UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

Francine Benetti

TESE DE DOUTORADO

Influência do peróxido de hidrogênio sobre a diferenciação celular e a mineralização do tecido pulpar após procedimento clareador. Estudo histológico e imunoistoquímico

ARAÇATUBA, SP

2018

UNIVERSIDADE ESTADUAL PAULISTA
"Júlio de Mesquita Filho"

Francine Benetti

Influência do peróxido de hidrogênio sobre a diferenciação celular e a mineralização do tecido pulpar após procedimento clareador. Estudo histológico e imunoistoquímico

Tese de doutorado apresentada à Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP como parte dos requisitos para obtenção do título de Doutor em Ciência Odontológica, área de concentração em Endodontia.

Orientador: Prof. Adj. Luciano Tavares Angelo Cintra

ARAÇATUBA, SP

2018

Catalogação na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

Benetti, Francine.

B465i Influência do peróxido de hidrogênio sobre a diferenciação celular e a mineralização do tecido pulpar após procedimento clareador. Estudo histológico e imunoistoquímico / Francine Benetti. - Araçatuba, 2018

124 f. : il. ; tab.

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba

Orientador: Prof. Luciano Tavares Angelo Cintra

Coorientador: Prof. André Luiz Fraga Briso

1. Clareamento dental 2. Estresse oxidativo 3. Peróxido de hidrogênio 4. Pulpite 5. Dentinogênese 6. Células-tronco I. T.

Black D24

CDD 617.67

Claudio Hideo Matsumoto – CRB-8/5550

Dados curriculares - Francine Benetti

Identificação

Nascida em **16 de dezembro de 1990** em Catanduva, SP.

Filiação: Joel de Freitas Benetti e Cacilda Apda. Porta Benetti

2009 – 2013 - Curso de Graduação

Concluiu o **Curso de Graduação em Odontologia**, na Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, em dezembro de 2013.

2014 – 2015 - Curso de Mestrado

Concluiu o **Curso de Mestrado em Ciência Odontológica, área de concentração Endodontia**, na Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, em setembro de 2015, sob orientação do professor Luciano Tavares Angelo Cintra, e coorientação do professor André Luiz Fraga Briso.

2016 – 2017 - Curso de Especialização

Concluiu o curso de **Especialização em Endodontia**, na Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP.

2015 – 2018 - Curso de Doutorado

Concluiu o **Doutorado em Ciência Odontológica, área de concentração Endodontia**, na Faculdade de Odontologia do Campus de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, sob orientação do professor Luciano Tavares Angelo Cintra, e coorientação do professor André Luiz Fraga Briso.

Dedicatória...

Dedicatória

Dedico este trabalho aos meus pais, **Joel** e **Cacilda**, meus maiores incentivadores.

Obrigada por sempre confiarem em mim, e sempre estarem ao meu lado. Obrigada por tanto amor, tanto cuidado, tanta dedicação.

Graças a você, pai, eu tenho ao meu lado a pessoa com o maior coração deste mundo. Aquele que daria tudo o que tem, mesmo que pouco, a quem estivesse precisando. Aquele que cuida mais do próximo, do que de si mesmo. Que possui uma fé que pode mover montanhas. A sua fé é o que tem de mais bonito, e é o maior exemplo que pode nos dar. Obrigada por tudo!

Obrigada, mãe, por resgatar forças quando muitas vezes não tem de onde a tirar. Por ser guerreira, por me ajudar tanto, em tudo. Por colocar os filhos sempre em primeiro lugar. Por muitas vezes dedicar sua vida à minha e a de meus irmãos. Por me fazer saber que sempre terei ao meu lado alguém que eu posso confiar, de todo o meu coração! Que um dia eu possa lhe retribuir tudo o que faz por mim!

Vocês são os responsáveis por este momento. Todas as minhas conquistas pertencem a vocês. Obrigada por tudo o que me ensinaram, pela base que me deram. É a vocês, com toda a certeza, que dedico este trabalho! Amo vocês!

Agradecimentos...

Agradeço a Deus

Agradeço a Deus, meu Pai,
por receber minhas orações, e por me guiar até o presente
momento. Por me fazer entender que Seus planos são
maiores que os meus, e que a maior vitória desta vida é ter o
Senhor ao nosso lado.

"O Teu manto de amor sinto estar sobre mim; Minha vida te entrego, Senhor; Viverei pela fé, protegido assim, Na presença de Deus, Criador. Meu destino de luz vejo ao longe, Senhor, E jamais olharei para trás; Minha crença em Ti guardarei, Redentor; Teu poder, virtuoso me faz."

.

Agradeço a Jesus, o
Caminho, a Verdade, e a Vida. O maior Professor, o maior
Exemplo, o melhor Amigo. Nosso Intercessor fiel.

"O Mestre que desceu dos céus, Jesus, o Redentor, Deixou exemplo aos santos Seus, Exemplo de amor: Curvado, lhes lavou os pés, E assim Se humilhou; Com essa obra que Ele fez, o amor nos ensinou. 'Amai-vos', disse o Redentor, Que teve morte atroz; Jesus, por seu divino amor, salvou a todos nós."

Agradecimentos

Aos meus irmãos, meus amigos, **Giovana** e **Victor Benetti**,

Vocês me dão alegria todas as vezes em que volto para "terrinha". Nos ajudamos, nos interessamos pelos problemas/alegrias uns dos outros, vibramos juntos... longe ou perto, sempre estamos presentes. Eu torço por vocês, como vocês torcem por mim. Agradeço muito por estarem sempre ao meu lado, e por me darem muito orgulho, a cada dia!
Contem comigo, eternamente!

Ao meu cunhado, **Allison Chivetta**,

Obrigada pela paciência que tem com nossa família. Obrigada por ser como um irmão, por querer o bem, por fazer o bem. Você merece o melhor que a vida pode proporcionar. Obrigada por ser parceiro, sempre!

Aos meus avôs, **Chico Porta**, **Idalina**, **Clair** e **Orávio Benetti** *(in memorian)*,

Obrigada pela alegria e amor incondicional que nos dão; por serem fonte de inocência, gratidão e amor. Quando estou ao lado de vocês, eu tenho paz!
Obrigada por serem a base de nossa família!

Eu amo vocês, minha família!

Agradecimentos

Agradecimento especial...

Ao meu orientador, Prof. Dr. Luciano Tavares Angelo Cintra,

Obrigada, professor, por me orientar não apenas nos projetos, mas na vida. Era ao professor que eu procurava em minhas grandes decisões e, em muitas vezes, era a única opinião que me importava. Obrigada por me ensinar, na clínica, nas salas de aula, no laboratório, nas pesquisas, e de uma forma muito especial, nas conversas. A cada conversa com o professor, era como se eu subisse mais um degrau no aprendizado da vida. Me fazia refletir no mundo e nas pessoas ao meu redor, no que estaria por vir, em quem eu era, mas principalmente, no tipo de pessoa que eu queria ser; me acrescentou honestidade e simplicidade, e me fez crescer muito.

Ter a chance de conhecer e aprender com o professor, é uma sorte, pois tem muito a ensinar, muito além do que cabe à um professor ou orientador... e por isso, te tenho como um "pai" dentro daquele departamento. O professor é meu exemplo de que trabalhar por amor, vale à pena. O gosto pelo o que faz te deixará eternizado no departamento de Endodontia da FOA, tenho certeza disso, pois o professor é espelho, aos alunos e aos demais professores, ainda que tão novo nesta caminhada.

Eu te admiro muito, e tenho muito orgulho por ser, ou ter sido, sua orientada. Torço para o professor, como torço para um amigo muito querido, e desejo que tenha a ciência de que suas conquistas são provas de que está no caminho certo.

Agradeço por ser paciente comigo, quando eu estava impaciente, e por tolerar minhas ansiedades. Deixo aqui meus sinceros e eternos agradecimentos ao professor, que me acolheu em 2011, na Iniciação Científica, me ensinou tudo o que sei, sempre me permitindo ter minha própria opinião; me ajudou, me orientou, me confiou projetos, acreditou em mim, e me incentivou, por pelo menos, até este momento. Minhas conquistas também são suas. Conte comigo sempre! Muito obrigada por tudo, mas principalmente, por ser minha maior inspiração. O professor estará sempre no meu coração!

"Bons pais corrigem erros, pais brilhantes ensinam a pensar."
Augusto Cury

Agradecimentos

À **Faculdade de Odontologia de Araçatuba**.

Agradeço à Faculdade de Odontologia de Araçatuba - FOA, da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, na pessoa seu Diretor Prof. Titular Wilson Roberto Poi e Vice-Diretor Prof. Titular João Eduardo Gomes Filho. Foi na FOA em que dei meus primeiros passos longe de casa. Eu aprendi, eu cresci, eu lutei, eu me superei, eu me desafiei. À FOA eu confiei toda a minha formação. Me deu a base, proporcionando minha formação como Cirurgiã-Dentista; neste caminho, me permitiu a Iniciação Científica, minhas primeiras bolsas, meu ingresso no mestrado, a minha especialidade, e me acolheu até aqui, término do meu doutoramento. Obrigada, FOA, por me dar o orgulho de dizer, onde quer que eu esteja, que vim da Faculdade de Odontologia de Araçatuba, e que sempre carregarei o nome desta instituição comigo, principalmente no meu coração!

Ao **Departamento de Odontologia Restauradora**.

Agradeço a este departamento e a todos que convivem nele, pelo acolhimento desde o início de minha jornada. Agradeço em especial à disciplina e **laboratório de Endodontia**, pela permissão e todo o suporte para realizar "minhas" pesquisas, e hoje, me permitir viver este momento. Há tanto o que agradecer, há tantas pessoas, tantos momentos, tanto crescimento! Fiz amigos, fiz colegas, fiz parceiros, fiz irmãos... Agradeço por todo o aprendizado que tive, e que espero também ter repassado. Foi neste laboratório em que passei a maior parte da minha vida durante esses 4 anos de pós-graduação, e assim se tornou como uma segunda casa para mim. Hoje não há dúvidas de que a Endodontia é parte da minha vida. Obrigada, ao laboratório de Endodontia da FOA, de todo o meu coração!

Agradecimentos

Agradeço à coordenação do **Programa de Pós-graduação em Ciência Odontológica** da Faculdade de Odontologia de Araçatuba – UNESP, representada pelo coordenador Prof. Adj. Luciano Tavares Angelo Cintra, e vice-coordenador Prof. Ass. Juliano Pelim Pessan, pelo impecável trabalho que desenvolvem visando o crescimento do programa.

Agradeço à **Fundação de Amparo à Pesquisa do Estado de São Paulo – Fapesp**, por me conceder bolsas de Iniciação Científica (projeto/renovação de bolsa num. 2011/13709-2, nos anos de 2011 a 2013), Mestrado (projeto num. 2013/25429-0, de março de 2014 a setembro de 2015) e Doutorado (projeto num. 2015/10825-2, de novembro de 2015 até este momento), e fornecer auxílio financeiro para a realização de nossos projetos. Agradeço ainda ao **Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq**, pelo auxílio disponibilizado a meu orientador, que também financiou nossas pesquisas.

Agradecimento aos demais queridos professores, que contribuíram para minha formação e para que este momento acontecesse.

Agradeço a meu coorientador, professor **André Luiz Fraga Briso**.

Professor, obrigada por acreditar tanto em mim! Obrigada por toda a força, e por ser um verdadeiro parceiro de nossas pesquisas! O professor é um dos responsáveis por termos formado esta equipe Endodontia e Dentística, e pela minha introdução nos trabalhos de clareação dentária e efeitos no tecido pulpar, e lhe sou eternamente grata por isso! Foi meu coorientador na Iniciação Científica, Mestrado e Doutorado, fazendo parte de toda minha formação. Obrigada por tudo o que me ensinou nesses anos, pela paciência, mas sobretudo, por todas as oportunidades que me concedeu. Tenho um enorme carinho pelo professor e uma grande admiração! Espero que nossa parceria, junto ao professor Luciano, seja eterna. O professor está no meu coração!

Agradeço ao meu professor e tutor, **Elói Dezan-Júnior**.

Professor, obrigada por todo incentivo, desde a minha graduação até os dias de hoje. Todas as palavras de carinho que o professor já se referiu a mim, ficam guardadas dentro do meu coração, eternamente. Obrigada pelo cuidado, pela atenção, por cada "empurrão" que me dá na vida, querendo que eu avance sempre mais degraus, principalmente na prática clínica. O professor me ensinou a lidar com os alunos da graduação e me permitiu dar as minhas primeiras aulas de verdade. Obrigada por se preocupar com minha saúde, por me chamar a atenção para me alimentar bem... rsrs. Obrigada por ser tão próximo, tão acessível às nossas incertezas, por esclarecer nossas dúvidas mais bobas, e por ser tão querido. Eu aprendi muito com o professor, e saiba que sempre terá um espaço guardado dentro do meu coração!

Agradecimentos

Agradeço ao professor **Edilson Ervolino**.

Professor, não tenho palavras para agradecer tudo o que me ajudou e me ensinou até este momento. Mesmo com tantos afazeres, o professor sempre propôs e aceitou nos auxiliar, sendo quase que como um coorientador nas "minhas" pesquisas de Iniciação Cientifica, Mestrado e Doutorado. Mas hoje agradeço em especial pela colaboração fundamental com este projeto de Doutorado, pelo auxílio na escolha dos marcadores, pelo auxílio durante as análises, até a obtenção dos resultados. Agradeço ainda por todas as vezes que me tranquilizou, quando eu procurava o professor com muitas dúvidas, mas sempre as solucionava rapidamente. Agradeço por me ensinar fazer as análises dos resultados, sempre com muita paciência, desenhando células em folhas de papel. O professor me proporcionou imenso aprendizado e segurança com meus trabalhos, e sou eternamente grata por isso. Obrigada mais uma vez por abrir as portas do departamento de Histologia para desenvolvermos os experimentos, e por toda confiança depositada em mim. Muito obrigada, de todo o meu coração!

Agradeço ao professor **João Eduardo Gomes-Filho**.

Muito obrigada, professor, por toda a paciência, apoio e pelas conversas atenciosas quando nos encontrávamos, seja no departamento ou na clínica de especialização. Obrigada por ser sempre tão gentil, calmo e educado. Uma simples conversa com o professor nos traz uma tranquilidade tamanha, tenha certeza disso. Obrigada por todas as dicas, pelos conselhos e pelas palavras firmes. E desejo de coração, que o professor nunca perca sua gentileza, pois é muito bonito de se ver.

Agradecimentos

Agradeço ao professor **Gustavo Sivieri de Araújo**.

Obrigada, professor, pela parceria no departamento. Por se interessar pelas minhas causas, por me dar apoio, por ser muito prestativo. Obrigada pela atenção e por todos os conselhos, sempre querendo o meu bem. O professor se tornou uma pessoa muito querida, e torço muito para o sucesso do professor. Espero que nunca desista de seus sonhos, assim como teve a coragem de batalhar pelos sonhos que já alcançou. E agradeço de uma forma especial, por nos emprestar o aparelho de Laser que possui, a qual sabemos que tem muita estima, e nos confiá-lo para realizar nossos trabalhos. Obrigada por tudo, sempre!

Agradeço ao professor **Rogério de Castilho Jacinto**.

Obrigada, professor, por compartilhar comigo o "vício" pelo café... rsrs Professor, lhe agradeço por ser sempre solícito. Por confiar em meu trabalho, por me permitir ajudar quando possível, e também pelas ajudas que o professor já me deu, em momentos específicos de minha vida. Agradeço por ser sempre gentil, paciente e compreensível. Agradeço por sempre dizer que tudo dará certo, que está sentindo. Que nosso "vício" pelo café continue nos proporcionando bons momentos de distração.

Agradeço aos demais professores do departamento, em especial ao professor **Ricardo Coelho Okida**, pelas palavras de apoio, pelo interesse, pela força, por ser muito querido; à professora **Ticiane Cestari Fagundes**, pela paciência de sempre, por me permitir ajudar quando possível, e por confiar em meu trabalho; ao professor **Paulo Henrique dos Santos**, por ser sempre muito querido e educado; e ao professor **Mauro Juvenal Nery**, pelas boas lembranças que guardo do professor e pelas palavras de carinho e incentivo que sempre dirigiu a mim durante minha graduação e início do mestrado. Os professores são muito queridos!

Agradecimentos

Agradeço ao professor **Francisley Ávila Souza**.

Obrigada a meu amigo e professor, Francisley. Seu apoio e consideração são muito especiais para mim. Serei eternamente grata por toda confiança que depositou em mim durante a minha graduação, por tudo o que me ajudou, e me ajuda até hoje. Tenho um carinho enorme pelo professor, e espero que sempre continue com este grande coração, que ajuda a todos a seu redor. Obrigada por tudo o que me ensinou! O professor é um grande exemplo de batalhador!

Agradeço aos demais professores que participaram da minha formação, seja na graduação ou na pós-graduação, pelo crescimento profissional e pessoal.

"Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo, e nunca se arrepende."

Leonardo da Vinci

Agradecimento aos funcionários da FOA.

Agradeço à técnica de departamento, amiga, "mãezona", **Nelci Vieira**. Nelzinha, muito obrigada por tudo! Por todo carinho que me proporciona, por sempre cuidar de mim. Obrigada pela paciência, pelos altos papos, por toda a consideração. Obrigada por todos os lanchinhos que você leva especialmente a mim no departamento, esconde na geladeira, ou fala bem alto que é para mim, para que ninguém tome posse... rsrs. Obrigada pelas vezes que não se importou pela minha impaciência, e por puxar minha orelha quando necessário. Obrigada, Nelzinha, por fazer eu me sentir em casa, por ter me ensinado tanto, e por confiar em mim! Você está no meu coração para sempre, tenha certeza disso!

Agradeço ao secretário do departamento, **Peterson**, por sempre me receber bem. Por perceber minhas ausências, por se importar, e ser sempre presente. Obrigada pela paciência, por tudo o que me ajuda, e pelas palavras de incentivo e força!

Agradeço ao técnico do departamento e parceiro, **João Rafael Amadeu**. Rafa, obrigada por ter se tornado uma pessoa tão querida. Obrigada por todas as caronas, pela companhia, e por levar um pouquinho mais de alegria ao nosso departamento. Desejo que tenha um futuro brilhante!

Agradeço às funcionárias da limpeza do departamento, **Andressa** e **Grazi**, pela simpatia comigo. Aos funcionários do biotério Central da FOA, **Camilo** e **João**, por toda paciência e compreensão. Aos porteiros da FOA, em especial, **Márcio** e **Carlão**, por se preocuparem, por me ajudarem, por torcerem por mim, e por compartilharem comigo momentos fora de hora na faculdade. Aos funcionários do **Staepe**, por serem extremamente prestativos, educados e atenciosos.

Agradeço às meninas da seção de pós-graduação, **Cristiane Lui Matos**, **Valéria de Queiroz M. Zagatto**, e **Lilian Sayuri Mada**, por toda a atenção, paciência e dedicação!

Agradecimentos

Agradecimento a meus eternos amigos e aos colegas de pós-graduação.

Deixo meus agradecimentos àquelas que estiveram ao meu lado durante a graduação, e que continuam comigo até os dias de hoje. **Rogéria Apda Agos Felipe** e **Bruna Barbieri Martins Borges**, muito obrigada! Obrigada por serem pessoas as quais eu sei que posso confiar de olhos fechados. Por quererem o meu bem, por lutarem por mim, por me apoiarem, por me ouvirem, me darem conselhos. Vocês são verdadeiros anjos em minha vida!

Obrigada, Ro, por toda sua amizade, compreensão, e por sempre me ouvir. Você sabe de todos os meus anseios, de todas as minhas angústias, de todas as minhas vitórias, assim como eu sei de todas as suas batalhas e todas suas conquistas. Sou eternamente grata por toda a paciência que tem comigo.

Obrigada, Feia, por me acolher, me aconselhar, por ser tão simples e tão determinada. Por dividir comigo casos de Endodontia, pelas nossas sábias discussões, e por compartilhar comigo seus maiores momentos. Eu não tenho palavras para te agradecer tudo o que faz por mim!

Vocês são verdadeiras irmãs, que sempre me ensinam, com gestos, com exemplos, e com palavras. Eu as admiro muito! Amo vocês!

Ao meu grande e querido amigo, **Leopoldo Cosme Silva**.

Leozinho, eu nunca poderia imaginar que nossa amizade cresceria tanto. Você que chegou ao departamento movimentando a todos, ocupando grande parte do "meu tempo", mas que aos poucos foi ocupando grande parte no meu coração, e no de cada um que convive com você. Obrigada, Leozinho, por ter se tornado tão importante na minha vida! Por ser aquele que me escuta, que me aconselha, que me alegra, que me faz rir... aquele que é parceiro para todas as horas, que se preocupa, que está SEMPRE ao meu lado, compartilhando várias "consultorias"! Eu não queria que você me fizesse falta nunca, porque queria você comigo sempre! Torço MUITO por você, por seus sonhos! Quero te ver crescer ainda mais do que já cresceu, quero ver você

conquistar o mundo, porque você é capaz disso! Você é como um irmão para mim, e eu amo você! Conte comigo para tudo, sempre, eternamente!

Às minhas grandes e eternas amigas do coração, **Vanessa Abreu Sanches Marques** e **Letícia Citelli Conti** .

Van, amiga, eu não tenho palavras para te agradecer por tudo! Eu não mereço ter ao meu lado alguém com coração tão bom e tão grande como o seu! Você é uma das pessoas mais verdadeiras que já conheci. Obrigada por me transmitir muita paz, harmonia, simplicidade e confiança extrema! Por ser alguém que eu sempre possa confiar, sem receios, sem medos. Por querer o bem de todos, e por nos ensinar sempre! Você nos ilumina! Obrigada por me permitir ser sua amiga! Eu amo você, migs!

Lezinha, obrigada por ter sido minha verdadeira parceira. Nossa amizade começou lentamente, e hoje já tem um tamanho enorme! Você me ajudou tanto, em tudo que precisei. Você foi e sempre será a melhor vizinha que já tive! Obrigada por tudo o que compartilhamos nesses últimos anos. Nossos desabafos, nossas conquistas, nossas tristezas, alegrias, nossos cafés, e nossas comidas... rsrs. Você me ensinou muito, com sua extrema educação e preocupação com os que estão ao seu redor. Eu amo você!

Agradeço em especial por vocês acreditarem mais em mim, do que eu mesma! Vocês foram as que mais me apoiaram e me deram forças nesses últimos anos. Graças ao apoio de vocês eu venci vários desafios, e espero que um dia possa retribuir tudo o que fizeram por mim. Vocês são as amigas que a Endodontia me deu!

Agradeço ao meu amigo e grande parceiro, **Carlos Roberto Emerenciano Bueno**.

Bueno, obrigada pelo companheirismo. Você é meu irmãozinho de Mestrado, que me acompanhou até este momento. Você me salvou de diversas situações (diversas!), você me ajudou, me ouviu, pegou minhas dores... você torna tudo mais simples sempre, descomplica, me anima, me apoia. Eu te admiro muito, pelo seu amor pela Endodontia, por sua simpatia, por sua

determinação. Obrigada por estar comigo desde o início, por ser meu parceiro de disciplinas, pelas nossas discussões construtivas no departamento, pelas trocas de experiência, e por me ensinar sempre! Você é um amigo que carrego no peito, e que levarei para sempre!

Agradeço à minha parceira, irmã e amiga, **Marjorie de Oliveira Gallinari**.

Obrigada, Jo! Iniciamos como amigas de experimento, e do nada, nossa amizade cresceu a um nível, que trato você como trato minha irmã (felizmente ou infelizmente... rsrs). Obrigada por me aturar, por dividir comigo nossos trabalhos, nossas pesquisas, nossos orientadores, e assim, as mesmas preocupações e as mesmas experiências. Obrigada por tudo o que me ensinou, me ajudou e me apoiou. Obrigada por permitir que nossa amizade fosse além do departamento, e que nos trouxesse ótimos momentos juntas. Torço muito para seu sucesso, e te admiro muito! Saiba que tem aqui uma amiga em que pode confiar e contar para toda a vida! Amo muito você!

Agradeço aos demais amigos do departamento de Odontologia Restauradora...

Amanda Caselato Andolfatto. Obrigada, Mandinha, por sua amizade! Obrigada por me transmitir alegria, sempre! Pelo seu alto astral, pelos seus abraços, e por tudo que compartilhamos no departamento. Você é extremamente querida para mim, e mora no meu coração! Amo você!

Renan Dal Fabbro. Obrigada, Re, pela parceria e amizade! Por ser uma pessoa sempre plena e calma. Obrigada pelos nossos momentos na especialização e no departamento. Torço muito para que alcance seus sonhos e saiba que tenho um carinho muito grande por você! Conte comigo sempre!

Morganna Borges de A. Souza. Obrigada, Morgs, pela sua amizade, sinceridade e companheirismo! Por sempre me ajudar quando precisei, e por ser alguém em quem eu possa confiar! Gosto muito de você!

Agradecimentos

Vanessa Rahal. Obrigada, Van, pela sua amizade! Obrigada por tudo o que compartilhamos juntas, pelos experimentos na histologia, pela parceria, por sempre torcer pela gente. Você está em meu coração!

Agradeço aos colegas e amigos **Camila Ambrósio Dias**, **Gabriely Cristinne Rezende**, **Bruno Guandaline Cunha (Jarbas)**, **Karina Sampaio Caiaffa**, **Ana Carolina**, **Úrsula Escalero Silva**, e **Caio Pavani**, pelo ótimo convívio de sempre, por todas as experiências, e pelas parcerias.

Agradeço àquelas que entraram posteriormente na pós-graduação, **Marina Talomey Cury**, **Ana Maria Veiga Vasques, Marina Carminatti, Carolina de Barros M. Cardoso, Caroline Loureiro,** e **Flavia Alfredo Plazza.** Torço para que cada uma atinja seus objetivos e alcance seus verdadeiros sonhos. Obrigada pela paciência de vocês comigo! Agradeço também ao **Pedro Chaves de Oliveira**, por me permitir ajudá-lo, por toda confiança depositada em mim, por todas as palavras de incentivo e carinho. Torço muito para que você seja feliz no caminho que escolheu. Conte comigo para o que precisar!

Agradeço aos que estavam na pós-graduação, e que me receberam no departamento ainda como aluna de Iniciação Científica. **Cristine Men**, **Annelise, Aguinaldo Facundo**, **Renata Oliveira, Mariane** e **Luciana Louzada**. Agradeço em especial à Lu, que foi minha parceira durante meu Mestrado. Obrigada por tudo!

Agradeço em especial às colegas **Índia Olinta de Azevedo Queiroz** e **Loiane Massunari**, pela parceria e por me ensinarem experimentos com cultura celular. Sou eternamente grata a vocês por isso, e por toda a paciência. Torço para que o futuro de vocês seja brilhante!

Agradeço ao meu colega e amigo, **Diego Valentim**! Obrigada, "Inútil", por tudo que me ajudou. Pelas vezes que em acolheu, me escutou, me aconselhou. Obrigada por tudo o que me ensinou,

desde a graduação, na Endodontia, e depois da pós-graduação. Obrigada por me ajudar dentro e fora do departamento. Você é muito querido para mim! Conte comigo, sempre!

Agradeço às minhas amigas do departamento de Odontopediatria da FOA, em especial, à **Lais Salomão Arias.** Obrigada, La, por ter sido sempre uma grande parceira, seja de espetinhos, seja de disciplinas, ou de representante discente da pós-graduação. Obrigada por ter sido minha amiga na maior viagem que já fiz (IADR – San Francisco), por ter me ajudado grandemente, e por ser companheira de desabafos. Eu não tenho palavras para te agradecer por tudo! Você é muito especial!

Agradeço a minha amiga, parceira, e dupla de especialização, **Marcela Ito Rey**, pela sua amizade, paciência e compreensão. Te admiro muito pela sua força, garra e determinação! Torço muito por você e desejo que seja muito feliz! Conte comigo sempre, amiga!

Agradeço aos meus amigos que se fazem sempre presente, **Cleidiel Araújo Lemos**, e **Fernanda Bernardi**. Vocês são especiais! Obrigada pela amizade sincera e pela torcida eterna! Cada um, de sua forma, é um grande exemplo para mim. Eu os admiro e os amo MUITO! Contem comigo eternamente!

Agradeço às esposas de meus professores, **Milena de Oliveira**, e **Vanessa Braga Dezan**. Vocês são pessoas extremamente queridas! Obrigada, Mi, por sempre nos receber na sua casa com tanta paciência, pelo seu alto astral, por suas conversas sempre animadas! Obrigada, Van, por se preocupar, por todos bolinhos de padaria que me deu, e por todas as vezes que abriu as portas de sua casa. Vocês ajudam aumentar os laços dentro do departamento! Obrigada por tudo!

Agradeço ao professor **Carlos Alberto de Souza Costa**, e à professora **Diana Gabriela Soares**, por terem aberto as portas do departamento de Patologia da Faculdade de Odontologia de

Araraquara, para que eu pudesse aprender um pouco da metodologia que desenvolvem. Obrigada, professor Carlos, por ser extremamente receptivo e atencioso! Sempre tive enorme admiração pelo professor! Obrigada, professora Diana, por ser espelho a todos. Saiba que o meu grande exemplo de pós-graduanda sempre foi você! Obrigada pelas conversas, conselhos, e tudo o que me ensinou. Te admiro grandemente!

E finalizo fazendo um agradecimento especial àquelas que tanto me ajudam, e que me dão o gostinho do que é ser "professor-orientador", "minhas" alunas de Iniciação Científica, **Juliana Maria de Araújo Lopes**, **Amanda Miyuki Terayama** e **Gabriela Aparecida Ramos**. Obrigada por serem minhas amigas, minhas parceiras, e muitas vezes, minhas confidentes. Eu fico feliz a cada conquista de vocês e a cada crescimento que temos juntas! Vocês superam minhas expectativas, e fazem jus às bolsas de vocês, o que me deixa muito orgulhosa! Obrigada por fazerem sempre mais! Obrigada pelas palavras amigas, por torcerem por mim! Eu adoro vocês, e saibam que podem contar comigo eternamente!! Agradeço também àquelas que já finalizaram seus projetos, e que foram as "minhas" primeiras orientadas, **Jéssica Galbiati**, **Lidiane Louzada**, e **Marina Carminatti**. Meu aprendizado com vocês foi eterno. Deixo meus agradecimentos também aos alunos Pibic Júnior, **Thalita, Leticia, Anna, Isabela, Renan e Suelen**, que me trouxeram e trazem imensa alegria!

Saibam que vocês moram no meu coração, e que estarei aqui para o que precisarem, sempre!

“Primeiro, lembre-se de olhar para as estrelas lá no alto, e não para os seus pés lá embaixo.
Depois, nunca desista do seu trabalho.
O trabalho lhe dá sentido e propósito, e a vida é vazia sem isso.”

Stephen Hawking

BENETTI, F. **Influência do peróxido de hidrogênio sobre a diferenciação celular e a mineralização do tecido pulpar após procedimento clareador. Estudo histológico e imunoistoquímico.** [Tese]. Faculdade de Odontologia, UNESP - Universidade Estadual Paulista. Araçatuba. 2018.

Resumo

Introdução Através de modelo experimental caracterizado por nosso grupo de pesquisa, e protocolo clareador adaptado, verificamos que o peróxido de hidrogênio (H_2O_2) contido no gel clareador pode gerar efeitos ao tecido pulpar, que ainda não estão completamente compreendidos. Outros estudos mostram uma indução à mineralização, levando à posterior calcificação de grande parte do tecido pulpar e à formação de nódulos.

Objetivos Os objetivos deste trabalho foram divididos em duas etapas:

1 – Verificar os efeitos do H_2O_2 na expressão de marcadores da mineralização no tecido pulpar, por meio da imunomarcação de osteocalcina (OCN) e osteopontina (OPN); e a presença de resposta celular específica ao estresse oxidativo, por meio de imunomarcação com anticorpo para espécies reativas de oxigênio (EROs);

2 – Determinar a capacidade de resposta ao estresse oxidativo gerado pelo H_2O_2 no tecido pulpar, por meio da imunomarcação de Heme-oxigenase-1 (HO-1); investigar os efeitos do gel clareador sobre a diferenciação odontoblástica, por meio da imunomarcação do fator de transcrição Jun-D; e a influência do estresse oxidativo gerado pelo H_2O_2 na identificação de células-tronco mesenquimais (CTMs) do tecido pulpar, por meio da técnica de imunofluorescência, com identificação concomitante de positividade celular para CD90, CD73, CD105 e negatividade para CD45.

Materiais e métodos Foram utilizados 60 ratos Wistar que tiveram os molares superiores direitos ou esquerdos clareados com 0,01 mL de H_2O_2 35%, em uma aplicação de 30 minutos, de forma randomizada. Os molares do lado não clareado serviram de controle. Após 0 horas, 2, 3, 7, 15 e 30 dias (n=10), os animais foram mortos e as maxilas processadas para avaliação histológica, imunoistoquímica (OCN, OPN, EROs, HO-1 e Jun-D) e de imunofluorescência (CD90, CD73, CD105, CD45). Os resultados foram submetidos a testes estatísticos específicos ($p<0,05$).

Resultados No tempo de 0 horas, houve necrose em toda a polpa coronária ($p<0,05$), e aos 2 e 3 dias, no terço oclusal ($p<0,05$); aos 7, 15 e 30 dias, não houve inflamação, assim como no controle ($p>0,05$). Dentina terciária estava presente aos 7 dias, aumentando em 15 e 30 dias ($p<0,05$). Em relação aos marcadores de mineralização, OCN foi ausente imediatamente após procedimento clareador, aumentando ao longo dos períodos, se tornando significativa aos 15 e 30 dias ($p<0,05$); OPN apresentou maior imunomarcação aos 7 e 15 dias no grupo clareado ($p<0,05$). A imunomarcação de EROs foi significativa em todos os terços da polpa coronária no grupo clareado aos 7 e 15 dias, e no terço cervical aos 2 e 30 dias, comparada ao controle ($p<0,05$). HO-1 revelou maior imunomarcação no grupo clareado nos terços médio e cervical da polpa coronária aos 2 e 3 dias, em todos os terços aos 7 dias, e no terço oclusal aos 15 dias, quando comparado ao grupo controle ($p<0,05$). Imunomarcação nuclear para Jun-D foi significativa no grupo clareado no terço cervical da polpa coronária aos 2 e 3 dias, e nos terços oclusal e médio aos 7 dias, quando comparado ao grupo controle ($p<0,05$), reduzindo nos demais períodos ($p>0,05$). Poucas células CD90+/CD73+/CD105+/CD45- foram observadas no tecido pulpar do grupo controle e do grupo clareado em todos os períodos de análise ($p>0,05$).

Conclusões Pode-se concluir que:

1 – A redução da inflamação e o processo de reparo pulpar após procedimento clareador está associado com o aumento de OCN, e OPN participa durante o processo de reparo; EROs está presente no processo de defesa celular contra o estresse oxidativo decorrente do H_2O_2.

2 – As células pulpares apresentam capacidade de resposta ao estresse oxidativo expressando HO-1 nos períodos onde há inflamação, até o início do reparo; Jun-D é presente no tecido pulpar durante a redução do processo inflamatório e início da produção de dentina terciária; a presença de estresse oxidativo não influencia o número de células CD90+/CD73+/CD105+/CD45- identificadas *in vivo* no tecido pulpar.

Palavras-Chave Clareação dentária, estresse oxidativo, resposta pulpar, peróxido de hidrogênio, dentinogênese, células-tronco.

BENETTI, F. **Influence of hydrogen peroxide on cell differentiation and mineralization of pulp tissue after bleaching. Histological and immunohistochemical study.** [Thesis, PhD]. School of Dentistry, UNESP - São Paulo State University. Araçatuba, SP, Brazil. 2018.

Abstract

Introduction Through an experimental model characterized by our research group, and adapted bleaching protocol, we verified that the hydrogen peroxide (H_2O_2) of bleaching gel can generate effects on the pulp tissue, which are not yet completely understood. Studies show an induction to mineralization, leading to subsequent calcification of a large part of the pulp tissue and to the formation of nodules.

Objectives The objectives of this study were divided into two stages:

1 – To verify the effects of H_2O_2 on the expression of mineralization markers in pulp tissue, through of immunolabelling of the osteocalcin (OCN) and osteopontin (OPN); and the presence of specific cellular response to oxidative stress, by immunolabeling with antibody to reactive oxygen species (ROS);

2 – To determine the capacity of response to oxidative stress generated by H_2O_2 in pulp tissue, through of immunolabelling of Heme-oxigenase-1 (HO-1); to investigate the effects of the bleaching gel on the odontoblastic differentiation, through the immunolabelling of the transcription factor Jun-D; and the influence of the oxidative stress generated by H_2O_2 on the identification of mesenchymal stem cells (MSCs) of the pulp tissue by the immunofluorescence technique, with the concomitant identification of cellular positivity for CD90, CD73, CD105 and negativity for CD45.

Materials and methods Sixty Wistar rats were used, and the right or left upper molars were bleached with 0.01 mL of 35% H_2O_2, in a direct application of 30 minutes, randomly. The molars on the unbleached side served as controls. After 0 hours, 2, 3, 7, 15 and 30 days (n=10), the animals were killed and the jaws processed for histological, immunohistochemical (OCN, OPN, ROS, HO-1 and Jun-D) and immunofluorescence (CD90, CD73, CD105, CD45) analysis. The results were submitted to specific statistical tests ($P<0.05$).

Results At 0 hours, there was necrosis throughout the coronary pulp ($P<0.05$), and at 2 and 3 days, in the occlusal third ($P<0.05$); at 7, 15 and 30 days, there was no inflammation, as well as in the control ($P>0.05$). Tertiary dentin was present at 7

days, increasing in 15 and 30 days (P<0.05). In relation to mineralization markers, OCN was absent immediately after bleaching procedure, increasing over the periods, becoming significant at 15 and 30 days (P<0.05); OPN has higher immunolabelling at 7 and 15 days in the bleached group (P<0.05). Immunolabelling of ROS was significant in all thirds of the coronary pulp in the bleached group at 7 and 15 days, and in the cervical third at 2 and 30 days, compared to the control group (P<0.05). HO-1 showed higher immunolabelling in the bleached group in the middle and cervical thirds of the coronary pulp at 2 and 3 days, in all thirds at 7 days, and in the occlusal third at 15 days, when compared to the control group (P<0.05). Nuclear immunolabelling for Jun-D was significant in the bleached group in the cervical third of the coronary pulp at 2 and 3 days, and in the occlusal and middle thirds at 7 days, when compared to the control group (P<0.05), reducing in the other periods (P>0.05). Low number of CD90+/CD73+/CD105+/CD45- cells were observed in the pulp tissue of the control group and the bleached group in all periods of analysis (
P>0.05).

Conclusions It is concluded that:

1 – The reduction of inflammation and the pulp repair process after bleaching is associated with increased of OCN, and OPN participates during the repair process; ROS are present in the cellular defence process against oxidative stress by H_2O_2.

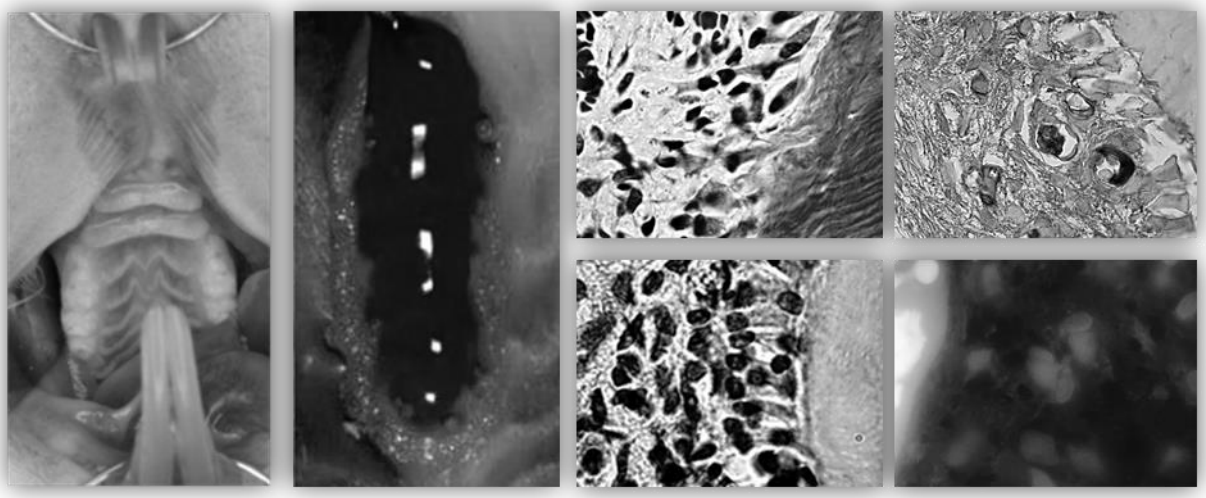
2 – The pulp cells had capacity to respond to oxidative stress expressing HO-1 in the periods where there is inflammation, until the beginning of the repair; Jun-D is present in the pulp tissue during the reduction of the inflammatory process and the beginning of the production of tertiary dentin; the presence of oxidative stress does not influence the number of CD90+/CD73+/CD105+/CD45- cells identified *in vivo* in the pulp tissue.

Keywords dental bleaching, oxidative stress, pulp response, hydrogen peroxide, dentinogenesis, stem cells.

S U M Á R I O

Páginas

1. Introdução e justificativa



I. Introdução e justificativa

A estética do sorriso tem atraído cada vez mais a atenção do público, levando a um aumento na busca pelo procedimento clareador dentário (Briso *et al*. 2015, Calderini *et al*. 2016). Apesar de ser um tratamento considerado conservador quando comparado a outras formas de tratamento para se obter dentes mais claros, esse procedimento causa alterações no tecido pulpar que ainda não estão completamente compreendidas (Seale *et al*. 1981, Lee *et al*. 2006a, Min *et al*. 2008, Costa *et al*. 2010, Cintra *et al*. 2013).

Estudos mostraram que o peróxido de hidrogênio (H_2O_2), componente ativo do gel clareador, pode causar alterações morfológicas na superfície dentária, como a redução da microdureza do esmalte e aumento da rugosidade (Chen *et al*. 2008, Briso *et al*. 2015). Ainda, espécies reativas de oxigênio (EROs), liberadas a partir do H_2O_2, possuem a capacidade de atravessar esmalte e dentina, levando a alterações no tecido pulpar, como inflamação, diminuição da celularidade e do metabolismo celular (Seale *et al.* 1981, Min *et al.* 2008, Cintra *et al.* 2013, Soares *et al.* 2014, Cintra *et al*. 2016a), além de desnaturação proteica (Caviedes-Bucheli *et al.* 2006, Camargo *et al.* 2007), e necrose tecidual (Costa *et al.* 2010, Cintra *et al.* 2013, Cintra *et al.* 2017, Benetti *et al*. 2018).

Em um estudo inicial, observamos que após uma sessão clareadora com alta concentração de H_2O_2 (3 aplicações de 15 min cada, H_2O_2 35%) o tecido pulpar de molares de ratos apresentava inflamação severa e áreas de necrose. Estes danos se intensificaram a medida que o número de sessões clareadoras foi aumentado (Cintra *et al.* 2013). Posteriormente, notamos que mesmo após intensa agressão pulpar, o tecido se apresentou organizado aos 30 dias do procedimento clareador (Cintra *et al*. 2016b). Entretanto, grande área da câmara pulpar estava ocupada por dentina terciária, sendo diretamente proporcional ao aumento da concentração e do tempo de aplicação do H_2O_2 sobre o esmalte dentário (Cintra *et al*. 2016b).

Observa-se que as células pulpares possuem a capacidade de promoverem a formação de tecido duro após o contato com o H_2O_2 (Hanks *et al.* 1993, Lee *et al.* 2006b, Matsui *et al.* 2009). Acredita-se que em baixas doses, o H_2O_2 seja capaz de estimular as células a se proliferarem e executarem suas funções (Burdon *et al.* 1989, Burdon *et al.* 1995, Wiese *et al.* 1995, Davies 1999), atuando como um agente

de sinalização para a mitose celular (Davies 1999), com papel importante na transdução do sinal que modula o comportamento das células (Chang & Karin 2001, Forman & Torres 2001, Liu *et al.* 2002, Runchel *et al.* 2011, Wu *et al.* 2013).

A imunomarcação do Antígeno Nuclear de Proliferação Celular (PCNA) revelou uma grande quantidade de células em proliferação no tecido pulpar de dentes clareados, principalmente nos terços mais profundos da polpa coronária (Benetti *et al.* 2017a). A presença de células apoptóticas no tecido pulpar também foi observada (Benetti *et al.* 2017a). A apoptose é crítica para o desenvolvimento dos tecidos e da recuperação destes após estímulos internos e externos (Vermelin *et al.* 1996, Mitsiadis & Rahiotis 2004, Mitsiadis *et al.* 2008). Assim, pode desempenhar papel na minimização da lesão à polpa dentária (Wu *et al.* 2013), e na formação de dentina terciária, durante um processo carioso, por exemplo, proporcionando espaço para a nova dentina e impedindo a ocorrência de necrose na tentativa de minimizar a inflamação (Mitsiadis *et al.* 2008).

Ainda, observamos que as citocinas pró-inflamatórias Fator de Necrose Tumoral (TNF)-α, interleucina (IL)-6 e IL-17 (Ferreira *et al*. 2017, Benetti *et al*. 2018) participam do processo inflamatório ocorrido no tecido pulpar após clareação dentária, particularmente nos períodos iniciais. Em adição, uma quantidade significativa de células positivas para o receptor CD5, presente em todos os timócitos ou linfócitos T maduros (Lozano *et al.* 2000, Brown & Lacey 2010, de Wit *et al.* 2011), foi observada nos períodos iniciais após o procedimento clareador, permanecendo nos períodos mais tardios quando alta concentração de H_2O_2 foi utilizada (Benetti *et al*. 2018). Estes resultados nos permitem concluir que o tecido pulpar tem a capacidade de se recuperar mesmo após os danos severos gerados pelo H_2O_2, mas os mecanismos celulares que envolvem esse processo são pouco conhecidos, e pouco se sabe sobre as consequências a longo prazo que o H_2O_2 pode ocasionar ao tecido pulpar.

É possível investigar *in vivo* o momento em que as células pulpares apresentam capacidade de defesa frente ao estresse oxidativo gerado pelo H_2O_2, e indiretamente, o período de permanência do estresse oxidativo no tecido pulpar. Para isso, podem ser utilizadas imunomarcação para Heme-oxigenase-1 (HO-1) e EROs. A HO-1 é uma proteína celular induzida em resposta ao estresse oxidativo, e está presente em vários mecanismos celulares reguladores, tendo função

importante na citoproteção e homeostase dos tecidos (Otterbein & Choi 2000, Cooper *et al.* 2007, Pae & Chung 2009). Tem capacidade de gerar monóxido de carbono (CO) para suprimir a sinalização inflamatória, e biliverdina e bilirrubina, que possuem ação antioxidante (Min *et al.* 2008, Pae & Chung 2009, Min *et al.* 2010).

Foi demonstrado que monômeros de resina são capazes de estimular a expressão de HO-1 via produção de EROs em células pulpares (Schweikl *et al.* 2004, Chang *et al.* 2005, Chang *et al.* 2009, Eckhardt *et al.* 2009), e que HO-1 desempenha papel fundamental na adaptação das células da polpa dentária humana às condições de estresse (Min *et al.* 2006, Min *et al.* 2006a, Lee *et al.* 2007, Pi *et al.* 2007, Min *et al.* 2008, Kook *et al.* 2009, Lee *et al.* 2009).

Outros estudos mostraram que HO-1 foi expressa em odontoblastos e células endoteliais após procedimento clareador em pré-molares humanos (Anderson *et al.* 1999). Segundo os autores, os odontoblastos da polpa coronária e células endoteliais subjacentes podem ter potencial de resposta ao estresse oxidativo aumentando a síntese de HO-1, representando uma resposta defensiva inicial, que antecedem vias inflamatórias clássicas.

Assim, a expressão de HO-1 em células do tecido pulpar de dentes clareados, pode indicar que estas células estejam sofrendo estresse oxidativo, causado pela presença de EROs derivadas do gel clareador, tendo a capacidade de se adaptarem através de mecanismos para sua proteção.

Ainda, a extensão de células atingidas pelas EROs liberadas pelo H_2O_2 também pode ser avaliada por meio de marcadores específicos de DNA de células danificadas pelas EROs, os anti-EROs. Há estudos na literatura que mostraram ser capaz a ação desses anticorpos em células atingidas pelo H_2O_2 (Ashok *et al.* 1997, Ashok *et al.* 1998, Mistry *et al.* 1999, Strollo *et al.* 2013), mas não encontramos estudos *in vivo* que avaliaram a extensão de células afetadas pelo H_2O_2 no tecido pulpar, após clareação dentária. Identificar estas marcações em cortes histológicos pode nos mostrar a real capacidade de penetração das EROs neste tecido e a extensão do tecido afetado após procedimento clareador.

Estudos mostraram que os danos causados pelo H_2O_2 do gel clareador ao tecido pulpar são posteriormente reparados, com a formação de novas células odontoblastóides e extensa formação de dentina terciária (Cintra *et al*. 2016b, Benetti *et al*. 2018). Assim, pode-se supor que ocorra alterações na expressão de

proteínas envolvidas no processo de dentinogênese, como osteocalcina (OCN) e osteopontina (OPN), e na expressão de fatores de transcrição envolvidos na diferenciação odontoblástica, como por exemplo, Jun-D. Ainda, células-tronco mesenquimais (CTMs) também podem atuar neste processo de reparo. No entanto, estudos são necessários para que se compreenda os mecanismos celulares envolvidos na reparação do tecido pulpar após clareação dentária.

Ao sofrer uma injúria, como por exemplo uma injúria química (caso do H_2O_2 do gel clareador), o tecido pulpar tem a reação defensiva de produzir dentina terciária (Torneck 1998). Quando o dano ocasionado ao tecido pulpar é leve, os próprios odontoblastos presentes no local são os responsáveis pela produção da dentina terciária, que recebe o nome de dentina reacional. Quando os danos ocorrem de forma severa, danificando os odontoblastos presentes, as células-tronco do tecido pulpar são recrutadas para a produção de novas células odontoblastóides, que respondem à injúria produzindo a dentina reparadora, o segundo sub-tipo de dentina terciária (Fitzgerald *et al.* 1990, Tornec 1998, Lee *et al.* 2006, Yuan *et al.* 2012). Assim, as células diferenciadas e não diferenciadas da polpa dentária podem contribuir para o processo de produção de dentina terciária (Agata *et al.* 2008).

Vários fatores de transcrição nucleares são considerados relevantes para a osteoblastogênese (Karsenty & Wagner 2002, Colucci *et al.* 2011). O gene Jun-D é conhecido por ter um papel chave no controle da diferenciação de osteoblastos (McCabe *et al.* 1995, Colucci *et al.* 2011), e foi demonstrado em osteoblastos completamente diferenciados (McCabe *et al.* 1996). Com base nas semelhanças morfológicas e funcionais entre osteoblastos e odontoblastos (TenCate 1998), muitos pesquisadores utilizam marcadores de diferenciação osteoblástica como marcadores terminais de odontoblastos (Hirata *et al.* 2005).

A expressão de Jun-D e osteocalcina (OCN) foi analisada na fase inicial de formação de dentina reacionária em ratos após a realização de um preparo do dente (Hirata *et al.* 2005), e Jun-D foi temporariamente expresso nos núcleos dos odontoblastos, em um e dois dias após o preparo. Já a OCN foi sintetizada e segregada para a matriz de dentina reacionária três dias após o preparo. Assim, a expressão de OCN está relacionada com a formação de dentina reacionária, e Jun-D está associado com a expressão de OCN em odontoblastos. Considera-se,

portanto, que Jun-D atua tanto como um fator de transcrição que controla a expressão de OCN, como um marcador de diferenciação de odontoblastos (Owen *et al.* 1990, Ducy *et al.* 1997, Kern *et al.* 2001).

A OCN, uma das diversas proteínas da linhagem dos osteoblastos, é expressa em odontoblastos envolvidos na formação da matriz de dentina reacionária (Hirata *et al.* 2005). Pode ser observada nos odontoblastos, em seus processos, e na matriz de dentina, mas não em pré-dentina (Matsui *et al.* 2009), o que indica que seja sintetizada dentro dos odontoblastos, transportada através de seus processos, e depositada na matriz de dentina (Bronckers *et al.* 1985, Okabe *et al.* 2006, Matsui *et al.* 2007, Wei *et al.* 2007, Matsui *et al.* 2008). Assim, a OCN é secretada por odontoblastos maduros e recém-formados, mas não está presente na pré-dentina (Matsui *et al.* 2009), o que nos leva a entender que a marcação de OCN antecede a mineralização da dentina (Yao *et al.* 1994).

Outra proteína que também desempenha papel importante nos processos que conduzem à mineralização é a osteopontina (OPN) (Matsui *et al.* 2009), uma glicoproteína fosforilada multifuncional (Gericke *et al.* 2005) presente na diferenciação dos odontoblastos, no crescimento e na regeneração óssea (Sodek *et al.* 2000, Matsui *et al.* 2009). Acredita-se que a OPN regula eventos inicias de remodelação óssea, tais como a adesão celular, o funcionamento dos osteoclastos, e a mineralização da matriz (Sodek *et al.* 2000). Ainda, pode ligar-se a uma grande quantidade de cálcio (Sodek *et al.* 2000), e, junto com a OCN, formar os cristais de hidroxiapatita (Gericke *et al.* 2005). Assim, tem papel não apenas na fase inicial de formação, mas também durante a calcificação da dentinogênese reparativa (Kuratate *et al.* 2008).

Assim, a dentina formada pelo tecido pulpar após procedimento clareador é constituída em parte, por dentina reparadora, formada por células odontoblastóides recém diferenciadas, e em parte, por dentina reacional, formada pelos odontoblastos presentes nos terços médio e cervical da polpa coronária, que produziram dentina como forma de defesa ao estresse oxidativo. Com a utilização de anti-OPN, acreditamos que esta proteína poderá ser expressa antes da expressão dos marcadores Jun-D e OCN, indicando a produção de dentina reacional, e expressa também após a marcação destes, indicando a formação de dentina reparadora.

Mesmo com a expressão destes marcadores imunoistoquímicos, acreditamos que a identificação de CTMs seja necessária para auxiliar no esclarecimento dos mecanismos que envolvem a dentinogênese e a proliferação celular após estresse oxidativo ao tecido pulpar pelo procedimento clareador.

Possivelmente, a menor concentração ou quantidade de H_2O_2 que atinge os terços médio e cervical da polpa coronária, seja capaz de ativar as células-tronco lá existentes, para que estas se diferenciem em novas células. Esta análise poderia nos levar ao entendimento de que o H_2O_2 do gel clareador possa ser capaz de ativar as células mesenquimais indiferenciadas em reposta ao estresse oxidativo gerado, o que levaria a um processo de reparo, mas também de envelhecimento precoce, levando a menor capacidade de reparo durante uma próxima injúria, uma vez que um grande número de CTMs, que seria utilizado durante toda a vida do elemento dentário, estaria sendo perdido (Basso *et al.* 2013, Soares *et al.* 2014).

Quando odontoblastos são perdidos devido às agressões ao dente, estes podem ser substituídos por células odontoblastóides derivadas de células indiferenciadas mesenquimais, ou CTMs, que residem na polpa, e também podem ser consideradas como células-tronco da polpa dentária (DPSC) (Gronthos *et al.* 2000, Gronthos *et al.* 2002), essenciais para a homeostasia do tecido pulpar.

As DPSC foram referidas como tendo uma grande capacidade de proliferação e de diferenciação (Nosrat *et al.* 2001, Tash *et al.* 2013), sendo capazes de gerar o complexo dentino-pulpar, o que conduz a sua utilização na regeneração deste complexo em aplicações clínicas durante um tratamento biológico para reparo do tecido pulpar danificado (Nakashima *et al.* 2004, Nakashima & Akamine 2005, Cordeiro *et al.* 2008, Huang 2009, Huang *et al.* 2009, Huang *et al.* 2009a).

Vários estudos têm documentado o potencial osteogênico *in vitro* e *in vivo* das DPSC (Laino *et al.* 2005, Carinci *et al.* 2008, Mori *et al.* 2010, Liu *et al.* 2011, Atari *et al.* 2012). Outros estudos detectaram os marcadores de CTMs STRO-1, CD90, CD105 e CD146 em tecidos pulpares inflamados por meio da análise de imunoistoquímica (Alongi *et al.* 2010). Marcadores como CD73, CD90, CD105, também foram observados em células pulpares que entraram em contato com sistemas adesivos (Trubiani *et al.* 2010). Assim, nosso último objetivo é verificar se

CTMs estão presentes em tecidos pulpares que sofrem estresse oxidativo após procedimento clareador.

Diversas são as abordagens para caracterizar as CTMs, dificultando a comparação entre resultados de estudos distintos (Fernandez-Vallone *et al.* 2013). Assim, em 2006, a Sociedade Internacional de Terapia Celular (ISCT) propôs critérios mínimos para definir CTMs humanas. Dentre estes critérios, estabeleceu-se que CTMs devem apresentar expressão para CD105, CD73 e CD90, e não expressão para, dentre outras, CD45 (Kawashima 2012, Fernandez-Vallone *et al.* 2013).

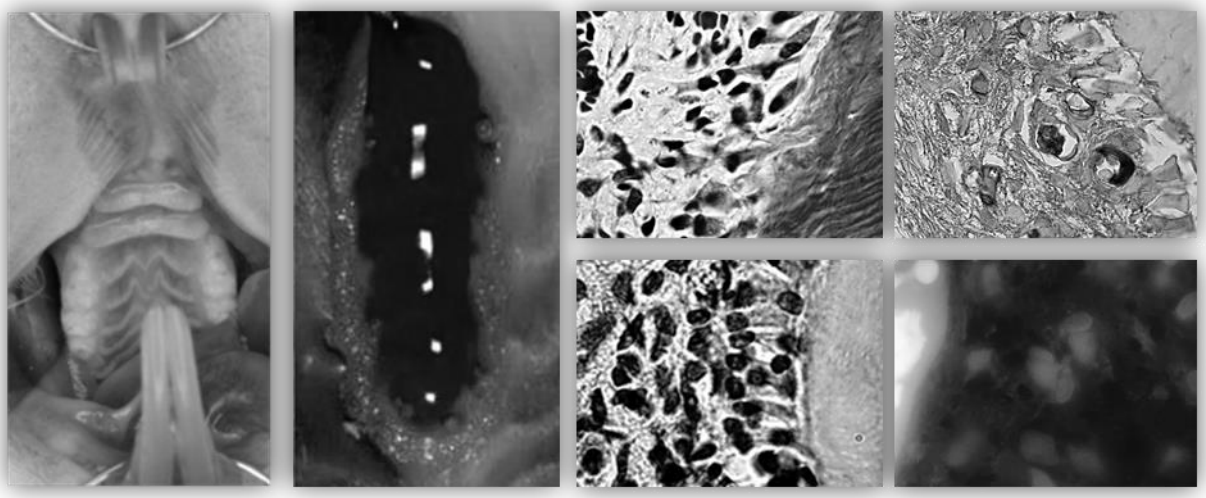
A CD90 (Thy-1) é uma glicoproteína expressa principalmente nos leucócitos e está envolvida em interações célula-célula e célula-matriz (Rege & Hagood 2006). Vários investigadores relataram a expressão de CD90 nas DPSCs (Huang *et al.* 2009a, Huang *et al.* 2009b, Balic *et al.* 2010, Karaoz *et al.* 2010, Pivoriuunas *et al.* 2010). A CD73 atua como uma molécula de adesão celular e intercede a ligação de linfócitos às células endoteliais (Airas *et al.* 1995, Fernandez-Vallone *et al.* 2013). Ainda, por ser uma molécula de adesão, age como um ativador de transdução de sinal durante a interação das CTMs com os demais componentes do microambiente do estroma, favorecendo os processos de proliferação e diferenciação (Barry *et al.* 2001). Vários estudos relataram sua expressão em DPSCs (Alongi *et al.* 2010, Pivoriuunas *et al.* 2010, Yamada *et al.* 2010). Já a CD105, conhecida como endoglina, está associada com o endotélio vascular humano (Rius *et al.* 1998), também sendo referida como expressa nas DPSCs em alta atividade de proliferação e migração (Huang *et al.* 2009b, Waddington *et al.* 2009, Alongi *et al.* 2010, Lee *et al.* 2010, Pivoriuunas *et al.* 2010).

Para a identificação de um marcador negativo para CTMs, podemos lançar mão da marcação de CD45, que é uma glicoproteína de superfície celular encontrada em todas as células da linhagem hematopoiética, exceto eritrócitos e plaquetas (Streuli *et al.* 1988, Rutz *et al.* 2007). Um estudo mostrou que polpas de incisivos murinos não erupcionados continham pouca marcação CD45+, e uma alta marcação CD90+/CD45-, sendo que este tecido exibiu uma rápida mineralização *in vitro* (Balic & Mina 2010). Essas características também foram observadas em molares humanos inclusos (Balic *et al.* 2010). Após erupção dos dentes, o perfil de marcação imunoistoquímica apresentou-se com maior quantidade de células

CD45+, e menor marcação para CD90+ (Balic & Mina 2010). Isso nos sugere que ambos, dentes de ratos e de humanos, apresentam maior marcação para células estaminais no momento do desenvolvimento dentário. Acreditamos que estas células voltem a se proliferar após o procedimento de clareação dentária. Assim, utilizaremos estes marcadores (CD90, CD105, CD73 e CD45) para a observação da resposta das CTMs em tecidos pulpares de dentes clareados.

Depreende-se dos estudos consultados na literatura que, até o momento, os mecanismos que envolvem as células após o contato com o H_2O_2 não estão completamente elucidados, principalmente no que diz respeito à dentinogênese reparadora. Vários estudos foram realizados para compreender os efeitos do H_2O_2 sobre o tecido pulpar, entretanto, muitos destes estudos se baseiam em culturas de células manipuladas *ex vivo*, o que leva a limitações significativas com relação à interação celular. Uma limitação deste modelo experimental é o fato dessas células não serem analisadas junto a um tecido organizado, restringindo a análise apenas à linhagem celular utilizada. A falta de estudos *in vivo,* que forneça informações detalhadas a respeito dos eventos celulares e moleculares que ocorrem após o procedimento clareador, é clara.

II. Objetivos



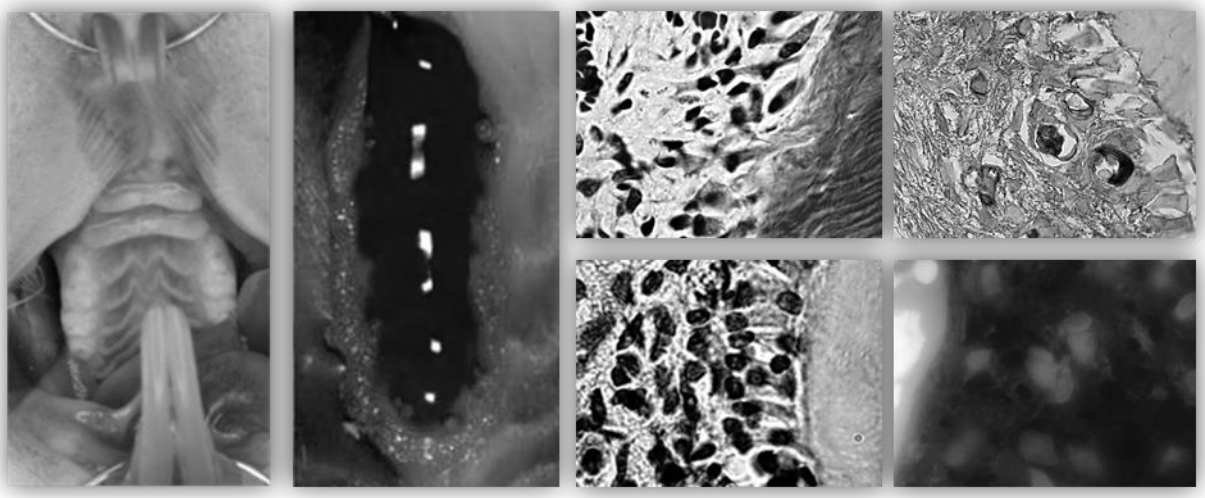
II. Objetivos

Os objetivos deste trabalho são:

1 – Verificar *in vivo* os efeitos do H_2O_2 do gel clareador na expressão de marcadores da mineralização no tecido pulpar, por meio da imunomarcação de osteocalcina (OCN) e osteopontina (OPN); e a presença de resposta celular específica ao estresse oxidativo, por meio de imunomarcação com anticorpo para espécies reativas de oxigênio (EROs);

2 – Determinar a capacidade de resposta ao estresse oxidativo gerado pelo H_2O_2 no tecido pulpar, por meio da imunomarcação de Heme-oxigenase-1 (HO-1); investigar os efeitos do gel clareador sobre a diferenciação odontoblástica, por meio da imunomarcação do fator de transcrição Jun-D; e a influência do estresse oxidativo gerado pelo H_2O_2 na identificação de células-tronco mesenquimais (CTMs) do tecido pulpar, por meio da técnica de imunofluorescência, com identificação concomitante de positividade celular para CD90, CD73, CD105 e negatividade para CD45.

III. Artigo 1



International Endodontic Journal

Presence of osteocalcin, osteopontin, and reactive oxygen species-positive cells in pulp tissue after dental bleaching

Abstract

Aim Apoptosis and lymphocyte-like cells are observed after tissue reorganisation and tertiary dentine formation in dental pulp previously damaged by hydrogen peroxide (H_2O_2). However, our understanding of the long-term effects of H_2O_2 is limited. This study analysed the influence of H_2O_2 on pulp repair through osteocalcin and osteopontin immunolabelling, and in cellular defence by using the anti-reactive oxygen species (ROS) antibody.

Methodology The maxillary molars of rats were treated with 35% H_2O_2 (Ble group) or placebo gel (control). At 0 h and 2, 7, 15, and 30 days (n=10 hemimaxillae), the rats were killed; pulp tissue was evaluated using inflammation and immunolabelling scores (osteocalcin/osteopontin); ROS-positive cells were counted. Friedman and Dunn tests or repeated-measures ANOVA was used ($P<0.05$).

Results The Ble group had necrosis in the coronary pulp at 0 h, and in the occlusal third of the coronary pulp at 2 days; at 7, 15, and 30 days, no inflammation was noted similar to the controls ($P>0.05$). Osteocalcin was absent in the Ble at 0 h, moderate at 2 days, and increased thereafter, differing from the controls at 15 and 30 days ($P<0.05$). Osteopontin was higher at 7 and 15 days in Ble group compared to controls ($P<0.05$). The Ble group had more ROS-positive cells in pulp at 7 and 15 days ($P<0.05$). Tertiary dentine was observed at 7 days, increasing thereafter ($P<0.05$).

Conclusions Post-bleaching pulp repair is associated with increased osteocalcin over time. Osteopontin also participates in this process, and anti-ROS is involved in cellular defence against H_2O_2.

Keywords Dental pulp, hydrogen peroxide, osteocalcin, osteopontin, reactive oxygen species, tertiary dentine.

Introduction

Hydrogen peroxide (H_2O_2) is widely used in dentistry as an active component of bleaching gels, because it is an excellent auxiliary material in dental aesthetic procedures (Briso *et al*. 2016, Cintra *et al*. 2016a, Benetti *et al*. 2017b). However, H_2O_2 is known to promote tooth sensitivity, even in intact teeth (Resende *et al*. 2016), and can have adverse effects on pulp tissue.

Several studies (Benetti *et al*. 2004, Soares *et al*. 2014, Cintra *et al*. 2016a) have showed that H_2O_2 releases reactive oxygen species (ROS), which can penetrate mineralised dental tissues and reach the pulp tissue (Costa *et al*. 2010, Cintra *et al*. 2013, Roderjan *et al*. 2015, Cintra *et al*. 2016b; 2017, Ferreira *et al*. 2018). The effects of this action range from inflammation, which may be minimal depending on the thickness of enamel and dentine (Kina *et al*. 2010, Benetti *et al*. 2017b), to pulp necrosis in teeth with less thick mineralised tissues (Costa *et al*. 2010, Cintra *et al*. 2013).

Previous studies have considered the necrosis generated after bleaching as an irreversible process (Costa *et al*. 2010, Roderjan *et al*. 2015). However, recent studies have shown that areas of necrosis observed in the bleached molars of rats were later replaced by tertiary dentine, and that pulp tissue recovered its cellular organisation (Cintra *et al*. 2016b, Benetti *et al*. 2017a; 2018). These results were observed even in diabetic rats (Cintra *et al*. 2017, Ferreira *et al*. 2018). A study on dogs also showed initial changes in the pulp which were reversible over time (Seale *et al*. 1981).

In vitro studies have shown that pulp cells can promote the formation of hard tissue after contact with H_2O_2 (Lee *et al*. 2006, Matsui *et al*. 2009). At low doses, H_2O_2 has been demonstrated to stimulate cell proliferation (Burdon & Rice-Evans 1989, Burdon 1995, Davies 1999, Benetti *et al*. 2017a), signal cell mitosis, and have an important action in cell signal transduction (Davies 1999, Chang & Karin 2001, Liu *et al*. 2002, Wu *et al*. 2013).

In a previous *in vivo* animal study, H_2O_2 induced cell proliferation in the pulp tissue, mainly in the third of the coronary pulp which was exposed to a lower concentration of H_2O_2 (Benetti *et al*. 2017a). On the other hand, studies have verified the presence of CD5-positive cells (Benetti *et al*. 2018), which may indicate the proliferation of thymocyte-like or mature T-lymphocyte cells and B cells (Lozano *et al*. 2000, Brown & Lacey 2010, de Wit *et al*. 2011). These cells were present in the pulp even 30 days after the bleaching procedure (Benetti *et al*. 2018). Presence of apoptotic cells in the pulp tissue of rat molars was also

observed after the use of higher concentrations of H_2O_2 in the later periods, where pulp tissue was already organised (Benetti *et al*. 2017a).

These results show that pulp tissue has the capacity to recover even after damage generated by H_2O_2, but the cellular mechanisms involved in this process are not fully understood. Thus, the objective of this study was to verify the effect of H_2O_2 in a bleaching gel on the repair process of pulp tissue over time, through the analysis of dentinogenesis markers, namely, osteocalcin and osteopontin. In addition, the study aimed to verify the period in which the cell became capable of responding to the effects of H_2O_2, as well as the duration of this effect, by using a specific antibody, the anti-reactive oxygen species (anti-ROS) antibody. Our hypothesis was that the effects of H_2O_2 contact with pulp tissue may be long lasting and may influence pulp tissue mineralisation over time, even after tissue organisation.

Materials and Methods

Fifty 2-month-old male Wistar albino rats (weighing approximately 280 g) were used. The sample size was established on the basis of the findings of previous studies (Cintra *et al*. 2013; 2016b, Benetti *et al*. 2017a; 2018). The animals were housed in a temperature-controlled environment (22°C ± 1°C, 70% humidity, and a 12-h light–dark cycle) and received water and food *ad libitum*. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The experimental protocol (CEUA-01053-2015) was approved by the local Ethics Committee.

Dental bleaching

The animals were anaesthetised by intramuscular injections of ketamine (80 mg/kg, Ketamina Agener 10%; União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) and xylazine (10 mg/kg, Xilazin; Syntec do Brasil LTDA, Cotia, São Paulo, Brazil). After the application and photo-activation of the resinous gingival barrier (Top Dam; FGM Dental Products, Joinville, SC, Brazil), the right or left maxillary molars of the rats were randomly treated with either bleaching gel (0.01 mL; 1 application of 30 min of the 35% H_2O_2; Whiteness HP Maxx; FGM Dental Products, Joinville, SC, Brazil) or placebo

gel (0.01 mL; 1 application of 30 min of the thickener of the bleaching gel) (Cintra *et al*. 2016b).

Histological and immunohistochemical analysis

The rats were killed immediately (0 h) and at 2, 7, 15, and 30 days (Hirata *et al*. 2005, Kina *et al*. 2010, Cintra *et al*. 2013, Cintra *et al*. 2016b) after dental bleaching, with an overdose of an anaesthetic solution (Thiopentax; Cristália—Produtos Químicos Farmacêuticos LTDA, Itapira, São Paulo, Brazil), resulting in n=10 hemimaxillae per bleaching group and 10 hemimaxillae per control group, in each period. The right and left hemimaxillae were separated, dissected, and fixed in a solution of 4% buffered formaldehyde for 24 h. The specimens were decalcified in a 10% ethylenediaminetetraacetic acid solution for 3 months and then dehydrated, clarified, and embedded in paraffin.

Serial histological sections of each specimen were selected from the point where the mesial root of the first molar was seen in all its longitudinal extension. Five-micron sections were cut in the vestibular–lingual plane and stained with haematoxylin–eosin (H.E.) or submitted to immunohistochemical analysis by using an indirect immunoperoxidase technique (Benetti *et al*. 2018, Ferreira *et al*. 2018) for osteocalcin, osteopontin, and ROS. The first blade obtained was selected for H.E. staining, and the next two for immunohistochemical analysis. This sequence was repeated, thus obtaining three blades for each staining.

For immunohistochemical reactions, the histological sections were deparaffinised in xylene and hydrated in a decreasing ethanol series. Antigen retrieval was achieved by immersing the histological slides in citrate buffer solution (Antigen Retrieval Buffer; Spring Bioscience, Pleasanton, CA, USA) in a pressurised chamber (Decloaking Chamber; Biocare Medical, Concord, CA, USA) at 95°C for 10 min. The slides were rinsed with phosphate-buffered saline at the end of each stage of the immunohistochemical reaction. The histological sections were immersed in 3% H_2O_2 solution for 1 h and 20 min and in 1% bovine serum albumin for 12 h to block the endogenous peroxidase activity and nonspecific sites, respectively. The histological slides were divided and incubated with one of the following primary antibodies: anti-osteocalcin (primary antibody goat, SC-18319; Santa Cruz Biotechnology, Dallas, Texas, USA), anti-osteopontin (primary antibody goat, SC-10593; Santa Cruz Biotechnology), or anti-ROS (primary antibody rabbit, orb13678;

Biorbyt Ltd., Cambridge, Cambridgeshire, UK). The primary antibodies were diluted (Antibody Diluent with Background Reducing Components; Dako Laboratories, Carpinteria, CA, USA) and placed in a moist chamber for 24 h. The histological sections were incubated with a biotinylated secondary antibody for 1 h and 30 min and were subsequently treated with streptavidin–horseradish peroxidase conjugate for 1 h and 30 min (Universal Dako Labelled Streptavidin-Biotin kit; Dako Laboratories). The slides were rinsed with phosphate-buffered saline, and the reaction was developed using the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB Chromogen kit; Dako Laboratories) and was counterstained with Harris's haematoxylin or Fast Green. The negative controls consisted of specimens submitted to the procedures previously mentioned but without the primary antibodies.

The analyses were performed by a single calibrated operator in a blinded manner under light microscopy (×400 magnification; DM4000 B; Leica, Wetzlar, Germany). The pulp chamber was divided into the occlusal, middle, and cervical thirds (Cintra *et al*. 2013, Benetti *et al*. 2017a), and inflammation was scored in each third as follows: 0, inflammatory cells absent or negligible in number; 1, mild inflammatory infiltrate (<25 cells per field); 2, moderate inflammatory infiltrate (between 25 and 125 cells per field); 3, severe inflammatory infiltrate (>125 cells per field); and 4, tissue necrosis (Benetti *et al*. 2017a; 2018).

The immunolabelling of osteocalcin and osteopontin was defined as the presence of a brownish colour in the cytoplasm of the cells and extracellular matrix. Because immunolabelling of both the cells and the extracellular matrix is of great importance for study, we performed semi-quantitative analysis, which provides information on the numbers of immunolabeled cells and immunolabelling intensity of the extracellular matrix (Ferreira *et al*. 2018). The scores were assigned as follows (Benetti *et al*. 2018, Ferreira *et al*. 2018): 0, immunolabelling missing; 1, low standard of immunolabelling; 2, moderate standard of immunolabelling; 3, severe standard of immunolabelling; and 4, very severe standard of immunolabelling. To analyse ROS, cells that had a brown nucleus were counted in each third of the pulp chamber of each specimen, and were analysed as the number of cells per mm^2 (Benetti *et al*. 2017a; 2018). The mean values of each third of each specimen were used for statistical analysis.

The mean of the central area of the pulp chamber and of each third of the pulp chamber was measured using an image processing software (Leica QWin V3, Leica Microsystems, Wetzlar, Germany) (Cintra *et al*. 2016b, Benetti *et al*. 2017a). By taking into account the obtained values, it was possible to calculate the reduction percentage in the central area of the pulp chamber in the treated groups considering the central area of the control group.

Statistical analysis

One-way repeated-measures ANOVA was used for parametrical data and Friedman repeated-measures statistical test was used for non-parametrical data. Significance was set at the level of 5% ($P<0.05$).

Results

Inflammatory infiltrate analysis

The representative images of the inflammatory response are shown in Figure 1 (a-f). The pulp tissue of the control group had normal conditions, with intact coronary pulp tissue, continuous odontoblast layer around the entire pulp, and absence of inflammatory cells. Immediately after bleaching, a large area of necrosis was observed in the coronary pulp. At 2 days, the area of necrosis was present in the occlusal third of the coronary pulp, and extended to the middle third in some specimens. Severe inflammatory infiltrate underlying the area of necrosis was observed. At 7 days, the pulp tissue showed no inflammation, and odontoblastoid cells appeared around the entire coronary pulp. At 15 and 30 days, the pulp tissue was organised, and the odontoblastoid cell layer had formed.

Table 1 shows the inflammatory infiltrate scores for the groups in each analysis period. The pulp tissue of the specimens analysed immediately and at 2 days after dental bleaching was different from that of the control specimens and the specimens of the bleached group analysed at 7, 15, and 30 days ($P<0.05$).

Immunohistochemical analysis

Immunolabelling of osteocalcin and osteopontin was observed in the extracellular matrix and cytoplasm of fibroblasts and odontoblast-like cells. Representative images are

shown in Figure 1 (osteocalcin, a1-f1; osteopontin, a2-f2). Table 1 shows the scores for the immunolabelling standard.

Low immunolabelling of osteocalcin was observed in the control group. In the Ble group, no immunolabelling of osteocalcin was observed immediately after bleaching. After 2 days, the immunolabelling was moderate; thereafter, it increased at 7, 15, and 30 days, ranging from moderate at 7 days, to severe at 15 days, and to very severe at 30 days. A significant difference was observed between the control and Ble groups at 15 and 30 days after bleaching ($P<0.05$).

Low standard of immunolabelling was observed for osteopontin in the control group. Immediately after bleaching, no immunolabelling was observed in the Ble group, and at 2 days, only moderate immunolabelling was observed. Immunolabelling of osteopontin increased at 7 days, where it was very severe. However, the immunolabelling reduced at 15 and 30 days after bleaching, but there was no difference compared to the immunolabelling at 7 days ($P>0.05$). A significant difference in relation to the control group was observed at 7 and 15 days after bleaching in the Ble group ($P<0.05$).

Table 1 Scores for inflammatory infiltrate and for immunohistochemical labeling of OCN and OPN, and central area of the pulp chamber (μm^2) in each period of analysis

Analysis		Scores	Groups						*P*
			Cont	Bleached					
				Oh	2d	7d	15d	30d	
H.E.	**Occlusal**	0	10/10	0/10	0/10	9/10	10/10	10/10	<0.001
		1	0/10	0/10	0/10	1/10	0/10	0/10	
		2	0/10	0/10	0/10	0/10	0/10	0/10	
		3	0/10	0/10	4/10	0/10	0/10	0/10	
		4	0/10	10/10	6/10	0/10	0/10	0/10	
		Median*	0^{a}	4^{b}	4^{b}	0^{a}	0^{a}	0^{a}	
	Middle	0	10/10	0/10	0/10	10/10	10/10	10/10	<0.001
		1	0/10	0/10	0/10	0/10	0/10	0/10	
		2	0/10	0/10	4/10	0/10	0/10	0/10	
		3	0/10	0/10	5/10	0/10	0/10	0/10	
		4	0/10	10/10	1/10	0/10	0/10	0/10	
		Median*	0^{a}	4^{b}	3^{b}	0^{a}	0^{a}	0^{a}	
	Cervical	0	10/10	0/10	0/10	10/10	10/10	10/10	<0.001
		1	0/10	0/10	3/10	0/10	0/10	0/10	
		2	0/10	0/10	6/10	0/10	0/10	0/10	
		3	0/10	2/10	1/10	0/10	0/10	0/10	
		4	0/10	8/10	0/10	0/10	0/10	0/10	
		Median*	0^{a}	4^{b}	2^{b}	0^{a}	0^{a}	0^{a}	
OCN		0	0/10	6/10	0/10	0/10	0/10	0/10	<0.001
		1	10/10	4/10	0/10	0/10	0/10	0/10	
		2	0/10	0/10	9/10	5/10	2/10	2/10	
		3	0/10	0/10	1/10	5/10	4/10	3/10	
		4	0/10	0/10	0/10	0/10	4/10	5/10	
		Median*	1^{ab}	0^{a}	2^{abc}	2^{bc}	3^{c}	4^{c}	
OPN		0	4/10	8/10	0/10	0/10	0/10	0/10	<0.001
		1	6/10	2/10	3/10	0/10	0/10	2/10	
		2	0/10	0/10	5/10	1/10	1/10	3/10	
		3	0/10	0/10	2/10	2/10	8/10	5/10	
		4	0/10	0/10	0/10	7/10	1/10	0/10	
		Median*	1^{a}	0^{a}	2^{ab}	4^{b}	3^{b}	3^{ab}	
Pulp chamber area (10^5)		Mean* (±SD)	21.89 $(\pm 1.18)^{a}$	21.78 $(\pm 2.64)^{a}$	23.79 $(\pm 4.19)^{a}$	14.18 $(\pm 9.90)^{b}$	12.30 $(\pm 5.25)^{b}$	6.65 $(\pm 2.47)^{c}$	<0.001

*Different letters in the same line indicate statistically significant differences between groups.

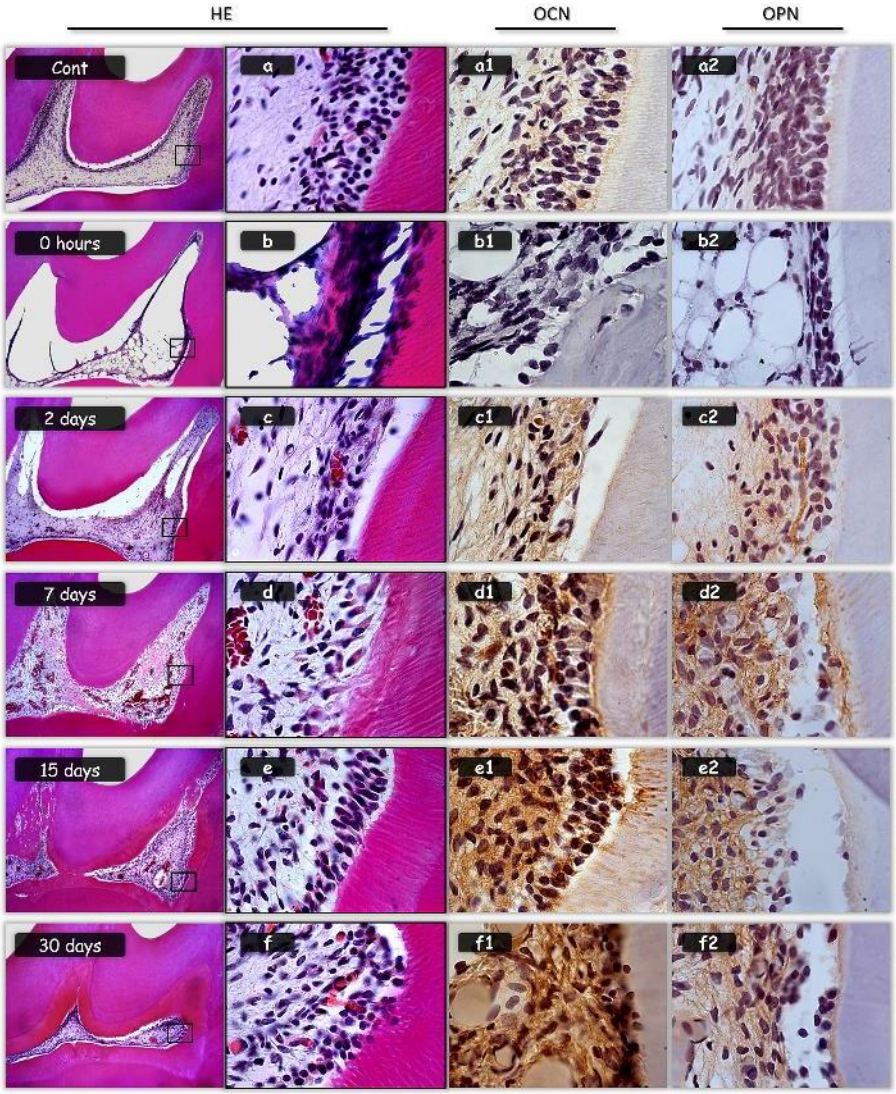


Figure 1 Representative images of histological and immunohistochemical analyses of osteocalcin and osteopontin. Control: panoramic microscopic aspect showing normal pulp tissue, (a) cervical third of the coronary pulp with organised pulp tissue, and low immunolabelling of (a1) osteocalcin and (a2) osteopontin. 0 h: panoramic microscopic aspect of the bleached group immediately after dental bleaching, showing an extensive area of necrosis and (b) absence of cellularity and immunolabelling of (b1) osteocalcin and (b2) osteopontin. 2 days: microscopic aspect of the bleached group at 2 days with regions of necrosis and pulp disorganisation, (c) evident tissue disorganisation, and moderate immunolabelling of (c1) osteocalcin and (c2) osteopontin. 7 days: microscopic aspect of the bleached group at 7 days showing organised pulp tissue and areas of tertiary dentine, (d) cervical third of the coronary pulp with the formation of odontoblastic-like cells, (d1) moderate immunolabelling of osteocalcin, and (d2) very severe immunolabelling of osteopontin. 15 days: microscopic aspect of the bleached group at 15 days with intact pulp tissue and presence of tertiary dentine, (e) higher magnification showing pulp organisation, and severe immunolabelling of (e1) osteocalcin and (e2) osteopontin. 30 days: microscopic aspect of the bleached group at 30 days with cellular and tissue organisation, (f) absence of inflammation and deposition of tertiary dentine, (f1) very severe immunolabelling of osteocalcin, and (f2) severe immunolabelling of osteopontin (100×, 1000×: haematoxylin–eosin [H.E.]; 1000×: immunohistochemical analysis of osteocalcin and osteopontin).

Figure 2 (a, a1, a2-f, f1, f2) shows representative images of the groups in relation to the immunolabelling of ROS, as well as the number of ROS-positive cells per mm^2 in each third of the coronary pulp. ROS-positive cells were absent in the control group and in the Ble group immediately after bleaching. At 2 days, the Ble group had a certain amount of immunoreactive cells, and compared to the control, their numbers were significant in the cervical third ($P<0.05$). However, greater immunolabelling was observed at 7 and 15 days, which was different from that in the control group in all thirds of the pulp chamber ($P<0.05$). At 30 days, despite the difference in relation to the control group in the cervical third ($P<0.05$), a small number of ROS-positive cells was observed in the Ble group.

Tertiary dentine analysis

Compared to the control group, the Ble group had a significant presence of tertiary dentine at 7 and 15 days after bleaching ($P<0.05$). This tertiary dentine gradually increased in thickness at 30 days after bleaching ($P<0.05$); at this time point, the pulp tissue of all the specimens was organised and showed no inflammation. Table 1 shows data on the reduction of the central area of the pulp chamber in each analysis period.

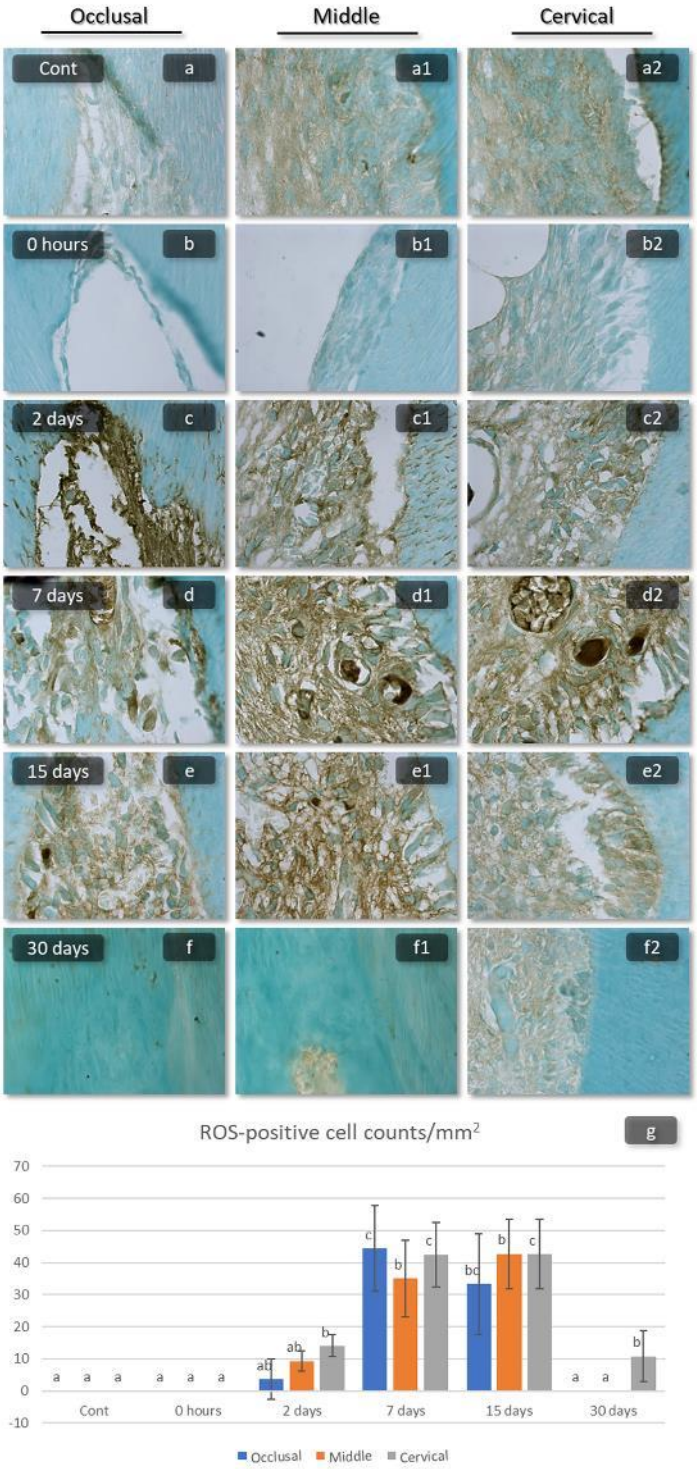


Figure 2 Representative images of immunohistochemical labelling of reactive oxygen species (ROS)-positive cells. Representative photomicrographs of the thirds of the coronary pulp of (a, a1, a2). Control without ROS-positive cells. (b, b1, b2) Bleaching groups at 0 h with no immunolabelled cells; (c, c1, c2) at 2 days with more ROS-positive cells in the cervical third; (d, d1, d2) at 7 days and (e, e1, e2) at 15 days with greater immunolabelling; and (f, f1, f2) at 30 days with lesser immunolabelling (immunolabelling of ROS: 1000×). The graph (g) shows the counts of ROS-positive cells, expressed as the number of cells per mm^2, and statistical analysis. Different superscript lowercase letters indicate statistically significant differences between groups at each third of the coronal pulp (P<0.05).

Discussion

This study evaluated *in vivo* the influence of H_2O_2 on the mineralisation and response capacity of pulp tissue of Wistar rats over time, and found that H_2O_2 induces the expression of osteocalcin and osteopontin in pulp tissue. The study also showed that the number of ROS-positive cells increased when these markers began to appear more intensely, as well as when the formation of tertiary dentine became significant. Thus, the hypothesis of the study was confirmed.

Some *in vitro* studies have previously shown the effects of H_2O_2 on the mineralisation and differentiation of different cell lines. The MDPC-23 odontoblast-like cell line treated with 0.2 and 0.3 mmol/L H_2O_2 for 14 days showed a significant increase in extracellular matrix mineralisation, number of calcification nodules, and alkaline phosphatase activity, indicating an alteration in the initial stage of odontoblast differentiation (Lee *et al*. 2006). The authors suggest a favourable H_2O_2 effect on odontoblasts to increase the capacity of dentine production (Lee *et al*. 2006). These effects were contrary to those on MC3TC-E1 pre-osteoblastic cells in which cell differentiation was inhibited (Lee *et al*. 2006).

Human dental pulp cells had initial reduction in the expression of odontoblastic markers and deposition of mineralised nodules after the application of H_2O_2 in artificial pulp chambers; however, the groups with a lower application time of H_2O_2 showed similar mineralisation to the control group during the 21-day period (Soares *et al*. 2015). These results agree with those of the present study regarding the immunolabelling of osteocalcin and osteopontin, whose expressions were inhibited after the initial contact with H_2O_2, but were expressed later. This fact shows that the dental pulp can recover and respond with an increase in these markers, which accelerate the process of dentinogenesis.

However, osteocalcin expression increased over time, whereas osteopontin expression was greater at 7 and 15 days and reduced over time. A previous study showed that a higher amount of osteocalcin was also expressed later than that of osteopontin in cell cultures after contact with minimal concentrations of H_2O_2 (Matsui *et al*. 2009). A significant importance of *in vitro* studies is their ability to demonstrate response of specific cell types to a material. However, *in vivo* studies allow the analysis of simultaneous reactions in an organised tissue with different cell types acting together in the response of that tissue to the aggressor agent (Cintra *et al*. 2016b). In our study, we could observe that significant levels

of osteocalcin and osteopontin expression were associated with the period in which the deposition of tertiary dentine was also observed.

Osteocalcin is a protein of the osteoblast lineage that is observed in odontoblasts involved in the formation of reactionary dentine (Hirata *et al*. 2005). Osteocalcin can also be observed in extensions of odontoblasts (Matsui *et al*. 2009), indicating that it is synthesised within the odontoblasts, transported through their processes, and deposited in the dentine matrix (Bronckers *et al*. 1985, Matsui *et al*. 2007, Wei *et al*. 2007, Matsui *et al*. 2008). However, this protein was not observed in pre-dentine (Matsui *et al*. 2009). In the present study, osteocalcin was present in the cytoplasm of fibroblasts and odontoblasts mainly in the period between 15 and 30 days. In contrast, immunolabelling of osteocalcin was negligible in the cytoplasm of odontoblast-like cells during differentiation at 7 days.

In a previous study, osteocalcin was observed in the initial phase of reactionary dentine formation in rats after tooth preparation (Hirata *et al*. 2005) and was synthesised and secreted to the reactionary dentine matrix 3 days after preparation. As greater osteocalcin expression was observed at 30 days in the present study, we believe that the production of dentine is still active in the later periods and is related to the presence of newly differentiated mature odontoblast-like cells, which produce the dentine matrix. Although Matsui *et al*. (2009) have shown a decrease in osteocalcin after its greater expression, we must consider that seeded cells tend to die after a certain period, which could have influenced their results. Thus, *in vivo* studies analysing the pulp at later periods than the present study may clarify the period in which the expression of osteocalcin still remains in the pulp tissue after bleaching.

Another important protein in the processes that lead to tissue mineralisation is osteopontin (Matsui *et al*. 2009), a multifunctional phosphorylated glycoprotein expressed by a wide variety of tissues and cells (Gericke *et al*. 2005), and present in the differentiation of odontoblasts, growth, and bone regeneration (Sodek *et al*. 2000, Matsui *et al*. 2009). Osteopontin has been shown to regulate early bone remodelling events, such as cell adhesion, osteoclast functioning, and matrix mineralisation (Sodek *et al*. 2000). Moreover, osteopontin can interact with a large amount of calcium (Sodek *et al*. 2000) and with osteocalcin, forming crystals of hydroxyapatite (Gericke *et al*. 2005). Thus, it plays a role not only in the initial formation phase, but also during the calcification of reparative dentinogenesis (Kuratate *et al*. 2008).

In this study, greater osteopontin immunolabelling occurred at 7 days after bleaching, at which time osteocalcin has not yet been expressed significantly compared to that in the control group. Thus, the greater expression of osteopontin before osteocalcin may suggest the presence of reactionary dentine, formed by odontoblasts, that resisted the effects of bleaching. The subsequent production of osteocalcin may suggest the formation of reparative dentine produced by newly differentiated mature odontoblast-like cells.

Studies have shown that pulp cells under oxidative stress, resulting from contact with H_2O_2 from the bleaching gel, were able to proliferate significantly over time, recovering their viability around 3-4 times, 3 days after bleaching (Soares *et al*. 2014). Benetti *et al*. (2017a) demonstrated *in vivo* cell proliferation in the pulp after bleaching, which was higher in the presence of lower H_2O_2 concentrations. In the present study, we evaluated cell reactivity against oxidative stress by using a specific marker, anti-ROS. Previous studies have shown the action of these antibodies on H_2O_2-affected cells (Ashok *et al*. 1997; 1998, Strollo *et al*. 2013), but no *in vivo* studies have evaluated the moment when the cells of the pulp tissue affected by H_2O_2 are able to react after dental bleaching.

We can observe that in the immediate period after contact with H_2O_2, the cells do not present reactivity to ROS, which occurs significantly in the cervical third of the coronary pulp at 2 days, in the other thirds at 7 and 15 days, and thereafter reducing by 30 days. This shows that the cellular reactivity to oxidative stress occurs during the initial period of repair of the pulp tissue, when the inflammation decreases.

Excessive production of ROS is considered an important factor in the formation of various disease-causing substances (Matsui *et al.* 2009). However, authors have suggested that small amounts of ROS have biodefense roles, in addition to enhancing the proliferative effect and differentiation of cells (Ueda *et al.* 1996, Schmitt *et al.* 2006, Matsui *et al.* 2007). Although ROS have been reported to cause tissue damage, such as apoptosis (Han *et al.* 2008, Benetti *et al*. 2017a), necrosis (Costa *et al.* 2010, Cintra *et al.* 2013), inflammation (Seale *et al.* 1981, Costa *et al.* 2010, Cintra *et al.* 2013, Ferreira *et al*. 2018, Benetti *et al*. 2018), or tissue aging (Cintra *et al*. 2017), they have also been found to have a more complex role in cell physiology (Scandalios 2005, Lee *et al.* 2006, Matsui *et al.* 2007, Matsui *et al.* 2009). The responsiveness of pulp tissue to oxidative stress and cell proliferation (Soares *et al*. 2014, Benetti *et al*. 2017a) and induction to the formation of hard tissue corroborates with these findings. However, high concentrations of H_2O_2 used during bleaching can cause

extreme damage to the pulp tissue; therefore, it is important that the healthy pulp is not affected by H_2O_2, since its aging can be accelerated.

In addition, cytokines such as interleukin-6 and interleukin-17 (Soares *et al*. 2015, Benetti *et al*. 2018, Ferreira *et al*. 2018) and tumour necrosis factor-α (Soares *et al*. 2015, Ferreira *et al*. 2018) are involved in the response of the pulp to the bleaching gel and decrease over time; however, CD5-positive cells, associated with a chronic inflammatory process, are observed for even long periods after dental bleaching with high concentrations of H_2O_2 (Benetti *et al*. 2018). In Europe, the minimum concentrations of H_2O_2 for use in dental bleaching have been determined (The Council of The European Union 2011).

The findings of this study suggest that although the necrosis caused by high concentrations of H_2O_2 may be reversible, pulp tissue suffers consequences that may extend in the long term, such as increased hard-tissue production, which may suggest a process of calcification of the pulp over time. Results observed in rat teeth cannot be extrapolated directly to the clinical setting (Cintra *et al*. 2016b). However, animal studies are very important in understanding the mechanism of repair of pulp tissue. Longitudinal studies in humans would be required to verify possible accelerated calcification over time. Nevertheless, these results reinforce that dental bleaching should be performed with caution, as it may result in adverse sequelae in the pulp tissue. Future studies, such as those analysing stem cell markers, for example, may help in understanding the effects of H_2O_2 on the pulpal response of bleached teeth.

Conclusion

The repair process of the pulp tissue after bleaching is associated with the increase of osteocalcin over time. Osteopontin also participates in this process, and ROS is involved in the cellular defence process against oxidative stress induced by H_2O_2.

Conflict of Interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

Acknowledgement

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (n. 2015/10825-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (n. 455943/2014-1).

References

Ashok BT, Ahmad J, Ali R (1998) Immunochemical detection of oxidative DNA damage in cancer and aging using anti-reactive oxygen species modified DNA monoclonal antibody. *The International Journal of Biochemistry & Cell Biology* **30**, 1367-77.

Ashok BT, Ahmad J, Qadri A, Ali R (1997) Anti-ROS-DNA monoclonal antibody as molecular probe for oxidative DNA damage. *Biochemistry and Molecular Biology International* **43**, 1219-29.

Benetti AR, Valera MC, Mancini MNG, Miranda CB, Balducci I (2004) *In vitro* penetration of bleaching agents into the pulp chamber. *International Endodontic Journal* **37**, 120-4.

Benetti F, Gomes-Filho JE, Ferreira LL, *et al*. (2017a) Hydrogen peroxide induces cell proliferation and apoptosis in pulp of rats after dental bleaching in vivo: Effects of the dental bleaching in pulp. *Archives of Oral Biology* **22**, 103-9.

Benetti F, Lemos CAA, de Oliveira Gallinari M, *et al*. (2017b) Influence of different types of light on the response of the pulp tissue in dental bleaching: a systematic review. *Clinical Oral Investigations. In press*. doi: 10.1007/s00784-017-2278-9.

Benetti F, Gomes-Filho JE, Ferreira LL, *et al*. (2018) Concentration-dependent effect of bleaching agents on the immunolabeling of interleukin-6, interleukin-17, and CD5-positive cells in the dental pulp. *International Endodontic Journal.* doi: 10.1111/iej.12891..

Briso AL, Fagundes TC, Gallinari MO, Moreira J, de Almeida L, Rahal V, Gonçalves RS, Santos PD (2016) An *in situ* study of the influence of staining beverages on color alteration of bleached teeth. *Operative Dentistry* **41**, 627-33.

Bronckers AL, Gay S, Dimuzio MT, Butler WT (1985) Immunolocalization of gamma-carboxyglutamic acid-containing proteins in developing molar tooth germs of the rat. *Collagen and Related Research* **5**, 17–22.

Brown MH, Lacey E (2010) A Ligand for CD5 is CD5. *Journal of Immunology* **185**, 6068–74.

Burdon RH (1995) Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. *Free Radical Biology and Medicine* **18**, 775-94.

Burdon RH, Rice-Evans C (1989) Free radicals and the regulation of mammalian cell proliferation. *Free Radical Research Communication* **6**, 345-8.

Chang L, Karin M (2001) Mammalian MAP kinase signaling cascades. *Nature* **410**, 37–40.

Cintra LT, Benetti F, Facundo ACS, *et al*. (2013) The number of bleaching sessions influences pulp tissue damage in rat teeth. *Journal of Endodontics* **39**, 1576-80.

Cintra LT, Benetti F, Ferreira LL, *et al*. (2016a) Penetration capacity, color alteration and biological response of two in-office bleaching protocols. *Brazilian Dental Journal* **27**, 169-75.

Cintra LT, Benetti F, Ferreira LL, *et al*. (2016b) Evaluation of an experimental rat model for comparative studies of bleaching agents. *Journal Applied of Oral Science* **24**, 95-104.

Cintra LT, Ferreira LL, Benetti F, *et al*. (2017) The effect of dental bleaching on pulpal tissue response in a diabetic animal model. *International Endodontic Journal.* **50**, 790-8.

Costa CA, Riehl H, Kina JF, Sacono NT, Hebling J (2010) Human pulp responses to in-office tooth bleaching. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics* **109**, e59-64.

Davies KJ (1999) The broad spectrum of responses to oxidants in proliferating cells: A new paradigm for oxidative stress. *IUBMB Life* **48**, 41–7.

de Wit J, Souwer Y, van Beelen AJ, *et al*. (2011) CD5 costimulation induces stable Th17 development by promoting IL-23R expression and sustained STAT3 activation. *Blood* **118**, 6107-14.

Ferreira LL, Gomes-Filho JE, Benetti F, Carminatti M, Ervolino E, Briso ALF, Cintra LT (2018) The effect of dental bleaching on pulpal tissue response in a diabetic animal model: Immunoregulatory cytokines study. *International Endodontic Journal* **51**, 347-6..

Gericke A, Qin C, Spevak L, *et al*. (2005) Importance of phosphorylation for osteopontin regulation of biomineralization. *Calcified Tissue International* **77**, 45–54.

Han YH, Kim SZ, Kim SH, Park WH (2008) Pyrogallol as a glutathione depletor induces apoptosis in HeLa cells. *International Journal of Molecular Medicine* **21**, 721–30.

Hirata M, Yamaza T, Mei YF, Akamine A (2005) Expression of osteocalcin and Jun D in the early period during reactionary dentin formation after tooth preparation in rat molars. *Cell and Tissue Research* **319**, 455-65.

Kina JF, Huck C, Riehl H, *et al*. (2010) Response of human pulps after professionally applied vital tooth bleaching. *International Endodontic Journal* **43**, 572-80.

Kuratate M, Yoshiba K, Shigetani Y, Yoshiba N, Ohshima H, Okiji T (2008) Immunohistochemical analysis of nestin, osteopontin, and proliferating cells in the reparative process of exposed dental pulp capped with mineral trioxide aggregate. *Journal of Endodontics* **34**, 970-4.

Lee DH, Lim BS, Lee YK, *et al*. (2006) Effects of hydrogen peroxide (H_2O_2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biology and Toxicology* **22**, 39–46.

Liu SL, Lin X, Shi DY, *et al*. (2002) Reactive oxygen species stimulated human hepatoma cell proliferation via cross-talk between PI3-K/PKB and JNK signaling pathways. *Archives of Biochemistry Biophysics* **406**, 173–82.

Lozano F, Simarro M, Calvo J, *et al*. (2000) CD5 signal transduction: positive or negative modulation of antigen receptor signaling. *Critical Reviews in Immunology* **20**, 347-58

Matsui S, Takahashi C, Tsujimoto Y, Matsushima K (2009) Stimulatory effects of low-concentration reactive oxygen species on calcification ability of human dental pulp cells. *Journal of Endodontics* **35**, 67–72.

Matsui S, Takeuchi H, Tsujimoto Y, Matsushima K (2008) Effects of Smads and BMPs induced by Ga-Al-As laser irradiation on calcification ability of human dental pulp cells. *Journal of Oral Science* **50**, 75– 81.

Matsui S, Tsujimoto Y, Matsushima K (2007) Stimulatory effects of hydroxyl radical generation by Ga-Al-As laser irradiation on mineralization ability of human dental pulp cells. *Biological & Pharmaceutical Bulletin* **30**, 27–31.

Rezende M, Ferri L, Kossatz S, Loguercio AD, Reis A (2016) Combined bleaching technique using low and high hydrogen peroxide in-office bleaching gel. *Operative Dentistry* **41**, 388-96.

Roderjan DA, Stanislawczuk R, Hebling J, Costa CA, Reis A, Loguercio AD (2015) Response of human pulps to different in-office bleaching techniques: preliminary findings. *Brazilian Dental Journal* **26**, 242-8.

Scandalios JG (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biolical Research* **38**, 995–1014.

Schmitt TL, Klein H, Droge W (2006) Hydrogen peroxide inhibits activity of the IGF-1 receptor kinase. *Redox Report* **11**, 105–9.

Seale NS, McIntosh JE, Taylor AN (1981) Pulpal reaction to bleaching of teeth in dogs. *Journal of Dental Research* **80**, 948-53.

Soares DG, Basso FG, Scheffel DS, Hebling J, de Souza Costa CA (2015) Responses of human dental pulp cells after application of a low-concentration bleaching gel to enamel. *Archives of Oral Biology* **60**, 1428-36.

Soares DG, Marcomini N, Basso FG, Pansani TN, Hebling J, de Souza Costa CA (2014) Indirect cytocompatibility of a low-concentration hydrogen peroxide bleaching gel to odontoblast-like cells. *International Endodontic Journal* **30**, [Epub ahead of print].

Sodek J, Ganss B, McKee MD (2000) Osteopontin. *Critical Reviews Oral Biology and Medicine* **11**, 279-303.

Strollo R, Ponchel F, Malmström V, *et al*. (2013) Autoantibodies to posttranslationally modified type II collagen as potential biomarkers for rheumatoid arthritis. *Arthritis Rheumatoid* **65**, 1702-12.

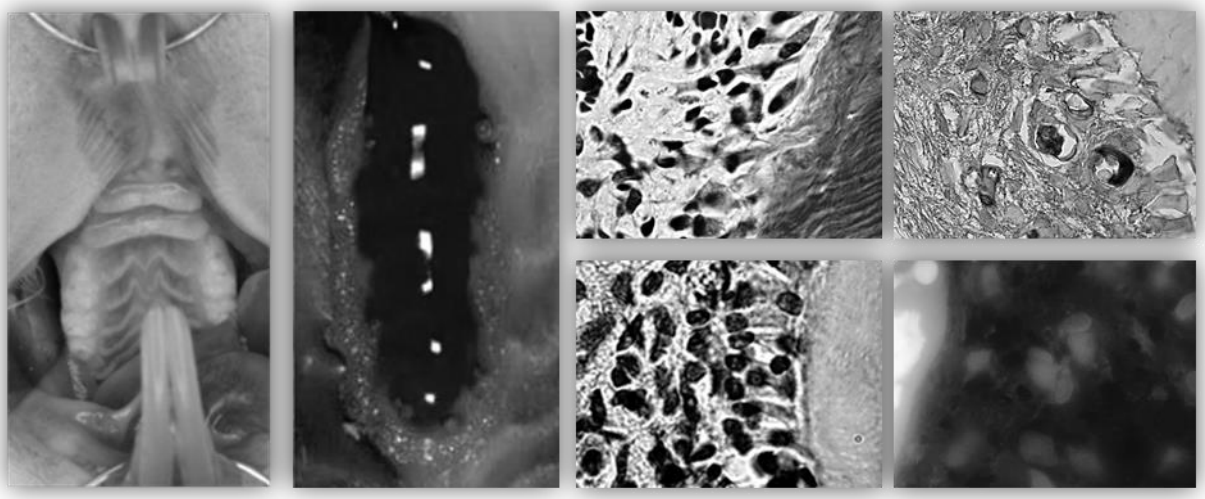
The Council of The European Union (2011) Council Directive 2011/84/EU, amending Directive 76/768/EEC concerning cosmetic products. *Official Journal of the European Union* **L283**, 36–8.

Ueda J, Saito N, Shimazu Y, Ozawa T (1996) A comparison of scavenging abilities of antioxidants against hydroxyl radicals. *Archives of Biochemical and Biophysical* **15**, 377– 84.

Wei X, Ling J, Wu L, Liu L, Xiao Y (2007) Expression of mineralization markers in dental pulp cells. *Journal of Endodontics* **33**, 703-8.

Wu TT, Li LF, Du R, Jiang L, Zhu YQ (2013) Hydrogen peroxide induces apoptosis in human dental pulp cells via caspase-9 dependent pathway. *Journal of Endodontics* **39**, 1151-5.

IV. Artigo 2



International Endodontic Journal

In vivo analysis of the presence of heme oxygenase-1, transcription factor Jun-D, and CD90+/CD73+/CD105+/CD45- cells in the pulp of bleached teeth

Abstract

Aim Very few *in vivo* studies have elucidated the cellular events occurring after tooth bleaching. This study used heme oxygenase-1 (HO-1) immunolabelling to investigate hydrogen peroxide (H_2O_2)-induced responsiveness in pulp cells, Jun-D immunolabelling to study the effects of H_2O_2 on odontoblastic differentiation, and CD90+/CD73+/CD105+/CD45- cell counting for *in vivo* identification of mesenchymal stem cells (MSCs) in the pulp.

Methodology The maxillary molars of 50 rats were treated with a bleaching gel (35% H_2O_2, 1 x 30 min) or placebo gel (control). At 2, 3, 7, 15, and 30 days after the treatment (n=10), inflammation was analysed by haematoxylin–eosin staining, HO-1– and Jun-D–immunolabelled cells were counted in each third of the pulp chamber, and the number of CD90+/CD73+/CD105+/CD45- cells was quantified by immunofluorescence. The results were assessed using the T-test or Mann-Whitney test ($P<0.05$).

Results Significant H_2O_2-induced inflammation was noted at 2 and 3 days ($P<0.05$), with tertiary dentine formation occurring from 7 days. The bleached specimens showed higher HO-1 immunolabelling in the middle and cervical thirds of the coronary pulp at 2 and 3 days, in all thirds at 7 days, and in the occlusal third at 15 days ($P<0.05$), and significant nuclear Jun-D immunolabelling in the cervical third at 2 and 3 days and in the occlusal and middle thirds at 7 days ($P<0.05$). Bleached and control groups showed low numbers of CD90+/CD73+/CD105+/CD45- cells in the pulp at all periods ($P>0.05$).

Conclusions Pulp cells show responsiveness to oxidative stress expressing HO-1 during the post-bleaching inflammation phase until the beginning of the repair phase. Jun-D expression occurs during the reduction of inflammation and the beginning of tertiary dentine production. Presence of oxidative stress does not influence the number of CD90+/CD73+/CD105+/CD45- cells identified *in vivo* in the dental pulp.

Keywords Dental pulp, heme oxygenase-1, hydrogen peroxide, Jun-D, stem cells, dental bleaching.

Introduction

Dental bleaching is frequently performed in dental offices (Grazioli *et al*. 2017), and the increasing demand for aesthetics has made this procedure popular, with several products available from different brands (Briso *et al*. 2016, Cintra *et al*. 2016b, Benetti *et al*. 2017b). Depending on the country, patients may have easy access to bleaching gels and may be able to use them without the supervision of a dentist or a professional qualified to offer this procedure to patients (Hasson *et al*. 2006, Demarco *et al*. 2009).

Hydrogen peroxide (H_2O_2), an active agent in bleaching gels, can reach the dentine–pulp complex (Cintra *et al*. 2016a). Like any other aggressor molecule, H_2O_2 affects the dental pulp, with the intensity of these effects increasing with H_2O_2 concentration and the degree of contact (Cintra *et al*. 2013, 2016b, Soares *et al*. 2016). Studies have shown changes in the pulp tissue after bleaching, such as intense cell proliferation (Benetti *et al*. 2017a), induction of mineralization (Lee *et al*. 2006, Soares *et al*. 2015b), and the presence of apoptotic cells (Benetti *et al*. 2017a, Kim *et al*. 2017), the T-helper CD5 receptor (Benetti *et al*. 2018), and proinflammatory cytokines such as tumour necrosis factor (TNF)-α (Ferreira *et al*. 2017), interleukin (IL)-6 (Soares *et al*. 2015, Ferreira *et al*. 2017, Benetti *et al*. 2018), and IL-17 (Ferreira *et al*. 2017, Benetti *et al*. 2018).

In addition to these changes, deeper changes may also occur in the pulp tissue of bleached teeth (Matsui *et al*. 2009). Heme oxygenase-1 (HO-1) is a cellular protein with cytoprotective activity that is induced in response to oxidative stress in various regulatory cellular mechanisms (Otterbein & Choi 2000, Pae & Chung 2009). Proinflammatory cytokines can induce the expression of HO-1 (Min *et al*. 2006b), and H_2O_2 also shows this ability (Min *et al*. 2008, Kim *et al*. 2017). However, the relationship between the expression of this protein and other cellular events that occur in pulp repair is poorly understood and lacks supporting histological data. In this regard, since HO-1 is an indirect indicator of oxidative stress, HO-1 immunolabelling can be used to verify the moment when the pulp cells respond to oxidative stress.

Since pulp tissue repair involves the differentiation of pre-odontoblastic cells and the formation of tertiary dentin (Lee *et al*. 2006, Yuan *et al*. 2012), some transcription factors must be present to facilitate the differentiation (Karsenty & Wagner 2002, Colucci *et al*. 2011). The Jun-D gene is known to play a key role in the control of osteoblast differentiation (McCabe *et al*. 1995, Colucci *et al*. 2011). Due to the morphological and functional similarities between osteoblasts and odontoblasts, markers of osteoblastic differentiation are

also used as odontoblast markers (Hirata *et al*. 2005). Jun-D was temporarily expressed in odontoblasts 1 and 2 days after preparation in rat teeth (Hirata *et al*. 2005). Thus, Jun-D is present in the pulp tissue during the repair process involving reactionary dentine formation, but its presence during the dentine formation stage after the severe loss of odontoblasts caused by intense H_2O_2-induced oxidative stress (Soares *et al*. 2011, Soares *et al*. 2015a, Cintra *et al*. 2017) has not yet been studied.

In addition, mesenchymal stem cells (MSCs) from the pulp are also recruited during repair of pulp tissue (Alongi *et al*. 2010). In 2006, the International Society of Cell Therapy proposed the minimum criteria for defining MSCs. One of these criteria was that these cells should show expression of certain markers such as CD105, CD73, and CD90, and no expression of some other markers such as CD45 (Kawashima 2012, Fernandez-Vallone *et al*. 2013). Studies have reported the presence of MSC markers in inflamed pulp tissues through immunohistochemical analysis (Alongi *et al*. 2010). CD73, CD90, and CD105 were also observed in pulp cells that came in contact with adhesive systems (Trubiani *et al*. 2010), but MSCs have not yet been identified *in vivo* in the pulp tissue after bleaching-induced damage.

There are very few *in vivo* studies providing detailed information regarding the cellular events that occur after bleaching. The objectives of this study were to investigate, using HO-1 immunolabelling, the progression of cellular defensive responses to oxidative stress, to identify the presence of Jun-D as a marker of odontoblastic differentiation, and to identify MSCs by counting the number of CD90+/CD73+/CD105+/CD45- cells in the pulp tissue of bleached teeth. Our null hypothesis was that H_2O_2 does not influence the expression of HO-1 and Jun-D and the identification of MSCs in the pulp tissue of bleached teeth.

Materials and Methods

A total of 50 two-month-old male Wistar albino rats (280 g) were used. The sample size was established on the basis of the findings of previous studies (Benetti *et al*. 2017a, 2018). The animals were housed in a temperature-controlled environment (22ºC ± 1ºC, 70% humidity) on a standard light/dark schedule with *ad libitum* access to food and water. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The experimental protocol was approved by the local Ethics Committee (CEUA-01053-2015).

Tooth bleaching

All rats were anaesthetised by intramuscular injections of ketamine (80 mg/kg, Ketamina Agener 10%; União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) and xylazine (10 mg/kg, Xilazin; Syntec do Brazil LTDA, Cotia, São Paulo, Brazil). After the application and photoactivation of the resinous gingival barrier (Top Dam; FGM Dental Products, Joinville, SC, Brazil), the right or left maxillary molars randomly received 0.01 mL of bleaching gel (35% H_2O_2, Whiteness HP Maxx; FGM Dental Products, Joinville, SC, Brazil) or placebo gel (thickener of the bleaching gel; Cintra *et al*. 2013) for 30 min. The volume of the applied bleaching or placebo gel was standardized by using 1.0-mL syringes (Benetti *et al*. 2018).

Histological analysis

At 2, 3, 7, 15, and 30 days (adapted from Hirata *et al*. 2005, Cintra *et al*. 2013, Benetti *et al*. 2018) after administration of the gels, the rats were killed with overdose of an anaesthetic solution (Thiopentax; Cristália—Produtos Químicos Farmacêuticos LTDA, Itapira, São Paulo, Brazil). The right and left hemimaxillae were separated, and 10 hemimaxillae were obtained per group for each time point (n = 10). The hemimaxillae were then dissected and fixed in a solution of 4% buffered formaldehyde for 24 h. The specimens were decalcified in a 10% ethylenediaminetetraacetic acid solution for 3 months and then dehydrated, clarified, and embedded in paraffin.

For each specimen, sections (5 μm) were selected from the point where the mesial root of the first molar could be seen with its complete longitudinal extension and were cut in the vestibular–lingual plane. Five-micron sections were stained with haematoxylin–eosin for analysis using light microscopy (×400 magnification; DM4000 B; Leica Microsystems, Wetzlar, Germany). The intensity of inflammation was scored according to the presence of the inflammatory infiltrate as follows: 0, inflammatory cells absent or negligible in number; 1, mild inflammatory infiltrate (<25 cells per field); 2, moderate inflammatory infiltrate (between 25 and 125 cells per field); 3, severe inflammatory infiltrate (>125 cells per field); and 4, tissue necrosis (Benetti *et al*. 2017a, 2018). The analyses were performed by a single calibrated operator in a blinded manner.

Immunohistochemical analyses for HO-1 and Jun-D

Other histological sections were obtained for immunohistochemical assessments with an indirect immunoperoxidase technique (Benetti *et al*. 2017a, Ferreira *et al*. 2017) for HO-1 and Jun-D. The sections were deparaffinised (xylene) and hydrated (decreasing ethanol series). Antigen retrieval was performed by immersing the histological slides in buffer citrate solution (Antigen Retrieval Buffer; Spring Bioscience, Pleasanton, CA, USA) in a pressurised chamber (Decloaking Chamber; Biocare Medical, Concord, CA, USA) at 95°C for 10 min, after which the slides were rinsed with phosphate-buffered saline (PBS). The histological sections were immersed in 3% H_2O_2 solution for 1 h and 20 minutes and in 1% bovine serum albumin for 12 h to block endogenous peroxidase activity and nonspecific sites, respectively, and divided and incubated with one of the following primary antibodies: anti-HO-1 (rabbit primary antibody; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) and anti-Jun-D (rabbit primary antibody; Sigma-Aldrich Co. LLC). The primary antibodies were diluted (Antibody Diluent with Background Reducing Components; Dako Laboratories, Carpinteria, CA, USA), and placed in a moist chamber for 24 h. The histological sections were incubated with a biotinylated secondary antibody for 1 h and 30 min and subsequently treated with streptavidin–horseradish peroxidase conjugate for 1 h and 30 min (Universal Dako Labelled Streptavidin-Biotin kit; Dako Laboratories). After rinsing with PBS, the reaction was developed using the chromogen 3,3′-diaminobenzidine tetrahydrochloride (DAB Chromogen kit; Dako Laboratories) and counterstained with *Fast Green*. The negative controls consisted of specimens that underwent the previously described procedures without treatment with the primary antibodies.

For analyses, cells with brown cytoplasm (HO-1 and Jun-D) or brown nucleus (Jun-D) were counted in each third of the pulp chamber of each specimen, and the data were presented as the number of cells per mm^2 (Benetti *et al*. 2017a, 2018). The mean values for each third of each specimen were used for statistical analysis.

Immunofluorescence analysis of CD90+/CD73+/CD105+/CD45- cells

Additional histological sections were selected for immunofluorescence analysis. In this assessment, two consecutives histological sections were obtained on the same slide to be later limited by PAP pen (Sigma-Aldrich Co. LLC) markings for immunostaining. The histological sections were deparaffinised (xylene) and hydrated (decreasing ethanol series). Antigen retrieval was achieved by immersing the histological slides in citrate buffer solution (Antigen Retrieval Buffer; Spring Bioscience) in a pressurised chamber (Decloaking

Chamber; Biocare Medical) at 95°C for 10 min (similar to immunohistochemistry method). The histological sections were then rinsed with PBS and immersed in 1% bovine serum albumin for 12 h to block the nonspecific sites. Marking with the PAP pen was performed, and the consecutives sections were divided into two allotments. Sections in allotment 1 were incubated with anti-CD73 (rabbit primary antibody, SC-25603; Santa Cruz Biotechnology, INC., Dallas, Texas, USA) and anti-CD90 (mouse primary antibody, SC-53116; Santa Cruz Biotechnology). Those in allotment 2 were incubated with anti-CD105 (rabbit primary antibody, orb124502; Biorbyt Ltd., San Francisco, CA, USA) and anti-CD45 (mouse primary antibody, SC-53047; Santa Cruz Biotechnology). The primary antibodies were diluted in PBS containing 0.1% Triton X-100 (PBS-TX) for 24 h in a humid chamber.

Sections from both allotments were then incubated with Cy3-conjugated anti-rabbit IgG secondary antibodies (1:200, Vector Lab. Inc., CA, USA) and fluorescein-conjugated anti-mouse IgG secondary antibodies (1:200, Jackson ImmunoResearch Inc., PA, USA). After three additional washes in deionized water, the slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 20 min and mounted with an aqueous solution of glycerol. The negative controls consisted of specimens subjected to the procedures described above without incubation with the primary antibodies.

After these treatments, cells expressing CD90 (allotment 1) and CD45 (allotment 2) would show green coloration, while those expressing CD73 (allotment 1) and CD105 (allotment 2) would show red coloration. The histological sections were analysed at a magnification of 400× throughout the coronary pulp, and only those that were CD90+/CD105+ in allotment 1 and CD73+/CD45- in allotment 2 were quantified. The absolute amounts of these cells in the coronary pulp were determined.

Statistical analysis

The T-test was used for parametric data and the Mann-Whitney statistical test was used for non-parametric data. Significance was set at the level of 5% ($P < 0.05$).

Results

Results

Histological analysis

Figure 1 shows the representative images of the pulp tissue in each group. The control group (Fig. 1A) showed normal histological conditions: there was intact pulp tissue throughout the

region of the coronary pulp; the odontoblast layer was present and continuous around the entire pulp; and there were no inflammatory cells.

In contrast, the bleached specimens (Fig. 1B-F) showed significant changes at 2 and 3 days ($P < 0.05$; Table 1). At these time points, necrosis with an underlying severe inflammatory infiltrate was mainly observed in the occlusal third of the coronary pulp; the coronary thirds did not contain an odontoblast layer and showed cellular degeneration (Fig. 1B, C). The inflammation in the pulp disappeared from 7 days, and the findings thereon were similar to those in the control group ($P > 0.05$). At 7 and 15 days post-bleaching (Fig. 1D, E), the pulp tissue was organised, and larger columnar and elongated odontoblasts-like cells were arranged beneath the dentine–pulp interface; tertiary dentine was present around the entire pulp tissue. This dentine gradually increased in thickness at 30 days, and pulp tissue was organised at this point (Fig. 1F).

Table 1 Scores for inflammatory infiltrate in each third of coronary pulp

Analysis		Scores	Groups						*P*
			Cont	Bleached					
				2d	3d	7d	15d	30d	
H.E.	**Occlusal**	0	10/10	0/10	0/10	10/10	10/10	10/10	ContXBle2d, ContXBle3d: <0.001
		1	0/10	0/10	0/10	0/10	0/10	0/10	
		2	0/10	0/10	0/10	0/10	0/10	0/10	
		3	0/10	5/10	6/10	0/10	0/10	0/10	
		4	0/10	5/10	4/10	0/10	0/10	0/10	
		Median	0	4*	3*	0	0	0	
	Middle	0	10/10	0/10	0/10	10/10	10/10	10/10	ContXBle2d, ContXBle3d: <0.001
		1	0/10	0/10	0/10	0/10	0/10	0/10	
		2	0/10	4/10	5/10	0/10	0/10	0/10	
		3	0/10	4/10	4/10	0/10	0/10	0/10	
		4	0/10	2/10	1/10	0/10	0/10	0/10	
		Median	0	3*	2*	0	0	0	
	Cervical	0	10/10	0/10	0/10	10/10	10/10	10/10	ContXBle2d, ContXBle3d: <0.001
		1	0/10	2/10	3/10	0/10	0/10	0/10	
		2	0/10	7/10	7/10	0/10	0/10	0/10	
		3	0/10	1/10	0/10	0/10	0/10	0/10	
		4	0/10	0/10	0/10	0/10	0/10	0/10	
		Median	0	2*	2*	0	0	0	

*The symbol in the same line indicate statistically significant differences compared to control group ($P<0.05$).

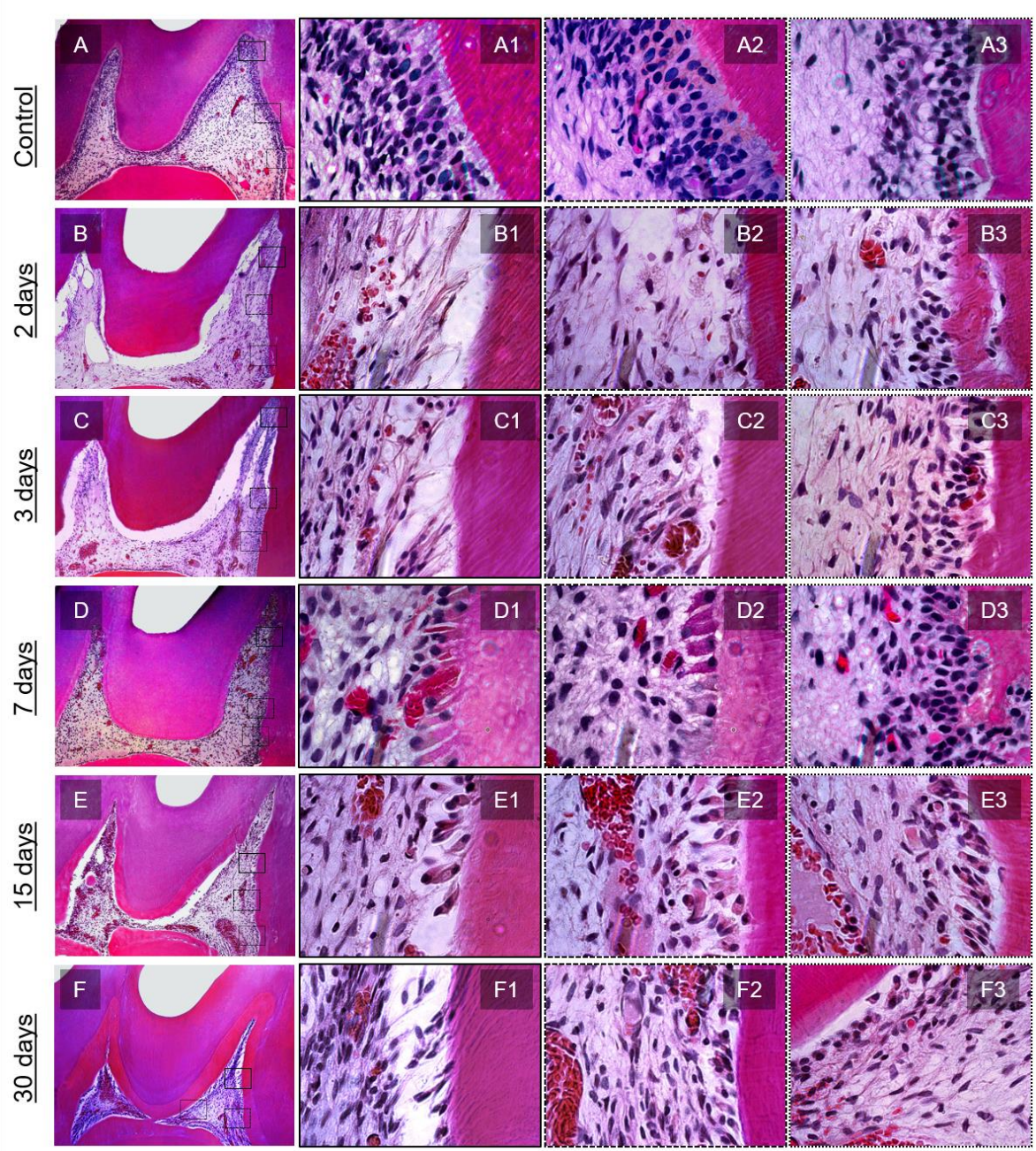


Figure 1. Representative images of histological analyses of (A) control and (B-F) bleached groups. (A) Panoramic microscopic aspect of the control group with normal pulp tissue, with the odontoblast layer in the (A1) occlusal, (A2) middle, and (A3) cervical third of the coronary pulp. (B, C) Bleached groups at (B) 2 and (C) 3 days post-bleaching showed necrotic areas in the cervical thirds and tissue disorganisation; (B1, C1) evident tissue disorganisation with cell degeneration in the occlusal third; (B2, C2) absence of the odontoblastic layer with presence of inflammatory cells in the middle third; and (B3, C3) tissue disorganisation in the cervical third. (D, E) Bleached group at (D) 7 and (E) 15 days post-bleaching, showing organised pulp tissue and deposition of tertiary dentine; (D1, E1) occlusal third and (D2, E2) middle third showing the presence of columnar and elongated odontoblast-like cells arranged beneath the dentin–pulp interface; (D3, E3) cervical third with an organised odontoblastic layer. (F) Bleached group at 30 days with tertiary dentin around almost all of the pulp tissue; (F1) the middle third and (F2, F3) cervical third showing the presence of an odontoblast-like layer. (100×, 1000×; haematoxylin–eosin [H.E.])

Immunohistochemical analysis for HO-1 and Jun-D

Figures 2 and 3 show representative images for HO-1 and Jun-D immunolabelling, respectively. Figure 4 shows the number of HO-1– (Fig. 4A) or Jun-D–positive cells (Fig. 4B, 4C) per mm^2 in each third of the coronary pulp.

In general, the maximum HO-1 immunolabelling was observed in the middle and cervical thirds at 2 and 3 days post-bleaching, compared to the control group ($P < 0.05$), in all thirds at 7 days ($P < 0.05$), and in the occlusal third at 15 days ($P < 0.05$). HO-1 was present in both fibroblasts and odontoblast-like cells. At 30 days, tertiary dentine was observed in the occlusal and middle thirds of the bleached group, and no HO-1–positive cells were found ($P < 0.05$).

Jun-D immunolabelling was observed in the cytoplasm and nucleus of fibroblasts and odontoblasts. In the control group, a number of cells with brown cytoplasm were observed (Fig. 4B), and these cells increased in number in all thirds of the coronal pulp at 7 days after bleaching ($P < 0.05$), and in the cervical third at 15 days after bleaching ($P < 0.05$).

Nuclear immunolabelling for Jun-D was mainly observed in odontoblasts. The control group showed almost no cells with brown nuclei (Fig. 4C), but the number of such cells was significant in the cervical third of the coronary pulp at 2 and 3 days after bleaching ($P < 0.05$) and in the occlusal and middle thirds at 7 days ($P < 0.05$). The degree of immunolabelling reduced over the course of the experimental period, eventually reaching levels similar to those observed in the control group ($P > 0.05$).

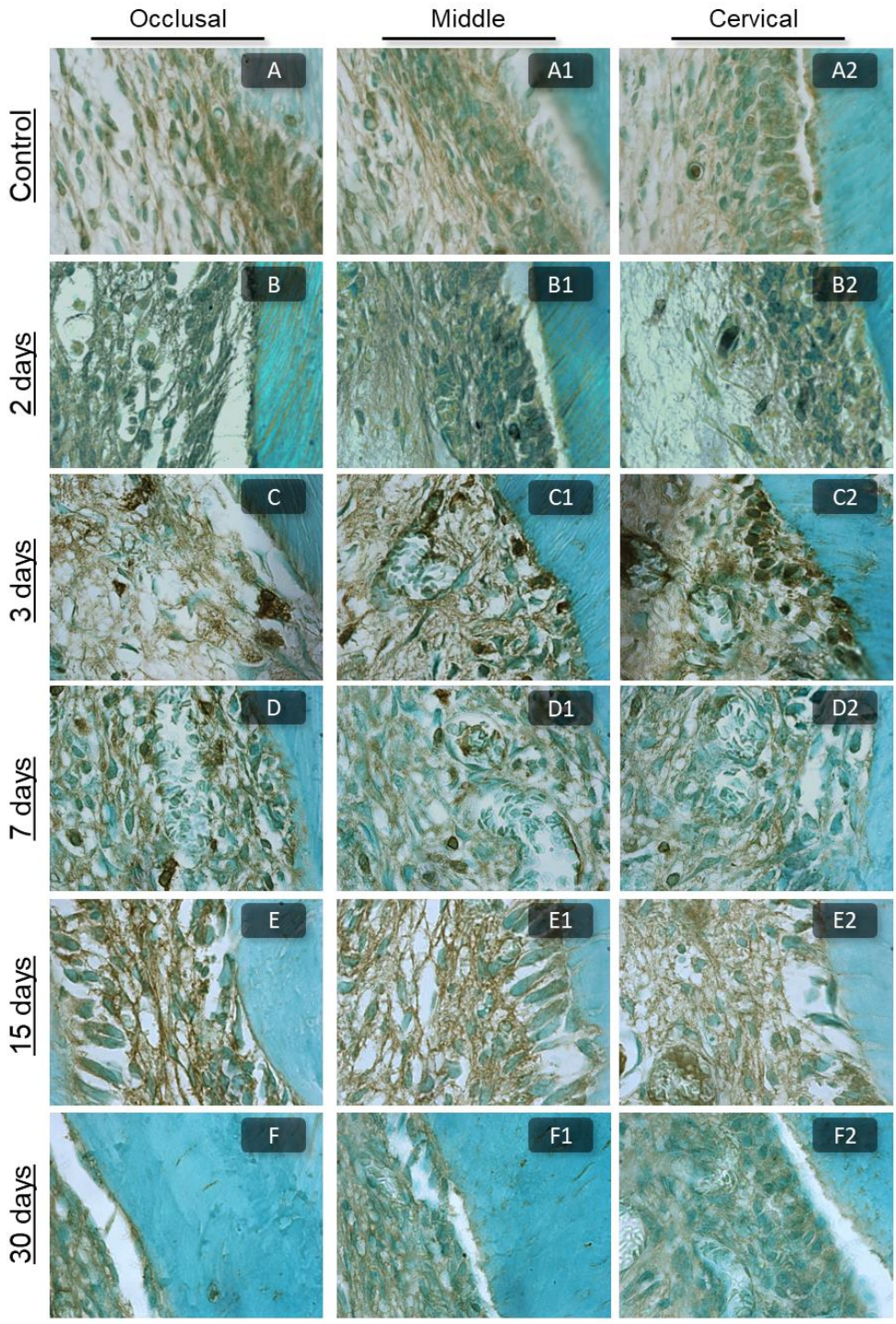


Figure 2. Representative images of HO-1 immunohistochemical analyses in the (A-F) occlusal, (A1-F1) middle, and (A2-F2) cervical thirds of each group. Presence of low immunolabelling in the thirds of the (A, A1, A2) control group and in the middle and cervical thirds of the bleached group at (E1, E2) 15 and all thirds at (F, F1, F2) 30 days, as well as in the occlusal third of the bleached group at (B) 2 and (C) 3 days post-bleaching. The immunolabelling was significant in the middle and cervical thirds of the bleached groups at (B1, B2) 2 and (C1, C2) 3 days post-bleaching, as well as in (D, D1, D2) all the thirds of the bleached group at 7 and (E) occlusal third at 15 days (1000×; immunohistochemical analysis of HO-1).

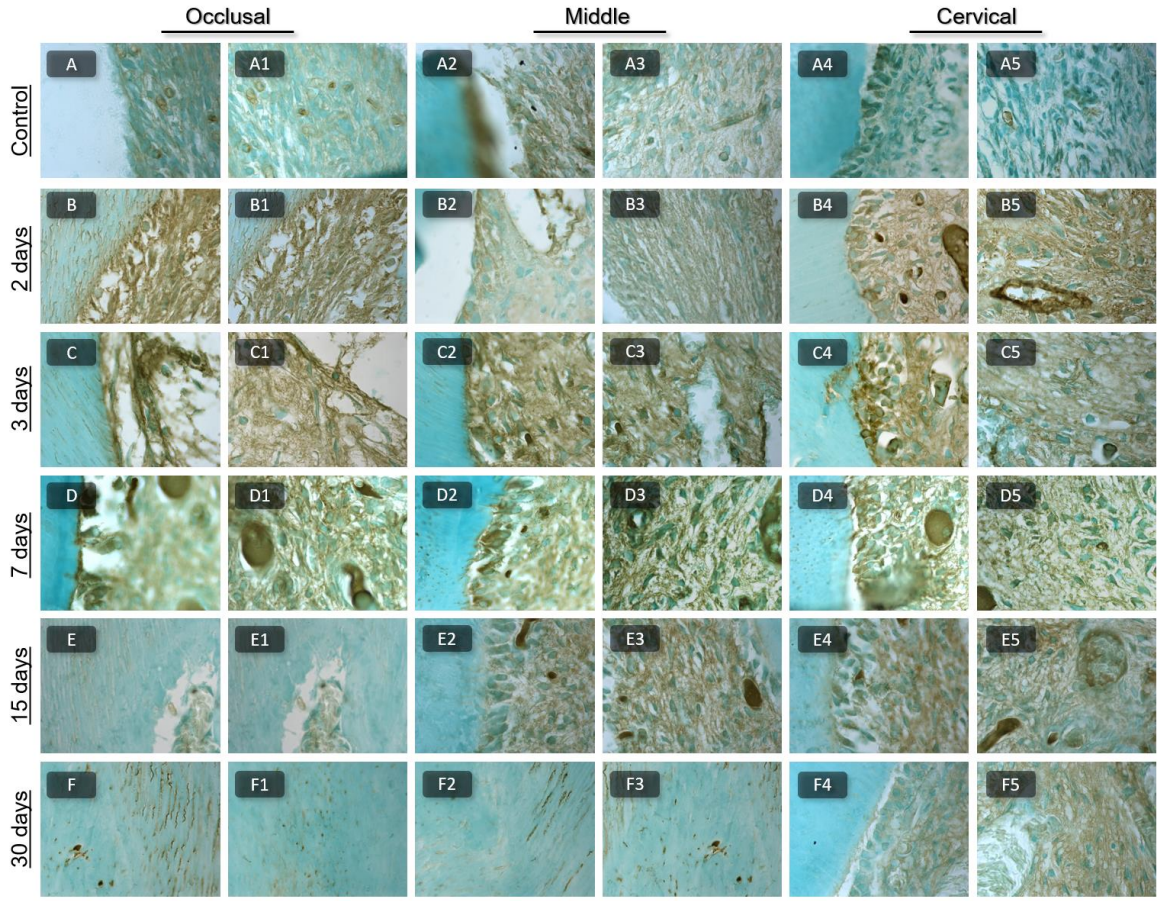


Figure 3. Representative images of immunohistochemical analyses of Jun-D in the (A, A2, A4-F, F2, F4) odontoblastic layer and (A1, A3, A5-F1, F3, F5) centre of the pulp chamber. Images of the (A, A1-F, F1) occlusal, (A2, A3-F2, F3) middle, and (A4, A5-F4, F5) cervical thirds of the pulp chamber of the (A-A5) control group and the bleached group at (B-B5) 2 days, (C-C5) 3 days, (D-D5) 7 days, (E-E5) 15 days, and (F-F5) 30 days after bleaching. (1000×; immunohistochemical analysis of Jun-D).

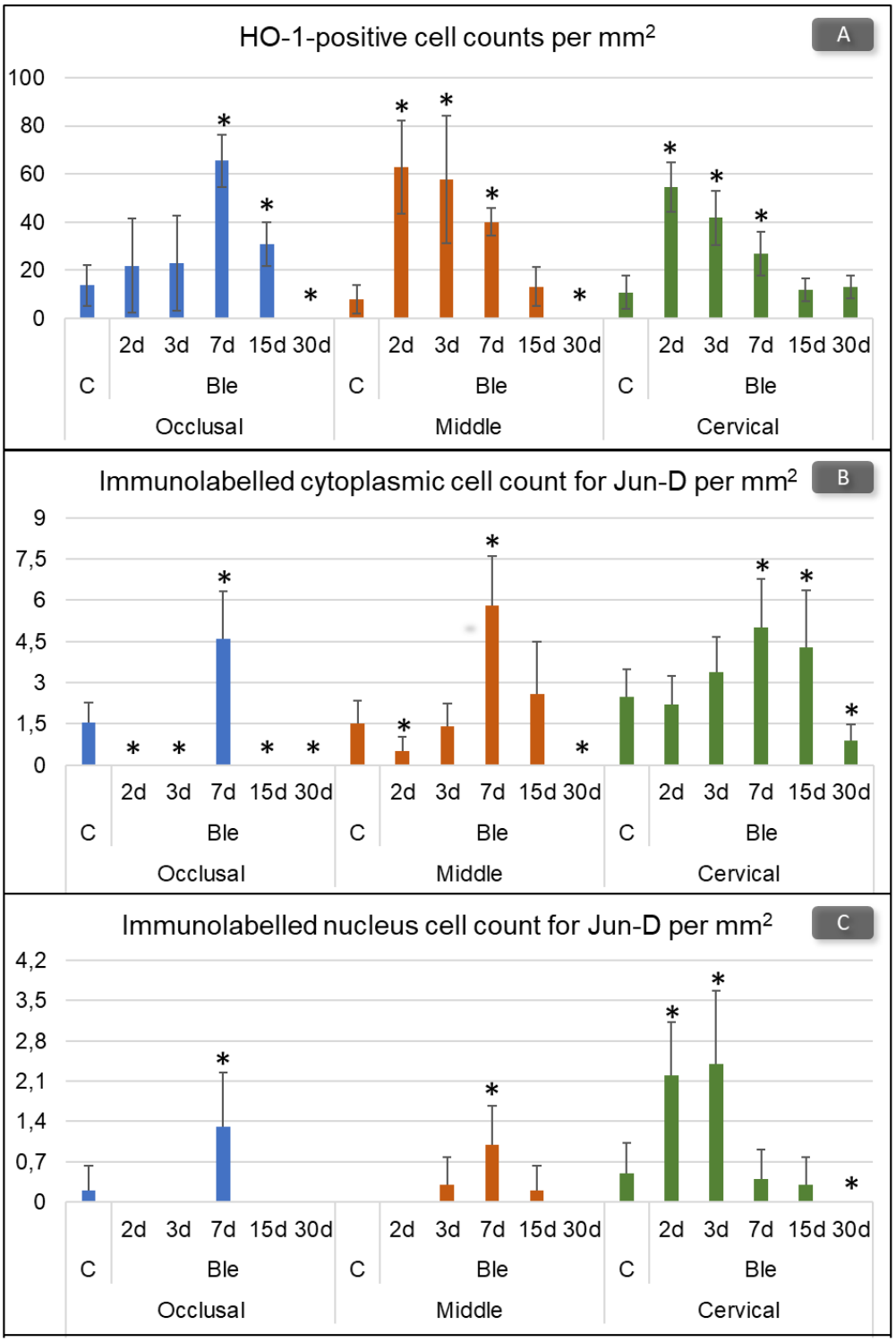


Figure 4. Representative graphs of the counts of immunolabelled cells in each third of the coronary pulp. The graphs show the number of cells immunolabelled per mm^2 for (A) HO-1 and (B, C) Jun-D. Immunolabelled cell counts for Jun-D are separated with respect to (B) cytoplasmic labelling and (C) nuclear labelling. The * symbol in the same graphic indicates statistically significant differences between the bleached and control groups for each third of the pulp chamber ($P < 0.05$).

Immunofluorescence analysis of CD90+/CD73+/CD105+/CD45- cells

Figure 5 shows representative images for identification of CD90-, CD73- and CD105-positive and CD45-negative cells, as well as a graph showing the number of these cells (Fig. 5G).

A relatively low number of these cells was observed within the coronary pulp, and when present, they were generally close to the blood vessels or the odontoblastic layer. Cells were observed in the control group (Fig. 5A1-A5) and in the bleached group at 2 days (Fig. 5B1-B5), 3 days (Fig. 5C1-C5), 7 days (Fig. 5D1-D5), 15 days (Fig. 5E1-E5), and 30 days (Fig. 5F1-F5), with no significant difference between groups ($P > 0.05$).

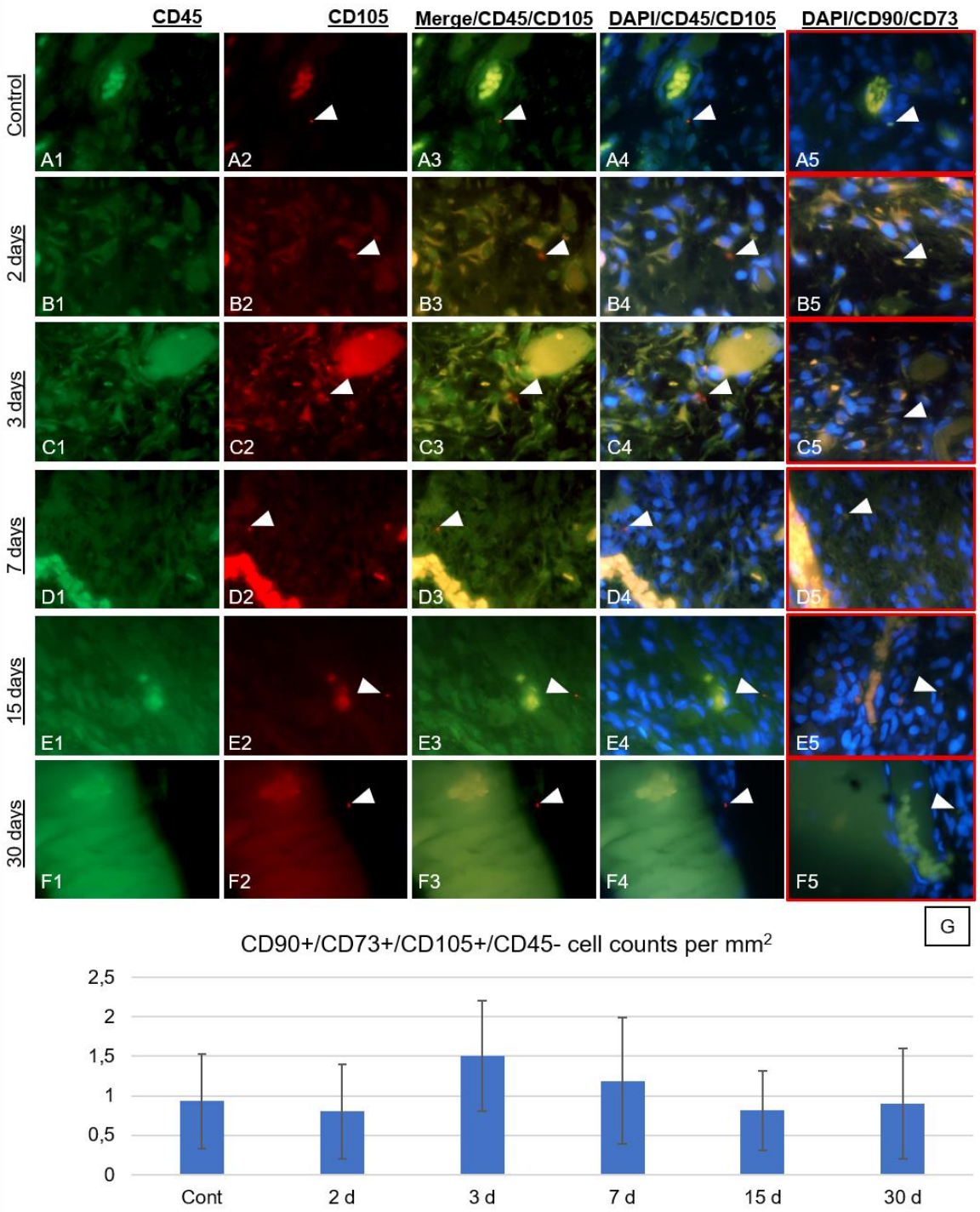


Figure 5. Representative immunofluorescence images of CD90+/CD73+/CD105+/CD45- cells. (A1-F1) Green images represent the immunofluorescence of the CD45 antibody; (A2-F2) red images represent the CD105 antibody; and (A3-F3) the merge image of CD45/CD105. (A4-F4) Merge image of CD45/CD105 and (A5-F5) merge image of CD90/CD73 with 4',6-diamidino-2-phenylindole (DAPI) staining. The arrowhead shows CD90+/CD73+/CD105+/CD45- cells, identified as red in merge image of CD45/CD105 and yellow in merge image of CD90/CD73. (G) The graph shows the cell count per mm^2 (1000×; immunofluorescence analysis).

Discussion

The present study investigated the effects of H_2O_2-induced oxidative stress on HO-1 immunolabelling in pulp tissue, which represented cellular defence capacity, Jun-D expression, which indicated odontoblastic differentiation, and the presence of CD90+/CD73+/CD105+/CD45- cells, which are indicative of MSCs. In general, H_2O_2 influenced HO-1 expression in the pulp during inflammation and at the beginning of repair and Jun-D expression during the reduction of inflammation and at the beginning of tertiary dentine production. Thus, the null hypothesis that H_2O_2 does not influence the expression of HO-1 and Jun-D in the pulp of bleached teeth was rejected. However, CD90+/CD73+/CD105+/CD45- cells were identified in relatively low numbers regardless of the presence of oxidative stress, and the part of the null hypothesis stating that H_2O_2 does not influence the identification of MSCs in pulp tissue after bleaching was accepted.

Injury to the dentine–pulp complex initiates an inflammatory process in the dental pulp (Massler 1967). Pulp tissue is surrounded by rigid and inextensible dentine walls, a characteristic that differentiates the pulp response from the responses of other connective tissues. This fact also reflects the importance of *in vivo* analyses of pulp tissue response to aggressive agents (Jeanneau *et al*. 2017).

Previous *in vivo* analyses have shown regions with high numbers of proliferating cells underlying the necrotic region of bleached teeth (Benetti *et al*. 2017a) and the presence of proinflammatory cytokines (Benetti *et al*. 2018). In fact, a series of cellular mechanisms occur in pulp tissue to restore its homeostasis, but these mechanisms are still poorly understood. Studies have shown that cells capable of responding to various stimuli, such as nitric oxide or proinflammatory cytokines, express the HO-1 protein (Min *et al*. 2006a, 2006b, 2008). This protein acts to generate carbon monoxide in order to suppress inflammatory signalling, in addition to presenting an indirect antioxidant effect by producing biliverdin and bilirubin (Min *et al*. 2008, 2010).

Our results show that HO-1 is primarily expressed 2 and 3 days after bleaching, during which the inflammatory process is present. This indicates that HO-1 participates in the inflammatory process generated in the pulp tissue of bleached teeth. Previous studies have reported that the proinflammatory cytokines TNF-α, IL-17, and IL-6 are present in significant amounts in pulp tissue at 2 days after bleaching (Ferreira *et al*. 2017, Benetti *et al*. 2018), confirming an inflammatory condition in this period. The treatment of pulp cells with only TNF-α could also increase HO-1 expression in these cells (Min *et al*. 2006),

showing that the inflammatory process alone could also stimulate HO-1 expression. These findings underline the importance of minimizing the inflammatory process in the repair of pulp tissue.

However, HO-1 was also present in all thirds of the coronary pulp at 7 days post-bleaching, in addition to the occlusal third at 15 days, when no inflammatory process was noted in the pulp. In a primary culture of human pulp cells, the induction of HO-1 by H_2O_2 was found to play a protective role against the cytotoxic effects of H_2O_2 and also stimulate odontoblast differentiation (Min *et al*. 2008). Thus, we suggest that the presence of HO-1 in these periods was a possible stimulus for odontoblastic differentiation, which was confirmed by the presence of significant levels of Jun-D at 7 days after bleaching. HO-1 expression was not significant in most thirds of the coronary pulp at 15 days and in the entire dental pulp at 30 days. These data indicate a significant reduction of oxidative stress in the pulp tissue in these periods. Furthermore, the HO-1 levels decreased significantly from the time when odontoblast differentiation was completed and no nuclear immunolabelling of Jun-D could be observed. This may suggest that HO-1 stimulates odontoblastic differentiation in response to the pulp inflammatory process but is not present after the differentiation of odontoblasts and reduction of the inflammatory process.

To our knowledge, this is also the first study to analyse the presence of Jun-D in the pulp tissue after exposure to H_2O_2. Osteoblastic differentiation is controlled by several transcription factors, including those of the Jun family (c-Jun, Jun B, and Jun-D) (Karsenty &Wagner 2002, Hirata *et al*. 2005). The genes of the Jun family have also been observed in odontoblasts, and each gene is related to different stages of dentin production (Kitamura & Terashita 1997); for example, Jun-D expression is observed in the initial phase of reactionary dentine formation in rats after of tooth preparation (Hirata *et al*. 2005).

Jun-D is considered active when observed in the nucleus of the cells, because it is a transcription factor (Klinz *et al*. 2013). In this study, nuclear immunolabelling for Jun-D was observed in the cervical third at 2 and 3 days after bleaching, when there was a reduction in the inflammatory process. This indicates the onset of odontoblastic differentiation, since the presence of Jun-D has been suggested to play a significant role in the differentiation of these cells (Hirata *et al*. 2005). Furthermore, nuclear immunolabelling for Jun-D was also observed at 7 days after bleaching, particularly in the occlusal and middle thirds of the coronary pulp, and reduced in the later periods, indicating that the differentiation of new odontoblasts had ceased. At 7 days, we observed the initiation of tertiary dentine formation.

A preliminary study corroborates our results, reporting that Jun-D was not observed in the mature odontoblasts (Hirata *et al*. 2005). Furthermore, one study observed that during osteoblast proliferation, the nuclear protein members of the activator of protein 1 (AP-1), such as Jun-D, are at their maximum levels, and their levels decrease in the maturation phase of the extracellular matrix (McCabe *et al*. 1996).

However, unlike the previous study (Hirata *et al*. 2005), we observed the expression of Jun-D in the control group, but in the cytoplasm, indicating their inactivity. A previous study with dental pulp cells observed the presence of other transcription factors of AP-1 (Fra-1 and c-Jun) in untreated cells (Liu *et al*. 2013). Further studies should be performed to understand the location and performance of Jun-D during odontoblastic differentiation.

This study also sought to identify the presence of MSCs in pulp tissue and to investigate *in vivo* the effects of H_2O_2 on these cells. We identified a low number of MSCs, regardless of the presence of oxidative stress. There are several approaches to characterize MSCs, which make it difficult to compare the results of different studies (Fernandez-Vallone *et al*. 2013). Numerous markers have been used to characterize these cells, including some negative markers whose absence indicates that the cells are MSCs (Kawashima 2012, Fernandez-Vallone *et al*. 2013). Among these markers, some have been extensively studied, such as CD90, CD73, and CD105 (Huang *et al*. 2009, Waddington *et al*. 2009, Alongi *et al*. 2010, Balic *et al*. 2010, Pivoriuunas *et al*. 2010), and thus were selected for the present study. As a negative marker, we opted for CD45, which is a cell surface glycoprotein found in all cells of the hematopoietic lineage, except erythrocytes and platelets (Rutz *et al*. 2007, Balic & Mina 2010).

The effect of different concentrations of H_2O_2 on the viability of human dental pulp stem cells and their recovery capacity was investigated *in vitro* in a previous study (Soares *et al*. 2015a). The study showed that immediate cytotoxicity was dependent on H_2O_2 concentration, with lower concentrations related to a greater late proliferative capacity of these cells. However, in the present study, few CD90-, CD73-, and CD105-positive cells were identified as CD45-negative in the pulp tissue, regardless of the period of analysis. Furthermore, although studies have indicated that the inflamed pulp contains a greater number of cells with markers of MSCs (Alongi *et al*. 2010, Kawashima 2012, Ma *et al*. 2012), in this *in vivo* study, there was no significant change in the presence of MSC marker-positive cells in the inflamed pulp tissue.

However, unlike these previous studies, our study considered only cells that showed positivity for three markers (CD90, CD73 and CD105), and negativity for a marker (CD45), according to the International Society of Cell Therapy standards. For example, Alongi et al. (2010) did not identify any negative markers in their study, so that they could consider the cells as MSCs. Still, a previous study stated that the stem cells in the dental pulp belong to rare populations with indefinite cytological markers and are thus poorly characterized *in vivo* (Kaneko *et al*. 2013). In addition, a small number of cells labelled with MSC markers were identified *in vivo* in the intact pulp tissue of rat molars (Kaneko *et al*. 2013), which corroborates our results.

In one study, non-erupted murine incisor pulps showed low CD45+ labelling and high CD90+/CD45- labelling and exhibited rapid *in vitro* mineralization (Balic & Mina 2010). The same findings were noted in human molars in another study (Balic *et al*. 2010). However, after eruption of the teeth, the immunohistochemical marking profile showed a higher number of CD45+ cells, and lower CD90+ labelling (Balic & Mina 2010). This suggests that both rat and human teeth has marking to stem cells at the period of dental development. These data may explain the difficulty in identifying cells with markers of positive MSCs in our study.

Furthermore, the ability of multipotent stem cells to replace damaged tissues has been shown to decrease with age (Jones & Rando 2011). Since recovery of pulp tissue homeostasis is observed even after the severe damage caused by H_2O_2, it can be assumed that the undifferentiated cells that rapidly replaced the lost tissue already had a certain level of differentiation, being ready for a rapid response in the pulp tissue. While many *in vivo* studies have investigated the role of stem cells from periradicular tissues in pulp regeneration (Huang *et al*. 2010, Tobias Duarte *et al*. 2014), and despite the large number of *in vitro* studies that have characterized MSCs from pulp tissue (Alongi *et al*. 2010, Balic *et al*. 2010, Balic & Mina 2010, Pivoriuunas *et al*. 2010, Kawashima *et al*. 2012, Ma *et al*. 2012), few studies have studied the mechanisms of *in vivo* actuation of these cells from the pulp in the repair of lost pulp tissue.

This study provides new data regarding the *in vivo* response profile of pulp tissue to H_2O_2. The findings showed that HO-1 is present during the inflammatory process and at the beginning of the pulp repair, and this is the first study to identify the Jun-D gene in odontoblastic differentiation after the damage caused by H_2O_2 in the pulp tissue. However, the difficulties encountered in *in vivo* identification of CD90+/CD73+/CD105+/CD45- cells

precluded a clarification of the mechanism by which these cells function in the recovery of pulp tissue and indicate the need for further studies on this topic.

Conclusions

Pulp cells show responsiveness to oxidative stress generated by bleaching and expressing HO-1 during the inflammation stage until the beginning of the repair. In addition, Jun-D nuclear immunolabelling is present in the pulp during the reduction of the inflammatory process and the beginning of tertiary dentine production. Presence of oxidative stress does not influence the number of CD90+/CD73+/CD105+/CD45- cells identified *in vivo* in the dental pulp.

Conflicts of interest

The authors explicitly state that there are no conflicts of interest in connection with this article.

Acknowledgement

This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (n. 2015/10825-2) and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (n. 455943/2014-1).

References

Agata H, Kagami H, Watanabe N, *et al*. (2008) Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells. *Differentiation* **76**, 981–93.

Alongi DJ, Yamaza T, Song Y, *et al*. (2010) Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. *Regenerative Medicine* **5**, 617–31.

Balic A, Aguila HL, Caimano MJ, *et al.* (2010) Characterization of stem and progenitor cells in the dental pulp of erupted and unerupted murine molars. *Bone* **46**, 1639–51.

Balic A, Mina M (2010) Characterization of Progenitor Cells in Pulps of Murine Incisors. *Journal of Dental Research* **89**, 1287-92.

Benetti F, Gomes-Filho JE, Ferreira LL, *et al*. (2017a) Hydrogen peroxide induces cell proliferation and apoptosis in pulp of rats after dental bleaching in vivo: Effects of the dental bleaching in pulp. *Archives of Oral Biology* **22**, 103-9.

Benetti F, Gomes-Filho JE, Ferreira LL, *et al*. (2018) Concentration-dependent effect of bleaching agents on the immunolabeling of interleukin-6, interleukin-17, and CD5-positive cells in the dental pulp. *International Endodontic Journal. In press.* doi: 10.1111/iej.12891.

Benetti F, Lemos CAA, de Oliveira Gallinari M, *et al*. (2017b) Influence of different types of light on the response of the pulp tissue in dental bleaching: a systematic review. *Clinical Oral Investigations. In press*. doi: 10.1007/s00784-017-2278-9.

Briso AL, Fagundes TC, Gallinari MO, *et al*. (2016) An *in situ* study of the influence of staining beverages on color alteration of bleached teeth. *Operative Dentistry* **41**, 627-33.

Cintra LT, Benetti F, Facundo ACS, *et al*. (2013) The number of bleaching sessions influences pulp tissue damage in rat teeth. *Journal of Endodontics* **39**, 1576-80.

Cintra LT, Benetti F, Ferreira LL, *et al*. (2016a) Penetration capacity, color alteration and biological response of two in-office bleaching protocols. *Brazilian Dental Journal* **27**, 169-75.

Cintra LT, Benetti F, Ferreira LL, *et al*. (2016b) Evaluation of an experimental rat model for comparative studies of bleaching agents. *Journal Applied of Oral Science* **24**, 95-104.

Cintra LT, Ferreira LL, Benetti F, *et al*. (2017) The effect of dental bleaching on pulpal tissue response in a diabetic animal model. *International Endodontic Journal* **50**, 790-798.

Colucci S, Brunetti G, Oranger A, *et al.* (2011) Myeloma cells suppress osteoblasts through sclerostin secretion. *Blood Cancer Journal* **1,** e27.

Demarco FF, Meireles SS, Masotti AS (2009) Over-the-counter whitening agents: a concise review. *Brazilian Oral Research* **23**, 64-70.

Fernández-Vallone VB, Romaniuk MA, Choi H, *et al*. (2013) Mesenchymal stem cells and their use in therapy: what has been achieved? *Differentiation* **85**, 1-10.

Ferreira LL, Gomes-Filho JE, Benetti F, *et al*. (2017) The effect of dental bleaching on pulpal tissue response in a diabetic animal model: Immunoregulatory cytokines study. *International Endodontic Journal, In press*.

Grazioli G, Valente LL, Isolan CP, *et al.* (2017) Bleaching and enamel surface interactions resulting from the use of highly-concentrated bleaching gels. *Archives of Oral Biology* **28**, 157-162.

Hasson H, Ismail AI, Neiva G (2006) Home-based chemically-induced whitening of teeth in adults (Review). *Cochrane Database Systematic Review* **4**, 1-43.

Hirata M, Yamaza T, Mei YF, Akamine A (2005) Expression of osteocalcin and Jun D in the early period during reactionary dentin formation after tooth preparation in rat molars. *Cell and Tissue Research* **319**, 455-65.

Huang GT, Yamaza T, Shea LD, *et al*. (2010) Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Engineering. Part A* **16**, 605-15.

Huang GTJ, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs from other sources: the biology and role in regenerative medicine. *Journal of Dental Research* **88**, 792–806.

Jones DL, Rando TA (2011) Emerging models and paradigms for stem cell ageing. *Nature Cell Biology* **13**, 506-12.

Jeanneau C, Laurent P, Rombouts C, *et al*. (2017) Light-cured Tricalcium Silicate Toxicity to the Dental Pulp. *Journal of Endodontics* **43**, 2074-2080.

Kaneko T, Arayatrakoollikit U, Yamanaka Y, Ito T, Okiji T (2013) Immunohistochemical and gene expression analysis of stem-cell-associated markers in rat dental pulp. *Cell and Tissue Research* **351**, 425-32.

Karsenty G, Wagner EF (2002) Reaching a genetic and molecular understanding of skeletal development. *Developmental Cell* **2**, 389–406.

Kawashima N (2012) Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? *Archives of Oral Biology* **57**, 1439-58.

Kim D, Kim H, Kim K, Roh S (2017) The Protective Effect of Indole-3-Acetic Acid (IAA) on H2O2-Damaged Human Dental Pulp Stem Cells Is Mediated by the AKT Pathway and Involves Increased Expression of the Transcription Factor Nuclear Factor-Erythroid 2-Related Factor 2 (Nrf2) and Its Downstream Target Heme Oxygenase 1 (HO-1). *Oxidative Medicine and Cellular Longevit* **2017**, 8639485.

Kitamura C, Terashita M (1997) Expressions of c-jun and jun-B proto-oncogenes in odontoblasts during development of bovine tooth germs. *Journal of Dental Research* **76**, 822-30.

Klinz FJ, Korkmaz Y, Cho B, Raab WH, Addicks K (2013) Transcription factor CREB is phosphorylated in human molar odontoblasts and cementoblasts *in vivo*. *Histochemistry and cell biology* **139**, 615-20.

Lee DH, Lim BS, Lee YK, *et al*. (2006) Effects of hydrogen peroxide (H2O2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. *Cell Biology and Toxicology* **22**, 39–46.

Liu Z, Jiang T, Wang X, Wang Y (2013) Fluocinolone acetonide partially restores the mineralization of LPS-stimulated dental pulp cells through inhibition of NF-κB pathway and activation of AP-1 pathway. *British Journal of Pharmacology* **170,** 1262-71.

Ma D, Gao J, Yue J, *et al*. (2012) Changes in proliferation and osteogenic differentiation of stem cells from deep caries in vitro. *Journal of Endodontics* **38**, 796-802.

Massler M (1967) Pulpal reactions to dental caries. *International Endodontic Journal* **17**, 441–460.

Matsui S, Takahashi C, Tsujimoto Y, Matsushima K (2009) Stimulatory effects of low-concentration reactive oxygen species on calcification ability of human dental pulp cells. *Journal of Endodontics* **35**, 67–72.

McCabe LR, Banerjee C, Kundu R, *et al*. (1996) Developmental expression and activities of specific Fos and Jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. *Endocrinology* **137**, 4398–408.

McCabe LR, Stein JL, Lian JB, *et al*. (1995) Selective expression of fos and jun related genes during osteoblast proliferation and differentiation. *Experimental Cell Research* **217**, 255–62.

Min KS, Hwang YH, Ju HJ, *et al*. (2006a) Heme oxygenase-1 mediates cytoprotection against nitric oxide-induced cytotoxicity via the cGMP pathway in human pulp cells. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **102**, 803-8.

Min KS, Kwon YY, Lee HJ, *et al.* (2006b) Effects of proinflammatory cytokines on the expression of mineralization markers and heme oxygenase-1 in human pulp cells, *Journal of Endodontics* **32**, 39-43.

Min KS, Lee HJ, Kim SH, *et al*. (2008) Hydrogen peroxide induces heme oxygenase-1 and dentin sialophosphoprotein mRNA in human pulp cells. *Journal of Endodontics* **34**, 983-9.

Min KS, Lee YM, Hong SO, Kim EC (2010) Simvastatin promotes odontoblastic differentiation and expression of angiogenic factors via heme oxygenase-1 in primary cultured human dental pulp cells. *Journal of Endodontics* **36**, 447e52.

Otterbein LE, Choi AE (2000) Hemeoxygenase: colors of defense against cellular stress. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **279**, 1029–37.

Pae HO, Chung HT (2009) Heme oxygenase-1: its therapeutic roles in inflammatory diseases. *Immune Network* **9**, 12e9.

Pivoriuunas A, Surovas A, Borutinskaite V, *et al*. (2010) Proteomic analysis of stromal cells derived from the dental pulp of human exfoliated deciduous teeth. *Stem Cells and Development* **19**, 1081–93.

Rutz JC, Hatton JF, Hildebolt C, *et al*. (2007) Localized Increases in Corticotropin-releasing Factor Receptors in Pulp after Dental Injury. *Journal of Endodontics* **33**, 1319-24.

Soares DG, Basso FG, Hebling J, de Souza Costa CA (2015a) Immediate and late analysis of dental pulp stem cells viability after indirect exposition to alternative in-office bleaching strategies. *Clinical Oral Investigations* **19,** 1013-1020.

Soares DG, Basso FG, Scheffel DS, *et al*. (2015b) Responses of human dental pulp cells after application of a low-concentration bleaching gel to enamel. *Archives of Oral Biology* **60**, 1428-36.

Soares DG, Marcomini N, Basso FG, *et al*. (2016) Indirect cytocompatibility of a low-concentration hydrogen peroxide bleaching gel to odontoblast-like cells. *International Endodontic Journal* **49**, 26-36.

Soares DG, Ribeiro AP, Sacono NT, *et al*. (2011) Transenamel and transdentinal cytotoxicity of carbamide peroxide bleaching gels on odontoblast-like MDPC-23 cells. *International Endodontic Journal* **44,** 116-125.

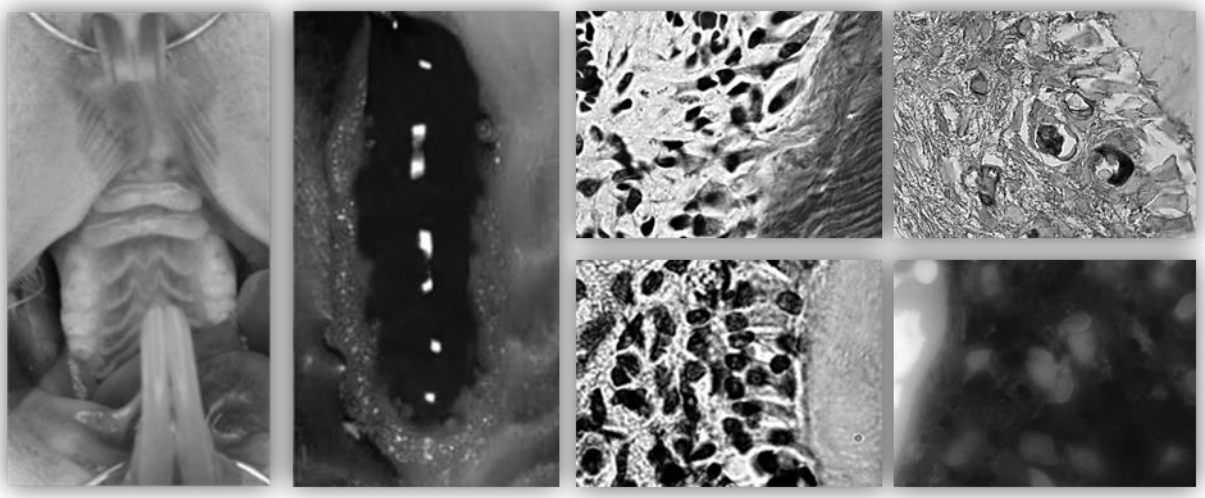
Tobias Duarte PC, Gomes-Filho JE, Ervolino E, *et al*. (2014) Histopathological condition of the remaining tissues after endodontic infection of rat immature teeth. *Journal of Endodontics* **40**, 538-42.

Trubiani O, Caputi S, Di Iorio D, *et al*. (2010) The cytotoxic effects of resin-based sealers on dental pulp stem cells. *International Endodontic Journal* **43**, 646-53.

Waddington RJ, Youde SJ, Lee CP, Sloan AJ (2009) Isolation of distinct progenitor stem cell populations from dental pulp. *Cells, Tissues, Organs* **189**, 268–74.

Yuan G, Yang G, Song S, Chen Z, Chen S (2012) Immunohistochemical localization of the NH2-terminal and COOH-terminal fragments of dentin sialoprotein in mouse teeth. *Cell Tissue Research* **349**, 605–14.

V. Considerações Finais



V. Considerações Finais

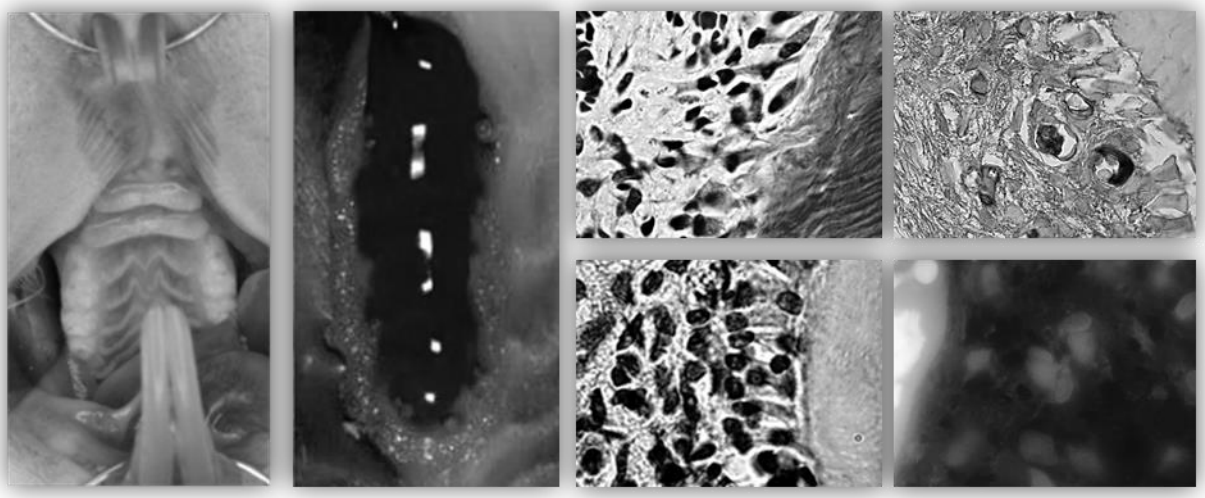
Assim como observado em nossos estudos anteriores, os achados deste estudo sugerem que géis clareadores com altas concentrações de H_2O_2 podem causar danos severos ao tecido pulpar, como a necrose. No entanto, o presente trabalho também sugere que estes danos podem ser reversíveis, mas que o tecido pulpar sofre consequências que podem se estender a longo prazo. Dentre essas consequências, estão a produção de dentina terciária e o aumento da produção de proteínas relacionadas à mineralização tecidual.

Apesar do fato de que resultados observados em dentes de ratos não podem ser extrapolados diretamente para a situação clínica, o modelo experimental caracterizado por nós para avaliar esta resposta é importante por trazer características que apenas estudos *in vivo* podem fornecer, como a resposta das células quando em meio a um tecido vivo, e não isoladas, como em estudos *in vitro*. Ainda, por se tratar da avaliação de um tecido conjuntivo que se encontra delimitado por paredes de dentina, o estudo *in vivo* se torna ainda mais relevante, pois demonstra a resposta do tecido pulpar sob pressão durante o processo inflamatório.

Foi observada capacidade de resposta frente ao estresse oxidativo, pela produção de HO-1 durante o processo inflamatório e também no início do reparo pulpar. Ainda, este foi o primeiro estudo a identificar o fator de transcrição Jun-D durante a diferenciação odontoblástica após o dano causado pelo H_2O_2, além de fornecer novas informações sobre a identificação de células-tronco mesenquimais (CTMs) na polpa dentária, sob influência ou não do estresse oxidativo.

Para a análise das CTMs, a seleção de vários marcadores positivos para estas células, assim como de ao menos um marcador negativo, se faz necessária. Optamos pela identificação de células CD90+/CD73+/CD105+/CD45-, e identificamos poucas destas células no tecido pulpar, independentemente da presença do estresse oxidativo. Assim, a dificuldade em identificar estas células impediram uma clarificação do mecanismo pelo qual as CTMs atuam na recuperação do tecido pulpar, indicando a necessidade de mais estudos sobre este tópico. No entanto, os resultados aqui encontrados reforçam que a clareação dentária deve ser realizada com cautela, pois pode resultar em sequelas adversas ao tecido pulpar.

VI. Referências



VI. REFERÊNCIAS

1. Agata H, Kagami H, Watanabe N, *et al.* Effect of ischemic culture conditions on the survival and differentiation of porcine dental pulp-derived cells. Differentiation 2008;76:981–93.
2. Airas L, Hellman J, Salmi M, Bono P, Puurunen T, Smith DJ, *et al.* CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. The J Exper Med 1995;182(5):1603–8.
3. Alongi DJ, Yamaza T, Song Y, *et al.* Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential. Regen Med 2010;5:617–31.
4. Anderson DG, Chiego DJ Jr, Glickman GN, McCauley LK. A clinical assessment of the effects of 10% carbamide peroxide gel on human pulp tissue. J Endod. 1999 Apr;25(4):247-50.
5. Ashok BT, Ahmad J, Ali R. Immunochemical detection of oxidative DNA damage in cancer and aging using anti-reactive oxygen species modified DNA monoclonal antibody. Int J Biochem Cell Biol. 1998 Dec;30(12):1367-77.
6. Ashok BT, Ahmad J, Qadri A, Ali R. Anti-ROS-DNA monoclonal antibody as molecular probe for oxidative DNA damage. Biochem Mol Biol Int. 1997 Dec;43(6):1219-29.
7. Atari M, Caballe-Serrano J, Gil-Recio C *et al.* The enhancement of osteogenesis through the use of dental pulp pluripotent stem cells in 3D. Bone. 2012;50:930–41.
8. Balic A, Aguila HL, Caimano MJ, Francone VP, Mina M. Characterization of stem and progenitor cells in the dental pulp of erupted and unerupted murine molars. Bone 2010;46(6):1639–51.
9. Balic A, Mina M. Characterization of Progenitor Cells in Pulps of Murine Incisors. J Dent Res 2010;89(11):1287-92.
10. Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human

mesenchymal stem cells. Bioch Bioph Res Communic 2001;289(2):519–24.

11. Basso FG, Pansani TN, Oliveira CF, Turrioni AP, Soares DG, Hebling J, *et al.* Cytotoxic effects of zoledronic acid on human epithelial cells and gingival fibroblasts. Braz Dent J 2013;24:551–8.
12. Benetti F, Gomes-Filho JE, Ferreira LL, Sivieri-Araújo G, Ervolino E, Briso ALF, Cintra LTA. Concentration-dependent effect of bleaching agents on the immunolabeling of interleukin-6, interleukin-17, and CD5-positive cells in the dental pulp. Int Endod J. 2018. *In press.*
13. Benetti F, Gomes-Filho JE, Ferreira LL, Ervolino E, Briso ALF, Sivieri-Araújo G, Dezan-Júnior E, Cintra LTA. Hydrogen peroxide induces cell proliferation and apoptosis in the dental pulp after bleaching *in vivo*. Arch Oral Biol, 2017a Sep;81:103-109.
14. Briso AL, Gonçalves RS, Costa FB, et al. Demineralization and hydrogen peroxide penetration in teeth with incipient lesions. Braz Dent J 2015;26:135-40.
15. Bronckers AL, Gay S, Dimuzio MT, Butler WT. Immunolocalization of gamma-carboxy glutamic acid-containing proteins in developing molar tooth germs of the rat. Coll Relat Res 1985;5:17–22.
16. Brown MH, Lacey E. A Ligand for CD5 is CD5. J Immunol. 2010 Nov;185(10) 6068–74.
17. Burdon RH, Rice-Evans C. Free radicals and the regulation of mammalian cell proliferation. Free Radic. Res. Commun. 1989;6:345-8.
18. Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic. Biol. Med. 1995;18:775-94.
19. Calderini A, Sciara S, Semeria C, Pantaleo G, Polizzi E. Comparative clinical and psychosocial benefits of tooth bleaching: different light activation of a 38% peroxide gel in a preliminary case-control study. Clin Case Rep. 2016 Jun 21;4(8):728-35.
20. Camargo SE, Valera MC, Camargo CH, Mancini MNG, Menezes MM. Penetration of 38% hydrogen peroxide into the pulp chamber in bovine and

human teeth submitted to office bleach technique. J Endod. 2007;33(9):1074-7.

21. Carinci F, Papaccio G, Laino G *et al.* Comparison between genetic portraits of osteoblasts derived from primary cultures and osteoblasts obtained from human pulpar stem cells. J Craniof Surg. 2008;19:616–25.
22. Caviedes-Bucheli J, Lombana N, Azuero-Holguin MM, Munoz HR. Quantification of neuropeptides (calcitonin gene-related peptide, substance P, neurokinin A, neuropeptide Y and vasoactive intestinal polypeptide) expressed in healthy and inflamed human dental pulp. Int Endod J. 2006;39:394–400.
23. Chang HH, Guo MK, Kastan FH, Chang MC, Huang GF, Wang YL, *et al.* Stimulation of glutathione depletion, ROS production and cell cycle arrest of dental pulp cells and gingival epithelial cells by HEMA. Biomaterials 2005;26:745e53.
24. Chang L, Karin M. Mammalian MAP kinase signaling cascades. Nature 2001;410: 37–40.
25. Chang MC, Lin LD, Chan CP, Chang HH, Chen LI, Lin HJ, *et al.* The effect of BisGMA on cyclooxygenase-2 expression, PGE2 production and cytotoxicity via reactive oxygen species- and MEK/ERK-dependent and independent pathways. Biomaterials 2009;30:4070e7.
26. Chen HP, Chang CH, Liu JK, Chuang SF, Yang JY. Effect of fluoride containing bleaching agents on enamel surface properties. J Dent. 2008 Sep;36(9):718-25.
27. Cintra LT, Benetti F, da Silva Facundo AC, Ferreira LL, Gomes-Filho JE, Ervolino E, Rahal V, Briso AL. The number of bleaching sessions influences pulp tissue damage in rat teeth. J Endod 2013 Dec;39(12):1576-80.
28. Cintra LT, Benetti F, Ferreira LL, Gomes-Filho JE, Ervolino E, Gallinari M de O, Rahal V, Briso AL. Penetration Capacity, Color Alteration and Biological Response of Two In-office Bleaching Protocols. Braz Dent J. 2016a Apr;27(2):169-75.

29. Cintra LT, Benetti F, Ferreira LL, Rahal V, Ervolino E, Jacinto R de C, Gomes Filho JE, Briso AL. Evaluation of an experimental rat model for comparative studies of bleaching agents. J Appl Oral Sci. 2016b Jan-Feb;24(1):95-104.
30. Cintra LTA, Ferreira LL, Benetti F, Gastélum AA, Gomes-Filho JE, Ervolino E, Briso ALF. The effect of dental bleaching on pulpal tissue response in a diabetic animal model. Int Endod J. 2017 Aug;50(8):790-798.
31. Colucci S, Brunetti G, Oranger A, Mori G, Sardone F, Specchia G, Rinaldi E, Curci P, Liso V, Passeri G, Zallone A, Rizzi R, Grano M. Myeloma cells suppress osteoblasts through sclerostin secretion. Blood Cancer J. 2011 Jun;1(6):e27.
32. Cooper KL, Liu KL, Hudson LG. Contribution of reactive oxygen species and mitogen-activated protein kinase signaling in arsenite-stimulate hemeoxygenase-1 production. Toxicol Appl Pharmacol 2007;218:119e27.
33. Cordeiro MM, Dong Z, Kaneko T, *et al.* Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J Endod 2008;34:962–9.
34. Costa CA, Riehl H, Kina JF, Sacono NT, Hebling J. Human pulp responses to in-office tooth bleaching. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010;109:e59-64.
35. Davies KJ. The broad spectrum of responses to oxidants in proliferating cells: A new paradigm for oxidative stress. IUBMB Life 1999;48:41–47.
36. de Wit J, Souwer Y, van Beelen AJ, et al. CD5 costimulation induces stable Th17 development by promoting IL-23R expression and sustained STAT3 activation. Blood. 2011 Dec 1;118(23):6107-14.
37. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 1997;89:747–54.
38. Eckhardt A, Gerstmayr N, Hiller KA, Bolay C, Waha C, Spagnuolo G, *et al.* TEGDMA-induced oxidative DNA damage and activation of ATM and MAP kinases. Biomaterials 2009;30:2006e14.
39. Fernández-Vallone VB, Romaniuk MA, Choi H, Labovsky V, Otaegui J, Chasseing NA. Mesenchymal stem cells and their use in therapy: what has been achieved? Differentiation. 2013 Jan;85(1-2):1-10.

40. Ferreira LL, Gomes-Filho JE, Benetti F, Carminatti M, Ervolino E, Briso ALF, Cintra LT. The effect of dental bleaching on pulpal tissue response in a diabetic animal model: Immunoregulatory cytokines study. Int Endod J. 2017. *In press*.
41. Fitzgerald M, Chiego DJ Jr, Heys DR. Autoradiographic analysis of odontoblast replacement following pulp exposure in primate teeth. Arch Oral Biol 1990;35: 707–15.
42. Forman HJ, Torres M. Redox signaling in macrophages. Mol Aspects Med 2001;22: 189–216.
43. Gericke A, Qin C, Spevak L, *et al.* Importance of phosphorylation for osteopontin regulation of biomineralization. Calcif Tissue Int 2005;77:45–54.
44. Gronthos S, Brahim J, Li W *et al.* Stem cell properties of human dental pulp stem cells. J Dent Res 2002;81:531–5.
45. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S: Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl Acad Sci 2000;97:13625–30.
46. Hanks CT, Fat JC, Wataha JC, *et al.* Cytotoxicity and dentin permeability of carbamide peroxide and hydrogen peroxide vital bleaching materials, in vitro. J Dent Res 1993;72:931–8.
47. Hirata M, Yamaza T, Mei YF, Akamine A. Expression of osteocalcin and Jun D in the early period during reactionary dentin formation after tooth preparation in rat molars. Cell Tissue Res. 2005 Mar;319(3):455-65.
48. Huang AH, Chen YK, Chan AW, Shieh TY, Lin LM. Isolation and characterization of human dental pulp stem/stromal cells from nonextracted crown-fractured teeth requiring root canal therapy. J Endod 2009b;35(5):673–81.
49. Huang G, Yamaza T, Shea LD *et al.* Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. Part A. Tissue Eng. 2009;16(2):605–15.
50. Huang GT: Pulp and dentin tissue engineering and regeneration: current
51. progress. Regen. Med. 2009;4:697–707.

52. Huang GTJ, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs from other sources: the biology and role in regenerative medicine. J Dent Res Crit Rev Oral Biol Med. 2009a;88:792–806.
53. Karaoz E, Dogan BN, Aksoy A, Gacar G, Akyuz S, Ayhan S, *et al.* Isolation and in vitro characterisation of dental pulp stem cells from natal teeth. Histochemistry and Cell Biology 2010;133(1):95–112.
54. Karsenty G, Wagner EF. Reaching a genetic and molecular understanding of skeletal development. Dev Cell 2002;2:389–406.
55. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Arch Oral Biol. 2012 Nov;57(11):1439-58.
56. Kern B, Shen J, Starbuck M, Karsenty G. Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. J Biol Chem 2001;276:7101–7.
57. Kook YA, Lee SK, Son DH, *et al.* Effects of substance P on osteoblastic differentiation and heme oxygenase-1 in human periodontal ligament cells. Cell Biol Int 2009;33: 424–8.
58. Kuratate M, Yoshiba K, Shigetani Y, Yoshiba N, Ohshima H, Okiji T. Immunohistochemical analysis of nestin, osteopontin, and proliferating cells in the reparative process of exposed dental pulp capped with mineral trioxide aggregate. J Endod. 2008 Aug;34(8):970-4.
59. Laino G, D'Aquino R, Graziano A *et al.* A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J Bone Min Res 2005;20:1394–402.
60. Lee DH, Lim BS, Lee YK, *et al.* Effects of hydrogen peroxide (H2O2) on alkaline phosphatase activity and matrix mineralization of odontoblast and osteoblast cell lines. Cell Biol Toxicol 2006a;22:39–46.
61. Lee SH, Ryu JS, Lee JW, Kwak DH, Ko K, Choo YK. Comparison of ganglioside expression between human adipose- and dental pulp-derived stem cell differentiation into osteoblasts. Arch Pharm Res 2010;33(4):585–91.
62. Lee SK, Choi HI, Yang YS, *et al.* Nitric oxide modulates osteoblastic differentiation with heme oxygenase-1 via the mitogen activated protein

kinase and nuclear factor-kappa B pathways in human periodontal ligament cells. Biol Pharm Bull 2009;32:1328–34.

63. Lee SK, Pi SH, Kim SH, *et al.* Substance P regulates macrophage inflammatory protein 3a/CCL20 with heme in human periodontal ligament cells. Clin Exp Immunol 2007; 150:567–75.
64. Lee YL, Liu J, Clarkson BH, *et al.* Dentin-pulp complex responses to carious lesions. Caries Res 2006b;40:256–64.
65. Liu HC, Ling-Ling E, Wang DS *et al.* Reconstruction of alveolar bone defects using bone morphogenetic protein 2 mediated rabbit dental pulp stem cells seeded on nanohydroxyapatite/collagen/poly (L-lactide). Part A. Tissue Engineering. 2011;17:2417–33.
66. Liu SL, Lin X, Shi DY, *et al.* Reactive oxygen species stimulated human hepatoma cell proliferation via cross-talk between PI3-K/PKB and JNK signaling pathways. Arch Biochem Biophys 2002;406:173–82.
67. Lozano F, Simarro M, Calvo J, Vilà JM, Padilla O, Bowen MA, Campbell KS. CD5 signal transduction: positive or negative modulation of antigen receptor signaling. Crit Rev Immunol. 2000;20(4):347-58.
68. Matsui S, Takahashi C, Tsujimoto Y, *et al.* Stimulatory effects of low-concentration reactive oxygen species on calcification ability of human dental pulp cells. J Endod 2009;35:67–72.
69. Matsui S, Takeuchi H, Tsujimoto Y, Matsushima K. Effects of Smads and BMPs induced by Ga-Al-As laser irradiation on calcification ability of human dental pulp cells. J Oral Sci 2008;50:75– 81.
70. Matsui S, Tsujimoto Y, Matsushima K. Stimulatory effects of hydroxyl radical generation by Ga-Al-As laser irradiation on mineralization ability of human dental pulp cells. Biol Pharm Bull 2007;30:27–31.
71. McCabe LR, Banerjee C, Kundu R, Harrison J, Dobner PR, Stein JL, Lian JB, Stein GS. Developmental expression and activities of specific Fos and Jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. Endocrinology 1996;137:4398–408.

72. McCabe LR, Stein JL, Lian JB, Bravo R, Stein GS. Selective expression of fos and jun related genes during osteoblast proliferation and differentiation. Exp Cell Res 1995;217:255–62.

73. Min KS, Hwang YH, Ju HJ, *et al.* Heme oxygenase-1 mediates cytoprotection against nitric oxide-induced cytotoxicity via the cGMP pathway in human pulp cells. Oral Surg Oal Med Oral Pathol Oral Radol Endod 2006;102:803–8.

74. Min KS, Kwon YY, Lee HJ, *et al.* Effects of proinflammatory cytokines on the expression of mineralization markers and heme oxygenase-1 in human pulp cells. J Endod 2006a;32:39–43.

75. Min KS, Lee HJ, Kim SH, Lee SK, Kim HR, Pae HO, Chung HT, Shin HI, Lee SK, Kim EC. Hydrogen peroxide induces heme oxygenase-1 and dentin sialophosphoprotein mRNA in human pulp cells. J Endod. 2008 Aug;34(8):983-9.

76. Min KS, Lee YM, Hong SO, Kim EC. Simvastatin promotes odontoblastic differentiation and expression of angiogenic factors via heme oxygenase-1 in primary cultured human dental pulp cells. J Endod 2010;36:447e52.

77. Mistry N, Evans MD, Griffiths HR, Kasai H, Herbert KE, Lunec J. Immunochemical detection of glyoxal DNA damage. Free Radic Biol Med. 1999 May;26(9-10):1267-73.

78. Mitsiadis TA, De Bari C, About I. Apoptosis in developmental and repair-related human tooth remodeling: a view from the inside. Exp Cell Res 2008;314:869–77.

79. Mitsiadis TA, Rahiotis C. Parallels between tooth development and repair: conserved molecular mechanisms following carious and dental injury. J Dent Res 2004;83: 896–902.

80. Mori G, Centonze M, Brunetti G *et al.* Osteogenic properties of human dental pulp stem cells. J Biol Reg Homeost Ag. 2010;24:167–75.

81. Nakashima M, Akamine A: The application of tissue engineering to regeneration of pulp and dentin in endodontics. J. Endod. 2005;31:711–8.

82. Nakashima M, Iohara K, Ishikawa M *et al.*: Stimulation of reparative dentin formation by ex vivo gene therapy using dental pulp stem cells

electrotransfected with growth/ differentiation factor 11 (Gdf11). Hum. Gene Ther. 2004;15:1045–53.

83. Nosrat IV, Widenfalk J, Olson L, Nosrat CA. Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. Dev Biol 2001;238:120–32.
84. Okabe T, Sakamoto M, Takeuchi H, Matsushima K. Effects of pH on mineralization ability of human dental pulp cells. J Endod 2006;32:198 – 201.
85. Otterbein LE, Choi AE. Hemeoxygenase: colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol 2000;279:L1029 –37.
86. Owen TA, Bortell R, Yocum SA, Smock SL, Zang M, Abate C. Coordinate occupancy of AP-1 sites in the vitamin D responsive and CCAAT box elements by Fos-Jun in the osteocalcin gene: model for phenotype suppression of transcription. Proc Natl Acad Sci 1990;87:9990–4.
87. Pae HO, Chung HT. Heme oxygenase-1: its therapeutic roles in inflammatory diseases. Immune Netw 2009;9:12e9.
88. Pi SH, Kim SC, Kim HT, *et al.* Defense of heme oxygenase-1 against cytotoxic and receptor of NF kappa-B ligand inducing effects of hydrogen in human periodontal ligament cells. J Periodontal Res 2007;42:331–9.
89. Pivoriuunas A, Surovas A, Borutinskaite V, Matuzeviccius D, Treigyte G, Savickiene J, *et al.* Proteomic analysis of stromal cells derived from the dental pulp of human exfoliated deciduous teeth. Stem Cells and Development 2010;19(7):1081–93.
90. Rege TA, Hagood JS. Thy-1 as a regulator of cell–cell and cell–matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. FASEB Journal 2006;20(8):1045–54.
91. Rius C, Smith JD, Almendro N, Langa C, Botella LM, Marchuk DA, *et al.* Cloning of the promoter region of human endoglin, the target gene for hereditary hemorrhagic telangiectasia type 1. Blood 1998;92(12):4677-90.
92. Runchel C, Matsuzawa A, Ichijo H. Mitogen-activated protein kinases in mammalian oxidative stress responses. Antioxid Redox Signal 2011;15:205–18.

93. Rutz JC, Hatton JF, Hildebolt C, Wells JE, Rowland KC. Localized Increases in Corticotropin-releasing Factor Receptors in Pulp after Dental Injury. J Endod. 2007 Nov;33(11):1319-24.
94. Schweikl H, Spangnuolo G, Schmalz G. Genetic and cellular toxicology of dental resin monomers. J Dent Res 2004;85:870e7.
95. Seale NS, McIntosh JE, Taylor AN. Pulpal reaction to bleaching of teeth in dogs. J Dent Res 1981;80:948-53.
96. Soares DG, Basso FG, Hebling J, de Souza Costa CA. Concentrations of and application protocols for hydrogen peroxide bleaching gels: effects on pulp cell viability and whitening efficacy. J Dent. 2014 Feb;42(2):185-98.
97. Sodek J, Ganss B, McKee MD. Osteopontin. Crit Rev Oral Biol Med 2000;11:279-303.
98. Streuli M, Morimoto C, Schrieber M, Schlossman SF, Saito H. Characterization of CD45 and CD45R monoclonal antibodies using transfected mouse cell lines that express individual human leukocyte common antigens. J Immunol 1988;141:3910–4.
99. Strollo R, Ponchel F, Malmström V, *et al.* Autoantibodies to post translationally modified type II collagen as potential biomarkers for rheumatoid arthritis. Arthritis Rheum. 2013 Jul;65(7):1702-12.
100. Tash PN, Tapşın S, Demirel S, Yalvaç ME, Akyuz S, Yarat A, Sahin F. Isolation and characterization of dental pulp stem cells from a patient with Papillon-Lefèvre syndrome. J Endod. 2013 Jan;39(1):31-8.
101. TenCate AR (1998) Hard tissue formation and destruction. In: TenCate AR (ed) Oral histology, 5th edn. Mosby, St. Louis, pp 69–78.
102. Torneck CD (1998) Dentin-pulp complex. In: TenCate AR (ed) Oral histology, 5th edn. Mosby, St. Louis, pp 150–196.
103. Trubiani O, Caputi S, Di Iorio D, D'Amario M, Paludi M, Giancola R, Di Nardo Di Maio F, De Angelis F, D'Arcangelo C. The cytotoxic effects of resin-based sealers on dental pulp stem cells. Int Endod J. 2010 Aug;43(8):646-53.
104. Vermelin L, Lecolle S, Septier D, *et al.* Apoptosis in human and rat dental pulp. Eur J Oral Sci 1996;104:547–53.

105. Waddington RJ, Youde SJ, Lee CP, Sloan AJ. Isolation of distinct progenitor stem cell populations from dental pulp. Cells Tissues Organs 2009;189(1–4):268–74.

106. Wei X, Ling J, Wu L, Liu L, Xiao Y. Expression of mineralization markers in dental pulp cells. J Endod 2007;33:703-8.

107. Wiese AG, Pacifici RE, Davies KJA. Transient adaptation to oxidative stress in mammalian cells. Arch. Biochem. Biophys. 1995;318:231-40.

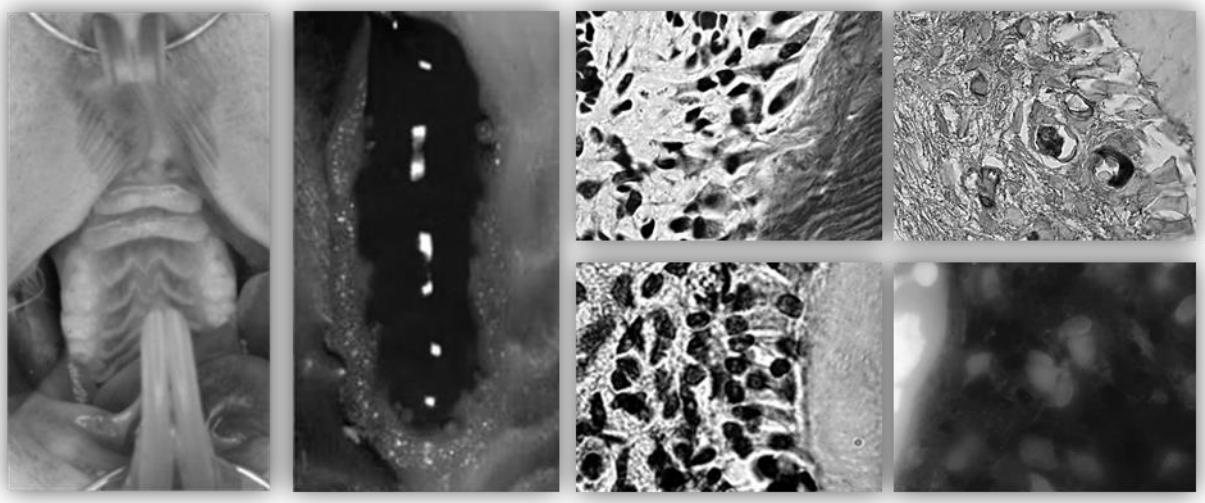
108. Wu TT, Li LF, Du R, Jiang L, Zhu YQ. Hydrogen peroxide induces apoptosis in human dental pulp cells via caspase-9 dependent pathway. J Endod. 2013 Sep;39(9):1151-5.

109. Yamada Y, Nakamura S, Ito K, Sugito T, Yoshimi R, Nagasaka T, *et al.* A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology. Tiss Engin Part A 2010;16(6):1891–900.

110. Yao KL, Todescan R Jr, Sodek J. Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture. J Bone Miner Res 1994;9:231-40.

111. Yuan G, Yang G, Song S, Chen Z, Chen S. Immunohistochemical localization of the NH2-terminal and COOH-terminal fragments of dentin sialoprotein in mouse teeth. Cell Tissue Res. 2012 Aug;349(2):605–14.

VII. Anexo I – Guia para submissão dos artigos



Guidelines for Publishing Papers in the International Endodontic Journal

1. GENERAL

International Endodontic Journal publishes original scientific articles, reviews, clinical articles and case reports in the field of Endodontology; the branch of dental sciences dealing with health, injuries to and diseases of the pulp and periradicular region, and their relationship with systemic well-being and health. Original scientific articles are published in the areas of biomedical science, applied materials science, bioengineering, epidemiology and social science relevant to endodontic disease and its management, and to the restoration of root-treated teeth. In addition, review articles, reports of clinical cases, book reviews, summaries and abstracts of scientific meetings and news items are accepted.

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

2. ETHICAL GUIDELINES

International Endodontic Journal adheres to the below ethical guidelines for publication and research.

2.1. Authorship and Acknowledgements

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

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

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

2.2. Ethical Approvals

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

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study. The authors MUST upload a copy of the ethical approval letter when submitting their manuscript. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

2.3 Clinical Trials

The International Endodontic Journal asks that authors submitting manuscripts reporting from a clinical trial to register the trials in any of the following public clinical trials registries: www.clinicaltrials.gov, https://www.clinicaltrialsregister.eu/, http://isrctn.org/. Other primary registries if named in the WHO network will also be considered acceptable. The clinical trial registration number and name of the trial register should be included in the Acknowledgements at the submission stage.

2.3.1 Randomised control clinical trials

Randomised control clinical trials should be reported using the guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.

2.3.2 Epidemiological observational trials

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

2.4 Systematic Reviews

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

2.5 DNA Sequences and Crystallographic Structure Determinations

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

2.6 Conflict of Interest and Source of Funding

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

The above policies are in accordance with the Uniform Requirements for Manuscripts Submitted to Biomedical Journals produced by the International Committee of Medical Journal Editors (http://www.icmje.org/).

It is the responsibility of the corresponding author to have all authors of a manuscript fill out a conflict of interest disclosure form, and to upload all forms individually (do not combine the forms into one file) together with the manuscript on submission. The disclosure statement should be included under Acknowledgements. Please find the form below:
Conflict of Interest Disclosure Form

2.7 Appeal of Decision
The decision on a paper is final and cannot be appealed.

2.8 Permissions
If all or parts of previously published illustrations are used, permission must be obtained from the copyright holder concerned. It is the author's responsibility to obtain these in writing and provide copies to the Publishers.

2.8 Copyright Assignment

If your paper is accepted, the author identified as the formal corresponding author for the paper will receive an email prompting them to login into Author Services; where via the Wiley Author Licensing Service (WALS) they will be able to complete the license agreement on behalf of all authors on the paper. Your article cannot be published until this has been done.

For authors choosing OnlineOpen
If the OnlineOpen option is selected the corresponding author will have a choice of the following Creative Commons License Open Access Agreements (OAA):
Creative Commons Attribution License OAA
Creative Commons Attribution Non-Commercial License OAA
Creative Commons Attribution Non-Commercial - No Derivs License OAA

To preview the terms and conditions of these open access agreements please visit the Copyright FAQs hosted on Wiley Author Services http://exchanges.wiley.com/authors/faqs-

--copyright-_301.html and visit http://www.wileyopenaccess.com/details/content/12f25db4c87/Copyright--License.html.

If you select the OnlineOpen option and your research is funded by certain funders [e.g. The Wellcome Trust and members of the Research Councils UK (RCUK) or the Austrian Science Fund (FWF)] you will be given the opportunity to publish your article under a CC-BY license supporting you in complying with Wellcome Trust and Research Councils UK requirements. For more information on this policy and the Journal's compliant self-archiving policy please visit: http://www.wiley.com/go/funderstatement.

3. OnlineOpen

OnlineOpen is available to authors of primary research articles who wish to make their article available to non-subscribers on publication, or whose funding agency requires grantees to archive the final version of their article. With OnlineOpen, the author, the author's funding agency, or the author's institution pays a fee to ensure that the article is made available to non-subscribers upon publication via Wiley Online Library, as well as deposited in the funding agency's preferred archive. For the full list of terms and conditions, see http://wileyonlinelibrary.com/onlineopen#OnlineOpen_Terms

Any authors wishing to send their paper OnlineOpen will be required to complete the payment form available from our website at:
https://authorservices.wiley.com/bauthor/onlineopen_order.asp

Prior to acceptance there is no requirement to inform an Editorial Office that you intend to publish your paper OnlineOpen if you do not wish to. All OnlineOpen articles are treated in the same way as any other article. They go through the journal's standard peer-review process and will be accepted or rejected based on their own merit.

3.1 MANUSCRIPT SUBMISSION PROCEDURE

Manuscripts should be submitted electronically via the online submission site http://mc.manuscriptcentral.com/iej. The use of an online submission and peer review site enables immediate distribution of manuscripts and consequentially speeds up the review process. It also allows authors to track the status of their own manuscripts. Complete instructions for submitting a paper is available online and below. Further assistance can be obtained from iejeditor@cardiff.ac.uk.

3.2. Getting Started

• Launch your web browser (supported browsers include Internet Explorer 5.5 or higher, Safari 1.2.4, or Firefox 1.0.4 or higher) and go to the journal's online Submission Site: http://mc.manuscriptcentral.com/iej

• Log-in, or if you are a new user, click on 'register here'.

• If you are registering as a new user.

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

- Enter your institution and address information as appropriate, and then click 'Next.'

- Enter a user ID and password of your choice (we recommend using your e-mail address as your user ID), and then select your areas of expertise. Click 'Finish'.

• If you are registered, but have forgotten your log in details, please enter your e-mail address under 'Password Help'. The system will send you an automatic user ID and a new temporary password.

• Log-in and select 'Author Centre '

3.3. Submitting Your Manuscript

• After you have logged into your 'Author Centre', submit your manuscript by clicking on the submission link under 'Author Resources'.

• Enter data and answer questions as appropriate. You may copy and paste directly from your manuscript and you may upload your pre-prepared covering letter.

• Click the 'Next' button on each screen to save your work and advance to the next screen.

• You are required to upload your files.

- Click on the 'Browse' button and locate the file on your computer.

- Select the designation of each file in the drop down next to the Browse button.

- When you have selected all files you wish to upload, click the 'Upload Files' button.

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

3.4. Manuscript Files Accepted

Manuscripts should be uploaded as Word (.doc) or Rich Text Format (.rft) files (not write-protected) plus separate figure files. GIF, JPEG, PICT or Bitmap files are acceptable for submission, but only high-resolution TIF or EPS files are suitable for printing. The files will be automatically converted to HTML and PDF on upload and will be used for the review process. The text file must contain the abstract, main text, references, tables, and figure legends, but no embedded figures or Title page. The Title page should be uploaded as a

separate file. In the main text, please reference figures as for instance 'Figure 1', 'Figure 2' etc to match the tag name you choose for the individual figure files uploaded. Manuscripts should be formatted as described in the Author Guidelines below.

3.5. Blinded Review

Manuscript that do not conform to the general aims and scope of the journal will be returned immediately without review. All other manuscripts will be reviewed by experts in the field (generally two referees). International Endodontic Journal aims to forward referees´ comments and to inform the corresponding author of the result of the review process. Manuscripts will be considered for fast-track publication under special circumstances after consultation with the Editor.

International Endodontic Journal uses double blinded review. The names of the reviewers will thus not be disclosed to the author submitting a paper and the name(s) of the author(s) will not be disclosed to the reviewers.

To allow double blinded review, please submit (upload) your main manuscript and title page as separate files.

Please upload:

• Your manuscript without title page under the file designation 'main document'

• Figure files under the file designation 'figures'

• The title page and Acknowledgements where applicable, should be uploaded under the file designation 'title page'

All documents uploaded under the file designation 'title page' will not be viewable in the html and pdf format you are asked to review in the end of the submission process. The files viewable in the html and pdf format are the files available to the reviewer in the review process.

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

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

3.7. E-mail Confirmation of Submission

After submission you will receive an e-mail to confirm receipt of your manuscript. If you do not receive the confirmation e-mail after 24 hours, please check your e-mail address carefully in the system. If the e-mail address is correct please contact your IT department. The error may be caused by some sort of spam filtering on your e-mail server. Also, the e-

mails should be received if the IT department adds our e-mail server (uranus.scholarone.com) to their whitelist.

3.8. Manuscript Status

You can access ScholarOne Manuscripts any time to check your 'Author Centre' for the status of your manuscript. The Journal will inform you by e-mail once a decision has been made.

3.9. Submission of Revised Manuscripts

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

4. MANUSCRIPT TYPES ACCEPTED

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

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

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

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

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

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

Letters to the Editor: are also acceptable.
Meeting Reports: are also acceptable.

5. MANUSCRIPT FORMAT AND STRUCTURE

5.1. Format

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

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

Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be

abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

5.2. Structure

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

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

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

• Aim: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.
• Methodology: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.
• Results: Give the main results of the study, including the outcome of any statistical analysis.
• Conclusions: State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.

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

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

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

• Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.
• Summary: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and analysis if any.
• Key learning points: Provide up to 5 short, bullet-pointed statements to highlight the key messages of the report. All points must be fully justified by material presented in the report.

Abstract for Clinical Articles should be no more than 250 words using the following structure:
• Aim: Give a clear statement of the main aim of the report and the clinical problem which is addressed.
• Methodology: Describe the methods adopted.
• Results: Give the main results of the study.
• Conclusions: State the primary conclusions of the study.

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

Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.
Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.
(i) Clinical Trials should be reported using the CONSORT guidelines available at www.consort-statement.org. A CONSORT checklist and flow diagram (as a Figure) should also be included in the submission material.
(ii) Experimental Subjects: experimentation involving human subjects will only be published if such research has been conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki (version 2008) and the additional requirements, if any, of the country where the research has been carried out. Manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and written consent of each subject and according to the above mentioned principles. A statement regarding the fact that the study has been independently reviewed and approved by an ethical board should also be included. Editors reserve the right to reject papers if there are doubts as to whether appropriate procedures have been used.

When experimental animals are used the methods section must clearly indicate that adequate measures were taken to minimize pain or discomfort. Experiments should be carried out in accordance with the Guidelines laid down by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with local laws and regulations.

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.
(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.
Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.
Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.
Conclusion: should contain a summary of the findings.

Main Text of Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should be focused to place the subject matter in context and to justify the need for the review. The Review section should be divided into logical sub-sections in order to improve readability and enhance understanding. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The use of tabulated and illustrative material is encouraged. The Conclusion section should reach clear conclusions and/or recommendations on the basis of the evidence presented.

Main Text of Mini Review Articles should be divided into Introduction, Review and Conclusions. The Introduction section should briefly introduce the subject matter and justify the need and timeliness of the literature review. The Review section should be divided into

logical sub-sections to enhance readability and understanding and may be supported by up to 5 tables and figures. Search strategies must be described and the use of state-of-the-art evidence-based systematic approaches is expected. The Conclusions section should present clear statements/recommendations and suggestions for further work. The manuscript, including references and figure legends should not normally exceed 4000 words.

Main Text of Clinical Reports and Clinical Articles should be divided into Introduction, Report, Discussion and Conclusion,. They should be well illustrated with clinical images, radiographs, diagrams and, where appropriate, supporting tables and graphs. However, all illustrations must be of the highest quality

Acknowledgements: International Endodontic Journal requires that all sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential conflicts of interest noted. Grant or contribution numbers may be acknowledged, and principal grant holders should be listed. Acknowledgments should be brief and should not include thanks to anonymous referees and editors. See also above under Ethical Guidelines.

5.3. References

It is the policy of the Journal to encourage reference to the original papers rather than to literature reviews. Authors should therefore keep citations of reviews to the absolute minimum.

We recommend the use of a tool such as EndNote or Reference Manager for reference management and formatting. The EndNote reference style can be obtained upon request to the editorial office (iejeditor@cardiff.ac.uk). Reference Manager reference styles can be searched for here: www.refman.com/support/rmstyles.asp

In the text: single or double authors should be acknowledged together with the year of publication, e.g. (Pitt Ford & Roberts 1990). If more than two authors the first author followed by et al. is sufficient, e.g. (Tobias et al. 1991). If more than 1 paper is cited the references should be in year order and separated by "," e.g. (Pitt Ford & Roberts 1990, Tobias et al. 1991).

Reference list: All references should be brought together at the end of the paper in alphabetical order and should be in the following form.

(i) Names and initials of up to six authors. When there are seven or more, list the first three and add et al.
(ii)Year of publication in parentheses
(iii) Full title of paper followed by a full stop (.)
(iv) Title of journal in full (in italics)
(v) Volume number (bold) followed by a comma (,)
(vi) First and last pages

Examples of correct forms of reference follow:
Standard journal article
Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. International Endodontic Journal 24, 8-14.

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

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

Books and other monographs
Personal author(s)
Gutmann J, Harrison JW (1991) Surgical Endodontics, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

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

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

Agency publication

Ranofsky AL (1978) Surgical Operations in Short-Stay Hospitals: United States-1975. DHEW publication no. (PHS) 78-1785 (Vital and Health Statistics; Series 13; no. 34.) Hyattsville, MD, USA: National Centre for Health Statistics.8

Dissertation or thesis

Saunders EM (1988) In vitro and in vivo investigations into root-canal obturation using thermally softened gutta-percha techniques (PhD Thesis). Dundee, UK: University of Dundee.

URLs

Full reference details must be given along with the URL, i.e. authorship, year, title of document/report and URL. If this information is not available, the reference should be removed and only the web address cited in the text.

Smith A (1999) Select committee report into social care in the community [WWW document]. URL http://www.dhss.gov.uk/reports/report015285.html

[accessed on 7 November 2003]

5.4. Tables, Figures and Figure Legends

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

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

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

the same amount and reproduced on the printed page at the smallest size at which essential details are visible.

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

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

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

Permissions: If all or part of previously published illustrations are to be used, permission must be obtained from the copyright holder concerned. This is the responsibilty of the authors before submission.

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

Further information can be obtained at Wiley Blackwell's guidelines for figures: http:/authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it:
http://authorservices.wiley.com/bauthor/eachecklist.asp.

5.5. Supporting Information

Publication in electronic formats has created opportunities for adding details or whole sections in the electronic version only. Authors need to work closely with the editors in developing or using such new publication formats.

Supporting information, such as data sets or additional figures or tables, that will not be published in the print edition of the journal, but which will be viewable via the online edition, can be submitted. It should be clearly stated at the time of submission that the supporting information is intended to be made available through the online edition. If the size or format of the supporting information is such that it cannot be accommodated on the journal's website, the author agrees to make the supporting information available free of charge on a permanent Web site, to which links will be set up from the journal's website. The author must advise Wiley Blackwell if the URL of the website where the supporting information is located changes. The content of the supporting information must not be altered after the paper has been accepted for publication.

The availability of supporting information should be indicated in the main manuscript by a paragraph, to appear after the References, headed 'Supporting Information' and providing titles of figures, tables, etc. In order to protect reviewer anonymity, material posted on the authors Web site cannot be reviewed. The supporting information is an integral part of the article and will be reviewed accordingly.

Preparation of Supporting Information: Although provision of content through the web in any format is straightforward, supporting information is best provided either in web-ready form or in a form that can be conveniently converted into one of the standard web publishing formats:

• Simple word-processing files (.doc or .rtf) for text.
• PDF for more complex, layout-dependent text or page-based material. Acrobat files can be distilled from Postscript by the Publisher, if necessary.
• GIF or JPEG for still graphics. Graphics supplied as EPS or TIFF are also acceptable.
• MPEG or AVI for moving graphics.

Subsequent requests for changes are generally unacceptable, as for printed papers. A charge may be levied for this service.

Video Imaging: For the on-line version of the Journal the submission of illustrative video is encouraged. Authors proposing the use such media should consult with the Editor during manuscript preparation.

6. AFTER ACCEPTANCE

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

6.1. Figures

Hard copies of all figures and tables are required when the manuscript is ready for publication. These will be requested by the Editor when required. Each Figure copy should be marked on the reverse with the figure number and the corresponding author's name.

6.2 Proof Corrections

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

6.3 Early Online Publication Prior to Print

International Endodontic Journal is covered by Wiley Blackwell's Early View service. Early View articles are complete full-text articles published online in advance of their publication in a printed issue. Early View articles are complete and final. They have been fully reviewed,

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

6.4 Online Production Tracking

Online production tracking is available for your article through Blackwell's Author Services. Author Services enables authors to track their article - once it has been accepted - through the production process to publication online and in print. Authors can check the status of their articles online and choose to receive automated e-mails at key stages of production. The author will receive an e-mail with a unique link that enables them to register and have their article automatically added to the system. Please ensure that a complete e-mail address is provided when submitting the manuscript. Visit http://authorservices.wiley.com/bauthor/ for more details on online production tracking and for a wealth of resources including FAQs and tips on article preparation, submission and more.

6.5 Author Material Archive Policy

Please note that unless specifically requested, Wiley Blackwell will dispose of all hardcopy or electronic material submitted two months after publication. If you require the return of any material submitted, please inform the editorial office or production editor as soon as possible.

6.6 Offprints

Free access to the final PDF offprint of your article will be available via Author Services only. Please therefore sign up for Author Services if you would like to access your article PDF offprint and enjoy the many other benefits the service offers.

Additional paper offprints may be ordered online. Please click on the following link, fill in the necessary details and ensure that you type information in all of the required fields: Offprint Cosprinters. If you have queries about offprints please email offprint@cosprinters.com

The corresponding author will be sent complimentary copies of the issue in which the paper is published (one copy per author).

6.7 Author Services

For more substantial information on the services provided for authors, please see Wiley Blackwell Author Services

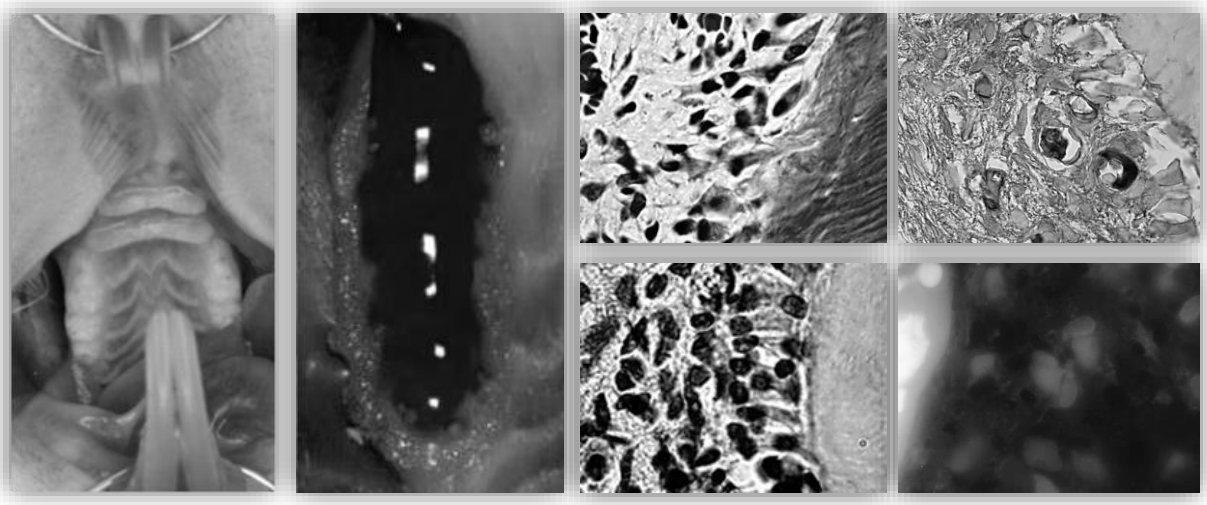
6.8 Note to NIH Grantees: Pursuant to NIH mandate, Wiley Blackwell will post the accepted version of contributions authored by NIH grant-holders to PubMed Central upon acceptance. This accepted version will be made publicly available 12 months after publication. For further information, see www.wiley.com/go/nihmandate

7 Guidelines for reporting of DNA microarray data

The International Endodontic Journal gives authors notice that, with effect from 1st January 2011, submission to the International Endodontic Journal requires the reporting of microarray data to conform to the MIAME guidelines. After this date, submissions will be assessed according to MIAME standards. The complete current guidelines are available at http://www.mged.org/Workgroups/MIAME/miame_2.0.html. Also, manuscripts will be published only after the complete data has been submitted into the public repositories, such as GEO (http://www.ncbi.nlm.nih.gov/geo/) or ArrayExpress (http://www.ebi.ac.uk/microarray/submissions_overview.html), in MIAME compliant format, with the data accession number (the identification number of the data set in the database) quoted in the manuscript. Both databases are committed to keeping the data private until the associated manuscript is published, if requested.

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

VIII. Anexo II – Certificado do Comitê de Ética no uso de animais



Certificado do Comitê de Ética no uso de animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

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

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Avaliação imunoistoquímica do tecido pulpar de ratos Wistar submetidos a procedimento clareador dentário"**, Processo FOA nº 01053-2015, sob responsabilidade de Luciano Tavares Angelo Cintra apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 09 de Dezembro de 2015.

VALIDADE DESTE CERTIFICADO: 10 de Dezembro de 2018.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 10 de Janeiro de 2019.

CERTIFICATE

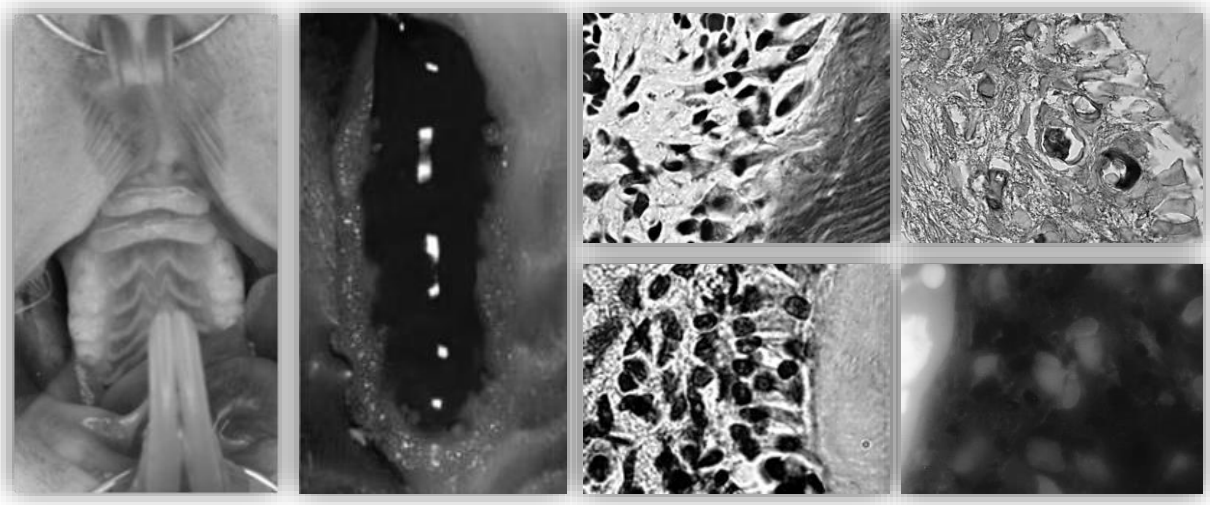
We certify that the study entitled **"Immunohistochemical evaluation of the pulp tissue of Wistar rats submitted to dental bleaching"**, Protocol FOA nº 01053-2015, under the supervision of Luciano Tavares Angelo Cintra presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on December 09, 2015.

VALIDITY OF THIS CERTIFICATE: December 10, 2018.
DATE OF SUBMISSION OF THE FINAL REPORT: January 10, 2019.

Profa. Adj. Maria Cristina Rosifini Alves Rezende
Vice-Coordenadora da CEUA
CEUA Vice-Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
Rua José Bonifácio, 1193 – Vila Mendonça - CEP: 16015-050 – ARAÇATUBA – SP
Fone (18) 3636-3234 Email CEUA: ceua@foa.unesp.br

IX. Anexo III - Carta de aceite referente ao Artigo 1



International Endodontic Journal

Carta de aceite referente ao Artigo 1

International Endodontic Journal

Decision Letter (IEJ-18-00021)

From: iejeditor@cardiff.ac.uk

To: francine_benetti@hotmail.com

CC: francine_benetti@hotmail.com, alfbriso@foa.unesp.br, andrebriso@terra.com.br, marina_carminatti@yahoo.com.br, julianam.lopes26@gmail.com, jeh.galbiati@hotmail.com, eervolino@foa.unesp.br, joao@foa.unesp.br, lucianocintra@foa.unesp.br

Subject: Manuscript ID IEJ-18-00021, International Endodontic Journal

Body: 20-Mar-2018

Dear Dr. Benetti

Manuscript ID: IEJ-18-00021

Manuscript Title: Presence of osteocalcin, osteopontin, and reactive oxygen species-positive cells in pulp tissue after dental bleaching

I am pleased to inform you that your manuscript has been accepted for publication in the International Endodontic Journal subject to major changes being made.

The comments of the referees are included at the bottom of this letter; please revise your paper taking into account any points they have raised. Also double check that in the body of the text and in the Reference section the names of authors are spelt correctly including any non-English characters where appropriate.

You will be unable to make your revisions online using the originally submitted version of the manuscript. Instead, revise your manuscript on your PC/MAC using your word processing programme and save it on your computer. Please highlight the changes to your manuscript within the document by using the "track changes" mode in MS Word or equivalent.

To upload your revised manuscript, log on to http://mc.manuscriptcentral.com/iej and enter your Author Centre, where you will find your original manuscript title listed under "Manuscripts with Decisions". Under "Actions," click on "Create a Revision". Your manuscript number has been appended to denote a revision.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees(s) in the space provided. You can use this space to document any changes you have made to the original manuscript. Please be as specific as possible in your response to the referee(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files that are to be replaced before completing the submission, for example, you would have to delete the file containing the main text and then upload the new version. You do not have to delete and upload files that are not being changed, for example, the Table or Figure files from the original version if they are to remain the same. Do NOT remove the Conflict of Interest forms these should remain with your submission throughout.

I look forward to receiving your revised manuscript.

Kind regards

Paul Dummer
Editor-in-Chief, International Endodontic Journal
iejeditor@cardiff.ac.uk