Paulo Carvalho Tobias Duarte

"AVALIAÇÃO HISTOLÓGICA DE ESTRATÉGIAS DE ENGENHARIA TECIDUAL PARA A TERAPIA ENDODÔNTICA"

ARAÇATUBA

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"AVALIAÇÃO HISTOLÓGICA DE ESTRATÉGIAS DE ENGENHARIA TECIDUAL PARA A TERAPIA ENDODÔNTICA"

Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção do título de ***Doutor*** *em Ciência Odontológica, Área de Concentração: Saúde Bucal da Criança.*

Orientador: Prof. Adj. João Eduardo Gomes Filho

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DEDICATÓRIA

Dedicatória

À Deus

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RESUMO

Duarte PCT. Avaliação histológica de estratégias de engenharia tecidual para a terapia endodôntica. [tese]. Araçatuba: Universidade Estadual Paulista, 2013.

RESUMO

O objetivo deste estudo foi avaliar a resposta tecidual ao implante de tubos de polietileno preenchidos com pasta triantibiótica (PTA) composta de ciprofloxacina, minociclina e metronidazol, veiculada com propilenoglicol e polietilenoglicol (macrogol), em tecido subcutâneo de ratos e, caracterizar, histologicamente, os tecidos neoformados pós-procedimento endodôntico regenerativo, empregando o coágulo ou o coágulo suplementado com plasma rico em plaquetas (PRP), aspirado de medula óssea (AMO), ou a mistura PRP/BMA, pós-desinfecção com PTA. **Estudo para avaliar a biocompatibilidade da pasta**: Trinta ratos receberam 2 implantes individuais de tubos de polietileno preenchidos com PTA ou pasta de hidróxido de cálcio (PHC) e um outro tubo vazio como controle. Trinta ratos adicionais receberam tubos de polietileno contendo os veículos da pasta propilenoglicol e macrogol e um procedimento somente de incisão, sem implante de tubo (Sham). Após 7, 15, 30, 60, e 90 dias, 12 animais foram sacrificados, e os tubos foram removidos e processados histologicamente pela técnica do glicolmetacrilato e corados com hematoxilina e eosina. Os scores variaram de 0 a 3, dependendo do teor de células inflamatórias, a cápsula fibrosa foi considerada fina ou espessa, e a necrose e a calcificação foram registradas como presentes ou ausentes. Os resultados foram analisados pelo teste de Kruskal-Wallis. Ambos os materiais induziram reações moderadas aos 7 e 15 dias sendo similares ao grupo controle ($p>0.05$) e reduziram para grau leve a

partir de 30 dias (p>0.05). Os veículos não interferiram na resposta da pasta. A pasta triantibiótica assim com seus respectivos veículos foram biocompatíveis em todos os períodos experimentais. **Estudo para análise histológica dos tecidos neoformados com diferentes estratégias de engenharia tecidual:** 20 dentes (40 raízes), de 2 cães da raça Beagle, foram expostos ao meio bucal pelo período de 90 dias para indução de lesão periapical. Os canais foram preparados até a barreira apical com limas Protaper #F3 e a barreira cementária apical foi penetrada com lima K #15, criando-se uma comunicação apical semelhante ao forame. A lima foi inserida 2 mm além da barreira apical a qual foi ampliada até a lima K #60. Após desinfecção com PTA por 28 dias, quatro grupos de tratamento foram estabelecidos: coágulo (C), C com gel de PRP, C com gel AMO, e C com gel de PRP/AMO. Controles negativos (sem tratamento) foram incluídos. Após 3 meses, os cães foram sacrificados e os dentes removidos e processados histologicamente. A análise revelou a presença de tecidos vitais (tecido conjuntivo, tecido semelhante ao cemento e tecido semelhante ao osso) em 23 das 32 raízes tratadas (71, 87%), porém nao houve diferença estatisticamente significativa entre os grupos. O grupo C com gel de PRP teve a maior formação de tecido e um maior limite crescimento interno do tecido, e o grupo C com gel de PRP/AMO, não apresentou tecido ósseo, porém isto nao foi estatisticamente significante (p>0.05). Novos tecidos vitais podem ser formados em dentes necróticos pós-desinfecção com PTA e ampliação do forame apical. Os novos tecidos formados foram caracterizados como tecido conjuntivo ou semelhante ao cemento ou osso, mas não como tecido pulpar.

ABSTRACT

Duarte PCT. Histological evaluation of tissue engineering strategies for endodontic therapy [thesis]. Araçatuba: São Paulo State University, 2013.

ABSTRACT

This study aimed to evaluate the tissue response to the implantation of polyethylene tubes filled with a triantibiotic paste (TAP) composed of ciprofloxacin, metronidazole and minocycline conveyed with propylene glycol and polyethylene glycol (macrogol) in subcutaneous tissue of rats and characterize histologically the newly formed tissues post regenerative endodontic procedure employing the blood clot (BC) or BC supplemented with platelet-rich plasma (PRP), bone marrow aspirate (BMA) or a mixture of PRP/ BMA, post-disinfection with TAP. **Study to evaluate TAP biocompatibility**: Thirty rats received two individual implants of polyethylene tubes filled with TAP or calcium hydroxide paste (CHP) and another empty tube as a control. Thirty other rats received additional polyethylene tubes implants containing the paste vehicles (propylene glycol and macrogol) and an incision procedure (Sham group). After 7, 15, 30, 60, and 90 days, 12 animals were killed, and the tubes were removed and processed for histological examination. The scores ranged from 0 to 3 depending on content of inflammatory cells, the fibrous capsule was considered thin or thick, and necrosis and calcification were recorded as present or absent. The results were analyzed using the Kruskal-Wallis test. Both materials induced moderate reactions at 7 and 15 days which was similar to the control group ($p > 0.05$) and reduced to mild after 30 days ($p > 0.05$). The vehicles did not affect the tissue response to the paste. The TAP and their respective vehicles were biocompatible in all experimental periods. **Study to**

histologically evaluate newly formed tissues after regenerative treatment: 20 teeth (40 roots), from 2 Beagle dogs, had their root canals exposed to the oral environment for 90 days to induce apical periodontitis. The canals were prepared until the apical barrier with Protaper files # F3 and the apical cementum barrier was penetrated with a #15 K-file, creating a foramen-like communication. The file was inserted 2 mm beyond the apical barrier which was enlarged to # 60 K-file. After disinfection with TAP for 28 days, 4 treatment groups were established: blood clot (BC), BC with PRP gel, BC with BMA gel, and BC with BMA/PRP gel. Negative controls (no treatment) were included. After 3 months, the dogs were sacrificed and the teeth removed for histological processing. The analysis revealed the presence of new vital tissues (connective tissue, cementum-like tissue and bone-like tissue) in 23 of the 32 treated roots (71, 87%), but there was no statistically significant difference between groups. The BC with PRP gel group had the highest percentual of tissue formation and tissue ingrowth limit, and group BC with PRP/BMA gel, showed no bone-like tissue formation, but this was not statistically significant ($p>0.05$). New vital tissues can be formed in necrotic mature teeth after disinfection with TAP and enlargement of the apical foramen. The new tissues were characterized as connective, cementum-like, or bone-like tissue but not as pulp tissue.

LISTA DE ABREVIATURAS

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%	Porcentagem
^{o}C	Grau Celsius
µm	Micrometro
µL	Microlitro
et al.	E colaboradores
Fig.	Figura
Ind.	Indústria
Inc.	Incorporation
AMO	Aspirado de medula óssea
BC	Blood clot
BMA	Bone marro aspirate
CHP	Calcium hydroxide paste
Com.	Comércio
CPDA-1	Citrate-phosphate-dextrose-adenine-1
CTMs	Célula-tronco mesenquimal
DPSC	Dental pulp stem cell
EDTA	Etilenodiaminotetracético
ERP	Endododontic regenerative procedure
G	Gravitacional
IL-10	Interleucin-10

IV	Intravenoso
K	Kerr
Ltda.	Limitada
MG	Minas Gerais
mg/kg	Miligrama por quilo
Ml	Mililitros
mm	Milímetro
MSC	Mesenchymal stem cell
NaCl	Cloreto de sódio
NB	Bone-like tissue
NC	Cementum-like tissue
NCT	Neoformed connective tissue
NIT	Neoformed intracanal tissue
NJ	New Jersey
OK	Oklahoma
p	nível de significância
PER	Procedimento endodôntico regenerativo
pH	Potencial hidrogeniônico
PHC	Pasta de hidróxido de cálcio
PPP	Plasma pobre em plaquetas
PRP	Plasma rico em plaquetas
PTA	Pasta triantibiótica
RJ	Rio de Janeiro

S.A.	Sociedade anônima
SCAP	Stem cell from apical papilla
SP	São Paulo
TAP	Triantibiotic paste
USA	United States of America
/	Dividido por
>	Maior
<	Menor
x	Vezes

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SUMÁRIO

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INTRODUÇÃO

INTRODUÇÃO

O advento da área de Medicina Regenerativa despertou o interesse dos endodontistas pela regeneração do complexo dentinopulpar fazendo surgir uma nova área - a Endodontia Regenerativa – voltada para a criação de tecidos que possam substituir a polpa doente, ausente ou traumatizada (1). A partir de então foram sendo desenvolvidas abordagens alternativas e biologicamente embasadas ao tratamento endodôntico convencional os quais foram denominados de procedimentos endodônticos regenerativos (PERs) (2). Ao contrário dos tratamentos regenerativos tradicionais, que utilizavam apenas materiais indutores e/ou biocompatíveis, os PERs incorporaram biofatores, células-tronco e scaffolds, mimetizando aspectos naturais da embriogênese tecidual (3).

Os PERs fundamentam-se nos princípios da engenharia tecidual, ciência que estuda o desenho e a manufatura de novos tecidos para substituição de órgãos perdidos por trauma ou doenças (4). Os três ingredientes chaves da engenharia tecidual regenerativa são uma fonte de células-tronco, uma estrutura física tridimensional adequada ao crescimento destas células e moléculas de sinalização que estimulem a proliferação e a diferenciação celular (2).

As estratégias de engenharia tecidual foram categorizadas em três classes: condutiva, indutiva e de transplante celular. A condutiva é realizada através da utilização de biomateriais, de forma passiva, facilitando o crescimento ou a regeneração de tecidos já existentes. A indutiva envolve a ativação de células próximas ao local do defeito, através de sinais biológicos específicos. Já o transplante de células utiliza células cultivadas e expandidas em laboratório e estas são levadas ao sítio desejado (5).

Os PERs podem ser aplicados em duas modalidades de engenharia de polpa: 1 – a regeneração parcial *in situ* de polpa: indicada quando parte de tecido sadio foi preservado e este intermedia a regeneração da porção perdida ou 2 - a nova síntese de polpa (*de novo synthesis*) indicada quando todo o tecido pulpar foi destruído e precisa ser regenerado em sua totalidade (3,6)

Entre os potenciais PERs dois se destacam: a revitalização via coágulo sanguíneo e o transplante de células-tronco. Historicamente, Ostby (7), em 1961, foi o primeiro a testar se o preenchimento do canal com coágulo levaria à regeneração pulpar. Em 1974, experimentos semelhantes foram conduzidos e geração de tecido conjuntivo frouxo foi observada, porém seu crescimento dentro dos canais foi de apenas 0.1 a 1.0 mm (8). 30 anos depois, Banchs e Trope (9), apresentaram um protocolo para revascularizar dentes imaturos, necróticos e portadores de periodontite apical através da desinfecção dos canais com pasta triantibiótica - ciprofloxacina, minociclina e metronidazol - proposta por Sato & Hoshino em 1996 (10,11) - e indução intracanal de coágulo. A eficiente desinfecção somada ao coágulo e ao selamento coronário efetivo, parece ter produzido o ambiente favorável para a revascularização bem sucedida demonstrada em múltiplos relatos de casos (12-14).

Pesquisas subsequentes confirmaram a eficácia antimicrobiana da pasta triantibiótica (15-16). Também foi ressaltada sua capacidade de conservar células-tronco presentes nos remanescentes da polpa ou papila apical, pós-infecção endodôntica, sugerindo sua excelente biocompatibilidade (17). Todavia, nenhum estudo avaliou a resposta tecidual à pasta triantibiótica que pode ser veiculada com propilenoglicol e polietilenoglicol (Macrogol) (9, 18-19) ou solução salina (12-14).

O coágulo, por conter fibrina, um arcabouço tridimensional, e plaquetas, ricas em fatores de crescimento, foi usado como matriz para regeneração em uma série de casos que resultaram em ausência de sintomatologia, reparo das lesões periapicais e aparente continuidade do desenvolvimento radicular (12-14). Um estudo em dentes imaturos de cães revelou superioridade dos grupos tratados quando o coágulo foi incorporado (20). O transplante de coágulo foi sugerido quando não for possível evocá-lo (21). Por último, foi demonstrado em um estudo em humanos haver um aumento na concentração de células-tronco nos canais imediatamente após indução (22). Todavia, análise histológica dos tecidos neoformados pela revascularização, revelou tecidos semelhantes ao cemento, osso ou ligamento periodontal, tratando-se de reparo e não regeneração (19, 20, 23). Portanto, o aparente aumento na espessura e comprimento radiculares pode estar relacionado à deposição de tecidos mineralizados nos canais e não à síntese de polpa e dentina (23).

Os PERs que utilizam do coágulo como estratégia para síntese da polpa de dentes imaturos não obtiveram êxito até o momento, abrindo o campo para o desenvolvimento de novos PERs que modifiquem o coágulo ou o excluam. A análise histológica de polpa de dente imaturo pós-tratamento regenerativo com plasma rico em plaquetas (PRP) sem coágulo, revelou presença de tecido semelhante ao pulpar e ausência de tecido ósseo (24,25). Entretanto, regeneração pulpar não ocorreu em dentes maduros de cães biopulpectomizados, nos quais foi injetado PRP, células-tronco da polpa dentária ou a mistura de ambos, sem indução coágulo (26).

A modificação do coágulo pelo PRP pode ser uma alternativa para potencializar a revascularização. Dentes humanos imaturos tratados via coágulo com adição de PRP

revelou radiograficamente maior aumento no comprimento e espessura das raízes comparativamente aos dentes tratados somente com coágulo (27).

O PRP, como o coágulo, contém a matriz de fibrina tridimensional (28) e fatores de crescimento de plaquetas, peptídeos que promovem proliferação, diferenciação, quimiotaxia e migração de várias células, tendo importante papel nos processos de reparo e regeneração (29). Todavia, no PRP existe maior concentração de fatores de crescimento e ausência de eritrócitos, os quais sofrem necrose logo após a formação do coágulo (2). Até o presente, nenhum estudo avaliou, histologicamente, o tipo de tecido formado com a estratégia do coágulo suplementado com PRP.

O PRP, por si só, não constitui o arcabouço ideal e sim a matéria prima para sua obtenção. Somente quando ativado ou coagulado constitui a estrutura tridimensional, a matriz de fibrina, apta a servir como scaffold (29). A mistura do PRP com trombina resultará na sua ativação, formando a solução viscosa do gel de plaquetas (gelação do PRP) e na liberação dos múltiplos fatores de crescimento contidos nos grânulos das plaquetas (29-31).

O método mais simples de transplante celular com potencial endodôntico regenerativo é a injeção intracanal de células-tronco pós-natais (1). O transplante de células, tanto de origem dentária como não dentária, para dentes maduros e vitais de cães, resultou em completa regeneração pulpar (32,33). Como a disponibilidade de tecidos pulpares autólogos declina com a idade, a busca por fontes alternativas de células-tronco mesenquimais (CTMs) para uso endodôntico deve ser incentivada (33).

Recentemente foi demonstrado que CTMs da medula óssea ou do tecido adiposo podem ser fontes alternativas para regeneração pulpar (33). O cultivo e a expansão de

células autólogas em laboratório, amplificando seu número, podem reduzir viabilidade, promover seleção indesejável ou reprogramação/ desdiferenciação celular (34), além de demandar tempo, existir maior risco de infecção e do custo elevado (35-36). Isto pode tornar o método inviável ou não competitivo com o tratamento endodôntico convencional ou implante dentário, oferecendo oportunidade única para o desenvolvimento de métodos de translação clínica, em curto prazo (37).

O uso do aspirado de medula óssea (AMO) por ser um método direto, mais rápido, com menor custo e risco de contaminação, tem sido proposto (38-39). No ambiente diverso da medula existem células multinucleares (já diferenciadas) e células mononucleares (indiferenciadas) ou células-tronco. Entre as células-tronco, ocorrem as hematopoiéticas, que dão origem a todos os tipos celulares do sangue e que são utilizadas nos tratamentos de doenças hematológicas pelo transplante de medula, e as não hematopoiéticas ou células estromais da medula, compostas pelas células-tronco mesenquimais (CTMS) e células-tronco endoteliais (38).

Recentemente foi sugerido que a utilização de aspirados totais como um coquetel de vários tipos celulares pode ser benéfico por conter “células trabalhadoras” que atuam sinergicamente promovendo a regeneração tecidual (35). Já foi demonstrado que células-tronco hematopoiéticas requerem o suporte das células estromais para diferenciação em outra linhagem (40). Esta interação pareceu beneficiar a cicatrização de defeitos ósseos preenchidos com concentrado de AMO associado a diferentes scaffolds (41). Todavia, até o presente, tal estratégia não foi avaliada em abordagens endodônticas regenerativas.

As inovações proporcionadas pela engenharia tecidual para o reparo de tecidos danificados justificam a realização de um estudo que analise os efeitos do gel de PRP, do

gel de AMO ou do gel composto PRP/AMO, como suplementos para o coágulo sanguíneo, na regeneração pulpar de dentes maduros e necróticos.

PROPOSIÇÃO

PROPOSIÇÃO:

Objetivo geral:

O objetivo geral deste trabalho foi avaliar *in vivo* diferentes Procedimentos Endodônticos Regenerativos (PERs).

Objetivos específicos:

1. Avaliar a biocompatibilidade da pasta triantibiótica que tem sido utilizada como medicação intracanal em PERs;
2. Avaliar a capacidade de diferentes PERs (Coágulo, Coágulo com gel de plasma rico em plaquetas, coágulo com gel de aspirado de medula óssea e coágulo com gel misto de plasma rico em plaquetas e aspirado de medula óssea) de regenerar o tecido pulpar em dentes de cães necróticos e completamente formados;
3. Avaliar as características histológicas do tecido neoformado após a utilização de diferentes PERs;

ARTIGO 1

Tissue Reaction to a Triantibiotic Paste Used for Endodontic Tissue Self-regeneration of Nonvital Immature Permanent Teeth

Tissue Reaction to a Triantibiotic Paste Used for Endodontic Tissue Self-regeneration of Nonvital Immature Permanent Teeth

Abstract

Introduction: The endodontic regenerative procedure (ERP), which is an alternative to calcium hydroxide-induced apexification, involves the use of a triple antibiotic paste (TAP) as a dressing material. The aim of this study was to evaluate the response of rat subcutaneous tissue to implanted polyethylene tubes that were filled with TAP or calcium hydroxide.

Methods: Thirty rats received 2 individual implants of polyethylene tubes filled with TAP or calcium hydroxide paste (CHP) and another empty tube as a control. Thirty additional rats received 2 individual implants, consisting of polyethylene tubes filled with dressing material carriers (macrogol and propylene glycol) and a sham procedure. After 7, 15, 30, 60, and 90 days, 12 animals were euthanized, and the tubes and surrounding tissue were removed and processed for histology by using glycol methacrylate and stained with hematoxylin and eosin. The histological score ranged from 0 to 3, depending on the content of inflammatory cells; the fibrous capsule was considered thin or thick, and necrosis and calcification were recorded as present or absent. The results were analyzed using the Kruskal–Wallis test.

Results: Both dressing materials induced moderate reactions at 7 and 15 days. These reactions were similar to the control ($p > 0.05$) and reduced in intensity (to mild) from day 30 onward ($p > 0.05$). The carriers did not interfere with the reaction of the dressing materials.

Conclusions: TAP and CHP were biocompatible over the different experimental periods examined.

Keywords: biocompatibility, connective tissue, triple antibiotic paste, calcium hydroxide

Introduction

Pulp necrosis is harmful for immature teeth; once tooth development ceases, viable pulp fails to develop, resulting in an open apex and thin dentin walls (1). Traditionally, endodontic treatment for immature permanent teeth with apical periodontitis includes calcium hydroxide-induced apexification (2). However, long-term use of calcium hydroxide can weaken the dentin (3). Recently, the placement of mineral trioxide-aggregate apical plugs has been proposed as an alternative treatment (4). Unfortunately, neither of these strategies can increase the root thickness and length that could be achieved by pulp regeneration into a canal, which is capable of promoting the continuation of normal root development (5).

Endodontic regenerative procedure (ERP)—an alternative clinical approach to apexification—has received great attention in recent years (6-13). This treatment protocol involves the use of a triple antibiotic paste (TAP), consisting of metronidazole, minocycline, and ciprofloxacin as a dressing, and the induction of bleeding to create a matrix for the ingrowth of new, vital tissue in the pulp canal space. Stem cells in the remaining vital pulp or apical papilla have been hypothesized to mediate tissue reconstitution (14). Many such cases have shown favorable results, including continued dentin pulp complex development shown by radiographic evidence, and no clinical symptoms (7, 15).

Several early studies documented the antimicrobial activity of TAP (16-19), but few described its biocompatibility (20). In a recent study in dogs, the pulp tissue survived in just 1 tooth after disinfection using TAP and blood clot formation (21). The authors speculated that the low rate of success could have resulted from the high concentration of TAP as a toxic material to the tissues (21)

Since the specific effect of TAP on tissues has not been examined, we evaluated the response at different time periods of rat subcutaneous tissue to implanted polyethylene tubes that were filled with TAP or calcium hydroxide paste (CHP).

Material and Methods

Sixty 4- to 6-month-old male Wistar Albino rats weighing 250–280 g were used. The animals were housed in temperature-controlled rooms and given food and water *ad libitum*. The animals were cared for as per the guidelines of the Araçatuba School of Dentistry, UNESP Ethical Committee, that approved the project before the experiments began.

Sixty 10.0-mm long polyethylene tubes with a 1.0-mm internal diameter and 1.6-mm external diameter (Abbott Lab, São Paulo, SP, Brazil) were filled with the dressing material. TAP comprised 5 parts of antibiotic mixture (equal amounts of 200 mg ciprofloxacin, 500 mg metronidazole, and 100 mg minocycline) and 1 part carrier (equal amounts of macrogol ointment and propylene glycol), with a creamy consistency, as previously described (22). CHP was prepared by mixing calcium hydroxide powder with saline. Both materials were inserted into polyethylene tubes using a lentulo spiral (Maillefer Dentsply, Tulsa, OK).

Furthermore, 30 additional polyethylene tubes were filled with equal amounts of macrogol ointment and propylene glycol, and 30 empty polyethylene tubes were used as controls.

The dorsal skins of 30 animals were shaved under anesthesia with xylazine (10 mg/kg) and ketamine (25 mg/kg) and disinfected with 5% iodine solution. A 2-cm incision was made on the shaved backs in a head-to-tail orientation, using a number 15 Bard Parker ™ blade (Franklin Lakes, NJ). The skin was reflected to create 2 pockets that were 6-cm apart on 1 side of the incision—1 in the cranial portion and 1 in the caudal portion—and an additional pocket on the opposite side of the incision. Tubes filled with TAP or CHP and the empty tubes were implanted into the spaces created by blunt dissection, and the skin was closed using 4/0 silk suture. Similarly, in the 30 additional animals, 2 pockets were created on each side of the incision, but only just 1 pocket received a tube filled with equal amounts of macrogol ointment and propylene glycol and the other was used as a sham. Sterile instruments and aseptic techniques were used throughout the experiments.

The animals were euthanized with an overdose of anesthetic solution at 7, 15, 30, 60, or 90 days after implantation, and the tubes and the surrounding tissues were removed and fixed in 10% buffered formalin at pH 7.0 (23, 24). The tubes were bisected transversely, and both halves were subsequently cut longitudinally with a sharp blade to allow the surfaces to maintain contact with the processing solutions. The specimens were embedded in glycol methacrylate, sectioned serially to 3-µm slices, and stained with hematoxylin and eosin (25).

Reactions in the tissues that were in contact with the material at the opening of the tube were scored as previously described (26): 0, no or few inflammatory cells and no

reaction; 1, less than 25 cells and mild reaction; 2, between 25 and 125 cells and moderate reaction; and 3, 125 or more cells and severe reaction. Fibrous capsules were classed as “thin” or “thick” if the thickness was <150 μm or >150 μm, respectively. Necrosis and calcification were recorded as present or absent. An average number of cells in each group was obtained from scoring 10 separate areas. The observer, who was familiar with this histological evaluation, was blinded to the treatment. The results were analyzed by Kruskal–Wallis test at a 5% significance level.

Results

On days 7 and 15, moderate chronic inflammatory cell infiltration by lymphocytes and macrophages was found in a fibrous capsule for TAP (Fig. 1 A–B), CHP (Fig. 1 F–G), and control (Fig. 1 K–L). The intensity of inflammation reduced on days 30, 60, and 90, at which point, a thin fibrous capsule that was almost void of inflammatory cells developed near the tubes for TAP (Fig. 1 C–E), CHP (Fig. 1 H–J), and control (Fig. 1 M–O). Dystrophic mineralization was only observed with calcium hydroxide.

On days 7 and 15, thin fibrous capsule formation and moderate cell inflammatory infiltration by lymphocytes and macrophages was observed with macrogol/propylene glycol (Fig. 2 A–B); however, mild inflammatory cell infiltration and a reduction in thickness of the fibrous capsule was evident from day 30 onward (Fig. 2 C–E).

Mild inflammatory cell infiltration was observed on day 7 in the sham group (Fig. 2 F), which reduced to mild infiltration with only few inflammatory cells from day 15 onward (Fig. G–J).

Comparison between the groups

The data were compared at each time point (Fig. 3). On days 7 and 15, the difference in scores between the groups (median score of 2) was insignificant ($p > 0.05$). Similarly, on days 30, 60, and 90, the difference in scores between the groups (median score of 1) was also insignificant ($p > 0.05$). An exception was the sham group, whose scores were significantly lower than the other groups at all periods ($p < 0.05$).

Discussion

Several methods have been used to evaluate the biocompatibility of endodontic materials, including subcutaneous implantation tests (23, 24). Implantation in the subcutaneous tissue of rats is one of the most appropriate tests to determine the local effects of compounds (27). In this study, empty polyethylene tubes in the control group induced few or no reactions in the subcutaneous tissue and allowed normal tissue repair, which is similar to the results of previous studies (28). The sham procedure showed mild reaction after 7 days and complete healing after 15 days, indicating no contamination and limited influence of the surgical procedures on tissue response to the materials.

A gentle treatment regimen (minimal or no instrumentation and an intracanal medication with TAP) prior to ERP may conserve any viable tissue that may remain in the canal system, harboring stem cells, i.e., SCAP in the apical papilla and DPSCs in the pulp (14). Calcium hydroxide can cause necrosis of the surrounding tissue, destroying remnant vital tissues that have the potential to differentiate into new pulp (7, 12). However, calcium hydroxide has been clinically employed as an intracanal dressing before ERP (8, 29),

because irrigation with NaOCl alone cannot render the canal free of bacteria (29), justifying its investigation in our study.

TAP and CHP induced moderate inflammatory reactions on days 7 and 15; however, this response waned, and the thickness of the fibrous capsule gradually reduced to a level similar to that of the TAP carrier and control. Our results for the CHP tissue reaction are consistent with previous results (28, 30). However, fewer studies have examined the effect of TAP (20), and the reaction of subcutaneous connective tissue to TAP at different times has not been assessed. In contrast to our results, TAP was speculated to be toxic to the tissues, once evidence of partial survival of pulp tissue was observed in 1 of 60 dogs that underwent TPA regeneration (19). Similarly, pulpal regeneration did not occur after endodontic treatment using apical negative pressure irrigation or conventional irrigation plus TAP intracanal dressing (31); the authors attributed these findings to periapical tissue irritation that was caused by the persistence of bacteria, extrusion of sodium hypochlorite, or TAP use.

Increase in root thickness and length, resembling normal maturation of the root, after treatment of immature teeth with TAP was observed in many clinical cases (8-10). A retrospective evaluation of radiographic outcomes in immature necrotic teeth also showed significant differences in root wall thickness after ERP with TAP (29); Thus, vital tissue from the pulp or apical papilla that harbored stem cells may have been present after disinfection and acted as the cellular source for healing (15, 21). Instrumentation beyond the confines of root canal, for inducing bleeding can transplant mesenchymal stem cells from bone into the canal lumen (9). However, new tissue formed in the teeth of dogs treated with ERP was considered periodontal tissue, which comprised cementum, bone, and periodontal ligament,

rather than pulp tissue (21). Therefore, further studies on the long-term outcome of new tissues in the canal space are required to explain clinical cases showing severe narrowing of the canal space.

The compounds in TAP may have positively influenced our results, as well as the results of clinical studies. Tetracyclines were shown to exert little or no cytotoxic effects, thus modulating host responses by inhibiting collagenases and matrix metalloproteinases, impeding osteoclastogenesis, and regulating angiogenesis (32,33). Furthermore, alternative systems may be controlled by tetracyclines, which could reduce the levels of elevated proinflammatory cytokines and increase the levels of IL-10, an anti-inflammatory cytokine (34). In addition, metronidazole, ciprofloxacin, and clindamycin generated viable fibroblasts in a cytotoxicity test (35).

We conclude that triantibiotic and calcium hydroxide pastes were biocompatible over the different experimental periods examined.

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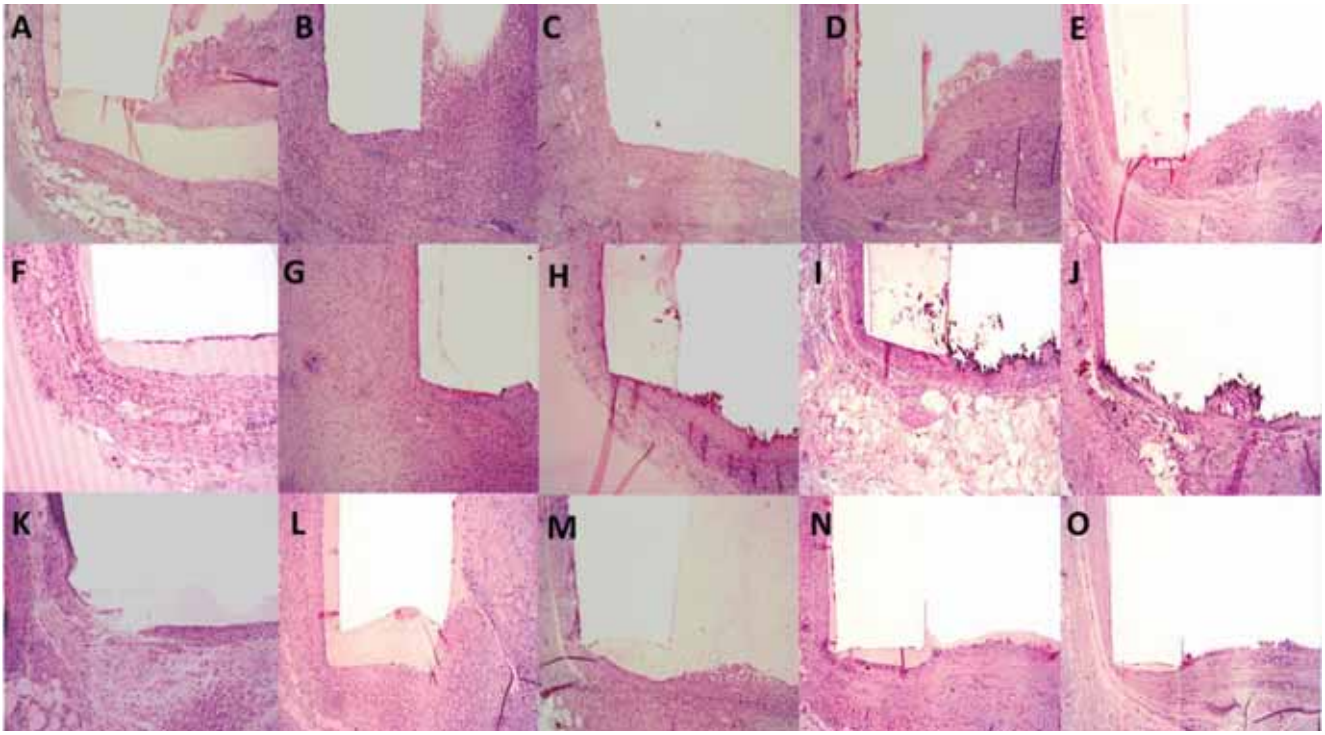
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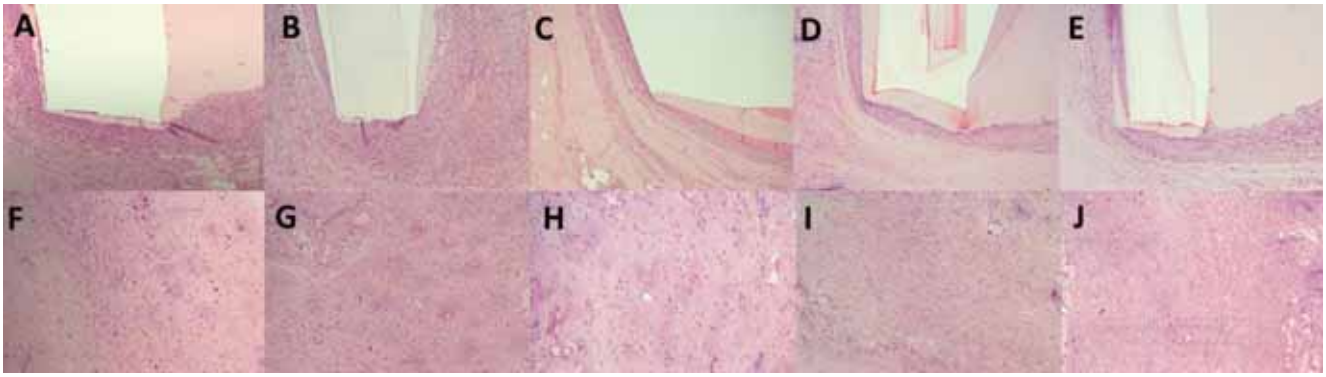
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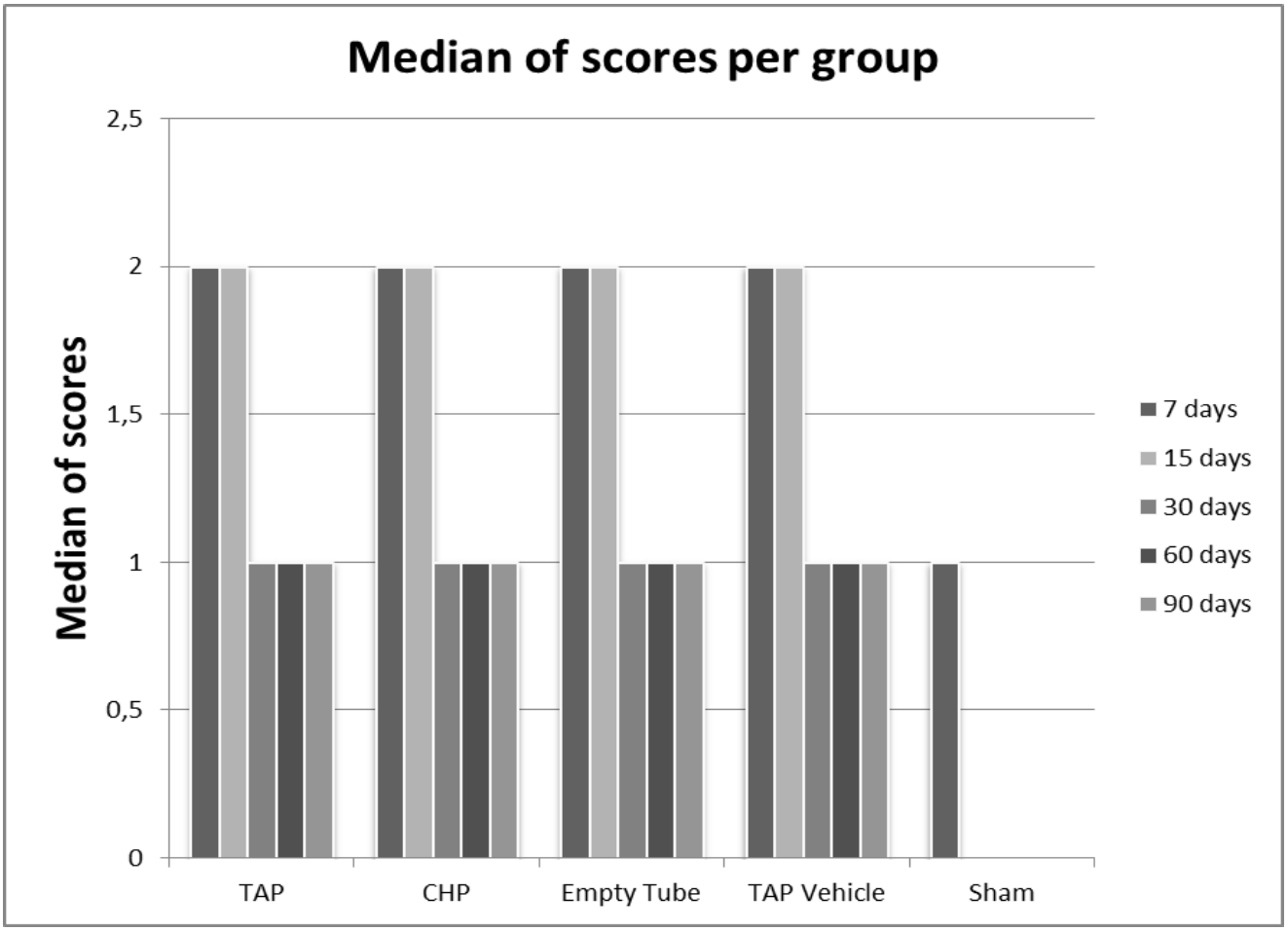
Median of scores per group
Median of scores
2,5
2
1,5
1
0,5
0
TAP
CHP
Empty Tube
TAP Vehicle
Sham
7 days
15 days
30 days
60 days
90 days

Legends

Fig. 1. Triantibiotic paste: (A–B) thick fibrous capsule formation and moderate cell inflammatory infiltration by lymphocytes and macrophages (7 and 15 days, respectively; hematoxylin-eosin staining, 10×); (C–E) mild inflammatory cell infiltration and reduction in thickness of fibrous capsule (30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×). Calcium Hydroxide: (F–G) moderate inflammatory cell infiltration and presence of a thick fibrous capsule (7 and 15 days, respectively; hematoxylin-eosin staining, 10×); (H–J) thin fibrous capsule formation, mild inflammatory cell infiltration, and dystrophic calcification in contact with the material (30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×). Control: (K–L) moderate chronic inflammatory cell infiltration with thick fibrous capsule formation (7 and 15 days, respectively; hematoxylin-eosin staining, 10×); (M–O) thin fibrous capsule surrounding the tube with few chronic inflammatory cells (30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×)

Fig. 2. Macrogol/propylene glycol: (A) thin fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (7 days; hematoxylin-eosin staining, 10×); (B–E) mild inflammatory cell infiltration and reduction in thickness of fibrous capsule (15, 30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×). Sham: (F) mild inflammatory cell infiltration (7 days; hematoxylin-eosin staining, 10×); (G–J) few inflammatory cells (15, 30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×)

Fig. 3. Median of inflammatory reaction scores per group (n=6). Observe that on days 7 and 15 the median values was not significantly different among the groups (median score of 2), except for the sham group. The same was observed on days 30, 60, and 90 (median score of 1). * statistical difference ($p<0.05\%$).

ARTIGO 2

Histologic characterization of engineered tissues in canal space of mature teeth with apical periodontitis

Histologic Characterization of engineered tissues in canal space of mature teeth with apical periodontitis

Abstract

Introduction: The conventional endodontic treatment does not restore the physiological, structural and mechanical integrity of the original dentinopulpar complex. Therefore, regeneration of pulp is considered an ideal treatment to preserve teeth. The aim of this study was to investigate the capacity of new tissue engineering strategies by a combination of induced blood clot, platelet-rich plasma (PRP) and bone marrow aspirate (BMA) to regenerate dental pulp in canine mature necrotic teeth.

Methods: Apical periodontitis was induced in 20 mature upper and lower premolars of 2 Beagle dogs. After biomechanical preparation, enlargement to a #60 file and disinfection with a triantibiotic paste for 28 days, four treatment groups were randomly assigned: blood clot alone (BC), BC with PRP gel, BC with BMA gel, and BC with BMA/PRP gel. Negative controls (untreated) were also included. The dogs were followed up for 3 months and killed. The jaws were fixed, demineralized and subjected to histological analysis.

Results: Histological analysis showed the presence of new vital tissues (connective, cement-like and bone-like tissue) in 23 of the 32 treated roots (71, 87%) with no statiscally significant difference between groups. The BC+PRP gel presented more tissue formation and a higher limit of tissue ingrowth, and in the BC with PRP/BMA gel, there was no bone tissue formation, but it was not statistically significant.

Conclusions: New vital tissues can be formed in necrotic permanent premolars teeth after disinfection and enlargement of apical foramen. The new tissues were characterized as connective, cementum-like, or bone-like tissue but not as pulp tissue.

Keywords: bone marrow aspirate, necrotic teeth, platelet-rich plasma, regenerative endodontics, tissue engineering, vital tissue growth.

Introduction

The complete restoration of the physiological, structural, and mechanical integrity of the original dentinopulpar complex is the primary goal of endodontic treatment (1). However, this goal cannot be achieved by radical endodontic treatment as it does not restore the vitality of the teeth, making them more susceptible to fractures and other complications (2). The advent of the field of regenerative medicine aroused interest in the possibility of pulpal regeneration, opening the field to create biological alternative procedures – endodontic regenerative procedures - ERP (3).

The ERP are based on the principles of tissue engineering, science that studies the design and manufacture of new tissues to replace organs lost to disease or trauma (1). The three key ingredients of regenerative tissue engineering are a source of stem cells, a physical three-dimensional structure suitable for the growth of these cells and signaling molecules that stimulate proliferation and cell differentiation (3).

Among the most used strategies are the revitalization via blood clot and cell transplantation. The induction of clot as single strategy did not promote the pulp regeneration in immature necrotic teeth (4-6) opening the field to develop new ERPs which

modify or remove the clot. The platelet-rich plasma (PRP) has been indicated in regenerative endodontics (3). A case treated with PRP showed the presence of tissue similar to pulp and lack of bone tissue (7), but no pulp synthesis was obtained by injecting PRP, dental pulp stem cells (DPSCs) or a mixture of both, inside the roots of mature teeth, without inducing a clot (8). Moreover, injection of PRP and blood clot revealed, radiographically, greater increase in root length and thickness in relation to patients treated only via blood clot strategy (9).

In the strategy of cell transplantation, injection of stem cells into the root canals seems to be the simplest option (10). The transplantation of stem cells, from dental source or not, which were cultured and expanded in laboratory, was effective for pulp synthesis (11-12). However, the cultivation and expansion have been linked to reduced viability, selection and unwanted reprogramming and / or cellular dedifferentiation (13), in addition to being time consuming, there is increased risk of infection and the high cost (14-15).

Bone marrow MSCs can be an alternative source for pulp synthesis (12). As the availability of pulp tissue decreases with age, alternative sources need to be evaluated (12). The use of bone marrow aspirate (BMA) as a straightforward and fast method, with lower cost and risk of contamination, has been proposed (16-17). The use of total aspirated as a cocktail of several cell types may be beneficial to contain “working cells” which can act synergistically promoting tissue regeneration (14). However, to date, this strategy has not been evaluated in regenerative endodontic approaches.

In this study, we aimed to histologically investigate the synergistic effects of the blood clot with PRP, BMA and PRP/BMA gels for pulp regeneration of mature and necrotic teeth, in a dog study model.

Material and Methods

Animals

Two male purpose-bred Beagles, aged 6 months were obtained from the Royal Institute (São Roque, São Paulo, Brazil). Animal care and handling was performed according to the guidelines of the Animal Research Ethics Committee of the Araçatuba School of Dentistry, São Paulo State University, Brazil, which approved the project before the beginning of the experiments. Forty roots from twenty mature second and third upper premolars and second, third and fourth lower premolars were used for this experiment.

Induction of Periapical Lesion

The animals received an intramuscular premedication composed by the association of 0,2% acepromazine (Acepran, Vetnil Ind. e Com. de Produtos Veterinários Ltda., Louveira, SP, Brazil) and morphine sulphate (Hipolabor Farmacêutica, Belo Horizonte, MG, Brazil) at doses of 0.04 mg/kg and 0.3 mg/kg, respectively followed by the passage of a catheter through the saphenous vein for conducting fluid with 0.9% NaCl (Glicolabor Indústria Farmacêutica Ltda., Ribeirão Preto, SP, Brazil). Then, the animals were induced with propofol (Propovan, Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil) 4 mg/kg associated with midazolam (Dormire, Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil) 0.3 mg/kg, both intravenously (IV), and subsequently were intubated with an endotracheal tube for delivering ventilatory support and maintenance of anesthesia with isoflurane (Isoforine, Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil).

All involved teeth were initially radiographed to confirm complete root formation and closed apices (Fig 1-A). In groups 1 to 4 after performing wear of the occlusal surfaces with a #3053 wheel type bur (KG Sorensen Ind. Com. Ltda., Barueri, São Paulo, Brazil), the teeth had their pulps mechanically exposed with a #1014 spherical diamond bur (KG Sorensen Ind. Com. Ltda., Barueri, São Paulo, Brazil) complemented with a # 3082 tapered diamond bur (KG Sorensen Ind. Com. Ltda., Barueri, São Paulo, Brazil) in a high speed handpiece under copious water cooling and nonaseptic conditions. Then, a sterile #15 K-file was used to disrupt the pulp tissue in the canals without removing it, followed by irrigation with sterile saline (0.9% NaCl, Glicolabor Indústria Farmacêutica Ltda., Ribeirão Preto, SP, Brazil). The root canals were left exposed to the oral cavity for 90 days until periapical radiolucencies were detected (Fig. 1B), indicating the development of apical periodontitis.

Root Canal Preparation and Intracanal Medication

After periapical periodontitis induction, under general and local anesthesia, all previously infected teeth were re-entered under aseptic conditions of rubber dam isolation with retractors (Ivory Company Inc., Philadelphia, USA) and surface disinfection with iodine solution (Riodeine, Indústria Farmacêutica Rioquímica Ltda., São José do Rio Preto, SP, Brazil). The canals were irrigated with 10 mL of 2,5% sodium hypochlorite (Impex, Labimpex Indústria e Comércio de Produtos para Laboratório Ltda., Diadema, SP, Brazil) and electronic odontometry was performed. The root canals were prepared until the apical barrier with Protaper rotary files # F3 (Dentsply Maillefer, Ballaigues, Switzerland) under constant irrigation with 2,5% NaOCl solution. The apical cemental barrier was penetrated with a #15 K-file to create an apical foramen-like communication and this instrument was

inserted 2mm beyond the apical barrier. This apical communication was widened to a #60 K-file. During the enlargement procedure only sterile saline was used to irrigate the canal which received a final irrigation with 10 mL of 2,5% sodium hypochlorite. The canals were dried with sterile paper points (Dentsply Ind. e Com. Ltda., Petrópolis, RJ, Brazil) and 17% ethylenediaminetetraacetic acid (EDTA) was applied in the canal space for 3 minutes. Subsequently, irrigation with 10 mL of 2,5% sodium hypochlorite and a final irrigation with 10 mL of sterile saline was performed. The canals were dried and a triple antibiotic paste (TAP), consisting of a mixture of equal parts of metronidazole, ciprofloxacin and minocycline mixed with macrogol and propylene glycol was applied to the canal spaces with a sterile lentulo spiral (Dentsply Ind. e Com. Ltda., Petrópolis, RJ, Brazil) in a slow speed handpiece. The teeth were closed temporarily with intermediate restorative material (Coltosol, Vigodent S.A. Indústria e Comércio, Bonsucesso, RJ, Brazil) and glass ionomer cement (Ketac™ Molar Easymix, 3M ESPE, Seefeld, Germany) for 4 weeks to allow disinfection of the root canals.

Preparation of platelet-rich plasma gel (PRP gel)

Using a disposable 10 mL syringe, containing 2 mL of CPDA-1, it was collected 8 mL of peripheral blood via the jugular vein, 5 ml of which was intended for preparation of PRP and other 5mL for the preparation of autologous thrombin. Using a refrigerated laboratory centrifuge (Hermle Z323K, Hermle Labortechnik GmbH, Wehingen, Germany), the tubes were initially centrifuged at 300 G for 10 minutes at a temperature of 22 ° C, resulting in three basic components: the upper (plasma), the intermediate portion (leukocytes) and lower portion (red cells). Then, the supernatant was pipetted over the whole intermediate

portion and 2 mm of the red blood cells layer, transferring the contents to another vacuum tube, to a second centrifugation at 640 G for 10 minutes. This second centrifugation resulted in two components: the platelet-poor plasma (PPP) located in the upper part of the tube and which was discarded, and the PRP (Fig. 1D) located in the bottom of the tube and whose final volume was 0.5 mL. A PRP Gel was prepared by activating the PRP with autologous thrombin in a 2:1 ratio.

Bone marrow aspirate gel (BMA gel)

After anesthetizing the animals, trichotomy and antisepsis of puncture area with 10% povidone-iodine solution was performed. Then, 3 mL of bone marrow aspirate (Fig. 1E) was collected from the iliac crest using a special needle for bone marrow aspiration (Komiyashiki, Japan) coupled to a 20 mL polyethylene syringe containing 1,5 mL of anticoagulant citrate-phosphate-dextrose-adenine - CPDA-1 (JP Indústria Farmacêutica S. A., Ribeirão Preto, São Paulo, Brazil). A BMA gel was prepared by activating the bone marrow aspirate with autologous thrombin in a 2:1 ratio

Preparation of autologous thrombin

For the preparation of autologous thrombin, the blood sample (5mL) was first centrifuged at 300 G for 10 minutes. Then, 250 µL was aspirated from the top layer (plasma) and transferred to another 5 mL vacutainer tube. To the plasma it was added 75µL of 10% calcium gluconate and the content was incubated in a water bath at 37°C for 15 minutes. After bathing, the sample was centrifuged at 640 G for 10 minutes and the resulting supernatant, the autologous thrombin, was ready for use.

Tissue Engineering Procedures

After disinfection, under the same conditions of asepsis and general and local anesthesia, the temporary restorations were removed from the experimental teeth. The triple antibiotic paste was removed from the canals with sterile saline, and then, 17% ethylenediaminetetraacetic acid (EDTA) was applied for 3 minutes in the canal space. Subsequently, the canals were dried with sterile paper points, and the experimental treatment groups were randomly assigned as follows: group 1 (Blood Clot, n= 8 roots), a sterile # 30 K-file was inserted 2mm past the canal terminus into the periapical tissues to induce bleeding to fill the canal space allowing the formation of a blood clot; group 2 (Blood Clot + PRP Gel, n = 8 roots), bleeding was evoked as described above and 20 µL of PRP Gel (PRP activated with autologous thrombin in a 2:1 ratio) was injected inside the roots using a flexible tip (Capillary Tips, Ultradent Products Inc., South Jordan, Utah, USA), coupled to a pipette, waiting for a gelation time of 2 minutes (Fig. 1C-D); group 3 (Blood Clot + Bone Marrow Aspirate Gel, n = 8 roots), after bleeding was induced in the canals, 20 µL of bone marrow aspirate, activated with autologous thrombin in a 2:1 ratio, was inserted into each root canal waiting for a gelation time of 2 minutes; group 4 (Blood Clot + PRP/BMA Gel, n = 8 roots), the bleeding was evoked, and 20 µL of PRP associated to BMA, activated with thrombin in a 2:2:1 ratio, was injected in the root canals waiting for a gelation time of 1 minute. All of these teeth were then closed with a triple coronal seal of white MTA, calcium hydroxide cement (Hidro C, Dentsply Indústria e Comércio Ltda., Petrópolis, RJ, Brazil) and composite (Dentsply Indústria e Comércio Ltda., Petrópolis, RJ, Brazil). In group 5 (negative controls, n = 4 teeth), the teeth were left untouched for comparison with the experimental teeth. All of the teeth were monitored radiographically after the treatment on a monthly basis

for 3 months before the animals were killed, and tissues were harvested for histologic processing.

Tissue preparation and Histological Processing

The animals were killed under general anesthesia provided by propofol (Propovan, Cristália, Produtos Químicos Farmacêuticos Ltda., Itapira, SP, Brazil) at 5mg/kg intravenously. The carotid arteries were exposed and cannulated and the animals were perfused with 4% buffered formalin. The jaws with the involved teeth were dissected and fixed in 4% formaldehyde for 48 hours, demineralized in 10% EDTA, dehydrated through ascending gradations of ethanol, embedded in paraffin, and sectioned on a Leica RM 2165 (Leica Microsystems, Wetzlar, Germany) microtome. Five-micrometer serial sections were longitudinally cut through the roots and stained with hematoxylin-eosin (HE), picrosirius red, and Brown &Brenn for disclosure of bacteria.

Histological Analysis

Each individual root was taken as an independent sample unit for the histological analysis which was performed under light microscope (Leica DM 4000 B, Leica Microsystems CMS GmbH, Germany) coupled to a Leica DFC 500 (Leica Microsystems CH-9435, Heerbrugg, Germany) camera, by two examiners blinded to the experimental groups. A detailed description of the histological characteristics of the neoformed intracanal tissues was performed. In addition, the intracanal and periapical tissues were analyzed in each section for determination of scores according to the histopathological parameters described in Table I.

Data Analysis

Statistical analysis of the scores for the histopathologic parameters was performed using the non-parametric Kruskal-Walis test followed by Dunn's test with the level of significance set at $P<0.05$.

Results

Neoformed intracanal tissues (NIT) occurred in 71.87% (23/32) of specimens subjected to various tissue engineering strategies, having occurred in 6 of 8 specimens in group BC; in 7 of 8 specimens in Group PRP; in 5 of 8 specimens in Group BMA, and in 5 of 8 specimens in Group PRP/BMA. The analysis of categorical data revealed no statistically significant difference between groups ($P=0.65$) (Fig. 1*F*). No tissue or just debris from the TAP was observed in 9 specimens where no tissue formation was found. The formed tissue types were: neoformed connective tissue (NCT), which occurred in 23 of the 23 specimens with newly formed tissues, cementum-like tissue (NC), which occurred in 23 of 23 specimens, and bone-like tissue (NB), which occurred in only 8 of the 23 specimens (Fig. 1*G*-*H*).

The three tissue types did not differ histologically in the different experimental groups. Considering the limit reached by the NIT, it was observed that in 8 specimens it reached the cervical third of the root (none in the BC group, 5 in the PRP group, 2 in the BMA group, and 1 in the PRP / BMA group), in 11 specimens it reached the middle third of the root (3 in the BC group, 2 in the PRP group, 2 in the BMA group, and 4 in the PRP / BMA group), and in 4 specimens it reached only the apical third of the root (3 in the BC group, 1 in the

BMA group and none in the PRP and PRP/BMA groups). There was no statistically significant difference between groups regarding the extent of newly formed tissue (P = 0.06) (Fig. 1*F*).

Histological appearance of the newly formed intracanal tissues (NIT)

Neoformed connective tissue (NCT)

This tissue occurred in all teeth with vital tissues and ranged from loose to moderately dense (Figs. 3*A*, 3*D*, 3*G*, 3*J*). In its composition fibroblast-like cells, stellate or spindle-like in appearance, were evenly distributed throughout the tissue. The extracellular matrix was comprised of thick collagen fibers, thin collagen fibers, and ground substance whose amount varied depending on the specimen. Some collagen fibers were inserted into the mineralized tissues (NC and / or NB) (Figs. 3*C*, 3*F*, 3*I*, 3*M*). An amount of blood vessels ranging from low to moderate occupied particularly the central portion of the NCT. Rare nerve bundles were found in this tissue.

Cementum-like tissue (NC).

This tissue occurred in all specimens with vital tissues and proved as a mineralized avascular structure, whose surface appeared covered with fibroblast-like and/or cementoblast-like cells with rare cementocytes-like cells embedded inside (Figs . 3*B-C*, 3*E-F*, 3*H-I*, 3*LM*). This tissue has been deposited as a layer juxtaposed to dentin along the inner walls of the channel, and in continuity with the extracanal cement. This tissue had a variable thickness, which generally diminished towards apex-crow. Hair-like projections were observed in the specimens when this tissue came off the dentin due to histological

processing (Figs. 3*B*, 3*E*, 3*L*). These projections seemed to be largely responsible for the connection between the cementum and dentin.

Bone-like tissue (NB)

This tissue occurred in 8 of the 23 revitalized roots (3 in the BC group, 4 in the PRP group, and 1 in the BMA group). However, no specimen in the PRP/BMA group had bony-like structures. In general, it appeared as small isolated islands within the NCT or adhered to the NC. In some specimens this tissue circumscribed medullary spaces. Its surface was covered with osteoblast-like cells, and osteocyte-like cells were uniformly distributed inside (Figs. 3*A*, 3*E-F*, 3*H-I*). In some specimens the NB was shown adhered to the NC.

Histological appearance of the intracanal tissues in the different experimental groups:

Group BC (Blood clot)

In the specimens in which there was the formation of the NCT, it occupied less than half of the root canal length and consisted of a connective tissue that ranged from loose to moderately dense *(Figs. 3A-C)*. A number of blood vessels ranging from low to moderate occupied particularly its central portion (Figs. *3A-B*). The presence of nerve bundles was not observed. Rare inflammatory cells were present in the NCT and there were no multinucleated giant cells. A layer of cementum-like tissue (Fig. 3*B*) was deposited on the root canal walls in 100% (6/6) of the specimens where the NCT occurred. Islands of bone-like tissue (Fig. 3*A-B*) of variable length, alone or in combination with NC were observed

within the NCT in 50% of the specimens (3/6). Discrete amount of TAP dispersed in the NCT was observed in 50% (3/6) of the specimens.

Group PRP (Platelet-rich plasma gel)

In the specimens in which there was the formation of the NCT, it reached the cervical third of the root in most specimens and was characterized by a connective tissue which ranged from loose to moderately dense (Figs. 3*D*-*F*). An amount of blood vessels ranging from low to high was observed in the central portion of NCT (Figs. 3*D*-3*E*). Rare nerve bundles were found in some specimens of this group. Few inflammatory cells were present in the NCT. The deposition of a layer of cementum-like tissue (Figs. 3*D*-3*E*) along the dentin walls occurred in all specimens where tissue neoformation occurred (7/7) while islands of bone-like tissue (Fig. 3*E*) occurred around (4/7) specimens. Areas of dentin resorption were not observed. Slight to moderate amount of TAP was dispersed in (2/7) of the specimens.

Group BMA (bone marrow aspirate gel)

In the specimens in which there was NCT presence, in most specimens it exceeded the middle third of the roots and consisted of a connective tissue which ranged from loose to moderately dense (Figs. *3G*-*I*). A low to moderate amount of blood vessels could be observed in the central portion of the NCT (Figs. 3*G*, 3*H*). Nerve bundles were observed in a single specimen of this group. Few inflammatory cells were present but no multinucleated giant cells could be observed. As in the other groups there was a cementum-like tissue deposition (Fig. 3*H*) in all specimens with vital tissues. Bone-like tissue (Fig. *3H*) occurred in only one specimen (1/5) in this group. Few areas of dentin resorption, mostly repaired, were

observed in this group. Discrete to large amount of TPA was observed in the NCT in (2/5) of the roots.

Group PRP / BMA (Platelet-rich plasma / bone marrow aspirate gel)

In the specimens in which there was NCT formation, in most specimens it exceeded the middle third of the roots and consisted of a connective tissue which ranged from loose to moderately dense (Fig. 3*J*-*M*). A number of blood vessels ranging from low to moderate (Fig. 3*J*-3*L*) could be observed. Few nerve bundles were observed in the specimens of this group. Rare inflammatory cells were present in the NCT and a slight amount of multinucleated giant cells was observed in a single specimen of the group. Deposition of cementum-like tissue (Fig. 3*J*-3*L*) was observed in all specimens with vital tissues. No bone-like tissue formation could be observed in the specimens of this group. Absence or just repaired resorption areas were observed in dentin. Slight to moderate amount of TPA was observed in the NCT (3/5) of the specimens.

Discussion

In the present study, the tissue engineering strategies (Groups 1-4), associated with tooth disinfection with a triantibiotic paste, resulted in the formation of vital tissues at about 71.87% (23/32) of previously infected roots. These results are important in demonstrating that the protocol adopted seems to have decontaminated the root canal systems and allowed the tissue ingrowth, confirming the biocompatibility of TAP, as demonstrated in our previous study (18), and its potent antimicrobial activity (19-20).

Our treatments produced mainly three types of tissues: neoformed connective tissue (NCT), cementum-like tissue (NC) and bone-like tissue (NB), confirming results of previous studies (4-6). The induction of bleeding and subsequent clot formation delivers immediately inside the root canals, the three-dimensional fibrin scaffold and mesenchymal stem cells present in periapical tissues nearby (21). Platelets, which are a big part of the clot, secrete various active growth factors, thus composing the triad for tissue engineering (22).

In the absence of pulp tissue, the source of stem cells responsible for the regeneration of mineralized tissues has not been fully clarified (6). They may be derived from peripheral blood (23), periodontal ligament (24), bone marrow (25), and even from granulation tissue (26) or periapical lesions (27). Molecular analysis of samples collected from the clot within the canal indicated a significant accumulation of stem cell markers CD73 and CD105 on 600x compared to the levels found in the bloodstream (21).

The mechanical irritation of the periapical tissue releases substantial amount of stem cells from their niches. Stem cells of the periodontal ligament (PDLSC) can play a significant role in the synthesis of newly formed tissues (5-6). The potential of PDLSC to differentiate into specific cell lineages producing cementum and / PDL-like structures was observed (24). As the cement is formed primarily from the periodontal ligament, this could explain the cement-like tissue deposition on dentin. The islands of bone tissue may have been formed from stem cells in the bone marrow in the periapical region (6).

We verified that maturation of newly formed tissues appeared incomplete by the presence of loose connective tissue that can be attributed to the experimental period of only

90 days. It was suggested that in the long term, these tissues are more organized and mature (5) and its deposition over time may lead to complete obliteration of the canal (6). Therefore, induction of endogenous mineralized structures within the canal from adjacent cells and tissues should be further evaluated.

The significance of the clot has been described in several studies (4, 6, 21, 28). In molars treated with PER in which induction of a blood clot was not feasible, root development was inferior to those with successful induction, leading the authors to recommend clot transplantation from one root to another (28). In a canine model, type I collagen sponge mixed with blood clot significantly increased the formation of mineralized tissues in relation to clot and sponge alone (6). So despite the clot alone does not provide the ideal conditions for pulp regeneration, its association with other strategies may be beneficial.

The platelet-rich plasma (PRP) has been suggested as a potential scaffold for the PERs, for forming a three-dimensional fibrin matrix and contains a high concentration of growth factors (3). However, there is little evidence about the use of PRP in PERs (7). Pulpal regeneration did not occur in vital mature in which was injected only PRP or PRP mixed with DPSCs (8). Moreover, the modification of the PRP by the blood clot resulted in an increase in the length and thickness of the roots, compared with blood clot alone (9). In our study, although with no significant difference, the association of blood clot with PRP resulted in the highest number of roots with vital tissues and in greater tissue growth among all groups. The association of clot with BMA and PRP/BMA also resulted in greater tissue invagination when compared to the clot alone, although this was not statistically significant.

In our study, the use of PRP or PRP / BMA, both associated with the clot, have not led to pulpal regeneration, confirming the results of Zhu et al (8) which failed in obtaining pulp synthesis through injection of PRP or PRP + DPSCs without inducing a clot. These are evidences that PRP may not promote pulp regeneration. On the other hand, when dental pulp $CD105^{+}$ stem cells were fractionated with the stromal cells derived factor 1 (SDF-1), in a collagen scaffold, complete pulp regeneration was achieved (11). When dental pulp, bone marrow or adipose tissue $CD31^{-}$ cells were delivered in a collagen scaffold associated with SDF-1, it also resulted in pulp synthesis (12).

In teeth with closed apex, the lowest blood supply may be a limitation for tissue engineering (3). It has been suggested that for avulsed teeth, the apices must present an apical opening of at least 1.0 mm so that the revascularization occurs after reimplantation (29). In our study, we performed the enlargement of the root canals only to 0.6 mm in diameter which did not limit the tissue formation and consisted the first evidence of revitalization of mature and necrotic teeth. This is consistent with other studies which obtained pulp regeneration by apical foramen enlargement up to 0.7 mm (11-12).

In our study, the absence of pulp regeneration can be related to the lack of stem cells derived from dental pulp. However, pulp synthesis was obtained from $CD105^{+}$ stem cells derived from adipose tissue (11) or a subpopulation of $CD31^{-}$ stem cells CD31-derived from bone marrow or adipose tissue (12), when collagen and the growth factor SDF-1 were included. Alternatively, we used the total bone marrow aspirate as a pool of cells from various lineages and/or PRP as a scaffold and source of growth factors for these cells with no success. Similarly, pulp synthesis was not showed when PRP or PRP + DPSCs were injected in the canals (8). Therefore, the success reached in other studies may not be

primarily related to the cell type, but to the fractionation of these cells and the appropriate growth factor and scaffold (8). This demonstrates the dynamic interactions between the elements of the triad of tissue engineering scaffold-cell-growth factor, which need to be better understood.

In the present study we also used EDTA as an adjunct to pulp regeneration. EDTA can promote the release of growth factors embedded inside dentin during dentinogenesis (30). EDTA may have influenced the increased formation of mineralized tissues, but its application has not promoted odontoblast differentiation, which coincides with previous data (6). Moreover, stem cells from the periodontal ligament (PDLSCs) or periapical region may be more likely to differentiate into cells similar to cementoblasts and / or osteoblasts when exposed to dentin growth factors (6).

New vital tissues can be formed in necrotic permanent premolars teeth after disinfection and enlargement of apical foramen. The new tissues were characterized as connective, cementum-like, or bone-like tissue but not as pulp tissue.

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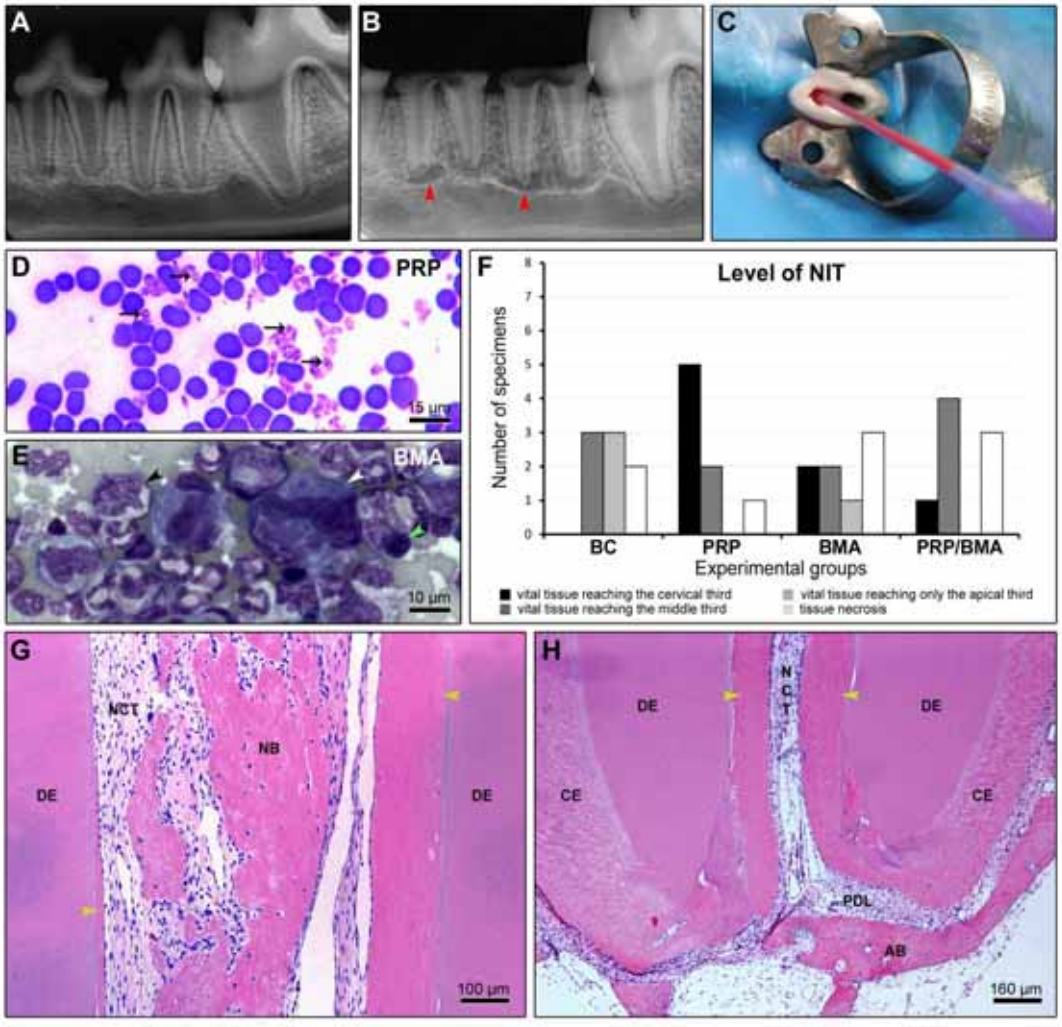
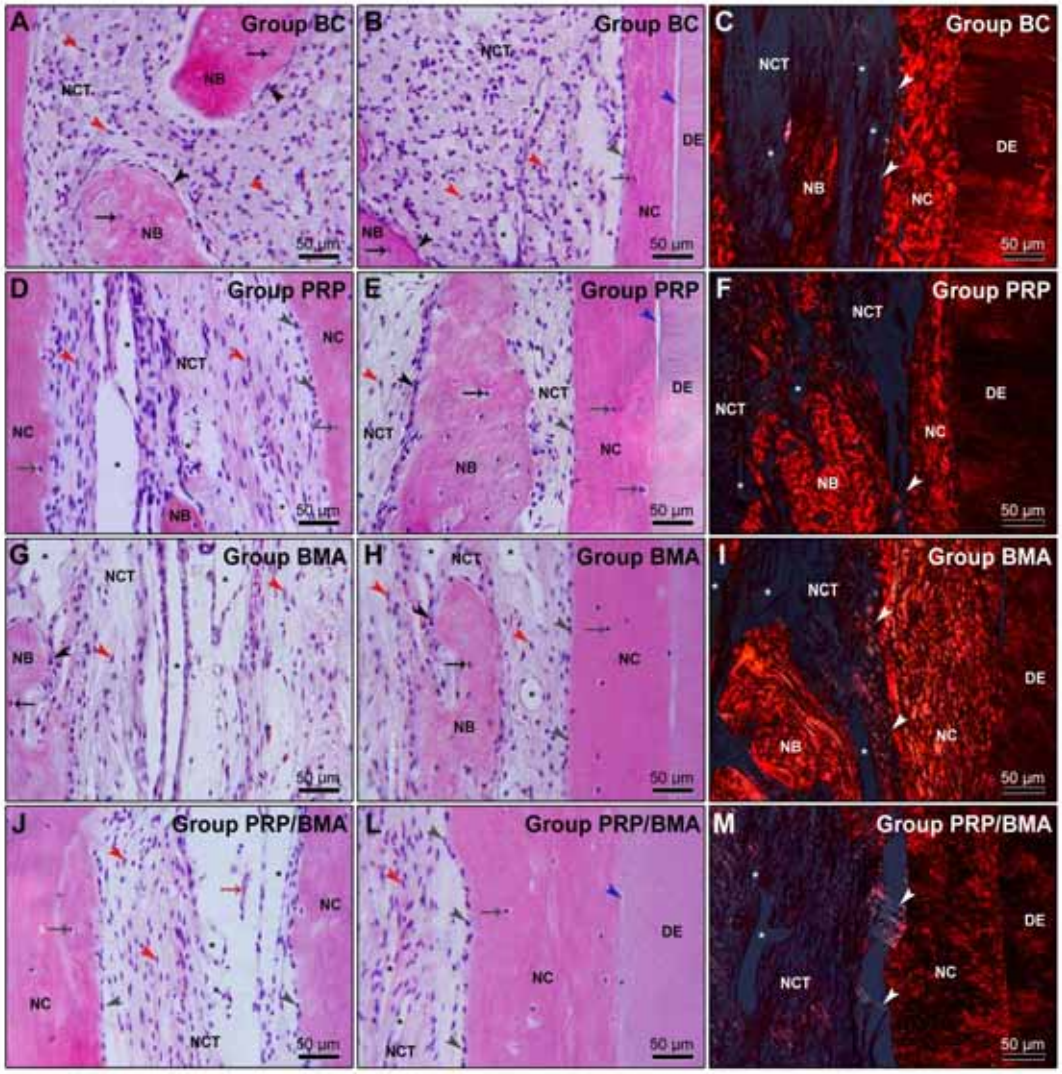

A
B
C
D
PRP
15 µm
E
BMA
10 µm
F
Level of NIT
Number of specimens
0
1
2
3
4
5
6
7
8
BC
PRP
BMA
PRP/BMA
Experimental groups
vital tissue reaching the cervical third
vital tissue reaching the middle third
vital tissue reaching only the apical third
tissue necrosis
G
NCT
NB
DE
DE
100 µm
H
DE
NCT
DE
CE
CE
PDL
AB
160 µm


A
Group BC
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Group PRP/BMA
DE
NCT
NC
50 μm

EVALUATED HISTOLOGIC PARAMETERS

NEOFORMED INTRACANAL TISSUES (NIT)

Scores for each parameter	Experimental groups			
	BC (n=8)	PRP (n=8)	BMA (n=8)	PRP BMA (n=8)
LIMITS OF THE NIT				
1 – reached the cervical third	0	5	2	1
2 – reached the middle third	3	2	2	4
3 – reached the apical third	3	0	1	0
4 – tissue necrosis	2	1	3	3
P=0.06	a	a	a	a
NEOFORMED CONNECTIVE TISSUE (NCT)				
1 – presence	6	7	5	5
4 – absence	2	1	3	3
P=0.65	a	a	a	a
NCT CELLULARITY PATTERN				
1 – large number of fibroblast-like cells	1	2	2	1
2 – moderate number of fibroblast-like cells	3	3	1	2
3 – discrete number of fibroblast-like cells	2	2	2	2
4 – tissue necrosis	2	1	3	3
P=0.69	a	a	a	a
NCT VASCULARIZATION PATTERN				
1 – high blood vessels density	0	1	0	0
2 – moderate blood vessels density	4	4	3	1
3 – low blood vessels density	2	2	2	4
4 – tissue necrosis	2	1	3	3
P=0.24	a	a	a	a
NCT INNERVATION PATTERN				
1 – moderate nervous bundles density	0	0	0	0
2 – low nervous bundles density	0	3	1	1
3 – absence of nervous bundles	6	4	4	4
4 – tissue necrosis	2	1	3	3
P=0.35	a	a	a	a
INTENSITY OF NCT INFLAMMATORY INFILTRATE				
1 – negligible number of inflammatory cells	6	6	5	5
2 – moderate number of inflammatory cells	0	1	0	0
3 – large number of inflammatory cells	0	0	0	0
4 – tissue necrosis	2	1	3	3
P=0.85	a	a	a	a
EXTENSION OF NCT INFLAMMATORY INFILTRATE				
1 – absence of inflammation	6	4	5	4
2 – extending up for about 1/3 of the NCT	0	3	0	1
3 – extending up for about 2/3 of the NCT	0	0	0	0
4 – tissue necrosis	2	1	3	3
P=0.85				
PRESENCE OF GIANT CELLS IN THE NCT				
1 – absent	6	7	5	4
2 – discrete: < 3 cells	0	0	0	1
3 – moderate to high: > 3 cells	0	0	0	0
4 – tissue necrosis	2	1	3	3
P=0.48	a	a	a	a
CEMENTUM-LIKE TISSUE (NC)				
1 – presence	6	7	5	5
4 – absence	2	1	3	3
P=0.65	a	a	a	a
BONE-LIKE TISSUE (NB)				
1 – presence	3	4	1	0
4 – absence	5	4	7	8
P=0.09	a	a	a	a
DENTIN RESORPTION				
1 – absence of resorption areas	7	7	4	6
2 – only repaired resorption areas	0	1	3	2
3 – few resorption areas	0	0	0	0
4 – many resorption areas	1	0	1	0
P=0.29	a	a	a	a
TRIANTIBIOTIC PASTE IN THE CANAL SPACE				
1 – absent	2	6	2	2
2 – discrete amount of paste	2	0	3	0
3 – moderate amount of paste	4	2	2	3
4 – large amount of paste	0	0	1	3
P=0.10	a	a	a	a
DEBRIS FROM BIOMECHANICAL PREPARATION				
1 – absent	7	7	5	5
2 – small amount of debris	1	1	3	3
3 – moderate amount of debris	0	0	0	0
4 – large amount of debris	0	0	0	0
P=0.46	a	a	a	a

Scores for each parameter	Experimental groups			
	BC (n=8)	PRP (n=8)	BMA (n=8)	PRP BMA (n=8)
PRESENCE OF BACTERIA				
1 – absence	4	7	5	5
4 – presence	4	1	3	3
P=0.47	a	a	a	a

PERIAPICAL TISSUES

Scores for each parameter	Experimental groups			
	BC (n=8)	PRP (n=8)	BMA (n=8)	PRP BMA (n=8)
INTENSITY OF THE INFLAMMATORY INFILTRATE				
1 – absence of inflammatory cells	3	5	3	1
2 – small number of inflammatory cells	3	3	2	6
3 – moderate number of inflammatory cells	2	0	3	1
4 – large number of inflammatory cells	0	0	0	0
P=0.27	a	a	a	a
PRESENCE OF GIANT CELLS				
1 – absent	6	8	5	3
2 – discrete	2	0	3	5
3 – moderate	0	0	0	0
4 – severe	0	0	0	0
P=0.06	a	a	a	a
EXTENSION OF THE INFLAMMATORY INFILTRATE				
1 – absent	2	4	2	0
2 – within the limits of the PDL	3	3	2	3
3 – beyond the limits of the PDL	1	1	3	4
4 – extending to bone and causing bone resorption	2	0	1	1
P=0.13	a	a	a	a
APICAL PDL THICKNESS				
1 – unchanged	4	5	3	2
2 – slightly increased	1	2	1	2
3 – moderately increased	2	1	3	3
4 – severely increased	1	0	1	1
P=0.37	a	a	a	a
APICAL PDL FUNCTIONALITY				
1 – cementum to bone insertion in all apical 1/3	4	3	2	1
2 – cementum to bone insertion in part of apical 1/3	0	1	1	2
3 – run parallel to tooth surface	2	3	2	1
4 – absence of organization	2	1	3	4
P=0.49	a	a	a	a
APICAL CEMENTUM RESORPTIONS				
1 – absent	6	6	4	7
2 – inactive resorption areas	2	2	4	1
3 – few active resorption areas	0	0	0	0
4 – many active resorption areas	0	0	0	0
P=0.42	a	a	a	a
REPAIR OF RESORPTION AREAS				
1 – nonexistent or totally repaired	8	8	5	8
2 – about 2/3 of the areas repaired	0	0	1	0
3 – less than 1/2 of the areas repaired	0	0	1	0
4 – non repaired	0	0	1	0
P=0.02	a	a	b	a
BONE RESORPTIONS				
1 – absent	6	4	3	2
2 – only inactive areas	1	4	3	6
3 – few active areas	1	0	2	0
4 – many active areas	0	0	0	0
P=0.36	a	a	a	a
TRIANTIBIOTIC PASTE IN THE PERIAPEX				
1 – absent	4	5	3	2
2 – small amount of paste debris	2	0	1	0
3 – moderate amount of paste	1	1	0	1
4 – large amount of paste debris	1	2	4	5
P=0.26	a	a	a	a
DEBRIS FROM BIOMECHANICAL PREPARATION				
1 – absent	8	8	8	8
2 – small amount of debris	0	0	0	0
3 – moderate amount of debris	0	0	0	0
4 – large amount of debris	0	0	0	0
P=1.00	a	a	a	a

Legends

Figure 01: (A) Periapical radiograph showing the radiographic appearance of normality of periapical dental tissues in the preoperative period. (B) Periapical radiograph showing the appearance and size of periapical lesion (red arrowheads) formed after 90 days of pulp exposure. (C) Demonstration of the clinical procedure of delivery of PRP and / or BMA in the root canal. (D) PRP smear indicating a large amount of platelets (black arrows) and erythrocytes. (E) BMA smear showing erythroid lineage cells (black arrowhead), myeloid lineage cells (green arrowhead) and megakaryocytes (white arrowhead). (F) Graph showing the level of newly formed intracanal tissues (NIT) or the occurrence of necrosis in the different experimental groups. (G) Photomicrographs of (NIT) at the middle third, where can be seen the neoformed connective tissue (NCT), the cementum-like tissue (NC) (yellow arrowheads) and bone-like tissue (NB). (H) Photomicrographs of the NIT at the apical level highlighting the newly formed connective tissue (NCT), continuous with the periodontal ligament (PDL) and cementum-like tissue (yellow arrowheads), in continuity with the extracanal cementum (CE). Abbreviations and symbols: AB, alveolar bone, CE, extracanal cementum, DE, dentin, BC, a group in which the root canal was filled only with blood clot; PRP group where the root canal was filled with blood clot and platelet rich plasma gel; BMA, group where the root canal was filled with blood clot and bone marrow aspirate gel; Group PRP / BMA, group where the root canal was filled with a mixture of blood clot and a pool of platelet-rich plasma and bone marrow aspirate, NB, tissue similar to bone, NC, cementum-like tissue; NCT, neoformed connective tissue; NIT, newly formed intracanal tissues; PDL, periodontal ligament, yellow arrowheads, cementum-like tissue; white arrowhead , megakaryocyte, black arrowhead, erythroid cell lineage; green arrowhead, cell of the myeloid lineage, red arrowheads, apical lesions, black arrows, platelets. Staining: in D-E,"Panótico" staining, in G-H, hematoxylin and eosin. Scale bars: in D, 15 µm; in E, 10 µm; in G, 100 µm; in H, 160 µm.

Figure 03: Histological appearance of tissue neoformed intracanal tissues (NIT) in groups BC (A-C), PRP (D-F), BMA (G-I) and PRP / BMA (J-M). (A, D, G and J) Photomicrographs showing the newly formed connective tissue (NCT) composed of a large number of fibroblast-like cells (red arrowheads), a moderate amount of collagen fibers and many blood vessels (asterisks). Adjacent to the NCT, it can be observed the presence of a cementum-like tissue (NC), and in some specimens the presence of islands of a bone-like tissue (NB). (B, E, H and L) Photomicrographs showing the NC, with cells similar to cementocytes (gray arrows) in its interior, and cells similar to cementoblasts (grey arrowheads) on their surface. In the specimens in which there is the presence of NB islands, these islands are present filled with osteocyte-like (black arrows) and osteoblast-like cells (black arrowheads), which are located inside and on the surface of the matrix bone, respectively. At the junction of dentin (DE) with NC it can be observed in some specimens hair-like projections (blue arrowheads). (C, F, I and F) Photomicrographs showing the NCT, NB, and NC observed in polarized light microscopy. It can be observed the NCT collagen fibers anchored in NC (Sharpey's fibers) (white arrowheads). Abbreviations and symbols: DE, dentin; Group BC, group where the root canal was filled only with the blood clot; Group PRP, group where the root canal was filled with blood clot and platelet-rich plasma gel; Group BMA, group where the root canal was filled with blood clot and bone marrow aspirate gel; Group PRP / BMA, group where the root canal was filled with blood clot and a pool of platelet-rich plasma and bone marrow aspirate gel; NB bone-like tissue; NC, cementum-like tissue; NCT, neoformed connective tissue; NIT, newly formed intracanal tissues; blue arrowheads, hair-like Projections; white arrowheads, Sharpey's fibers; gray arrowheads, cementoblast-like cells; black arrowheads, osteoblast-like cells, red arrowheads, fibroblast-like cells; gray arrows, cementocyte-like cells; black arrows, osteocyte-like cells; red arrow, nerve fiber bundle; asterisks, blood vessels. Staining: A, B, D, E, G, H, J and L, Hematoxylin and Eosin; In C, F, I, M, Pricrosirus red viewed in polarized light microscopy. Scale bars: 50 microns.

CONCLUSÃO

CONCLUSÃO:

1. A pasta triantibiótica que tem sido utilizada como medicação intracanal em PERs foi biocompatível;
2. Nenhum dos PERs utilizados foi capaz de regenerar o tecido pulpar em dentes com polpa necrótica de dentes de cães completamente formados;
3. Os tecidos neoformados foram caracterizados como tecido conjuntivo, semelhante ao cemento ou semelhante ao osso;

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ANEXO 1

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL
(CEEA)

CERTIFICADO

Certificamos que o Projeto ***"ANÁLISE HISTOPATOLÓGICA DA BIOCOMPATIBILIDADE DA PÁSTA TRI-ANTIBIÓTICA À BASE DE CIPROFLOXACINA, MINOCICLINA E METRONIDAZOL EM TECIDO SUBCUTÂNEO DE RATOS"*** sob responsabilidade do ***Prof. Dr. João Eduardo Gomes Filho*** está de acordo com os Princípios Éticos da Experimentação Animal (COBEA) e foi aprovado pela CEEA número de protocolo número 002315-2010.

Araçatuba, 24 de Março de 2010

Prof.ª Adj. Tereza Cristina Cardoso da Silva
Presidente da CEEA- FOA/UNESP

Faculdade de Odontologia e Curso de Medicina Veterinária – Departamento de Apoio , Produção e Saúde Animal. Rua Clóvis Pestana, 793 CEP 16050-680 Araçatuba – SP
Tel (18) 3636- 1350 Fax (18) 3636- 1352 E-mail: dapsa@fmva.unesp.br

ANEXO 2

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

CERTIFICADO

Certificamos que o Projeto ***"EFEITO DA TERAPIA ENDODÔNTICA COM CÉLULAS-TRONCO NA REGENERAÇÃO PULPAR"*** sob responsabilidade do(a) ***Pesquisador(a) João Eduardo Gomes Filho*** e o colaborador **Paulo Carvalho Tobias Duarte** está de acordo com os Princípios Éticos da Experimentação Animal (CONCEA) e foi aprovado pela CEUA em 01/07/2011 de acordo com o Processo 1263/2011.

Araçatuba, 04 de Julho de 2011

João Eduardo Gomes Filho
Presidente da CEUA

FAC/selvm.

Faculdade de Odontologia e Curso de Medicina Veterinária – Departamento de Apoio , Produção e Saúde Animal, Rua Clóvis Pestana, 793 CEP 16050-680 Araçatuba – SP
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ANEXO 3

Diretrizes para publicação na revista Journal of Endodontics (JOE)

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 developthe 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.

ANEXO 4

Tissue Reaction to a Triantibiotic Paste Used for Endodontic Tissue Self-regeneration of Nonvital Immature Permanent Teeth

João Eduardo Gomes-Filho, PhD, Paulo Carvalho Tobias Duarte, MSc, Claudiel Batista de Oliveira, DDS, Simone Watanabe, MSc, Carolina Simonetti Lodi, MSc, Luciano Tavares Ângelo Cintra, PhD, and Pedro Felício Estrada Bernabé, PhD

Abstract

Introduction: The endodontic regenerative procedure (ERP), which is an alternative to calcium hydroxide–induced apexification, involves the use of a triple antibiotic paste (TAP) as a dressing material. The aim of this study was to evaluate the response of rat subcutaneous tissue to implanted polyethylene tubes that were filled with TAP or calcium hydroxide. **Methods:** Thirty rats received 2 individual implants of polyethylene tubes filled with TAP or calcium hydroxide paste (CHP) and another empty tube as a control. Thirty additional rats received 2 individual implants consisting of polyethylene tubes filled with dressing material carriers (macrogol and propylene glycol) and a sham procedure. After 7, 15, 30, 60, and 90 days, 12 animals were euthanized, and the tubes and surrounding tissue were removed and processed for histology by using glycol methacrylate and stained with hematoxylin and eosin. The histological score ranged from 0 to 3 depending on the content of inflammatory cells; the fibrous capsule was considered thin or thick, and necrosis and calcification were recorded as present or absent. The results were analyzed using the Kruskal-Wallis test. **Results:** Both dressing materials induced moderate reactions at 7 and 15 days. These reactions were similar to the control ($P > .05$) and reduced in intensity (to mild) from day 30 onward ($P > .05$). The carriers did not interfere with the reaction of the dressing materials. **Conclusions:** TAP and CHP were biocompatible over the different experimental periods examined. *(J Endod 2012;38:91–94)*

Key Words

Biocompatibility, calcium hydroxide, connective tissue, triple antibiotic paste

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Pulp necrosis is harmful for immature teeth; once tooth development ceases, viable pulp fails to develop, resulting in an open apex and thin dentin walls (1). Traditionally, endodontic treatment for immature permanent teeth with apical periodontitis includes calcium hydroxide–induced apexification (2). However, the long-term use of calcium hydroxide can weaken the dentin (3). Recently, the placement of mineral trioxide aggregate apical plugs has been proposed as an alternative treatment (4). Unfortunately, neither of these strategies can increase the root thickness and length that could be achieved by pulp regeneration into a canal, which is capable of promoting the continuation of normal root development (5).

The endodontic regenerative procedure (ERP), an alternative clinical approach to apexification, has received great attention in recent years (6–13). This treatment protocol involves the use of a triple antibiotic paste (TAP) consisting of metronidazole, minocycline, and ciprofloxacin as a dressing and the induction of bleeding to create a matrix for the ingrowth of new, vital tissue in the pulp canal space. Stem cells in the remaining vital pulp or apical papilla have been hypothesized to mediate tissue reconstitution (14). Many such cases have shown favorable results, including continued dentin pulp complex development shown by radiographic evidence and no clinical symptoms (7, 15).

Several early studies documented the antimicrobial activity of TAP (16–19), but few described its biocompatibility (20). In a recent study in dogs, the pulp tissue survived in just 1 tooth after disinfection using TAP and blood clot formation (21). The authors speculated that the low rate of success could have resulted from the high concentration of TAP as a toxic material to the tissues (21). Because the specific effect of TAP on tissues has not been examined, we evaluated the response at different time periods of rat subcutaneous tissue to implanted polyethylene tubes that were filled with TAP or calcium hydroxide paste (CHP).

Materials and Methods

Sixty 4- to 6-month-old male Wistar Albino rats weighing 250 to 280 g were used. The animals were housed in temperature-controlled rooms and given food and water ad libitum. The animals were cared for as per the guidelines of the Araçatuba School of Dentistry, UNESP Ethical Committee, which approved the project before the experiments began.

Sixty 10.0-mm-long polyethylene tubes with a 1.0-mm internal diameter and 1.6-mm external diameter (Abbott Laboratories, São Paulo, SP, Brazil) were filled with the dressing material. TAP comprised 5 parts of antibiotic mixture (ie, equal amounts of 200 mg ciprofloxacin, 500 mg metronidazole, and 100 mg minocycline) and 1 part carrier (equal amounts of macrogol ointment and propylene glycol), with a creamy consistency, as previously described (22). CHP was prepared by mixing calcium hydroxide powder with saline. Both materials were inserted into polyethylene tubes using a lentulo spiral (Maillefer Dentsply, Tulsa, OK). Furthermore, 30 additional polyethylene tubes were filled with equal amounts of macrogol ointment and propylene glycol, and 30 empty polyethylene tubes were used as controls.

The dorsal skins of 30 animals were shaved under anesthesia with xylazine (10 mg/kg) and ketamine (25 mg/kg) and disinfected with 5% iodine solution. A

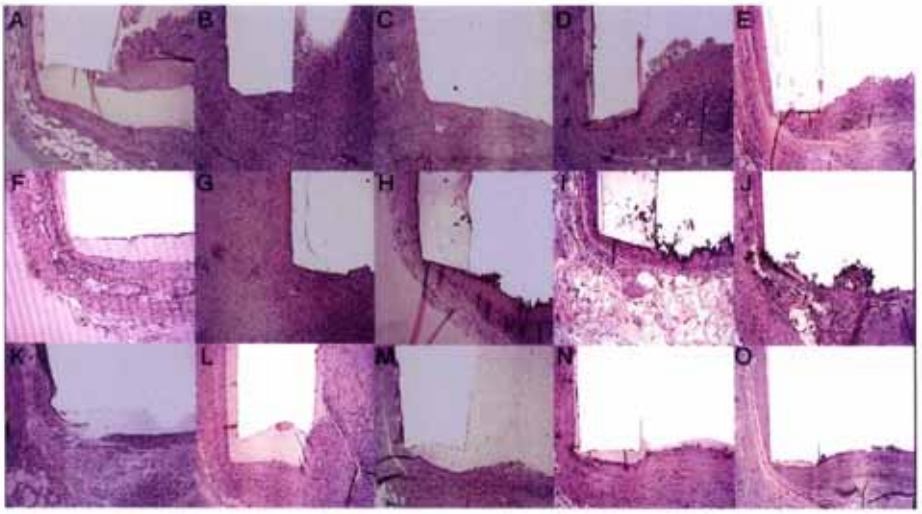

Figure 1. Triantibiotic paste: (*A* and *B*) thick fibrous capsule formation and moderate cell inflammatory infiltration by lymphocytes and macrophages (7 and 15 days, respectively; hematoxylin-eosin staining, 10×) and (*C–E*) mild inflammatory cell infiltration and reduction in thickness of the fibrous capsule (30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×). Calcium hydroxide: (*F* and *G*) moderate inflammatory cell infiltration and presence of a thick fibrous capsule (7 and 15 days, respectively; hematoxylin-eosin staining, 10×) and (*H–J*) thin fibrous capsule formation, mild inflammatory cell infiltration, and dystrophic calcification in contact with the material (30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×). Control: (*K–L*) moderate chronic inflammatory cell infiltration with thick fibrous capsule formation (7 and 15 days, respectively; hematoxylin-eosin staining, 10×) and (*M–O*) thin fibrous capsule surrounding the tube with few chronic inflammatory cells (30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×).

2-cm incision was made on the shaved backs in a head-to-tail orientation using a number 15 Bard Parker blade (Franklin Lakes, NJ). The skin was reflected to create 2 pockets that were 6 cm apart on 1 side of the incision, 1 in the cranial portion and 1 in the caudal portion, and an additional pocket on the opposite side of the incision. Tubes filled with TAP or CHP and the empty tubes were implanted into the spaces created by blunt dissection, and the skin was closed using a 4/0 silk suture. Similarly, in the 30 additional animals, 2 pockets were created on each side of the incision, but only 1 pocket received a tube filled with equal amounts of macrogol ointment and propylene glycol and the other was used as a sham. Sterile instruments and aseptic techniques were used throughout the experiments.

The animals were euthanized with an overdose of anesthetic solution at 7, 15, 30, 60, or 90 days after implantation, and the tubes and the surrounding tissues were removed and fixed in 10% buffered formalin at a pH of 7.0 (23, 24). The tubes were bisected transversely, and both halves were subsequently cut longitudinally with a sharp blade to allow the surfaces to maintain contact with the processing solutions. The specimens were embedded in glycol methacrylate, sectioned serially to 3-μm slices, and stained with hematoxylin and eosin (25).

Reactions in the tissues that were in contact with the material at the opening of the tube were scored as follows (26): 0, no or few inflammatory cells and no reaction; 1, less than 25 cells and mild reaction; 2, between 25 and 125 cells and moderate reaction; and 3, 125 or more cells and severe reaction. Fibrous capsules were classed as "thin" or "thick" if the thickness was <150 μm or >150 μm, respectively. Necrosis and calcification were recorded as present or absent. An average number of cells in each group was obtained from scoring 10 separate areas. The observer, who was familiar with this histological evaluation, was blinded to the treatment. The results were analyzed by the Kruskal-Wallis test at a 5% significance level.

Results

On days 7 and 15, moderate chronic inflammatory cell infiltration by lymphocytes and macrophages was found in a fibrous capsule for TAP (Fig. 1*A* and *B*), CHP (Fig. 1*F* and *G*), and control (Fig. 1*K* and *L*). The intensity of inflammation reduced on days 30, 60, and 90, at which point, a thin fibrous capsule that was almost void of inflammatory cells developed near the tubes for TAP (Fig. 1*C–E*), CHP (Fig. 1*H–J*), and control (Fig. 1*M–O*). Dystrophic mineralization was only observed with calcium hydroxide.

On days 7 and 15, thin fibrous capsule formation and moderate cell inflammatory infiltration by lymphocytes and macrophages was observed with macrogol/propylene glycol (Fig. 2*A* and *B*); however, mild inflammatory cell infiltration and a reduction in the thickness of the fibrous capsule was evident from day 30 onward (Fig. 2*C–E*). Mild inflammatory cell infiltration was observed on day 7 in the sham group (Fig. 2*F*), which reduced to mild infiltration with only few inflammatory cells from day 15 onward (Fig. 2*G–J*).

Comparison between the Groups

The data were compared at each time point (Fig. 3). On days 7 and 15, the difference in scores between the groups (median score of 2) was insignificant ($P > .05$). Similarly, on days 30, 60, and 90, the difference in scores between the groups (median score of 1) was also insignificant ($P > .05$). An exception was the sham group, whose scores were significantly lower than the other groups at all periods ($P < .05$).

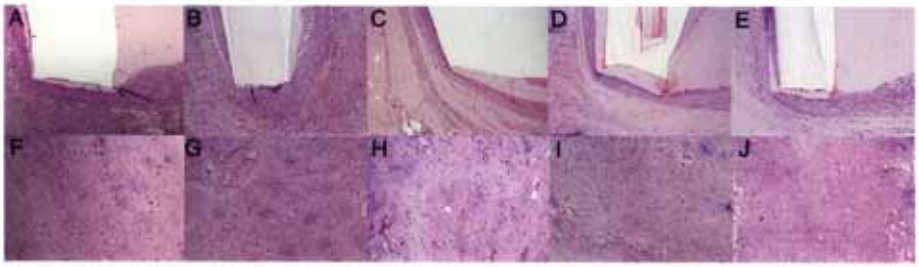

Figure 2. Macrogol/propylene glycol: (*A*) thin fibrous capsule formation and moderate cell inflammatory infiltration consisting of lymphocytes and macrophages (7 days; hematoxylin-eosin staining, 10×) and (*B–E*) mild inflammatory cell infiltration and reduction in thickness of fibrous capsule (15, 30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×). Sham: (*F*) mild inflammatory cell infiltration (7 days; hematoxylin-eosin staining, 10×) and (*G–J*) few inflammatory cells (15, 30, 60, and 90 days, respectively; hematoxylin-eosin staining, 10×).

Discussion

Several methods have been used to evaluate the biocompatibility of endodontic materials, including subcutaneous implantation tests (23, 24). Implantation in the subcutaneous tissue of rats is one of the most appropriate tests to determine the local effects of compounds (27). In this study, empty polyethylene tubes in the control group induced few or no reactions in the subcutaneous tissue and allowed normal tissue repair, which is similar to the results of previous studies (28). The sham procedure showed a mild reaction after 7 days and complete healing after 15 days, indicating no contamination and limited influence of the surgical procedures on tissue response to the materials.

A gentle treatment regimen (minimal or no instrumentation and an intracanal medication with TAP) before ERP may conserve any viable tissue that may remain in the canal system harboring stem cells (ie, SCAP in the apical papilla and dental pulp stem cells in the pulp) (14). Calcium hydroxide can cause necrosis of the surrounding tissue, destroying remnant vital tissues that have the potential to differentiate into new pulp (7, 12). However, calcium hydroxide has been clinically used as an intracanal dressing before ERP (8, 29) because irrigation with NaOCl alone cannot render the canal free of bacteria (29), justifying its investigation in our study.

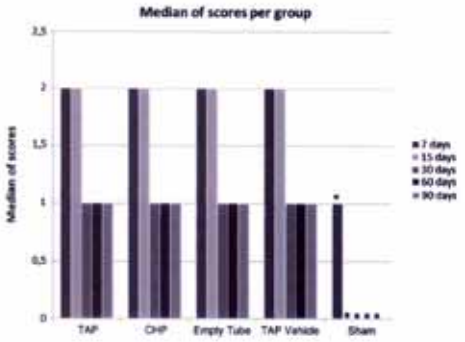

Figure 3. The median of inflammatory reaction scores per group ($n = 6$). Observe that on days 7 and 15 the median values was not significantly different among the groups (median score of 2) except for the sham group. The same was observed on days 30, 60, and 90 (median score of 1). *Statistical difference ($P < .05$).

TAP and CHP induced moderate inflammatory reactions on days 7 and 15; however, this response waned, and the thickness of the fibrous capsule gradually reduced to a level similar to that of the TAP carrier and control. Our results for the CHP tissue reaction are consistent with previous results (28, 30). However, fewer studies have examined the effect of TAP (20), and the reaction of subcutaneous connective tissue to TAP at different times has not been assessed. In contrast to our results, TAP was speculated to be toxic to the tissues once evidence of partial survival of pulp tissue was observed in 1 of 60 dogs that underwent TPA regeneration (19). Similarly, pulpal regeneration did not occur after endodontic treatment using apical negative pressure irrigation or conventional irrigation plus TAP intracanal dressing (31); the authors attributed these findings to periapical tissue irritation that was caused by the persistence of bacteria, the extrusion of sodium hypochlorite, or TAP use.

An increase in root thickness and length, resembling normal maturation of the root, after the treatment of immature teeth with TAP was observed in many clinical cases (8–10). A retrospective evaluation of radiographic outcomes in immature necrotic teeth also showed significant differences in root wall thickness after ERP with TAP (29); thus, vital tissue from the pulp or apical papilla that harbored stem cells may have been present after disinfection and acted as the cellular source for healing (15, 21). Instrumentation beyond the confines of root canal for inducing bleeding can transplant mesenchymal stem cells from bone into the canal lumen (9). However, new tissue formed in the teeth of dogs treated with ERP was considered periodontal tissue, which comprised cementum, bone, and periodontal ligament rather than pulp tissue (21). Therefore, further studies on the long-term outcome of new tissues in the canal space are required to explain clinical cases showing severe narrowing of the canal space.

The compounds in TAP may have positively influenced our results as well as the results of clinical studies. Tetracyclines were shown to exert little or no cytotoxic effects, thus modulating host responses by inhibiting collagenases and matrix metalloproteinases, impeding osteoclastogenesis, and regulating angiogenesis (32, 33). Furthermore, alternative systems may be controlled by tetracyclines, which could reduce the levels of elevated proinflammatory cytokines and increase the levels of interleukin-10, an anti-inflammatory cytokine (34). In addition, metronidazole, ciprofloxacin, and clindamycin generated viable fibroblasts in a cytotoxicity test (35). We conclude that triantibiotic and calcium hydroxide pastes were biocompatible over the different experimental periods examined.

Acknowledgments

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

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ANEXO 5

Técnica para inclusão em Glicol Metacrilato (Leica – Historesin)

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 autopolimerizante e aguardar a polimerização.

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

Peças cortadas em 3 µm.
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.

ANEXO 6

Anexo 6

I - Protocolo de preparo dos componentes a partir do sangue periférico

1 - Preparo da trombina autóloga

Usando uma seringa descartável de 10mL, contendo 2mL de CPDA-1, foram coletados 8 mL de sangue periférico, via veia jugular, dos quais 5mL foi destinado ao preparo da trombina autóloga e os outros 5mL ao preparo do PRP. Para o preparo da trombina autóloga, a amostra de sangue (5mL) foi inicialmente centrifugada a 300 G por 10 minutos. Da camada superior (plasma) foi aspirado 250 µL o qual foi transferido para outro tubo vacutainer com capacidade para 5mL. Ao plasma, adicionou-se 75µL de gluconato de cálcio a 10%, sendo o conteúdo incubado em banho maria a 37º C por 15 minutos. Após o banho, a amostra foi centrifugada a 640 G por 10 minutos, estando o sobrenadante, trombina autóloga, pronta para o uso.

2 - Preparo do plasma rico em plaquetas (PRP) e do gel de PRP

A partir do restante da amostra de sangue periférico coletada (5 Ml), o preparo do PRP foi feito de acordo com uma adaptação do protocolo de DE ROSSI et al., utilizando-se uma centrífuga laboratorial refrigerada (Hermle Z323K, Hermle Labortechnik GmbH., Wehingen, Alemanha). Inicialmente, os tubos foram centrifugados a 300 G por 10 minutos, à temperatura de 22º C, resultando em três componentes básicos: porção superior (plasma), porção intermediária (Leucócitos) e porção inferior (hemácias) (Figura 9). Em seguida, foi pipetado todo o sobrenadante mais a porção intermediária e 2 mm da camada de hemácias, transferindo-se o conteúdo para um outro tubo a vácuo, para uma segunda centrifugação a 640 G por 10 minutos. Esta segunda centrifugação resultou em dois componentes: o plasma pobre em plaquetas (PPP), localizado na parte superior do tubo e que foi descartado, e o PRP, localizado no fundo do tubo e cujo volume

final foi de 0,5mL (Figura 10). Para o preparo do gel de PRP, o PRP foi ativado, no momento de sua injeção nos condutos radiculares, com trombina autóloga na proporção de 2:1, aguardando-se o tempo de geleificação de 2 minutos.

II - Protocolo de preparo dos componentes a partir do AMO

1 - Coleta do aspirado de medula óssea (AMO) e preparo do gel de AMO

Após a anestesia dos animais, procedeu-se a tricotomia e antissepsia da área de punção com solução de iodopovidona a 10%. Em seguida, aspirou-se 3 mL de medula da crista do osso ilíaco, utilizando-se uma agulha especial para punção de medula óssea (Komiyashiki, Japão) acoplada a uma seringa de polietileno de 20 mL (Figura 8), contendo como anticoagulante 1,5 mL de CPDA-1 (JP Indústria Farmacêutica S. A., Ribeirão Preto, São Paulo, Brasil).

Para o preparo do gel de aspirado de medula óssea, o AMO foi ativado com trombina autóloga na proporção de 2:1 imediatamente antes de ser levado aos canais, aguardando-se o tempo de geleificação de 2 minutos.

2 - Preparo do gel misto (PRP/AMO)

Para o preparo do gel misto de PRP/AMO, o PRP foi associado ao AMO e o conjunto foi ativado com trombina autóloga, na proporção de 2:2:1, imediatamente antes de sua aplicação nos canais radiculares, aguardando-se o tempo de geleificação de 1 minuto.