NATÁLIA APARECIDA GOMES

FOTOATIVAÇÃO MOBILIZA PERICITOS E CÉLULAS DE ORIGEM NEURAL NO REPARO DA POLPA DENTÁRIA

Faculdade de Odontologia
Universidade Federal de Minas Gerais
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FOLHA DE APROVAÇÃO

FOTOATIVAÇÃO DE PERICITOS NESTIN GFP/NG2 DsRED EM INJÚRIA DA POLPA DENTÁRIA

NATÁLIA APARECIDA GOMES

Dissertação submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Odontologia, como requisito para obtenção do grau de Mestre, área de concentração Clínica Odontológica.

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*“*O que precisamos é de mais pessoas especializadas no impossível*.”*

Theodore Roethke

RESUMO

Introdução: O laser tem se mostrado capaz de fotoativar células-tronco endógenas induzindo sua diferenciação em múltiplos tecidos, além de modular processos inflamatórios e imunes em microambientes injuriados. Essa terapia é hoje chamada de terapia por fotobiomodulação (PBM) ou simplesmente fotoativação. Células da glia e pericitos – células perivasculares –, por sua vez, têm sido sugeridas como as principais células de reparo da polpa dentária. No entanto, os efeitos da PBM sobre essas células nunca foram explorados utilizando técnicas elegantes (*state-of-art*) de rastreamento, as quais poderiam prover informações relevantes sobre as interações do laser com os componentes celulares marcados. **Objetivos**: Este estudo visou verificar a capacidade estimuladora da PBM na mobilização de pericitos e células de origem neuralno reparo da polpa dentária após injúria tecidual *in vivo.* Para isso, foi utilizado um modelo murino(NG2-DsRed/Nestin-GFP) com transgene para pericitos (NG2) e células neurais indiferenciadas (Nestina). **Métodos**: As polpas dentárias dos primeiros molares superiores dos camundongos (n =12) foram expostas utilizando broca #1190F e lima K #20. O grupo PBM (n = 6) foi tratado imediatamente, 24h, 48h e 72h após a injúria com laser de diodo (InGaAlP; 660nm; 20mW; 5J/cm^2; 0,71 W/cm^2; 7s; modo contínuo e em contato) e o outro grupo foi mantido como controle(n = 6) sem qualquer tratamento. No 4º dia, os animais foram eutanasiados e os efeitos da aplicação da PBM sobre a mobilização dos pericitos e no reparo da polpa dentária foram verificados por microscopia confocal e por análises histológicas (H&E e azul de toluidina). Polpas dentárias saudáveis foram utilizadas como parâmetro de normalidade (n = 6). Os dados foram analisados pelo teste de ANOVA seguido pelo *post-hoc* de Tukey (α = 0,05). **Resultados**: A terapia promoveua mobilização significativa de pericitos e células indiferenciadas nos cornos pulpares coronários contíguos à região injuriada em relação ao controle (p<0,05). Além disso a PBM mostrou intensaproliferação de capilares terminais nos dentes fotoativados (p<0,05), enquanto manteve sinais de vitalidade pulpar nos terços coronários adjacentes à injúria. Embora não identificados na polpa dentária em nenhum grupo estudado, mastócitos puderam ser observados intactos ou degranuladosnos tecidos orais moles adjacentes ao dente fotoativado (p<0,05). **Conclusão:** A PBM estimulou a a neoformação da microvasculatura tecidual local e contribuiu para o influxo de células potentes para a região injuriada. A PBM pode ser considerada uma terapia adjuvante promissora em tratamentos endodônticos regenerativos da polpa dentária.

Palavras-chave: Fotobiomodulação. Pericitos. Células-tronco mesequimais. Polpa dentária.

ABSTRACT

Photoactivation mobilizes experts and cells of neural origin in the dental pulp repair

Introduction: Laser light has proven to be capable of photoactivate endogenous stem cells inducing their differentiation into multiple tissues, in addition to modulating inflammatoryand immune processes in injured microenvironments. This therapy is now called photobiomodulation therapy (PBM). Glial cells and pericytes – the perivascular cells – have been identified as the true stem cells of our body. However, the effects of PBM on these cells have never been explored using elegant (state-of-art) tracking techniques, which could providerelevant information about the laser's interactions with these labeled cellular components. **Objectives**: The goal of this study is to verify the stimulating capacity of PBM in the mobilization of pericytes and other endogenous cells in the repair of the dental pulp upon tissueinjury *in vivo* using a murine model (NG2-DsRed/Nestin-GFP) with transgenes for pericytes (NG2) and undifferentiated cells (Nestin). **Methods**: The dental pulps of the animals' first upper molars (n = 12) were exposed using drill 3195 and K#20 file. The PBM group (n = 6) was treated for 3 consecutive days with a diode laser (InGaIP; 660nm; 20mW; 5J/cm^2; 0.71 W/cm^2;7s; continuous and in contact) and the other group was maintained as control (n = 6) without any treatment. On the 4th day, the animals were euthanized and the effects of the application ofPBM on the mobilization of pericytes and on the dental pulp repair were verified by confocal microscopy and histological analysis (H&E and toluidine blue), respectively. Healthy dental pulps were used as a normality parameter (n = 6). The data were analyzed by the ANOVA testfollowed by the Tukey's poshoc (α = 0.05). Results: PBM showed an intense proliferation of terminal capillaries in the photoactivated teeth (p<0.05), while signs of pup vitality were observed in the coronal thirds adjacent to the injury site. In addition, the therapy promoted significant mobilization of pericytes and undifferentiated cells in the coronary pulp horns contiguous to the injury concerning the control group (p<0.05). Mast cells could not be identified in the dental pulp of any of the studied groups but could be seen mostly degranulated in the smooth tissues adjacent to the mesial root of the teeth of the photoactivated group (p<0.05). Conclusion: PBM stimulates the pulp tissue microvasculature neoformation and contributes to the influx of potent cells into the injured site. PBM may be a promise adjunct tool in regenerative endodontic procedures of the pulp tissue.

Keywords: Photobiomodulation. Pericytes. Wound healing. Dental pulp.

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LISTA DE ABREVIATURAS E SIGLAS

αSMA	α-Actina de Músculo Liso *(α-Smooth Muscle Actin)*
Bsp	Sialoproteína óssea *(Bone sialoprotein)*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA	Comissão de Ética no Uso de Animais
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CONCEA	Conselho Nacional de Controle de Experimentação Animal
CV	Coeficiente De Variação
DAPI	*4',6'-diamino-2-fenil-indol*
DsRed	*Red Fluorescent Protein*
Dspp	Sialoproteína fosfodentinária *(Dentin sialophosphoprotein)*
DSPCs	Células-tronco da Polpa Dentária *(Dental Pulp Stem Cells)*
FGF	Fator de Crescimento de Fibroblastos (*Fibroblast Growth Factor*)
FAPEMIG	Fundação de Amparo à Pesquisa do Estado de Minas Gerais
GFP	*Green Fluorescent Protein*
FUNDEP	Fundação de Desenvolvimento da Pesquisa
H&E	Hematoxilina e Eosina
IC	Intervalo de Confiança

ICB	Instituto de Ciências Biológicas
InGaAlP	Indium-gallium-aluminum-phosphide
IP	Intraperitoneal
MSCs	Células-tronco mesenquimais (Mesenchymal Stem Cells)
NG2	Neural/glial antigen 2
OCT	Optimum Cutting Temperature
PBM	Terapia por Fotobiomodulação (Photobiomodulation)
PBS	Tampão fosfato-salino (Phosphate *Buphered Saline)*
PDGFR-β	Receptor β do fator de crescimento derivado de plaquetas (Platelet-derived Growth Factor Receptor-β)
SCs	Células-tronco (Stem Cells)
TBO	Azul de Toluidina (Toluidine Blue O)
TER	Terapia Endodôntica Radical
TGFβ1	Fator de Crescimento Transformante β1 (Transforming Growth Factor β1)
UV	Ultravioleta
VEGF	Fator de crescimento endotelial vascular (Vascular Endotelial Growth Factor)

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1 CONSIDERAÇÕES INICIAIS

Diante de injúrias dentárias, como lesões cariosas ou traumas, o dano tecidual pode ser tão severo que se torna letal aos odontoblastos presentes na polpa. Nesse cenário, uma cadeia de eventos celulares dá início à reparação tecidual através da ativação de genes envolvidos na determinação do destino de células indiferenciadas da polpa dentária, ou das chamadas células-tronco mesenquimais (MSCs, do inglês *Mesenchymal Stem Cells*) adultas. De fato, essas células proliferam e migram para o local da injúria, onde, em cooperação com outras células locais, participam da reparação do dente reestabelecendo a camada de odontoblastos e formando uma barreira mineralizada denominada dentina terciária (FENG *et al*., 2011; LØVSCHALL *et al*., 2007; SMITH, 1995; TZIAFAS, 1995).

As MSCs estão presentes em todos os tecidos do nosso corpo, sendo seus nichosverdadeiros reservatórios de células preparadas para atuar em situações de dano tecidual. São células capazes de se diferenciar em vários outros tipos celulares, além de apresentarem atividadeimunoreguladora (GLENNIE *et al*., 2005; GRONTHOS *et al*., 2000; SPAGGIARI *et al*., 2006). Essa última propriedade, em particular, é importante para resolução dos quadros de injúria tecidual, uma vez que um sistema imune permissivo é necessário na modulação dos processos inflamatórios em direção ao reparo. A natureza ubíqua das MSCs em tecidos adultos, inclusive na polpa dentária, sugere que essas células possuam um progenitor comum e que sua fonte primária,ou nicho, seria perivascular (KERN *et al*., 2006; SHI, GRONTHOS, 2003). Nesse contexto, a atenção sobre a origem dessas células tem se voltado para os pericitos, células que dividem a localização ao redor de vasos sanguíneos junto das células endoteliais e que, portanto, apresentamampla distribuição em todos os órgãos (ALLIOT-LICHT *et al*., 2001; NEHLS, DRENCKHAHN, 1993; PRAZERES *et* al., 2018; SHI, GRONTHOS, 2003).

Os pericitos são células que apresentam alta plasticidade, sendo capazes de se diferenciarem em múltiplos tipos celulares incluindo osteoblastos, condroblastos, fibroblastos, adipócitos (BIRBRAIR *et al*., 2017; NEHLS, DRENCKHAHN,1993), células nervosas e odontoblastos (ALLIOT-LICHT *et al*.,

2001; FENG *et al*., 2011; SHI; GRONTHOS, 2003). De fato, Løvschall *et al*. (2007) conseguiram verificar a presença de pericitos na polpa dentária através de um estudo sobre a odontogênese em roedores e análise de dentes injuriados humanos. No entanto, somente em 2011, Feng *et al.* demonstraram de maneira elegante (utilizando um modelode rastreamento genético – LacZ), a presença de pericitos no plexo vascular de incisivos de camundongos e sua participação direta em situações de injúria da polpa dentária. Foi visto que um pequeno número de pericitos povoa o mesênquima dentário durante o desenvolvimento, onde eles permanecem essencialmente quiescentes até ocorrerem danos à camada odontoblástica. Na presença de dano, há estímulos à proliferação desses pericitos, os quais aumentam em número e efetivamente contribuem para a formação de novos odontoblastos (FENG *et al*., 2011). Por outro lado, os autores também identificaram que os pericitos contribuem apenas com um pequeno percentual desses novos odontoblastos, sugerindo que a reposição dessas células também possa ser mobilizada de outras fontes (FENG *et al*., 2011).

De fato, Kaukua e colaboradores (2014) descreveram que as células de Schwann e/ou seus precursores, oriundas do sistema nervoso periférico, dariam de fato origem a maior parte das MSCs da polpa dentária e dos odontoblastos em dentes adultos. Na verdade, as células de Schwann e seus precursores seriam células da crista neural dormentes, as quais podem ser recrutadas dos nervos contribuindo para a manutenção dos tecidos periféricos (KAUKUA *et al*., 2014). Nesse mesmo estudo, os pericitos foram excluídos como intermediários para as MSCs da polpa e odontoblastos derivados de células de Schwann e/ou seus precursores, sugerindo que eles seriam uma fonte adicional e independente de células indiferenciadas capazes de repor odontoblastos perdidos.

Alguns efeitos da PBM sob o tecido pulpar já foram demonstrados. Arany *et al*. em 2014,reportaram que a PBM pode estimular a formação de dentina terciária através da via deativação deTGFβ1 (do inglês, *Transforming Growth Factor β1*). Em 2017, Moreira e colaboradores demonstraram que a polpa dentária de molares de ratos tratados endodonticamente pode ser completamente regenerada por meio da estimulação de sangramento periapical e aplicação subsequente da PBM (MOREIRA *et al*., 2017). No entanto, o mecanismo através do qual esse processo ocorreu ainda necessita ser esclarecido. Em decorrência da indução do

sangramento parao interior do canal radicular, é possível que pericitos constituam parte das MSCs endógenas que repovoaram o canal radicular. Considerando o novo tecido pulpar formado observado nesse estudocom a presença de vasos e nervos, a hipótese seria que esses pericitos tenham colaborado com a regeneração desses componentes teciduais devido sua natureza pró-angiogênica e de neoformaçãode tecidos neurais. Dessa forma, o objetivo deste trabalho foi verificar a capacidade estimuladorada PBM na mobilização de pericitos e no reparo da polpa dentária após injúria tecidual *in vivo*. Utilizando um modelo de camundongo da linhagem C57BL/6 com transgenes fluorescentes para as proteínas nestina (GFP) – marcador de células indiferenciadas – e NG2 (DsRed) – marcador de pericitos.

2 OBJETIVOS

2.1 Objetivo geral

Verificar a capacidade estimuladora da PBM na mobilização de pericitos e células neuraisno reparo da polpa dentária após injúria tecidual *in vivo* utilizando um modelo murino (NG2- DsRed/Nestin-GFP) com transgenes fluorescents para pericitos (NG2) e células indiferenciadas (Nestina).

2.2 Objetivos específicos

1) Caracterizar a distribuição e quantificar células transgenicamente marcadas na polpa dentária íntegra;

2) Caracterizar, quantificar e comparar a mobilização de pericitos e suas subpopulações (Tipo 1 e Tipo 2), e demais células indiferenciadas, nas polpas íntegras einjuriadas tratadas ou não com a PBM utilizando microscopia confocal;

3) Avaliar histologicamente o microambiente pulpar quanto à presença de vasos sanguíneos e sinais inflamatórios nas polpas dentárias íntegras e injuriadas tratadas ou não com PBM; e

4) Quantificar a presença de mastócitos degranulados e não-degranulados nos tecidosadjacentes ao dente e nas polpas dentárias íntegras e injuriadas tratadas ou não com PBM.

3 METODOLOGIA EXPANDIDA

3.1 Aspectos éticos

Este estudo foi submetido e aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Minas Gerais (protocolo# CEUA 47/2019). Para o cálculo amostral foi utilizada uma das variáveis mais instáveis correspondente aos pericitos, o PDGFR-β, com coeficiente de variação de 23% (KYYRIAINEN *et al*., 2017). Para a garantia de uma viabilidade experimental segura, consideramos um intervalo de confiança de 20% [dentro do limite máximo de 30% e adequado ao coeficiente de variação da variável (SAMPAIO, 2007) De acordo com Sampaio, 2007, a fórmula utilizada foi:

$$n = (2XCV)^2 / IC^2$$

Em que, n = número de camundongos por grupo; CV = coeficiente de variação; IC = intervalo de confiança.

Substituindo os valores das incógnitas:

$$n = (2XCV)^2 / IC^2 n = (2x23)^2/20^2$$

$$n = 2116/400$$

$n = 5,29$ ou $n = 6$

Arredondando o n encontrado para um valor inteiro, temos **n=6**. É importante mencionarque a base do cálculo amostral foi o coeficiente de variação de uma proteína não quantificada no estudo – o PDGFR-β. Entretanto, no momento de concepção do projeto almejávamos conduzir esses experimentos. De toda forma, o fato de não termos executado esses ensaios não invalida o cálculo

amostral uma vez que todas as outras variáveis testadas têm coeficientes de variação menores. No futuro, a avaliação do PDGFR-β ainda poderá ser conduzida.

3.2 Expansão das colônias de Nestin-GFP/NG2-DsRed

Os animais transgênicos com o fenótipo esperado Nestin-GFP/NG2-DsRed foram obtidos pelo cruzamento de machos Nestin-GFP/NG2-DsRed com fêmeas Nestin-GFP. Os animais homozigotos positivos para um dos genes foram usados em outros cruzamentos com menor probabilidade de obtenção do fenótipo ou eutanasiados de acordo com as normas do CONCEA e foram desconsiderados para inclusão no presente estudo. Doze animais duplo positivos (Nestin- GFP/NG2-DsRed) obtidos foram utilizados nesse estudo. Devido à raridade do fenótipo Nestin-GFP/NG2-DsRed, maxilas com molares hígidos foram obtidas de animais Nestin-GFP/NG2- DsRed utilizados como controles de outras pesquisas aprovadas pela CEUA com outras finalidadese que teriam as cabeças descartadas (CEUA #19/2018). Todos os animais foram alojados em condições controladas de temperatura (24 ºC) e sob ciclo claro-escuro de 12/12 horas e alimentados*ad libitum.*

3.3 Procedimentos *in vivo*

Camundongos fêmeas e machos de 8-10 semanas de idade e pesando de 15-20 gramas foram anestesiados (100mg/Kg Ketamina + 10mg/Kg Xilazina, via IP) e adaptados em uma mesacirúrgica personalizada desenvolvida em colaboração com o Ohlab (Associação Mineira deReabilitação, Belo Horizonte, Brasil), baseada numa publicação prévia (MARCHEZAN *et al*., 2018). A mesa cirúrgica foi impressa tridimensionalmente (3D Fortus 380 MC, Stratasys, EUA) em filamento de nylon 12 (Figura A). Essa ferramenta otimizou o acesso intrabucal aos primeirosmolares do camundongo, considerando a restrita abertura de 0,5 a 0,8 cm, e perímetro reduzido da mesa occlusal dos molares que possuem aproximadamente 1,5mm de comprimento no sentido mésio-distal.

O procedimento cirúrgico nos primeiros molares superiores esquerdo e direito dosanimais foi feito utilizando uma broca #1190F (KG Sorensen, Cotia, Brasil) acoplada a um contra- ângulo e motor de bancada (Beltec, São Paulo, Brasil). Incialmente, foi feito um desgastesuperficial na fossa mesial dos primeiros molares superiores de forma a criar-se um nicho (FiguraB). Em seguida, uma Lima K #20 foi utilizada com pressão apical e movimentos rotacionais alternados para direita e para a esquerda de forma a penetrar na dentina e na câmara pulpar. A limafoi introduzida aproximadamente 0,5mm.

O sinal clínico que indicava que a profundidadealmejada havia sido atingida era o travamento da lima no orifício criado sem a necessidade de segurá-la. Ao retirar a lima, o orifício formado podia ser visualizado, embora não houvesse sinal de sangramento pulpar (Figura C). Os dentes foram mantidos abertos por todo o tempo experimental. Essa conduta foi optada por dois motivos: (1) a PBM, por seus efeitos vasculares já conhecidos, poderia levar a uma dilatação tecidual de proporções danosas ao tecidopulpar que se encontra confinado entre paredes dentinárias rígidas; (2) qualquer material restaurador utilizado no selamento da cavidade poderia confundir a resposta pulpar promovida exclusivamente pela PBM. No pós-operatório, os animais foram medicados com analgésico opioide (Tramadol, 5mg/Kg de 12:12h) e mantidos com ração pastosa para facilitar a alimentação.Não houve restrição de água ou de movimentação.

Figura A – Mesa cirúrgica personalizada confeccionada através da impressão 3D.

Fonte: Arquivo Pessoal

Figura B – Primeiro passo na realização da injúria pulpar.

Fonte: Arquivo Pessoal

Figura C – Segundo passo na realização da injúria pulpar.

Fonte: Arquivo Pessoal

3.4 Photobiomodulação – PBM

A PBM foi realizada com laser de diodo (InGaAlP personalizado; DMC Equipamentos, São Carlos, Brasil), em modo de operação contínuo e aplicação pontual e em contato. Osparâmetros de dosimetria foram os mesmos utilizados no estudo anterior (DINIZ *et al*., 2018): 660 nm, 20 mW, 0,71 W/cm^2, área do feixe de 0,028 cm^2, 7 s, 0,14 J de energia total por ponto, 5J/cm^2. A PBM não causa dor, nem aumento de temperatura significativo – 0,1°C nos parâmetros utilizados. A primeira fotoativação foi realizada no transoperatório. As demais fotoativações foramrealizadas sempre no mesmo horário da primeira e apenas com contenção manual dos animais.

3.5 Eutanásia e Preparo da Amostra para Análise Histoquímica e Microscopia Confocal

Os animais foram eutanasiados com sobredose anestésica (300mg/Kg Cetamina + 30mg/Kg Xilazina, via IP), seguida de deslocamento cervical no quarto dia experimental.Os espécimes foram fixados em paraformaldeído 4%, por 48h. Em seguida, foramdesmineralizados em EDTA 10% durante 28 dias, e preparados para a análise em microscopia confocal e ou histoquímica. Dessa forma, como os animais tinham ambos os primeiros molars superiores direito e esquerdo operados, uma amostra de cada animal foi utilizada na análise confocal e a outra metade nas análises histológicas.

Para as análises histoquímicas, as amostras foram desidratadas em série ascendente de álcool, diafinizadas em xilol e incluídas em parafina. Foram então utilizadas seções de 5 µm de espessura dos blocos cortados sagitalmente no sentido vestíbulo-lingual em micrótomo (Leica, Wetzlar, Alemanha), os quais foram posteriormente corados com H&E ou Azul de Toluidina O (para marcação de mastócitos). Para análise no microscópio confocal, após a desmineralização, as amostras foram imersas overnight em sacarose 30%. Em seguida, as amostras foram incluídas em composto Optimum Cutting Temperature (OCT) e congeladas à -20ºC (Figura D). Os blocos foram então cortados em criostato a 20 µm de espessura.

Figura D – OCT, cassete para inclusão das amostras e criostato.

3.6 Avaliação histológica

Dez campos histológicos dos cornos distais, médios e mesiais (região injuriada) de cada grupo experimental foram selecionados aleatoriamente e analisados (400×) da seguinte maneira:

Vitalidade pulpar: vitalidade total da polpa dentária coronária (++), vitalidade parcial da polpa dentária coronária (+) e não vital (-). Polpa com tecido necrótico ou ausência de tecido dentro da câmara pulpar foram consideradas não vitais, enquanto amostras com tecido pulpar representadas por tecido conjuntivo vascularizado foram vitais; (2) Sinais inflamatórios avaliados em cada região da polpa dentária coronária: apenas as amostras classificadas como vitais (++ e +) foram avaliadas para a condição do tecido pulpar distante da injúria, designadas como: presença de apenas um dos sinais inflamatórios (+); dois sinais de inflamação (++); mais de dois sinais inflamatórios (+++); ou ausência de inflamação (-). Os sinais inflamatórios foram: vasos sanguíneos dilatados e congestionados; glóbulos vermelhos fora dos vasos; infiltração de células inflamatórias (como leucócitos polimorfonucleares e neutrófilos); e microabcessos.

O tamanho (diâmetro) e o número dos vasos sanguíneos terminais e centrais também foramquantificados nessas imagens pelo software Fiji (NIH). Todas as amostras foram observadas em um microscópio óptico (Standard 25, Carl Zeiss,

Göttingen, Alemanha) e todas as análises histológicas foram realizadas por examinador calibrado e cego.

Para a quantificação de mastócitos, os cortes foram corados com Azul de Toluidina O a 1% (89640-5G, Sigma Aldrich, Missouri, EUA) em água destilada por 2 minutos (BAYAT et al., 2008). Para cada seção histológica, três campos histológicos consecutivos na coroa com aumento de 40× foram selecionados para a análise. Outros seis campos foram analisados nas regiões adjacentes ao dente. A contagem de mastócitos foi realizada de acordo com Sawasaki et al. (2009) e realizada às cegas pelo mesmo examinador.

3.7 Análise confocal

Para a análise confocal, os cortes foram contra-corados com DAPI em meio de montagem (Abcam, Cambridge, EUA). A localização dos pericitos na polpa dentária foi observada em microscópio confocal usando um Eclipse Ti com um cabeçote confocal A1R (Nikon, Tóquio, Japão) equipado com quatro lasers diferentes (excitação em quatro comprimentos de onda: 405, 488, 546 e 647 nm) e filtros passa banda de emissão em 450/50, 515/30 , 584/50 e 663/738 nm (MARQUES et al., 2015; ANTUNES et al., 2018) no Center for Gastrointestinal Biology, sob coordenação do Prof. Gustavo B Menezes, no Instituto de Ciências Biológicas. Foram utilizadas as objetivas Plan Apo 20× e 40×. As células indiferenciadas (GFP) e a população total de pericitos (DsRed), assim como as subpopulações de pericitos Tipo 1 (colocalização DsRed e DAPI em rosa) e Tipo 2 (colocalização de GFP, DsRed e DAPI em amarelo) foram contadas pela intensidade de fluorescência (valores médios de cinza), após ajuste manual de threshold para todas as amostras, usando o Photoshop (Adobe, San Jose, EUA) ou o software Fiji (NIH, Bethesda, EUA).

3.8 Análise estatística

Os dados foram tabulados e analisados pelo ANOVA seguido pelo post-hoc de Tukey ao nível de significância de 0,05.

4 RESULTADOS

Os resultados e discussão serão apresentados na forma de artigo científico.

4.1 Artigo

Artigo a ser submetido ao periódico Journal of Dental Research (Qualis A1) após as considerações da banca.

Photoactivation of NG2 and Nestin cells in dental pulp injury *in vivo*

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Abstract

Pericytes are already known to collaborate in dental tissue repair. Cell-based therapies that stimulate these perivascular components may be of relevance for tissue regeneration. The goal of this study was to examine the early effect of photobiomodulation (PBM) in labeled pericytes (NG2) and undifferentiated cells (Nestin) after dental pulp exposure in vivo. We descriptively compared the in vivo cell tracking in NG2-DsRed/Nestin-GFP transgenic mice after an experimental dental pulp injury model in phototactivated and non-photoactivated samples. We report overall mobilization of cells in the pulp tissues treated by PBM compared to the controls. Moreover, we showed that PBM remarkedly stimulated terminal blood vessels sprouting while maintaining pulp vitality in the areas adjacent to the exposure site. Pericytes were shown to contribute to the dental pulp repair in both groups and were predominantly located close do the injury site. However, an increased number and a redistribution pattern of these perivascular cells in the cell rich zone in the photoactivated samples was also observed. PBM promotes important vascular changes with intense flow of pericytes and undifferentiated cells in the photoactivated dental pulps and may be a relevant tool for dental pulp conservative treatments.

Keywords: pulp biology, perivascular cells, pericytes, odontoblasts, stem/progenitor celltherapy, photobiomodulation.

Introduction

Upon dental pulp injuries, the damage may be severe enough to become lethal to the odontoblasts. In this scenario, a chain of cellular events initiates tissue repair through the activation of mesenchymal stem cells (MSCs). These cells proliferate and migrate from their niches to the injured site, where – in cooperation with other local cells – they participate in the dental pulp tissue repair by differentiating into “odontoblast-like cells” and, sometimes, forming a mineralized barrier called osteodentin (SHI and GRONTHOS 2003; TZIAFAS 1995; FENG et al., 2011).

Elegant genetic line-tracing studies have demonstrated that the nerve bundles are the major source of dormant neural crest cells, i.e., Schwann cells and their precursors, which are thus responsible for the maintenance of peripheral tissues in the pulp tissue (KAUKUA et al., 2014; COUVE et al., 2017). Despite that, the pericytes were set as an independent source of responsive cells able to replace lost odontoblasts (KAUKUA et al., 2014). The pericyte mobilization toward dental pulp repair was previously reported in numerous studies (LØVSCHALL et al., 2007, FENG et al., 2011, KAUKUA et al., 2014; ZHAO et al., 2014). There is recent evidence about the major contribution of resident perivascular cells expressing α smooth muscle actin (αSMA), stimulated or not by Fibroblast Growth Factor 2 (FGF2), to the reparative dentinogenesis process (VIDOVIC et al., 2017; VIDOVIC-ZDRILIC et al., 2018).

Pericytes are particular components of the local microvasculature, which are responsible for blood vessel formation and stabilization (BIRBRAIR et al., 2013, 2015). These cells can control the blood flow (PALLONE et al., 2003) and modulate the immune system (NAVARRO et al., 2016). Noteworthy, two pericytes subpopulations were described in the skeletal muscle interstice and other organs, the Type-1 (Nestin GFP-/NG2 DsRed) and the Type-2 (Nestin GFP+/NG2 DsRed+) subsets (BIRBRAIR et al., 2013). Type 2 pericytes are more potent and can differentiate into the neural lineage and also skeletal muscle and blood vessel tissues, while Type 1 pericytes are prone to differentiate into adipose or fibrous tissues (BIRBRAIR et al., 2013, 2015). In truth, pericytes and MSCs are ubiquitous in the mesenchymal compartment of tissues and may be expanded to give other tissue phenotypes depending on how they are stimulated (SHARPE and YIANNI 2019).

Accordingly, cell-based therapies that target these perivascular

components may be of relevance for tissue regeneration. Our group has demonstrated that photobiomodulation (PBM) could be an adjuvant candidate for dental pulp regeneration by stimulating endogenous cells to repopulate the root canals (MOREIRA et al., 2017). Previously, Arany et al. (2014) have shown enhanced tertiary dentin formation triggered by the TGFβ1 activation in photoactivated rat tooth. As such, PBM could be used as a low-cost single strategy to promote vascular changes and further influx of potent cells in partially vital pulp tissue. Herein, we used the Nestin-GFP/NG2-DsRed transgenic mouse model to localize and quantify pericytes (NG2) and undifferentiated cells (Nestin) upon healthy and injured conditions. Pericytes were increased in the dental pulp microenvironment e mainly distributed nearby the injury site. Moreover, PBM stimulated new terminal capillaries sprouting and has driven pericytes and other cells to collaborate in the dental pulp tissue repair.

Material and Methods

Animal Model

The Animal Care and Use Committee at Universidade Federal de Minas Gerais approved animal handling and procedures in this work (protocol #47/2019). Experiments were conducted according to the ARRIVE guidelines. Colonies of transgenic animals with the expected Nestin- GFP/NG2-DsRed phenotype were obtained as previously described (BIRBRAIR et al., 2013). All animals were housed under controlled conditions and under light-dark cycle of 12:12 hours and fed ad libitum.

In vivo procedures

Eight to ten-week old male or female Nestin-GFP/NG2-DsRed mice (n = 12; 6 per group) were used in this study. In regard to the rarity of the phenotype and following the 3R concept, the healthy samples were collected from mice with the same age used in other approved experiments with discharged head (protocol #19/2018). A mouse dental bed was created by three-dimensional (3D) printing in nylon filament 12 (Figure S1) (3D Fortus 380 MC, Stratasys, USA) with a head support design (adapted

from Marchesan et al. 2018). The experimental pulp exposure on the mice maxillary first molars was performed as described in Figure S1. The teeth were left opened for 3 days. Postoperatively, the animals were medicated with opioid analgesic (Tramadol, 5mg/kg, every 12 hours for 24h) and maintained with pasty food and water ad libitum.

Photobiomodulation (PBM)

PBM was performed transoperativelly and on the next 3 days with an indium-gallium- aluminum-phosphide (InGaAlP) customized diode laser, in continuous operation. The dosimetry parameters were as follows: 660 nm, 20 mW, 0.028 cm2 spot area, 0.71 W/cm2, 7 s, 5 J/cm2, 0.14 J total energy per point, in punctual and contact modes (Diniz et al., 2018).

Histology

On the 4th post-operative day, the animals were euthanized, and specimens were fixed in 4% formaldehyde for 48 hours. Then, samples were demineralized in 10% EDTA for 21-28 days, and half of the samples were prepared for confocal microscopy and the other half for histological analysis. For confocal analysis, samples were immersed overnight in sucrose 30% and then embedded in optimum cutting temperature (OCT) compound. Sections of 20 µm thickness were counterstained with DAPI in mounting medium (Abcam, Cambridge, UK) and analyzed. After paraffin embedding, blocks were serially sectioned using a microtome at 6 µm sections (Leica) and were either stained for hematoxylin and eosin (H&E) or Toluidine Blue O (TBO). Protocols for H&E, TBO and histomorphometric analysis are described as Supplementary Material and methods.

Confocal analysis

In vivo tracking of labelled cells in the dental pulp were observed in microscope by using an Eclipse Ti with an A1R confocal head (Nikon, Tokyo, Japan) (Antunes et al., 2018). Plan Apo 20× and 40× objectives were used. A scan large image

was performed for each sample under the 10x objective. Each channel was counted separately, except for Type 1 and Type 2 pericytes subpopulations that were counted in the merged images. A threshold was manually adjusted and the fluorescence area (mean gray values) was calculated by using the Fiji software (NIH).

Statistical Analysis

Statistical significance between groups was calculated using one-way ANOVA followed by Tukey's post-hoc using the GraphPad Prism 8.0 Software (GraphPad Prism Version 8.0c fo Mac, GraphPad Software, La Jolla, USA). Data were expressed as means ± standard error. The differences among the groups were considered significant when $p<0.05$ in all experiments.

Results

An increased number of total cells was observed throughout the coronary dental pulp treated with PBM

To observe the early effect of PBM on the injured pulp tissue, we have exposed the first molar dental pulp and photoactivated it for 3 consecutive days (Figure 1). We initially sought whether PBM would be able to attract cells to the injury site (Figure 2). DAPI staining quantification showed increased overall number of cells in PBM-treated samples ($p > 0.05$). Total cells were slightly greater in the sham compared to the healthy group, but this difference was not statistically different ($p < 0.05$) (Figure 2).

PBM enhanced the number of pericytes and undifferentiated cells in the damaged pulp tissue We next searched what type of cells were particularly present in the PBM scenario. First,we examined the PBM stimulus in vivo by quantifying the mean fluorescence of cells expressing only the NG2-DsRed (total pericyte population) or the Nestin-GFP (undifferentiated cells) transgene (Figure 2). We found that both pericytes and undifferentiated cells were increased in the photoactivated wounds in comparison to the sham and healthy groups ($p < 0.05$).

Noteworthy, the number of Nestin-GFP cells was similar between sham and healthy groups ($p > 0.05$). Otherwise, the number of NG2-DsRed cells was increased in the sham damaged pulp tissuein relation to the healthy tooth ($p < 0.05$).

We also looked for the cells coexpressing NG2-DsRed and Nestin-GFP transgene, or for the cells expressing only the NG2-DsRed transgene, both colocalized with DAPI (Figure 2). We observed that PBM has attracted both pericytes subpopulations to the injury site, while in the sham group only the Type 1 subset was increased in relation to the healthy group ($p < 0.05$).

Pericytes and undifferentiated cells present a different pattern of distribution upon photoactivation

We next sough for a distribution pattern of the labeled cells in the damaged (Figures 2 and 4) and healthy dental pulp tissue microenvironment (Figure 3). Undifferentiated cells were clearly disposed on the periphery of the pulp tissue in the healthy tissues (Figures 2 and 3), whereas upon photoactivation, these cells could be seen all over the coronary pulp tissue, including the central part of the pulp, or were frequently concentrated close to structures suggestive of vessels (Figures 2 and 4). Pericytes, instead, were found sparsely distributed or sometimes concentrated in the central region of the coronary dental pulp tissue in all group

(Figure 2). Constitutively, both pericyte subpopulations (Type 1 or Type 2) were observed in the upper region of the pulp horns sharing their localization with numerous Nestin cells in the odontoblast lining layer (Figure 3). Otherwise, particularly in the PBM-treated groups, the NG2 cells tended to accumulate on the periphery of the exposure site or in close relationship with Nestin positive cells in the cell rich zone of the pulp tissue (Figure 4). Figure 4 depicts two sagittal sections of the same tooth in different regions upon PBM treatment. The distribution pattern of pericytes far from the injury site was similar to the healthy samples. NG2 positive cells were observed in the odontoblastic layer and presented a morphology typically odontoblastoid. Otherwise, nearby the injury site, the pericytes have accumulated around the necrotic tissue and were also sparsely distributed among the Nestin positive cells in the periphery of the pulp tissue. Structures suggestive of blood vessels were also observed in the central pulp tissue.

Histological examination showed terminal blood vessels sprouting in the photoactivated

samples

Histological examination showed that all treated groups presented necrosis (or the tissue was absent) in the mesial horns – the site of the injury (Figure 5). In the middle horns, although PBM samples presented two or three inflammatory signs (ISs) in 63.7% of the samples, none of them lost vitality thoroughly. In the sham group middle horns, 40% of the samples presented necrosis. From the total of vital samples, 66.6% showed two or three ISs in the sham samples. The distal horns presented one or two IS in more than 80% of PBM samples against 60% in the sham group. Otherwise, 18% of the PBM-treated samples and 40% of the sham group presented two or three ISs in the furthest region of the exposure site.

Blood vessels quantification showed increased number of terminal vessels in the photoactivated dental pulps while the number of core vessels were similar to the sham and healthy groups ($p > 0.05$) (Figure 5). Moreover, congested blood vessels were frequently seen in the PBM-treated samples.

Although vascular changes were pronounced in the pulp tissue, no masts were found in any studied group by using the Toluidine Blue O staining technique (Figure S2). Instead, in the adjacent gingival tissue, masts were found in its non-degranulated/intact form in the healthy and sham groups ($p > 0.05$). In the PBM-treated samples, beyond the presence of intact mast cells, a higher number of degranulated masts was counted ($p < 0.05$).

Discussion

Caries lesions and trauma may damage the dental pulp sometimes resulting in infection or necrosis. However, the following endodontic treatment with bionert dental materials fails to restore tooth sensitivity and increases the risk of tooth fracture (GONG et al., 2016). Instead, cell therapies may be promising strategies to obtain partial or whole dental pulp regeneration. In the present study, we examined the expression of NG2 and Nestin reporters in the teeth during an acute inflammatory process in the coronal pulp tissue. As such, Nestin is an intermediate filament protein

expressed in neural progenitors during the early development of neural tissues; while the NG2 is a well-recognized marker for pericytes. Our characterization studies have shown that both cells are compartmentalized in the pulp tissue and is also suggestive that they share spot in the odontoblast lining layer. The labelled cells tracking experiments showed that the early and frequent exposure of the pulp cells to PBM resulted in overall influx of NG2 and Nestin cells in the coronary compartment. Moreover, we showed that PBM remarkably stimulated terminal blood vessels sprouting while maintaining pulp vitality in the areas adjacent to the exposure site.

A recent study has beautifully characterized the close relationship between nerve bundles and vascular networks in the human pulp tissue (FRANÇA et al., 2019). Progress about the contribution of neural and vascular progenitors toward pulp tissue repair is setting these components as the main targets in endodontic regenerative procedures (FENG et al., 2011; KAUKUA et al., 2014; COUVE et al., 2017). Accordingly, the current literature presents numerous studies that look particularly for the vascular stimulation of the dental pulp aiming cell- based regenerative therapies (IOHARA et al., 2009; SOURON et al., 2014). There is evidence showing that vascular endothelial growth factor (VEGF) signaling induces differentiation of dental pulp stem cells (DPSCs) into vascular endothelial cells and also odontoblasts (SAKAI et al., 2010; BENTO et al., 2013; ZHANG et al., 2016, SASAKI et al., 2020). Moreover, it has also been reported that DPSCs facilitate vascular formation by directing endothelial cells and stabilizing newly formed blood vessels (JANEBODIN et al., 2013; ROMBOUTS et al., 2017).

Pericytes are known components of the dental pulp microenvironment responsible for the pulp tissue repair upon injury, at least partially (LØVSCHALL et al., 2007; FENG et al., 2011; KAUKUA et al., 2014; YIANNI and SHARPE 2019). These cells may present characteristics similar to the multipotent stem cells (SCs) since they are capable of differentiating into angiogenic, neurogenic, myogenic, and several other tissues and cells – for example, odontoblasts (BIRBRAIR et al., 2013, 2015; FENG et al., 2011). Considered their shared role with some DPSCs phenotypes in the dental pulp, there is strong evidence that some of those DPSCs are, in fact, pericytes (YIANNI and SHARPE 2019). The Mina´s group observations have shown that perivascular cells expressing αSMA constitute a population of mesenchymal progenitors in dental pulp capable of giving rise to Dspp+ odontoblasts and Bsp+ osteoblasts in reparative

dentin (VIDOVIC et al., 2017). Moreover, αSMA+ progenitors stimulated by Fibroblast Growth Factor 2 (FGF2) can differentiate into odontoblasts (VIDOVIC-ZDRILIC et al., 2018).

A matter of debate is whether all pericytes, irrespective of tissue of isolation, have equal differentiation potential (YIANNI and SHARPE 2019). The present study is the first that differentiates two types of pericytes subpopulations in the dental pulp, the Type 1 and Type 2. In all studied groups, the Type 1 pericytes were much more evident than the Type 2 in the healthy and injured pulp tissues. Noteworthy, upon photoactivation, the number of Type 2 pericytes increased significantly in relation to the healthy tissues but not than the sham groups. Type 2 pericytes, in turn, are less committed than the Type 1 ones (BIRBRAIR et al., 2013). Yet, it remains to be discovered what are the key roles of these two pericytes subsets in the dental pulp tissue in healthy and injured conditions. Previous studies highlight that some dental pulp pericytes are already precommitted to an odontoblast fate characterized by overrepresentation of key odontogenic genes (SUBRAMANIAN et al., 2005). Herein, our reporter model allowed us to observe the exclusive expression of the NG2 protein in cells morphologically similar to the odontoblasts in the root and coronary sites, although we cannot confirm their perivascular origin.

In regard to Nestin cells, in the present study they were seen in the periphery of the pulp tissue in the healthy tissues, mostly in the region known as the cell-rich zone. However, Nestin cells were also seen in the odontoblast lining layer. A previous histochemical study in mice has observed, from different confirmatory experiments, the same correspondent location of Nestin cells (QUISPE et al., 2002; NAKATOMI et al., 2018). Nakatomi et al. 2018, in turn, found that the expression of Nestin was found to be restricted to the coronal pulp of molars. Otherwise, in the present study, was possible to identify Nestin cells even in the radicular pulp tissue of the mice maxillary molars (shown for PBM group and healthy samples).

PBM is capable of modulating cell signaling and metabolism in an injured milieu through the generation of reactive oxygen species and energy (ARANY et al., 2014). It is also known to activate critical endogenous molecules such as VEGF, FGF and TGFβ1 (GAENGEL et al., 2009; ARANY et al., 2014; de FREITAS and HAMBLIN, 2016; VITOR et al., 2020), all growth factors closely related to pericytes angiogenic differentiation. In truth, there is accumulated evidence. showing that PBM at 660 nm

can increase local blood circulation and inflammatory cells influx (KIMIZUKA et al., 2018; JIN et al. 2018). Previously, a preliminary mouse skin study using immunohistochemical techniques showed an increased number of NG2 and αSMA positive cells in the PBM-treated groups with dosimetry parameters similar to the ones of the current study (MEDRADO et al., 2010). Herein, the significative mobilization of perivascular cells by PBM in the dental pulp was explored for the first time. PBM has shown increased number of small blood vessels throughout the adjacent compartments of the coronary pulp tissue adjacent to the exposure site, thus suggesting a possible role on vessel sprouting upon photoactivation. Exuberant structures suggestive of blood vessels were observed in some PBM-treated samples and core vessels were, indeed, frequently shown congested with blood cells upon photoactivation.

We next evaluated whether PBM was capable of guiding the labeled cells to the injurysite. An intense influx of total cells including pericytes and undifferentiated cells was observed in the PBM-treated groups in relation to the healthy and sham control groups. Pericytes were found sparsely distributed in the dental pulp tissue in the sham and in the healthy tissues, while tended to accumulate on the periphery of the exposure site and in the cell rich zone in the PBM-treated injuries. Cells coexpressing both αSMA-tdTomato and Col2.3-GFP transgenes were also reported as early as 2 days in close proximity to the site a pulp injury treated with FGF2 in a model similar to the present study (VIDOVIC-ZDRILIC et al., 2018).

Our histological experiments for mast cell detection using the toluidine blue O staining was not able to identify any masts within the dental pulp tissue in none of the studied groups. More accurate techniques are though important to confirm this particular feature. Noteworthy, the gingival tissue adjacent to the treated tooth has shown remarked number of mast cells in the photoactivated specimens, and this increase was conferred mostly by degranulated masts. Mast cells, in truth, have been correlated as the main cellular components responsible for the vascular effects triggered by PBM. Curiously, our dental pulp tissue studies have shown that the local microvasculature effects of PBM may be not due to the mast cells.

Regenerative procedures combining stem cells, scaffolds, and growth factos or by simply stimulating cell homing were successfully reported in the current literature (SHARPE and YELLICK 2019; YIANNI and SHARPE 2019). In this context,

blood vessels may present a crucial migratory surface that guides glial progenitors as a road during axonal regeneration (CATTIN and LLOYD 2016) and support DPSCs with nutrients and oxygen during pulp tissue repair (SHARPE and YELLICK 2019). Despite the dental pulp is a highly vascular tissue, a posed issue is that, currently, is hard to relate pulpal vascularity with specific markers for dental pulp stem cells (KRIVANEK et al., 2017). The Nestin-GFP/NG2-DsRed transgenic model may provide further comprehensive studies to understand the differential activation of pericytes and undifferentiated cells in the dental pulp and possible effects of PBM on the dentinogenic differentiation of dental pulp stem/progenitors cells. Taken together, our observations support the key roles of PBM in the dental pulp local blood vessels and, particularly, in perivascular cells expressing NG2, which may contribute to the success of regenerative endodontic procedures.

Author contributions

I. M. A. Diniz, N. A. Gomes, T. A. Silva, I. B. do Valle, M. M. Marques contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript; S. R. Oliveira, H. M. C. Oliveira, C. B. S. Gomes, P. H. R. G. Reis, L. S. Castilho contributed to data acquisition and interpretation, performed statistical analyses, drafted and critically revised the manuscript; G. B. Menezes, R. A. Mesquita and C. S. Magalhães contributed to conception, design, and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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Figure legends

Figure 1. *Labeled cell tracking in healthy and after pulp exposure in sham and PBM groups at day 4 post-surgery*. (A) Bright field (a) and epifluorescence (b-e) representative sagittalsections through a healthy maxillary first molar. Note that NG2 positive cells can be observed dispersed in the central part of the pulp tissue; whereas Nestin positive cells are observed predominantly in the periphery. (B) Bright field and epifluorescence (b-e) representative sagittal sections through the injured sham maxillary first molars. Note that pericytes appear increased in the central part of the pulp tissue and in intimacy with the damaged pulp tissue (d,e). The images of panel (C) represent bright field (a) and epifluorescence (b-e) sagittal sections through the injuredmaxillary first molar treated with PBM. Also note the same continuous layer of Nestin positive cells of the healthy sample, now disposed either in the periphery or below the damaged pulp tissueresembling a biological barrier. Pericytes appear in close relationship with the pulp tissue at the injury site and also sparsely distributed in the central part of the pulp tissue. In all images, the dental pulp is denoted by dotted lines. The injury site is represented by asterisks (*). D = Dentin; PT = Pulp Tissue; AB = Alveolar Bone. Scale bar = 20 µm.

Figure 2. *Labeled cell quantification in healthy and at the injury site of PBM and sham*

groups.

(A) Epifluorescence images (a-d) of healthy (A), sham (B) and PBM-treated molars. Panels A, B and C depict small magnification images of split channels DAPI (a), GFP (b), and Dsred (c). In all panels, higher magnification of DsRed (red) and DAPI (blue) merged channels
(d) showing Type 1 pericytes in pink (yellow arrow). Higher magnification of GFP (green) and DsRed merged channels (e) showing Type 2 pericytes in yellow (white arrows). Note that Nestin cells present a peripheric distribution, mainly in the healthy (A) and PBM groups (B). Note the increased cellularity in the photoactivated sample. Also note the increased presence of Nestin andNG2 cells in the PBM-treated samples. (D) Bar graphs showing the total cells (DAPI), total pericytes (NG2-DsRed), undifferentiated cells (Nestin-GFP), Type 1 (NG2-DsRed and DAPI colocalization – pink) and Type 2 pericytes (Nestin-GFP/NG2-DsRed colocalization - yellow) mean fluorescence quantification. In all images, the dental pulp is denoted by dotted lines. Data are shown as the mean ± SEM, n = 6 for each group. D = Dentin; PT = Pulp Tissue. Scale bar = 20µm. 42

Figure 3. *Labelled cells characterization in a healthy maxillary molar.* Epifluorescence images of 20× (a,d,g,j,a´) and 40× of distal (b,e,h,k,b´) and mesial (c,f,i,l,c´) horns of a healthy second maxillary molar sagittal sections. Higher magnifications of pink and blue boxed areas (a) show the distribution of Nestin cells predominantly in the periphery of the dental pulp tissue, possibly composing

the cell-rich zone – white dotted lines – in the distal (b) and mesial horns (c), respectively. Both pericyte subpopulations (Type 1 or Type 2), but particularly the Type 1, were observed in the upper region of the pulp horns sharing their localization with numerous Nestin cells in the odontoblast lining layer (e,f,h,i,k,l). Yellow dotted lines are delimiting the pulp chamber (a-c) or the central pulp tissue (j-l). Yellow dotted rectangles show Nestin positive cells located in the odontoblast lining layer. Structures suggestive of blood vessels are marked with asterisks (*). D = Dentin; PT = Pulp Tissue. Scale bar = 20 µm.

Figure 4. *Two sagittal sections of the same tooth in different regions treated with PBM.* One section shows in a region far from the injury site (A), while the other depicts a view from a region close to the pulp exposure site on the mesial horn (B). In panel A, note that the pulp tissueinside the root channel presents NG2 positive cells with an odontoblastoid morphology (white arrows) (c). Also note that far from the injury site, Nestin cells appear concentrated in the peripheryof the pulp tissue, whereas pericytes are observed mostly in the upper region of the pulp horns, similarly to the healthy tissues (d). In panel B, the radicular pulp tissue still shows structures suggestive of blood vessels (*) and NG2 positive cells in the central part and in the periphery of the pulp are sharing space with Nestin cells in a region compatible with the odontoblast lining layer (c). Note that, in a closer view, exuberant structures suggestive of blood vessel are seen in the central part of the pulp (*) (d). Also note that some NG2 cells are now seen in the cell-rich zone (dotted yellow arrows) in the injured third (d). The dental pulp is denoted by dotted lines. D
= Dentin; PT = Pulp Tissue; AB = Alveolar Bone; PL = Periodontal Ligament. Scale bar = 20 µm.

Figure 5. *Histological evaluation of pulp vitality, inflammatory signs and blood vessel quantification.* Small magnification photomicrographs showing sagittal sections of maxillary molars of healthy (A), PBM treated (B) and sham groups (C). (A) The distal horn (a) of a healthy molar presents dental pulp consisting of highly cellularized connective tissue, with vessels of varying diameters and congestion. At the periphery of this tissue, a lining layer of polarized cells arranged in palisade is in close contact with a pre-dentin layer, which in turn is surrounded by dentin. The middle (b) and mesial (c) thirds present histological characteristics verysimilar to those of the distal third. The images in figure (B) illustrate the histological findings of the sham group represented by altered pulp tissue. The distal horn (a) presents pulp tissue represented by swollen connective tissue with infiltration of mononuclear inflammatory cells anda large number of congested vessels. The middle horn (b) presents dental pulp with intense mononuclear inflammatory infiltrate, disruption of the odontoblast layer, mainly on its mesial surface and small areas of necrosis. The mesial horn (c) shows an area of pulp tissue exposure; the dental pulp is represented by a necrotic tissue. (C) Represents the histological findings of the micepulps exposed and treated with PBM. The molar shows pulp tissue with inflammatory changes, especially in the middle horn adjacent to the pulp exposure site. The distal horn (a) presents pulp tissue with characteristics similar to the ones described for the healthy group. The middle horn (b)has also preserved dental pulp and is rich in vessels. The mesial horn (c) presents rupture of the dentin wall and the remaining dental pulp tissue

appears represented by accumulations of inflammatory cells dispersed in a necrotic tissue. Blood vessels of variable diameters were observed in all groups (D). Larger vessels were observed regularly in all groups (E) whereas smallvessels were predominant in the photoactivated ones (F). Data are shown as the mean ± SEM, n=5 for each group. N.S. = non-significant. Scale bar = 20 µm.

Supplementary Figures

Figure S1. *Experimental procedures in vivo.* The mouse dental bed was designed to achieve mouse maximum aperture of the mouth; but also to hold the mouse head at a stable positionwhile performing the experimental pulp exposure procedure (A). Pulp exposure on the mice maxillary first molars was performed in two steps. First, the superficial enamel on the mesial fossawas rough with a #3195 drill (KG Sorensen, Cotia, Brazil) coupled to a contra-angle (Kavo, Biberach, Germany) and bench motor at 5,000 rpm (Micro Motor LB100 Beltec, São Paulo, Brazil) to create a niche (B). Then, a K#20 file was apically pressured with alternating rotational movements to the right and left to penetrate the dentin and the pulp chamber (C). The desired depthwas reached by the locking of the file into the created hole (approximately 0.5mm). Representative bright field photomicrography showing the injury site (*) (D). D = Dentin; PT = Pulp Tissue; PL
= Periodontal Ligament; AB = Alveolar Bone. Scale bar = 20 µm.

Figure S2. *Mast cells in response to PBM therapy.* Representative Toluidine Blue O (TBO) stained sections of the distal, middle and mesial horns of the dental pulp tissue in all groups(A-C). Note that mast cells are not identified in the pulp tissue by the histochemical technique. Inthe areas of smooth tissues adjacent to the maxillary first molar mesial root mast cells could be detected and counted (D). Note the presence of non-degranulated/intact (pink arrow) and degranulated (green arrows) mast cells in the lamina propria of the representative gingival tissue. Bar graphs showing cellular quantification of mast cells (E) in healthy, sham and PBM groups, whether intact or degranulated. Data are shown as the mean ± SEM, n= 6 for each group. N.S. = non-significant. Scale bar = 20 µm.

Supplementary Materials and Methods

Pulp vitality and inflammatory signs (ISs)

H&E staining process was performed according to Diniz et al. 2018. Ten histological fields of the distal, middle and mesial horns (injured site) of the coronal dental pulp of each experimental group were randomly selected and analyzed (40×), as follows: (1) Pulp vitality: vital(+),(++), partially vital (+/-) or non-vital (-). Pulp with necrotic or absent tissue inside the pulp chamber were considered non-vital, whereas samples with pulp tissue represented by vascularizedconnective tissue were vital; the presence of necrotic tissue alongside vascularized connective tissue was considered partially vital; (2) Inflammatory signs (IS): presence of only one IS (+); twoIS (++); more than two IS (+++); or absence of inflammation (-). The inflammatory signs were dilated and congested blood vessels; red blood cells out of the blood vessels; infiltration of inflammatory cells (such as polymorphonuclear leukocytes and neutrophils); and microabscesses. Only horns with vital or partially vital pulp tissues were classified for the ISs.

Blood vessels quantification

Blood vessels were defined as a tube-like structure with a lumen, regardless it contained or not red blood cells. Terminal capillaries (< 10µm in diameter) and core vessels (10 – 50 µm) (França et al. 2020) were quantified in H&E images by using the Fiji (NIH) software (NIH, Bethesda, USA).

Mast cell quantification

For mast cells quantification, the sections were stained with Toluidine Blue 1% O (89640-5G, Sigma Aldrich, Missouri, USA) in distilled water for 2 minutes (adapted from Bayat et al., 2008). The coronal pulp tissue and five other consecutive histological fields of 40× magnificationin the smooth tissues adjacent to the first superior molar mesial root were selected for the analysis.Masts were located and classified as degranulated or non-degranulated/intact cells (adapted from SAWASAKI *et al*., 2009). All samples were analyzed with an optical microscope (Standard 25, Carl Zeiss, Göttingen, Germany) and all histological analysis were undertaken by blinded examiner.

FIGURES

Figure 1. *Labeled cell tracking in healthy and after pulp exposure in sham and PBM groups at day 4 post-surgery*.

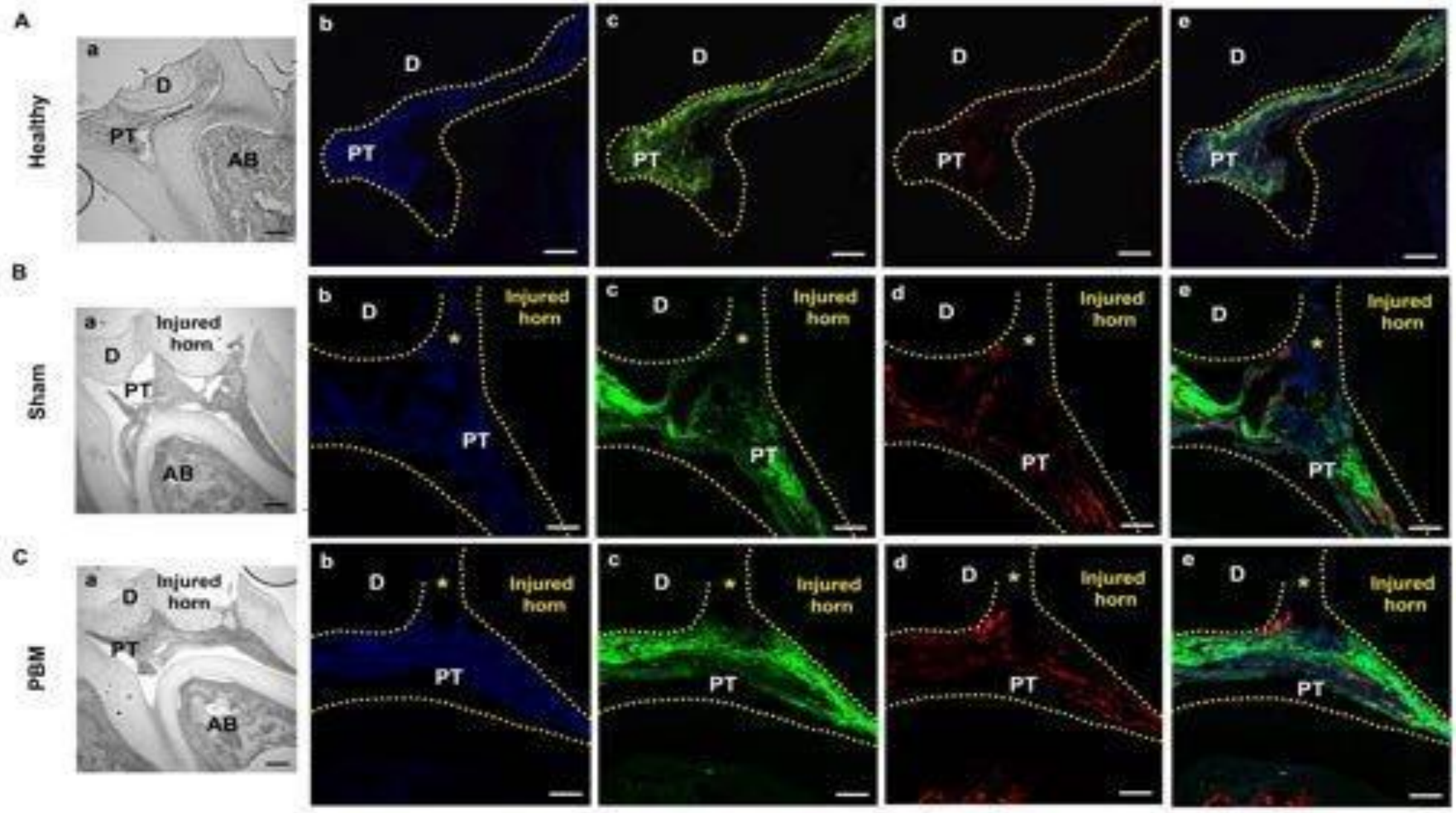


Figure 2. *Labeled cell quantification in healthy and at the injury site of PBM and sham groups.*

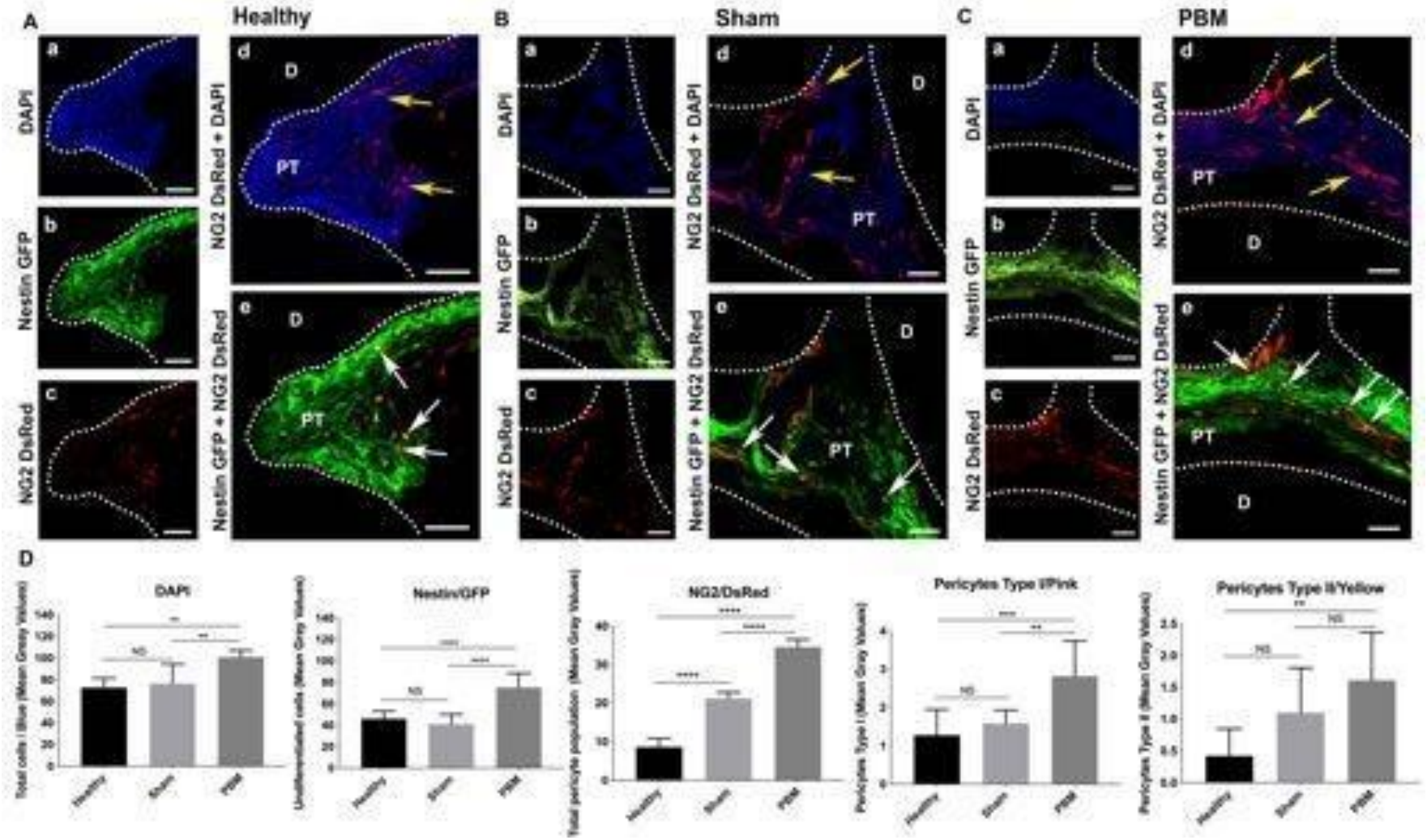


Figure 3. *Labelled cells characterization in a healthy maxillary molar.*

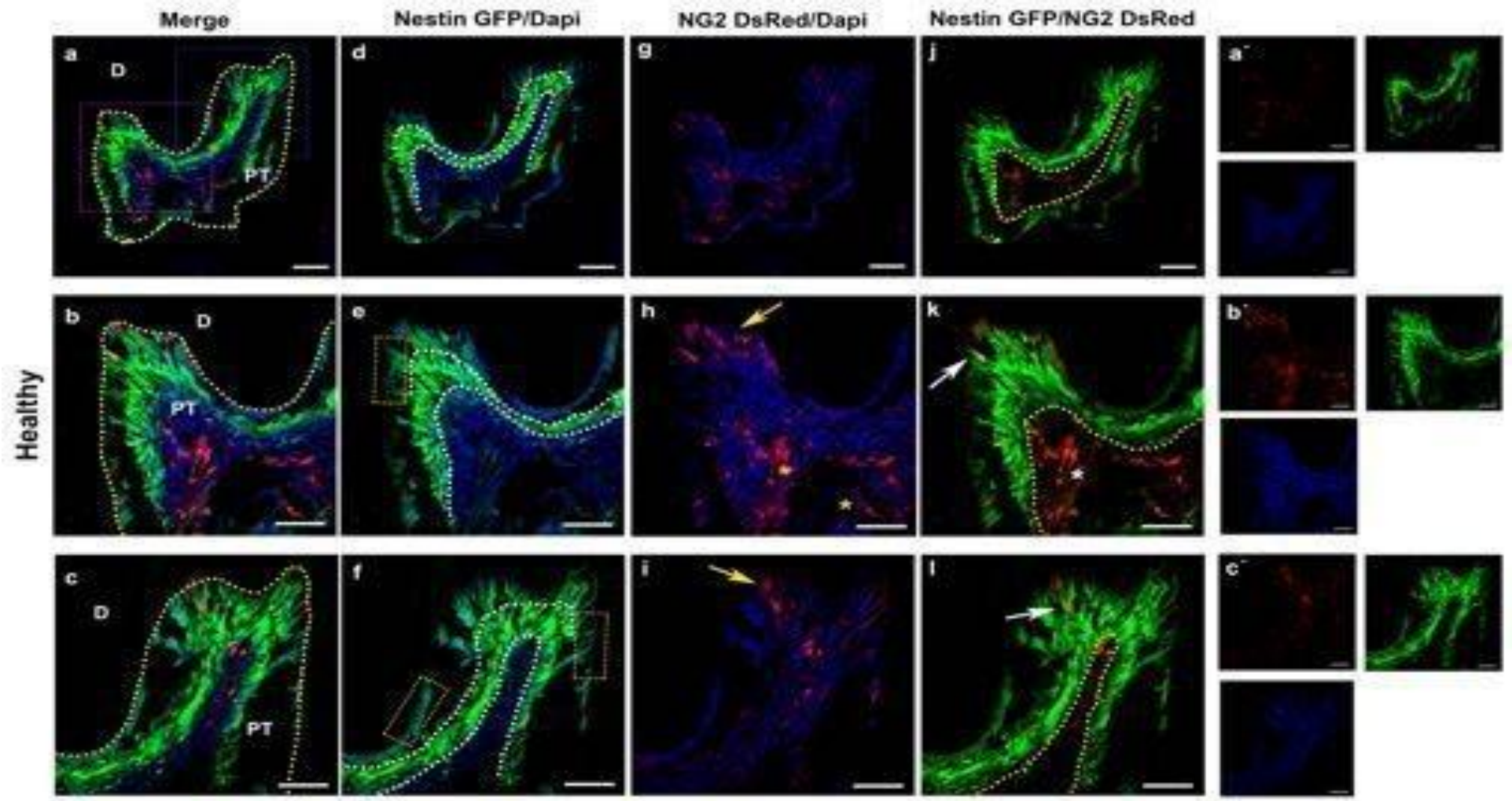


Figure 4. *Two sagittal sections of the same tooth in different regions treated with PBM.*

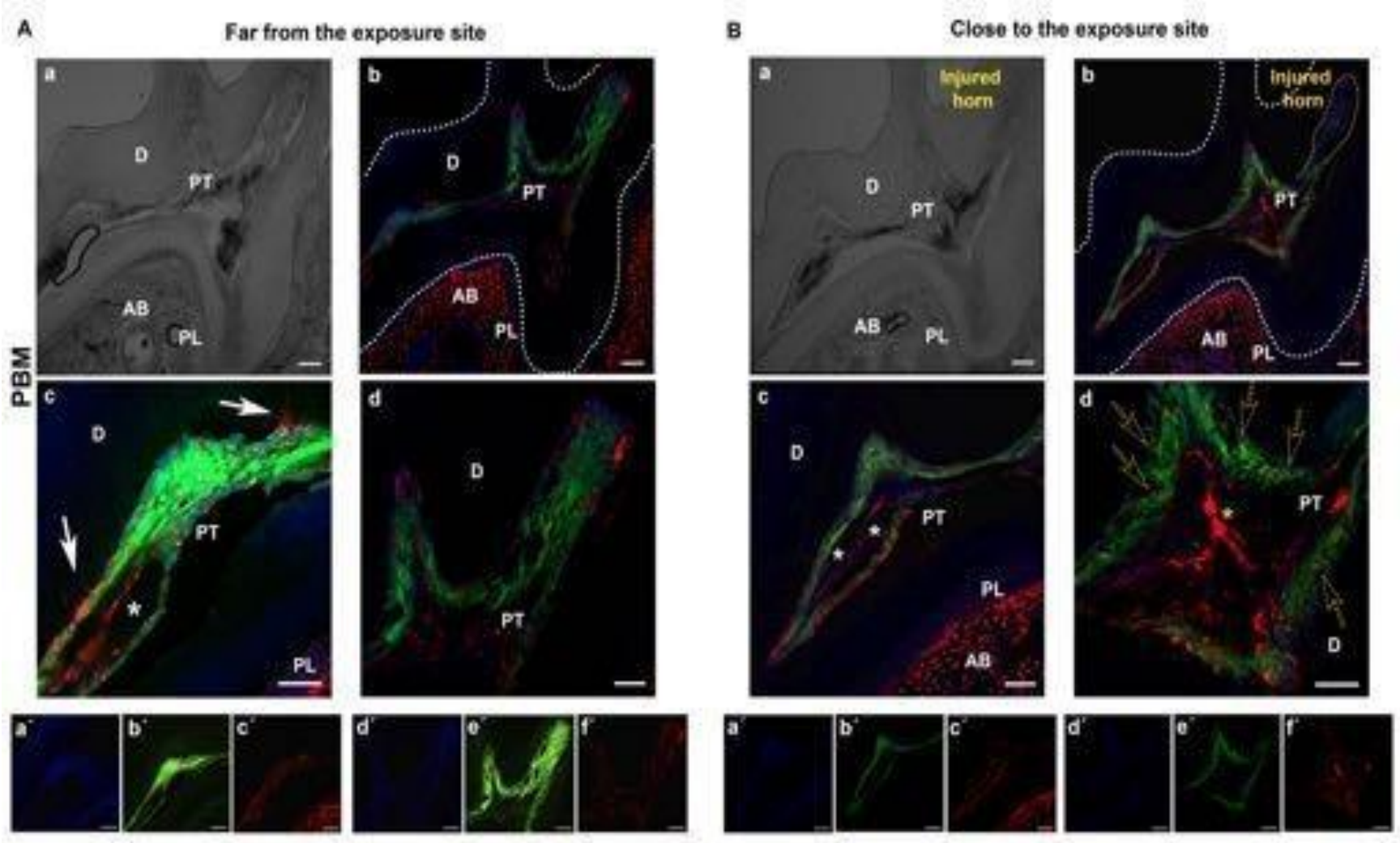


Figure 5. *Histological evaluation of pulp vitality, inflammatory signs and blood vessel quantification.*

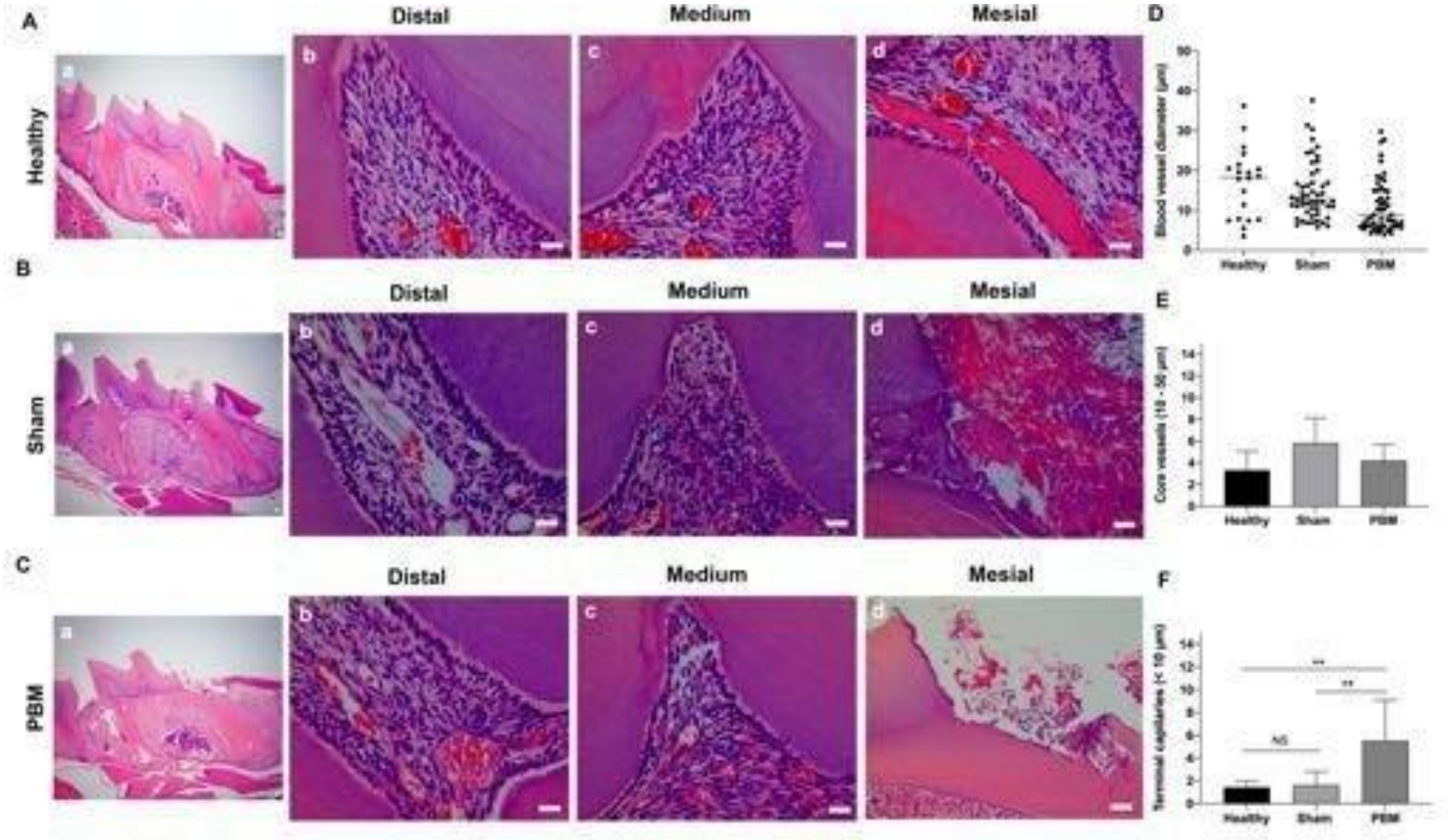


Figure S1. *Experimental procedures in vivo.*

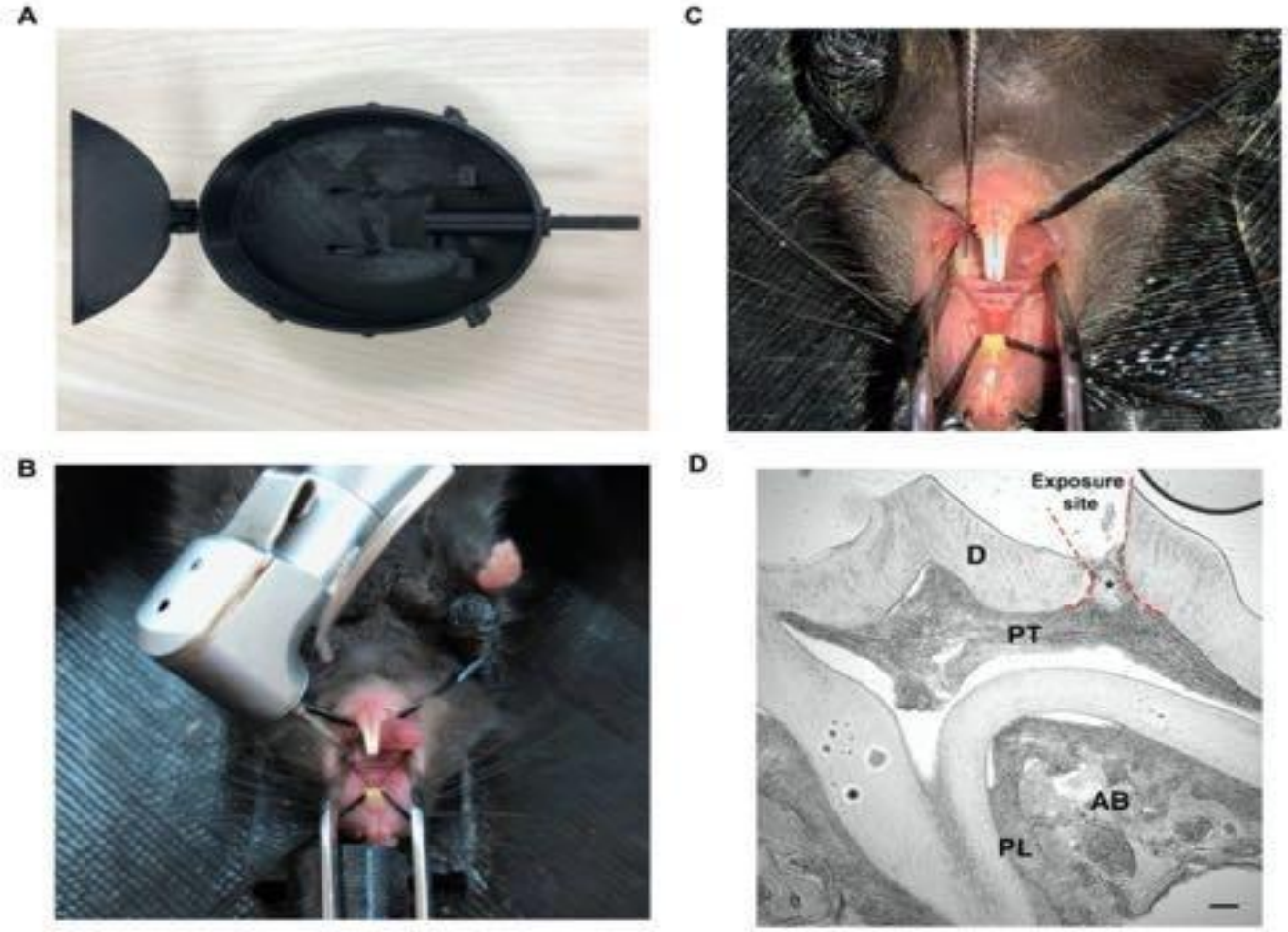
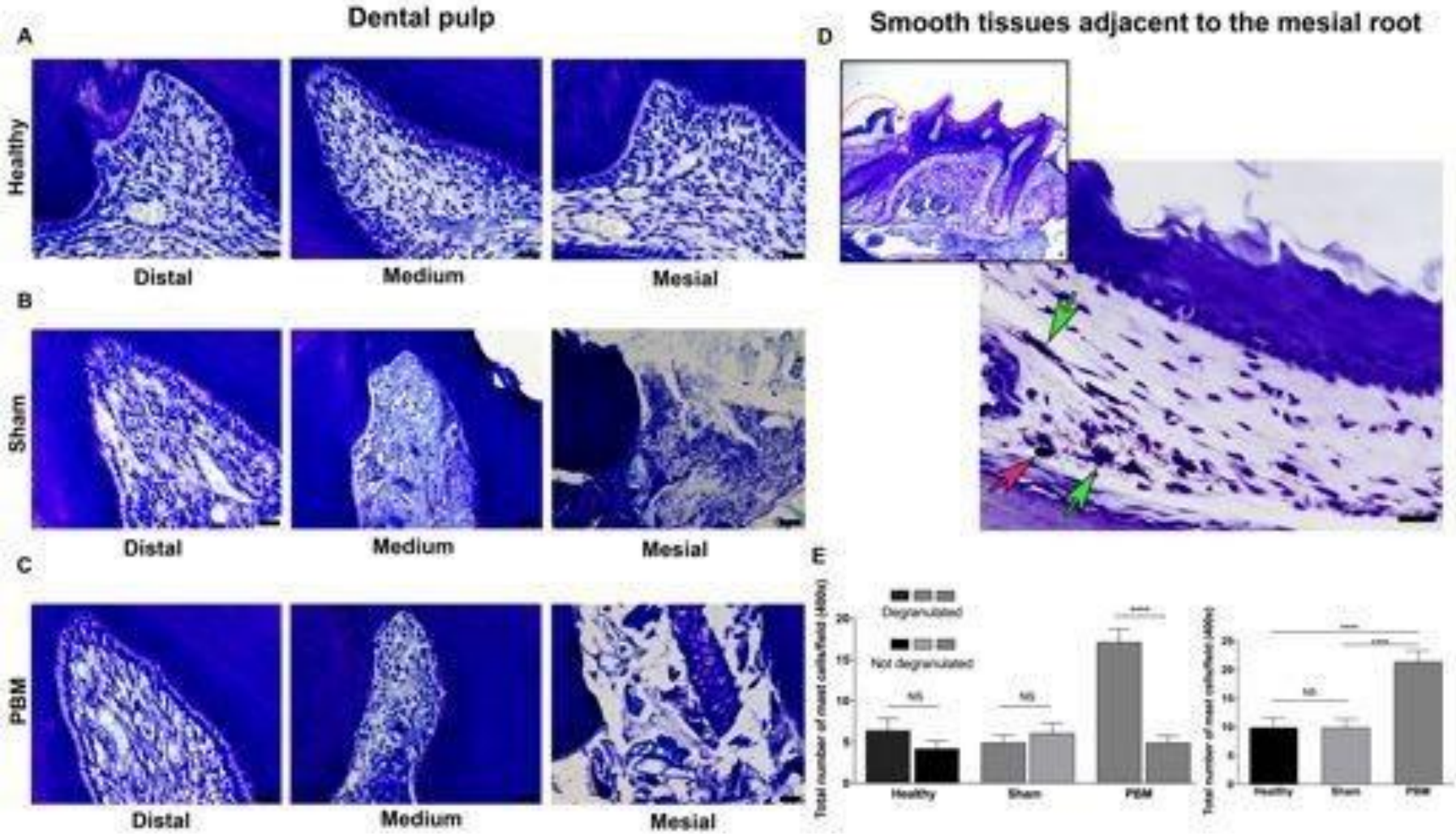


Figure S2. *Mast cells in response to PBM therapy.*



5 CONSIDERAÇÕES FINAIS

O objetivo deste estudo foi verificar a capacidade estimulatória da PBM na modulação depericitos e no reparo da polpa dentária após injúria tecidual por meio de um modelo murino (Nestin-GFP/NG2-DsRed) com transgene para pericitos (NG2) e células indiferenciadas (Nestina). Tal modelo permitiu que este estudo fosse o primeiro a diferenciar dois tipos de subpopulações de pericitos na polpa dentária. Além disso, permitiu que rastreássemos os pericitos e observássemos que, parte deles, apresenta morfologia semelhantes aos odontoblastos.

Observamos também que a PBM apresentou quesitos importantes para manipular pericitos. Demonstramos que eles são influenciados por essa terapia e parecem atuar no processode reparação do tecido pulpar. Neste estudo, a PBM aumentou de forma geral a população celular – inclusive de pericitos – além de modular o processo inflamatório na polpa injuriada. Dessa maneira, a PBM parece atuar como gatilho para ativação dessas células. Ademais, os pericitos podem ser a chave para o entendimento acerca de alguns efeitosbenéficos provocados pelo laser.

Nesse sentido, o modelo transgênico Nestin-GFP/NG2-DsRed poderá propiciar estudos futuros para entender a ativação diferencial de pericitos e células indiferenciadas na polpa dentáriae os possíveis efeitos do PBM na diferenciação de células-tronco / progenitores da polpa dentária.Em conjunto, nossas observações suportam o papel da PBM nos vasos sanguíneos da polpa dentária e, particularmente, nas células perivasculares que expressam NG2, o que pode contribuirpara o sucesso dos procedimentos endodônticos regenerativos.

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ANEXO A – Aprovação da comissão de ética no uso de animais

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA: 47/2019
Título do projeto: FOTOATIVAÇÃO DE PERICITOS NESTIN GFP+/NG2 DsRED EM LESÕES DA POLPA DENTÁRIA
Finalidade: Pesquisa
Pesquisador responsável: Ivana Marcia Alves Diniz
Unidade: Faculdade de Odontologia
Departamento: Departamento de Odontologia Restauradora

Situação atual: Decisão Final - Aprovado

Aprovado na reunião do dia 08/04/2019. Validade: 08/04/2019 à 07/04/2024
Belo Horizonte, 08/04/2019.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

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