive cells to OCN and OPN showed dark brown color confined to cytoplasm, however, extracellular matrix also showed a weak markup.

No immunostaining for all biomarkers in CONTROL and IRM groups, regardless of experimental period or blood pressure level ($p<0.05$).

The results for RUNX-2 are shown in Figure 1. For RUNX-2 protein, WHITE and GREY MTA groups, both in normotensive and hypertensive condition, moderate pattern of immunostaining prevailed after 7 days, same to WHITE and GREY MTA groups into normotensive condition at 30 days ($p>0.05$). At same experimental period but into hypertensive condition, WHITE and GREY MTA groups presented lower standard comparing to normotensive condition and comparing to period of 7 days ($p<0.05$), ranged from low to moderate standard immunostaining.

The results for OPN and OCN are shown in Figure 2 and 3, respectively. For both biomarkers, OPN and OCN proteins, WHITE and GREY MTA groups, regardless of systemic condition, prevailed a low standard of immunostaining after 7 days, differing statistically from the analysis after 30 days in normotensive condition ($p<0.05$). At 30 days, WHITE and GREY MTA groups into normotensive condition was ranged from moderate to high for both biomarkers. In hypertension condition, for WHITE MTA group at 30 days the response was lower ($p<0.05$), standard immunostaining ranged from low to moderate, while for MTA GREY group was low standard ($p<0.05$).

DISCUSSION

This study evaluated the effect of hypertension on MTA mineralization ability by RUNX-2, OPN and OCN immunostaining. According to the results, we rejected null hypothesis, because hypertension decreases immunostaining for these proteins, influencing negatively on MTA mineralization capacity.

The following 3 biomarkers, RUNX-2, OPN and OCN were chosen for examination of osteogenic differentiation in the present study. RUNX-2 is a transcription factor involved in

osteoblastic differentiation and skeletal morphogenesis (24). This protein is known to be early marker of osteoblastic differentiation (24). RUNX-2 transcription factor has been shown to effectuate the expression of OPN and OCN, which are expressed later in the differentiation process (25).

The immunohistochemical technique used to detect RUNX-2, OPN and OCN showed high specificity in the detection of such proteins, proved by total staining absence in negative control of immunohistochemistry reaction.

MTA is formulated with calcium oxide, calcium phosphate, arsenic, tetracalcium aluminoferrite, iron, cadmium, and chromium, and they are responsible to stimulate mineralization (5,6). Calcium oxide reacts with water in tissue fluids to form calcium hydroxide, which further dissociates into calcium and hydroxyl ions (26). The calcium ions react with carbon dioxide in tissues to form crystals of calcite, a polymorph of calcium carbonate, which will stimulate the deposition of hard tissue (27).

Despite these MTA properties, under hypertensive condition in 30 days, the pattern of immunostaining for RUNX-2 decreased compared to normal blood pressure and to 7 days. This result indicates that hypertension not allows early osteogenic differentiation. RUNX-2 can directly stimulate the transcription of osteoblast-related genes, such as those encoding OPN and OCN (24,25). In our study, in the presence of MTA, OPN and OCN protein expression was considered low under 7 or 30 days for hypertensive condition, different result for normal blood pressure in 30 days, which presented high expression.

These findings can be explained because hypertension can affect bone mineral density, mineralization and bone modeling (20) due to Angiotensin II mediation which lead to abnormal metabolism of 1,25-dihydroxy vitamin D3 and, as consequence, calcium is excreted by the body.

Also, Angiotensin II can activate osteoclasts by up-regulating RANKL expression, which binds to RANK - a receptor expressed on the surface of osteoclast precursors – initiating a signaling cascade, which will result in activation of NF-κB and differentiation and activation of osteoclasts (28). By this way, with more presence of osteoclast, less presence of osteoblast.

Regardless of experimental period or blood pressure level, no immunostaining for all biomarkers were seen in CONTROL and IRM groups. This result was expected, once both groups do not have mineralization induce products. Control group consist in empty tubes implanted in subcutaneous mice and IRM is basically composed of Zinc Oxide, Polymethyl Methyl, Eugenol and Acetic Acid (3).

CONCLUSION

In conclusion, hypertension can jeopardize MTA mineralization ability. Thus, hypertension may have a negatively impact endodontic treatment outcome.

Acknowledgement: The authors deny any conflicts of interest.

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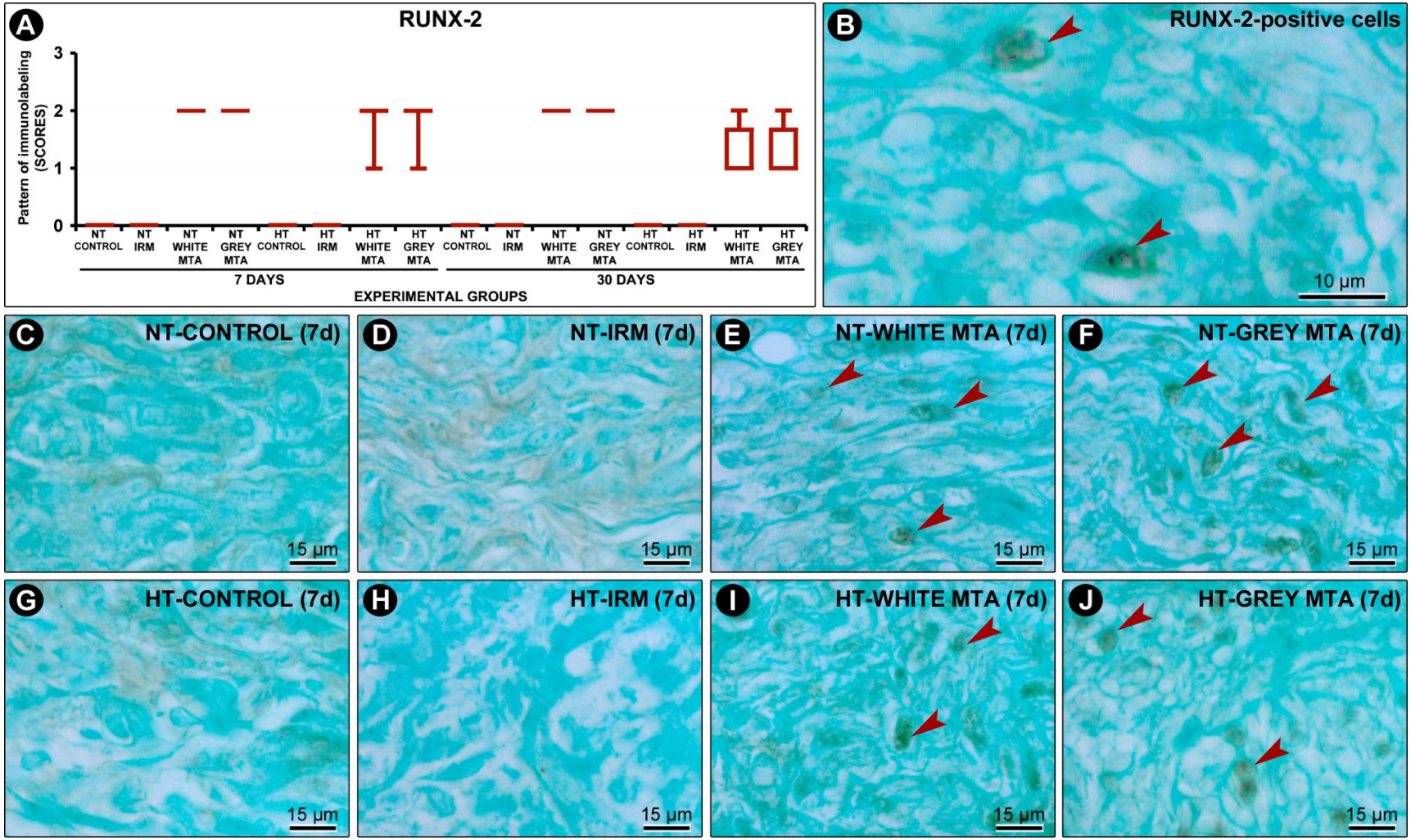
A
RUNX-2
Pattern of immunolabeling (SCORES)
3
2
1
0
NT CONTROL
NT IRM
NT WHITE MTA
NT GREY MTA
HT CONTROL
HT IRM
HT WHITE MTA
HT GREY MTA
NT CONTROL
NT IRM
NT WHITE MTA
NT GREY MTA
HT CONTROL
HT IRM
HT WHITE MTA
HT GREY MTA
7 DAYS
30 DAYS
EXPERIMENTAL GROUPS
B
RUNX-2-positive cells
10 µm
C
NT-CONTROL (7d)
15 µm
D
NT-IRM (7d)
15 µm
E
NT-WHITE MTA (7d)
15 µm
F
NT-GREY MTA (7d)
15 µm
G
HT-CONTROL (7d)
15 µm
H
HT-IRM (7d)
15 µm
I
HT-WHITE MTA (7d)
15 µm
J
HT-GREY MTA (7d)
15 µm

Figure 1. RUNX-2 immunostaining pattern. (A) Graph about the pattern of immunelabeling in differents experimental groups for RUNX-2 protein. **(B)** Photomicrograph showing RUNX-2 cells - immunoreactive (RUNX2-IR) (red arrows). **(C-J)** Photomicrographs showing immunostaining for RUNX-2 at 7 days post-implantation of the empty tube or cement content in NT-CONTROL (C), SHR-CONTROL (D), NT-IRM (E), SHR-IRM (F), NT-WHITE MTA (G), SHR-WHITE MTA (M), NT-GREY MTA (I) and SHR-GREY MTA (J). **Original magnification:** B: 5000x; C-J: 1000x. Scale bars: B: 10 micrometres; C-J: 25 micrometers. **Counter-staining:** *Fast Green*.

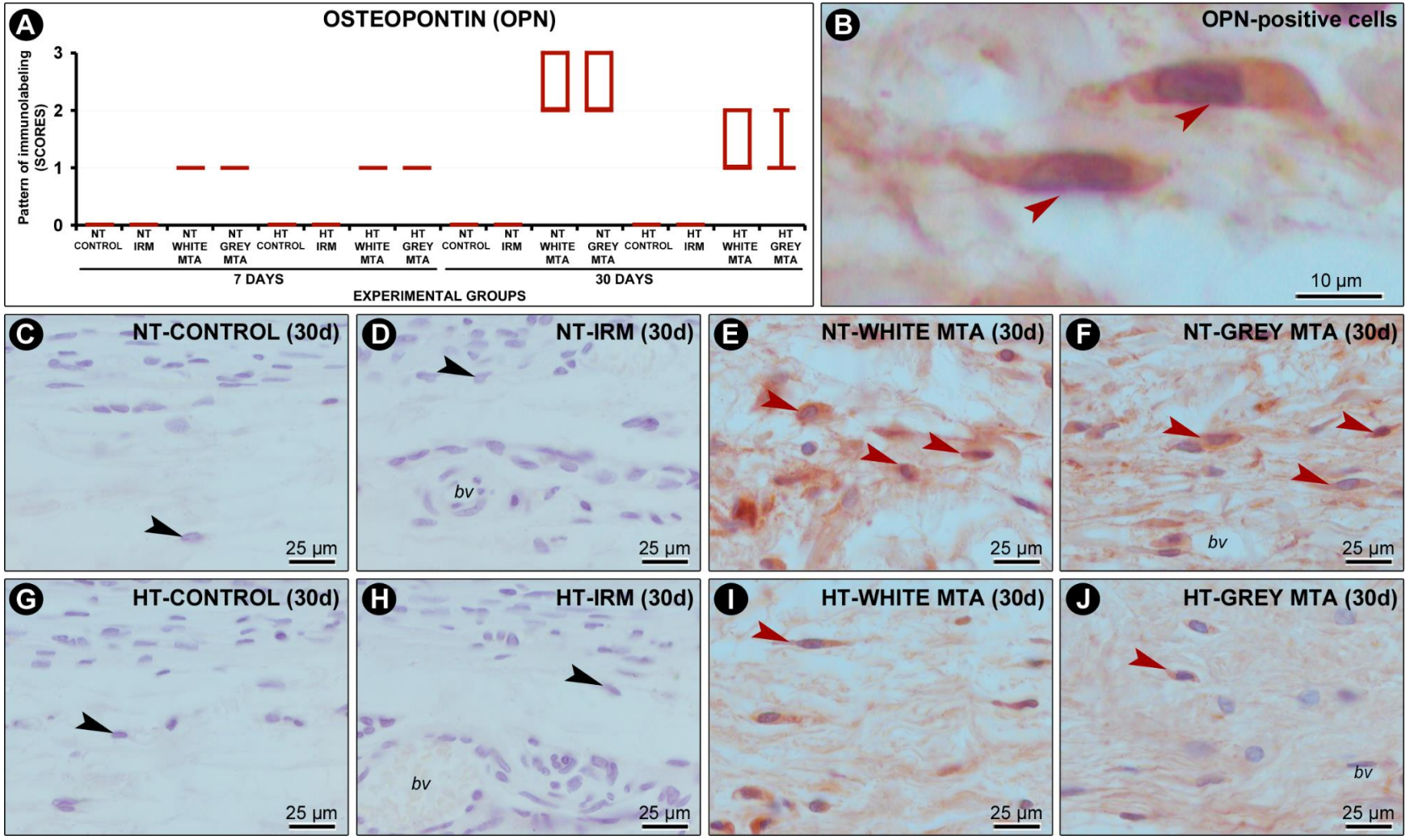
A
OSTEOPONTIN (OPN)
Pattern of immunolabeling (SCORES)
3
2
1
0
NT CONTROL
NT IRM
NT WHITE MTA
NT GREY MTA
HT CONTROL
HT IRM
HT WHITE MTA
HT GREY MTA
NT CONTROL
NT IRM
NT WHITE MTA
NT GREY MTA
HT CONTROL
HT IRM
HT WHITE MTA
HT GREY MTA
7 DAYS
30 DAYS
EXPERIMENTAL GROUPS
B
OPN-positive cells
10 µm
C
NT-CONTROL (30d)
25 µm
D
NT-IRM (30d)
bv
25 µm
E
NT-WHITE MTA (30d)
25 µm
F
NT-GREY MTA (30d)
bv
25 µm
G
HT-CONTROL (30d)
25 µm
H
HT-IRM (30d)
bv
25 µm
I
HT-WHITE MTA (30d)
25 µm
J
HT-GREY MTA (30d)
bv
25 µm

Figure 2. OPN immunostaining pattern. (A) Graph about the pattern of immunelabeling in differents experimental groups for OPN protein. **(B)** Photomicrograph showing osteopontin cells - immunoreactive (OPN-IR) (red arrows). **(C-J)** Photomicrographs showing immunostaining for OPN at 30 days post-implantation of the empty tube or cement content in NT-CONTROL (C), SHR-CONTROL (D), NT-IRM (E), SHR-IRM (F), NT-WHITE MTA (G), SHR-WHITE MTA (M), NT-GREY MTA (I) and SHR-GREY MTA (J). **bv:** blood vessels. **Original magnification:** B: 5000x; C-J: 1000x. Scale bars: B: 10 micrometres; C-J: 25 micrometers. **Counter-staining:** *Harris hematoxylin*.

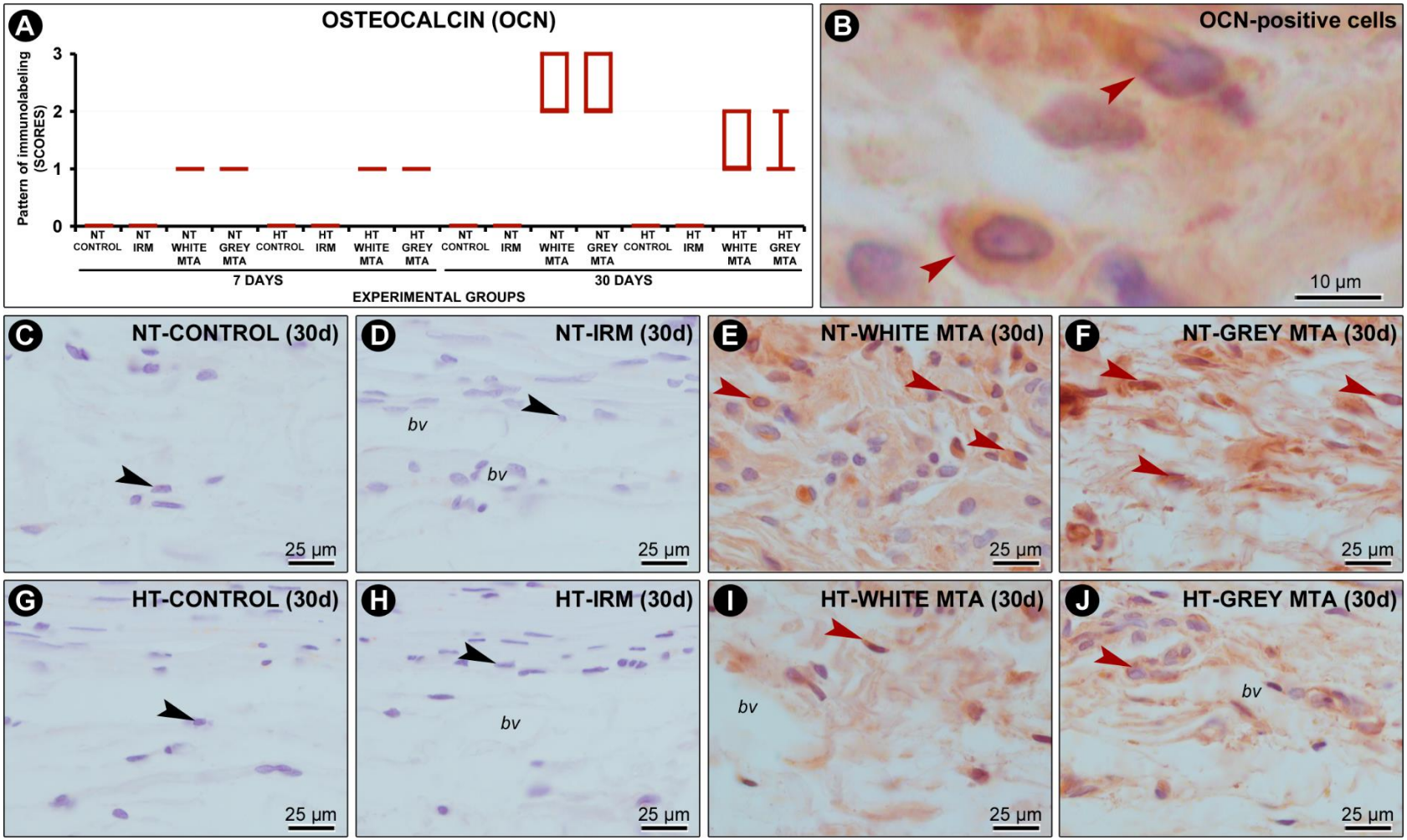
A
OSTEOCALCIN (OCN)
Pattern of immunolabeling (SCORES)
3
2
1
0
NT CONTROL
NT IRM
NT WHITE MTA
NT GREY MTA
HT CONTROL
HT IRM
HT WHITE MTA
HT GREY MTA
NT CONTROL
NT IRM
NT WHITE MTA
NT GREY MTA
HT CONTROL
HT IRM
HT WHITE MTA
HT GREY MTA
7 DAYS
30 DAYS
EXPERIMENTAL GROUPS
B
OCN-positive cells
10 µm
C
NT-CONTROL (30d)
25 µm
D
NT-IRM (30d)
bv
bv
25 µm
E
NT-WHITE MTA (30d)
25 µm
F
NT-GREY MTA (30d)
25 µm
G
HT-CONTROL (30d)
25 µm
H
HT-IRM (30d)
bv
25 µm
I
HT-WHITE MTA (30d)
bv
25 µm
J
HT-GREY MTA (30d)
bv
25 µm

Figure 3. OCN immunostaining pattern. (A) Graph about the pattern of immunelabeling in differents experimental groups for OCN protein. **(B)** Photomicrograph showing osteocalcin cells - immunoreactive (OCN-IR) (red arrows). **(C-J)** Photomicrographs showing immunostaining for OCN at 30 days post-implantation of the empty tube or cement content in NT-CONTROL (C), SHR-CONTROL (D), NT-IRM (E), SHR-IRM (F), NT-WHITE MTA (G), SHR-WHITE MTA (M), NT-GREY MTA (I) and SHR-GREY MTA (J). **bv**: blood vessels. **Original magnification:** B: 5000x; C-J: 1000x. Scale bars: B: 10 micrometres; C-J: 25 micrometers. **Counter-staining:** *Harris hematoxylin*.

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Anexo I –

Metodologias Detalhadas

1- Manejo dos camundongos

Foram utilizadas duas linhagens diferentes de camundongos. A linhagem com a pressão arterial normal (BPN/3J) e a linhagem com a pressão arterial elevada (BPH/2J) que foram adquiridos do Jackson's Laboratory (Bar Harbor, Maine, USA).

Os camundongos foram mantidos em microisoladores com alimentação balanceada e água filtrada (Figura 1).

Foram adquiridos dois camundongos machos e duas fêmeas de cada linhagem diferente. Esses camundongos foram cruzados a fim de se conseguir a quantidade necessária para a realização dos experimentos.

Quando do nascimento dos novos camundongos, os machos foram separados e colocados em gaiolas diferentes. A fêmea foi mantida com a sua prole até a quarta semana, quando, então, foram separados. Vale ressaltar que durante o manejo da progenitora e dos bebês deve-se ter muito cuidado, pois o contato humano na prole pode fazer com que a progenitora entenda que os bebês estão alterados, fazendo com que a fêmea mate seus filhos.

Figura 1: Manejo dos camundongos. Disposição dos microisoladores e a gaiola dos camundongos.

2- Metodologia de diferenciação de osteoclastos a partir de células de medula óssea

Células de medula óssea (Bone Marrow Cells) foram isoladas a partir do fêmur dos camundongos das diferentes linhagens, normotensos (BPN/3J) e hipertensos (BPH/2J). Os camundongos tinham 7 semanas de idade.

Os ratos foram sacrificados em câmara de gás e após o sacrifício os ratos foram descontaminados com álcool 70% e a extração celular foi realizada em fluxo laminar.

Para dissecar o fêmur, inicialmente deve-se cortar a pele e divulsionar o músculo do osso. Dessa forma encontrar-se-á a junção do fêmur com a pelvis, que deve ser cortada delicadamente a fim de se conseguir separar o osso do restante do corpo. Deve-se remover a maior quantidade possível do músculo do fêmur e encontrar a junção tíbia e fíbula e separar esses ossos. Deve-se tomar muito cuidado para não quebrar o fêmur. Após isso, deve ser realizado o corte das extremidades do osso com uma tesoura para ter acesso a medula óssea.

Após isso, deve-se lavar a medula óssea usando uma agulha e seringa contendo alpha-MEM suplementado com 10% de soro fetal bovino. A quantidade de meio a ser utilizado por fêmur é de 1mL, sendo metade em uma direção e a outra metade na outra direção. Colocar esse meio que lavou a medula em um tubo falcon.

As células devem ser mantidas em gelo até o posterior processamento, mas evitar ficar muito tempo esperando. Quando for realizar o processamento, deve-se completar o 1mL de meio com mais 4, a fim de ficar em um total de 5mL por tubo. Esses tubos devem ser centrifugados durante 5 minutos em 1500 RPM. Deve-se verter o tubo e adicionar 5mL de solução de ACK e esperar 3-5 min a temperatura ambiente, para eliminar possíveis hemácias e outras células. Centrifugar novamente por 5 minutos a 1500 RPM. Deve-se ressuspender as células em 5mL de alpha-MEM suplementado com FBS e posteriormente colocar 5mL de Histopaque no fundo do

tubo, para que por densidade as células sejam isoladas. Centrifugar novamente por 20 minutos com 1500 RPM. Deve-se transferir o pelet formado para um novo tubo falcon e lavar as células para remover o histopaque que é tóxico. Para a lavagem, ressuspender as células com 5mL de PBS e centrifugar novamente por 5 min a 1500 RPM. Verter o tubo, resusspender as células em 5mL de alpha-MEM com FBS e 50ng/mL de M-CSF e fazer a contagem celular para o ajuste da concentração com o auxílio da câmara de Neubauer. A concentração final deve ser de 1,5x106 células/µL.

Lembrar que todos os materiais devem estar estéreis.

As células, nessa concentração celular, foram distribuídas em 40 poços da placa de 96 poços. As células foram então incubadas durante 13 dias, sendo que nos 5 primeiros dias em α-MEM contendo 10% de FBS inativado com M-CSF (50 ng/mL). Após a incubação, as células aderentes foram considerados macrófagos-derivados da medula óssea (BMMs). Os BMMs foram cultivadas na presença de M-CSF (25 ng/ml) e RANKL (100 ng/ml) durante 8 dias. A diferenciação osteoclástica foi avaliada através da medição da atividade de fosfatase ácida resistente a tartarato (TRAP) como um marcador de diferenciação precoce.

Para a coloração com o TRAP, deve-se realizar a solução de coloração: 10mL de solução de ácido acético; 0,1mL de solução de NNDimetil-Formamide; 1mg de ASMX; 5mg de fast red. Misturar todas essas soluções com um agitador até a solução se tornar homogênea.

Enquanto mistura essa solução, preparar as células para a coloração.

Deve-se lavar uma vez as células com PBS. Para isso, deve-se remover quase todo o meio das células (80%) e adicionar os 80% de PBS. Esse procedimento deve ser feito bem lentamente, para não desgrudar as células do fundo dos poços. Além disso, não se deve fazer os 40 poços de

uma só vez, pois dessa forma as células ficam muito secas e podem desgrudar do fundo do poço. Fazer no máximo 5 poços por vez.

Após isso, remover todo o PBS e adicionar lentamente o paraformoldeído e manter por 5 minutos. Lavar as células com PBS por duas vezes, no entanto, como as células estão fixadas, pode-se retirar todo líquido e adicionar novo PBS para lavar. Deve-se colocar a solução 1:1 de etanol-acetona e deixar durante 1 minuto. Deve-se remover a solução e esperar 1 minuto para a secagem dos poços.

Neste momento, as células estão preparadas para receber a coloração de TRAP. Então, deve-se adicionar a solução de TRAP que estava sendo agitada e deixar agir por 10 min. Findado esse processo, as células estão coradas e as células que possuíam mais de três núcleos TRAP-positivas foram consideradas osteoclastos (Figura 2).

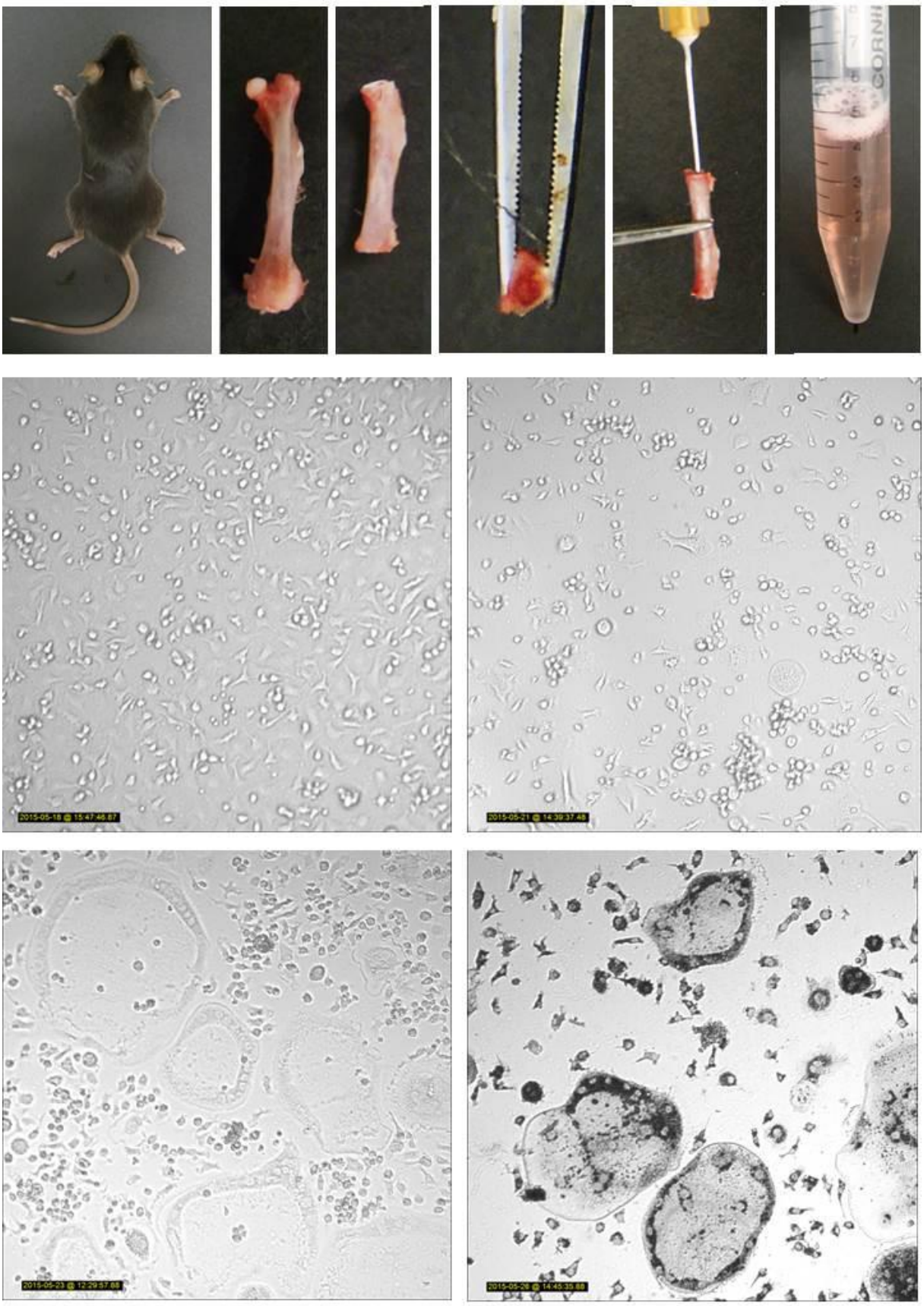

Figura 2: Metodologia de diferenciação de osteoclastos a partir de células de medula óssea. Camundongo sacrificado; Osso longo fêmur; Fêmur com as extremidades cortadas; Observação da medula óssea; Lavagem da medula óssea com o meio de cultura celular; Meio de cultura celular contendo as células em tubo falcon; Células indiferenciadas após o ajuste da concentração celular; Células após 5 dias de incubação com meio de cultura alpha-MEM, FBS e M-CSF; Células após 8 dias de incubação com estímulo do RANKL; Osteoclastos diferenciados após a coloração com TRAP.

3- Estimulação lesão periapical

Foram usados camundongos fêmeas e machos com 7 semanas de idade. Os ratos foram anestesiados através de injeção intraperitoneal com cetamina (80 mg/kg) e xilazina (10 mg/kg) e foram colocados sobre uma mesa de retração mandibular para manter a boca do camundongo aberta. As polpas dentárias de ambos os primeiros molares inferiores foram expostas usando uma peça de mão dentária elétrica com uma broca de número 1/4 broca redonda sob um microscópio cirúrgico. As câmaras pulpares foram abertas até que as entradas dos canais pudessem ser visualizadas. As polpas radiculares foram desorganizadas com uma lima endodôntica tamanho 6.

Após 21 dias da exposição pulpar, os ratos foram sacrificados utilizando a câmara de gás de CO2 e as mandíbulas foram isoladas e dissecadas a fim de que se ficasse livre de tecido mole. As hemimandíbulas direitas foram fixadas em paraformaldeído a 4% e posteriormente em PBS. As amostras foram digitalizados por Microtomografia computadorizada para analisar o tamanho da lesão periapical. As hemimandíbulas esquerdas foram imediatamente congeladas para posterior extração de proteínas e análise da expressão de citocinas pró-inflamatórias (Figura 3).

Figura 3: Estimulação da lesão periapical. Camundongo anestesiado e posicionado na mesa de retração mandibular; Seleção do primeiro molar inferior; Polpa do primeiro molar inferior exposta; Mandibula dissecada após 21 dias da exposição pulpar; Hemimandíbula; Microtomografia computadorizada destacando a abertura coronária suficiente e a formação da lesão periapical.

4- Análise do osso geral e fenótipo lesão periapical

Após a fixação, o paraformaldeído foi reduzido por meio da remoção dele e da colocação da peça em água destilada por pelo menos 1 hora antes do escaneamento. As amostras foram

escaneadas em Microtomografia computadorizada. Os ângulos da imagem foram ajustados utilizando o programa ImageJ e o tamanho da lesão periapical foi medido pelo programa Adobe Photoshop C56, no qual o tamanho da lesão periapical foi medido em micrômetros quadrados (Figura 4).

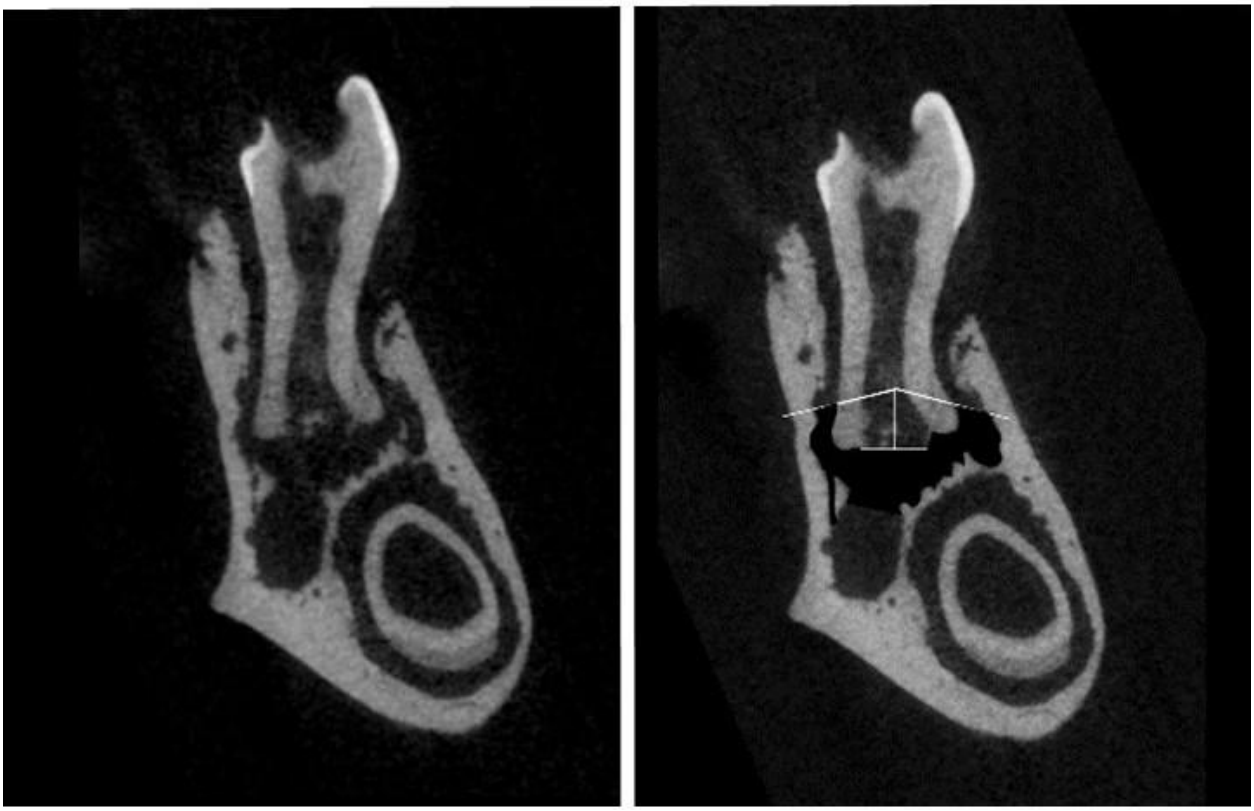

Figura 4: Análise do osso geral e fenótipo lesão periapical. Após o ajuste dos ângulos para o detalhe da raíz distal do primeiro molar inferir; Template para a análise e mensuração do tamanho da lesão periapical.

5- Expressão de citoquinas pró-inflamatórias de proteínas lesão periapical

Foram utilizadas 5 amostras de cada estado sistêmico. Cada hemimandíbula após dissecada foi imediatamente congelada (-80) em tubos de ependorff. Para a extração de proteínas, uma amostra de cada vez foi colocada em gelo seco (-60) e foi feita a redução da peça para que a peça

ficasse mais permeável ao Prechill RNA late. Cortar metade do ramo ascendente da mandíbula e o incisivo. A peça foi retornada ao ependorff e adicionado 300-500µL de Prechilll RNA late. Manter as amostras overnight em freezer -20.

Após esse período, é necessária a realização da remoção do tecido mole e da maior redução da peça. Para isso, as amostras que extavam no freezer são colocadas em uma caixa com gelo. No microscópio invertido, colocar uma bolsa de gelo para manter a peça fria durante o procedimento. Colocar uma placa sobre a bolsa de gelo e comece a remoção do tecido mole. Com o bisturi, corte o tecido mole abaixo da gengiva livre, remova o tecido da mandíbula na porção vestibular, remova o tecido da porção lingual e remova a gengiva ao redor dos dentes. Se esse procedimento demorar muito, coloque 50µL do RNA prechill late, para a peça não ficar seca.

Após isso, deve-se reduzir ainda mais a peça. Para isso deve-se cortar no terceiro molar e um pouco distante primeiro molar (dessa forma a raiz distal do primeiro molar fica no centro da peça). Retornar a peça ao Precill RNA late.

Pode-se prosseguir a extração de proteína no mesmo dia. Deve-se pesar as amostras. Para isso, pesa-se o tubo ependorff vazio e depois contendo a peça e por conta simples de subtração, se tem o peso das peças.

Para a extração de proteína, pegar um tubo contendo grânulos no interior, colocar duas cápsulas de ¼ Ceramic SPhere, adicionar a sua peça e 800µL de solução Lysis contendo gentamicina (Lysis a 1% e gentamicina 50µg/mL). Após isso, ir à máquina para quebrar a peça. As especificações da máquina são: 410m/s; CY 24x2 por 20 segundos. Tem que fazer isso duas vezes, e esperar 5 minutos entre as vezes e as peças devem ser mantidas em gelo durante esse tempo.

Após isso, centrifugar a 1500RPM por 1 minuto, para reduzir as bolhas. Adicionar 500µL de água pura, homogeneizar e colocar no vórtex – isso para diminuir a concentração da Lysis. Trasferir 500µL dessa solução para um tubo que contem membrana para filtrar e centrifugar por apenas 15 segundos. Adicionar mais 500µL restante nesse tubo e centrifugar novamente.

Fazer alíquotas dessa solução. A melhor forma é colocar as alíquotas em uma placa de fazer ELISA, colocando 300µL em cada poço e congelar em -80. Com essas amostras, pode-se fazer o teste de ELISA. Para o teste de ELISA, não é necessário fazer em triplicata, pois o valor será exato.

Com o sobrenadante que foi recolhido e congelado após centrifugação, foram realizados ensaios de citocinas por meio dos kits de ELISA obtidos a partir de R & D Systems (DuoSet) e foram usadas de acordo com as instruções do fabricante para avaliar os níveis das interleucinas IL-1α, IL-1β e TNF-α nos os tecidos periapicais. A concentração de cada citocina foi calculada com referência a uma curva padrão construída usando citocinas recombinantes proporcionadas em cada kit. Os resultados foram expressos como picogramas de citocinas por miligramas de tecidos periapicais (Figura 5).

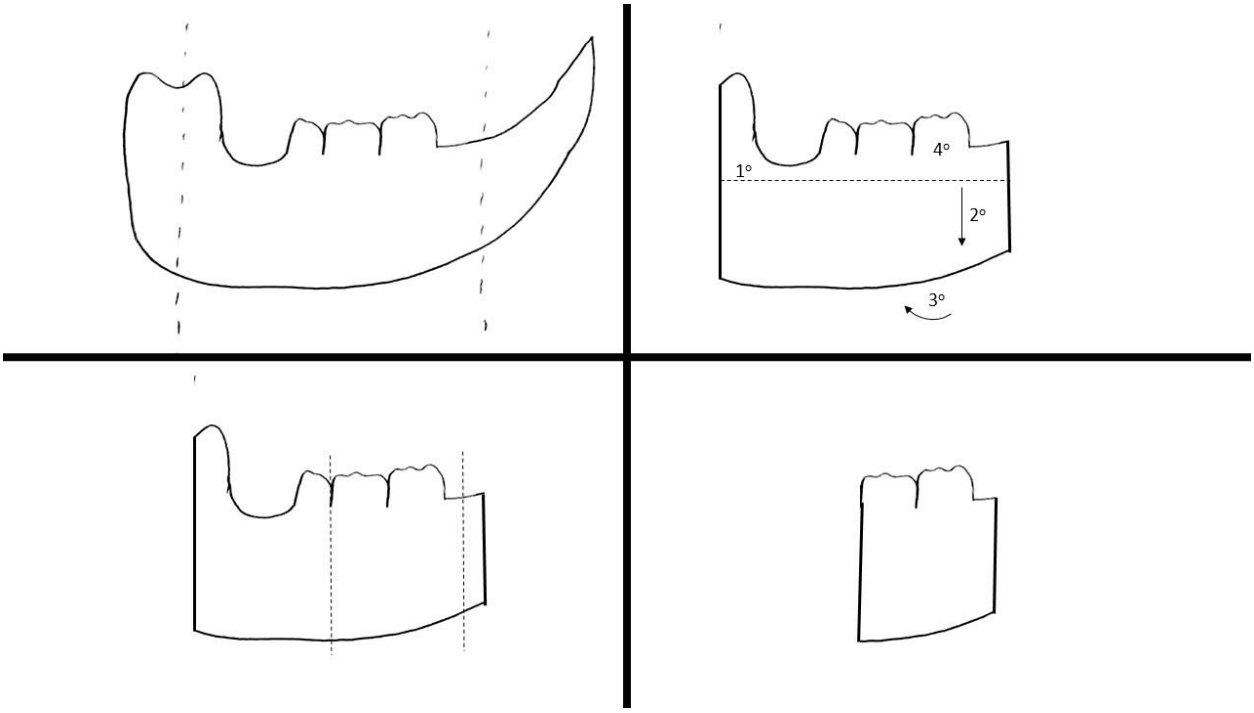

Figura 5: Expressão de citoquinas pró-inflamatórias de proteínas lesão periapical. Hemimandíbula com esquema mostrando o local da primeira redução da peça; Esquema mostrando a sequência para a remoção dos tecidos moles; Esquema mostrando os locais da segunda redução da peça; Peça pronta para ser avaliada.

6- Manejo dos ratos

Foram utilizados ratos machos espontaneamente hipertensos (SHR) e ratos machos normotensos Wistar, com idades aproximadas de 30 dias, pesando aproximadamente 250g, sendo provenientes do biotério da Faculdade de Odontologia de Araçatuba UNESP.

Os animais foram mantidos em ambientes com temperatura entre 22°C e 24°C, com ciclo de luz controlada (12 horas claro e 12 horas escuro) e em gaiolas coletivas, contendo seis ratos por gaiola. No interior das gaiolas havia cama de serragem, a qual era trocada diariamente. Esses

animais foram alimentados antes e durante todo o período experimental, com ração sólida triturada e água “ad libitum”, exceto nas primeiras 12 horas pré e pós-operatórias.

7- Materiais Experimentais

Foram empregados os cimentos reparadores MTA Branco e Cinza da Ângelus® e o cimento IRM®.

O Ângelus MTA® é um cimento composto de óxidos minerais na forma de finas partículas hidrofílicas. Apresenta na sua composição SiO2, K2O, Al2O3, Na2O, Fe2O3, SO3, CaO, Bi2O3 e MgO. Além de resíduos insolúveis de sílica cristalina, óxido de cálcio e sulfatos de potássio e sódio. O seu tempo de presa inicial, segundo o fabricante, é de aproximadamente 10 minutos e o final de 15 minutos.

O cimento IRM® é composto por pó e líquido, sendo que o pó apresenta Óxido de Zinco e Polimetacrilato de Metila e o líquido compreende Eugenol 99,5% e Ácido Acético 0,5%. Para seu preparo, a proporção de 6/1 em peso deve ser mantida. Para a espatulação, use uma técnica que misture rápida e completamente 50% do pó com o líquido. Leve o pó remanescente à mistura em 2 ou 3 acréscimos e espatule completamente. A mistura será bastante consistente e deverá ser esfregada vigorosamente por 5 a 10 segundos. Assim processada, a mistura terá uma ótima consistência de trabalho, sendo macia e adaptável. A espatulação deverá ser completada em aproximadamente 1 minuto.

8- Tubos de Polietileno

Foram utilizados tubos de polietileno estéreis com 1,0 mm de diâmetro interno e 1,6 mm de diâmetro externo e 10,0 mm de comprimento. Os materiais, depois de manipulados segundo as

recomendações dos fabricantes, foram introduzidos nos tubos de polietileno de forma a preenchê-los completamente. No grupo controle, os tubos permaneceram vazios (Figura 6).

9- Cirurgia de implante dos tubos do dorso dos ratos

Foram empregados como sedativo Dopaser, a base de xilazina (relaxante muscular, analgésico e sedativo) na dosagem de 10 mg/Kg de peso corporal e como anestésico o Vertanacol, a base de cloridrato de ketamina a 5% na dosagem de 25 mg/Kg de peso corporal.

Para a realização das intervenções cirúrgicas, os animais foram inicialmente pesados e anestesiados. O período de trabalho com cada animal não ultrapassou 5 minutos para não haver necessidade de complementação anestésica.

Após a anestesia de todos os ratos, foi feita a tricotomia da região dorsal e a lavagem da área com a finalidade de se evitar contaminação do campo cirúrgico pela presença de pêlos. A anti-sepsia da área desprovida de pêlos foi efetuada esfregando uma gaze embebida em solução aquosa 10% de PVPI por dois minutos. Em seguida, iniciou-se o procedimento cirúrgico, fazendo-se uma incisão com lâmina de bisturi número 155, no dorso do animal, (tendo a coluna vertebral como linha média de marcação). Foram criadas quatro bolsas por divulsionamento, sendo duas para o lado direito e duas para o lado esquerdo da incisão. De cada lado, por tanto, foram criadas duas bolsas sendo uma na porção cranial e outra na porção caudal do animal. Com auxílio de uma pinça reta, cada bolsa recebeu um tubo contendo um dos três materiais. A quarta bolsa recebeu um tubo vazio como controle. Assim, cada animal recebeu quatro tubos, três preenchidos com os materiais a serem testados (MTA Ângelus® Branco e Cinza e IRM®) e um tubo vazio (controle). O tecido foi suturado com fio de seda (4,0) não reabsorvível e anti-sepsia final realizada com PVPI 10%.

Os animais foram acompanhados até se recuperarem da anestesia antes de retornarem ao biotério (Figura 6).

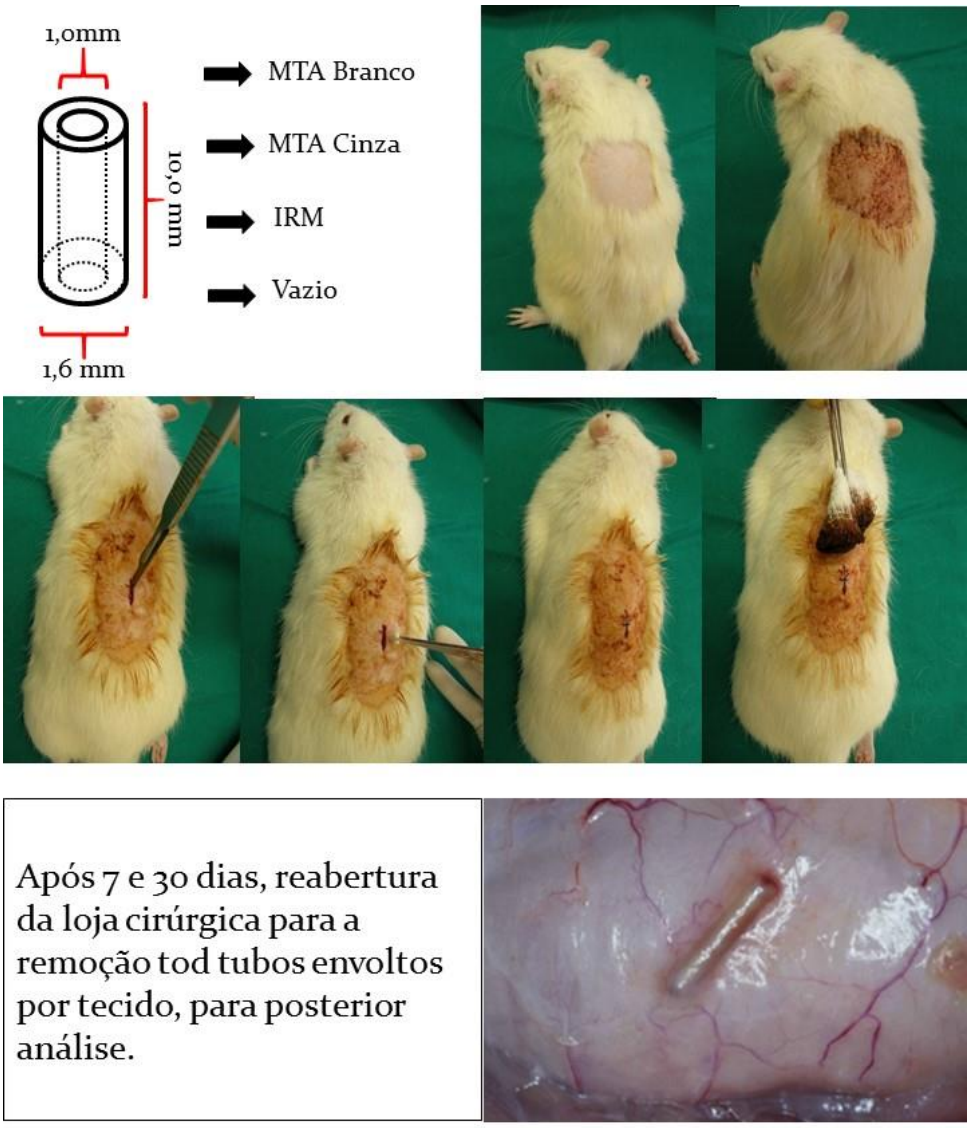

Figura 6: Cirurgia de implante dos tubos do dorso dos ratos. Esquema do tubo de polietileno que foram preenchidos pelos materiais MTA Branco, MTA Cinza e IRM; Tricotomia; Antissepsia; Incisão; Implante dos tubos; Sutura; Antissepsia final; Sacrifício dos ratos após 7 e 30 dias; Remoção dos tubos envoltos por tecido para análise.

10- Processamento Laboratorial

Os períodos de avaliação serão de 07 e 30 dias (American National Standards Institute, 1979; Federation Dentaire International, 1980). A cada tempo experimental, metade dos animais de cada grupo (hipertenso e normotenso) foram sacrificados por meio de uma dose excessiva de anestésico tiopental sódico na dosagem de 100 mg/kg de peso. Após o sacrifício, foi realizada novamente uma tricotomia da região dorsal, e anti-sepsia da área com PVPI 10%. Logo após, realizou-se uma nova incisão com lâmina de bisturi número 15, tendo a coluna vertebral como linha média de marcação, os tubos foram localizados e removidos juntamente com os tecidos que os envolvem e foram fixados em solução formalina 10% pH 7.

11- Protocolo para inclusão em historesina

Após fixar as peças em formol por no mínimo 48horas, lavar as peças em água corrente por no mínimo 5 e no máximo 7horas. Deve-se escorrer a água e deixar no álcool 70 overnight. Iniciar as trocas de álcoois a cada 30 minutos: álcool 90% 1, álcool 90% 2, álcool 90% 3, álcool 95% 1, álcool 95% 2, álcool 95% 3. Fazer a solução de infiltração, e colocar as peças por 72 horas nessa solução. Fazer a solução de inclusão e terminar a inclusão das peças em historesina.

Após isso, foram feitos cortes histológicos para as análises histomorfológicas qualitativas do processo inflamatório, que consistiu na descrição dos fenômenos inflamatórios observados microscopicamente nos cortes teciduais representativos de cada grupo e tempos pós-operatórios.

Foram feitos cortes histológicos em 3 μm para serem coradas com hematoxilina e eosina; 10 μm para serem coradas de acordo com a técnica de Von Kossa ou permanecerem sem coloração para serem observadas sob luz polarizada.

12- Análise Histológica para Hematoxilina Eosina

Para análise, foram contadas as células inflamatórias de 10 áreas ao redor do tubo e foi feita a média da quantidade de células, para posterior análise estatística.

13- Análise Histológica para Von Kossa

Para análise, foram medidas as áreas coradas ao redor do tubo e foi feita a média, para posterior análise estatística.

14- Análise Histológica para Luz Polarizada

Para análise, foram medidas as áreas birrefringentes à luz polarizada ao redor do tubo e foi feita a média, para posterior análise estatística.

15- Protocolo para inclusão em parafina

Deve-se deixar as peças em formol por no máximo 24horas. Lavar por 12 horas as peças em água corrente. Realizar as sucessivas trocas de álcool e xilol, sendo 1hora em cada um dos seguintes álcoois na sequencia: álcool 70%, álcool 80%, álcool 95%, álcool 100% 1, álcool 100% 2, álcool 100% 3. Após isso, 1hora no álcool/xilol seguido de 1hora no xilol 1, 30 minutos no xilol 2 e 30 minutos em xilol 3. A inclusão na parafina é feita na sequência, sendo 1hora na parafina 1, 1hora na parafina 2 e 1 hora na parafina 3. Incluir as peças embebidas na parafina com o auxílio da máquina.

16- Análise Imunoistoquímica Qualitativa

Esta análise permite a demonstração de antígenos nos cortes histológicos por meio da ligação com anticorpos específicos, após a reação histoquímica com coloração visível em microscopia ótica. Para isso, foram realizados os procedimentos para bloqueio de reações inespecíficas para posterior incubação do anticorpo primário. Os anticorpos primários utilizados foram os anticorpos policlonais, produzidos em cabras: anti-runx2, anti-osteocalcina, anti-osteopontina. Como anticorpo secundário, foi utilizado o anticorpo biotinilado anti-cabra.

As reações obtidas foram amplificadas pela reação Streptavidina-Biotina (DAKO) e reveladas utilizando a Diaminobenzidina (DAKO) como cromógeno. Ao término das reações imunostoquímicas, as lâminas receberam contra-coloração pela Hematoxilina de Harris e passaram pela etapa de desidratação para montagem das lamínulas com Permount.

Três lâminas de cada espécime foram selecionadas para analisar, a expressão de runx2, osteocalcina e osteopontina pelo método de imunoperoxidase. Três cortes representativos de cada proteína em cada espécime foram capturados por uma câmara digital (Axio Cam MRc5; Carl Zeiss do Brasil Ltda., Rio de Janeiro, RJ, Brazil) acoplado a um estereomicroscopio (Stemi 2000-C; Carl Zeiss do Brasil Ltda) com aumento do 1:100 para a análise. A marcação de cada proteína foi classificada por 01 examinador calibrado de acordo com a seguinte escala semiquantitativa (Heymann et al. 2008): (-) marcação ausente ou insignificante, (+) marcação leve, (++) marcação moderada, (+++) marcação severa. Para facilitar a comparação entre os grupos, os escores foram convertidos em: 1 para ausência ou marcação desprezível (-), 2 para marcação leve (+), 3 para marcação moderada (++) e 4 para marcação severa (+++).

Anexo II –

Carta de aceite do Artigo 1 na *Brazilian Oral Research*

onbehalfof+editor.chief.bor+gmail.com@manuscriptcentral.com (onbehalfof+editor.chief.bor+gmail.com@manuscriptcentral.com)

02/03/2016

Para: christinemen@hotmail.com, cmartins@forsyth.org

02-Mar-2016

Dear Mr. Martins:

It is a pleasure to accept your manuscript entitled "Relationship between hypertension and periapical lesion: an in vitro and in vivo study" in its current form for publication in the Brazilian Oral Research. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Brazilian Oral Research, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Giuseppe Alexandre Romito
Editor-in-Chief, Brazilian Oral Research
editor.chief.bor@gmail.com

Associate Editor Comments to Author:

Associate Editor
Comments to the Author:
The authors answer all the questions from reviewers and improved the text.

Anexo III –

Carta de aceite do Artigo 2 no *Journal of Endodontics*

Date: Jan 11, 2016

To: "João Eduardo Gomes-Filho" joao@foa.unesp.br,jegomesfilho@yahoo.com.br

From: "The Journal of Endodontics" ees.joe.0.368fdb.d434204f@eesmail.elsevier.com

Subject: Acceptance of JOE Manuscript

Ref.: Ms. No. JOE 15-934R2 Hypertension undermines mineralization-inducing capacity of and tissue response to mineral trioxide aggregate endodontic cement

Dear Dr. Gomes-Filho,

I am pleased to inform you that your manuscript has now been accepted for publication in Journal of Endodontics.

You will soon be contacted by our publisher to review the galley proofs.

When your paper is published on ScienceDirect, you want to make sure it gets the attention it deserves. To help you get your message across, Elsevier has developed a new, free service called AudioSlides: brief, webcast-style presentations that are shown (publicly available) next to your published article. This format gives you the opportunity to explain your research in your own words and attract interest. You will receive an invitation email to create an AudioSlides presentation shortly. For more information and examples, please visit http://www.elsevier.com/audioslides

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Thank you for submitting this manuscript. I look forward to seeing it published soon.

With kind regards,

Ken Hargreaves Editor Journal of Endodontics

Anexo IV –

Guia para submissão do *International Endodontic Journal*

Artigo a ser enviado para análise e apreciação do *International Endodontic Journal*, cujo link de guia para submissão http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1365-2591/homepage/ForAuthors.html

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Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance

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International Endodontic Journal adheres to the definition of authorship set up by The International Committee of Medical Journal Editors (ICMJE). According to the ICMJE, authorship criteria should be based on 1) substantial contributions to conception and design of, or acquisiation of data or analysis and interpretation of data, 2) drafting the article or revising it critically for important intellectual content and 3) final approval of the version to be published. Authors should meet conditions 1, 2 and 3.

Acknowledgements: Under acknowledgements please specify contributors to the article other than the authors accredited. Please also include specifications of the source of funding for the study and any potential conflict of interests if appropriate. Please find more information on the conflict of interest form in section 2.6.

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

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Randomised control clinical trials should be reported using the guidelines available atwww.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material. It is also mandatory for all authors submitting clinical trail manuscripts to *The International Endodontic Journal* to ensure that the trial is registered in any of the following free, public clinical trials registries: www.clinicaltrials.gov and http://isrctn.org/. The clinical trial registration number and name of the trial register will then be published with the paper.

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It is the responsibility of the corresponding author to have all authors of a manuscript fill out a conflict of interest disclosure form, and to upload all forms together with the manuscript on submission. The disclosure statement should be included under Acknowledgements. Please find the form below:

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Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the abstract, main text, references, tables, and figure legends, but no embedded figures or Title page. The Title page should be uploaded as a separate file. In the main text, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.
3.5. Blinded Review
Manuscript that do not conform to the general aims and scope of the journal will be returned immediately without review. All other manuscripts will be reviewed by experts in the field (generally two referees). International Endodontic Journal aims to forward referees´ comments and to inform the corresponding author of the result of the review process. Manuscripts will be considered for fast-track publication under special circumstances after consultation with the Editor.
International Endodontic Journal uses double blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper and the name(s) of the author(s) will not be disclosed to the reviewers.
To allow double blinded review, please submit (upload) your main manuscript and title page as separate files.
Please upload:
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All documents uploaded under the file designation 'title page' will not be viewable in the html and pdf format you are asked to review in the end of the submission process. The files viewable in the html and pdf format are the files available to the reviewer in the review process.

3.6. Suspension of Submission Mid-way in the Submission Process

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

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After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your e-mail server. Also, the e-mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

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4. MANUSCRIPT TYPES ACCEPTED

Original Scientific Articles: must describe significant and original experimental observations and provide sufficient detail so that the observations can be critically evaluated and, if necessary, repeated. Original Scientific Articles must conform to the highest international standards in the field.

Review Articles: are accepted for their broad general interest; all are refereed by experts in the field who are asked to comment on issues such as timeliness, general interest and balanced treatment of controversies, as well as on scientific accuracy. Reviews should generally include a clearly defined search strategy and take a broad view of the field rather than merely summarizing the authors´ own previous work. Extensive or unbalanced citation of the authors´ own publications is discouraged.

Mini Review Articles: are accepted to address current evidence on well-defined clinical, research or methodological topics. All are refereed by experts in the field who are asked to comment on timeliness, general interest, balanced treatment of controversies, and scientific rigor. A clear research question, search strategy and balanced synthesis of the evidence is expected. Manuscripts are limited in terms of word-length and number of figures.

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

Case Reports: illustrating unusual and clinically relevant observations are acceptable but they must be of sufficiently high quality to be considered worthy of publication in the Journal. On rare occasions, completed cases displaying non-obvious solutions to significant clinical challenges will be considered. Illustrative material must be of the highest quality and healing outcomes, if appropriate, should be demonstrated.

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

Letters to the Editor: are also acceptable.

Meeting Reports: are also acceptable.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited. A list of independent suppliers of editing services can be found athttp://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication

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

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

5.2. Structure

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

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

Abstract for Original Scientific Articles should be no more than 250 words giving details of what was done using the following structure:

• **Aim**: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.

• **Methodology**: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.

• **Results**: Give the main results of the study, including the outcome of any statistical analysis.

• **Conclusions**: State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.

Abstract for Review Articles should be non-structured of no more than 250 words giving details of what was done including the literature search strategy.

Abstract for Mini Review Articles should be non-structured of no more than 250 words, including a clear research question, details of the literature search strategy and clear conclusions.

Abstract for Case Reports should be no more than 250 words using the following structure:

• **Aim**: Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• **Summary**: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.

• **Key learning points**: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:

• **Aim:** Give a clear statement of the main aim of the report and the clinical problem which is addressed.

• **Methodology:** Describe the methods adopted.

• **Results:** Give the main results of the study.

• **Conclusions:** State the primary conclusions of the study.

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

Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.
Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.
(i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.
(ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.
When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.
All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.
(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.
Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.
Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.
Conclusion: should contain a summary of the findings.
Main Text of Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented.
Main Text of Mini Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should briefly introduce the subject matter and justify the need and timeliness of the literature review. The Review section should be divided into logical sub-sections to enhance readability and understanding and may be supported by up to 5 tables and figures. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The Conclusions section should present clear statements/recommendations and suggestions for further work. The manuscript, including references and figure legends should not normally exceed 4000 words.
Main Text of Clinical Reports and Clinical Articles should be divided into Introduction, Report, Discussion and Conclusion,. They should be well illustrated with clinical images, radiographs, diagrams and, where appropriate, supporting tables and graphs. However, all illustrations must be of the highest quality
Acknowledgements: *International Endodontic Journal* requires that all sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential conflicts of interest noted. Grant or contribution numbers may be acknowledged, and principal grant holders should be listed. Acknowledgments should be brief and should not include thanks to anonymous referees and editors. See also above under Ethical Guidelines.

5.3. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.
We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. The EndNote reference style can be obtained upon request to the editorial office (iejeditor@cardiff.ac.uk). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.asp
In the text: single or double authors should be acknowledged together with the year of publication, e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by *et al.* is sufficient, e.g. (Tobias *et al.* 1991). If more than 1 paper is cited the references should be in year order and separated by "," e.g. (Pitt Ford & Roberts 1990, Tobias *et al*. 1991).
Reference list: All references should be brought together at the end of the paper in alphabetical order and should be in the following form.
(i) Names and initials of up to six authors. When there are seven or more, list the first three and add *et al.*
(ii)Year of publication in parentheses
(iii) Full title of paper followed by a full stop (.)
(iv) Title of journal in full (in italics)
(v) Volume number (bold) followed by a comma (,)
(vi) First and last pages
Examples of correct forms of reference follow:

Standard journal article

Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. *International Endodontic Journal* **24**, 8-14.

Corporate author

British Endodontic Society (1983) Guidelines for root canal treatment. *International Endodontic Journal* **16**, 192-5.

Journal supplement

Frumin AM, Nussbaum J, Esposito M (1979) Functional asplenia: demonstration of splenic activity by bone marrow scan (Abstract). *Blood* **54** (Suppl. 1), 26a.

Books and other monographs

Personal author(s)

Gutmann J, Harrison JW (1991) *Surgical Endodontics*, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Chapter in a book

Wesselink P (1990) Conventional root-canal therapy III: root filling. In: Harty FJ, ed. *Endodontics in Clinical Practice*, 3rd edn; pp. 186-223. London, UK: Butterworth.

Published proceedings paper

DuPont B (1974) Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the Third Annual Meeting of the International Society for Experimental Rematology; pp. 44-46. Houston, TX, USA: International Society for Experimental Hematology.

Agency publication
Ranofsky AL (1978) Surgical Operations in Short-Stay Hospitals: United States-1975. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8
Dissertation or thesis
Saunders EM (1988) In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis). Dundee, UK: University of Dundee.
URLs
Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text.
Smith A (1999) Select committee report into social care in the community [WWW document]. URL http://www.dhss.gov.uk/reports/report015285.html [accessed on 7 November 2003]

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Tables: Tables should be double-spaced with no vertical rulings, with a single bold ruling beneath the column titles. Units of measurements must be included in the column title.

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5 column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same typeface should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction, symbols should be at least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected; each panel of a multipart figure should be sized so that the whole figure can be reduced by the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

Figures should be on a white background, and should avoid excessive boxing, unnecessary colour, shading and/or decorative effects (e.g. 3-dimensional skyscraper histograms) and highly pixelated computer drawings. The vertical axis of histograms should not be truncated to exaggerate small differences. The line spacing should be wide enough to remain clear on reduction to the minimum acceptable printed size.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same typesize as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by a thin space (1 000). Unusual units or abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general, visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc.)

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7 Guidelines for reporting of DNA microarray data

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Prospective authors are also encouraged to search for previously published microarray data with relevance to their own data, and to report whether such data exists. Furthermore, they are encouraged to use the previously published data for qualitative and/or quantitative comparison with their own data, whenever suitable. To fully acknowledge the original work, an appropriate reference should be given not only to the database in question, but also to the original article in which the data was first published. This open approach will increase the availability and use of these large-scale data sets and improve the reporting and interpretation of the findings, and in increasing the comprehensive understanding of the physiology and pathology of endodontically related tissues and diseases, result eventually in better patient care.