

Índia Olinta de Azevedo Queiroz

Tese de Doutorado

**Análise de propriedades biológicas do MTA
em condição normal e hiperglicêmica**

Orientador: Prof. Titular João Eduardo Gomes Filho

Araçatuba – SP
2017

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**Análise de propriedades biológicas do MTA
em condição normal e hiperglicêmica**

Tese apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP como parte dos requisitos para obtenção do título de Doutor em Endodontia.

Orientador: Prof. Titular João Eduardo Gomes Filho

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2017

Catálogo na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

- Q3a Queiroz, Índia Olinta de Azevedo.
Análise de propriedades biológicas do MTA em condição normal e hiperglicêmica : influência do diabetes na biomineralização do MTA / Índia Olinta de Azevedo Queiroz. – Araçatuba, 2017
184 f. : il. ; tab.
- Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientador: Prof. João Eduardo Gomes Filho
1. Diabetes mellitus 2. Inflamação 3. Cimentos dentários
4. Calcificação fisiológica I. T.

Black D24
CDD 617.67

Dados Curriculares

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Sociedade Brasileira de Pesquisa Odontológica

International Association of Dental Research

American Association of Dental Research

Dedicatória

Dedico este trabalho...

A Deus

Pela bênção de viver.....

*"A vontade de Deus nunca irá leva-lo aonde a
Graça de Deus não possa protegê-lo."*

Francisco Cândido Xavier

A minha família...

À minha mãe, **Maria Suelly de Souza Azevedo Queiroz**, “Uma mãe é capaz de ensinar mais do que cem professores.” A minha inspiração, meu alicerce e meu exemplo de vida. Simplesmente a pessoa que não mede esforços para me ver feliz. Obrigado por todos os momentos dedicados a mim, pelos conselhos, pelo amor, pela honestidade e pelo afeto. Mãe, sem você isso não seria possível!!! Amo você!!!

Ao meu pai, **João Queiroz Pinto**, “Na longa jornada da vida muitos mestres encontramos, alguns seguimos, outros abandonamos, dentre todos, um deles é o que mais amamos”. Ao meu herói e meu maior exemplo de simplicidade e bondade. Obrigada por todos os ensinamentos passados, pelo amor, carinho, respeito e por sempre cuidar de mim... Amo você!!!

À minha irmã, **Indira Augusta de Azevedo Queiroz**, “A felicidade está em nossas mãos”. Sua simplicidade, sensibilidade e o jeito como você consegue abrir mão das coisas em função dos outros me fazem enxergar outro lado da vida. Obrigada pelo carinho, atenção e amor... Amo você!!!

À minha irmã, **Indiane Souza de Azevedo Queiroz**. “Pessoas grandes são aquelas que lutam por ideais”. Sua força de vontade, determinação e coragem me inspiram a cada dia. Obrigada por compartilhar comigo sua vida e sempre acreditar em mim. Tenho muito orgulho de ser sua irmã!!! Amo você!!!

Agradecimentos

Especiais

Aos professores responsáveis diretos pelo desenvolvimento deste trabalho...

Prof. Dr. João Eduardo Gomes Filho

Por simplesmente “ser você”. Por ser não somente um orientador e sim um mentor. Pela preocupação, paciência, respeito, calma e atenção que teve comigo durante toda a minha formação (mestrado e doutorado). Por acreditar em mim mesmo com toda a minha inexperiência. Pela liberdade que sempre me proporcionou para que fizesse minhas próprias escolhas e desenvolvesse minhas atividades. Por todas as vezes que dividir com você meus medos e anseios estando sempre disposto a me ajudar e ouvir. Obrigada por me fazer querer ser uma pessoa melhor. Ter você como meu orientador por todos esses anos foi uma honra e privilégio. Muito obrigada por tudo!!!

Prof. Dr. Edilson Ervolino

“Meu coorientador”, pela forma responsável e atenciosa com que me acolheu desde o meu mestrado, pela maneira como me ajudou, conduzi e orientou em todas as etapas deste trabalho. Muito obrigado pelos ensinamentos em biologia óssea, seu entusiasmo foi um dos motivos que me fizeram procurar um laboratório de biologia óssea nos EUA. Por todas as vezes que em que pode me escutar e me ajudar. Obrigada pela sua amizade e por dividir comigo parte do seu conhecimento.

Prof. Dra. Sandra Helena Penha de Oliveira

Por ser uma inspiração para mim. Pela forma carinhosa como sempre me acolheu e me ensinou, bem como nos momentos onde fui “desabafar” e estava disposta a me ouvir. Pela prontidão em me atender em todos os momentos que precisei. Por confiar e acreditar em mim e por me oferecer uma oportunidade que jamais vou esquecer. Muito obrigada pela amizade, carinho e respeito. Admiro muito você!!!

Prof. Dr. Ivo Kalajzic

Rigor, exigência e competência te definem. Pela maneira como compartilhou comigo seus conhecimentos e experiências em biologia óssea através das aulas, reuniões e experimentos. Pela forma acolhedora como me recebeu

durante meu doutorado sanduíche, tentando do seu jeito fazer com que minha experiência em outro país fosse a melhor possível. Pelos “happy hour” no The Half Door, pelas conversas, conselhos e risadas no “lab time” (especialmente nas sextas-feiras). Muito obrigada por tudo!!!

Aos os grandes responsáveis por “**hold me**” todos esses anos, sem vocês não seria nada...

Thiago Machado, “Algumas vezes na vida, você encontra uma pessoa especial; a que muda sua vida simplesmente por estar nela; a que te faz rir até você não poder mais parar; a que te faz acreditar que realmente tem algo bom no mundo.” Pelo companheirismo, amizade, atenção, carinho, paciência e amor. Por estar sempre ao meu lado, principalmente nos momentos em que mais precisei. E por nunca desistir de mim...Muito obrigada por fazer parte da minha vida!!!

Loiane Massunari, “Ninguém cruza nosso caminho por acaso e nós não entramos na vida de alguém sem nenhuma razão. “Fioite”, às vezes eu me pergunto o que seria de mim sem você na minha vida para “puxar meu freio” rrsrrsrs.. Loi, sua amizade foi um dos melhores presentes que a pós-graduação me ofereceu. Muito obrigada, pela amizade, confiança, apoio, carinho e atenção que sempre teve comigo. Pelos nossas comilanças nos jantares, almoços e gelatos.. hehehe.. Adoro você “fioite”!!!

Renata Oliveira Samuel, “Amigo é coisa para se guardar do lado esquerdo do peito...” pelos momentos de carinho e amizade que divididos desde o primeiro dia do mestrado. Pelo tempo que passamos juntas, dividindo experiências, dificuldades, sonhos, anseio, desejos e aflições. Pelo seu jeito corajoso de ir atrás de seus sonhos. Por ser a melhor “roommate”. Pelas gargalhadas e lágrimas. Pelas noites regadas a vinho e pizzas. Pelas nossas noites solteiras. Pelas nossas viagens. Pelo carinho com que sempre teve comigo, mesmo com meus defeitos. Obrigada por todos esses anos!!!

Nelci Vieira, “Minha segunda mãe”. A mãezona, a que me escuta, me ajuda, me ensina, me aconselha. Nel, eu não tenho palavras para te descrever, ou descrever o que você representou e representa na minha vida. Obrigada por tudo minha mãezona!!! Amo você!!!

Simone Watanabe, minha eterna amiga/supervisora/orientadora. Japinha, eu devo a você parte da minha formação. Muito obrigada por me ensinar, me apoiar e estar sempre ao meu lado. Serei eternamente grata...

Gabrielly Cristinni Rezende, pela amizade, compreensão e ajuda. Muito obrigada pelo carinho com que sempre me acolheu, principalmente quando estava em Santa Fé. Por me escutar, pelas viagens, pelos tempos alegres/malucos na micro, pelas festas no rancho e pelas nossas comilanças nos jantares, almoços e gelatos...hehehe... Adoro você Gaby!!!

Ludmila Santos, “A vida é marcada pela presença de pessoas queridas e que apesar da distância, ficam sempre em nossos corações”. Lud, esse tempo que estive em Araçatuba, você foi capaz de me ajudar a viver novamente e a lembrar das minhas origens. Muito obrigada por tudo, pela amizade, alegria e carinho. Sinto sua falta...

Gustavo Sivieri de Araújo, “o amigo”, uma pessoa de uma bondade e natureza simples que poucos conhecem, escondido atrás do Professor Sivieri...hehehe...Muito obrigada por ter me deixado conhecer esse seu outro lado e por dividir uma parte da sua vida comigo nos trabalhos, festas, happy hours, drinks e viagens...

Agradecimientos

À Faculdade de Odontologia de Araçatuba, da Universidade Estadual Paulista “Júlio de Mesquita filho” – UNESP, na pessoa seu Diretor **Prof. Titular Wilson Roberto Poi** e Vice-Diretor **Prof. Titular João Eduardo Gomes Filho**, pelo empenho e dedicação com que o conduz.

Ao programa de Pós-Graduação em Ciência Odontológica da Faculdade de Odontologia de Araçatuba – UNESP representado pelo seu coordenador **Prof. Dr. Luciano Tavares Ângelo Cintra**, pela competência e afinco na condução do programa de pós-graduação.

À **Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP**, pelo total apoio financeiro para a realização deste trabalho através da concessão da Bolsa de Doutorado (processo nº 2013/06641-8) e da Bolsa de Doutorado sanduíche (BEPE) (processo nº 2014/13750-0).

Aos docentes da disciplina de Endodontia da Faculdade de Odontologia de Araçatuba – UNESP, **Prof. Prof. Dr. Luciano Tavares Ângelo Cintra**, **Dr. Rogério de Castilho Jacinto**, **Prof. Dr. Gustavo Sivieri de Araújo**, **Prof. Dr. Elói Dezan Júnior**, **Prof. Dr. José Arlindo Otoboni Filho**, **Prof. Dr. Mauro Juvenal Nery**, e novamente ao meu orientador **Prof. Dr. João Eduardo Gomes Filho** pelo aprendizado, apoio e contribuição durante minha formação.

Ao Departamento de Ciências Básicas da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP, representado pelos **Prof. Dr. Edilson Ervolino**, pela oportunidade de realizar o processamento laboratorial imunoistoquímico; pela **Profa. Dr. Sandra Helena Penha de Oliveira**, por me proporcionar realizar todo o meu experimento *in vitro*; e pela **Profa. Dr. Rita Cássia Menegati Dornelles** pela disponibilidade de ceder todo material necessário para análises bioquímicas.

Ao Departamento de Odontologia Infantil e Social da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP, representado pelo **Prof. Dr. Alberto Carlos Botazzo Delbem**, por

disponibilizar o laboratório e equipamentos necessários para realizar para análises bioquímicas.

Ao **Prof. Dr. Luciano Tavares Ângelo Cintra**, pelas conversas, conselhos e por sempre disposto a ensinar/ajudar. Por dividir seus conhecimentos, experiências e sempre buscar tirar o melhor de cada pessoa. Luciano, obrigada pelos ensinamentos/lições e por ter sido esse exemplo de professor/conselheiro/orientador durante minha formação; e ao **Prof. Dr. Eloi Dezan Júnior**, pela simplicidade, generosidade e paciência em dividir seus conhecimentos clínicos. Pelo seu jeito extrovertido, atencioso e por sempre “pensar” nos seus alunos, buscando ferramentas/meios de nos incentivar a sermos profissionais melhores.

Aos amigos **Wagner Garcez de Melo**, você realmente é o que podemos chamar de um excelente Profissional/Professor. Muito obrigada pela inestimável ajuda durante todos os meus experimentos nos finais de semana, na redação do texto, nas análises estatísticas e no mais importante em dividir comigo seus conhecimentos de forma tão gentil e espontânea; e **Luis Gustavo Narciso**, pela preciosa ajuda durante as coletas sanguíneas.

Aos amigos **Marcos Frozoni** e **Guilherme Bonduki**, pela confiança e por me ajudarem na realização do meu doutorado sanduíche, me proporcionando um crescimento pessoal e profissional. Serei eternamente grata a vocês pelo que fizeram e pela amizade dispensada à minha pessoa.

Aos amigos do programa de pós-graduação Ciência Odontológica da Faculdade de Odontologia de Araçatuba – UNESP, Área de Concentração de Endodontia, **Diego Valentim, Ludmila Santos, Loiane Massunari, Gabrielly Rezende, Paulo Tobias, Renata Samuel, Marcelo Wayama, Mariane Azuma, Luciana Louzada, Annelise Katrine, Francine Beneti, Carlos Bueno, Christine Mem Martins, Renan Dal Fabro, Leticia, Camila, Amanda e Vanessa**, e da iniciação científica **Luanna Gonçalves, Larissa Gonçalves e Aline Ávila**, pela amizade e pelos momentos de alegria, conversas, brincadeiras e descontrações proporcionadas.

Aos amigos do Laboratório de Farmacologia do Departamento de Ciências Básicas da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP, **Aline Takamiya, Victor Balera, Dayane Queiroz, Leticia, Carluci Beltran, Maria Fernanda Lopes, Fernanda Demarqui**, pela paciência, apoio, ensinamentos e por sempre estarem dispostos a me ajudar e ensinar. Vou sentir saudades da “Farmaco B” que com certeza é a melhor!!!

Aos funcionários do Departamento de Odontologia Restauradora da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP, **Nelci Vieira, Cláudia Neves Corrêa, Elaine Cristina Francischini Ferreira e Peterson Moura**, pela amizade, paciência e colaboração, apoio e incentivo. O que seria desse departamento sem a presença de vocês!!!

Aos funcionários do Departamento de Cirurgia e Clínica Integrada, **Odair, Dirce e Paulo** da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP, pelo carinho, dedicação e simpatia com que sempre me atenderam todas às vezes. Muito obrigada por me ensinarem e me ajudarem durante as etapas desse trabalho.

Aos funcionários da Seção Técnica de Graduação e Pós-Graduação da Faculdade de Odontologia de Araçatuba - UNESP, **Valéria Queiroz Marcondes Zagatto, Lílian Sayuri Mada e Cristiane Regina Lui Matos**, pela eficiência e presteza de sempre. Obrigada pela ajuda durante minha representação discente e por tudo o que fizeram por mim durante esses anos.

À **Alice e família**, pela amizade e pela forma como me acolheram em Araçatuba; Aos meus amigos de “Moc”, **Amanda Normanha, Geraldo Edson, Rafael Santiago, Paulo Sergio, Lara Mota, Mariana Silveira e Swed**, pela amizade, apoio e carinho sempre.

A todos aqueles que, direta ou indiretamente, contribuíram para a realização deste trabalho!!!

To the responsible for my USA life experience...

To **University of Connecticut Health Center – UCHC**, specially to Center of Regenerative Medicine and Skeletal Development, Department of Reconstructive Sciences of School of Dental Medicine for received and given me the opportunity to develop my PhD and improve my knowledge.

To Professor Dr. **Ivo Kalajzic**, Center of Regenerative Medicine and Skeletal Development, for open his lab and share with me your knowledge and experiences, to be a mentor that gave me the opportunity to know more about bone biology. Thank you for everything!!

To Professor Dr. **Mina Mina** and **Ivana Vidovic**, Department of Craniofacial sciences of School of Dental Medicine, for always be receptive, for the gentleness in share hers knowledge and for all the help in the *in vivo* experiment.

To my co-workers, **Emilie Roeder**, **Xi Wang**, **Paola Vizzarri**, **Brya Matthews**, **Devin Shaheen** and **Mara** for friendship, affection, dedication and sympathy that always had with me. Thanks for sharing yours expertise with me and help me all time and for the fun times together. I hope to see you all again.

To friends from Department of Reconstructive Sciences and Department of Craniofacial sciences of School of Dental Medicine, **Lipin**, **Yalin**, **Zhihua Wu (Lisa)**, **Bharbara** and **Anu** for friendship and attention that always had with me.

To **Dipika Gupta**, **Nidhi Gupta**, **Tulika Sharma**, **Marian George** and **Bandita Adhikari**, my “american/indian family”, thank you for taking care of me during my USA life, to make my life more happy, to teach me another culture and for the drinks and party that we shared. I miss our house, girls!!

To **Dipika Gupta**, roommate and friend, the girl that loves her job, the inspiration for everyone close to her and an example of researcher. A friend that listened me when I was crying and happy, holding me when I was drunk (hehe)

and capable to do everything to see a friend happy. Dipiii, I miss you so much, thanks for everything and as I said I will see you again soon.

To **Emilie Roeder**, for make my life in the lab easier, better and unforgettable, for sharing and teach me in the lab. For the trips together, for your patience, friendship and be an amazing friend....

To **Mariana Quezado**, for help and take care of me when I arrived, for the New York days, for all the happy hours together and for teach me chemistry and mathematics. See you soon in BH, Mari.

To the three Musketeers, **Fani Memi** and **Debargha Bassuli**, for the friendship, for listen me in the bad and good times, for trying to speaking and learning Portuguese, for all the drinks, talking, happy hours, for the new year's day/eve and for be the two most incredible friend that I knew...

To all my other USA/UCHC/World friend, **Fabiana Saoki**, **Martinna Bertolini**, **Alexandro Lima**, **Ivana Vidovic**, **Nilse dos Santos**, **Melissa Car**, **Hank Hrdlicka**, **Yulia Pustovalova**, **Alexandra Pozhidaeva**, **Anushree Vk**, **Guilia Vigone**, **Igor Matic**, **Ryan Russell**, **Sara Acevedo**, **Guivini Gomes**, **Alberto Ortega**, **Scotti Danger Adamson**, **Tosin Quadri**, **Nicole Glidden** and **Anilei Hoare**, **Luciana Arraes**, **Anthar Darwish**, **Jelena Vidas** and **Candace Reeve**, for friendship and all the good/funny times that we spend together.

Epígrafe

"Cada sonho que você deixa para trás é um pedaço do seu futuro que deixa de existir."

Steve Jobs

Resumo

Queiroz, IOA. **Análise de propriedades biológicas do MTA em condição normal e hiperglicêmica.** [Tese]. Araçatuba: UNESP – Univ. Estadual Paulista; 2017.

O objetivo deste estudo foi analisar as propriedades biológicas do MTA em condição normal e hiperglicêmica. Para tanto, esse trabalho foi dividido em duas partes, sendo que a primeira teve como objetivo avaliar o efeito do MTA no processo de reparo do Ligamento Periodontal (PDL) e na diferenciação de células mesenquimais progenitoras do PDL (PDSCs) e da Medula Óssea (BMSCc) após injúria dental. Uma perfuração na região de furca do primeiro molar superior de camundongos transgênicos (α SMACreERT2/Ai9/Col2.3GFP) foi realizada e os efeitos do MTA após 2, 17 e 30 dias de lesão, foram examinados e comparados com resina composta (AS) utilizando análise histológica e epifluorescência. Além disso, BMSCs e PDSCs desses camundongos foram isoladas, cultivadas e os efeitos do MTA na proliferação celular e diferenciação osteogênica foram avaliados. Os resultados indicaram que o MTA promoveu a regeneração do PDL e do osso alveolar na área da injúria dental. No entanto, demonstrou efeitos negativos na diferenciação osteogênica de PDSCs e BMSCc. A segunda parte, teve como objetivo avaliar a influência da Diabetes Mellitus na proliferação celular, produção de citocinas, resposta tecidual, capacidade de mineralização e na expressão local e sistêmica de marcadores ósseos. Para alcançar esses objetivos, células de linhagem fibroblástica L929 foram cultivadas em alta concentração de glicose e a influência do MTA na proliferação celular e na produção de citocinas das IL-1 β , IL-6 e TNF- α foram observados às 6, 24, 48 e 72 horas; tubos de polietileno foram implantados no tecido subcutâneo de ratos normais e diabéticos (induzidos pelo Aloxano) e a influência do MTA na resposta tecidual, produção de citocinas e na capacidade de mineralização em condição diabética foram observadas através de técnicas histológicas e imunistoquímicas aos 07 e 30 dias; análises bioquímicas para Cálcio, Fósforo e Fosfatase Alcalina e imunistoquímica para osteocalcina e osteopontina, aos 07 e 30 dias, também foram realizadas com a finalidade de verificar a influência do MTA na expressão local e sistêmica de marcadores ósseos. O quadro hiperglicêmico promoveu, *in vitro*, um aumento da produção

de IL-6 e comprometeu a proliferação celular após 72hs. Independente da condição diabética, a resposta tecidual e a capacidade de produção de IL-1 β , IL-6 e TNF- α de ambos MTA não foi alterada, embora uma redução na intensidade de fluorescência do MTA Branco foi observada aos 14 dias em animais diabéticos. Por outro lado, o quadro hiperglicêmico inibiu a produção local de osteocalcina e osteopontina na presença dos dois MTA e aumentou os níveis séricos de Fósforo e Fosfatase Alcalina. Assim, concluiu-se que, o MTA promoveu a regeneração do PDL e do osso alveolar na área da injúria dental, contudo, apresentou um efeito negativo com relação à diferenciação osteogênica e, que em condições hiperglicêmicas, o MTA Cinza melhores resultados biológicos quando comparado ao MTA Branco.

Palavras-chaves: Diabetes Mellitus, Inflamação, Cimentos Dentários, Calcificação Fisiológica.

Abstract

Queiroz, IOA. **Analysis of biological properties of MTA in normal and hyperglycemic conditions.** [Thesis]. Araçatuba: UNESP – Univ. Estadual Paulista; 2017.

The aim of this study was to analyze the biological properties of MTA in normal and hyperglycemic conditions. Therefore, this study were divided into two parts; the first part aim was to evaluate MTA effect on healing of periodontal ligament (PDL) and differentiation of mesenchymal progenitor cells in PDL (PDSCs) and bone marrow stromal cells (BMSCc) following dental injury. Perforation on the pulp floor in the furcation area in the first maxillary molars of transgenic mice (α SMA $CreERT2/Ai9/Col2.3GFP$) were performed and the effects of MTA after 2, 17, 30 days of injury, were examined and compared to AS using histological and epifluorescence analysis. Additionally, BMSCs and PDSCs from these mice were isolated, cultured and the effects of MTA on cell proliferation and osteogenic differentiation were evaluated. The results indicated that MTA promoted regeneration of injured PDL and alveolar bone in the area of dental injury. However, it has demonstrated negative effects on the osteogenic differentiation of PDSCs and BMSCs. The aim of second part was to evaluate the influence of Diabetes Mellitus on cell proliferation, cytokine production, tissue response, mineralization ability and local and systemic expression of bone markers. To achieve these goals, L929 fibroblasts cell line were cultured under high glucose concentration and the influence of MTA on cell proliferation and production of cytokine IL-1 β , IL-6 and TNF- α were observed at 6, 24, 48 and 72 hours; polyethylene tubes were implanted in the subcutaneous tissue of normal and diabetic rats (induced by Alloxan) and the influence of MTA on tissue response, cytokines production and mineralization ability in diabetic condition were observed by histological and immunohistochemical techniques at 07 and 30 days; biochemical analysis for Calcium, Phosphorus and Alkaline Phosphatase and immunohistochemistry for osteocalcin and osteopontin were also performed, at 07 and 30 days, in order to verify the influence of MTA on the local and systemic expression of bone markers. The hyperglycemic state promoted an increase on IL-6 production and impaired L929 proliferation after 72hs. Independent of the diabetic condition, the tissue response and ability to produces IL-1 β , IL-6 and TNF- α by both MTA was not change, although a

reduction on fluorescence intensity of White MTA was observed after 14 days in diabetic animals. Moreover, hyperglycemia state inhibited the local production of osteocalcin and osteopontin in the presence of both MTA and increased serum levels of Phosphorus and Alkaline Phosphatase. Thus, it was concluded that MTA promoted regeneration of PDL and alveolar bone in the area of dental injury, moreover, it had a negative effect in relation to osteogenic differentiation; and under hyperglycemic condition, Gray MTA showed better biological results when compared with White MTA.

Keywords: Diabetes Mellitus, Inflammation, Dental Cements, Physiological Calcification.

*Lista de
Abreviaturas*

LISTA DE ABREVIATURAS

ADA – Associação Americana de Diabetes
Al₂O₃ – Óxido de Alumínio
ALP – Fosfatase Alcalina
ANOVA – Análise de Variância
AS – Compósito resinoso autoadesivo
BMSCs – Células Mesenquimais da Medula Óssea.
BSP – Sialoproteína Óssea
Ca – Cálcio
CaP – Cálcio Fosfato
cDNA – Ácido Desoxirribonucleico complementar
CEUA – Comissão de Ética no Uso Animal
CO₂ – Gás carbônico
DM – Diabetes Mellitus
DMEM – Meio Essencial Mínimo de Dulbecco
DNA – Ácido Desoxirribonucleico
EDTA – Ácido Etilenodiaminotetracético
ELISA – Ensaio de Imunoabsorção Enzimática
FAPESP = Fundação de Amparo à Pesquisa do Estado de São Paulo
FBS – Soro Fetal Bovino
FeO – Óxido de Ferro
Fe₂O₃ – Óxido Férrico
Fig. – Figura
g – Gramas
GAPDH – Gliceraldeído-3-fosfato desidrogenase
GFP - Proteína Verde Fluorescente
GMA – Glicol metacrilato
g/mL– Gramas por Mililitros
GMTA – Mineral Trióxido Agregado Cinza
h – Horas
H&E – Hematoxilina e Eosina
IDF – Federação Internacional de Diabetes
IL1-β – Interleucina 1 beta

IL-6 – Interleucina 6
IP – Intraperitoneal
IR – Imunorreatividade
Kg – Quilogramas
L-929 – Células de Linhagem Fibroblástica L-929
M – Molar
Mg – Magnésio
mg – Miligramas
mg/dL – Microgramas por Decilitros
mg/kg – Miligramas por Quilogramas
mg/ml – Miligramas por Mililitros
MgO – Óxido de Magnésio
min – minutos
mL – Mililitros
mm – Milímetro
mM – Milimolar
MSCs – Células mesenquimais indiferenciadas
MTA – Mineral Trióxido Agregado
MTA-CM – Meio de cultura condicionado com Mineral Trióxido Agregado
nm – Nanômetro
OC – Osteocalcina
OCN – Osteocalcina
OH⁻ - Hidroxila
OPN – Osteopontina
OZE – Óxido de Zinco e Eugenol
P – Fósforo
PBS – Tampão fosfato-salino
PDL – Ligamento Periodontal
PDSCs – Células Progenitoras do Ligamento Periodontal.
pH – potencial Hidrogeniônico
RNA – Ácido Ribonucleico
rpm – Rotação por minuto
RT-qPCR – Reação em Cadeia de Polimerase com transcriptase reversa em tempo real

Runx-2 – Fator de transcrição relacionado ao Runt 2
s – Segundos
Si – Silício
TM – Tamoxifeno
TNF- α – Fator de necrose tumoral alfa
UV- Luz Ultravioleta
U/L – Unidades por Litro
U/mL – Unidades por Mililitros
VH – Veículo
VK – Von Kossa
vs – versus
WMTA – Mineral Trióxido Agregado Branco
ZOE – Óxido de Zinco e Eugenol
% – Por cento
°C – Graus Célsius
® – Marca registrada
 α – alfa
 β – Beta
x – Vezes
n – Tamanho da amostra
 α -MEM – Meio Essencial Mínimo alfa
 α -SMA – Actina de Músculo Liso alfa
 α -SMACreERT2/Ai9/Col2.3GFP – Animal Triplo Transgênico
 μ g – Microgramas
 μ m – Micrômetros
 μ m² – Micrometros quadrados
 μ g/g – Microgramas por gramas
 μ g/ml – Microgramas por Mililitros

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Introdução

Introdução

A "Medicina Endodôntica" visa estudar a relação e/ou associação entre doenças sistêmicas e as de origem endodônticas (1-4). Entre elas, a Diabetes Mellitus (DM) que é uma doença complexa, progressiva e debilitante de origem metabólica caracterizada por um quadro de hiperglicemia crônica que promove alterações no metabolismo dos carboidratos, lipídios, proteínas, água e eletrólitos resultantes da insuficiente secreção/ação do hormônio insulina (5).

DM é considerada como um fator modulador das infecções endodônticas (6). No entanto, esta relação ainda não está completamente elucidada, estudos mostram que as alterações na resposta imune e a persistência do estado inflamatório associadas a DM podem interferir e comprometer o reparo dos tecidos periapicais (7-9).

A hiperglicemia crônica decorrente da DM promove a ativação de vias que aumentam a inflamação (3,5). Assim, a elevação dos níveis inflamatórios sistêmicos altera diversas funções do sistema imune (10,11) como o comprometimento da resposta leucocitária e o aumento da expressão de citocinas pró-inflamatórias, promovendo uma redução da capacidade de defesa celular e aumentando a susceptibilidade à infecção e inflamação, afetando diretamente a integridade dos tecidos pulpare e periapicais e interferindo no processo de reparo (3, 12-14).

DM também tem sido associada com alterações no processo de reparo ósseo (15,16), onde mecanismos fisiopatológicos relacionados à perda óssea como a redução da atividade osteoblástica, diminuição da síntese de colágeno e alterações no metabolismo do cálcio e fósforo e na expressão de marcadores de formação óssea tem sido observados em indivíduos diabéticos (17,18). Entretanto, os mecanismos pelos quais a DM interfere no metabolismo ósseo e, portanto, no processo de reparo/cicatrização ainda precisam ser esclarecidos, sabe-se, que controle da inflamação é essencial para que o processo de reparo ocorra, uma vez que, na presença de um quadro hiperglicêmico, a persistência da inflamação leva uma estimulação, pelos neutrófilos, da condrogênese e inibição da osteogênese (19,20).

O osso é um tecido dinâmico que está em constante remodelação e a diferenciação osteoblástica é regulada por uma série de hormônios, citocinas e múltiplos fatores de transcrição (21-23) e que podem ser inibidos e/ou alterados pelo quadro hiperglicêmico (24). Deste modo, a estimulação da reabsorção óssea, através da inibição da osteogênese, acarreta no aumento da reabsorção óssea periapical (25,26). Além disso, em indivíduos diabéticos, a redução da capacidade de reparo também está associada a diminuição da resistência à infecção bacteriana e maior susceptibilidade as infecções endodônticas (6, 27, 28).

A infecção endodôntica é tratada através da eliminação dos micro-organismos patogênicos e o restabelecimento da normalidade dos tecidos apicais e periapicais afetados, bem como da utilização de materiais capazes de promover reações de teciduais favoráveis, apresentarem adequadas propriedades físicas e químicas, que sejam indutores de mineralização e que possam favorecer e contribuir para a reparo periapical (29,30).

Uma vez que, os cimentos reparadores e obturadores estão em íntimo contato com tecidos perirradiculares, sua composição química, bem como, compostos tóxicos liberados pelos mesmos podem interferir na resposta inflamatória e, conseqüentemente, no processo de reparo (31-33). Assim, materiais com as mais variadas bases: óxido de zinco e eugenol, resina epóxica, ionômero de vidro, hidróxido de cálcio e Agregado Trióxido Mineral (MTA), podem ser encontrados.

Óxido de Zinco e Eugenol (ZOE) é um cimento composto de um pó de Óxido de Zinco e um líquido o Eugenol, utilizado que nos mais diversos procedimentos endodônticos: proteção pulpar direta, em selamento provisório, como obturador endodôntico, em revestimento de cavidades profundas. É um cimento que apresenta ação antimicrobiana (34), bom selamento marginal (35), ação anestésica e anti-inflamatória local (36). No entanto, devido ao seu componente líquido: Eugenol quando aplicado diretamente sobre os tecidos pode desencadear uma resposta inflamatória crônica dos tecidos periapicais (37) e danos sobre o tecido pulpar (38).

MTA é um cimento reparador à base de silicato de cálcio que foi introduzido em 1993 por Torabinejad (39) com a finalidade de proporcionar o

selamento de comunicações patológicas ou iatrogênicas entre o dente e sua superfície externa (49,41). Entretanto, devido às suas excelentes propriedades físicas, químicas e biológicas (42,43) este passou a ser rotineiramente utilizado nas mais diversas situações clínicas (pulpotomias, capeamentos pulpare, apicogêneses, apicificações e obturação dos canais radiculares) (41).

Estudos, *in vitro* e *in vivo*, mostram que o MTA é um material bioativo (44); biocompatível (45); promove a proliferação e diferenciação de células mesenquimais/progenitoras da polpa dentária (46) e ligamento periodontal (47), além de induz dentinogênese (48), cementogênese (49) e osteogênese (50). MTA também é capaz de estimular a produção de citocinas (51,52) e a expressão marcadores ósseos (49, 53). Inclusive em condições hiperglicêmicas o MTA mostrou-se biocompatível, promoveu mineralização (54) e foi capaz de induzir a formação de ponte de dentina (55,56).

MTA encontra-se atualmente disponível sob duas formas MTA Cinza e MTA Branco, onde a principal diferença entre ambos está na redução das concentrações de Al_2O_3 , MgO, e FeO encontras no MTA Branco (57). Apenas poucos estudos comparando MTA Cinza e MTA foram realizados, alguns mostrando semelhanças; são biocompatíveis (58), capazes de induzir a proliferação celular (59) e de estimular a formação de ponte de dentina (60), e outros diferenças; MTA Cinza favorece a adesão osteoblástica (61), porém cementoblastos e queratinócitos crescem melhor na superfície do MTA Branco (62). Embora, o MTA Branco tenha sido introduzido como uma alternativa para evitar a pigmentação dental produzida pelo MTA Cinza, estudos *in vitro* e em *in vivo*, também verificaram pigmentação dental causada pelo MTA Branco (63,64).

Recentemente, o MTA também começou a ser empregado em procedimentos endodônticos regenerativos (65,66), uma vez que os tecidos dentários (polpa dentária, ligamento periodontal e osso alveolar) são fonte rica e acessível de células mesenquimais/progenitoras (67,68), e tais procedimentos envolvem a interação e diferenciação das células mesenquimais/progenitoras, bem como a utilização de biomateriais (69). No entanto, os mecanismos envolvidos nessa interação ainda não estão totalmente explicados.

A diferenciação de células osteoprogenitoras é um principais processos responsáveis pela formação e remodelação óssea, com isso, torna-se um pré-requisito compreender e analisar as vias envolvidas no desenvolvimento ósseo (70). Em função disso, investigações tem utilizando animais transgênicos e marcadores visuais (GFP - proteína verde fluorescente) sob o controle da actina de músculo liso (α -SMA) (promoter) e do colágeno tipo I 2.3kb (promoter) expressado por osteoblastos maduros com a finalidade de identificar a subpopulação de células progenitoras que expressam α -SMA e exibem um potencial osteogênico (71-73).

Deste modo, como uma das propriedades do MTA é induzir a mineralização e promover o reparo nos tecidos onde é aplicado, torna-se relevante compreender e verificar a influência do MTA no processo de diferenciação osteogênica de células mesenquimais, bem como, no processo de reparo dos tecidos periodontais após injúria dental, através da utilização de animais transgênicos. Ao mesmo tempo, como a DM é uma desordem metabólica que altera a resposta inflamatória e, portanto, afeta o processo de mineralização, justifica-se o estudo da influência dos MTA Cinza e MTA Branco na viabilidade celular, resposta tecidual, produção de citocinas, capacidade de mineralização e na expressão local e sistêmica de marcadores ósseos em condição diabética.

Proposição

Proposição

O presente trabalho teve o intuito de avaliar as propriedades biológicas do MTA em condição normal e hiperglicêmica.

Os objetivos específicos foram:

✓ Avaliar, *in vivo*, os efeitos do MTA na reparação dos tecidos periodontais e ósseo após injúria dental (perfuração) usando animais transgênicos (α SMACreERT2/Ai9/Col2.3GFP);

✓ Avaliar, *in vitro*, a influência do MTA na proliferação e diferenciação de células progenitoras da Medula Óssea e do Ligamento Periodontal utilizando linhagem de animais transgênicos (α SMACreERT2/Ai9/Col2.3GFP);

✓ Avaliar, *in vitro*, a influência do MTA na viabilidade celular e na produção de citocinas IL-1 β , IL-6 e TNF- α em condição hiperglicêmica;

✓ Avaliar, *in vivo*, a influência do MTA na resposta tecidual, na produção de citocinas IL-1 β , IL-6 e TNF- α e capacidade de mineralização em condição diabética;

✓ Avaliar, *in vivo*, a influência do MTA na produção local (osteocalcina e osteopontina) e sistêmica (Cálcio, Fósforo e Fosfatase Alcalina) de marcadores de formação óssea em condição diabética.

Artigo 1

Artigo 1: Mineral Trioxide Aggregate improves healing response of periodontal tissue to injury

Abstract

Objectives and Background: Mineral Trioxide Aggregate (MTA) a biomaterial used in endodontic procedures as it exerts beneficial effects on regenerative processes. In this study we evaluate MTA effect on healing of PDL and differentiation of mesenchymal progenitor cells in PDL and bone marrow stromal cells following periodontal ligament and alveolar bone injury.

Materials and Methods: We used an inducible Cre-loxP *in vivo* fate mapping approach to examine the effects of MTA on the contributions of descendants of cells expressing α SMA-CreERT2 transgene to the PDL and alveolar bone after experimental injury to the root furcation on the maxillary first molars. The effects of MTA after 2, 17, 30 days of injury, were examined and compared to AS using histological and epifluorescence analysis. The effects of two dilutions of MTA (MTA 1:5 and MTA 1:50) on proliferation and differentiation of mesenchymal progenitor cells derived from bone marrow (BMSC) and periodontal ligament (PDSCs) from α SMA CreERT2;Ai9/Col2.3GFP were examined using presto blue assay, alkaline phosphatase and Von Kossa staining. The expression of markers of differentiation were assessed by real time PCR

Results: Histological and epifluorescence analyses showed better repair of injury in teeth restored with MTA as shown by greater expansion of SMA9+ and 2.3GFP+ cells as compared to AS. We also observed positive effect on alveolar bones and apical region on distant from the site of injury. The *in vitro* data showed that MTA supported viability of the PDL fibroblasts but not their differentiation. MTA did not exert effect on BMSCs viability during the 9 days in cultures, but resulted in significant decreases in von Kossa staining and levels of expression of OC and Bsp as compared to OM and control media. In BMSCs and PDL cells grown in presence of MTA there were marked decrease in SMA9-stained and 2.3GFP-stained areas as compared to OM indicating the reduced levels of expression of markers of osteogenesis.

Conclusion: MTA promotes regeneration of injured PDL and alveolar bone reflected as contribution of progenitors (SMA9+ cells) into osteoblasts (Col2.3+

cells). In vitro effects of MTA are supportive to viability of the PDL progenitor but have negative effects on osteogenic differentiation of both PDL and BMSCc.

Keywords: MTA, injury, periodontal ligament, progenitor cells, differentiation

Introduction

Mineral Trioxide Aggregate (MTA), a calcium silicate-based cement, is a bioactive biomaterial used extensively in almost all endodontic therapies including root perforation repair, apexification, apexogenesis, pulpotomy and root-end filling (1). MTA has been used extensively in regenerative endodontic procedures (2,3). MTA has been reported to have low cytotoxicity, and well as the ability to promote proliferation and differentiation of stem/progenitor cells resulting in cementogenesis, dentinogenesis and osteogenesis (4-9).

Dental tissues are a rich source of mesenchymal stem cells (MSCs) that participate in healing and regeneration following injury or infection (10-12). Previous in vivo lineage tracing studies in our laboratory showed that alpha-smooth muscle actin (α SMA) expressing cells residing in perivascular areas within a number of tissues including PDL, dental pulp, bone marrow and periosteum represent a population of mesenchymal progenitor cells (12-16). Following periodontal injury, α SMA⁺ cells expand and differentiate into osteoblasts in the alveolar bone, fibroblasts in the PDL and cementoblasts (16). Our studies also showed that this population is capable of giving rise to a second generation of odontoblasts during reparative dentinogenesis (15).

Despite numerous studies on the effects of MTA on various dental tissues, the underlying mechanisms of the effects of MTA on regeneration of periodontal tissues and surrounding alveolar bone and its effects on differentiation of stem/progenitor cells are not fully understood. We designed the present study to gain insight into the effects of MTA on perivascular cells expressing α SMA during repair of the periodontium and surrounding alveolar bone using cell lineage-tracing experiments in developing mouse molars. We utilized the previously characterized α SMA^{CreERT2};Ai9/Col2.3GFP transgenic animal in which α SMA serves as a marker of progenitor cells in PDL. In these transgenic animals Col2.3GFP transgene serves as a marker for identification of PDL cells, mature osteoblasts and cementoblasts.

Materials and Methods

Transgenic mice

The α SMACreERT2;Ai9/Col2.3GFP mice have been previously described (13). For in vivo and in vitro lineage tracing experiments α SMACreERT2;Ai9 (cross between α SMACreERT2 Cre reporter mice with Ai9 mice from Jackson Labs, Bar Harbor, ME, USA) and α SMACreERT2;Ai9/Col2.3GFP (cross between α SMACreERT2;Ai9 with Col2.3GFP mice) were used. Animal protocols were approved by the Institutional Animal Care Committee.

Tooth injury in vivo

Four to six weeks old transgenic mice were injected with corn oil (vehicle, VH) or tamoxifen (TM) (75 ug/g body weight) twice in 24h intervals. Two days later, mice were anesthetized with an intraperitoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg) and experimental pulp perforations on maxillary first molars were performed as previously described (17). Briefly, class I cavity was prepared with a carbide round burr (diameter 0.40 mm) on the occlusal surface of first maxillary molars. Pulp chambers were opened, and coronal pulp tissues were removed with pulp extractor (VDW[®] STERILE Barbed Broaches, VDW GmbH, Munich, GE) up to the root canal orifices. A perforation was created in the center of the floor of the pulp chamber using an endodontic hand file number #15 (Dentsply, Tulsa, OK, USA) (Supplemental Figure 1).

The perforation area was filled with one-step self-etching Adhesive System (AS) (Clearfill SE Bond; Kuraray, Okayama, JP) (AS, controls) or White ProRoot MTA (Dentsply), prepared according to the manufacturer recommendations. The cavities were then sealed with a light-cured composite resin (SDI wave restorative system, SDI Wave, SDI Inc, Itasca, IL, USA) in both groups. Animals were sacrificed by intra-cardiac perfusion with 4% paraformaldehyde in PBS (17) at various time points (2, 17 and 30 days). The maxillary arches were isolated, cleaned of soft tissue, fixed in 4% paraformaldehyde solution for additional 24h and then, decalcified with 14% EDTA for 7 days. Decalcified tissues were placed in 30% sucrose solution overnight and embedded in cryomatrix (Thermo Shandon, Pittsburg, PA, USA). Seven-micrometer sections were obtained using the Leica cryostat and

mounted using a CryoJane tape transfer system (Instrumedics, St Louis, MO, USA). Sections were imaged using a AxioScan.Z1 (Carl Zeiss). Adjacent sections were processed for hematoxylin/eosin staining and analyzed by light microscopy.

Cell isolation and culture

Primary bone marrow stromal cells (BMSCs) were prepared from the hind limbs of 4-6 week old α SMACreERT2;Ai9/Col2.3GFP mice as previously described (18, 19). The cells were plated in 12 well culture plates at a density of $10^6/\text{cm}^2$ for 7 days in basal medium: α -modified essential medium (α -MEM), 10% fetal bovine serum (FBS, Life Technologies, Carlsbad, CA, USA) and 100 U/ml of penicillin, 100 mg/ml of streptomycin (1% PS). Cre activity was induced by $1\mu\text{M}$ of 4-OH-Tamoxifen added at days 2 and 4 of culture.

At day 7, cells were grown in 4 different conditions including control basal medium, dilutions of MTA-CM in basal medium and osteogenic media (OM) (α -MEM 10% FBS + 50ug/ml ascorbic acid + 8mM β -glycerophosphate). Medium was changed every two days.

PDL cells were isolated from 4-6 week old α SMACreERT2;Ai9/Col2.3GFP mice as previously described (12). Briefly, the mandibles and maxilla were dissected from the surrounding tissues, rinsed in 0.12% chlorhexidine digluconate (Clorhexidina, Villevie, Joinville, SC, BRA), for 30 secs, and washed in PBS. Molars with the adherent PDL were removed from the surrounding alveolar bone and digested in Dulbecco's modified Eagle's medium (DMEM) with 2 mg/ml Collagenase P (Sigma-Aldrich, Saint Louis, MO, USA) and 0.25% trypsin (Life Technologies), and digested at least for 2 h at 37°C . Following washing, PDL cells were seeded in DMEM 20% FBS + 1% PS and cultured in 5% oxygen for 7 days. The medium was changed every 2 days and the Cre activity was induced by $1\mu\text{M}$ of 4-OH-Tamoxifen added at days 3 and 5 of culture. At day 7, the cells were transferred to normoxic conditions until confluence (around day 11) and then trypsinized and plated in 24 well plates at a density $10^5/\text{cm}^2$. Medium was changed to different treatments the following day.

MTA Conditioned Medium

White ProRoot MTA was mixed with sterile water according to the manufacturer's instructions. MTA discs were prepared under aseptic conditions as described previously with minor modifications (20). Briefly, the discs were created by using a sterile cylindrical polyethylene tube (diameter: 6mm; height: 3mm). The MTA discs were kept a 5% CO₂ incubator at 37°C for 6 hours for setting. After 6 hours, the discs were sterilized by ultraviolet (UV) light for 1 hour. The discs were incubated in α -MEM 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂ for 3 days (1 mL of α -MEM 10% FBS for each disc). After 3 days, the supernatants were collected, filtered through a sterile 0.22 μ m filter (Sigma-Aldrich, Saint Louis, MO, USA). The supernatant collected was referred as MTA conditioned media (MTA-CM). Two different dilutions of MTA-CM were used (high dilution, 1:50 and low dilution, 1:5).

Cell viability assay

Cell viability was determined using a Presto Blue assay (Thermo Fisher Scientific, Waltham, MA, USA). At various time points the Presto blue reagent was added to the cell medium, incubated for 2 hours and the fluorescence intensity was measured (560nm excitation/590nm emission). The experiments were performed in triplicate.

Histochemical analysis of cell cultures

The histochemical staining for alkaline phosphatase (ALP) was performed on cultures fixed in 10% formalin for 5 minutes using 86-R Alkaline Phosphatase kit (Sigma Aldrich) according to the manufacturer instructions. The number of ALP positive colonies per well was counted. Mineralization was assessed after 21 days of culture using von Kossa staining as described previously (21). Plates were imaged on a flat bed scanner and mineralized area was quantified using ImageJ.

Detection of epifluorescence

Expression of GFP and tdTomato was imaged on an inverted Observer Z1 microscope (Carl Zeiss). The region scanned covered approximately half the

well area. Fluorescent area proportion for each channel was quantified using ImageJ.

RNA extraction and gene expression

RNA was extracted using Trizol reagent (Life Technologies) (19). Reverse transcription was performed using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The expression of osteogenic genes osteocalcin (Oc, Mm03413826_mH), bone sialoprotein (Bsp, Mm00492555_m1) was assessed by RT-qPCR (14). The gene expression was normalized by the expression of a housekeeping gene (GAPDH).

Statistics

Data were subjected to statistical analysis by using the GraphPad Prism (version 5.0) software. For all parametric data, ANOVA followed by Tukey's test was used. The p value was considered significant at 0.05.

Results

Effects of MTA on periodontal tissue regeneration

In the present study we used the previously characterized α SMA9-CreERT2;Ai9/Col2.3GFP mouse to examine the effects of MTA on regeneration of periodontal tissue following injury. Adhesive system (AS) without MTA has been used as a control for the effects of MTA on healing. In these experiments perforation on the pulp floor in the furcation area in the first maxillary molars was performed on 4-6 weeks old TM- injected α SMA9-CreERT2;Ai9/Col2.3GFP mice. In uninjured tissue, Col2.3GFP expression (referred to as 2.3GFP) was detected in osteoblasts, osteocytes within the alveolar bone, cementoblasts on the root surface, odontoblasts lining the pulp chamber and roots and in PDL fibroblasts surrounding the roots in the remaining areas of the teeth (Figure 1B-E). Histological analysis of the injured area showed that perforation at the pulpal floor resulted in local destruction of dentin, odontoblasts, PDL in the furcation area and the underlying alveolar bone as evident by the lack of 2.3GFP expression in these locations (Figure 1). Examination of the area underneath the injury showed presence of a very few α SMA9-tdTomato⁺ (referred as SMA9) and 2.3GFP⁺ cells in teeth filled with AS

and MTA (Figure 1). At this time the number of SMA9⁺ in bone marrow of the alveolar bone and in dental pulp were increased as compared to uninjured controls (Figure 1). Examination of the area of injury, 17 to 30 days following injury showed increase in expression of SMA9⁺, 2.3GFP⁺ and cells co-expressing SMA9⁺ and 2.3GFP⁺ at the site of injury in teeth filled with AS and MTA (Figure 2). Histological analysis in teeth filled with AS showed that the area underneath the injury contained necrotic tissue that was separated from alveolar bone with a relatively large fibrous area filled with small and elongated 2.3GFP⁺ fibroblasts, some of which were also SMA9⁺, oriented parallel to the bone surface (Figure 2A). A few cells co-expressing both transgenes were detected at day 17 but not at day 30 after injury (Figure 2B).

Histological examination of teeth filled with MTA showed that the area underneath the injury contained dentin chips, PDL-like fibroblasts and well-organized alveolar bone. SMA9⁺ and 2.3GFP⁺ cells were detected in the PDL-like fibroblasts and the alveolar bone in the area of repair. A few cells co-expressing both transgenes (SMA9⁺/2.3GFP⁺) were detected in the area of repair and underlying alveolar bone at day 17 (Figure 2A). The number of SMA9⁺/2.3GFP⁺ cells in PDL-like cells and alveolar bones increased at day 30 (Figure 2B). These observations together indicated contribution of SMA9⁺ cells in the organized PDL-like fibroblasts and underlying alveolar bone in teeth filled with MTA, but limited differentiation of SMA9⁺ cells to mature lineages in injuries filled with AS (Figure 2).

Further examination of these teeth showed that injury at the pulp floor and restorative materials also had a significant effect on the expression of these transgenes in the apical regions (Figure 3). Examination at day 30, showed significant expansion in the SMA9⁺ cells in the apical regions in injured teeth as compared to uninjured teeth (Figure 3). The apical region of teeth filled with MTA showed organized structure containing SMA9⁺ cells and 2.3GFP⁺ osteoblasts and cementoblasts. There were numerous double labeled cells in this area indicating the contribution of SMA9⁺ cells to osteoblasts and cementoblasts. Unlike the periapical region of teeth filled with MTA, the periapical regions of teeth filled with AS was very disorganized with significantly lower numbers of SMA9⁺ and 2.3GFP⁺ and no double labeled cells. These data

indicate that application of MTA resulted in better repair of the PDL in the area under the injury as well as in the apical region.

Effect of MTA-CM on PDLC In vitro

To gain a better understanding of the underlying process mediated by MTA, we examined the effects of media conditioned with MTA (MTA-CM) on the cell viability, presence of SMA9⁺ cells and differentiation of PDL progenitors. In these experiments the effects of two different concentrations of MTA-CM were compared to osteogenic media (OM) and control media. Presto Blue assay showed that OM media increased the viability of the PDL cells as compared to controls at day 7. However, MTA at both concentrations had negative effects on cell viability at day 7, although this effect was greater at the lower concentration (Figure 4A).

We also examined the effect of MTA-CM on osteogenic differentiation, by ALP staining and gene expression. In PDL cells, OM did not affect the ALP staining (Figure 4B) but resulted in increased levels of expression of OC and BSP as compared to controls. Both high and low concentrations of MTA-CM decreased the number of ALP⁺ colonies compared to both control and OM. MTA-CM also failed to induce expression of OC and BSP compared to control (Figure 4C). These observations showed that MTA-CM had negative effects on both viability and differentiation of PDL fibroblasts.

Effect of MTA-CM on Bone marrow stromal cells (BMSC) In vitro

We observed significant effects of MTA application on the alveolar bone underneath the site of injury as well as in the apical region. Therefore, we also examined the effects of MTA-CM on BMSCs. Our results showed that OM increased viability or cell number in the cultures while MTA-CM had a slight but significant negative effect on viability at both concentrations (Figure 5A). Treatment with OM increased von Kossa staining and increased levels of OC and BSP expression in BMSCs as compared to controls. Both concentrations of MTA-CM resulted in significant decreases in von Kossa staining and levels of expression of OC and BSP as compared to OM and control media (Figure 5C-D).

Effects of MTA-CM on the SMA9⁺ progenitors and their osteogenic differentiation in vitro

BMSCs and PDL cells grown in OM showed marked increases in SMA9⁺ and 2.3GFP⁺ areas as compared to controls indicating the positive roles of osteogenic media on progenitor cells and their differentiation (Figure 6A-B). In BMSCs and PDL cells grown in both concentrations of MTA-CM SMA9⁺ and 2.3GFP⁺ areas were significantly lower compared to OM, and 2.3GFP⁺ area was reduced compared to control (Figure 6A-B). Reduced levels of expression of markers of osteogenesis in these cultures were therefore related to reduced number of progenitor cells giving rise to osteoblasts.

Discussion

Despite the progress made in understanding the biological effects of MTA, the mechanism of its effects on wound healing and the nature of hard-tissue formation remain unclear. Therefore, our study focused on the effect of MTA on mesenchymal progenitor cells during repair of PDL and surrounding tissues. We showed that placement of MTA in site of injury at the furcation area can significantly improve the healing process. Compared to AS-filled teeth, where the injured area contained necrotic tissue and a large fibrous layer typical of scarring, in MTA filled teeth PDL-like fibroblasts and well-organized tissue were present. These observations are consistent with previous observations that have shown bacteria invasion and necrosis of tissues in teeth filled with AS due to its inadequate sealing properties (22,23). On the other hand, it is well documented that MTA is a commonly used restorative material because of its biological and sealing properties that reduce bacterial invasion (24).

Previous studies showed contribution of SMA9⁺ cells and their ability to differentiate into mature cell types, including PDL fibroblasts, osteoblasts and cementoblasts during growth and in repair after PDL injury (16). Our lineage tracing study showed significant expansion of SMA9⁺ cells, 2.3GFP⁺ cells and cells co-expressing SMA9/2.3GFP at the perforation site, confirming a biological response and healing process promoted by MTA. Furthermore, MTA induced contribution of SMA9⁺ cells in repair of alveolar bone underlying injured PDL, indicating that MTA promotes bone repair. Following PDL regeneration and alveolar bone healing, MTA showed a positive effect on distant root periodontal

complex. Unlike the disorganized apical region in AS capped teeth, in teeth filled with MTA this area showed organized structure containing differentiated SMA9⁺/2.3GFP⁺ osteoblasts and cementoblasts. These observations suggest that MTA mediates regeneration through interactions with periodontal ligament and alveolar bone progenitor/stem cells.

In contrast to our *in vivo* observations, *in vitro* results showed that MTA conditioned media does not support differentiation of BMSCs and PDLCs. The lack of differentiation in our *in vitro* studies is also different from previously reported positive effects of MTA on the formation of mineralized nodules and expression of cemento/osteoblastic marker genes in PDLCs and BMSCs (9,25,26). Explanation for these differences can be the amount of calcium, aluminum, bismuth and silicon ions released by MTA into media and its variation depending of concentration utilized, which might inhibit or suppress cell growth and functions resulting in changes in the cell response, such as proliferation and differentiation (27-29). It has been also reported that MTA enhances proliferation of human dental pulp cells through sustained release of calcium ions (30). In contrast, others have shown that rate of calcium ion release from MTA were higher during the first three hours with subsequent decreases thereafter (31). Furthermore, high concentrations of MTA have been shown to exert cytotoxic effects on human PDL fibroblasts (32). Taken together, these findings may explain decreased in viability when MTA-CM was used in PDLC and BMSC cultures. Although the outcomes of the *in vitro* experiments may depend on the cell type, different culture systems and concentrations of MTA, the most likely explanation for these differences is the lack of mineralization inducing reagents such as ascorbic acid and β -glycerophosphate in media used to examine the effects of MTA on differentiation.

Although cell studies results are relevant, it is not possible to assess the complex interactions between materials and host. Therefore, we primarily evaluated the *in vivo* effects of the MTA on the progenitor lineages. Collectively, our findings showed contribution of SMA9⁺ cells in soft tissue repair and newly calcified bone matrix formation as well as positive effect of MTA on PDL and alveolar bone injury.

Acknowledgments

We would like to thank all individuals who provided reagents, valuable input and technical assistance in various aspects of this study, including Drs. Emilie Roeder and Xi Wang. This work was supported by R01-DE016689 (MM), R01-AR055607 (IK) & R90-DE022526 grants from National Institute of Health (NIDCR); by #2014/13750-0 scholarship from São Paulo Research Foundation (FAPESP). BGM is supported by Connecticut Stem Cell grant 14-SCA-UCHC-02. The authors declare no conflicts of interest.

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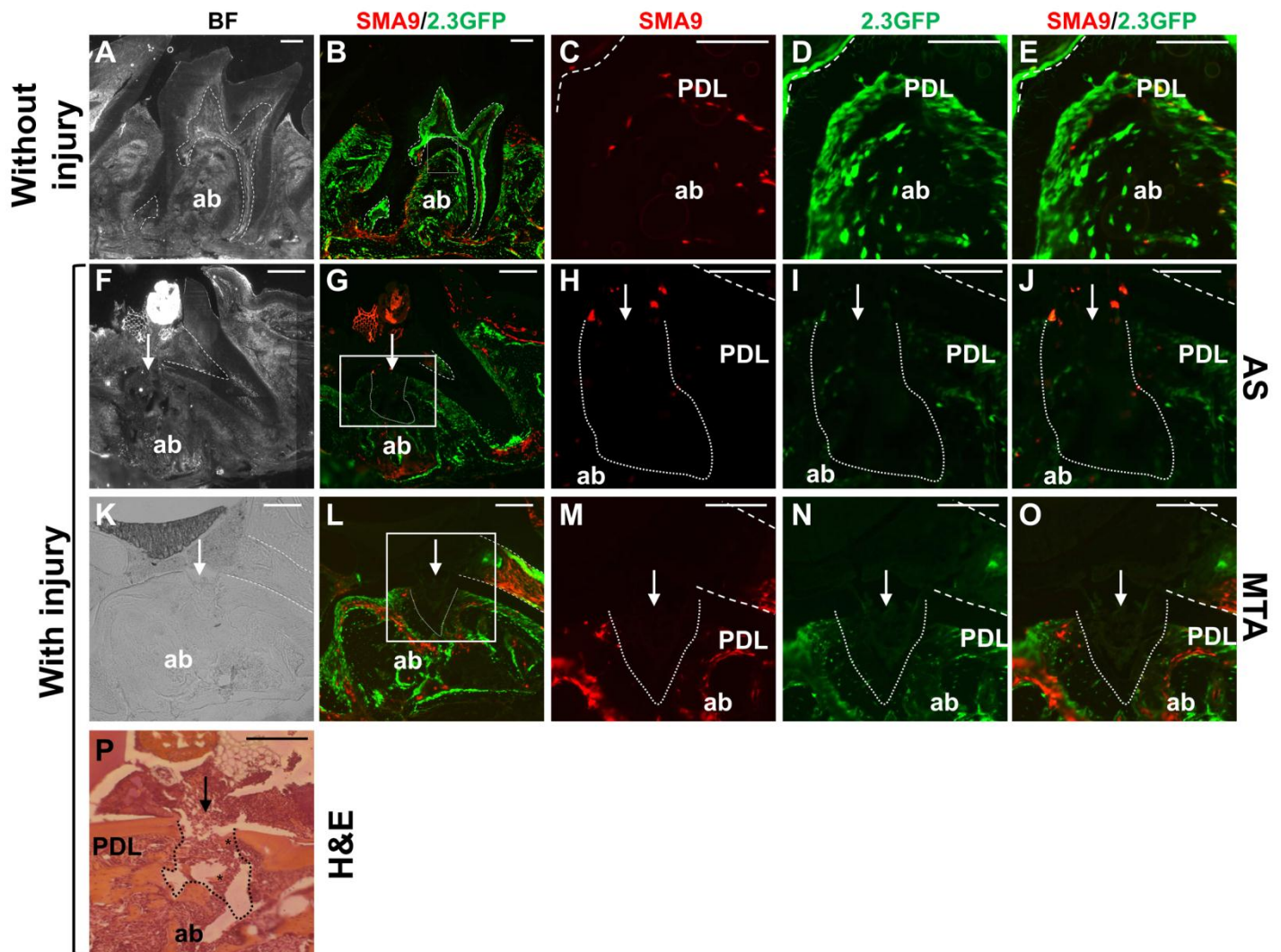


Figure 1: Effects of experimental perforation of the integrity of PDL and alveolar bone. Images of sagittal sections through maxillary molars from α SMACreERT2;Ai9/Col2.3GFP mice are shown. In all images the dental pulp is denoted by dashed lines, the site of injury by an arrow and the injury to the PDL and underlying alveolar bone by dotted lines. **(A – E)** Bright field **(A)** and epifluorescence **(B)** images of intact molars isolated 14 days post TM injection. **C - E** are higher magnification of boxed area shown in **B**. Note the expression of SMA9⁺ cells (red) and 2.3GFP⁺ cells (green) in dental pulp, periodontal ligament (PDL) and alveolar bone (ab). Also note a few cells co-expressing SMA9/Col2.3-GFP (yellow) detected in PDL indicating the differentiation of SMA9 into PDL fibroblasts. **(F – O)** Bright field **(F, K)** and epifluorescence **(G, L)** images of sections through injured maxillary molars filled with AS **(F-J)** and MTA **(K-O)** isolated 2 days post PDL injury. **H – J** are higher magnification of boxed area shown in **G**. **M – O** are higher magnification of boxed area shown in **L**. Note the lack of detectable SMA9⁺ and 2.3GFP⁺ cells at the sites of injury. Also note expansion of SMA9⁺ and 2.3GFP⁺ cells in bone marrow of the alveolar bone surrounding injury in molars restored with MTA **(L – O)**. **P** is an H&E stained section of a maxillary molar isolated 2 days after injury (indicated by arrow). Note the destruction of dentin in the pulpal floor, PDL and alveolar bone at the site of injury. Also note residual dentin chips (*). Scale bars=100 μ m.

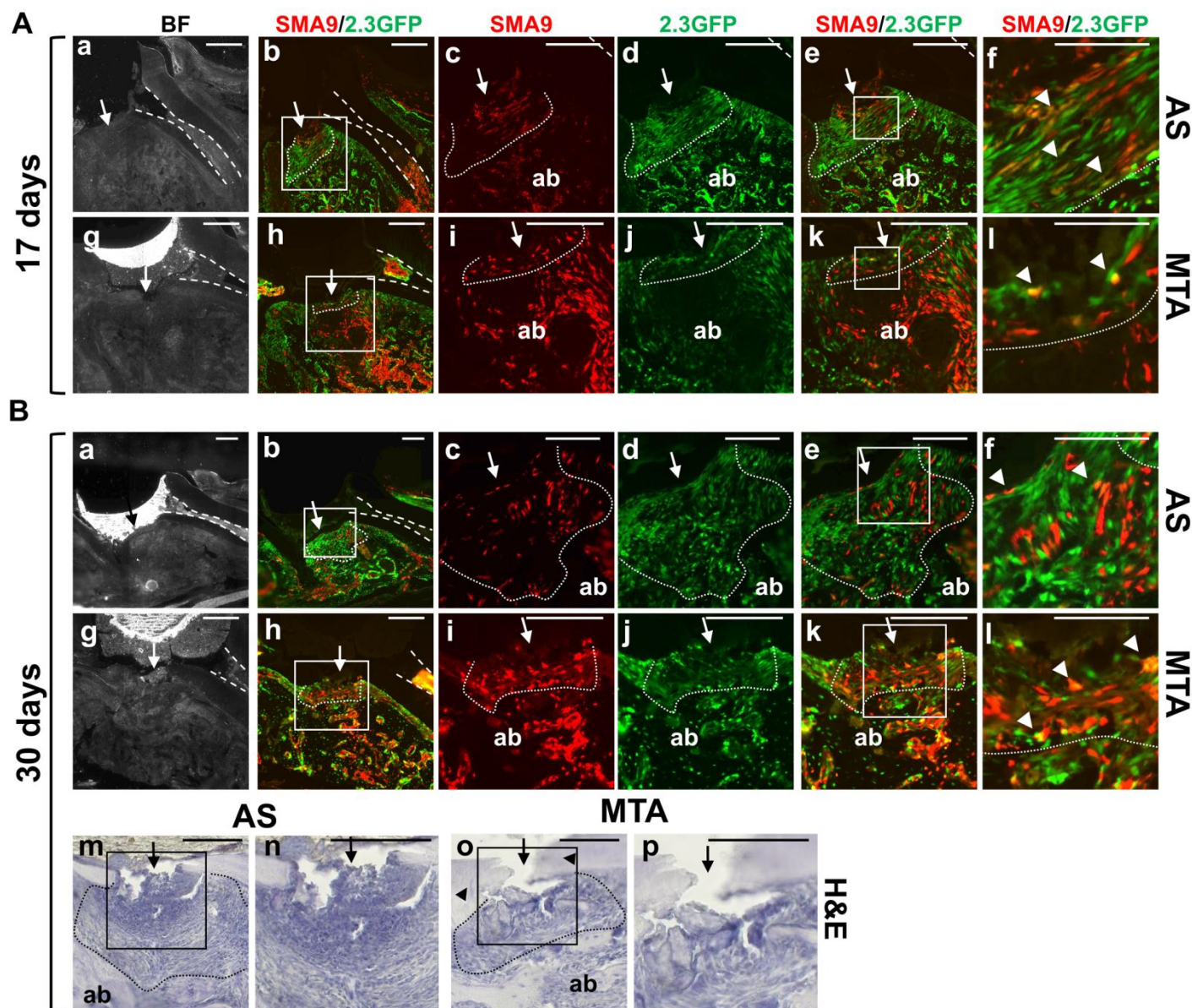


Figure 2. Effect of MTA on regeneration of PDL and the underlying alveolar bone. Representative bright field and epifluorescence images of sagittal sections through injured maxillary molars from α SMACreERT2;Ai9/Col2.3GFP animals. In all images the dental pulp is denoted by dashed lines, the site of injury by an arrow and the sites of repair by dotted lines. **(A)** Histology of injured molars 17 days after injury and restoration with AS (a – f) and MTA (g – l). c – f are higher magnification of the boxed area outlined in b and i – l are higher magnification of boxed area in h. SMA9⁺ cells are present in PDL region (outlined by dotted lines) and alveolar bone (ab) in both groups (c and i). Thick layer of cells expressing 2.3GFP are evident in AS restored molars (d). Co-expression of SMA9 and 2.3GFP (yellow, arrowheads) is indicated. **(B)**. Images of molars 30 days after injury and restoration with AS (a – f) and MTA (g – l). c – f are higher magnification of the boxed area outlined in b and i – l are higher magnification of boxed area in h. Note increase in number of SMA9⁺ cells in repaired PDL and alveolar bone in molars restored with MTA (i) as compared to molars restored with AS (c). Also note increase in co-expression of SMA9⁺ and 2.3GFP⁺ cells (arrowheads) in repaired PDL of molars restored with MTA (f) as compared to molars restored with AS (l). Scale bar=50 μ m, (f and l) in A and B=25 μ m. (m - p) are images of H&E stained section of maxillary molars 30 days after injury (indicated by arrow). n and p are higher magnifications of boxed areas in m and o, respectively. Repaired PDL and PDL region are outlined with dotted line. Note the lack of alveolar bone repair and large fibrous area containing fibroblastic cells in PLD region in molars restored with AS (m and n). Also note well-organized repaired alveolar bone(ab), PDL with PDL-like fibroblasts, and partial dentin repair (arrowheads) in molars restored with MTA (n and o). Scale bar=100 μ m.

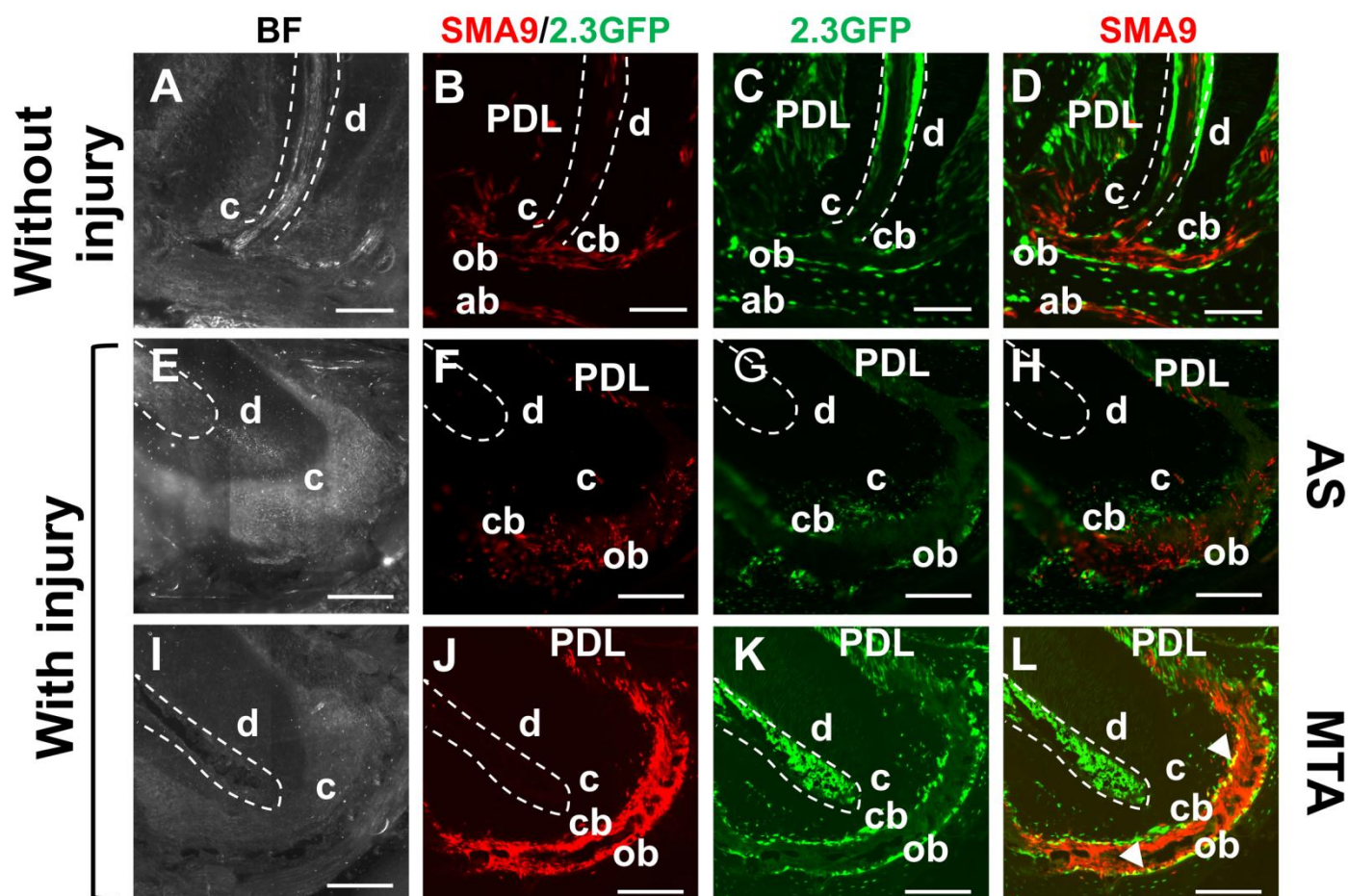


Figure 3. Effect of MTA on apical region. A – D are representative bright field and fluorescent images of a sagittal section through the root of an uninjured maxillary molar at day 30 post TM injection showing expression of SMA9⁺ (B) and 2.3GFP⁺ (C) cells in dental pulp (outlined with dotted line), alveolar bone (ab), periodontal ligament (PDL), cementoblasts (cb) and osteoblasts (ob) in apical area. Note co-expressing SMA9/Col2.3-GFP (yellow) detected in cementoblasts and osteoblasts. Scale bar=100µm. E – L are representative images of the apical root region at day 30 post injury restored with AS (E – H) and MTA (I – L). Note significant expansion of SMA9⁺ (J) and 2.3GFP⁺ (K) cells in apical area of MTA restored molars as compared to AS restored molars (F-G). Also note lack of co-expressing cells in apical cementoblasts and osteoblasts in molars restored with AS (H) as compared to MTA restored molars where co-expression of SMA9 and 2.3GFP is present in both cementoblasts and osteoblasts (L). Note increase in co-expressing cells in apical region of molars restored with MTA as compared to control without injury. Scale bar=50µm.

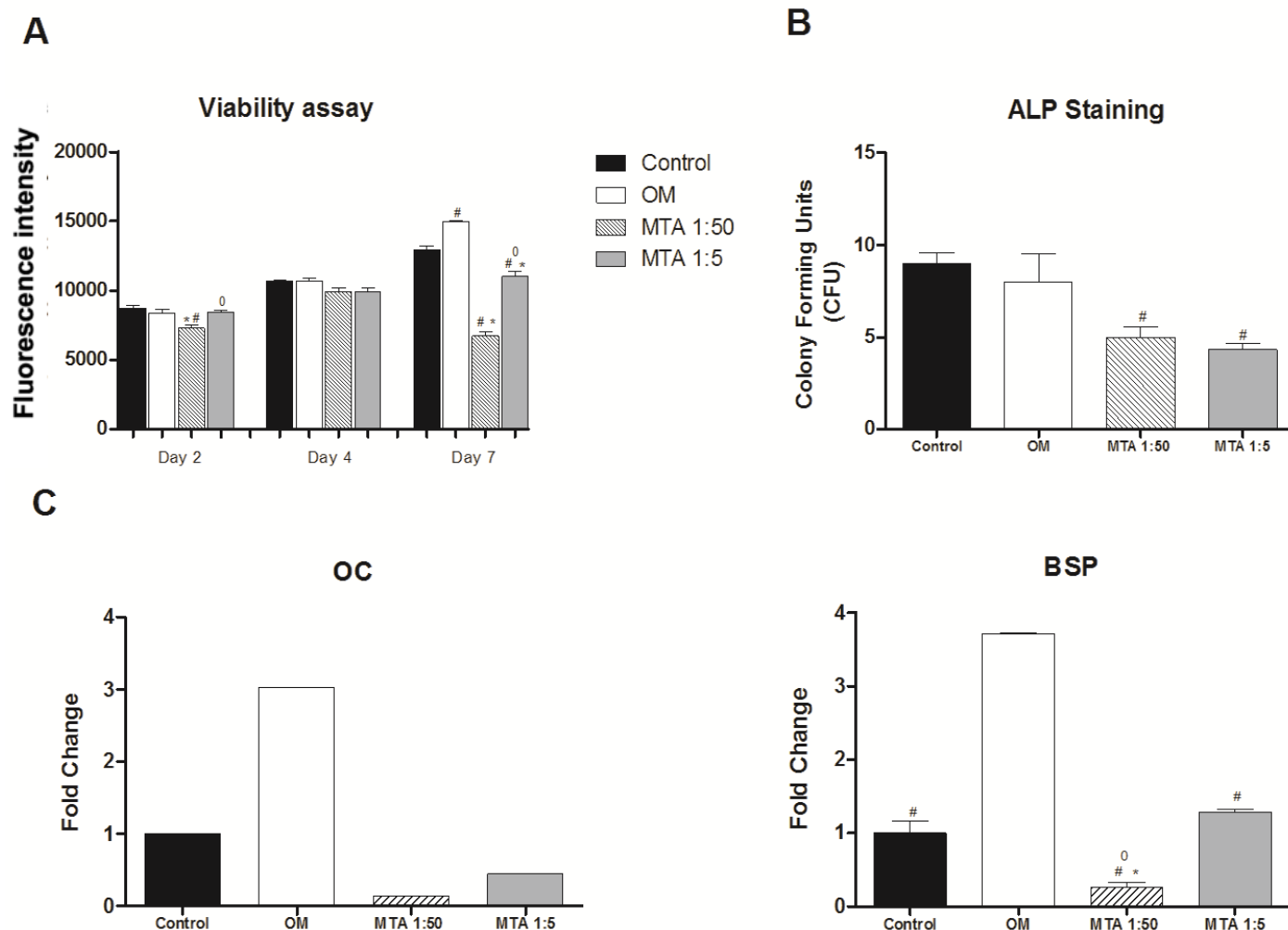


Figure 4. Effect of MTA-CM on cell viability and osteogenesis of PDLCs. PDLC were cultured in control medium, osteogenic medium (OM) or MTA-CM diluted 1:50 or 1:5. (A) Cell viability was determined by using Presto blue reagent at days 2, 4 and 7 after treatment initiation. (B) ALP staining was performed on day 7 of treatment and positive colonies were counted. (C) Expression of OC and BSP was determined at day 7 of treatment, normalized to GAPDH expression. Results represent mean \pm SEM values from three independent experiments. #: $p \leq 0.05$ (vs. Osteogenic media); *: $p < 0.05$ (vs. Control); 0: $p < 0.05$ (vs. MTA 1:50).

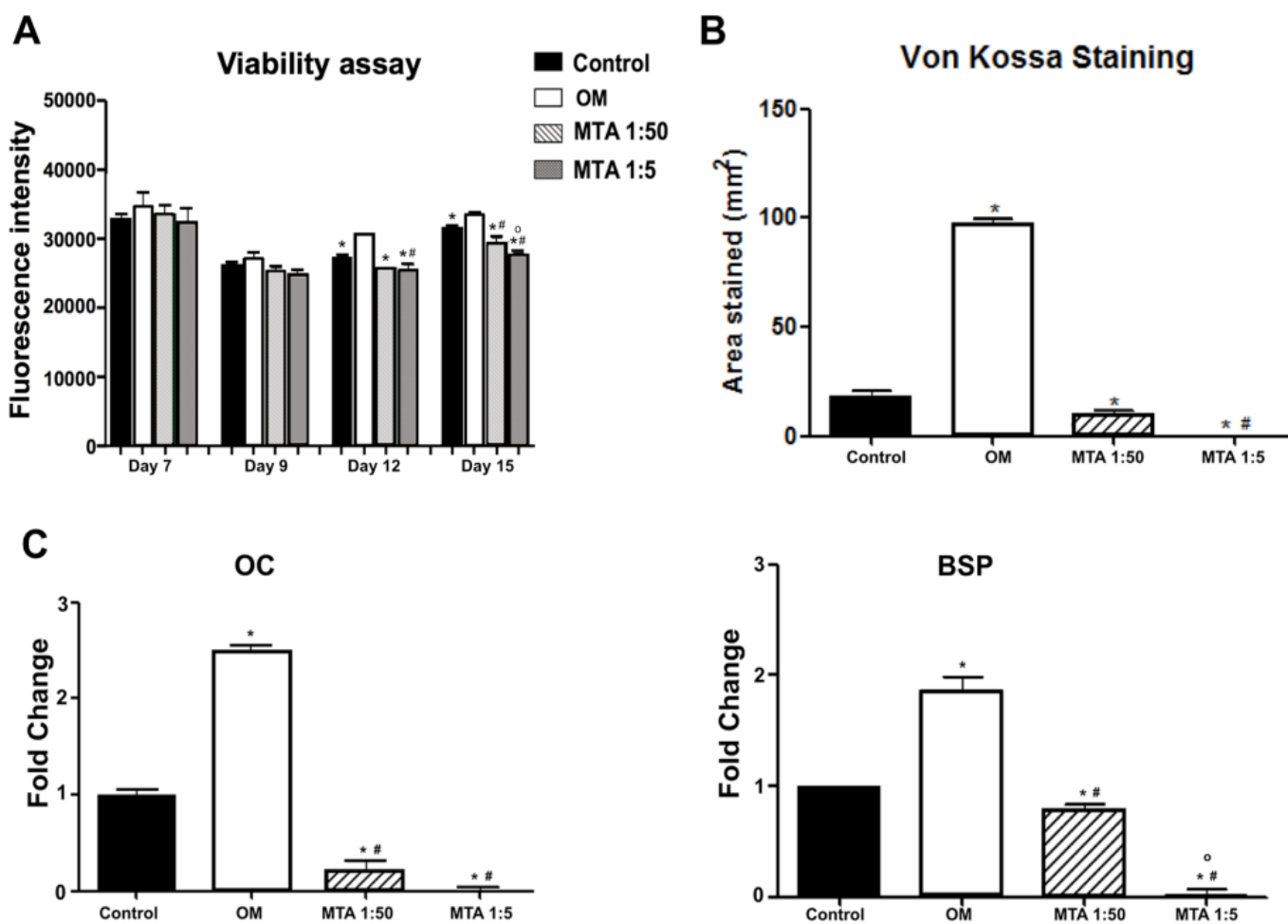


Figure 5. Effect of MTA-CM on cell viability and osteogenesis of BMSC cultures. BMSC were cultured in control medium, osteogenic medium (OM) or MTA-CM diluted 1:50 or 1:5. (A) Cell viability was determined by using Presto blue reagent at days 7 (before MTA-CM), 9, 12 and 15. (B) Mineralization by von Kossa staining was assessed on day 21 and expressed as area stained. (C) Expression of OC and BSP was determined at day 21 of culture, normalized to GAPDH expression. Results represent mean \pm SEM values from three independent experiments. #: $p \leq 0.05$ (vs. Osteogenic media); *: $p < 0.05$ (vs. Control); 0: $p < 0.05$ (vs. MTA 1:50)

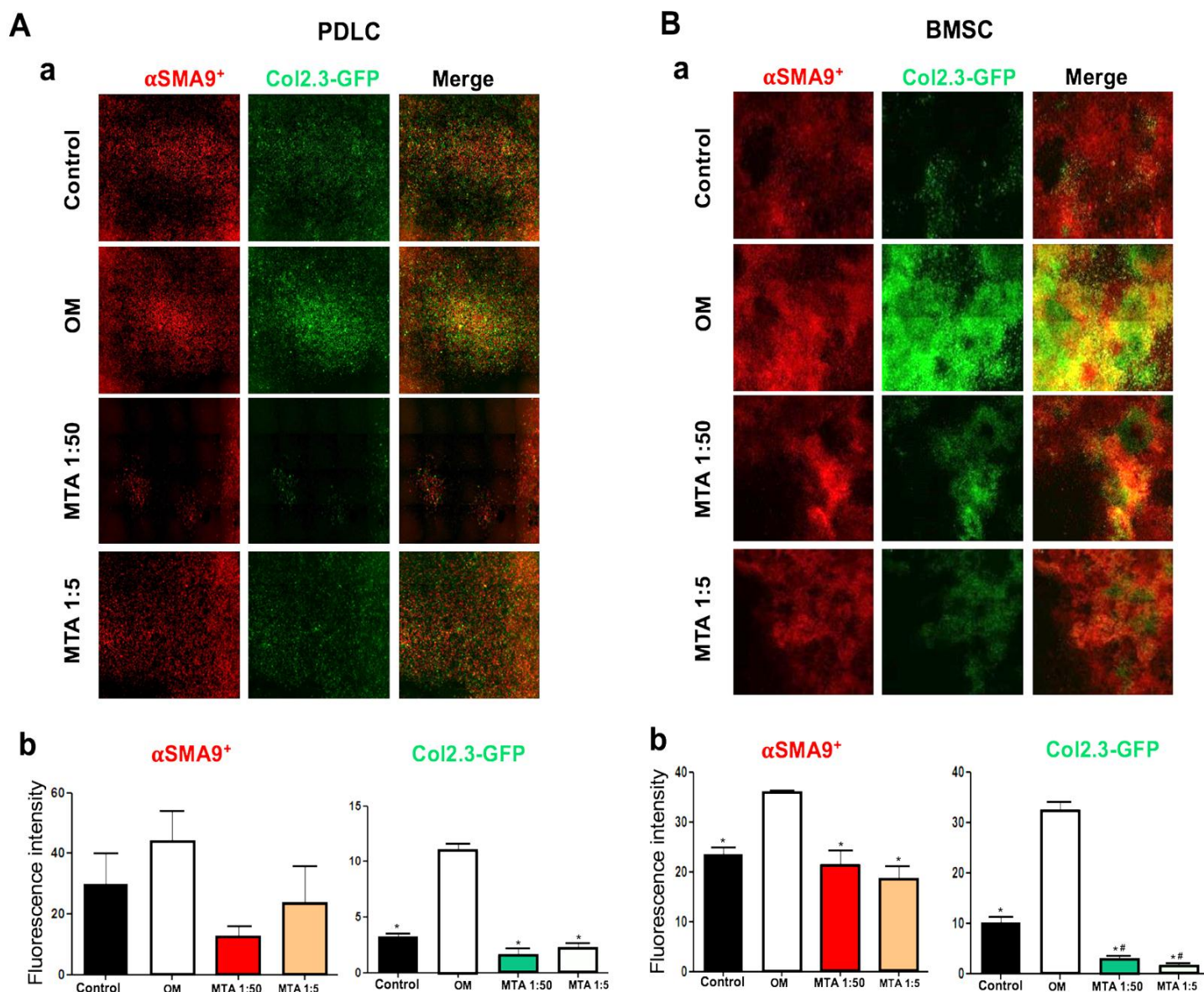
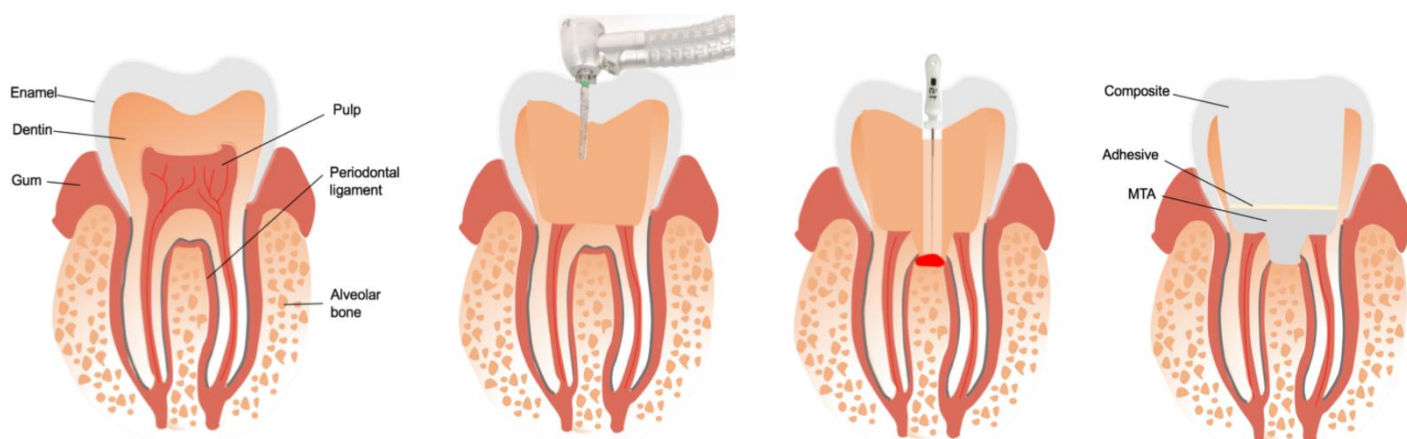
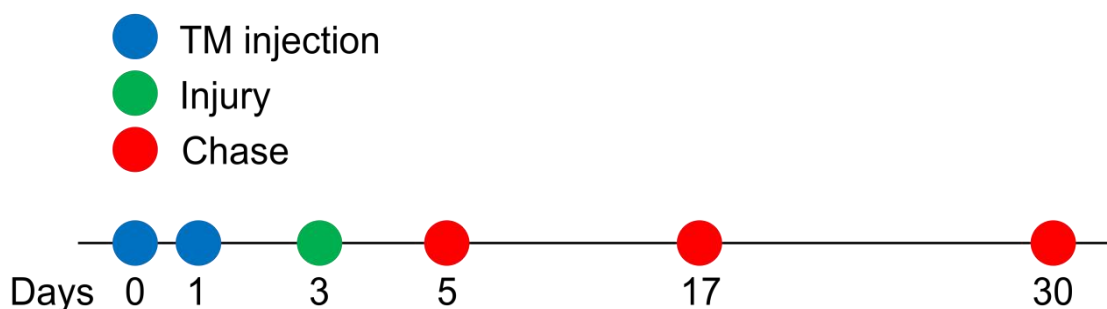


Figure 6. Effects of MTA-CM on the SMA9⁺ progenitors and their osteogenic differentiation in vitro. (A-B) Images of scanned PDLC and BMSC cultures analyzed at the end point (day 7 for PDLC and day 21 for BMSC). Increase of the SMA9 and 2.3GFP positive areas in OM group as compared to control in both PDLC and BMSC cultures was observed. Also, note significant decreases in SMA9⁺ and 2.3GFP⁺ areas in both concentrations of MTA-CM as compared to OM in both cultures. Image analysis was completed on whole cell culture wells and area of fluorescence was measured. The images are generated from one representative of three biological replicates.



Supplemental Figure 1. Schematic representation of experimental PDL injury. A. Scheme of lineage tracing experiments where 4-6 weeks old α SMACreERT2/Ai9 transgenic mice were injected with tamoxifen (TM) twice in 24- hour interval. PDL injury was performed 48 hours after second injection and animals were chased at indicated time points after PDL injury. B. Schematic representation of experimental PDL injury where access cavity preparation was performed using dental carbide round bur. Access through the floor of dental pulp chamber to PDL was created using endodontic K file size 15 and PDL with surrounding alveolar bone was injured with the same instrument. In experimental group injury was capped with MTA and composite associated with adhesive system (AS). In control group injury was capped with AS and tooth was restored with composite.

Artigo 2

Artigo 2: Hyperglycemic condition interferes on cell proliferation and IL-6 production stimulated by Gray MTA

Abstract

Introduction: Diabetes mellitus (DM) affects inflammatory and immune responses and impairs healing processes. We investigated DM influence on cell proliferation and cytokine production induced by Gray Mineral Trioxide Aggregate (GMTA) and White MTA (WMTA).

Methods: L929 fibroblasts were cultured under high or normal glucose concentration. Effects of GMTA and WMTA on cell proliferation and IL-1 β , IL-6, and TNF- α production were investigated using Alamar Blue assay and ELISA, respectively, at 6, 24, 48, and 72h. Moreover, polyethylene tubes containing GMTA, WMTA, and empty tubes (control) were implanted into dorsal connective tissues of Wistar rats previously assigned normal and diabetic groups (Alloxan induced). After 7 and 30 days, the tubes with surrounding tissues were removed, fixed, and subjected to immunohistochemical analysis of IL-1 β , IL-6, and TNF- α . Nonparametric and parametric data were statistically analyzed ($p < 0.05$).

Results: *In vitro* assays showed no detectable production of IL-1 β and TNF- α . The hyperglycemic condition promoted IL-6 up-regulation production ($p < 0.05$) and impaired cell proliferation at 72h ($p < 0.05$). Under high glucose condition, GMTA induced greater cytotoxicity and IL-6 production than WMTA did. *In vivo* assay showed no differences in IL-1 β , IL-6, and TNF- α production between both systemic conditions in presence of GMTA and WMTA at all time points.

Conclusion: Hyperglycemic conditions interfered on cell proliferation and IL-6 production stimulated by GMTA. Moreover, no up-regulation of inflammatory cytokines in diabetic animal tissues was observed in the presence of GMTA and WMTA.

Keywords: Diabetes Mellitus; Cytokines; Gray MTA; White MTA

Introduction

Diabetes mellitus (DM) is a metabolic disease considered as a modulator of endodontic infections (1). The hyperglycemic state promoted by DM can alter immune functions through impairment of leucocyte responses and cellular defense capacity reduction, leading to increased susceptibility to infection and inflammation and a direct effect on dental pulp integrity and periapical healing process (2-7).

During periapical healing, proinflammatory cytokines IL-1 β , IL-6, and tumor necrosis factor alpha (TNF- α) play an essential role in inflammatory response development (8,9). Moreover, tissue response to biomaterials depends on innate and nonspecific immune responses (5,8,10). The root end filling material is set in close contact with periapical tissue and its chemical composition can interfere on inflammatory response and consequently affect repair process (9,11).

Mineral Trioxide Aggregate (MTA) has been widely studied as an endodontic material since it was introduced (12). Both *in vivo* and *in vitro* investigations showed that MTA is biocompatible, shows low cytotoxicity, and stimulates hard tissue formation (13,14). Besides, MTA stimulates the release of IL-1 β , IL-6, TNF- α , and growth factors from cells (15-17). Even in diabetic conditions, MTA does not alter tissue response, promotes mineralization and induces dentin bridge formation (6,14,18). Despite these investigations, the biological mechanism of MTA, especially its relationship with systemic conditions, is still not completely elucidated.

DM induces alterations in immune cell function and stimulates expression of proinflammatory cytokines (3), thus compromising dental pulp and periapical response. In this study, we aimed to evaluate *in vitro* and *in vivo* effects of both Gray and White MTA on cell viability and cytokine production under diabetic conditions.

Materials and Methods

In vitro study

Cell Culture

L929 mouse fibroblasts were grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO

BRL, Gaithersburg, MD) streptomycin (50 g/mL), and 1% antibiotic/antimycotic cocktail (300 U/mL penicillin, 300 µg/mL streptomycin, 5 µg/mL amphotericin B) (GIBCO BRL, Gaithersburg, MD) under standard cell culture conditions (37°C, 100% humidity, 95% air, and 5% CO₂).

The hyperglycemic condition was simulated with cell culture media supplemented with a high concentration of glucose (25 mM glucose).

MTA conditioned media

Gray and White MTA Angelus[®] (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil) were mixed according manufacturer's instructions. MTA discs were prepared as previously described (19) with some modifications. Briefly, the discs were formed using a sterile polyethylene tube (5 mm in diameter and 3 mm in height) and kept in a 5% CO₂ incubator at 37°C to setting for 6h. Then, the discs were removed from the mold and sterilized in ultraviolet (UV) light for 1h. Each disc was immersed in 1 mL of DMEM with 10% FBS and incubated in a humidified atmosphere containing 5% CO₂. After 3 days, the discs were discarded and supernatants (extract) were collected and filtered through a sterile 0.22 µm filter (Sigma-Aldrich, Saint Louis, MO, USA). The collected supernatants were referred as the GMTA extract or WMTA extract. An extract dilution of 1:50 was used in this study.

Cell proliferation assay

Cell proliferation was determined using Alamar Blue reduction assay (Alamar Blue[®] Cell Viability Reagent, Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. L929 fibroblasts were seeded into 24-well plates (10⁴ cells/mL medium per well) and incubated for 24h in a humidified air atmosphere of 5% CO₂ at 37°C. After, both MTA extract (1:50) and Alamar Blue reagent (1:10) was added to the culture medium and after 6h, 24h, 48h, and 72h, 200 µl of medium was transferred to a 96 well plate. Optical density (OD) was measured at 570 nm and 600 nm. Alamar blue reduction was calculated with a manufacturer provided formula. The percentage of reduction level reflects the cell proliferation. The controls were cultured in media without MTA extracts. The experiments were performed in triplicate.

Inflammatory cytokine production assay

For cytokine assay, L929 fibroblasts were seeded into 24-well plates (10^4 cells/mL medium per well) and incubated for 24h in a humidified atmosphere of 5% CO₂ at 37°C. After incubation, both MTA extracts were added to cells at the dilution of 1:50. After 6, 24, 48, and 72h of extracts addition, the culture media were collected and levels of IL-1 β , IL-6, and TNF- α were evaluated using DuoSet ELISA kits according to manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA). Cells cultured without MTA extracts served as controls.

In vivo study***Animals***

Twenty male Wistar albino rats weighing 250–280 grams and within 3 to 4 months-old were used in this study. The animals were divided in two main groups: normal and diabetic. The diabetic condition was induced as described previously (14). The study was approved and performed according to the guidelines of Ethical Committee (protocol number 00557-2013).

Surgical procedure and immunohistochemical analyses

Polyethylene tubes (Abbott Labs of Brazil, São Paulo, SP, Brazil) filled with Gray and White MTA Angelus or empty tubes were implanted in the dorsal connective tissue of rats (14). On 7 and 30 days after implantation, six animals from each group were euthanized and the implanted tubes along with the surrounding tissues were excised, fixed, processed and embedded in paraffin. The tissues were then sliced into 5 μ m semi-serial sections and submitted to immunohistochemistry using an indirect immunoperoxidase technique for detecting IL-1 β (Rabbit anti-IL-1 β SC 7884), TNF- α (Goat anti-TNF- α SC 1348), and IL-6 (Rabbit anti-IL-6 SC 1265).

Cytokine production near the tube opening was analyzed at 400x magnification (Leica Microsystems, Wetzlar, Germany). A semi-quantitative immunostaining analysis was performed and the criteria for establishing immunoreactivity patterns were adapted from Garcia *et al.* (20): score 0: total absence of immunoreactivity (IR); score 1 (low IR pattern): IR in approximately 25% of cells per field; score 2 (moderate IR pattern): IR in approximately 50% of

cells per field; score 3 (high IR pattern): IR in approximately 75% of cells per field.

Statistical analysis

Data were subjected to statistical analysis by using the GraphPad Prism (version 5.0) software. For nonparametric data, Kruskal-Wallis test was used followed by Dunn test, and for parametric data, ANOVA followed by Bonferroni's correction. The p value was considered significant at 5%.

Results

Effects of MTA on cell proliferation under high glucose concentration

The effects of MTA on proliferation of L929 fibroblasts grown at high or normal glucose concentration are shown in Figure 1. In normal conditions, L929 proliferation was not affected by GMTA and WMTA. In contrast, under hyperglycemic conditions, GMTA treatment showed reduced cell proliferation at 24h ($p<0.05$).

Comparison between all groups in both conditions indicated that hyperglycemic conditions impaired the cell proliferation at 72h ($p<0.05$).

Influence of DM on cytokine production upon MTA treatment

ELISA was used to assess IL-1 β , TNF- α , and IL-6 production by L929 fibroblasts grown under high or normal glucose concentration after MTA treatment. Irrespective of hyperglycemic condition, no production of IL-1 β and TNF- α was observed in the presence of both MTAs.

However, significant effects on IL-6 production were observed as shown in Figure 2. In normal conditions, Control group released more IL-6 than that by the GMTA-treated group at 6 and 48h ($p<0.05$). At 48h, IL-6 production upon WMTA treatment significantly reduced compared to that in Control group ($p<0.05$). Under hyperglycemic condition, at 6 h, Control group secreted more IL-6 than GMTA-treated group ($p<0.05$). However, at 48h, WMTA-treated group showed more IL-6 production than Control group ($p<0.05$). Differences between both MTAs were observed only at 24h, when GMTA-treated cells showed more IL-6 production than WMTA-treated cells ($p<0.05$). Additionally, hyperglycemic conditions promoted up-regulation of IL-6 production in all groups ($p<0.05$).

In contrast, *in vivo* experiment revealed the presence of IL-1 β , TNF- α , and IL-6 positive cells in all groups evaluated on day 7 and 30. However, no significant difference was detected among all groups in both systemic conditions. Cells stained positively for IL-1 β , TNF- α , and IL-6 are shown in Figures 3 and 4.

Discussion

Determination of relationship between systemic diseases and their oral manifestation, especially with regard to tissue response to endodontic materials is challenging, and few studies have emphasized this relationship (6,14,18). DM is a systemic disease associated with impaired cell proliferation and raise on proinflammatory cytokine production (3, 21). In this study, we aimed to evaluate the effect of both Gray and White MTA on cell proliferation and cytokine production under hyperglycemic conditions. Additionally, to simulate hyperglycemic condition, we used glucose concentrations corresponding to those observed in patients with poorly controlled diabetes (25mM) (22).

It has been previously reported that glucose induces alteration of gene regulation, differentiation, and cell proliferation (21). Cell growth is dependent on glucose concentration, and high glucose concentration is known to impair cell proliferation (21,22). In our study, cell proliferation was affected by high glucose concentration only at 72h. This result is in agreement with the report by Li et al. (21) that evaluated effect of high glucose concentrations (25mM) in two different types of cell and demonstrated that cell proliferation varies according to evaluated cell type and the glucose exposure time, where increasing exposure time results on decreasing cell proliferation.

L929 proliferation was not inhibited in normal conditions by both MTAs, thus confirming low cytotoxicity of these materials as reported previously (16,23). In contrast, Haglund et al. (24) reported that MTA caused inhibits growth of L929 fibroblasts. However, under hyperglycemic conditions, GMTA showed increased cytotoxicity only at 24h. DM is associated with altered calcium homeostasis (25) and the difference in chemical composition of both MTAs (26) results in release of more Ca⁺² ions with GMTA treatment in initial periods (27), which could increase intracellular calcium levels and alter cell functions. These facts in relation to impaired cell proliferation induced by

hyperglycemic state and observation that related cell cultures show better growth upon treatment with WMTA (28), could explain the obtained results. Moreover, no difference in cytotoxicity and cell proliferation between GMTA and WMTA has been described in literature (23).

Although studies have shown release of cytokines IL-1 β , TNF- α , and IL-6 by cells grown in presence of both MTAs (15-17), no expression of IL-1 β and TNF- α was observed with both tested materials, irrespective of diabetic condition. However, IL-6 production was observed at all time points evaluated. IL-6 is a pro- and anti-inflammatory cytokine released by several cell types in response to irritants (29) and can be correlated with suppression of IL-1 β and TNF- α transcription (30). Besides, IL-1 β and IL-6 were not previously detected in presence of MTA probably owing to cell type and time of exposure to MTA (24). In addition, the effect of MTA on cytokine production depends of cell culture type, MTA composition, and specific cytokines investigated (13). In this study, MTA extract was used to avoid direct contact between the test material and cells, which could change both properties of material and cell response (19,31).

IL-6 expression has significant anti-inflammatory effects in modulating infection-stimulated bone destruction (32). In normal conditions, ELISA revealed IL-6 production in the presence of both MTAs corroborating with previous studies (15,16). In addition, Control group produced more IL-6 compared to that with GMTA at 6h and 48h, and with WMTA at 48h. These findings can be explained by studies that reported an anti-inflammatory effect for MTA resulting in decreased production of some proinflammatory cytokines (8,33).

Meanwhile, the average IL-6 production was higher in hyperglycemic condition than that under normal conditions. Glucose intake is reported to cause oxidative stress and inflammatory changes at cellular and molecular levels, which promotes upregulation of IL-6 and other cytokines (34), thus corroborating our findings. Furthermore, at 24h, GMTA-treated cells showed more IL-6 production than WMTA-treated cells did, and at 48h, WMTA-treated cells showed more IL-6 production than Control group did. The ionic dissociation of GMTA may be changed by its high concentration of Fe₂O₃ (26), resulting in release of more Ca⁺² ions at the initial time compared to that by WMTA (27). Because an increase in Ca⁺² ions may act as a tissue irritant and

IL-6 being a cytokine released in response to irritants, we considered that these facts might explain our results in the diabetic conditions.

Although cell culture studies are widely used to investigate the cytotoxicity of a biomaterial, they cannot be used to examine host-biomaterial interaction. Thus, to better understanding the DM influence on cytokine production of both MTAs, a subcutaneous implantation study was performed.

Irrespective of diabetic condition, IL-1 β , IL-6, and TNF- α immune-reactive cells were identified at all time points evaluated in presence of both MTAs. In fact, previous studies reported IL-1 β , IL-6, and TNF- α production in presence of MTA (8,33). However, no difference was observed between both MTAs and systemic conditions. Although DM induced an inflammatory response and cytokines release (3,34), no evidence of a direct correlation between DM and production of IL-1 β , IL-6, and TNF- α upon MTA treatment was established. In addition, no reports evaluating such cytokine production were found in existing literature. The ability of MTA to precipitate apatite during acute phase of inflammation, together with its alkalinity, might induce modifications in gene expression and signaling pathways (35). These facts together with anti-inflammatory effect of MTA (8,33) and its capacity to promote no significant tissue inflammation (14) could affect these cytokines production and explain ours findings. Besides, earlier reports have shown no difference in inflammatory response to MTA in diabetic animals (14,18).

Therefore, it is clear that further investigations are necessary to clarify the correlation between DM and inflammatory and immune response to endodontic materials.

On basis of these results, we concluded that hyperglycemic condition interfered on cell proliferation and IL-6 production observed with GMTA treatment at 24hs. However, IL-1 β , IL-6, and TNF- α production in tissue of diabetic animals was not altered in presence of both MTAs.

Conflicts of interest

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

Acknowledgments

This study was financially supported by FAPESP (São Paulo Research Foundation) #2013/06641-8 and #2014/25475-4.

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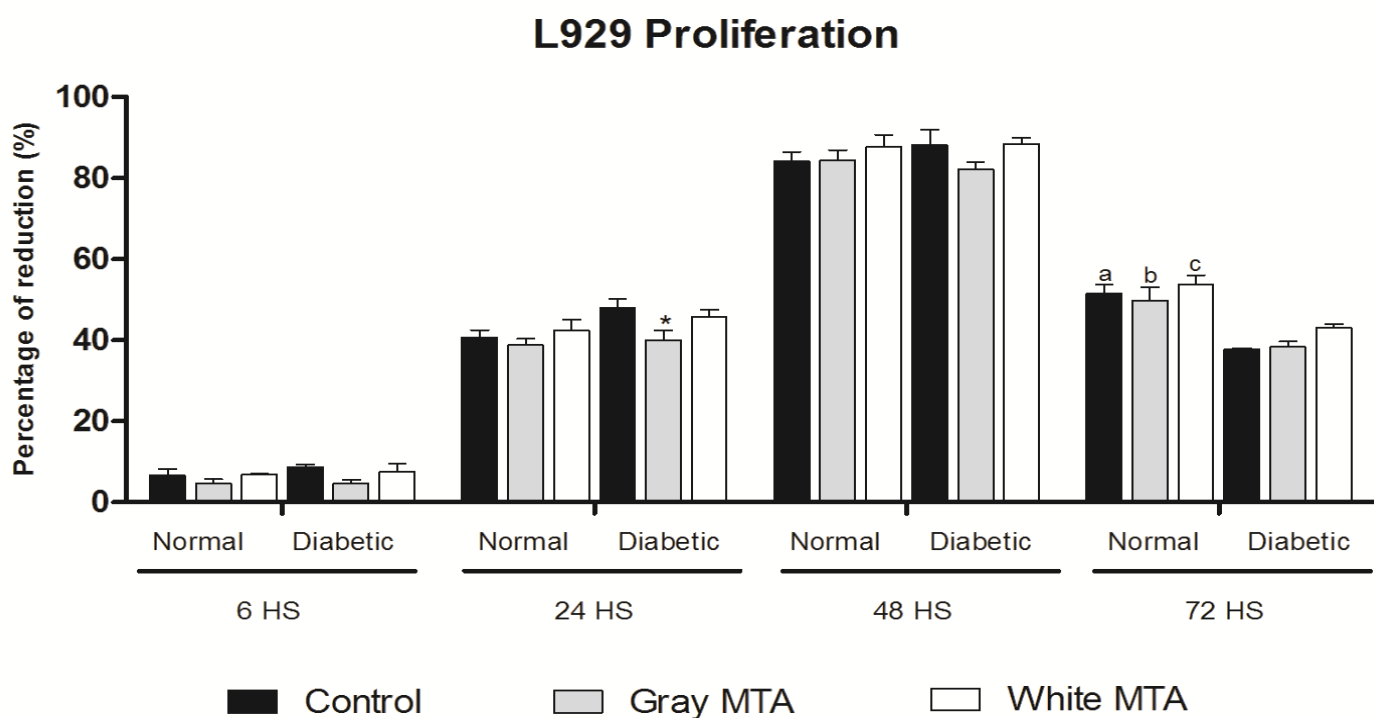


Figure 1: Effect of both Gray MTA and White MTA extract on L929 proliferation under high or normal glucose concentration after 6, 24, 48, and 72 h were determined using the Alamar blue reduction assay. The hyperglycemic conditions impaired cell proliferation at 72 h ($p < 0.05$). Symbols: *: $p < 0.05$ vs. Control. The letters indicate statistical difference between the normal and hyperglycemic conditions. Letters: a: $p < 0.05$ vs. Control diabetic; b: $p < 0.05$ vs. Gray MTA diabetic; c: $p < 0.05$ vs. White MTA diabetic.

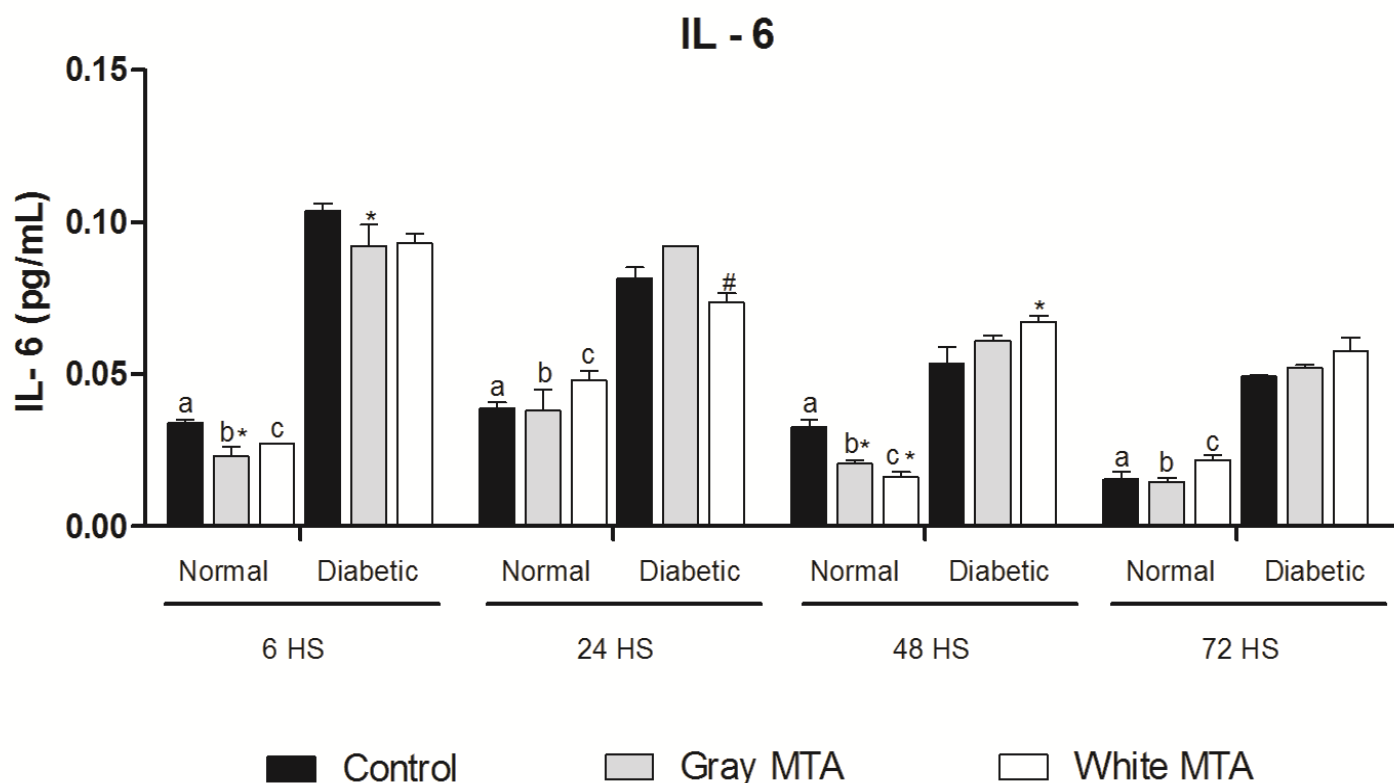


Figure 2: Influence of hyperglycemic condition on IL-6 production by fibroblasts upon MTA treatment. The symbols indicate the statistical difference under each systemic condition. The hyperglycemic conditions resulted in upregulated IL- 6 production. Symbols: *: $p < 0.05$ vs. Control; #: $p < 0.05$ vs. WMTA. The letters indicate statistical difference between the normal and hyperglycemic conditions. Letters: a: $p < 0.05$ vs. Control diabetic; b: $p < 0.05$ vs. Gray MTA diabetic; c: $p < 0.05$ vs. White MTA diabetic.

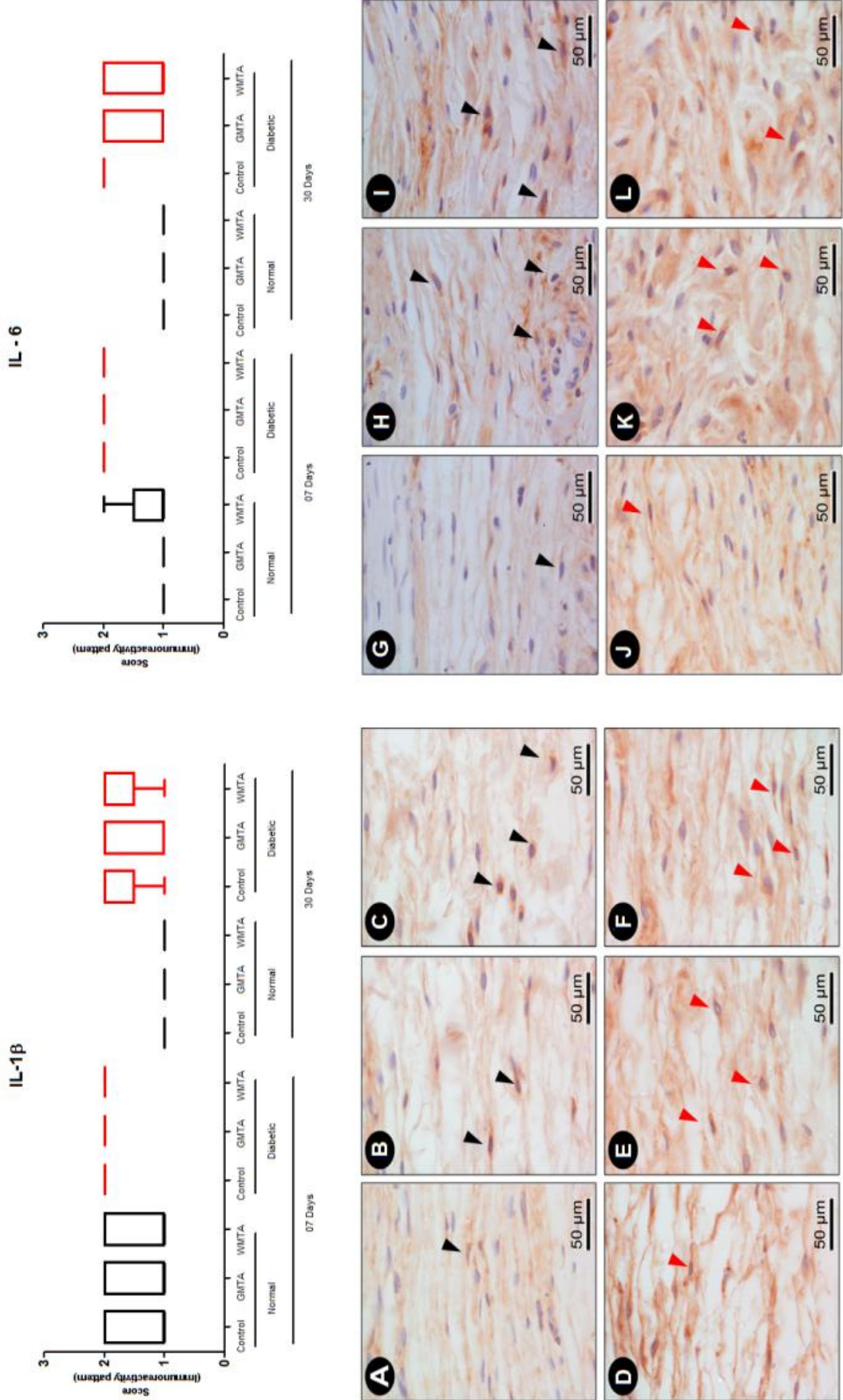


Figure 3: Graph showing immunostaining patterns for IL-1 β and IL-6 observed in normal and diabetic groups. Photomicrographs showing the immunoreactive cells (arrowheads) for IL-1 β and IL-6 in the normal and diabetic groups at 7 days. IL-1 β : A: Control normal; B: White MTA normal; C: Gray MTA normal; D: Control diabetic; E: White MTA diabetic; F: Gray MTA diabetic. IL-6: G: Control normal; H: White MTA normal; I: Gray MTA normal; J: Control diabetic; K: White MTA diabetic; L: Gray MTA diabetic. Harris-hematoxylin counterstaining. Scale bars: 50 μ m. Original magnification: 1000x.

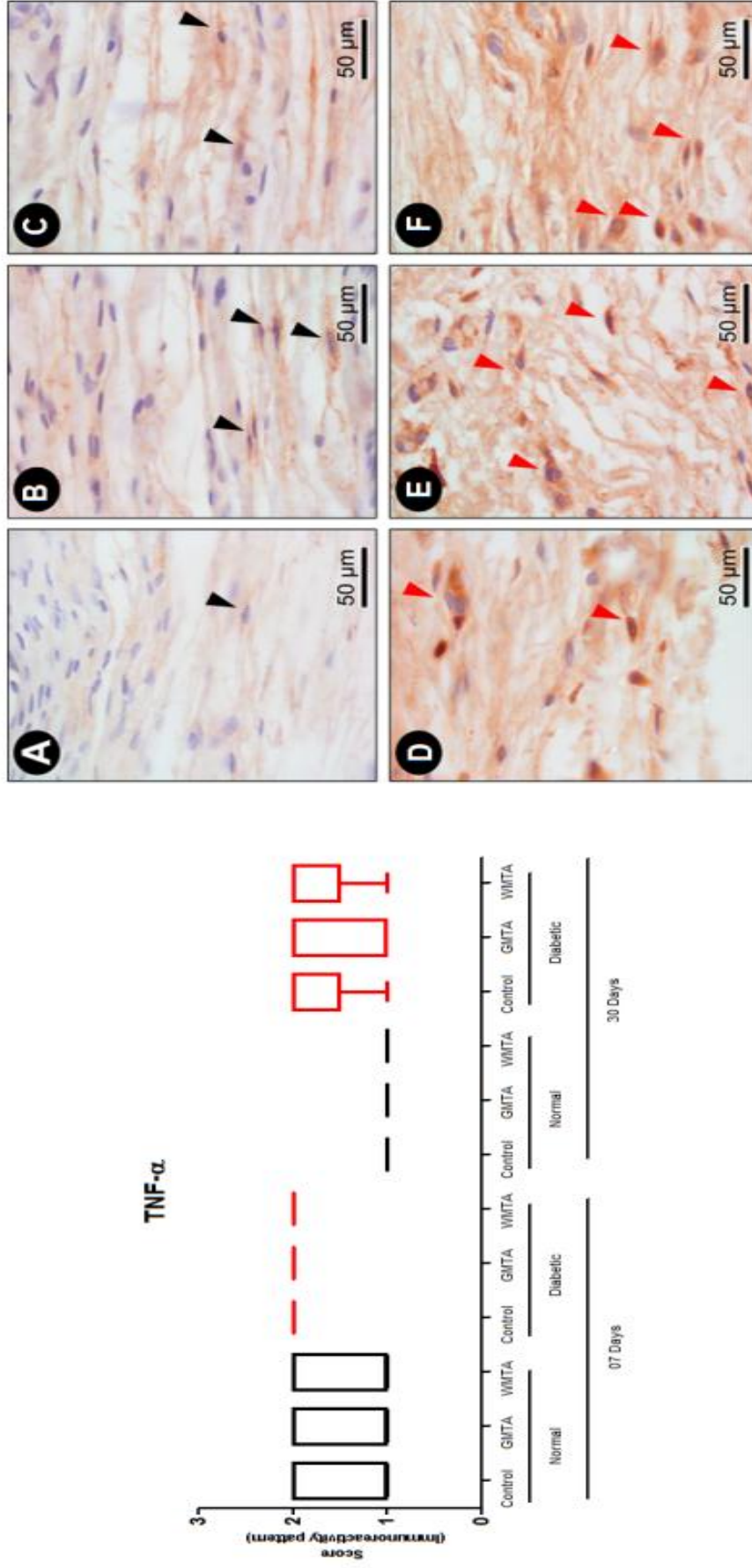


Figure 4: Immunostaining patterns for TNF-α observed in the normal and diabetic groups. Photomicrographs showing the immunoreactive cells (arrowheads) for TNF-α in the normal and diabetic groups at 7 days. A: Control normal; B: White MTA normal; C: Gray MTA normal; D: Control diabetic; E: White MTA diabetic; F: Gray MTA diabetic. Harris-hematoxylin counterstaining. Scale bars: 50 μm. Original magnification: 1000x.

Artigo 3

Artigo 3: Diabetes mellitus affects mineralization ability of white mineral trioxide aggregate

Abstract

Introduction: Diabetes mellitus (DM) is a metabolic disorder that affects tissue repair capacity. Therewith, the aim of this study was to evaluate the influence of DM on the tissue response and mineralization ability of gray (GMTA) and white mineral trioxide aggregate (WMTA).

Methods: Twenty-four Wistar rats were divided into two groups: normal and diabetic (Alloxan-induced). Four polyethylene tubes were implanted in the dorsal connective tissue of each rat, three containing GMTA, WMTA, zinc oxide and eugenol (ZOE) and one empty tube. Six animals from each group received injections of calcein, alizarin, and oxytetracycline on day 7, 14, and 21, respectively. The animals were killed after 7 and 30 days of experimentation and the tubes with surrounding tissues were removed and fixed. All specimens were prepared for histological analysis with either hematoxylin and eosin or von Kossa staining, or remained unstained for polarized light or fluorescence analysis.

Results: All materials in both systemic groups caused moderate reactions after 7 days, which decreased with time. Structures that were positive for von Kossa or birefringent to polarized light were observed in response to both MTAs but not to ZOE, regardless of the diabetic condition and time of evaluation ($p < 0.05$). WMTA exhibited higher fluorescence intensity than GMTA after 14 days in the normal group ($p < 0.05$). The fluorescence intensity was lower in the diabetic group for WMTA after 14 days ($p < 0.05$).

Conclusion: It was concluded that diabetic conditions altered the fluorescence intensity of WMTA but not the inflammatory response.

Key Words: Diabetic mellitus; Mineralization; Gray MTA; White MTA

Introduction

The possible association between systemic conditions and oral diseases has been emphasized (1), especially in relation to diabetes mellitus (DM) (2), which is a metabolic disease characterized by chronic hyperglycemia that affects an estimated 382 million people worldwide (3). If uncontrolled, DM leads to complications that have been associated with activation of pathways that increase inflammation, oxidative stress, and apoptosis (4).

Considering the relationship between DM and oral diseases, it was shown that DM can be a modulating factor of endodontic infections and may compromise the healing process of periapical tissues (5). Hyperglycemia may increase the inflammatory state because of elevated levels of systemic inflammation markers (6), and the tissue repair capacity including dental pulp healing can be hindered (7). Thus, it is paramount to understand the correlation between systemic and oral diseases, including the effect of DM on tissue response to endodontic materials.

Mineral trioxide aggregate (MTA) is a root-end filling material used for the repair of lateral perforations and because of its adequate physical, chemical, and biological properties (8), it has been used in pulpotomy, pulp capping, and the treatment of open apices (9). White MTA (WMTA) was introduced as an alternative to gray MTA (GMTA), aiming to avoid tooth staining. The main difference between the two materials is the lower concentration of Al_2O_3 , MgO, and FeO in WMTA (10). The reduction in FeO results in a reduction of the aluminoferrite phase, which is responsible for the gray color of GMTA (11). However, studies have also verified *in vitro* staining caused by WMTA, where gray-colored alteration was observed in contact with dentin (12), even in clinical application of WMTA (13).

Studies comparing WMTA and GMTA showed some similarities; both materials are non-toxic and induce cell proliferation when hydrated (14). Both are able to stimulate dentinal bridge formation when used as pulp capping materials (15). Both materials are considered biocompatible (16, 17). However, some differences have also been reported. Osteoblast adherence is not favored by WMTA (18), and cementoblasts and keratinocytes grow better on the surface of WMTA than on GMTA (19). Moreover, previous data revealed no difference

in tissue response and the mineralization potential of GMTA in diabetic conditions (20).

Although the differences between WMTA and GMTA have already been widely explored, there is no study in the literature comparing the tissue response to these materials in diabetic conditions, which can modify the systemic and local inflammatory responses. Hence, the aim of this study was to compare the subcutaneous tissue response and mineralization ability of WMTA and GMTA in diabetic rats.

Materials and Methods

The present study was approved and performed according to the guidelines of the Ethical Committee (protocol number 00557-2013). Twenty-four male albino Wistar rats, aged between 3 and 4 months and weighing around 250–280 g, were used in the study. The rats were divided into two groups of 12 animals each: normal and diabetic.

Diabetes induction

For the diabetes induction, 12 animals received 150 mg/kg of Alloxan monohydrate (Sigma Aldrich Corp., St. Louis, MO, USA) in a single intraperitoneal dose. Blood glucose was measured three days after injection to confirm hyperglycemia. Animals were considered diabetic when they showed glucose levels higher than 250 mg/dL, and the third day after injection was considered the first day of diabetes (20).

Sample preparation and surgical procedures

Sterile polyethylene open-ended tubes (Abbott Labs of Brazil, São Paulo, SP, Brazil) with an internal diameter of 1.0 mm, an external diameter of 1.6 mm, and a length of 10.0 mm were used for the experiments. Gray MTA, white MTA (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil), and zinc oxide Eugenol[®] (SS White, Artigos Dentários, Rio de Janeiro, RJ, Brazil) were prepared according to the manufacturers' recommendations and inserted into the twenty-four tubes with a lentulo spiral (Maillefer Dentsply, Tulsa, OK, USA). Twenty-four tubes remained empty and these were used as controls.

On the first day of diabetes, subcutaneous implantation was performed following previous studies (16, 20).

Fluorochrome injection

The fluorescent markers calcein, alizarin red S, and oxytetracycline hydrochloride (Sigma Aldrich Corp., St Louis, MO, USA) were injected intramuscularly at a dose of 20 mg/kg 7, 14, and 21 days after implantation, respectively, only in 12 animals (six from each group) (20). The fluorochromes were used to evaluate the time sequence of calcium deposition in the tissue.

Histological procedures and analysis

Seven and 30 days after the implantation, the animals' blood glucose levels were measured to confirm diabetic conditions. The animals were killed by an anesthetic overdose, and the tubes with the surrounding tissues were removed and fixed in 10% formalin solution. The specimens were processed and embedded in glycol methacrylate, serially sectioned into 3- μm cuts, and stained with hematoxylin-eosin. The 10- μm cuts were stained using the von Kossa technique or were kept unstained for polarized light visualization. The 50- μm cuts remained unstained and these were observed with fluorescence (20).

Inflammatory reactions in the tissues close to the material were evaluated according to ISO/TR 7405-1997 (21) as: 0, no or few inflammatory cells and no reaction; 1, fewer than 25 cells and light reaction; 2, between 25–125 cells and moderate reaction; and 3, 125 or more cells and severe reaction. Fibrous capsules were considered to be thin when the thickness was $<150\mu\text{m}$ and thick at $>150\mu\text{m}$. Necrosis and mineralization areas were recorded in μm^2 using Leica Qwin software (Leica Microsystems, Wetzlar, Germany). An average value for each material was obtained from the sum of cells counted in ten separate fields (400 \times magnification). Fluorescence intensity was measured using a fluorescence light microscopy (DM 4000B, Leica Microsystems, Wetzlar, Germany) (534 nm for calcein, 357 nm for alizarin, and ultraviolet 368 nm for oxytetracycline). A measuring area (150 μm \times 700 μm) in the middle of the tube opening was used, and fluorescence intensity was determined using the software LAS v4.1 (Leica Applications Suite – version 4.1; Leica Microsystems, Wetzlar, Germany).

Statistical analysis

The data for inflammatory response were analyzed using Kruskal-Wallis tests. The results of mineralization and fluorescence intensity were analyzed by ANOVA and Mann Whitney tests ($p < 0.05$).

Results

Figures 1 and 2 show the inflammatory response found in the normal and diabetic group at 30 days. Data comparing inflammatory responses are shown in Table 1. The presence of mineralization as determined by von Kossa staining and the fluorescence intensity of calcein, alizarin, and oxytetracycline staining are shown in Table 2.

Diabetes did not modify the inflammatory response of any of the tubes including the empty tubes (negative control). After 7 days, there was no significant difference among the scores of the different groups (median score 2). At 30 days, a decrease in inflammatory response was observed for all materials (median score 1), independent of the diabetic condition. There was no statistically significant difference among the materials for necrotic areas ($p > 0.05$), which were absent on day 30.

There were significant differences between all materials and the positive control ($p < 0.05$) in all mineralization parameters, independent of the diabetic status. Mineralized areas were observed in both groups at 7 and 30 days. These areas were absent in contact with ZOE at 7 and 30 days, independent of the diabetic condition.

Significant differences were found in the fluorescence intensity between each material and the control group, independent of the fluorochrome and diabetic condition ($p < 0.05$). The positive control showed baseline fluorescence intensity.

In the normal animals, WMTA showed higher fluorescence intensity than GMTA at 14 days ($p < 0.05$). Moreover, in diabetic animals, the fluorescence intensity of WMTA was lower than that in normal animals within the same experimental period ($p < 0.05$). Additionally, the ZOE group showed lower fluorescence intensity than both MTAs for all fluorochromes, regardless of the diabetic status and observation period ($p < 0.05$).

Discussion

Subcutaneous tissue reaction is one of the *in vivo* biocompatibility tests that have been used to determine the local effects and biocompatibility of materials (16,17). The host response to materials is complex and dependent on the innate and non-specific immune response that, depending on the chemical and physical properties of the material, might evoke a signaling pathway of inflammatory cascade, inactivating the inflammatory process, and contributing or not to a wound healing process (22).

Diabetes mellitus is a disorder that when uncontrolled might lead to oral problems such as changes in pulp tissues (7). DM changes the inflammatory response (7) once it reduces the functions of polymorphonuclear leukocytes (23) and alters the immune responses (24), resulting in increased susceptibility to infections (25) and delayed healing processes (23). Additionally, DM has been associated with altered calcium homeostasis (26), which leads to a lower capacity of bone formation and mineralization.

Both MTAs evoked a moderate inflammatory response in 7 days, which decreased after 30 days, regardless of the diabetic condition. The current results corroborate with those of other studies (16, 20, 27), indicating that the tissue response are similar between GMTA and WMTA. However, the present findings are in conflict with others that showed differences in the intensity of inflammatory responses between GMTA and WMTA in a 7-day period (17). These differences may be attributed to the method used to evaluate the biocompatibility of dental materials. Whereas previous results were obtained based on the number of inflammatory cells, the present study is based on the evaluation of the density of inflammatory cells.

Despite this, GMTA releases more Ca^{+2} ions than WMTA does, with decreasing ion concentrations with time (28). In this study, WMTA exhibited higher fluorescence intensity than GMTA did in the normal group at 14 days. This was possibly due to the different chemical compositions of WMTA and GMTA, especially the higher concentration of Fe_2O_3 present on GMTA (10) that could interfere in the ionic dissociation and affect calcium release. Besides, WMTA has been associated with higher calcium release compared with GMTA (29), which might explain these results. Calcium phosphate and calcium oxide are released by MTA. Calcium oxide reacts with water in tissue fluids to form

calcium hydroxide, which further dissociates into Ca^{+2} and OH^- ions. The Ca^{+2} ions react with the carbon dioxide that is present in the tissues and form calcium carbonate granulations, which would stimulate the deposition of hard tissues (27). Moreover, Ca^{+2} ions can react with tissue fluids and produce hydroxyapatite crystals. This ability makes MTA not only biocompatible but also bioactive, and these properties can be seen in both MTAs (30).

The present results did not show evidence of the direct correlation between DM and inflammatory response for both MTAs. However, WMTA exhibited lower fluorescence intensity in diabetic animals on the 14th day. The stimulation of repair by deposition of mineralized tissue is associated with the ability of a biomaterial to release calcium ions (16, 27, 28). The lower presence of metallic oxides in WMTA (10) can promote slow calcium release. On the other hand, DM affects the microcirculation (31) and promotes abnormal metabolism of calcium (26). Thus, we believe that once DM changes the inflammatory response and alters calcium homeostasis, it could have interfered with the response on the 14th day, which may justify our findings.

The chemical composition of sealers can positively or negatively affect the result of biocompatibility tests (22). Thus, ZOE was used as a positive control, since the toxicity of zinc oxide and eugenol sealers has been demonstrated both *in vivo* and *in vitro* (32, 33). In this study, ZOE presented the same biological behavior as both MTAs after 30 days, independent of the diabetic condition. Such results disagree with studies that showed tissue irritation (32, 33), probably because of ZOE liquid/powder proportion. The high concentration of eugenol in the tissue is sufficient to inhibit cell respiration and thus kill cells (34). The fluorescence intensity observed could be explained by the capacity of ZOE to release calcium ions (35).

It is evident that other studies using different methods are necessary to elucidate the relationship between DM and the biocompatibility of endodontic materials, as the correlation is not yet completely understood.

In conclusion, the present results indicate that in general, the inflammatory response to the materials did not change by diabetic conditions, but the fluorescence intensity of WMTA at 14th days was altered in diabetic animals.

Conflicts of interest

The authors deny any conflicts of interest.

Acknowledgments

The authors thank FAPESP (São Paulo Research Foundation) for financial support. Process number: 2013/06641-8.

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Tables

Table 1: Inflammatory scores specimens stained with hematoxylin-eosin

Normal	07 days				30 days			
	Control	GMTA	WMTA	ZOE	Control	GMTA	WMTA	ZOE
<i>Inflammatory response</i>								
0 Absent	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
1 Mild	1/6	2/6	1/6	2/6	6/6	6/6	6/6	3/6
2 Moderate	5/6	4/6	5/6	4/6	0/6	0/6	0/6	2/6
3 Severe	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6

Diabetic	07 days				30 days			
	Control	GMTA	WMTA	ZOE	Control	GMTA	WMTA	ZOE
<i>Inflammatory response</i>								
0 Absent	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
1 Mild	2/6	1/6	1/6	1/6	6/6	5/6	4/6	3/6
2 Moderate	4/6	5/6	5/6	5/6	0/6	1/6	2/6	2/6
3 Severe	0/6	0/6	0/6	0/6	0/6	0/6	0/6	1/6

Inflammation Score: 0: none or few inflammatory cells and no reaction; 1: <25 cells and mild reaction; 2: between 25 and 125 cells and moderate reaction; 3: 125 or more cells and severe reaction. GMTA: Gray MTA; WMTA: White MTA; ZOE: Zinc Oxide and Eugenol.

Table 2: Medium of samples in Each Group categorized necrosis, presence of mineralization and Fluorescence intensity.

Groups/ Time	Material	Necrosis	Presence of Mineralization			
			VK	F		
				C	A	O
Normal (7days)	Control	0 ¹	0 ¹	-	-	-
	Gray MTA	11597.1 ²	4132.53 ²	-	-	-
	White MTA	12302.3 ²	4396.49 ²	-	-	-
	ZOE	13212.8 ²	0 ¹	-	-	-
Diabetic (7days)	Control	0 ¹	0 ¹	-	-	-
	Gray MTA	17017.3 ²	3312.26 ²	-	-	-
	White MTA	15076.1 ²	2776.01 ²	-	-	-
	ZOE	18873.0 ²	0 ¹	-	-	-
Normal (30 days)	Control	0 ¹	0 ¹	44.065 ^{a1}	35.074 ^{a1}	30.895 ^{a1}
	Gray MTA	0 ¹	3454.06 ²	63.822 ^{a2}	59.775 ^{a2}	68.916 ^{a2}
	White MTA	0 ¹	4722.99 ²	93.833 ^{a2}	95.810 ^{a3}	95.173 ^{a2}
	ZOE	0 ¹	0 ¹	57.419 ^{a3}	46.835 ^{a4}	44.256 ^{a3}
Diabetic (30 days)	Control	0 ¹	0 ¹	43.469 ^{a1}	37.245 ^{a1}	29.050 ^{a1}
	Gray MTA	0 ¹	4738.95 ²	52.272 ^{a2}	50.557 ^{a2}	41.598 ^{a2}
	White MTA	0 ¹	5612.71 ²	53.777 ^{a2}	56.073 ^{a5}	43.439 ^{a2}
	ZOE	0 ¹	0 ¹	48.509 ^{a3}	45.098 ^{a4}	38.063 ^{a3}

ZOE: Zinc oxide and eugenol; VK: Von Kossa; F: Fluorescence intensity; C: Calcein; A: Alizarin; O: Oxytetracycline.

The areas of mineralization and necrosis were measured in μm^2 .

*Same letters and numbers indicate no statistical difference among the groups in row and columns respectively ($p>0.05$).

Figure 1

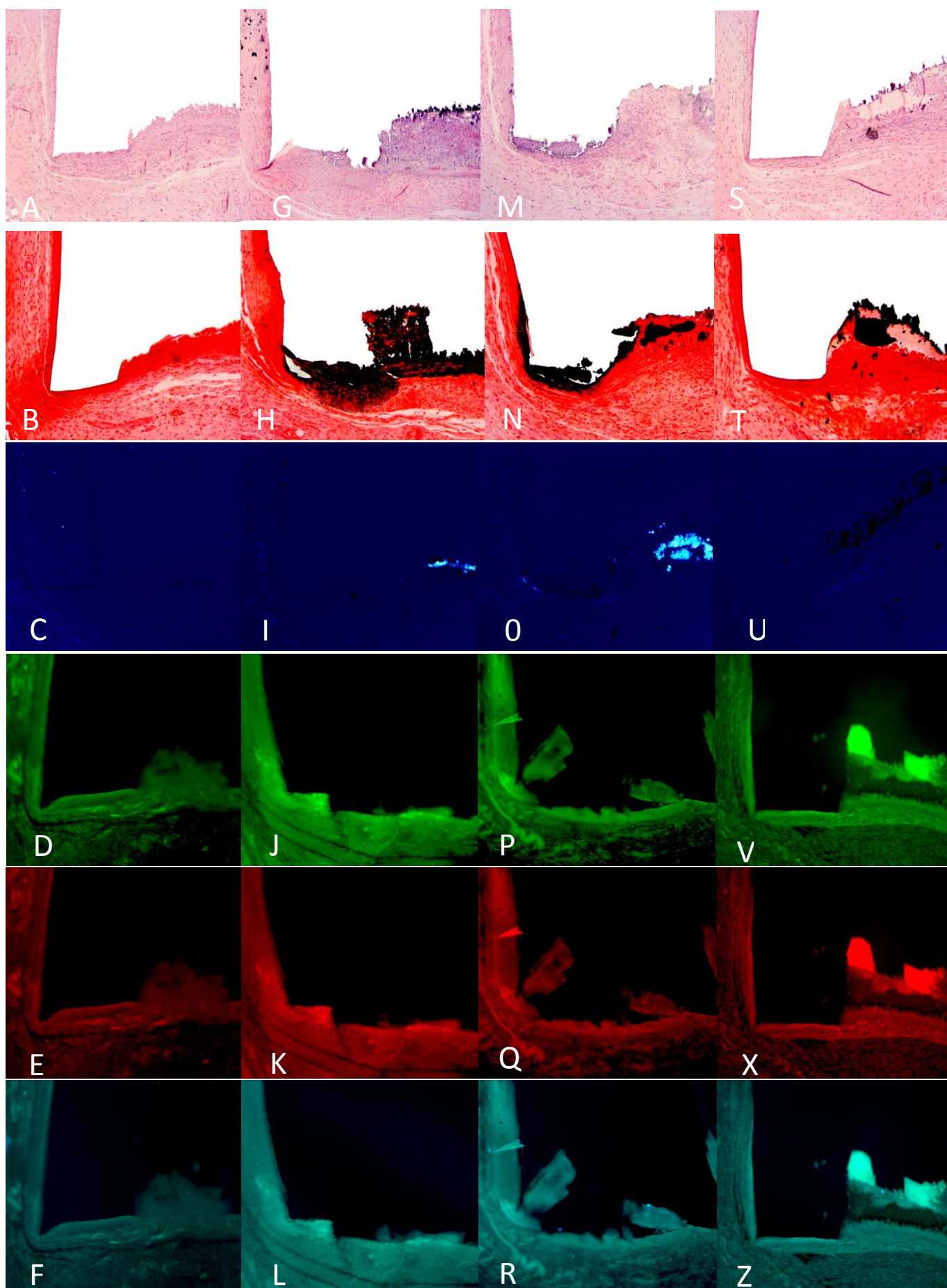


Figure 1: Response found in normal group at 30 days. **Control:** (A) specimens showing thin fibrous capsule formation and mild inflammatory cell infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin, 10×); (B) absence of mineralization (von Kossa, 10×); (C) absence of birefringent structures to polarized light (polarized light, 10×); (D–F) absence of calcification areas in close contact with the tube opening (fluorochrome analysis, 10×) for calcein, alizarin, and oxytetracycline, respectively. **Gray MTA and white MTA, respectively:** (G and M) thick fibrous capsule formation and mild inflammatory cell infiltration (hematoxylin-eosin, 10×); (H and N) presence of dystrophic calcification on the tube opening (von Kossa, 10×); (I and O) presence of birefringent structures to polarized light (polarized light, 10×); (J–L and P–R) calcification areas in close contact with the tube opening (fluorochrome analysis, 10×) for calcein, alizarin, and oxytetracycline, respectively. **ZOE:** (S) presence of mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10×); (T) absence of mineralization (von Kossa, 10×). Note: dark color observed represent the ZOE sealer, not mineralization areas; (U) absence of birefringent structures to polarized light (polarized light, 10×); (V–Z) fluorescence intensity (fluorochrome analysis, 10×) for calcein, alizarin, and oxytetracycline, respectively.

Figure 2

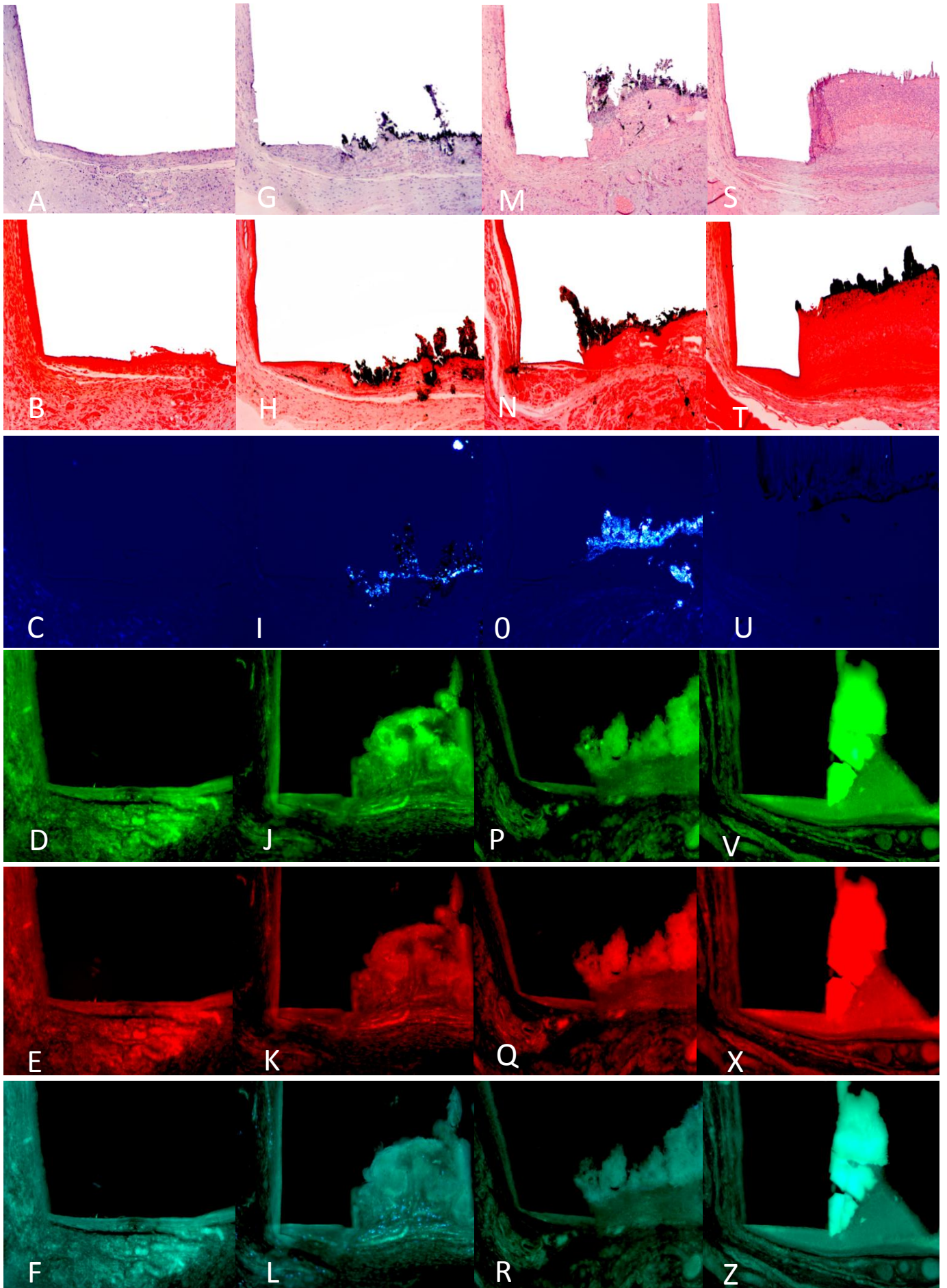


Figure 2: Response found in diabetic group at 30 days. **Control:** (A) specimens showing thin fibrous capsule formation and mild inflammatory cell infiltration consisting of macrophages and lymphocytes (hematoxylin-eosin, 10×); (B) absence of mineralization (von Kossa, 10×); (C) absence of birefringent structures to polarized light (polarized light, 10×) (D–F); absence of calcification areas in close contact with the tube opening (fluorochrome analysis, 10×) for calcein, alizarin, and oxytetracycline, respectively. **Gray MTA and white MTA, respectively:** (G and M) thick fibrous capsule formation and mild inflammatory cell infiltration (hematoxylin-eosin, 10×); (H and N) presence of dystrophic calcification on the tube opening (von Kossa, 10×); (I and O) presence of birefringent structures to polarized light (polarized light, 10×); (J–L and P–R) calcification areas in close contact with the tube opening (fluorochrome analysis, 10×) for calcein, alizarin, and oxytetracycline, respectively. **ZOE:** (S) presence of mild inflammatory cell infiltration consisting of macrophages and lymphocytes after 30 days (hematoxylin-eosin, 10×); (T) absence of mineralization (von Kossa, 10×) Note: dark color observed represent the ZOE sealer, not mineralization areas; (U) absence of birefringent structures to polarized light (polarized light, 10×); (V–Z) fluorescence intensity (fluorochrome analysis, 10×) for calcein, alizarin, and oxytetracycline, respectively.

Artigo 4

Artigo 4: Effect of *Diabetes Mellitus* on local and systemic bone marker expression induced by Gray versus White Mineral Trioxide Aggregate

Abstract

Aim: This study aimed to investigate the relationship between Diabetes Mellitus (DM) and the local/systemic effects of Gray Mineral Trioxide Aggregate (GMTA) and White Mineral Trioxide Aggregate (WMTA) on bone marker expression.

Methodology: Wistar rats were divided into two groups (healthy and diabetic), which were further divided into three subgroups (control, GMTA and WMTA). Polyethylene tubes filled with sealers were implanted in dorsal connective tissue. Empty tubes were used as control. On days 7 and 30, blood samples were collected for calcium, phosphorus and alkaline phosphatase (ALP) measurement. The animals were euthanized and the implanted tubes with surrounding tissues were removed, fixed and processed for immunohistochemical analysis of osteopontin (OPN) and osteocalcin (OCN). Nonparametric and parametric data were statistically analysed.

Results: No difference in systemic serum calcium levels between both groups was observed. On day 7, serum phosphorus level of WMTA group of healthy rats was higher than that of the diabetic group. On day 30, healthy rats exhibited lesser phosphorus levels than the diabetic ones. At both time points, the diabetic group showed more ALP activity than the healthy group. Immunohistochemical analyses of healthy rats revealed OCN and OPN positive cells in presence of both MTAs. However, under diabetic condition, both OCN and OPN were absent.

Conclusion: Both MTAs caused an increase in serum calcium, phosphorus and ALP, suggesting a potential systemic effect, along with induction of OCN and OPN production. Moreover, DM was capable of inhibiting OCN and OPN production.

Key Words: Bone markers, Diabetes Mellitus, Mineral Trioxide Aggregate, Systemic effect

Introduction

Endodontic medicine is a new area of research that studies the relationship between endodontic variables and systemic health (Segura-Egea *et al.* 2015). Fouad (2003) proposed that DM could be a potential modulating factor for endodontic infections. However, the relationship between these infections and systemic diseases has not been completely understood, and systemic alterations arising due to correlation between endodontic diseases and DM are being investigated (Cintra *et al.* 2013, Cintra *et al.* 2014). As described previously, the hyperglycaemic state due to DM diminishes or alters the immune response, decreases the leucocyte function and upregulates the expression of pro-inflammatory cytokines, leading to reduction in the tissue repair capacity and healing process of the dental pulp and periapical tissues (Delamaire *et al.* 1997, Iacopino 2001, Fouad 2003).

Root-end filling and repair materials should be biocompatible with the periradicular tissues, and have suitable chemical and physical properties to promote complete tissue repair (Saxena *et al.* 2013). MTA is a calcium silicate-based cement, developed as a root-end filling material and used in different clinical procedures (Parirokh & Torabinejad 2010) owing to its attractive properties (Torabinejad *et al.* 1995, Holland *et al.* 1999). Both Gray MTA (GMTA) and white MTA (WMTA) are known, and studies comparing them have been conducted (Camilleri *et al.* 2004, Bozeman *et al.* 2006, Bidar *et al.* 2011).

Studies have reported increased levels of ALP, OCN and OPN expression, and stimulation of cytokine and calcium ion (Ca^{2+}) release upon MTA treatment (Thomson *et al.* 2003, Chen *et al.* 2009). Additionally, diabetic condition negatively affects the tissue response and the mineralization ability of MTA (Gomes-Filho *et al.* 2015).

Ca^{2+} released upon MTA treatment is an important ion involved in the regulation of various cellular functions, such as increasing the expression of bone markers (Rashid *et al.* 2003, Tada *et al.* 2010) by active osteoblasts during different phases of their development, which reflects bone formation (Seibel 2005, Eriksen 2010). Additionally, MTA promotes alkalization by increasing the pH due to the release of hydroxyl ions (OH^-) (De Vasconcelos *et al.* 2009). Alkaline pH activates ALP, which is a phosphate-releasing enzyme

involved in the mineralization process and is considered an important indicator of bone formation (Seibel 2005, Eriksen 2010).

DM is characterized by abnormalities in the metabolism of carbohydrates, proteins, lipids, water and electrolytes (ADA 2013). Furthermore, it is also associated with altered calcium homeostasis and the expression of bone markers, which results in bone loss and reduced bone formation (Levy *et al.* 1994, McCabe 2007). Therefore, the aim of the present study was to investigate the potential relationship between DM, and the local and systemic effects of subcutaneous implantation of GMTA and WMTA on bone marker expression in rats, by immunohistochemical and biochemical analyses.

Material and Methods

Animals

Wistar albino rats, aged 3 to 4 months, weighing 250–280 g were procured from the School of Dentistry of Araçatuba/UNESP. The animals were divided in two groups: Healthy and diabetic (n=48 per group). Each group was further divided in three subgroups: Control, GMTA and WMTA (n=8 per group). The present study was approved and performed according to the guidelines of the ethical committee (protocol number 00557-2013).

Induction of Diabetes

Animals belonging to the diabetic group received a single dose (150 mg/kg bw) of Alloxan monohydrate (Sigma Aldrich Corp., St. Louis, MO, USA) for the induction of diabetes (Gomes-Filho *et al.* 2015). The animals that showed blood glucose levels higher than 250 mg/dL were considered diabetic and used for the study.

Sample preparation and surgical procedure (Subcutaneous implantation)

Sterile polyethylene tubes (Abbott Labs of Brazil, São Paulo, SP, Brazil) with 1 mm internal diameter, 1.6 mm external diameter, and 10 mm length were used. Gray and White MTA Angelus[®] (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil) were prepared according to the manufacturer's recommendations and inserted in the tubes with a lentulo spiral (Maillefer Dentsply, Tulsa, OK, USA). Empty tubes were used as controls.

The surgical procedure for implantation of the polyethylene tubes in the dorsal connective tissue was performed according to previous reports (Holland *et al.* 1999, Gomes-Filho *et al.* 2015).

Blood collection and biochemical assays

On days 7 and 30 after implantation, the animals were anaesthetized for blood collection. Cardiac puncture was performed to collect 5 mL blood from each animal. Blood was centrifuged at 3000 rpm for 20 min at 20°C, and the plasma was stored at -20 °C for the measurement of calcium, phosphorus and ALP.

Calcium and phosphorus levels were determined using the colorimetric Calcium Liquiform assay Kit (Labtest Diagnostica SA, MG, Brazil) and the Phosphorus UV Liquiform assay Kit (Labtest Diagnostica SA, MG, Brazil), which exhibited sensitivity of 0.1 mg/dL and 0.14 mg/dL respectively. As a marker for bone formation, serum ALP levels were determined using a commercially available kit (Labtest Diagnostica SA, MG, Brazil) with sensitivity of 0.125 U/L. Sample absorbance was read using a spectrophotometer (Hitachi, model U 1100, Japan).

Immunohistochemical processing

After blood collection, the animals were euthanized with an overdose of the anaesthetic and the implanted tubes along with the surrounding tissues were removed and fixed in 10% formalin. The formalin-fixed specimens were processed and embedded in paraffin, sliced into 5-µm semi-serial sections and subjected to immunohistochemistry using an indirect immunoperoxidase staining technique for the detection of OPN (using goat anti-osteopontin antibody, SC10593; Santa Cruz Biotechnology, Santa Cruz, CA) and OCN (using rabbit anti-osteocalcin antibody, SC18319; Santa Cruz Biotechnology, Santa Cruz, CA) following the method by Garcia *et al.* (2014).

OPN and OCN production around the opening of the tube was analysed using a microscope under 400x magnification (Leica Microsystems, Wetzlar, Germany). Semi-quantitative immunohistochemistry analysis was performed. The criteria for establishing the immunoreactivity (IR) score was adapted from

Garcia *et al.* (2014): score of 0 (no IR); 1 (low, IR in <25% cells); 2 (moderate, IR in ~50% cells); 3 (high, IR in ~75% cells).

Statistical analysis

GraphPad Prism 5.0 software was used for performing statistical analysis. Kruskal-Wallis followed by the Dunn multiple comparison test was performed for nonparametric data, and analysis of variance (ANOVA) followed by the Tukey's test for parametric data. The difference was considered significant at $p < 0.05$.

Results

MTA increases calcium, phosphorus and ALP serum levels

In normal conditions, on day 7, the GMTA and WMTA groups exhibited significantly higher calcium levels as compared to the control group ($p < 0.05$), which significantly decreased by day 30 ($p < 0.05$). The plasma phosphorus level for all the groups was higher on day 7, and significantly reduced by day 30 ($p < 0.05$). The ALP activity for control and GMTA groups was significantly lower than that of the WMTA group on day 7 ($p < 0.05$).

However, under diabetic condition, the GMTA group exhibited significantly higher calcium and phosphorus levels than the WMTA group on day 7 ($p < 0.05$). Moreover, the serum phosphorus levels for all the groups increased on day 30 only ($p < 0.05$). In addition, ALP activity was significantly higher on day 30 for the control and WMTA groups as compared to day 7 ($p < 0.05$).

Comparison between the GMTA and WMTA groups under both the systemic conditions (normal and diabetic), showed no difference in the calcium levels. However, WMTA-treated from normal group exhibited significantly higher phosphorus levels compared to the diabetic group on day 7 ($p < 0.05$). Moreover, on day 30, both GMTA and WMTA-treated healthy rats exhibited significantly lower phosphorus levels than the diabetic group ($p < 0.05$). Furthermore, on both the days, the diabetic group showed significantly higher ALP activity than the healthy group ($p < 0.05$).

Biochemical analyses showing the serum levels of calcium, phosphorus and ALP, under both the systemic conditions are represented in Figures 1 and 2.

Diabetes Mellitus inhibits OCN and OPN production of MTA

The immunoreactive cells showed a dark brown staining confined to the cytoplasm. In healthy rats, immunohistochemical analyses showed the absence of OCN- and OPN-positive cells in the control group on days 7 and 30. On the other hand, the GMTA and WMTA groups displayed OCN- and OPN-positive cells on both the days (Fig. 3). However, under diabetic conditions, no immunoreactive cells were observed in the control, GMTA and WMTA groups on both the days (Fig. 3).

Discussion

Subcutaneous implantation is a method for studying the process of osteogenesis or for evaluating the osteogenic potential of a given implant (Asatrian *et al.* 2014). Besides, animal models have been used for understanding the pathogenesis of diseases such as DM, a chronic metabolic inflammatory disease associated with altered calcium homeostasis and bone marker expression (Levy *et al.* 1994, McCabe 2007). The aim of this study was to investigate the relationship between DM, and the systemic and local effects of GMTA and WMTA treatment on the expression of bone markers. While the systemic effects of MTA treatment are already documented (Khalil *et al.* 2013, Simsek *et al.* 2016, Demirkaya *et al.* 2016), no study has addressed its systemic effects on the expression of bone markers under diabetic conditions.

The ability of MTA to release Ca^{+2} upon hydration has been previously reported (De Vasconcelos *et al.* 2009). In this study, the serum calcium levels of both the MTA groups was higher than that of the control group on day 7, while it decreased on day 30. These findings are in agreement with previous investigations that measured the release of Ca^{2+} from polyethylene tubes filled with both the MTAs and immersed in deionized water. The results showed higher Ca^{2+} release during the initial period, followed by subsequent reduction with time (Duarte *et al.* 2003, De Vasconcelos *et al.* 2009). However, Ca^{2+} leaching from the MTAs into simulated body fluids increased with time (Camilleri

2011). Demirkaya *et al.* (2016) reported that the substances released by MTA in the surrounding tissues can enter the bloodstream and can be transported to distant sites upon tissue damage. Traces of Ca^{2+} released from the MTA has been detected in organs like liver, kidney and brain (Simsek *et al.* 2016). Calcium homeostasis is controlled by the influx of ions across the electrical and chemical gradients through calcium channels, once the physical mechanisms for solute selectivity favours the transport of ions through the interstitial fluid in the blood (Rizzoli & Bonjour 2006, Guyton & Hall 2011). Thus, the ability of MTA to induce the contraction of blood vessels mediated by the influx of calcium (Tunca *et al.* 2007) supports our data that Ca^{2+} released from the MTA can enter the systemic circulation.

The total phosphate is expressed in the serum as phosphorus (Guyton & Hall 2011). The serum phosphorus levels were higher in all the groups on day 7 compared to that on day 30. Torabinejad *et al.* (1995) reported calcium and phosphorus as the main components of MTA. However, recent studies have demonstrated that both GMTA and WMTA do not contain phosphorus; their major elements include calcium, silicon and bismuth (Asgary *et al.* 2006). However, phosphorus was observed in the analysis of set MTA, since MTA forms an amorphous structure containing phosphate (49%) when mixed with water (Camilleri *et al.* 2007). In addition, in the presence of phosphate-containing body fluids, MTA has the ability to precipitate calcium phosphate (CaP) (Tay *et al.* 2007) and induce the formation of hydroxyapatite (HA) (Bozeman *et al.* 2006). However, the concentration of phosphate ions present in HA, as well as the ions derived from MTA leaching tends to decrease with time (Sarkar *et al.* 2005, Reyes-Carmona *et al.* 2009, Camilleri 2011). Furthermore, it is possible to correlate these results with the calcium levels because once the Ca^{2+} released in the extracellular fluid reacts with the phosphate ions present in the tissue fluids, it precipitates as CaP. As a result, the increase in Ca^{+2} will be proportional to the CaP precipitate, resulting in an increase in the phosphate ions. The level of extracellular phosphate is determined by the balance between the dynamic fluxes from and into the intracellular compartments (Rizzoli & Bonjour 2006, Guyton & Hall 2011), including the precipitation of CaP and the transfer of phosphate ions from the HA crystals into the interstitial fluid and the blood, as supported by our study.

Hyperglycaemia results in metabolic changes due to insulin deficiency, impairment in parathyroid hormone and alteration of vitamin D metabolism leading to abnormal plasma levels of calcium, phosphorus and alkaline phosphatase (Joshi *et al.* 2013). In this study, under diabetic conditions, the calcium and phosphorus levels in GMTA group were higher than that in the WMTA group on day 7. On the other hand, only the phosphorus level was high on day 30 for all the groups. De Vasconcelos *et al.* (2009) measured the release of Ca^{2+} from both the MTAs and showed that GMTA exhibited higher values. Meanwhile, Garcia *et al.* (2014a) reported that WMTA is associated with greater Ca^{2+} release than GMTA on day 7. The authors proposed that higher concentration of Fe_2O_3 in GMTA may have an influence on Ca^{2+} release. Furthermore, GMTA produced more CaP precipitate than WMTA (Bozeman *et al.* 2006), which correlated with the phosphorus levels. However, studies have shown that the phosphate ions released after CaP precipitation reduced with time (Sarkar *et al.* 2005, Reyes-Carmona *et al.* 2009). At the same time, an increase in the serum phosphate level in diabetes has been reported (Raskin & Pak 1981). DM affects the blood vessels thereby compromising the microcirculation (Domingueti *et al.* 2016), and promoting abnormal metabolism of microelements, such as calcium and phosphorus (ADA 2013). Masuda *et al.* (2005) showed that MTA has a positive effect on revascularization of the connective tissue. The above mentioned facts may help in explaining our findings under diabetic conditions.

No differences were observed in the calcium levels between normal and diabetic groups. Levy *et al.* (1985) also reported no differences in the level of calcium in the plasma of diabetic animals. Similarly, normal plasma calcium levels were reported in diabetic rats, despite an increase in the renal calcium excretion (Anwana & Garland 1990). Nevertheless, elevated calcium levels due to an increase in the intracellular influx of calcium were observed in the diabetic condition (Levy *et al.* 1986, Gilon *et al.* 2014). In contrast, phosphorus levels of the WMTA group of normal rats was higher than that of the diabetic group on day 7. Alterations in electrolyte metabolism due to DM may modify the composition of the interstitial fluid, which can affect the rate of chemical reactions leading to the gradual release of calcium hydroxide, which may interfere with its own ionic dissociation and CaP precipitation. Moreover, on day

30, both GMTA and WMTA group of normal group exhibited lower phosphate levels than the diabetic group. Raskin & Pak (1981) reported that an increase in the rate of renal phosphate reabsorption, coupled with a decrease in the urinary phosphorus loss, could be the reason behind the increase in the phosphorus levels in diabetes. On the other hand, reduced levels of serum phosphorus in diabetic rats (Xiao *et al.* 2015) along with no differences in its level between the nondiabetics and diabetics have also been described (Levy *et al.* 1985, Levy *et al.* 1986).

ALP is an enzyme present in almost all the tissues of the body (Millán 2006). In healthy adults, this enzyme is mainly present in the liver and bones, and is elevated in the serum when the tissue is subjected to metabolic stimulation (Limdi & Hyde 2003). Polymorphonuclear leukocytes, neutrophils and lymphocytes contain ALP (Smith *et al.* 1985, Grozdea *et al.* 1991), which is released in the inflammatory exudate during acute inflammation (Shinozaki *et al.* 1998). Likewise, increase in the pH of the extracellular fluid improves mineral precipitation (Ramp *et al.* 1994) and activates ALP (Eriksen 2010).

Ionic dissociation of MTA leads to the release of OH^- , which is responsible for alkalization of the medium and activation of ALP (Holland *et al.* 1999, De Vasconcelos *et al.* 2009). On day 7, the WMTA group showed higher ALP level than the GMTA group. Borges *et al.* (2010) reported no pH differences between the GMTA and WMTA groups, even though the pH was highly alkaline at the initial time point. In contrast, evaluation of OH^- released from the polyethylene tubes filled with the MTAs showed lower pH values in the presence of WMTA (De Vasconcelos *et al.* 2009). In addition, difference in the biocompatibility between both MTAs on day 7 (Shahi *et al.* 2006) can be associated with the higher concentration of metallic oxide in GMTA (Asgary *et al.* 2006), which interferes with ionic dissociation. Khalil *et al.* (2013) proposed that MTA induces changes in the liver and kidney, including inflammation, apoptosis and vascular congestion. Investigations also revealed that the level of trace metal ions from MTA increased in the organs, such as liver, in the rats (Simsek *et al.* 2016, Demirkaya *et al.* 2016). Demirkaya *et al.* (2016) suggested that these ions could be naturally absorbed in the body, which may result in alteration of the metabolism in these organs. Thus, we propose that the differences in the ionic dissociation between both MTAs can induce different

intensities of inflammation in the liver, thereby intensifying the release of granules containing ALP in the inflammatory exudate. Furthermore, alterations in the liver metabolism can lead to increased expression and release of ALP in the circulation, which may explain the above results.

ALP levels, under diabetic condition, increased in the WMTA and control groups on day 30. These results differ from earlier studies that evaluated the release of OH⁻ from both the MTAs and verified the reduction in alkalinity (De Vasconcelos *et al.* 2009) or maintaining stability (Borges *et al.* 2010) over time. On the other hand, the lower occurrence of metallic oxides in WMTA (Asgary *et al.* 2006) may affect its OH⁻ release potential. Additionally, alterations in the inflammatory response due to DM (Delamaire *et al.* 1997), along with the differences in the tissue response of both the MTAs (Shahi *et al.* 2006), can enhance the release of granules containing ALP into the inflammatory exudate, thereby increasing the ALP activity. Moreover, an increase in the level of serum ALP was reported in diabetic rats (McCracken *et al.* 2000). These evidences therefore support our data.

ALP activity was higher in the diabetic group as compared to normal. These results disagree with earlier studies that reported a decrease in ALP activity in diabetes (Xiao *et al.* 2015). In contrast, a systemic increase in the serum ALP levels in diabetic rats was reported (McCracken *et al.* 2000). Additionally, higher concentration of glucose is associated with increased ALP activity in the rat dental pulp cells (Inagaki *et al.* 2010).

Important proteins such OPN and OCN are present in mineralized connective tissues (Seibel 2005, Eriksen 2010). In this study, both MTAs revealed the ability to induce the production of OPN and OCN in subcutaneous tissue. These data can be supported by previous studies, which revealed that Ca²⁺ released by MTA promotes OPN and OCN expression (Thomson *et al.* 2003, Chen *et al.* 2009).

OCN and OPN were not produced upon MTA treatment in diabetic condition, indicating a direct correlation between DM and the local production of bone markers. It is possible to correlate these findings with earlier investigations which showed that the hyperglycaemic state can suppress OCN expression (Botolin & McCabe 2006). Also, OCN expression is depressed after titanium implantation in diabetic condition (von Wilmsky *et al.* 2011, Colombo *et al.*

2011). Besides, lower levels of serum OCN have been reported in diabetic patients (Achemlal *et al.* 2005). On the other hand, Nakajima *et al.* (2013) reported that advanced glycation end-products (AGEs) promoted an upregulation of OCN expression in dental pulp.

OPN is a multifunctional protein (Denhardt *et al.* 2001, Lund *et al.* 2009), whose upregulation is associated with acute and chronic inflammatory conditions such as DM (Lund *et al.* 2009, Kahles *et al.* 2014). Our data differ from earlier studies, which reported an increase in OPN expression in diabetic condition (Inagaki *et al.* 2010, Colombo *et al.* 2011). Moreover, no inhibitory effect was established on the inflammatory response by MTA in diabetic condition (Gomes-Filho *et al.* 2015). It is necessary to conduct more studies to substantiate and understand the systemic effects of these endodontic materials.

Conclusion

Based on these results, it was concluded that both MTAs increased the calcium, phosphorus and ALP serum levels, suggesting a possible systemic effect. They also promoted OCN and OPN production. Additionally, DM was capable of inhibiting the MTA-induced production of OCN and OPN.

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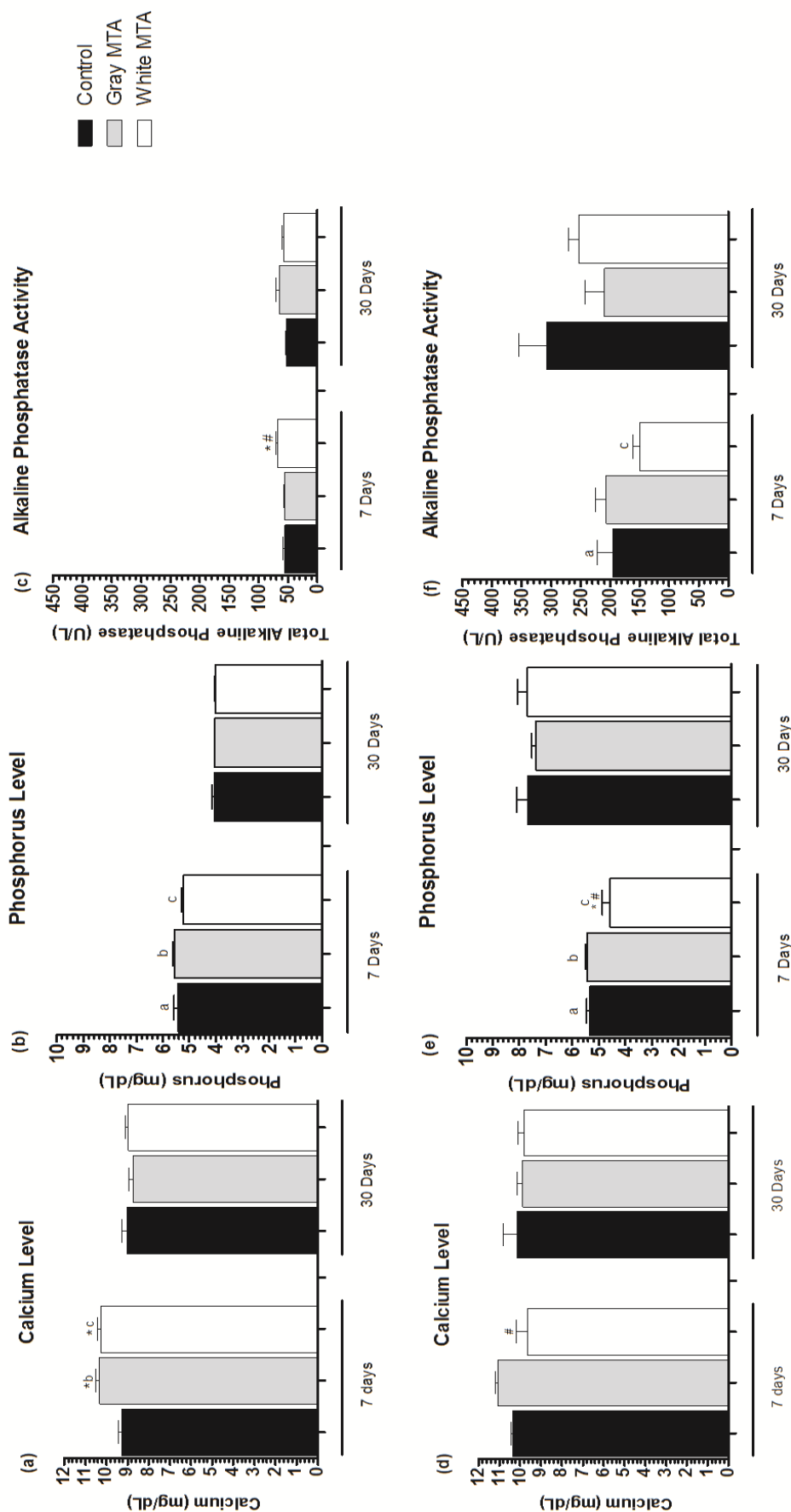


Figure 1: Graph showing the serum levels of calcium, phosphorus and alkaline phosphatase for healthy group (a, b, c) and diabetic group (d, e, f) on days 7 and 30. The symbols indicate statistical difference between each time point. Symbols: *: $p < 0.05$ vs. Control; #: $p < 0.05$ vs. Gray MTA. The letters indicate statistical difference between days 7 and 30. Letters: a: $p < 0.05$ vs. Control; b: $p < 0.05$ vs. GMTA; c: $p < 0.05$ vs. WMTA.

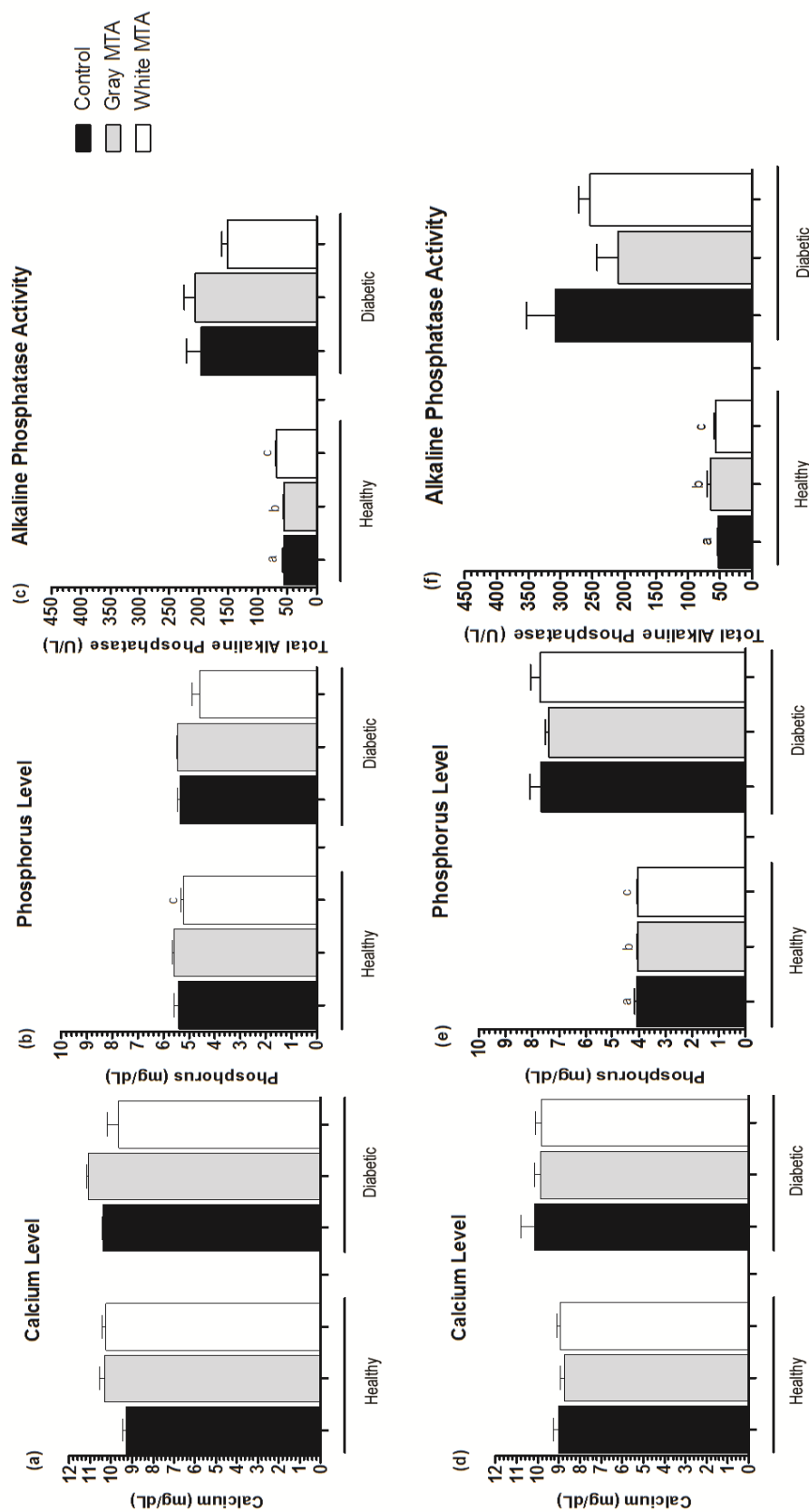


Figure 2: Graph showing comparison between the calcium, phosphorus and alkaline phosphatase serum levels in healthy and diabetic groups on day 7 (a, b, c) and 30 (d, e, f). The symbols indicate statistical difference between each time. Symbols: *: p<0.05 vs. Control; #: p<0.05 vs. Gray MTA. The letters indicate statistical difference between days 7 and 30. Letters: a: p<0.05 vs. Control diabetic group; b: p<0.05 vs. GMTA diabetic group; c: p<0.05 vs. WMTA diabetic group.

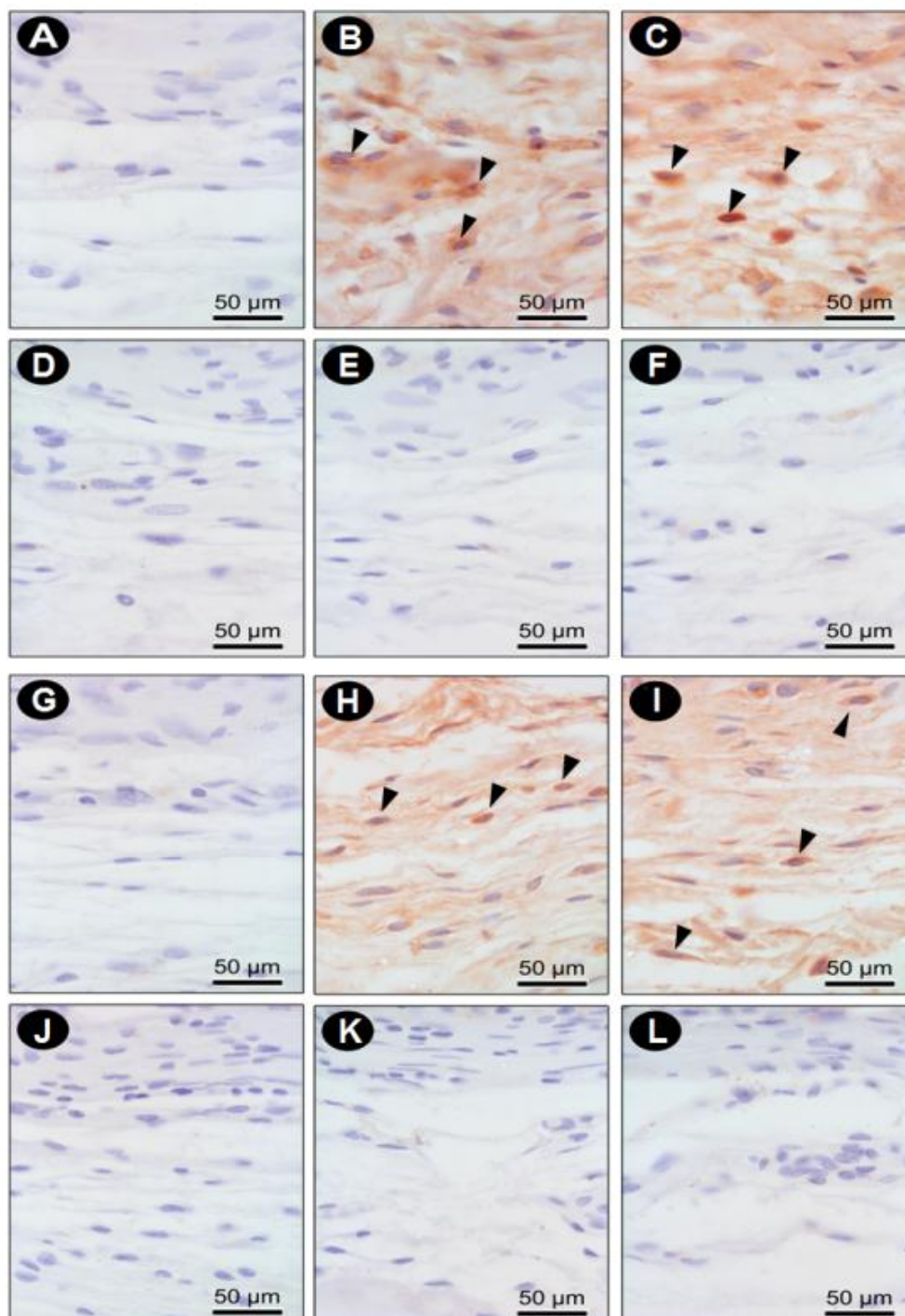


Figure 3: Photomicrographs showing the histological appearance of immunolabelling for OCN and OPN found in healthy and diabetic groups on day 30. **OCN:** **A:** Control normal; **B:** WMTA healthy; **C:** GMTA healthy; **D:** Control diabetic; **E:** WMTA diabetic; **F:** GMTA diabetic. **OPN:** **G:** Control normal; **H:** WMTA healthy; **I:** GMTA healthy; **J:** Control diabetic; **K:** WMTA diabetic; **L:** GMTA diabetic. Harris hematoxylin counterstaining. (Scale bars, 50 µm; Original magnification, 1000x).

Conclusão

Conclusão

Concluiu-se, assim, que com base nos objetivos propostos e metodologias empregadas que:

✓ MTA promoveu a regeneração do Ligamento Periodontal e do osso alveolar na área da injúria dental (perfuração), favorecendo a formação de ponte de dentina;

✓ MTA promoveu a proliferação células mesenquimais progenitoras do Ligamento Periodontal; no entanto, demonstrou efeitos negativos na diferenciação osteogênica de PDSCs e BMSCs.

✓ A condição hiperglicêmica interferiu na proliferação celular e produção de IL-6 do MTA Cinza *in vitro*;

✓ *In vivo*, a hiperglicemia não produziu alteração na resposta tecidual e na capacidade de produção de citocinas pro-inflamatórias, no entanto, reduziu a intensidade de fluorescência do MTA Branco aos 14 dias;

✓ A hiperglicemia impediu a produção de marcadores ósseos pelo MTA e aumentou os níveis séricos de Fósforo e Fosfatase Alcalina, principalmente, aos 07 dias na presença do MTA Cinza;

✓ Em condições diabéticas o MTA Cinza demonstrou resultados biológicos mais favoráveis quando comparado ao MTA Branco.

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Referências

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Anexos

Anexos

Anexo 1 – Comitê de Ética

 **UNIVERSIDADE ESTADUAL PAULISTA**
"JÚLIO DE MESQUITA FILHO" 

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

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

CERTIFICADO

Certificamos que o Relatório Final do trabalho intitulado "**Avaliação comparativa da resposta tecidual de ratos diabéticos ao MTA Cinza e MTA Branco**", Processo FOA nº 2013-00557, sob responsabilidade de João Eduardo Gomes Filho e colaboração de India Olinta de Azevedo Queiroz foi aprovado pela CEUA em 28 de Abril de 2016.

CERTIFICATE

We certify that the study entitled "**Comparative evaluation of tissue response to Grey and White MTA in diabetic rats**", Protocol FOA nº 2013-00557, under the supervision of João Eduardo Gomes Filho and collaboration of India Olinta de Azevedo Queiroz had its the Final Report approved by the CEUA on April 28, 2016.


Profa. Dra. Maria Gisela Laranjeira
Coordenadora da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
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Anexo 2 – Protocolos experimentais – In vitro

Protocolo para isolar e para cultivar células mesenquimais da medula óssea (BMSC)

Materiais: gelo, PBS estéril gelado, EtOH, tubo de Falcon 50 mL, tesoura (grande e pequeno) e fórceps

1. Matar os animais, remover os ossos longos (fêmur e tíbia) e colocar em PBS gelo-frio
2. No fluxo laminar, limpar os ossos, removendo os músculos e epífise
3. Coloque 12 ml de α MEM contendo 10% FBS (Soro Fetal Bovino) em tubo falcon
4. Enxágue a medula óssea com α MEM (agulha e seringa)
5. Ressuspender as células com maior agulha
6. Filtrar as células através da malha do filtro de células em um novo tubo Falcon
7. Contar as células vivas, não-eritrócitos e placa de 10 milhões de células em 2 ml por poço (placa de 6 poços)
8. Após 4 dias de cultura, aspirar metade do meio e adicionar 1 ml de meio fresco
9. Após 7 dias aspirar todo o meio e adicionar meios de diferenciação osteogênica ou meio com MTA
10. Realizar troca meio a cada 2-3 dias

Meio de diferenciação osteogênico

α MEM contendo 10% FBS e antibióticos + 50 ug/ml ácido ascórbico + 8 mM de β -glicerofosfato

Soluções Estoques: ácido ascórbico: 25 mg / ml (500x)

β -glicerofosfato: 1 M ou 800 mM (100x)

**Protocolo para isolar e para cultivar células progenitoras
periodontais (PDSC)**

Materiais: gelo, PBS estéril gelado, EtOH, tubo de Falcon 50 mL, tesoura (grande e pequeno) e fórceps

1. Preparar as soluções:
 - a. Clorexidina 0,12%
 - b. Digestion Buffer: Colagenase P(20mg) + PBS(10ml)+ Tripsina(0.05%)
 - c. Meio de cultura: DMEM + 20% PBS
2. Matar os animais, remover a maxila e mandíbula, remover os tecidos adjacentes, realizar a hemisseccção e colocar em PBS gelo-frio
3. Colocar as peças seccionadas em solução de Clorexidina 0,12% for 30 segundos. Logo após lavar as peças com PBS
4. Colocar as peças em uma nova solução de PBS e realizar a limpeza das peças (remover tecido gengival) com a ajuda do microscópio
5. Realizar a extração dos molares superiores e inferiores com o auxílio dos fórceps. Manter os dentes em PBS até a remoção de todos.
6. Colocar os dentes na solução (digestion buffer) por 90min a 37⁰C em constante agitação.
7. Retirar o sobrenadante e acrescentar ao mesmo DMEM+20%FBS (inativar a ação da tripsina) e centrifugar por 5min/1576rpm ou 500G
8. Coletar o sobrenadante com cuidado para não remover o pellet.
9. Resuspender o pellet em 1ml de meio de cultura DMEM + 20% PBS. Adicionar 9ml de meio e cultivar (placa petri) em condições de hipóxia
10. Após 4 dias, remover 5ml do meio de cultura e acrescentar 5ml de novo meio de cultura.
11. Após 7 dias, remover todo o meio de cultura e acrescentar novo meio. Transferir as células para condições de normoxia.
12. Trocar o meio a cada dois dias até as células atingirem a confluência. Realizar a passagem das células (placa 24 poços) e adicionar o meio osteogênico ou meio com MTA.
10. Realizar troca meio a cada 2-3 dias.

Protocolo para preparação do meio contendo MTA (MTA-CM ou MTA extract)

1. Manipular o MTA com água estéril de acordo com as instruções do fabricante e coloca-lo em discos de silicone esteireis.

Obs.: tubos de polietileno com confeccionados nas dimensões (5mm de diâmetro e 3mm altura)

2. Deixar na estufa a 37°C (5% de CO₂, 37°C) por 6 horas para tomar presa.

3. Retirar da estufa e remover os discos dos tubos de silicone

4. Adicionar 1ml de α MEM contendo 10% FBS e antibióticos para cada disco confeccionado em um tubo falcon

5. Incubar por 72 horas na estufa a 37°C (5% de CO₂, 37°C) para se obterem os ingredientes bioativos do MTA.

6. Após a incubação, remover o sobrenadante e filtrar através de um filtro de 45 μ m. O sobrenadante filtrado será o meio de MTA.

Protocolo para coloração celular com Von Kossa

1. Lavar as células com PBS

2. Adicionar formol a 10% e esperar por 5min para fixar as células

3. Adicionar 5% de nitrato de prata

4. Coloque no reticulador UV (Botão de reticulação automática duas vezes 12 segundos)

obs.: não tampar a placa

5. Caso não tenha reticulador UV, deixar a placa sobre a luz da bancada por no mínimo 30 minutos

6. Remover o nitrato de prata

7. Lavar com água e deixar secar

Protocolo para coloração celular com Fosfatase Alcalina

1. Lavar as células com PBS;

2. Adicionar formol a 10% e esperar por 5min para fixar as células

3. Usar o Kit 86RT da Sigma Aldrich
 4. Para fazer 4mL de solução, por exemplo:
 - a. Pegar um eppendorf e misturar: 100 µl de nitrato de sódio (solução 1)
100 µl de Solução FRV (solução 2)
 - b. Pegar 4,5 mL de água destilada, misturar com a solução do eppendorf.
 - c. Adicionar 100 µl de Naphtol (solução 3) e misturar – cor ficará amarela
 5. Coloque 1mL da solução final em cada poço
 6. Guardar a placa no escuro (longe da luz) por 30min.
 7. Remover o nitrato de prata
 8. Lavar com água e deixar secar
- Obs.: para cada 4ml de solução usar 100ul das soluções do kit

Protocolo para extração de RNA

Materiais: PBS, EtOH, H₂O, Falcon tubo 15 mL, seringa, agulha

1. Lavar as células com PBS
- Obs.: em todas as fases deve-se usar gelo
2. Adicionar 1 mL de trizol em cada poço
 3. Realizar a remoção das células com o trizol e coloca-las em um eppendorf
 4. Utilizar a Polytron por 20 segundos
 5. Adicionar 200µl de clorofórmio
 6. Agitar vigorosamente por 15 seg.
 7. Centrifugar 12 000 rpm / 15 min / 4⁰C
 8. Transferir a fase clara superior para um novo tubo eppendorf
 9. Adicionar 500µl de isopropanol (1 volume) e agitar vigorosamente
 10. Armazenar no - 20⁰C por no mínimo uma noite
 11. Após o tempo overnight, centrifugar 10 000 RPM / 10 min / 4⁰C
 12. Descartar o sobrenadante e colocar no gelo
 13. Colocar 1mL de EtOH a 80% e transfira para um novo tubo de 1,5 mL
 14. Centrifugar na velocidade máxima / 5 min / 4⁰C
 15. Aspirar líquido e deixar o pellet seco no gelo
 16. Centrifugar (centrifugação curta) para eliminar o restante EtOH

17. Adicionar 100 µL de água livre de RNase
18. Aquecer a 55°C / 5 min, vortex, e repetir 55 °C / 5 min
19. Medir a concentração no nanodrop.

Protocolo do ensaio proliferação - Alamar Blue

1. Adicionar alamar Blue na proporção 1:10 no meio de cultura das células em cultivo. Deixar poços com meio de cultura somente para a leitura do branco (Blank) e poços sem célula com meio + alamarBlue (controle negativo).
2. Incubar a 37°C e aguardar a partir de 1h para quantificar por absorbância (570 e 600 nm) ou fluorescência (excitação 530-560 nm e emissão 590 nm).
3. A avaliação pode ser feita por endpoint (semelhante ao desenho experimental de MTT, por exemplo) ou por cinética. Neste caso, considerar plaquear as células em pequena densidade devido à saturação do reagente no decorrer do tempo.
4. Após a finalização do experimento, as células podem ser recuperadas para outro ensaio.

Protocolo de preparo Meio de Cultura

DMEM suplementado com 10% FBS - 1L

Materiais:

- 850mL de H₂O Miliq
 - 10,03g de Dulbecos Medium Eagles (DMEM)
 - 3,7g de bicarbonato de sódio
 - 100mL de soro fetal bovino
 - 10mL de penicilina/streptomina
 - 1mL de gentamicina
 - 300µL de fungisone
 - 20mL de glutamina
1. Colocar H₂O Miliq em um bécker, diluir o pacote de DMEM inteiro, acrescentar o bicarbonato e o restante da água.
 2. Colocar o bécker no agitador e ajustar pH 6,9.

3. Dentro do fluxo, acrescentar antibióticos, soro fetal, glutamina (dentro do fluxo) e filtrar (bomba vácuo).

Meio hiperglicêmico

1. Adicionar ao meio de cultura DMEM com 10% FBS glicose na seguinte concentração:

Glicose – 0,225g em 50mL

Anexo 3 – Protocolos experimentais – In vivo

Protocolo de indução de diabetes

Material: ALLOXAN MONOHYDRATE, 25 G

Referência do Produto: A7413

Marca: Sigma Aldrich

1. Realizar a pesagem e a dosagem da glicemia dos animais;
2. Preparo da solução de Alozano de acordo com o número de animais utilizados para o experimento.

Ex.: Para indução de 50 animais pesando 200g cada

Em um recipiente coloca-se solução salina 0,9% estéril na proporção de 0,5ml para cada animal: 50 animais = 25 ml de solução salina

Realiza a pesagem da droga para ser utilizada na proporção de 150mg/kg. Cada animal pesa 200g, logo 50 animais = 10000g

$$\begin{array}{l} 150\text{mg/kg: } 150\text{mg (droga)} \text{ ----- } 1000\text{g (droga)} \\ \quad \quad \quad X \text{ mg (droga)} \quad \quad \quad \text{-----} \quad 10000\text{g (peso total animais)} \end{array}$$

$$X = 500\text{mg (peso total da droga)}$$

Em béquer colocar 25 ml da solução salina e ir acrescentando aos poucos a droga (500mg) com agitação constante usando um agitador magnético até a completa diluição.

3. Realizar a injeção por via intraperitoneal na dose única de 150mg/kg, proporcional ao peso do animal com agulha e seringa de insulina;

$$\text{Dose} = \frac{\text{peso do rato} \times \text{volume solução (25ml)}}{\text{dose droga (500mg)}}$$

4. Após a indução deixar somente água e passadas 06 horas oferecer aos animais glicose a 10% (água com açúcar) e comida;
5. Retirar água com açúcar após 24hs.
6. Após o 3º dia realizar a dosagem da glicemia dos animais.

Protocolo para injeção dos Fluorocromos

Material: Calcein, 10 G

Referência do Produto: C0875

Marca: Sigma Aldrich

Alizarin Red S, 25 G

Referência do Produto: A5533

Marca: Sigma Aldrich

Oxytetracycline hydrochloride, 10 G

Referência do Produto: O5875

Marca: Sigma Aldrich

1. Realizar a pesagem dos animais;
2. Preparo da solução dos fluorocromos de acordo com o número de animais utilizados para o experimento.

Ex.: Para indução de 10 animais pesando 300g cada

Em um recipiente coloca-se solução salina 0,9% estéril na proporção de 1ml para cada animal: 10 animais = 10 ml de solução salina

Realiza a pesagem da droga para ser utilizada na proporção de 20mg/kg. Cada animal pesa 300g, logo 10 animais = 3000g

20mg/kg: 20mg (droga) ----- 1000g (droga)

X mg (droga) _____ 3000g (peso total animais)

X = 60mg ou 0,0060g (peso total da droga)

Em béquer colocar 10 ml da solução salina e ir acrescentando aos poucos a droga (60mg ou 0,0060g) com agitação constante usando um agitador magnético até a completa diluição.

3. Realizar a injeção por via intramuscular na dose única de 20mg/kg com agulha e seringa de insulina. Injetar 1ml da solução para cada animal.

Protocolo para dosagem sanguínea de Cálcio

Material: Cálcio Liquiform (Kit Labtest)

Cálcio está envolvido no processo de mineralização óssea. Para determinação deve-se preparar o reagente de trabalho.

Reagentes do kit (100 testes)

- R1 – Reagente 1 (tampão)
- R2- Reagente 2 (cuidado, contém HCl)
- R3- Padrão (10mg/dL) (Contém formol, cuidado com evaporação)

Material necessário

- Pipeta automática que libere 20 μ L
- Pipeta automática que libere 1mL ou repipetador automático
- Ponteiras
- Placa para leitura na leitora Elisa (template)
- Béqueres para colocar os reagentes a serem pipetados
- Amostra (plasma, que não seja coletado com EDTA ou fluoreto)
- Tubos de ensaio em duplicata para as amostras
- Tubo para padrão e branco
- Estante para tubos

Obs: é importante que os tubos estejam bem limpos, de preferência lavados com HCl e muito bem enxaguados com água destilada.

Procedimento

1. Primeiramente deve-se preparar o reagente de trabalho, onde se mistura 3 volumes do reagente 1 (R1) com 1 volume do reagente 2 (R2), calcular a quantidade de reagente que será necessário já que se usa 1mL do mesmo por amostra. No kit a proporção do R1 para o R2 já está certa, então caso vá fazer 60 testes (incluindo padrão e branco), é só misturar o conteúdo todo dos dois reagentes. Toma-se cuidado para não preparar o reagente de trabalho em excesso, pois sua estabilidade é de apenas 8 horas, tendo que ser descartado caso não utilizado.

2. Nos tubos de teste adicionar 20 μ L do plasma e o padrão no tubo correspondente e em todos os tubos pipetar 1mL do reagente de trabalho.

Agitar a estante levemente para misturar o reagente com a amostra. A estabilidade da cor não é citada na bula, porém recomenda-se fazer a leitura o mais rápido possível, para evitar alguma perda. Se a leitura for feita no espectrofotômetro manual (570nm) adicionar o branco na cubeta e zerar o aparelho. Proceder as leituras das amostras. Caso a leitura for feita na leitora Elisa, deve-se pipetar 300µL da reação em cada poço, de acordo com a ordem definida (A1- Branco/ A2- Padrão/ A3- Padrão/ A4- amostra).

3. Ligar o computador, entrar no programa KC Junior, open protocol, Cálcio labtest, modify protocol e arrumar o template de acordo com o que pipetou na placa. Não esquecer de conferir o comprimento de onda (570nm). Fazer uma leitura sem placa, só para calibrar o aparelho (read plate). Depois fechar os resultados (file- close results).

4. Colocar a placa na leitora e realizar a leitura (read plate), salvando com ID (Cálcioseunome/orientador- dd/mm/AA). Após abrirá a leitura das absorbâncias, então abra o Excel e copie as informações (template e absorbâncias menos o branco).

Cálculos

Cálcio (mg/dL) = Abs teste (amostras)/abs padrão x 10

Pode-se fazer o cálculo usando o fator, onde: fator=10/abs padrão

Cálcio (mg/dL)= abs teste x fator

Caso deseje pode se realizar o cálculo no Excel, recomenda-se usar o fator, multiplicando-o pelas absorbâncias da amostras.

Exemplos de cálculos:

Fator=10/1,346 =7,43

Cálcio = 1,270 x 7,43 = 9,4mg/dL

Protocolo para dosagem sanguínea de Fósforo

Material: Fósforo UV Liquiform (Kit Labtest)

Fósforo está envolvido no processo de mineralização óssea. Sua absorbância é determinada em comprimento de onda de 340nm sendo necessário fotômetro que tenha leitura na faixa ultravioleta.

Reagentes do kit (100 testes)

- R1 – Reagente de cor (cuidado, cáustico)
- R2- Padrão (5,0mg/dL)

Material necessário

- Pipeta automática que libere 10 μ L
- Pipeta automática que libere 1mL ou repipetador automático
- Ponteiras
- Cronômetro
- Banho Maria (37°C)
- Placa para leitura na leitora Elisa (template)
- Béqueres para colocar os reagentes a serem pipetados
- Amostra (plasma)
- Tubos de ensaio em duplicata para as amostras
- Tubo para padrão e branco
- Estante para tubos

Obs: é importante que os tubos estejam bem limpos, sem resíduos de detergentes, uma vez que o fósforo inorgânico é componente comum da maioria dos detergentes.

Procedimento

1. Nos tubos de teste adicionar 10 μ L do plasma e em todos os tubos pipetar 1mL do reagente de cor (R1), de preferência com o repipetador pelo tempo de estabilidade. Agitar a estante levemente para misturar o reagente com a amostra. Levar ao banho maria que já deve estar na temperatura (37°C), deixar por 5 minutos. Após deve-se retirar a estante do banho e secá-la. A absorbância é estável somente por 30 minutos, recomendando-se leitura imediata. Se a leitura for feita no espectrofotômetro manual (340nm) adicionar o branco na cubeta e zerar o aparelho. Proceder as leituras das amostras. Caso a leitura for feita na leitora Elisa, deve-se pipetar 300 μ L da reação em cada poço, de acordo com a ordem definida (A1- Branco/ A2-Padrão/ A3-Padrão/ A4-amostra).
2. Ligar o computador, entrar no programa KC Junior, open protocol, Fósforo labtest, modify protocol e arrumar o template de acordo com o que pipetou na placa. Não esquecer de conferir o comprimento de onda (340nm). Fazer uma

leitura sem placa, só para calibrar o aparelho (read plate). Depois fechar os resultados (file- close results).

3. Colocar a placa na leitora e realizar a leitura (read plate), salvando com ID (Fósforo se nome/orientador- dd/mm/AA). Após abrir a leitura das absorbâncias, então abra o Excel e copie as informações (template e absorbâncias menos o branco).

Cálculos

Fósforo inorgânico (mg/dL) = Abs teste (amostras)/abs padrão x 5

Pode-se fazer o cálculo usando o fator, onde: fator=5/abs padrão

Fósforo inorgânico(mg/dL)= abs teste x fator

Caso deseje pode se realizar o cálculo no Excel, recomenda-se usar o fator, multiplicando-o pelas absorbâncias da amostras.

Exemplos de cálculos:

Fator=5/0,247 =20,2

Fósforo inorgânico = 0,190 x 20,2 = 3,8mg/dL

Protocolo para dosagem sanguínea de Fosfatase Alcalina

Material: Fosfatase Alcalina (Kit Labtest)

Fosfatase alcalina óssea é uma enzima, envolvida no processo de mineralização, portanto não é possível analisar sua quantidade e sim sua atividade.

Reagentes do kit (100 testes)

- R1 – Substrato
- R2 – Tampão (pH 10,1- básico “cuidado”)
- R3- Reagente de cor (reagente de parada- também alcalino “cuidado”)
- R4- Padrão 45U/L

Material necessário

- Pipeta automática que libere 50µL
- Pipeta automática que libere 500 µL e 2mL ou repipetador automático (duas ponteiros)
- Ponteiros

- Cronômetro
- Banho Maria (37°C)
- Placa para leitura na leitora Elisa (template)
- 3 béqueres para colocar os reagentes a serem pipetados
- Amostra (plasma)
- Tubos de ensaio em duplicata para as amostras
- Tubo para padrão e branco
- Estante para tubos

Procedimento

1. Em todos os tubos pipetar 0,5mL (500µL) do tampão (R2) e 50 µL do substrato (R1). Levar ao banho maria que já deve estar na temperatura (37°C), deixar por 2 minutos. Os passos seguintes sugere-se fazer em duas pessoas, pois a reação exige extrema atenção quanto ao tempo.
2. A adição da amostra (50µL) deve ser feita com intervalos (de 20 em 20 segundos) de acordo com a prática de pipetar de quem manuseia a amostra, importante que seja calculado de forma que a última amostra seja pipetada antes de completar o tempo da reação (10 minutos) não se esquecer de pipetar 50µL padrão (R4) no tubo correspondente.
3. Após os 10 minutos da adição da primeira amostra deve-se adicionar 2mL do reagente de parada (R3) , procedendo sua adição de acordo com o tempo em que foram adicionadas as amostras (de 20 em 20 segundos), desta forma a reação em todos os tubos ocorrerá no tempo de 10 minutos. A reação toda ocorre dentro do banho maria.
4. Após a adição do R3 em todos os tubos, deve-se retirar a estante do banho e secá-la. A cor é estável durante 120 minutos. Se a leitura for feita no espectrofotômetro manual (590nm) adicionar o branco na cubeta e zerar o aparelho. Proceder as leituras das amostras. Caso a leitura for feita na leitora Elisa, deve-se pipetar 300µL da reação em cada poço, de acordo com a ordem definida (A1- Branco/ A2- Padrão/ A3- Padrão/ A4- amostra).
5. Ligar o computador, entrar no programa KC Junior, open protocol, fosfatase alcalina labtest, modify protocol e arrumar o template de acordo com o que pipetou na placa. Não esquecer de conferir o comprimento de onda (590nm). Fazer uma leitura sem placa, só para calibrar o aparelho (read plate). Depois fechar os resultados (file- close results).

6. Colocar a placa na leitora e realizar a leitura (read plate), salvando com ID (FAseunome/orientador- dd/mm/AA). Após abrirá a leitura das absorbâncias, então abra o Excel e copie as informações (template e absorbâncias menos o branco).

Cálculos

Fosfatase alcalina (U/L) = Abs teste (amostras)/abs padrão x 45

Pode-se fazer o cálculo usando o fator, onde: fator=45/abs padrão

Fosfatase alcalina (U/L)= abs teste x fator

Caso deseje pode se realizar o cálculo no Excel, recomenda-se usar o fator, multiplicando-o pelas absorbâncias da amostras.

Exemplos de cálculos:

Fator=45/0,360 =125

Fosfatase alcalina = 0,295 x 125 = 37 U/L

Metodologia

Roy modificado

Técnica para inclusão em Glicol Metacrilato **(Leica – Historessin)**

1. Lavar em água corrente no mínimo 5 horas;
2. Desidratar em álcool 70% por uma noite;
3. No dia seguinte:
 - a. Álcool 90% por 30 minutos;
 - b. Álcool 90% por 30 minutos;
 - c. Álcool 90% por 30 minutos;
 - d. Álcool 95% por 30 minutos;
 - e. Álcool 95% por 30 minutos;
 - f. Álcool 95% por 30 minutos;
4. Colocar as peças desidratadas na solução A ativada e deixar por 72 horas (temperatura ambiente);
5. Isolar os moldes de plástico próprio para inclusão com spray de teflon, colocar as peças no interior dos moldes, cobrir as peças com resina para

inclusão (leica – historesin), depois de polimerizada, preencher com resina acrílica auto polimerizante e aguardar a polimerização.

Técnica para Coloração em Hematoxilina e Eosina (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 3 μ m.

1. Hidratar rapidamente em água destilada;
2. Colocar na Hematoxilina – 30 minutos;
3. Lavar em água corrente até remover o excesso de corante;
4. Colocar na Eosina – 5 minutos;
5. Lavar em água corrente até remover o excesso de corante;
6. Deixar secar na estufa, montar com Entelan e lamínula.

Técnica para Coloração em Von Kossa (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 10 μ m.

1. Hidratar rapidamente em água destilada;
2. Colocar sobre os cortes nitrato de prata 1% e deixar sob a luz solar por no mínimo 15 minutos;
3. Lavar em água corrente para remover o excesso de nitrato de prata;
4. Colocar no tiosulfato de sódio 5% - 30 segundos;
5. Lavar em água corrente - 1 minuto;
6. Colocar na safranina 0,5% - 30 segundos;
7. Lavar em água corrente para remover o excesso do corante.
8. Deixar secar na estufa, montar com Entelan e lamínula.

Técnica para Luz Polarizada (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 10 μ m.

1. Lâminas secas e montar com Entelan e lamínula.

Técnica para Luz Polarizada (para peças incluídas em Glicol Metacrilato)

Peças cortadas em 50µm.

1. Após o corte, deixar secar por poucos minutos
2. Lâminas secas e montar com meio de montagem para fluorescência à base de glicerol e lamínula.
3. Armazenar em geladeira

Técnica para inclusão em Parafina

1. Lavar em água corrente por 12 horas;
2. Realizar as trocas de Álcool e Xilol na sequência:
 - a) Álcool 70% por 1 hora;
 - b) Álcool 80% por 1 hora;
 - c) Álcool 95% por 1 hora;
 - d) Álcool 100% (I) por 1 hora;
 - e) Álcool 100% (II) por 1 hora;
 - f) Álcool 100% (III) por 1 hora;
 - g) Álcool/Xilol (50/50) por 1 hora;
 - h) Xilol 100% (I) por 1 hora;
 - i) Xilol 100% (II) por 30 minutos;
 - j) Xilol 100% (III) por 30 minutos;
 - k) Parafina (I) por 1 hora;
 - l) Parafina (II) por 1 hora;
 - m) Parafina (III) por 1 hora;
3. Retirar as peças da parafina (III) e inclui-las embebidas com o auxílio da máquina.

Protocolo para Imunoistoquímica

1º Dia

1. Desparafinização

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

2º. Ciclo: Bateria:

- a) Xilol I – 5 minutos
- b) Xilol II – 5 minutos
- c) Xilol III – 10 minutos
- d) Álcool 100 I – 2 minutos
- e) Álcool 100 II – 2 minutos
- f) Álcool 100 III – 2 minutos
- g) Álcool 95 – 2 minutos
- h) Álcool 70 – 2 minutos

2. Lavagens

- a) PBS – 5 minutos
- b) PBS – Triton x100 – 5 minutos
- c) PBS – 5 minutos

3. Recuperação Antigênica

198 ml de água destilada }
2 ml de tampão citrato 100x } 5 minutos – 95 ° C

4. Lavagens

- a) PBS – 5 minutos
- b) PBS – Triton x100 – 5 minutos
- c) PBS – 5 minutos

5. Bloqueio da Peroxidase Endógena

180 mL de PBS }
20 mL de H₂O₂ } 1 hora

6. Lavagens

- a) PBS – 5 minutos
- b) PBS – Triton x100 – 5 minutos
- c) PBS – 5 minutos

7. Bloqueio da Biotina Endógena

200 mL de PBS
9 g de leite em pó (molico) } 1 hora

8. Lavagem

a) PBS – 5 minutos

9. Bloqueio dos sítios inespecíficos

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

2º Dia

1. Incubação anticorpo primário

200 µL de solução contendo anticorpo primário - 24 horas

3º Dia

1. Lavagens

a) PBS – 5 minutos

b) PBS – Triton x100 – 5 minutos

c) PBS – 5 minutos

2. Incubação anticorpo Secundário Biotinilado

200 µL de solução contendo anticorpo secundário Biotinilado - 1 hora

3. Lavagens

a) PBS – 5 minutos

b) PBS – Triton x100 – 5 minutos

c) PBS – 5 minutos

4. Incubação na Estreptavidina Conjugada com HRP

200 µL de estreptavidina conjugada com HRP - 1 hora

5. Lavagens

a) PBS – 5 minutos

b) PBS – Triton x100 – 5 minutos

c) PBS – 5 minutos

6. Revelação com Cromógeno – DAB

200µL de DAB substrato (2,5 mL)
2 gotas de DAB cromógeno } 200µl por lâmina

7. Lavagens
 - a) PBS – 5 minutos
 - b) Água destilada – 5 minutos
8. Contra-coloração: O corante muda dependendo do marcador. Para hematoxilina
 - a) Hematoxilina de Harris (tempo variável)
 - b) Água destilada – 1 minuto
 - c) Álcool 70% – 2 minutos
 - d) Álcool 95% – 2 minutos
 - e) Álcool 100% I – 2 minutos
 - f) Álcool 100% II – 2 minutos
 - g) Xilol – 2 minutos
 - h) Xilol I – 5 minutos
 - i) Xilol II – 5 minutos
 - j) Xilol III – 10 minutos
9. Deixar secar na estufa, montar com Entelan e lamínula.

Técnica para Coloração em Hematoxilina e Eosina (para frozen sections)

Montar os slides cortados numa lâmina nova e deixar secar

1. Lavar com PBS por 5min e depois secar a lâmina
2. Colocar na Hematoxilina (Mayers Modified Haematoxylin) por 30 segundos;
3. Lavar em água corrente até remover o excesso de corante;
4. Colocar Eosina (Shandon Bluing Reagent) por 30segundos;
5. Lavar em água corrente até remover o excesso de corante;
6. Montar com glicerol/PBS (solução 50/50) ou glicerol/PBS (solução 50/50) e lamínula.

Anexo 4 - Diretrizes para publicação dos trabalhos

Guidelines for Publishing Papers in the Journal of Periodontal Research

1. GENERAL

The *Journal of Periodontal Research* is an international research periodical the purpose of which is to publish original clinical and basic investigations and review articles concerned with every aspect of periodontology and related sciences. Reports of scientific meetings in periodontology and related fields are also published.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in the *Journal of Periodontal Research*. Authors are encouraged to visit Wiley Blackwell's Author Services for further information on the preparation and submission of articles and figures.

2. ETHICAL GUIDELINES

The *Journal of Periodontal Research* adheres to the below ethical guidelines for publication and research.

2.1. Authorship and Acknowledgements

Authors submitting a paper do so on the understanding that the manuscript have been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal.

The *Journal of Periodontal Research* 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 acquisition 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.

It is a requirement that all authors have been accredited as appropriate upon submission of the manuscript. Contributors who do not qualify as authors should be mentioned under Acknowledgements.

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2.2. Ethical Approvals

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.

2.3 Photographs of People

The *Journal of Periodontal Research* follows current HIPAA guidelines for the protection of patients/subject privacy. If an individual pictured in a digital image or photograph can be identified, his or her permission is required to publish the image. The corresponding author may submit a letter signed by the patient authorizing the *Journal of Periodontal Research* to publish the image/photo. Or, a form provided by the *Journal of Periodontal Research* (available by clicking the “Instructions and Forms” link in Manuscript Central) may be downloaded for your use. This approval must be received by the Editorial Office prior to final acceptance of the manuscript for publication. Otherwise, the image/photo must be altered such that the individual cannot be identified (black bars over eyes, tattoos, scars, etc.). The *Journal of Periodontal Research* will not publish patient photographs that will in any way allow the patient to be identified, unless the patient has given their express consent.

2.4 Clinical Trials

Clinical trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist should also be included in the submission material. The *Journal of Periodontal Research* encourages

authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following free, public clinical trials registries: www.clinicaltrials.gov, <http://clinicaltrials.ifpma.org/clinicaltrials/>, <http://isrctn.org/>. The clinical trial registration number and name of the trial register will then be published with the paper.

2.5 Conflict of Interest and Source of Funding

Please disclose information concerning sources of institutional, private and corporate financial support for the work within the manuscript be fully acknowledged, and any potential conflicts of interest under Acknowledgements

2.6 Appeal of Decision

The decision on a paper is final and cannot be appealed.

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3.1. Getting Started

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After clicking on 'register here', enter your name and e-mail information and click 'Next'. Your e-mail information is very important.

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- Review your submission (in HTML and PDF format) before sending to the Journal. Click the 'Submit' button when you are finished reviewing.

3.3. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc/.docx) or Rich Text Format (.rtf) 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 entire manuscript including title page, abstract, text, references, figure legends and tables but *no* embedded figures. Figure tags should be included in the file. Manuscripts should be formatted as described in the Author Guidelines below.

3.4. Blinded Review

All manuscripts submitted to the Journal of Periodontal Research will be reviewed by two experts in the field. The Journal of Periodontal Research uses single blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper.

3.5. Suggest a Reviewer

The Journal of Periodontal Research attempts to keep the review process as short as possible to enable rapid publication of new scientific data. In order to facilitate this process, please suggest the names and current email addresses

of 2 potential international reviewers whom you consider capable of reviewing your manuscript.

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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To submit your revised manuscript, locate your manuscript under 'Manuscripts with Decisions' and click on 'Submit a Revision'. Please remember to delete any old files uploaded when you upload your revised manuscript.

4. MANUSCRIPT TYPES ACCEPTED

Original 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 articles must conform to the highest international standards in the field.

Review Articles: are selected for their broad general interest; all are refereed by experts in the field. Reviews should take a broad view of the field rather than merely summarizing the authors' own previous work, so extensive citation of the authors' own publications is discouraged.

Mini Reviews are covering a smaller area and may be written in a more free format.

Short Communications: Short communications, limited to 1-3 pages, including illustrations and references, will be considered for rapid publication. Such papers must be based on work that is of special importance or having the potential for great impact, or a body of work that is complete but of insufficient scope to warrant a full-length paper. Short communications need not follow the usual divisions.

Meeting Reports: Reports of scientific meetings in periodontology and related fields are also published.

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Articles exceeding 7 published pages (including figures and tables) are subject to a charge of GBP70.00 per additional page. For guidance purposes, one published page amounts approximately to 5,500 characters; text should be reduced if figures/tables are included within the 7 pages. If authors are unable to pay additional page fees they will need to reduce the length of their articles.

5.2. Format

Language: The language of publication is English. Authors for whom English is a second language must have their manuscript professionally edited by an English speaking person before submission to make sure the English is of high quality. It is preferred that manuscript is professionally edited. A list of independent suppliers of editing services can be found at http://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

Abbreviations and symbols: Abbreviations should be in accordance with Guidelines laid down by the American Society of Microbiology. Unless they are in common usage (e.g. DNA), all terms must be displayed in full in the key words, and the first time that they appear in the abstract, the main text, tables and figures, followed by the abbreviation in parentheses. If an abbreviation is used in the body of figure or table only it must be defined in the figure legend or table footnotes. The symbol % is to be used for percent, h for hour, min for minute, and s for second. *In vitro* and *in vivo* are to be italicized. Use only standard abbreviations. All units will be metric. Use no roman numerals in the text. In decimals, a decimal point, and not a comma, will be used. In cases of

doubt, the spelling orthodoxy of *Webster's Third New International Dictionary* will be adhered to.

Scientific Names: Proper names of bacteria should be binomial and should be singly underlined in the typescript. The full proper name (e. g. *Streptococcus sanguis*) must be given upon first mention. The generic name may be abbreviated thereafter with the first letter of the genus (e. g. *S. sanguis*). If abbreviation of the generic name could cause confusion, the full name should be used. If the vernacular form of a genus name (e. g. streptococci) is used, the first letter of the vernacular name is not capitalized and the name is not underlined. Use of two letters of the genus (e. g. *Ps.* for *Peptostreptococcus*) is incorrect, even though it might avoid ambiguity. With regard to drugs, generic names should be used instead of proprietary names. It is strongly recommended that all abbreviations be introduced in the first paragraph in Materials and Methods. Alternatively, define each abbreviation and introduce it in parentheses the first time it is used; e.g., "Cultures were grown in Eagle minimal essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

5.3. Structure

All manuscripts submitted to the Journal of Periodontal Research should include: Title page, abstract, main text, references and tables, figures and figure legends were appropriate.

Title Page: should contain the title of the article, name(s) of the author(s), initials, and institutional affiliation(s), a running title not to exceed 40 letters and spaces, and the name and complete mailing address, including email address, of the author responsible for correspondence. We accept only one corresponding author per manuscript and in case of co-correspondence a foot note will be added indicating 'Both the authors have contributed equally to the work'. The author must list 4 keywords for indexing purposes.

Abstract: The abstract should consist of 1) the objective 2) the background data discussing the present status of the field 3) methods 4) results 5) conclusion.

Main Text of Original Research Articles

Introduction: Summarize the rationale and purpose of the study, giving only strictly pertinent references. Do not review existing literature extensively.

Material and methods: Materials and methods should be presented in sufficient detail to allow confirmation of the observations. Published methods should be referenced and discussed only briefly, unless modifications have been made.

Results: Present your results in a logical sequence in the text, tables, and illustrations. Do not repeat in the text all of the data in the tables and illustrations. Important observations should be emphasized.

Discussion: Summarize the findings without repeating in detail the data given in the Results section. Relate your observations to other relevant studies and point out the implications of the findings and their limitations. Cite other relevant studies.

Main Text of Reviews, Short Communications and Meeting Reports

These need not follow the usual divisions.

Acknowledgements: Acknowledge only persons who have made substantive contributions to the study. Authors are responsible for obtaining written permission from everyone acknowledged by name because readers may infer their endorsement of the data and conclusions. Sources of financial support must be acknowledged.

5.4. References

References should be numbered consecutively in the order in which they appear in the text, and should be kept to a pertinent minimum. References should include the beginning and ending page numbers. Identify references in the text, tables, and figure legends by Arabic numerals in parentheses. References cited only in the tables or figure legends should be numbered in accordance with a sequence established by the first notation of that figure or table in the text. Use the style of the examples below, which is based on *Index Medicus*. Manuscripts accepted but not published may be cited in the reference list by placing "in press" after the abbreviated title of the journal. Abstracts and manuscripts not yet accepted may be cited in full in the text but not in the reference list. References must be verified by the author(s) against the original documents. We recommend the use of a tool such as Reference Manager for reference management and formatting. Reference Manager reference styles can be searched for here: <http://refman.com/downloads/styles>

Examples:

- (1) *Standard journal article* (List all authors up to 6; for 7 or more list the first 3 and add "et al.") Dockrell H, Greenspan JS. Histochemical identification of T-cells in oral lichen planus. *Oral Surg* 1979; 48: 42-49. Thomas Y, Sosman J, Yrigoyen O, et al. Functional analysis of human T- cell subsets defined by monoclonal antibodies. I. Collaborative T-T interactions in the immunoregulation of B-cell differentiation. *J Immunol* 1980; 125: 2402-2405.
- (2) *Corporate author*. The Royal Marsden Hospital Bone- Marrow Transplantation Team. Failure of syngeneic bone- marrow graft without preconditioning in post- hepatitis marrow aplasia. *Lancet* 1977; 2: 628-630.
- (3) *No author given* Anonymous. Coffee drinking and cancer of the pancreas [Editorial]. *Br Med J* 1981; 283: 628-635.
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Health Statistics, 1978; DHEW publication no. (PHS) 78-1785. (Vital and health statistics; series 13; no. 34.)

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5.5. Tables, Figures and Figure Legends

Tables: Tables should be numbered consecutively with arabic numerals. Use titles which are self explanatory. Due regard should be given to the proportions of the printed page.

Figures: All figures should clarify the text and their number should be kept to a minimum. Details must be large enough to retain their clarity after reduction in size. Illustrations should preferably fill a single column width (54 mm) after reduction, although in some cases 113 mm (double column) and 171 mm (full page) widths will be accepted. Micrographs should be designed to be reproduced without reduction, and they should be dressed directly on the micrograph with a linear size scale, arrows, and other designators as needed.

Preparation of Electronic Figures for Publication: Although low quality images are adequate for review purposes, print publication requires high quality images to prevent the final product being blurred or fuzzy. Submit EPS (lineart) or TIFF (halftone/photographs) files only. MS PowerPoint and Word Graphics are unsuitable for printed pictures. Do not use pixel-oriented programmes. Scans (TIFF only) should have a resolution of 300 dpi (halftone) or 600 to 1200 dpi (line drawings) in relation to the reproduction size (see below). EPS files should be saved with fonts embedded (and with a TIFF preview if possible).

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5.6. Supporting Material

Supporting Material, such as data sets or additional figures or tables, that will not be published in the print edition of the journal, but which will be viewable via the online edition, can be submitted. It should be clearly stated at the time of submission that the Supporting Material is intended to be made available through the online edition. If the size or format of the Supporting Material is such that it cannot be accommodated on the journal's Web site, the author agrees to make the Supporting Material available free of charge on a permanent Web site, to which links will be set up from the journal's website. The author must advise Wiley Blackwell if the URL of the website where the Supporting Material is located changes. The content of the Supporting Material must not be altered after the paper has been accepted for publication. The availability of Supporting Material should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting Material' and providing titles of figures, tables, etc. In order to protect reviewer anonymity, material posted on the authors Web site cannot be reviewed. The Supporting Material is an integral part of the article and will be reviewed accordingly.

6. AFTER ACCEPTANCE

Upon acceptance of a paper for publication, the manuscript will be forwarded to the Production Editor who is responsible for the production of the journal.

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The corresponding author will receive an email alert containing a link to a web site. A working email address must therefore be provided for the corresponding author. The proof can be downloaded as a PDF (portable document format) file from this site. Acrobat Reader will be required in order to read this file. This software can be downloaded (free of charge) from the following Web site: www.adobe.com/products/acrobat/readstep2.html. This will enable the file to be opened, read on screen, and printed out in order for any corrections to be added. Further instructions will be sent with the proof. Hard copy proofs will be posted if no e-mail address is available; in your absence, please arrange for a colleague to access your e-mail to retrieve the proofs. Proofs must be returned to the Production Editor within three days of receipt. Excessive changes made by the author in the proofs, excluding typesetting errors, will be charged separately. Other than in exceptional circumstances, all illustrations are retained

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The Journal of Periodontal Research is covered by Wiley Blackwell's Early View service. Early View articles are complete full-text articles published online in advance of their publication in a printed issue. Early View articles are complete and final. They have been fully reviewed, revised and edited for publication, and the authors' final corrections have been incorporated. Because they are in final form, no changes can be made after online publication. The nature of Early View articles means that they do not yet have volume, issue or page numbers, so Early View articles cannot be cited in the traditional way. They are therefore given a Digital Object Identifier (DOI), which allows the article to be cited and tracked before it is allocated to an issue. After print publication, the DOI remains valid and can continue to be used to cite and access the article.

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Guidelines for Publishing Papers in the Journal of Endodontics

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

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

2. General Points on Composition

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

a. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section “c”, below), or sentences with little to no transition within a paragraph.

b. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors' names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland et al., reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as

a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al., 1983)". In this example, the paragraph's subject is sodium hypochlorite and sentences should focus on this subject.

c. Sentences are stronger when written in the active voice, i.e., the subject performs the action. Passive sentences are identified by the use of passive verbs such as "was," "were," "could," etc. For example: "Dexamethasone was found in this study to be a factor that was associated with reduced inflammation", can be edited to: "Our results demonstrated that dexamethasone reduced inflammation". Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.

d. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph.(This section was written deliberately with sentences of varying length to illustrate this point.)

e. Use parallel construction to express related ideas. For example, the sentence, "Formerly, Endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method", can be edited to "Formerly, Endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation". The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.

f. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used", can be edited to "Caution must be used when conclusions are drawn from the results of this study".

g. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.

3. General Points on the Organization of Original Research Manuscripts

Please Note: Starting in 2009, all abstracts should be organized into sections that start with a one-word title (in bold), i.e., Introduction, Methods, Results, Conclusions, etc., and should not exceed more than 250 words in length.

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

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

c. Introduction: The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address. The purpose of the study, the tested hypothesis and its scope should be described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the JOE. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to provide sufficient detail. However, many successful manuscripts require no more than a few paragraphs to accomplish these goals.

d. Material and Methods: The objective of the methods section is to permit other investigators to repeat your experiments. The three components to this section are the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the particular aspects used in the present study. The inclusion of a “methods figure” will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a commercial product, the manuscript should state that they either followed manufacturer’s protocol or specify any changes made to the protocol. Studies on humans should conform to the Helsinki Declaration of 1975 and state that the institutional IRB approved the protocol and that informed consent was obtained. Studies involving animals should state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis, drop-outs from clinical trials, etc.

e. Results: Only experimental results are appropriate in this section (i.e., neither methods nor conclusions should be in this section). Include only those data that are critical for the study. Do not include all available data without justification, any repetitive findings will be rejected from publication. All Figs./Charts/Tables should be described in their order of numbering with a brief description of the major findings.

f. Figures: There are two general types of figures. The first type of figure includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (i.e., about 175 mm wide X 125 mm high when published in the JOE), you would be able to place about 12 panels of photomicrographs (or radiographs, etc.) as an array of four columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing on your part given the small size of each panel, you will

only be able to illustrate the most important feature of each photomicrograph. Remember that each panel must be clearly identified with a letter (e.g., “A”, “B”, etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Chang, et al, (JOE 28:90, 2002), Hayashi, et al, (JOE 28:120, 2002) and by Davis, et al (JOE 28:464, 2002). At the Editor’s discretion, color figures may be published at no cost to the authors. However, the Editor is limited by a yearly allowance and this offer does not include printing of reprints.

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

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

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

Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl

(N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).

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

h. References: The reference style follows Index Medicus and can be efficiently learned from reading past issues of the JOE. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

Guidelines for Publishing Papers in the International Endodontic Journal

1. GENERAL

International Endodontic Journal publishes original scientific articles, reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic well-being and health. Original scientific articles are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and to the restoration of root-treated teeth. In addition, review articles, reports of clinical cases, book reviews, summaries and abstracts of scientific meetings and news items are accepted. Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in *International Endodontic Journal*. Authors are encouraged to visit Wiley Author Services for further information on the preparation and submission of articles and figures.

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Authors submitting a paper do so on the understanding that the manuscript has been read and approved by all authors and that all authors agree to the submission of the manuscript to the Journal. *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 acquisition 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. **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.

2.2. Ethical Approvals

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. The authors **MUST** upload a copy of the ethical approval letter when submitting their manuscript. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

2.3 Clinical Trials

2.3.1. Randomised control clinical trials should be reported using the guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material. The International Endodontic Journal asks that authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following public clinical registries: www.clinicaltrials.gov, <https://www.clinicaltrialsregister.eu/>,

<http://isrctn.org/>. Other primary registries if named in the WHO network will also be considered acceptable. The clinical trial registration number and name of the trial register should be included in the Acknowledgements at the submission stage.

2.3.2 Epidemiological observational trials

Submitting authors of epidemiological human observations studies are required to review and submit a 'strengthening the reporting of observational studies in Epidemiology' (STROBE) checklist and statement. Compliance with this should be detailed in the materials and methods section. (www.strobe-statement.org)

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Systematic reviews should be reported using the PRISMA guidelines available at <http://prisma-statement.org/>. A PRISMA checklist and flow diagram (as a Figure) should also be included in the submission material.

2.5 DNA Sequences and Crystallographic Structure Determinations

Papers reporting protein or DNA sequences and crystallographic structure determinations will not be accepted without a Genbank or Brookhaven accession number, respectively. Other supporting data sets must be made available on the publication date from the authors directly.

2.6 Conflict of Interest and Source of Funding

International Endodontic Journal requires that all authors (both the corresponding author and co-authors) disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise that might be perceived as influencing an author's objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker's fees from a company. If authors are unsure whether a past or present affiliation or relationship should be disclosed in the manuscript, please contact the editorial office atiejeditor@cardiff.ac.uk. The existence of a conflict of interest does not preclude publication in this journal. The above policies are in accordance with the Uniform Requirements for Manuscripts Submitted to

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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.

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Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission. Log-in, or if you are a new user, click on 'register here'. If you are registering as a new user. After clicking on 'register here', enter your name and e-mail information and click 'Next'. Your e-mail information is very important. Enter your institution and address information as appropriate, and then click 'Next'. Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID), and then select your areas of expertise. If you are registered, but have forgotten your log in details, please enter your e-mail address under 'Password Help'. The system will send you an automatic user ID and a new temporary password. Log-in and select 'Author Center'

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3.4. Manuscript Files Accepted

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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: Your manuscript without title page under the file designation 'main document'. Figure files under the file designation 'figures'. The title page and Acknowledgements where applicable, should be uploaded under the file designation 'title page'. 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.

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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.

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Letters to the Editor: are also acceptable.

Meeting Reports: are also acceptable.

5. MANUSCRIPT FORMAT AND STRUCTURE

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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 at http://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

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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]

5.4. Tables, Figures and Figure Legends

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.)

Figure legends: Figure legends should begin with a brief title for the whole figure and continue with a short description of each panel and the symbols used; they should not contain any details of methods.

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The *International Endodontic Journal* gives authors notice that, with effect from 1st January 2011, submission to the *International Endodontic Journal* requires the reporting of microarray data to conform to the MIAME guidelines. After this date, submissions will be assessed according to MIAME standards. Also, manuscripts will be published only after the complete data has been submitted into the public repositories, such as GEO (<http://www.ncbi.nlm.nih.gov/geo/>) or Array Express (http://www.ebi.ac.uk/microarray/submissions_overview.html), in MIAME compliant format, with the data accession number (the identification number of the data set in the database) quoted in the manuscript. Both databases are committed to keeping the data private until the associated manuscript is published, if requested. 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.