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

Mariane Maffei Azuma

TESE DE DOUTORADO

INFLUÊNCIA LOCAL E SISTÊMICA DA SUPLEMENTAÇÃO ALIMENTAR COM OS ÁCIDOS GRAXOS ÔMEGA-3 EM RATOS COM INFECÇÃO ENDODÔNTICA

Araçatuba, SP

2017

Mariane Maffei Azuma

Influência local e sistêmica da suplementação alimentar com os ácidos graxos ômega-3 em ratos com infecção endodôntica

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 odontógica, com área de concentração em endodontia.

Orientador: Prof. Adj. Luciano Tavares Angelo Cintra

Co-orientador: Prof. Dr. Edilson Ervolino

Araçatuba- SP

2017

Catalogação na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

Azuma, Mariane Maffei.
A997i Influência local e sistêmica da suplementação alimentar com os ácidos graxos ômega-3 em ratos com infecção endodôntica / Mariane Maffei Azuma. - Araçatuba, 2017
110 f. : il. ; tab.

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientador: Prof. Luciano Tavares Angelo Cintra
Coorientador: Prof. Edilson Ervolino

1. Periodontite periapical 2. Ácidos graxos ômega-3 4. Endodontia I. T.

Black D24
CDD 617.67

DADOS CURRICULARES

Mariane Maffei Azuma

Nascimento	17-02-1990 Urânia-SP
Filiação	Milton Hiroshi Azuma Silmara de Fátima Maffei Azuma
2008/2011 .	Curso de Graduação em Odontologia- Faculdade de Odontologia de Araçatuba- Universidade Estadual Paulista "Júlio de Mesquita Filho".
2012/2014	Curso de Pós-graduação em Ciência Odontológica, área de concentração em Endodontia, nível de Mestrado- Faculdade de Odontologia de Araçatuba- Universidade Estadual Paulista "Júlio de Mesquita Filho".
2015-2016	Curso de especialização em Endodontia- Faculdade de Odontologia de Araçatuba- Universidade Estadual Paulista "Júlio de Mesquita Filho".

Dedicatória

Dedico este trabalho

Aos meus pais, **Silmara e Milton**, por todo o amor e dedicação ao longo da minha vida. Por acreditarem no meu potencial, muitas vezes, até mais do que eu mesma. Pelo incentivo e suporte necessários para que todos os meus objetivos fossem realizados. Sem o apoio de vocês, a realização desse sonho não seria possível. Essa conquista é nossa!

À minha irmã, **Letícia**, por ser meu porto seguro e por cuidar tão bem dos nossos pais durante a minha ausência. Minha vida seria muito incompleta sem você!

À minha tia, madrinha, melhor amiga e mãe de alma, **Marlene Maffei** (in memorian), por ser minha principal referência de mulher forte. Pelo amor incondicional, apoio, incentivo e dedicação ao longo dos 19 anos em que convivemos aqui na terra. Mesmo após ter deixado este plano, é você quem eu reconheço nos meus gestos, nas minhas atitudes e quem guia meus passos. Essa conquista é nossa!

Amo vocês!

Agradecimentos

A Deus, pelo dom da vida, por iluminar meus passos e minhas escolhas e por ter colocado pessoas maravilhosas ao meu lado.

À direção da Faculdade de Odontologia de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho", na pessoa do seu diretor **Prof. Dr. Wilson Roberto Poi.**

À secção técnica de pós-graduação representada pela **Valéria Queiroz Marcondes Zagatto, Cristiane Regina Lui Matos e Lilian Sayuri Mada.**

À **Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP)**, por ter concedido a minha bolsa de doutorado (2013/26390-0) e por ter fomentado meu maior sonho, que era o de fazer pesquisa no exterior, por meio da bolsa estágio de pesquisa no exterior (2015/07107-0).

Aos docentes da disciplina de Endodontia da Faculdade de Odontologia de Araçatuba – UNESP, **Prof. Dr. Luciano Tavares Ângelo Cintra, Prof. Dr. Rogério de Castilho Jacinto, Prof. Dr. Gustavo Sivieri de Araújo, Prof. Dr. Eloi Dezan Júnior, Prof. Dr. José Arlindo Otoboni Filho, Prof. Dr. Mauro Juvenal Nery, Prof. Dr. João Eduardo Gomes Filho**, pelos ensinamentos, incentivo e grande contribuição durante minha formação.

Aos meus colegas e amigos de pós-graduação **Annelise Prieto, Renata Samuel, Aguinaldo Facundo, Diego Valentim, Loiane Massunari, Índia Olinta, Marcelo Wayama, Carlos Bueno, Luciana Louzada, Christine Men Martis, Renan Dal Fabro, Ana Maria Vasques, Leticia Conti, Vanessa Marques, Marina Cury, Marina Carminatti, Francine Benetti, Gabrielly Resende, Camila Ambrósio, Ludmilla Santos, Amanda Andolfatto, Leopoldo Cosme e Carolina Barros,** pela troca de conhecimentos e bons momentos vividos.

Aos alunos de iniciação científica, **Camila Pipa, Carol Barros, Juliana Nobre, Jéssica Cordeiro e Pedro Chaves**, por toda a ajuda, amizade, carinho e confiança depositada em mim. Vocês são muito especiais e trabalhar com vocês foi um grande presente! Contem sempre comigo!

Aos funcionários do departamento de Odontologia Restauradora, **Nelci Vieira, Cláudia Neves, Elaine e Peterson Moura**, pela amizade e por não medirem esforços para nos ajudar. Conviver com vocês foi um grande presente!

Ao Departamento de Ciências Básicas da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" - UNESP, representado pelo **Prof. Dr. Edilson Ervolino** e pela **Prof. Dra. Sandra Helena Penha de Oliveira**, pela oportunidade de realizar parte do processamento laboratorial.

Aos pós-graduandos do laboratório de farmacologia, **Victor Balera, Carluci Beltran e Dayane Queiroz**, por nunca terem medido esforços para me ajudar todas as vezes que precisei. Muito obrigada!

À **Prof. Dra. Suely Regina Mogami Bomfim**, por toda a disponibilidade para a realização deste trabalho, bem como pela parceria em todos os outros trabalhos desenvolvidos ao longo do meu curso de doutorado. Muito obrigada!

Ao pós-graduando, **Luis Gustavo Narciso**, por toda a colaboração e por não ter medido esforços para nos ajudar.

Agradecimentos especiais

Agradeço, especialmente, àquelas pessoas que me deram suporte científico e emocional ao longo da minha jornada

Prof. Dr. Luciano Tavares Angelo Cintra

Meu orientador de TCC, mestrado, especialização e doutorado. Obrigada por ter sido o meu "pai científico", supervisor e amigo. Você foi, ao longo desses seis anos de convivência, meu grande exemplo de profissional competente, perfeccionista, dedicado e motivado. Obrigada por ter sido um orientador sempre acessível, presente e por nunca ter medido esforços para me ajudar toda as vezes que precisei. Agradeço toda a confiança depositada no meu trabalho, em especial nesse projeto do "ômega-3". Com certeza nossa parceria foi a melhor que eu poderia ter tido durante minha formação profissional. Muito obrigada por tudo e conte comigo sempre!

Prof. Dr. Edilson Ervolino

Meu co-orientador. Obrigada pela grande contribuição para a realização deste trabalho. Agradeço a competência, a paciência e o perfeccionismo!

Prof. Dr. Mauro Juvenal Nery

Meu querido professor de endodontia e exemplo de motivação e alegria em tudo o que faz. Obrigada por ter me introduzido à endodontia de forma tão cativante e pela amizade ao longo desses anos. Você é ESPETACULAR!!!

Prof. Dr. Eloi Dezan Júnior

Meu eterno tutor do PET- Odonto-FOA e professor de endodontia. Obrigada por todos os ensinamentos em clínica, broncas e amizade. Com certeza você teve papel fundamental na minha formação e tenho muito orgulho de ter sido sua aluna.

Prof. Dra. Sandra Helena Penha de Oliveira

Minha querida professora de farmacologia. Obrigada por sempre manter o seu laboratório disponível e de portas abertas para mim. Obrigada pelo carinho, conselhos, ensinamentos e amizade. Admiro muito a sua competência, força e caráter. Ter você por perto é um grande privilégio!

Prof. Dra. Sônia Regina Panzarini

Querida professora de clínica integrada. Obrigada pelos ensinamentos, amizade, exemplo de profissionalismo e dedicação. Sua maneira de cativar os alunos com educação, atenção e seriedade é um grande exemplo para mim!

Camila de Souza Fernandes

Minha amiga de infância que, mesmo distante, sempre me dá suporte emocional e vibra com as minhas conquistas. Obrigada pelos 21 anos de amizade, companheirismo e carinho! Você é especial!

Ana Clara Fuzissima, Bruna Rodrigues e Jakeline Carvalho

Minhas "bests", amigas que estão presentes na minha vida desde o ensino médio. Vocês tiveram grande influência na minha formação. Obrigada por serem minhas psicólogas, ombro amigo, porto-seguro, inspiração e por me incentivarem a ser sempre uma pessoa melhor. Amo vocês!

Annelise Katrine Carrara Prieto

Minha "irmã Wistar". Sua amizade foi o melhor presente que eu poderia ter ganho durante o mestrado. Obrigada pela paciência, conselhos, amizade, carinho e alto-astral! Você fez com que meu período na pós-graduação se tornasse muito mais leve e divertido. Amo você e conte sempre comigo!

Diego Valentim

Meu veterano desde sempre. Obrigada por toda a ajuda e parceria durante a graduação, mestrado e doutorado. Admiro muito você e conte sempre comigo!

Renata Oliveira Samuel

Minha parceira de pós-graduação e "ELISAs da vida". Obrigada por ter me acolhido desde o momento em que entrei no mestrado, amizade, parceria, conselhos e toda ajuda. Aprendi muito com você e admiro a sua determinação e foco para alcançar seus objetivos. Conte sempre comigo!

Marcela Ito Rey, Camila Barbosa Pipa e Clícia Ribeiro

Presentes que ganhei de Araçatuba e da disciplina de Endodontia. Muito obrigada por todo o suporte emocional, amizade e torcida! A amizade, apoio e alegria de vocês fizeram toda a diferença durante minha estadia em Araçatuba. Vocês são especiais!

Carolina de Barros Morais Cardoso

"Meu orgulhinho" e presente que ganhei no início do doutorado. Obrigada pela confiança depositada em mim durante os três anos em que supervisionei você na iniciação científica. Ser sua colega de pós-graduação e amiga nesta etapa final me enche de orgulho! Obrigada por ter sido meu ombro amigo, incentivadora e por ter feito com que o desenvolvimento deste trabalho fosse muito mais divertido. Você fez toda a diferença! Conte sempre comigo!

Amanda Andolfatto

Minha amiga durante os cursos de especialização e doutorado. Obrigada pelo carinho, conversas, apoio e confiança! Sua amizade é um grande presente!

Milena Xavier de Oliveira

Esposa do meu orientador e amiga. Obrigada por ter me acolhido durante esses anos em que fui orientada do Prof. Luciano, Mi. Agradeço a companhia, risadas, happy hours e conselhos. Sua amizade fez toda a diferença e trouxe ótimos momentos durante minha jornada na pós-graduação.

Ao querido, Ricardo Honda, obrigada por todo o cuidado e incentivo ao longo da minha jornada. Você é especial e teve papel fundamental para que eu chegasse até aqui. Muito obrigada!

Acknowledgement

The Forsyth Institute/Harvard Dental School, specially to department of Immunology and Infectious Diseases for the opportunity to improve my knowledge.

I would like to express my sincere gratitude to my supervisor **Dr. Toshi Kawai** for the continuous support during my great experience at his laboratory. Thanks for your patience, motivation, and immense knowledge. I could not have imagined having a better supervisor during my experience abroad.

Besides my advisor, I would like to thank **Dr. Xiaozhe Han** and **Dr. Hajime Sasaki** for all the support during my last months at the Forsyth Institute.

My sincere thanks also go to my colleguagues from Forsyth Institute, **Kenji Egashira, Wichaya Wisitrasameewong, Alex Movila, Jaeyoung Kim, Montse Torruella, Satoru Shindo, Abdullah Albassam, Atsushi Ikeda, Hajme Yamazaki,** and **Kimito Hirai**. Thanks for the stimulating discussions, scientific support, "lunch time" and "dinner time", and all the fun we have had together. I am so lucky for have worked with such brilliant researchers like you!

I would like to express all my gratitude to my Brazilian friends that I have met at the Forsyth Institute, **Ana Cristina Andrada, Daniele Kaiser, Márcia Tanaka, Vanessa Tubero, and Mauro Abreu**, for all the suport, and for have made me feel at home.

I would like to express all my gratitude to the resposible for my great and unforgetable experience in USA...

My landlords, **Bruce and Barry**. Thanks for provided me such a great time in Boston. Because of you, my experience in Boston was better than I was expecting. You made me feel as a member of your family and introduced me to the american culture on the best way. I'll never forget that! I hope to see you soon!

My rommates, **Damla Mergen**, **Melih Kassap**, **Alp Ilgenli**, **Mai Kameda**, **Florentine Barbey**, **Ralitsa Mitrakieva**, **Tom Steyer**, **Patrick Ober**, **Marcia Tanaka**, **Patricia Polednia**, and **Veronica Fracastoro**. Thanks for the great time we had together! You guys made all the diference during my time in Boston!

In particular, I would like to express my gratitude to my soul sister, **Damla Mergen**, for being such a wonderful friend. I have learned with you that a big smile and positive energies have the power to change everything. Thanks for all the support and ice cream! No matter the distance, our hearts will always beat together. Love you, sister!

I would like to express my gratitude to my best Italian friend, **Isabella Gosetto**, for being my best psychologist, and for all the support and great time that we had together. Because of you, I know that true friendship continues to grow even over the longest distance. Love you!

E, por fim, gostaria de agradecer os meus amigos brasileiros que foram os responsáveis pelo meu suporte emocional durante a minha estadia em Boston, **Ivanete Schneider Hahn, Viviane Patricia Romani, Juliana Gabrielle Martins, Guilherme Uzejka, Cristiano Costa e Márcia Tanaka.** Obrigada por todos os momentos maravilhos que compartilhamos, bem como pelo ombro amigo nos momentos difíceis. Vocês são especiais!

“To remain indifferent to the challenges we face is indefensible. If the goal is noble, whether or not it is realized within our lifetime is largely irrelevant. What we must do therefore is to strive and persevere and never give up”.

(Dalai Lama)

RESUMO

AZUMA MM. **Influência local e sistêmica da suplementação alimentar com os ácidos graxos ômega-3 em ratos com infecção endodôntica**. 2017. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2017.

O objetivo deste trabalho foi avaliar a influência da suplementação alimentar com os ácidos graxos ômega-3 no processo inflamatório local e sistêmico de ratos com periodontite apical (PA). Foram utilizados 40 ratos da linhagem Wistar distribuídos em quatro grupos (n= 10): ratos controle (C); ratos suplementados com ômega-3 (C-O); ratos com PA induzida (PA); ratos com PA induzida suplementados com ômega-3 (PA-O). Para a indução da PA, as polpas dentárias foram expostas ao meio bucal durante 30 dias. O ômega-3 foi administrado pelo método gavage, uma vez ao dia, 15 dias antes da exposição pulpar e 30 dias depois da exposição pulpar. Após este período, o sangue foi coletado, por meio de punção cardíaca, para a realização do hemograma e quantificação sérica das citocinas pró-inflamatórias fator de necrose tumoral alfa (TNF-α), Interleucina 6 (IL-6) e Interleucina 17 (IL-17), pelo método ELISA. Após a coleta sanguínea, os ratos foram eutanasiados e as maxilas e mandíbulas foram coletadas e submetidas a processamento para análise histológica, em hematoxilina e eosina, bem como análise imunoistoquímica para os marcadores fosfatase ácida resistente ao tartrato (TRAP), osteocalcina (OCN), TNF-α, IL-6, IL-17, Interleucina 1β (IL-1β) e Interleucina 10 (IL-10). Os resultados foram analisados por testes estatísticos específicos para cada caso ($p<0,05$). O grupo PA apresentou infiltrado inflamatório mais intenso, bem como maior área de reabsorção óssea na região periapical quando comparado ao grupo PA-O ($p<0,05$). Além disso, observou-se maior número de osteoclastos e citocinas pró-inflamatórias, representadas por TNF-α, IL-1β, IL-6 e IL-17, bem como menor número de osteoblastos e da citocina anti-inflamatória IL-10 no grupo PA quando comparado ao grupo PA-O ($p<0,05$). Em relação às alterações sistêmicas, a presença de quatro focos de PA aumentou os níveis séricos de leucócitos, linfócitos, monócitos, TNF-α e IL-6

(p<0,05) e a suplementação alimentar com os ácidos graxos ômega-3 diminuiu os níveis séricos de leucócitos e linfócitos de ratos com PA (p<0,05). Conclui-se que a suplementação alimentar com os ácidos graxos ômega-3 reduz o processo inflamatório e a reabsorção óssea da região periapical, bem como as células inflamatórias do sangue, de ratos com infecção endodôntica.

Palavras-chave: Periodontite apical, ácidos graxos ômega-3, medicina endodôntica

ABSTRACT

AZUMA MM. **Local and systemic influence of the dietary supplement with omega 3 fatty acids in rats with endodontic infection.** 2017. Doctoral thesis-School of Dentistry, Sao Paulo State University, Araçatuba, 2017.

The aim of this study was to evaluate the effects of the dietary supplement omega 3 polyunsaturated fatty acids on the local and systemic inflammatory response of rats with apical periodontitis (AP). Forty male rats were divided into groups: control untreated rats (C), control rats treated with omega 3 alone (C-O), rats with pulp exposure-induced apical periodontitis (AP), and rats with pulp exposure-induced AP treated with omega 3 (AP-O). The pulps were exposed to the oral environment during 30 days for the development of AP. Omega 3 was administered by gavage method, once a day, for 15 days before pulp exposure and, subsequently, 30 days after pulp exposure. After 30 days, blood samples were collected by cardiac puncture and subjected to blood count and the serum levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), and Interleukin 17 (IL-17) were measured using ELISA method. Subsequently, rats were killed and maxillae and jaws were collected and subjected to histologic analysis, using hematoxylin and eosin staining. Immunohistochemistry was performed in order to detect TRAP-positive osteoclast cells (TRAP), Osteocalcin-positive osteoblasts cells (OCN), TNF-α, IL-6, IL-17, Interleukin 1β (IL-1β) and Interleukin 10 (IL-10). Results from the different analysis were subjected to statistical analysis, using specific statistical methods according to each analysis ($p<0.05$). AP group showed more severe inflammatory infiltrate as well as higher area of bone resorption in the periapical region compared to AP-O group ($p<0.05$). Moreover, the number of TRAP-positive cells as well as proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-17 were higher in AP group compared to AP-O group ($p <0.05$). In addition, the number of OCN-positive cells and the anti-inflammatory cytokine IL-10 decreased in AP group compared to AP-O group ($p<0.05$). Regarding systemic alterations, the presence of four AP focus increased the serum levels of leukocytes, lymphocytes, monocytes, TNF-α, and IL-6 ($p<0.05$). The dietary

supplement with omega 3 decreased the serum levels of leukocytes and lymphocytes of rats with AP ($p<0.05$). It may be concluded that the dietary supplement with omega 3 fatty acids decrease the inflammatory process and the pathological bone resorption in the periapical region, as well as serum inflammatory cells of rats with apical periodontitis.

Key-words: Apical periodontitis, omega 3 fatty acids, endodontic medicine.

S U M Á R I O

Páginas

I. INTRODUÇÃO

I. INTRODUÇÃO GERAL E REVISÃO DE LITERATURA

A periodontite apical (PA) é uma patologia caracterizada por reabsorção óssea, em resposta à infecção bacteriana oriunda dos canais radiculares (Kawashima et al., 1996). A resposta do hospedeiro é dependente da participação de diversas células inflamatórias, que podem ser representadas, principalmente, por linfócitos T, linfócitos B, macrófagos (Liapatas et al., 2003), além de células ósseas específicas, como osteócitos, osteoblastos e osteoclastos (Xiong et al., 2009; Silva et al., 2012). Neste cenário, vários mediadores inflamatórios são produzidos localmente, com a finalidade de modular a resposta imune. Dentre eles, a expressão de citocinas tem papel fundamental para que ocorra a indução e manutenção da resposta inflamatória (Xiong et al., 2009; Colic et al., 2009; Fan et al., 2011) e o processo de reabsorção óssea (Siqueira&Roças, 2007; Silva et al., 2012).

O tratamento endodôntico convencional, que visa a remoção mecânica e química do conteúdo necrótico dos canais radiculares tem sido o procedimento padrão para o tratamento da PA, o que, consequentemente, reduz o estímulo antigênico dos tecidos periapicais, diminuindo a intensidade da resposta inflamatória do local afetado, e estimulando o processo de reparo dos tecidos periapicais. Entretanto, até o momento, a literatura está escassa de estudos a respeito de outros fatores influentes neste processo de reabsorção óssea de dentes com infecção endodôntica (IE). Assim, um dos focos deste trabalho foi o de avaliar a influência de um suplemento, comumente utilizado por pacientes, sobre o processo de reabsorção óssea dos tecidos periapicais decorrente da infecção endodôntica.

O ômega-3, que é o principal ácido graxo poli-insaturado, tem como componentes os ácidos eicosapentanóico (EPA) e docosahexaenóico (DHA), que competem com o ácido araquidônico nas vias lipoxigenase (LOX) e ciclo-oxigenase (COX), o que resulta na redução da síntese de metabólitos pró-inflamatórios (Calder, 2006). Assim, o ômega-3 tem sido estudado por ter um papel importante na modulação da resposta inflamatória (Bittiner et al., 1988;

Ariza et al., 1998; Vardar et al., 2004). O DHA tem mostrado efeitos anti-inflamatórios, visto que sua presença pode diminuir a produção de interleucina 1 e interferir nas vias de sinalização que conduzem a indução da ciclo-oxigensase-2 em células endoteliais (Massaro et al., 2006). O EPA reduz os níveis de ácido araquidônico disponíveis e compete com o mesmo, o que diminui a produção de seus metabólitos, as prostaglandinas e os leucotrienos e, como consequência, ocorre a diminuição da resposta inflamatória (Mukaro et al., 2008). Além disso, tem sido demonstrado que a dieta com ômega-3 protege camundongos contra infecção de inúmeros patógenos bacterianos extracelulares, regula os níveis de triglicérides e colesterol no soro, altera funções celulares de leucócitos polimorfonucleares, modula a proliferação de linfócitos e produção de citocinas, e aumenta a capacidade anti-oxidante endógena (Blok et al., 1992; Fernandes & Venkataraman, 1993). Estes aspectos demonstram que o ômega-3 possui efeitos anti-inflamatórios, anti-trombótico, anti-arritmico, hipolipemiantes e vasodilatadores. Por causa de suas propriedades anti-inflamatórias, o ômega-3 tem sido aceito como uma terapia complementar ao tratamento convencional de doenças inflamatórias crônicas, como a artrite reumatóide (Lee et al., 2012; Miles et al., 2012), doenças cardiovasculares (Dinocolantonio et al., 2014; Jain et al., 2015) e diabetes (Farsi et al., 2014; Elwakeel & Hazaa, 2015). Além disso, sabe-se que os ácidos graxos e mediadores lipídicos derivados do ômega-3 tem um papel importante na regulação do metabolismo ósseo (Hogstrom et al., 2007; Maggio et al., 2009).

Em odontologia, o ômega-3 tem sido estudado como uma terapia complementar ao tratamento de doenças inflamatórias, como gengivite, periodontite e estomatite aftosa recorrente (Araghizadeh et al., 2013; Bendik et al., 2009; El Khouli & El-Gendy, 2014). Sabe-se que a suplementação alimentar com ômega-3 reduz a quantidade de mediadores pró-inflamatórios, Interleucina-1β (IL-1β) e fator de necrose tumoral-alfa (TNF-α), bem como o infiltrado inflamatório dos tecidos gengivais de ratos com gengivive (Araghizadeh et al., 2013). Na periodontite, os resultados do ômega-3 estão associados com a diminuição de perda óssea (Kesavalu et al., 2006; Bendik et al., 2009), diminuição de mediadores pró-inflamatórios dos tecidos gengivais

(Vardar et al., 2004; Vardar et al., 2006; Kesavalu et al., 2007), e melhora dos sintomas clínicos, como redução da inflamação gengival e da profundidade de bolsa periodontal, e aumento do nível de inserção óssea (Deore et al., 2014).

Diante dos resultados positivos associados ao ômega-3 no tratamento de doenças inflamatórias que acometem a cavidade bucal, achamos oportuno investigar se o mesmo poderia exercer efeitos no controle da inflamação e no processo de reabsorção óssea dos tecidos periapicais de ratos com IE. O artigo 1 desta dissertação aborda os efeitos do ômega-3 no processo de reabsorção óssea, por meio da observação dos osteoclastos, células responsáveis pela reabsorção óssea, e dos osteoblastos, células responsáveis pelo processo de formação óssea. O artigo 2 aborda os efeitos do ômega-3 no processo inflamatório, por meio da observação da expressão de mediadores pró-inflamatórios, representados por TNF-α, IL-1β, Interleucina-6 (IL-6) e Interleucina-17 (IL-17), bem como pela expressão do mediador anti-inflamatório Interleucina-10 (IL-10), nos tecidos periapicais de ratos com IE.

Além das injúrias locais, sabe-se que a PA pode trazer consequências indesejáveis sistemicamente, comprometendo a saúde geral do indivíduo. Por mais de um século, a relação bidirecional entre doenças locais e sistêmicas tem sido alvo de intensos debates e controvérsias. No início dos anos 1900s, a teoria da infecção focal se tornou popular, o que ocasionou medo na população daquela época, visto que a teoria sugeria que muitas enfermidades sistêmicas eram consequência de infecções locais, cuja origem era a boca (Miller et al., 1891; Billings, 1914). Devido a isso, a exodontia era o primeiro tratamento de escolha para qualquer doença que acometesse a estrutura dentária (Pallasch & Wahl, 2003). Apesar de sua popularidade, a teoria da infecção focal foi desacreditada em meados de 1900s, devido a falta de evidências científicas que a comprovasse (Newman, 1996). Entretanto, alguns pesquisadores continuaram intrigados sobre as possíveis conexões entre doenças bucais e sistêmicas e, no início do século XXI, estudos sobre o assunto em questão voltaram a surgir, principalmente na área de periodontia. Apesar da endodontia e periodontia terem a patogênese semelhante em relação a resposta inflamatória frente a microorganismos, com consequente processo inflamatório e destruição de tecido ósseo, pode-se notar que a "Medicina Endodôntica" não tem sido abordada com a mesma abrangência que

a “Medicina Periodontal”. Desde 2012, a “American Heart Association” considera que a doença periodontal e a doença vascular aterosclerose estão inter-relacionadas (Lockhart et al., 2012). Em contrapartida a isso, no mesmo ano, a “American Association of Endodontists” escreveu uma nota esclarecendo que não existe nenhuma evidência científica válida de que existe uma inter-relação entre infecção endodôntica e doenças sistêmicas (American Association of Endodontists, 2012). Após 2012, as publicações a respeito do assunto aumentaram, entretanto, várias lacunas a respeito da conexão entre infecção endodôntica e doenças sistêmicas precisam ser preenchidas.

Sabe-se que a PA é capaz de potencializar alterações sistêmicas causadas pela diabetes mellitus em ratos, aumentando os níveis séricos de triglicérides (Cintra et al., 2013 a), creatinina (Cintra et al., 2013 b), hemoglobina glicada (Cintra et al., 2014 c), mediadores pró-inflamatórios, como a IL-17 (Cintra et al., 2014 b), além de alterar alguns parâmetros do hemograma, com destaque à série branca (Cintra et al., 2014 a). Além disso, a PA potencializa os efeitos da diabetes nos parâmetros oxidativos, por meio do aumento dos níveis séricos de ácido úrico e dimuição dos níveis de albumina (Prieto et al., 2017). Em humanos, verificou-se que a PA está associada ao descontrole glicêmico e aumento da proteína C reativa de pacientes diabéticos tipo 2 (Sanchéz-Dominguéz et al., 2015; Al-Zahrani et al., 2017).

Embora nos últimos anos a correlação entre doenças cardiovasculares e IE tenha sido controversa (Berlin-Broner et al., 2016), estudos realizados em 2014 e 2016 em humanos, concluíram que a PA está associada, de forma independente, à presença de doença arterial coronariana, especialmente à síndrome coronariana aguda (Costa et al., 2014; Liljestrand et al., 2016).

Em humanos sem doenças sistêmicas, a presença de PA está associada com o aumento do estresse oxidativo (Inchingolo et al., 2013), em animais, observou-se que a PA está relacionada com o aumento de mediadores pró-inflamatórios no cérebro de fetos cujas mães possuem PA (Bain et al., 2013), aumento sérico dos mediadores pró-inflamatórios TNF-α, IL-6, IL-17, Interleucina 23 (IL-23), Interferon gama (IFN-γ) e óxido nítrico (Cintra et al., 2016), além de promover uma diminuição do sinal insulínico no sangue e

músculo esquelético (Astolphi et al., 2013; Astolphi et al., 2015; Pereira et al., 2016; Pereira et al., 2017), o que pode predispor o desenvolvimento de doenças metabólicas, como a diabetes mellitus.

Diante dos resultados que a literatura nos fornece até o momento sobre o possível impacto que a PA pode causar na saúde geral, o terceiro foco deste trabalho, abordado no artigo 3 desta tese, foi o de investigar se a suplementação alimentar com ômega-3 seria capaz de amenizar as alterações sistêmicas, representadas pelos níveis séricos de células e mediadores inflamatórios, decorrentes da infecção endodôntica.

II. OBJETIVOS

Objetivo Geral

O objetivo geral deste estudo foi avaliar a influência da suplementação alimentar com os ácidos graxos ômega-3 no processo inflamatório local e sistêmico de ratos com e sem periodontite apical (PA), consequente da infecção endodôntica.

Objetivos específicos

1. Avaliar os efeitos da suplementação alimentar com os ácidos graxos ômega-3 na área de reabsorção óssea de ratos com infecção endodôntica, por meio de análise histológica e histométrica, bem como pela imunomarcação de células TRAP- postivias e OCN-positivas.

2. Avaliar os efeitos da suplementação alimentar com os ácidos graxos ômega-3 no processo inflamatório dos tecidos periapicais de ratos com infecção endodôntica, por meio da imunomarcação dos mediadores inflamatórios TNF-α, IL-1β, IL-6, IL-17 e IL-10.

3. Avaliar, sistemicamente, os efeitos da PA e os efeitos da suplementação alimentar com os ácidos graxos ômega-3, por meio da análise do perfil hematológico e quantificação das citocinas pró-inflamatórias IL-6, IL-17, TNF-α no sangue, pelo método ELISA.

III. Artigo 1

Omega 3 fatty acids reduce bone resorption while promoting bone generation in rat periapical periodontitis

§ Artigo publicado no periódico *Journal of Endodontics*

Abstract

Introduction: This study evaluated the effects of the dietary supplement omega 3 polyunsaturated fatty acids (ω-3 PUFAs) on pulp exposure-induced apical periodontitis (AP) in rats. **Methods:** Twenty eight male rats were divided into groups: control untreated rats (C), control rats treated with ω-3 PUFAs alone (C-O), rats with pulp exposure-induced apical periodontitis (AP), and rats with pulp exposure-induced AP treated with ω-3 PUFAs (AP-O). ω-3 PUFAs was administered orally, once a day, for 15 days before pulp exposure and, subsequently, 30 days after pulp exposure. Rats were sacrificed 30 days after pulp exposure, and jaws were subjected to histological and immunohistochemical analyses. Immunohistochemical analyses were performed to detect TRAP-positive osteoclasts and osteocalcin-positive osteoblasts on the bone surface of periapical area. Results were statistically evaluated using ANOVA and Tukey's HSD, and $P<.05$ was considered statistically significant. **Results:** The bone resorption lesion was significantly larger in the AP group compared to C, C-O and AP-O groups ($P < .05$). The level of inflammatory cell infiltration was significantly elevated, and the number of TRAP-positive osteoclasts was significantly higher in the periapical lesion of the AP group compared to AP-O, C and C-O groups ($P < .05$). The number of osteocalcin-positive osteoblasts was significantly increased in the AP-O group compared to the AP group ($P > .05$). **Conclusion:** Supplementation with ω-3 PUFAs not only suppresses bone resorption, but also promotes new bone formation in the periapical area of rats with apical periodontitis in conjunction with downregulation of inflammatory cell infiltration into the lesion.

Keywords: apical periodontitis; omega 3 fatty acids, endodontic infection; osteocalcin

Introduction

Apical periodontitis (AP) is an inflammatory disease characterized by inflammatory bone destruction in response to intracanal bacterial infection (1). Bone remodeling processes, which are mediated by specific bone cells, including osteocytes, osteoblasts and osteoclasts, appear to be dysregulated in the periapical periodontitis lesion (2). Currently, mechanical removal of infected dentin of root canals, accompanied by chemical disinfection, has been the standard procedure to treat periapical periodontitis to reduce the level of inflammation at the affected site. To date, however, alternative therapies able to reduce bone resorption in periapical periodontitis are not available.

Omega 3 polyunsaturated fatty acids (ω-3 PUFAs), as represented by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been accepted as an adjuvant therapy in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis (3), cardiovascular disease (4) and diabetes (5). In addition, ω-3 PUFAs and lipid mediators derived from ω-3 PUFAs are reported to have important roles in the prevention of pathogenic bone resorption (6). These findings indicate that dietary supplement with ω-3 PUFAs inhibits the activation of proinflammatory arachidonic acid cascade, downmodulates acute inflammatory response by polymorphonuclear (PMN) leukocytes, and suppresses proliferation of lymphocytes and their production of proinflammatory cytokines (7,8). The production of proinflammatory factors, such as prostaglandin E2 (PGE2), Interleukin- 1β (IL-1β) and tumor necrosis factor alpha (TNF-α), as a consequence arachidonic acid cascade, acute inflammatory response by PMN, and lymphocyte activation, is known to promote osteoclastogenesis, while suppressing osteoblastogenesis (9,10). Many studies have reported the positive effects of ω-3 PUFAs as an adjunct therapy for periodontal disease (11-15). However, to the best of our knowledge, none of these studies has ever investigated the effect of ω-3 PUFAs on periapical bone resorption processes in either human patients with periapical periodontitis or animal models of endodontic infection.

Osteocalcin (OCN) plays a key role as a calcium-binding protein and is one of the most abundant non-collagenous proteins in bone tissue (16). The expression of OCN is considered to be a biomarker of bone formation (17).

Especially, OCN is produced by osteoblast cells in their late stage of differentiation (18). While it has been reported that ω-3 PUFAs can promote homeostatic osteoblastogenesis-mediated new bone formation (19), the effects of ω-3 PUFAs on the expression of OCN, as well as on pathogenic bone remodeling, remain unclear.

Therefore, this study aimed to evaluate the effect of the dietary supplement ω-3 PUFAs on the pathogenic bone resorption induced in a rat model of periapical periodontitis by pulp exposure-elicited endodontic infection. In order to evaluate the impact of ω-3 PUFAs on dysregulated bone remodeling processes, TRAP-positive osteoclast cells, as well as OCN-positive osteoblasts, were monitored through histological and immunohistochemical analyses.

Materials and methods

Experimental animals

The experimental protocol was approved by the Institutional Ethics Committee (CEUA 2014-00550) of UNESP-Univ Estadual Paulista, Sao Paulo, Brazil, and conducted in accordance with relevant guidelines. Twenty eight six-week-old male Wistar rats (*Rattus norvegicus albinus*) weighing 200-250g each were used in this study. The rats were housed in a mini-isolator (Alesco, São Paulo, SP, Brazil) in temperature-controlled rooms and given ad libitum access to water and food.

The rats were randomly assigned into four groups (seven rats/group): 1) control untreated rats (C), 2) healthy rats treated with ω-3 PUFAs (C+O), 3) rats with pulp exposure-induced apical periodontitis (AP), and 4) rats with pulp exposure-induced AP treated with ω-3 PUFAs (AP+O).

Supplementation with ω-3 PUFAs

Rats in the C+O and AP+O groups were orally gavaged with ω-3 PUFAs (Omega 3 Catarinense-Laboratório Catarinense S.A, Joinville, SC, Brazil) (water solution, 40mg/kg; 60% EPA and 40% DHA, once a day), while rats in groups C and AP received control distilled water during 15 days before AP induction (prophylactic administration) and 30 days after AP induction

(therapeutic administration). As a consequence, rats were either treated with ω-3 PUFAs or control water for a total period of 45 days (12,13).

Induction of apical periodontitis (AP)

Rats were anesthetized with ketamine (87 mg/kg; Francotar; Virbac do Brazil Ind. e Com. Ltda., Roseira, SP, Brazil) and xylazine (13 mg/kg; Rompun; Bayer S. A., São Paulo, SP, Brazil). The pulp of the right mandibular first molar was exposed using a dental round bur (Broca Ln Long Neck; Maillefer, Dentisply Ind e Com Ltda, Petrópolis, SP, Brazil) (groups AP and AP-O) for the development of AP. The coronal pulp tissue was exposed to the oral cavity for 30 days.

Immunohistological analyses

Mandibles sampled from sacrificed rats were decalcified in 10% EDTA for 30 days and submitted to conventional histologic processing for the creation of paraffin-embedded tissue section. Semi-serial sections (4μm) were performed in the laterolateral direction, allowing sectioning of the mandibular first molar in its longitudinal axis. Sections were stained with hematoxylin-eosin (H&E) or submitted to immunohistochemistry by using an indirect immunoperoxidase technique for tartrate-resistant acid phosphatase (TRAP) (primary antibody goat anti-TRAP SC 30832; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and primary antibody rabbit anti-osteocalcin SC-30044 for osteocalcin, following the previously described protocol (20). Histopathologic, histometric, and immunohistochemical analyses were performed by a certified histologist (E.E.) who was blinded to the experimental groups.

Histologic analysis was conducted by using the following parameters: nature and extension of inflammation, presence and extension of necrosis, state of vasculature, and pattern of cellularity of dental and periapical tissues.

The intensity of inflammatory infiltration was graded as follows: absent (0 to few inflammatory cells: score 1); mild (< 25 inflammatory cells: score 2),

moderate (25-125 inflammatory cells: score 3), or severe (>125 inflammatory cells: score 4).

For AP and AP-O groups, the area of periapical lesion associated with the distal root of the mandibular first molars was histometrically measured. The area was calculated by rounding up the lesion boundary, considering the outer external surface of alveolar bone, and it was expressed in square micrometers. For each rat, 7 serial histologic sections were measured histometrically using an image processing system, which consisted of a light microscope (DM 4000 B Leica), a color camera (DFC 500, Leica, Wetzlar, Germany), a color image processor (Leica Qwin V3 software, Leica, Wetzlar, Germany), and a personal computer (Intel Corel I5, Windows 10). The apical periodontitis areas were determined for each side, and the average value (mean ± standard deviation) was calculated for each experimental group.

The number of osteoclasts and osteoblasts was analyzed in the histological section used for histometric analysis. The perimeter was calculated by contouring the boundary of the apical periodontitis with the aid of Leica Microsystems software. The number of TRAP-positive multinucleated cells as well as OCN-positive cells was calculated in the perimeter and expressed in cells/millimeter.

Statistical analysis

The number of TRAP-positive cells, OCN-positive cells and lesion size were statistically determined using analysis of variance for multiple comparisons, and the Tukey test was used for pairwise comparisons at 5% significance. Histologic findings were analyzed with the Kruskal-Wallis test. Dunn's method was used for pairwise comparisons at 5% significance. Statistical analyses were performed by using SigmaPlot software (San Jose, CA, USA).

Results

Histopathologic and histometric Analysis

To evaluate the effects of ω-3 PUFAs on cell infiltrate in the apical periodontitis, histologic images of H&E-stained periapical area in the different experimental groups of rats sacrificed at day-30 were performed and are shown in Figure 1. No sign of inflammation was noted in the periapical tissue of C or C-O groups (Figures 1A, 1B, 1E and 1F). However, in the AP and AP-O groups, the dental pulp showed signs of total necrosis at 30 days after pulp exposure, and the enlargement of periapical space between bone and cementum of tooth apex was observed, indicating that bone surrounding apex was pathogenically resorbed in response to pulp exposure (Figures 1C, 1D, 1G and 1H). Furthermore, these lesions were primarily composed of inflammatory infiltrates, including neutrophils and mononuclear cells. Statistical analysis showed that the magnitude of bone loss was higher in the AP group compared to the AP-O, C-O, and C groups ($P < .05$). The intensity of inflammatory infiltration was greater in the AP-O group (Figures 1G and 1H) compared to C (Figures 1A and 1B), C-O (Figures 1E and 1F) and AP groups (Figures 1C and 1D) ($P < .05$) (Table 1).

To evaluate the effects of ω-3 PUFAs in the bone resorption, the area of AP was measured histometrically. The area of periapical lesion of mandibular first molar was larger in the AP-O (Figures 1G and 1H) and AP (Figures 1C and 1D) groups when compared with the C (Figures 1A and 1B) and C+O groups (Figures 1E and 1F) ($P <.05$). In addition, apical periodontitis was more evident in the AP group when compared with the AP-O group ($P <.05$) (Figures 1C, 1D, 1G, 1H and Table 1).

Table 1. Scores and median of the Intensity of inflammatory cells and apical periodontitis area (μm2) according to the groups

Histologic parameters	Experimental groups				Statistical analysis
Intensity of inflammatory infiltration	C	C-O	AP	AP-O	
1: absent	7/7	7/7	0/7	5/7	
2: mild	0/7	0/7	0/7	2/7	Kruskal-Wallis
3: moderate	0/7	0/7	3/7	0/7	P < .05
4: severe	0/7	0/7	4/7	0/7	
Median*	1^{a}	1^{a}	4^{b}	1^{a}	
Apical periodontitis ($x10^4$ μm² ± SD*)	13.20 ± 1.67^{a}	12.78 ± 1.71^{a}	129.08 ±19.3^{b}	43.94 ± 27.90^{c}	Tukey P < .05

*Different superscript letters indicate significant statistical differences in raws (P < .05).

C, control untreated rats; C-O, healthy rats treated with ω-3 PUFAs; AP, rats with pulp exposure-induced apical periodontitis; AP-O, rats with pulp exposure-induced AP treated with ω-3 PUFAs.

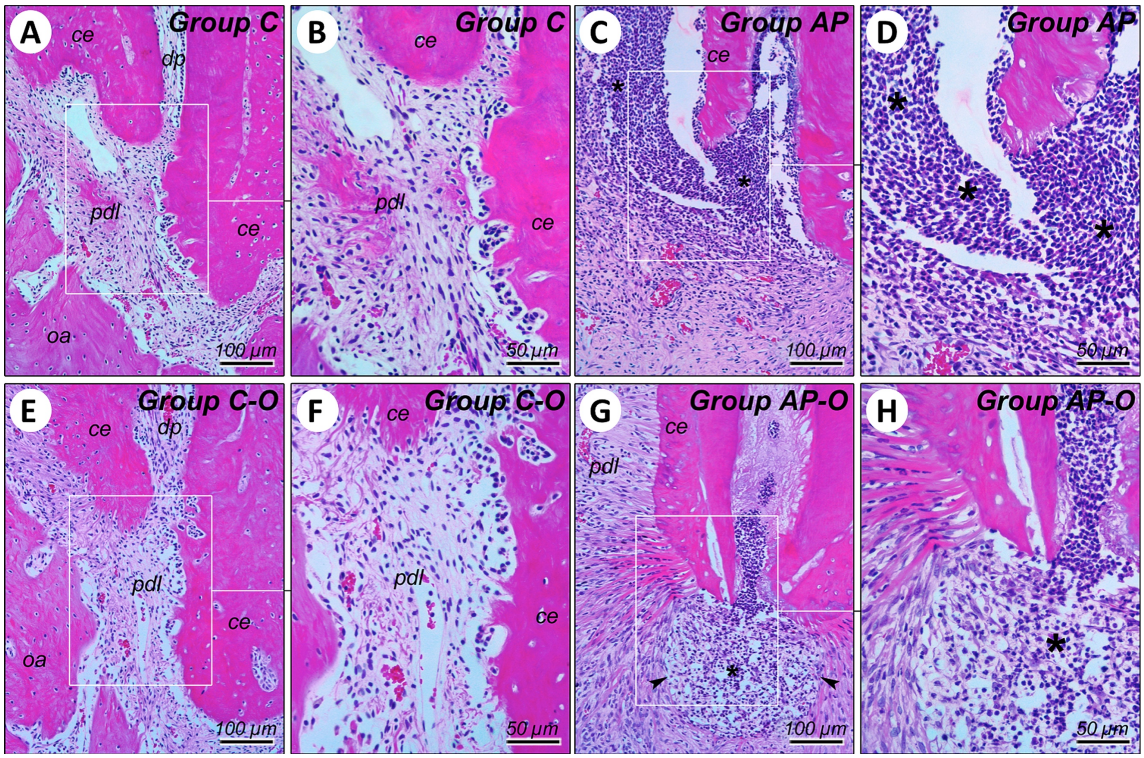

Figure 1. Histologic images of periapical lesions at 30 days after pulp exposure (H&E staining). (A and B) Group C. Periapical region is free of inflammatory infiltrates (original magnification, x100 and x200, respectively). (E and F) Group C-O. Similar to C, the periapical region is free of inflammatory infiltrates (original magnification, x100 and x200, respectively). (C and D) Group AP. Intense infiltration of inflammatory cells in the area surrounding tooth apex can be observed. Severe disorganization of the periodontal ligament and large bone resorption area are visible (hematoxylin; original magnification, x100 and x200, respectively). (G and H) Group AP-O. Compared with AP, a decreased level of inflammatory infiltrates is observed in the periodontal ligament region. The size of bone resorption is also diminished compared to the AP group (hematoxylin; original magnification, x100 and x200, respectively). *Arrowheads* indicate margins of apical periodontitis. dp, dental pulp; pdl, periodontal ligment; ce, cementum; ao, alveolar bone. * Site of infiltration of inflammatory cells.

Histologic evaluation of TRAP-positive osteoclasts and OCN-positive cells that emerged in periapical lesion

To evaluate bone remodeling and effect of w-3 PUFAs on osteoclastogenesis, we performed TRAP-staining.

The histochemistry images of TRAP-staining are shown in Figure 2. The number of TRAP-positive multinucleated cells per millimeter on the alveolar bone surface of rats induced of apical periodontitis was higher in the AP and AP-O groups compared to the C or C+O groups ($P < .05$). In addition, the AP group showed a higher number of TRAP-positive cells when compared with the AP-O group ($P < .05$) (Figure 2).

In order to evaluate bone remodeling and effect of w-3 PUFAs on osteoblastogenesis, we perfomed histochemistry to detect OCN-positive cells. The histochemistry images of OCN-positive cells are shown in Figure 3. The number of OCN-positive cells per millimeter on the alveolar bone surface of rats induced of apical periodontitis was higher in the C and C-O groups compared to the AP or AP-O groups ($P <.05$). In addition, the AP-O group showed a higher number of OCN-positive cells when compared with the AP group ($P <.05$) (Figure 3).

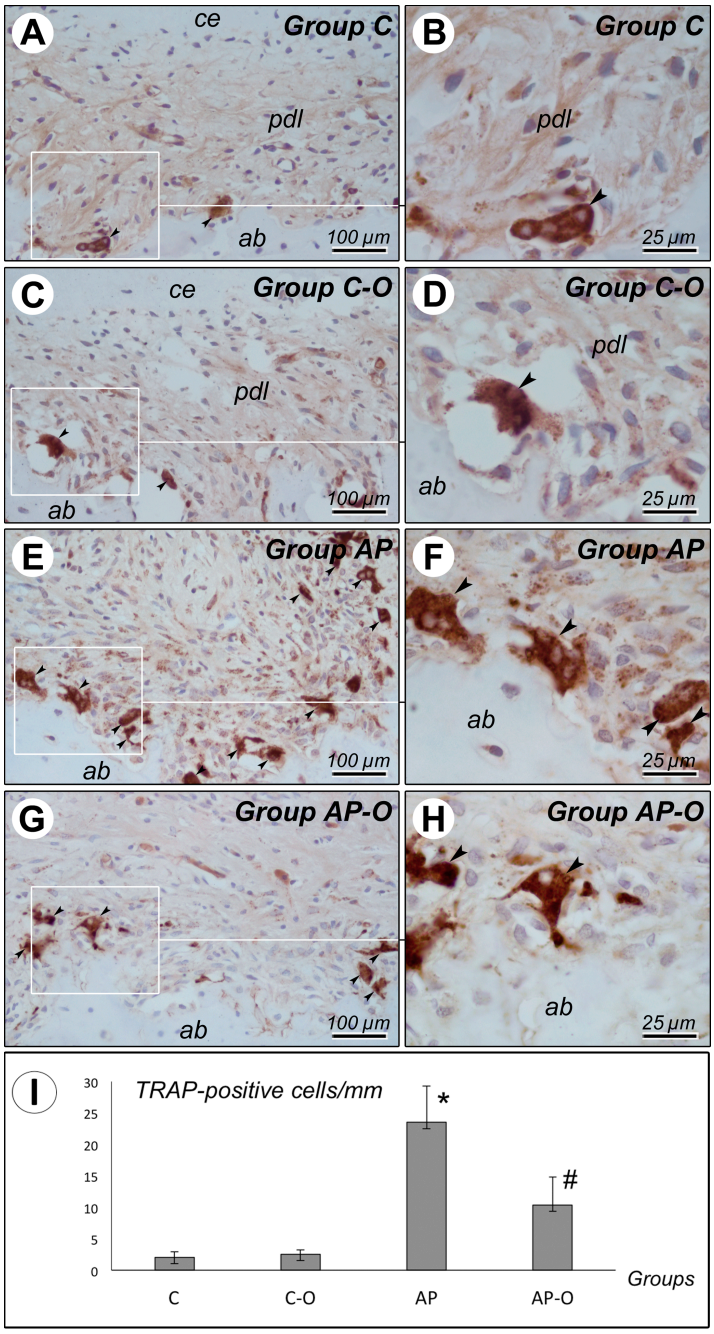

Figure 2. Histological images and number (media and standard deviation) of TRAP-positive multinucleated cells (arrowheads) stained in the periapical bone at 30 days after pulp exposure groups. Representative images control groups C (A and B) and C-O (C and D), as well as periapical periodontitis groups AP (E and F) and AP-O (G and H), are shown [ab, alveolar bone; pdl, periodontal ligament]. After staining the section with TRAP, nuclei were counterstained with hematoxylin. Original magnification: x100 (A, C, E and G) and x400 (B, D, F, H). The number of TRAP-positive cells per mm in the perimeter of apical periodontitis in the distal root of the mandibular first molar in the experimental groups are shown (I). *Statistically difference between the indicated group and C, C-O and AP-O groups (P<.05). #Statistically difference between the indicated group and C, C-O and AP groups (P<.05).

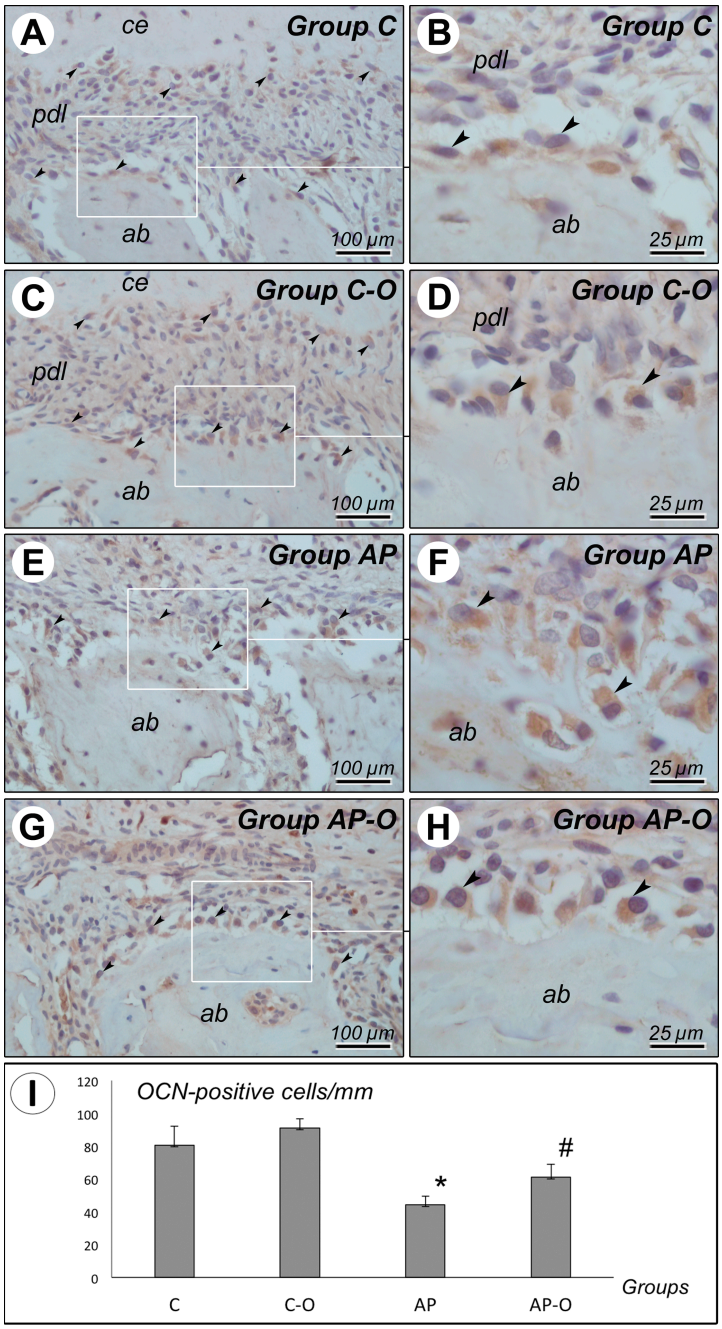

Figure 3. Histological images and number (media and standard deviation) of osteocalcin (OCN) expressed in osteoblasts (arrowheads). Representative images of non-exposed control groups C (A and B) and C-O (C and D), as well as periapical periodontitis groups AP (E and F) and AP-O (G and H), are shown [ab, alveolar bone; pdl, periodontal ligament]. After staining the section with OCN, nuclei were counterstained with hematoxylin. Scale bars: Original magnification: C–E, x100 (A, C, E and G) and x400 (B, D, F, H). The number of OCN-positive cells per mm in the perimeter of apical periodontitis in the distal root of the mandibular first molar in the experimental groups are shown (I). *Statistically difference between the indicated group and C, C-O and AP-O groups (P<.05). #Statistically difference between the indicated group and C, C-O and AP groups (P<.05).

Discussion

In the present study, systemic administration of ω-3 PUFAs not only decreased osteoclastogenesis, but also promoted osteoblastogenesis in the context of bone resorption lesion of periapical periodontitis, suggesting that ω-3 PUFAs can act on two different bone cells and ameliorate the pathogenic bone loss outcomes of this disease. The pathogenic bone resorption induced in the AP group by pulp exposure was significantly reduced by systemic administration with ω-3 PUFAs in the AP-O group. This result agrees with previous studies performed in the rat model of periodontitis and human patients with periodontitis (14, 21, 22). However, other studies concluded that dietary supplement with ω-3 PUFAs had no effect on bone resorption in human patients with periodontitis (12, 13, 23). Nonetheless, none of these studies has ever addressed the effects of ω-3 PUFAs on pathogenic bone outcomes of periapical periodontitis.

Rats that received prophylactic administration of ω-3 PUFAs in addition to therapeutic administration showed better outcomes in suppressing the bone resorption induced in apical periodontitis compared to rats that received therapeutic administration alone (12, 13). To explain, prophylactic administration of ω-3 PUFAs acid for 2 weeks allowed the accumulation of EPA and DHA in the cell membrane (24), eventually promoting the robustness of membrane stability and fluidity in response to inflammatory challenge by the induction of periodontitis. It would be intriguing to examine in a future study if prophylactic administration of ω-3 PUFAs can also increase the efficacy of ω-3 PUFAs on pathogenic bone loss induced by pulp exposure.

The present study corroborates the findings of (25) in which rats induced of endodontic infection were supplemented with fish oil. Compared to experimental rats that received placebo treatment, a decreased number of osteoclasts was seen in periapical lesion of rats that received ω-3 PUFAs. Some *in vitro* studies have also demonstrated that ω-3 PUFAs could decrease osteoclastogenesis (26-29). It is well established that the metabolites of polyunsaturated fatty acids, e.g., prostaglandin, hydroxyeicosatetraenoic acids, and leukotrienes, act on osteoclast progenitors and suppress their RANKL-mediated differentiation and activation (30, 31). According to an *in vitro* study,

the effects of DHA on osteoclastogenesis are, at least in part, mediated by lipoxygenase, a metabolic derivative of DHA (32).

The level of OCN is considered a marker of new bone formation (19). Thus, our results showing an elevated number of OCN-positive osteoblasts in the ω-3 PUFAs-treated AP rats suggested that ω-3 PUFAs could also improve new bone formation, even in the context of inflammatory bone resorption lesion. Further studies to elucidate the molecular mechanisms of bone formation should be performed to verify this hypothesis.

In addition, we found a greater number of inflammatory infiltrates in the periapical area of the AP group, causing more severe inflammatory infiltration, when compared to AP-O and C groups. Inflammation is characterized by a complex sequence of events involving alterations in the inflammatory network, as well as rearrangement of innate immune cell populations and changes in their activation status (33). Therefore, the presence of inflammatory cells in the AP and AP-O groups indicates inflammatory reaction to bacterial challenge in the periapical area. Reduced inflammatory status in the AP-O group supports some studies showing the effects of ω-3 PUFAs during inflammation (34, 35). These studies suggested that ω-3 PUFAs can competitively inhibit the production of arachidonic acid metabolites via the cyclooxygenase (COX) and lypoxigenase (LOX) pathways, thus reducing proinflammatory arachidonic mediators (34) and decreasing inflammatory response (35).

The findings here in presented indicate that systemic supplementation with ω-3 PUFAs can decrease bone resorption in the periapical area of rats with periapical periodontitis, as well as the number of infiltrating inflammatory cells and the number of osteoclasts. Furthermore, ω-3 PUFAs promoted osteoblastogenesis in the lesion. In summary, this study lays the groundwork for further investigation of ω-3 PUFAs as a therapeutic regimen for periapical periodontitis.

Acknowledgments

This study was supported by FAPESP (2013/26390-0 and 2015/03054-0).

The authors declare no conflicts of interest.

References

1. Kawashima N, Okiji T, Kosaka T, et al. Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: a quantitative immunohistochemical study. J Endod 1996; 22:311–16.
2. Xiong H, Wei L, Peng B. Immunohistochemical localization of IL-17 in induced rat periapical lesions. J Endod 2009;35:216–20
3. Lee YH, Bae SC, Song GG. Omega-3 polyunsaturated fatty acids and the treatment of rheumatoid arthritis: a meta-analysis. Arch Med Res. 2012;43:356-62.
4. Jain AP, Aggarwal KK, Zhang PY. Omega-3 fatty acids and cardiovascular disease. Eur Rev Med Pharmacol Sci. 2015;19:441-45.
5. Farsi PF, Djazayery A, Eshraghian MR, et al. Effects of supplementation with omega-3 on insulin sensitivity and non-esterified free fatty acid (NEFA) in type 2 diabetic patients. Arq Bras Endocrinol Metabol. 2014;58:335-40.
6. Maggio M, Artoni A, Lauretani F, et al. The impact of omega-3 fatty acids on osteoporosis. Curr Pharm Des 2009;15:4157–64.
7. Alam SQ, Bergens BM, Alam BS. Arachidonic acid, prostaglandin E2 and leukotriene C4 levels in gingival and submandibular salivary glands of rats fed diets containing n-3 fatty acids. Lipids. 1991;26:895-900.
8. Fernandes G, Venkataraman J. Role of omega-3 fatty acids in health and disease. Nutr Res 1993; 13:19-45.
9. Shen CL, Peterson J, Tatum OL, et al. Effect of long-chain n-3 polyunsaturated fatty acid on inflammation mediators during osteoblastogenesis. J Med Food. 2008;11:105-10.
10. Nakanishi A, Iitsuka N, Tsukamoto I. Fish oil suppresses bone resorption by inhibiting osteoclastogenesis through decreased expression of M-CSF,

PU.1, MITF and RANK in ovariectomized rats. Mol Med Rep. 2013;7:1896-903.

11. Vardar S, Buduneli E, Türkoğlu O, et al. Therapeutic versus prophylactic plus therapeutic administration of omega3 fatty acid on endotoxin-inducedperiodontitis in rats. J Periodontol 2004; 75:1640-6.
12. Vardar-Sengül S, Buduneli N, Buduneli E, et al. Dietary supplementation of omega-3 fatty acid and circulating levels of interleukin-1beta, osteocalcin, and C-reactive protein in rats. J Periodontol 2006; 77:814-20.
13. Kesavalu L, Vasudevan B, Raghu B, et al. Omega-3 fatty acid effect on alveolar bone loss in rats. J Dent Res. 2006; 85:648-52.
14. Iwasaki M, Yoshihara A, Moynihan P, et al. Longitudinal relationship between dietary ω3 fatty acids and periodontal disease. Nutrition. 2010; 26:1105-9.
15. Deore GD, Gurav AN, Patil R, et al. Omega 3 fatty acids as a host modulator in chronic periodontitis patients: a randomised, double-blind, palcebo-controlled, clinical trial. J Periodontal Implant Sci. 2014; 44:25-32.
16. Lian JB, Gundberg CM. Osteocalcin: Biochemical considerations and clinical applications. Clin Orthop Relat Res 1988;226:267-91.
17. Shibutani T, Inuduka A, Horiki I, et al. Bisphosphonate inhibits alveolar bone resorption in experimentally-induced peri-implantitis in dogs. Clin Oral Implants Res 2001;12:109-14.
18. Zoch ML, Clemens TL, Riddle RC. New insights into the biology of osteocalcin. Bone. 2016;82:42-9.
19. Fong L, Muhlhausler BS, Gibson RA, et al. Perinatal maternal dietary supplementation of ω3-fatty acids transiently affects bone marrow microenvironment, osteoblast and osteoclast formation, and bone mass in male offspring. Endocrinology. 2012;153:2455-65.
20. Garcia VG, Longo M, Gualberto Junior EC, et al. Effect of the concentration of phenothiazine photosensitizers in antimicrobial photodynamic therapy on bone loss and the immune inflammatory response of induced periodontitis in rats. J Periodontal Res 2013;49:584–94
21. Elkhouli AM. The efficacy of host response modulation therapy (omega-3 plus low-dose aspirin) as an adjunctive treatment of chronic periodontitis (clinical and biochemical study). J Periodontal Res. 2011;46:261-68.

22. Bendyk A, Marino V, Zilm PS, et al. Effect of dietary omega-3 polyunsaturated fatty acids on experimental periodontitis in the mouse. J Periodontal Res. 2009; 211-16.
23. Martinez GL, Koury JC, Brito F, et al. The impact of non-surgical periodontal treatment on serum levels of long chain-polyunsaturated fatty acids: a pilot randomized clinical trial. J Periodontal Res. 2014; 49:268-74.
24. Croft KD, Beilin LJ, Loece FM, et al. Effects of diet enriched eicosapentaenoic or docosahexaenoic acid or prostanoid metabolism in the rat. Lipids 1987; 22:647-50.
25. Indahyani DE, Pudyani PS, Santoso AL, et al. The effect of fish oil on bone resorption following pulp exposure in rats. Dent Traumatol. 2002;18:206-11.
26. Sakaguchi K, Morita I, Murota S. Eicosapentaenoic acid inhibits bone loss due to ovariectomy in rats. Prostaglandins Leukot Essent Fatty Acids 1994; 50:81-4
27. Kruger MC, Coetzer H, de Winter R, et al. Calcium, gamma-linolenic acid and eicosapentaenoic acid supplementation in senile osteoporosis. Aging (Milano) 1998;10:385–94.
28. Fernandes G, Lawrence R, Sun D. Protective role of n-3 lipids and soy protein in osteoporosis. Prostaglandins Leukot Essent Fatty Acids 2003;68:361–72
29. Sun L, Tamaki H, Ishimaru T, et al. Inhibition of osteoporosis due to restricted food intake by the fish oils DHA and EPA and perilla oil in the rat. Biosci Biotechnol Biochem 2004;68:2613–5.
30. Yasuda H, Shima N, Nakagawa N, et al. A novel molecular mechanism modulating osteoclast differentiation and function. Bone 1999; 25:109–13.
31. Bezerra MC, Carvalho JF, Prokopowitsch AS, et al. RANK, RANKL and osteoprotegerin in arthritic bone loss. Braz J Med Biol Res 2005;38:161–70
32. Yuan J, Akiyama M, Nakahama K, et al. The effects of polyunsaturated fatty acids and their metabolites on osteoclastogenesis in vitro. Prostaglandins Other Lipid Mediat. 2010; 92:85-90.
33. Yang KK, Dorner BG, Merkel U, et al. Neutrophil influx in response to a peritoneal infection with Salmonella is delayed in lipopolysaccharide-binding protein or CD14-deficient mice. J Immunol 2002;169:4475–80.

34. Calder PC. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids 2006;75:197–202.
35. Mukaro VR, Costabile M, Murphy KJ, et al. Leukocyte numbers and function in subjects eating n-3 enriched foods: selective depression of natural killer cell levels. Arthritis Res Ther 2008;10:R57.

IV. Artigo 2

Omega 3 fatty acids reduce inflammation in rat apical periodontitis

§ Artigo submetido para o periódico *Journal of Endodontics*

Abstract

Introduction: The effects of omega 3 polyunsaturated fatty acids (ω-3 PUFAs) on pro- and anti-inflammatory mediators were evaluated in a rat model of pulp exposure-induced apical periodontitis (AP). **Methods:** Twenty-eight male Wistar rats were divided into four groups: control untreated rats (C), control rats treated with ω-3 PUFAs (C-O), rats with pulp exposure-induced apical periodontitis (AP), and rats with pulp exposure-induced apical periodontitis treated with ω-3 PUFAs (AP-O). Omega 3 PUFAs were administered by gavage method once a day for 15 days prior to the pulp exposure which was continued for 30 days after pulp exposure. Rats were sacrificed 30 days after pulp exposure, and dissected jaws were subjected to immunohistochemical analysis to detect immunoreaction to tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-1β (IL-1β), Interleukin-17 (IL-17) and Interlukin-10 (IL-10) on the bone surface of periapical area. Results were statistically evaluated using the Mann-Whitney test, and $P < .05$ was considered statistically significant. **Results:** Immunoreaction to proinflammatory cytokines TNF-α, IL-6, IL-1β and IL-17 was higher in the AP group compared to AP-O, C and C-O groups ($P < .05$). Immunoreaction to the anti-inflammatory cytokine IL-10 was lower in the AP group compared to AP-O ($P < .05$). **Conclusion:** Supplementation with ω-3 PUFAs can modulate inflammatory response in rat apical periodontitis, decreasing levels of TNF-α, IL-6, IL-1β, IL-17 expression, and increasing the level of IL-10 expression.

Keywords: apical periodontitis, omega 3 fatty acids, endodontic infection, cytokines, bone resorption.

Introduction

Apical periodontitis (AP) may be histologically characterized by the presence of fibrous and granulated tissue and different inflammatory cell infiltrates, including T lymphocytes, B lymphocytes and macrophages (1). This periapical inflammatory process is a host immune response against bacterial infection present in the necrotic pulp. When the host defense mechanism is not able to eradicate the infection over time, chronic AP ensues, leading to destruction of the bone structures in the periapical area (2).

In AP, several proinflammatory cytokines are produced locally to mediate immune response. The production of proinflammatory cytokines has a particular importance in the induction of bone resorption (3,4). Based on current evidence, proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-1β (IL-1β), and Interleukin-17 (IL-17) may stimulate osteoclastogenesis (OC-genesis) and bone resorption (5). Such "cytokine storm" is directly related to the development of AP (4,5). On the other hand, some studies suggest that Interleukin-10 (IL-10), which can suppress the production of proinflammatory cytokines and chemokines, such as IL-1, IL-6 and TNF-α (6,7), is associated with down-modulation of AP development (5). This suggests the importance of developing therapeutic regimens able to downregulate proinflammatory response, but increase anti-inflammatory response, during apical periodontitis to reduce the bone loss.

Omega 3 polyunsaturated fatty acids (ω-3 PUFAs), as represented by eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been studied as an adjuvant therapy in the treatment of oral diseases, including gingivitis (8), periodontitis (9-11), apical periodontitis (12) and recurrent aphthous stomatitis (13). Related findings have indicated that diet supplemented with ω-3 PUFAs may inhibit the production of arachidonic acid metabolites via the cyclooxygenase (Cox2) and lipoxigenase pathways, thus reducing the expression of proinflammatory response (Calder 2006), while decreasing inflammatory symptoms (13). A previous study showed that ω-3 PUFAs reduce bone resorption and promote bone generation on pulp exposure-induced apical periodontitis in rats, leading to a decrease in TRAP-positive cells and increase of osteocalcin-positive cells (12). However, to the

best of our knowledge, no study has ever investigated the effects of ω-3 PUFAs on cytokine expression in either human patients with apical periodontitis or animal models of endodontic infection.

Therefore, this study aimed to evaluate the effects of the dietary supplement ω-3 PUFAs on the inflammatory process and pathogenic bone resorption induced in a rat model of pulp exposure-induced apical periodontitis. To accomplish this, the effects of ω-3 PUFAs on the production of proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-17 and the anti-inflammatory cytokine IL-10 were monitored in rat periapical tissues through immunohistochemical analysis.

Materials and methods

Experimental animals

The experimental protocol was approved by the Institutional Ethics Committee (CEUA 2014-00550) of the Universidade Estadual Paulista (UNESP), Sao Paulo, Brazil, and it was conducted in accordance with relevant guidelines. Six-week-old male Wistar rats (Rattus norvegicus albinus) (n=28), weighing 200-250g each, were used in this study. The rats were housed in mini-isolators (Alesco, São Paulo, SP, Brazil) in temperature-controlled rooms and given ad libitum access to water and food.

The rats were randomly assigned into four groups (n=7/group): 1) control untreated rats (C), 2) control rats treated with ω-3 PUFAs (C-O), 3) rats with pulp exposure-induced apical periodontitis (AP), and 4) rats with pulp exposure-induced AP treated with ω-3 PUFAs (AP-O).

Supplementation with ω-3 PUFAs

Rats in the C-O and AP-O groups were administered by an oral gavage with ω-3 PUFAs (Omega 3, Catarinense Pharma-Laboratório Catarinense S.A, Joinville, SC, Brazil) (water solution, 40mg/kg; 60% EPA; 40% DHA, once a day), while rats in groups C and AP received control distilled water

during 15 days before AP induction (prophylactic administration) and 30 days after AP induction (therapeutic administration). As a consequence, rats were either treated with ω-3 PUFAs or control water for a total period of 45 days (9,12,14).

Induction of apical periodontitis (AP)

Rats were anesthetized with ketamine (87 mg/kg; Francotar, Virbac do Brazil Ind. e Com. Ltda., São Paulo, SP, Brazil) and xylazine (13 mg/kg; Rompun, Bayer S. A., São Paulo, SP, Brazil). The pulp of the right mandibular first molars was exposed using surgical round burs (Broca Ln Long Neck; Maillefer, Dentisply Ind e Com Ltda, Petrópolis, SP, Brazil) (groups AP and AP-O) for the development of AP. The coronal pulp tissue was exposed to the oral cavity for 30 days (12,15,16).

Immunohistological analyses

Mandibles sampled from sacrificed rats were decalcified in 10% EDTA for 30 days and subjected to conventional histologic processing to make paraffin-embedded tissue sections. Semi-serial sections (4μm) were performed in the laterolateral direction, allowing sectioning of the mandibular first molar in its longitudinal axis. Sections were submitted to immunohistochemistry by using an indirect immunoperoxidase technique for the cytokines TNF-α, IL-6, IL-1β, IL-17 and IL-10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), following a previously described protocol (12,15,17). Immunohistochemical analyses were performed by a certified histologist (E.E.) blinded to the experimental groups. Semiquantitative immunolabeling of TNF-α, IL-6, IL-1β, IL-17 and IL-10 was performed by a certified histologist who was blinded to the treatments. Three histologic sections were used for each animal, and positive immunoreactivity (IR) was defined as the presence of a brownish color in the cytoplasm of the cells and extracellular matrix. Because immunolabeling of both cells and extracellular matrix is important to this study, we performed semiquantitative analysis to determine the number of immunoreactive cells and immunolabeling intensity of the extracellular matrix. The scores were assigned as follows (15,17): 1, complete absence of immunoreactive cells; 2 (low IR), a few

immunoreactive cells and weak labeling of the extracellular matrix (approximately one quarter of the immunoreactive cells); 3 (moderate IR), a moderate number of immunoreactive cells and moderate labeling of the extracellular matrix (approximately one half of the immunoreactive cells); and 4 (high IR), a large number of immunoreactive cells and strong labeling of the extracellular matrix (approximately three quarters of the immunoreactive cells).

Statistical Analysis

Statistical analysis and data tabulation were performed using SigmaPlot software (Systat Software Inc., San Jose, CA, USA). Nonparametric data were analyzed by performing comparisons with the Mann-Whiteney Rank Sum test. Significance was set at 5%.

Results

To evaluate the effects of ω-3 PUFAs on pro- and anti-inflammatory cytokine expression relative to apical periodontitis, cytokine immunoreactivity (IR) was measured. Photomicrographs showing the IR pattern for TNF-α, IL-6, IL-1β, IL-17 and IL-10 are shown in Figure 1.

Sections of teeth stained to detect TNF-α showed complete absence of immunoreactive cells in groups without AP (C and C-O groups) (score 1). Comparing AP and AP-O groups, the AP group predominantly showed moderate IR (score 3), while the AP-O group predominantly showed low IR (score 2) ($P < .05$) (Figure 1 and Table 1).

For IL-1β and IL-6 IR, groups C and C-O showed complete absence of IR (score 1). The AP and AP-O groups were compared, and the AP group showed moderate to high IR (scores 3 and 4), while the AP-O group showed low to moderate IR (scores 2 and 3) ($P < .05$).

For IL-17 IR, groups C and C-O showed complete absence of IR (score 1). When the AP group was compared to the AP-O group, the AP group showed low to moderate IR (scores 2 and 3), while the AP-O group showed no to low IR (scores 1 and 2) ($P < .05$).

Moreover, IL-10 was not detected in groups C and C-O (score 1). Comparing AP and AP-O groups, the AP group showed no to low IR (score 1 and 2), while the AP-O group showed low IR (score 2) ($P < .05$) (Figure 1).

Table 1. Scores observed for TNF-∝, IL-1β, IL-17 and IL-10 immunoreaction according to different groups.

Cytokines	**Experimental groups**				
TNF-α	**C**	**C-O**	**AP**	**AP-O**	
1: absent	7/7	7/7	0/7	0/7	Mann-Whitney test C x C-O= p>0.05 C x AP x AP-O= p<0.05
2: low	0/7	0/7	1/7	6/7	
3: moderate	0/7	0/7	6/7	1/7	
4: hight	0/7	0/7	0/7	0/7	
Median	1^a	1^a	3^b	2^c	
IL-1β					
1: absent	7/7	7/7	0/7	0/7	Mann-Whitney test C x C-O= p>0.05 C x AP x AP-O= p<0.05
2: low	0/7	0/7	0/7	2/7	
3: moderate	0/7	0/7	3/7	5/7	
4: hight	0/7	0/7	4/7	0/7	
Median	1^a	1^a	4^b	3^c	
IL-6					
1: absent	7/7	7/7	0/7	0/7	Mann-Whiteney test C x C-O= p>0.05 C x AP x AP-O= p<0.05
2: low	0/7	0/7	0/7	3/7	
3: moderate	0/7	0/7	3/7	4/7	
4: hight	0/7	0/7	4/7	0/7	
Median	1^a	1^a	4^b	3^c	
IL-17					
1: absent	7/7	7/7	0/7	4/7	Mann-Whitney test C x C-O x AP-O= p>0.05 AP x AP-O= p<0.05
2: low	0/7	0/7	5/7	3/7	
3: moderate	0/7	0/7	2/7	0/7	
4: hight	0/7	0/7	0/7	0/7	
Median	1^a	1^a	2^b	1^a	
IL-10					
1: absent	7/7	7/7	5/7	0/7	Mann-Whitney test C x C-O x AP= p>0.05 AP x AP-O= p<0.05
2: low	0/7	0/7	2/7	7/7	
3: moderate	0/7	0/7	0/7	0/7	
4: hight	0/7	0/7	0/7	0/7	
Median	1^a	1^a	1^a	2^c	

Different letters in the raws mean that there is statistical significance difference, p<0.05

C, control untreated rats; C+O, control rats treated with ω-3 PUFAs; AP, rats with pulp exposure-induced apical periodontitis; AP-O, rats with pulp exposure-induced AP treated with ω-3 PUFAs

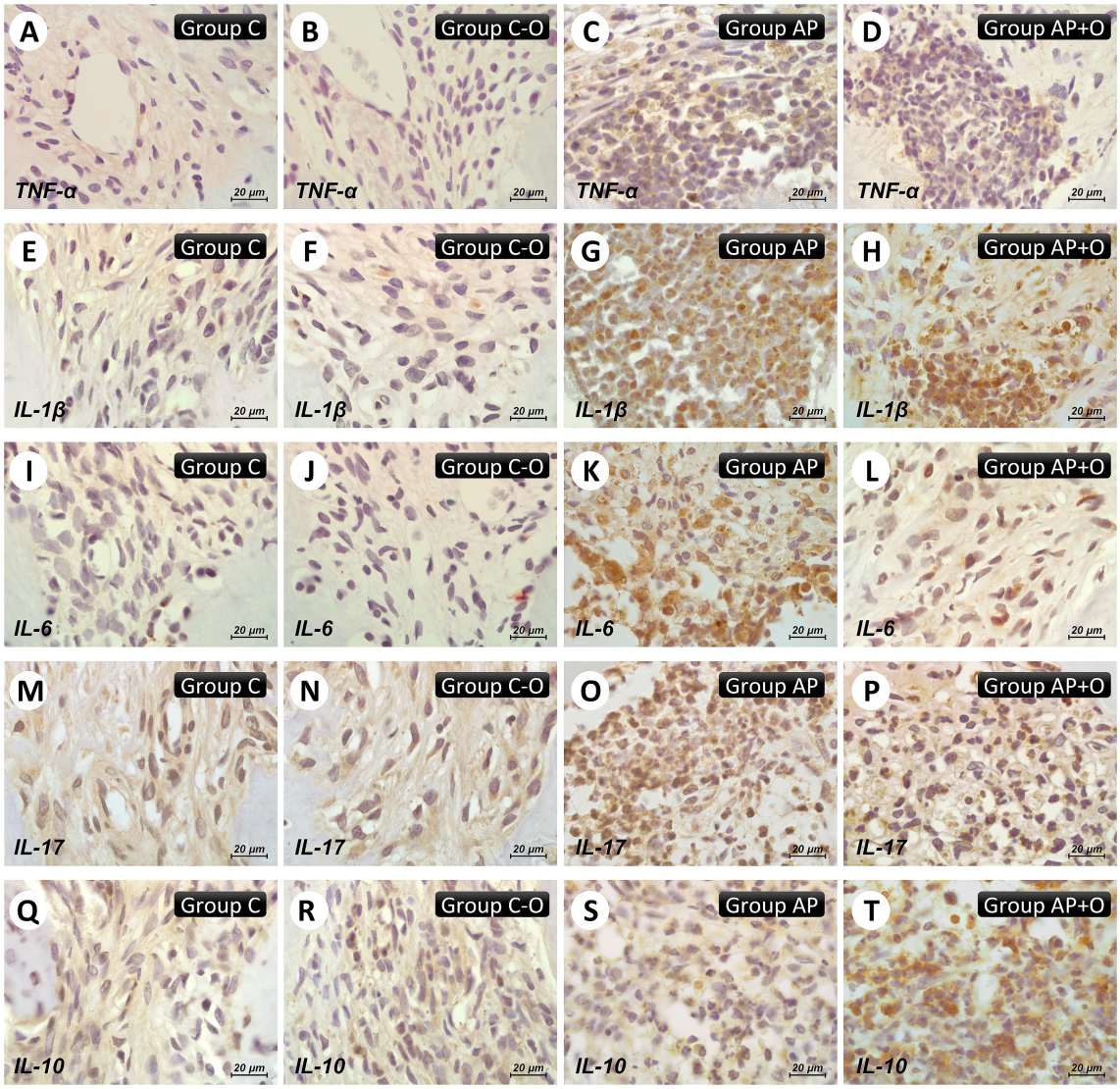

Figure 1. Immunoreaction of TNF-α, IL-1β, IL-6, IL-17 and IL-10 in the periapical bone at 30 days after pulp exposure groups. Representative images of TNF-α immunoreaction among the groups: C (A), C-O (B), AP (C), AP-O (D). Representative images of IL-1β immunoreaction among the groups: C (E), C-O (F), AP (G), AP-O (H). Representative images of IL-6 immunoreaction among the groups: C (I), C-O (J), AP (K), AP-O (L). Representative images of IL-17 immunoreaction among the groups: C (M), C-O (N), AP (O), AP-O (P). Representative images of IL-10 immunoreaction among the groups: C (Q), C-O (R), AP (S), AP-O (T). Original magnification x400.

Discussion

In the present study, systemic oral administration of ω-3 PUFAs decreased the expression of the proinflammatory cytokines TNF-α, IL-1β, IL-6 and IL-17, and it increased the expression of the anti-inflammatory cytokine IL-10 in the context of bone resorption lesion of AP.

This study complements the results of a previous study, which showed that systemic oral administration of ω-3 PUFAs suppresses bone resorption and promotes bone generation, thus decreasing OC-genesis, while increasing osteoblastogenesis (OB-genesis), to maintain normal bone remodeling homeostasis (12). The present data indicated that the expressions of proinflammatory and anti-inflammatory cytokines may be associated with OC-genesis and OB-genesis, respectively, in APs.

During AP formation and development, inflammatory cells, including T cells, B cells and macrophages, are recruited to periapical tissues in order to eliminate infection. This results in a classical “cytokine storm” (1,4) in which TNF-α plays a key role in increasing osteoclast (OC) sensitivity to nuclear factor kappa-B ligand (RANKL) (18), which, together with its receptor RANK, upregulates osteoclast formation, activation and survival in a variety of pathologic bone resorption conditions including AP (4). In addition, it is well established that TNF-α, IL-6 and IL-1β act as proinflammatory mediators during bone resorption in apical periodontitis (19). Other mediators, such as IL-1β, IL-6 and Cox2, are also regulated by NF-$\kappa\beta$ activation (20). Importantly, many studies reported that EPA and DHA can inhibit the production of TNF-α by preventing Nuclear Factor kappa B (NF-κB) activation (21,22). Therefore, it is plausible that the lower expression of TNF-α, IL-1β and IL-6 in AP of rats supplemented with ω-3 PUFAs occurred through NF-$\kappa\beta$ modulation by ω-3 PUFAs.

Another study used *Porphyromonas gingivalis* (*Pg*)-induced experimental periodontal disease in rats, and results showed that supplementation with ω-3 PUFAs decreased the expression of TNF-α, IL-1β and IL-6 in periodontal tissues (23). These results corroborate those of the present report. In addition, while several studies have reported on the expression of IL-17 in apical periodontitis (24,25), none has confirmed its role in the development of disease. IL-17 appears to exacerbate chronic inflammation, and it plays a role in cyst formation (24). We found that supplementation with ω-3 PUFAs decreased the expression of IL-17 in AP. Therefore, it is hypothesized that DHA suppresses the activity of IL-17-producing CD4(+) T cells, as reported previously (26). However, more studies should be performed to better elucidate the role of IL-17 in AP of rats supplemented with ω-3 PUFAs.

It is well established that IL-10 is an anti-inflammatory cytokine and that it can regulate B-cell proliferation and differentiation (27). Moreover, IL-10 directly affects OC-genesis as a suppressor of bone loss in periodontal disease (28) and apical periodontitis (29). In the present study, we found that the anti-inflammatory cytokine IL-10 could suppress bone loss in rat AP. Furthermore, IL-10 expression did not demonstrate significant variations among groups C, C-O and AP, which is consistent with previous studies that found no differences in IL-10 expression in the presence of AP in wild-type mice (28). However, our study found higher expression of IL-10 in the AP-O group compared to AP ($P < .05$). This result is contrary to that of previous studies which found no differences in IL-10 expression in individuals with periodontal disease, either with or without ω-3 PUFAs supplementation (23). This discrepancy could be attributed to differences in dietary composition or experimental time. Studies have further demonstrated that anti-inflammatory effects associated with ω-3 PUFAs are dose- and time-dependent (30).

The findings here in presented indicate that systemic oral administration with ω-3 PUFAs can modulate the inflammation in rat apical periodontitis, decreasing TNF-α, IL-6, IL-1β, IL-17 and increasing IL-10 expression. Future studies should explore the capacity of ω-3 PUFAs to generate endogenous metabolites involved in inflammation resolution, including resolvins, docosatrienes and neuroprotectins, as previously shown in studies involving other inflammatory diseases (31-33). Notwithstanding this, the present study supports further investigation of ω-3 PUFAs as a therapeutic regimen for apical periodontitis.

Acknowledgments

This study was supported by FAPESP (2013/26390-0).

The authors declare no conflicts of interest.

References

1. Liapatas S, Nakou M, Rontogianni D. Inflammatory infiltrate of chronic periradicular lesions: an immunohistochemical study. Int Endod J. 2003;36:464-71.

2. Gazivoda D, Dzopalic T, Bozic B, et al. Production of proinflammatory and immunoregulatory cytokines by inflammatory cells from periapicallesions in culture. J Oral Pathol Med 2009;38:605-11.

3. Siqueira JF Jr, Rocas IN. Bacterial pathogenesis and mediators in apical periodontitis. Braz Dent J 2007;18: 267-80.

4. Silva MJ, Kajiya M, AlShwaimi E, et al. Bacteria-reactive immune response may induce RANKL-expressing T cells in the mouse periapical bone loss lesion. J Endod 2012;38:346-50.

5. Araujo-Pires AC, Francisconi CF, Biguetti CC, et al. Simultaneous analysis of T helper subsets (Th1, Th2, Th9, Th17, Th22, Tfh, Tr1 and Tregs) markers expression inperiapical lesions reveals multiple cytokine clusters accountable for lesions activity and inactivity status. J Appl Oral Sci 2014;22:336-46.

6. Houri-Haddad Y, Soskolne WA, Halabi A, et al. IL-10 gene transfer attenuates P. gingivalis-induced inflammation. J Dent Res 2007;86:560-564.

7. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. Immunol Rev 2008;226:205-18.

8. Araghizadeh N, Paknejad M, Alaeddini M, et al. The efficacy and prophylactic characteristics of omega-3 fatty acids in experimental gingivitis in rats. Iran J Basic Med Sci 2014;17:87-92.

9. Kesavalu L, Vasudevan B, Raghu B, et al. Omega-3 fatty acid effect on alveolar bone loss in rats. J Dent Res 2006;85:648-52.

10. Vardar-Sengul S, Buduneli E, Turkoglu O et al. The effects of selective COX-2 inhibitor/celecoxib and omega-3 fatty acid on matrix metalloproteinases, TIMP-1, and laminin-5gamma2-chain immunolocalization in experimental periodontitis. J Periodontol 2008;79:1934-41.

11. Deore GD, Gurav AN, Patil R, et al. Omega 3 fatty acids as a host modulator in chronic periodontitis patients: a randomised, double-blind, palcebo-controlled, clinical trial. J Periodontal Implant Sci 2014; 44: 25-32.

12. Azuma MM, Gomes-Filho JE, Ervolino E, et al. Omega 3 Fatty Acids Reduce Bone Resorption While Promoting Bone Generation in Rat Apical Periodontitis. J Endod 2017;43:970-976.

13. El Khouli AM, El-Gendy EA. Efficacy of omega-3 in treatment of recurrent aphthous stomatitis and improvement of quality of life: arandomized, double-blind,

placebo-controlled study. Oral Surg Oral Med Oral Pathol Oral Radiol 2014;117:191-196.

14. Vardar-Sengül S, Buduneli N, Buduneli E, et al. Dietary supplementation of omega-3 fatty acid and circulating levels of interleukin-1beta, osteocalcin, and C-reactive protein in rats. J Periodontol 2006;77:814-20.

15. Cintra LT, Samuel RO, Azuma MM, et al. Multiple Apical Periodontitis Influences Serum Levels of Cytokines and Nitric Oxide. J Endod 2016;42:747-51.

16. Cintra LT, da Silva Facundo AC, Prieto AK, et al. Blood profile and histology in oral infections associated with diabetes. J Endod 2014;40:1139-44.

17. Garcia VG, Longo M, Gualberto Junior EC, et al. Effect of the concentration of phenothiazine photosensitizers in antimicrobial photodynamic therapy on bone loss and the immune inflammatory response of induced periodontitis in rats. J Periodontal Res 2013;49:584-94.

18. Weitzmann MN, Pacifici R. The role of T lymphocytes in bone metabolism. Immunol Rev 2005;208:154-68.

19. Martinho FC, Chiesa WM, Leite FR, et al. Correlation between clinical/radiographic features and inflammatory cytokine networks produced bymacrophages stimulated with endodontic content. J Endod 2012;38:740-5.

20. Mullen A, Loscher CE, Roche HM. Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages. J Nutr Biochem 2010;21:444-450.

21. Novak TE, Babcock TA, Jho DH, et al. NF-κB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF-α-transcription. Am J Physiol Lung Cell Mol Physiol 2003; 284:L84–L89

22. Vedin I, Cederholm T, Freund Levi Y, et al. Effects of docosahexaenoic acid–rich n − 3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study. Am J Clin Nutr 2008; 87:1616–1622.

23. Kesavalu L, Bakthavatchalu V, Rahman MM, Su J, Raghu B, Dawson D, Fernandes G, Ebersole JL. Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in Porphyromonas gingivalis-induced experimental periodontal disease. Oral Microbiol Immunol 2007;22:232-9.

24. Ajuz NC, Antunes H, Mendonça TA, Pires FR, Siqueira JF Jr, Armada L. Immunoexpression of interleukin 17 in apical periodontitis lesions. J Endod 2014;40:1400-3.

25. Yang S, Zhu L, Xiao L, et al. Imbalance of interleukin-17+ T-cell and Foxp3+ regulatory T-cell dynamics in rat periapical lesions. J Endod 2014;40:56-62.

26. Kong W, Yen JH, Ganea D. Docosahexaenoic acid prevents dendritic cell maturation, inhibits antigen-specific Th1/Th17 differentiation and suppresses experimental autoimmune encephalomyelitis. Brain Behav Immun 2011;25:872-82.

27. Volk H, Asadullah K, Gallagher G, et al. IL-10 and its homologs: important immune mediators and emerging immunotherapeutic targets. Trends Immunol 2001;22:414-7.

28. Sasaki H, Okamatsu Y, Kawai T, et al. The interleukin-10 knockout mouse is highly susceptible to Porphyromonas gingivalis-induced alveolar bone loss. J Periodontal Res 2004;39:432-441.

29. De Rossi A, Rocha LB, Rossi MA. Interferon-gamma, interleukin-10, Intercellular adhesion molecule-1, and chemokine receptor 5, but not interleukin-4, attenuate the development of periapical lesions. J Endod 2008;34:31-8.

30. Kremer JM, Lawrence DA, Jubiz W et al. Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis. Clinical and immunologic effects. Arthritis Rheum 1990;33:810-20.

31. Calder PC. The relationship between the fatty acid composition of immune cells and their function. Prostaglandins Leukot Essent Fatty Acids. 2008;79:101-108.

32. Chapkin RS, Kim W, Lupton JR, et al. Dietary docosahexaenoic and eicosapentaenoic acid: emerging mediators of inflammation. Prostaglandins Leukot Essent Fatty Acids 2009;81:187-191.

33. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual antiinflammatory and pro-resolution lipid mediators. Nat Rev Immunol 2008 8:349-361.

V. Artigo 3

Omega 3 fatty acids alter systemic inflammatory mediators resulting from apical periodontitis

§ Artigo formatado segundo às normas do periódico *Clinical Oral Investigations*

Abstract

Objectives This study investigated the effects of omega 3 polyunsaturated fatty acids (ω-3 PUFAs) on the systemic health of rats with pulp exposure-induced apical periodontitis (AP).

Materials and Methods Forty male Wistar rats were divided into groups: control untreated rats (C), control rats treated with ω-3 PUFAs alone (C-O), rats with pulp exposure-induced apical periodontitis (AP), and rats with pulp exposure-induced apical periodontitis treated with ω-3 PUFAs (AP-O). The ω-3 PUFAs were administered orally once a day for 15 days before pulp exposure and subsequently 30 days after pulp exposure. Rats were killed 30 days after pulp exposure, and blood and jaw samples were collected. A blood profile was conducted to determine the total number of leukocytes, comprising the neutrophils, monocytes, and lymphocytes. Proinflammatory cytokines tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), and Interleukin-17 (IL-17) were quantified with enzyme-linked immunosorbent assays (ELISA). In addition, histological analysis was performed to confirm the development of AP. The data were statistically analyzed ($p < 0.05$).

Results AP was larger in the AP group compared with the AP-O group ($p < 0.05$). Furthermore, the AP and AP-O groups showed higher numbers of lymphocytes, leukocytes, monocytes, and eosinophils and greater expressions of TNFα and IL-6 than the C and C-O groups did ($p < 0.05$). The presence of leukocytes and lymphocytes as well as the expression of IL-6 decreased in the AP-O group compared with the AP group ($p < 0.05$).

Conclusions Omega 3 polyunsaturated fatty acid (ω-3 PUFA) supplementation decrease the bone resorption area of AP, as well as reduces the systemic

effects of AP, thus decreasing the number of leukocytes, lymphocytes, and IL-6 in rat's blood.

Clinical Relevance Omega 3 polyunsturated fatty acids reduce the number of systemic proinflammatory cells and mediators resulting from apical periodontitis.

Key-words: Apical periodontitis. Endodontic medicine. Endodontic infection. Omega 3 fatty acids

Introduction

Many researchers and clinicians have studied the possible bidirectional relationship between endodontic infection and systemic diseases [1-4]. Previous studies have reported that apical periodontitis (AP), which is a consequence of endodontic infection, may potentiate the effects of periodontal disease in the blood, including changes in the lipid profile [5], and an increase in the number of inflammatory cells [6] and mediators [7]. In addition, the presence of four AP foci is able to increase the number of proinflammatory mediators in the blood systemically, which may alter the blood's homeostasis [8]. Moreover, many observational studies in humans relate the presence of AP to systemic diseases, such as cardiovascular disease [9], diabetes [10], chronic liver disease [11], and inherited coagulation disorders [12].

Considering that AP occurred in the Brazilian population with an average of 2.7 lesions per subject [13], it is suitable to study the impact of multiple foci of AP in systemic health. The main concern about an increase in inflammatory cells and mediators in the blood is that it may potentiate local inflammatory processes, such as those that occurr in AP [14]. In addition, higher levels of inflammatory mediators can accelerate the pathogenesis of autoimune diseases [15].

Currently, conventional endodontic treatment, comprising the mechanical and chemical desinfection of infected dentin from root canals, has been the standard procedure for treating AP. However, it would be interesting to find complementary alternative therapies that could minimize the local and

systemic effects related to AP. One of the goals is to reduce the number of inflammatory cells, including lymphocytes, monocytes, neutrophils, and eosinophils, as well as proinflammatory mediators, such as tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6), and Interleukin-17 (IL-17), which those cells produce, and which are related to AP pathogenesis [8,16,17].

To date, ω-3 PUFAs have been demonstrating positive effects as an adjuvant therapy in the treatment of oral diseases, including periodontitis [18,19], gingivitis [20], recurrent aphthous stomatitis [21], and apical periodontitis [22]. The positive effects related to ω-3 PUFAs may be because diets with ω-3 PUFAs inhibit the synthesis of the lipid mediators of inflammation, alter the cellular functions of polymorphonuclear leukocytes, and modulate lymphocyte proliferation and cytokine production [23-25]. Moreover, a previous study showed that supplementation with ω-3 PUFAs decreased osteoclastognesis and increased osteoblastogenesis, thus decreasing bone resorption and promoting bone regeneration in the AP scenarios of rats with endodontic infection [22]. Thus, our hypothesis was that if ω-3 PUFAs can modulate AP locally, it is possible that diets with ω-3 PUFAs may also influence the systemic changes related to AP.

Therefore, the aim of this study was to evaluate the effects of the dietary supplement of ω-3 PUFAs on inflammatory cells and proinflammatory mediators in the blood of rats with AP.

Materials and methods

Experimental animals

The Institutional Ethics Committee (CEUA 2014-00550) of UNESP-Universidade Estadual Paulista, Sao Paulo, Brazil, approved the experimental protocol, which was conducted in accordance with relevant guidelines. Forty male Wistar rats (*Rattus norvegicus albinus*), weighing 200-250 g each, were used in this study. The rats were housed in mini-isolators (Alesco, São Paulo, SP, Brazil) in temperature-controlled rooms and given *ad libitum* access to water and food.

The rats were randomly assigned to four groups (10 rats/group):

1. Control: untreated rats (C)
2. Rats treated with ω-3 PUFAs (C-O)
3. Rats with pulp exposure-induced AP (AP)
4. Rats with pulp exposure-induced AP treated with ω-3 PUFAs (AP-O)

Supplementation with ω-3 PUFAs

Rats in the C-O and AP-O groups were orally gavaged with ω-3 PUFAs (Omega 3 Catarinense- Laboratório Catarinense S.A, Joinville, SC, Brazil) (water solution, 40 mg/kg; 60% EPA and 40% DHA, once a day), whereas rats in the C and AP groups received control distilled water during 15 days before pulp exposure (prophylactic administration) and 30 days after AP induction (therapeutic administration). As a consequence, rats were treated either ω-3 PUFAs or control water for a total period of 45 days [18,22].

Induction of AP

Rats were anesthetized with ketamine (87 mg/kg; Francotar; Virbac do Brazil Ind. e Com. Ltda, Roseira, SP, Brazil) and xylazine (13 mg/kg; Rompun; Bayer S.A, Sao Paulo, SP, Brazil). The pulps of the right upper first and second molars as well as the right lower first and second molars were exposed by using a dental round bur (Broca Ln Long Neck; Maillefer, Dentisply Ind e Com Ltda, Petrópolis, RJ, Brazil) (groups AP and AP-O) for the development of AP. The coronal pulp tissue was exposed to the oral cavity for 30 days [26-28].

Blood sample collection and determination of hematologic parameters

At 30 days, the rats were anesthetized, and a cardiac puncture was performed to collect 5 mL of blood from each rat [26]. The samples were placed in EDTA, homogenized, and immediately transferred to a technician, who was blinded to the case status, for processing. The following parameters were analyzed using an automatic analyzer (ABX Micros ABC Vet; Horiba ABX Diagnostics, Montpellier, France): neutrophils, leukocytes, lymphocytes, and monocytes.

Assessment of serum levels of TNF-α, IL-6, and IL-17

Serum cytokine levels were assessed [7,8] by performing capture enzyme-linked immunosorbent assays (ELISAs) using commercial kits (rat TNF ELISA set BD OptEIA, cat #558535; BD Biosciences, San Diego, CA; rat IL-6 ELISA set BD OptEIA, cat #550319; BD Biosciences; rat IL-17A ELISA MAX Deluxe, cat #437904; Biolegend, San Diego, CA), according to the manufacturers' instructions.

Histopathological analysis

Mandible samples from the killed rats were removed, postfixed in neutral buffered formalin for 48 hours, and then decalcified in buffered (pH=8) 17% EDTA (Sigma Chemical Co, St Louis, MO). Subsequently, they were rinsed again with sterile water before being dehydrated with ethanol, cleared in xylene, and finally embedded in paraffin. Serial slices (6μm) were prepared in the mesiodistal plane and stained with hematoxylin-eosin. The slices were examined sequentially under an optical microscope (DM 4000 B; Leica, Wetzlar, Germany).

Inflammatory infiltrate was evaluated for its intensity and extension. The average number of cells per field was noted as well as whether the inflammation extended beyond the apical foramen. For each experimental group, the number of cells was calculated as the average of 10 separate areas (x400 magnification) [6,22].

The intensity of the inflammatory infiltrate was graded as follows: absent (zero to few inflammatory cells: score 1); mild (< 25 inflammatory cells: score 2), moderate (25-125 inflammatory cells: score 3), or severe (>125 inflammatory cells: score 4). A 0.5-mm square area of the periapical portion of the distal root of the first right lower molar was examined, and the cell count per unit area was calculated. For the AP and AP+O groups, the area of the periapical lesion was histometrically measured. For each rat, seven serial histologic sections were measured histometrically using an image processing system, which consisted of a light microscope (DM 4000 B Leica), a color camera (DFC 500, Leica), a color image processor (Leica Qwin V3 software,

Leica), and a personal computer (Intel Corel I7,, Windows 10). The apical periodontitis area in μm2 was determined for each side [6, 8, 22].

Statistical analysis

Statistical analysis was performed by using SigmaPlot software (Systat Software Inc, San Jose, CA). Nonpametric data were analyzed by performing multiple comparisons with the Kruskal-Wallis test followed by the Dunn test. Parametric data were analyzed by performing an analysis of variance followed by the Tukey test. The level of significance was set at 5%.

Results

Blood profile

To evaluate the effects of ω-3 PUFAs on inflammatory cells in the blood of rats with four AP foci, the number of leukocytes, represented by lymphocytes, neutrophils, monocytes, and eosinophils were counted, and the results are shown in Table 1.

The total number of leukocytes and lymphocytes were significantly higher in the AP and AP-O groups when compared with the C group ($p < 0.05$). However, the number of leukocytes and lymphocytes decreased in the AP-O group compared with the AP group ($p < 0.05$). The total number of monocytes and eosinophils were higher in the AP and AP-O groups compared with the C and C-O groups ($p < 0.05$). Meanwhile, the total number of monocytes and eosinophils were higher in the AP and AP-O groups than in the C group ($p < 0.05$). No statistically significant difference was found in the total number of neutrophils among the groups ($p > 0.05$).

Table 1 - Mean and Standard Deviation of Blood Cell Parameters.

Hematologic Parameters (x10^3 cells/μl)	**Groups (Mean** ± SD)*			
	C	**C-O**	**AP**	**AP-O**
Leukocytes	5.63 ± 1.31^a	7,01 ± 1.43^{ab}	8.84 ± 1.45^c	7.32 ± 1.37^b
Lymphocytes	3.83 ± 1.31^a	5.12 ± 1,32^b	7.13± 1,05^c	5.70 ± 1,33^{bd}
Neutrophils	1.55 ± 0,42^a	1.28 ± 0,48^a	1.67 ± 0,39^a	1.58 ± 0,25^a
Eosinophils	0.084 ± 0,030^a	0.17 ± 0,08^b	0.18 ± 0.03^b	0.18 ±0.10^b
Monocytes	0.083 ± 0.03^a	0.14 ± 0.09^{ab}	0.17 ± 0.03^b	0.14 ± 0.04^b

*Different letters indicate significant statistical differences in rows ($p < 0.05$).

Serum levels of TNF-α, IL-6, and IL-17

To evaluate the effects of ω-3 PUFAs on pro-inflammatory cytokines in the blood of rats with four AP foci, ELISA was performed to detect TNF-α, IL-6, and IL-17 in rat blood, and the results are shown in Table 2.

Serum levels of TNF-α were higher in the AP and AP-O groups compared with the control ($p < 0.05$). However, the dietary supplement with ω-3 PUFAs did not decrease the levels of TNF-α among the groups with AP ($p > 0.05$). Serum levels of IL-6 were higher in the AP group compared with the C, C-O, and AP-O groups ($p < 0.05$). No statistical difference was found in the IL-17 levels among the groups ($p > 0.05$).

Table 2 - Mean and Standard Deviation of TNF-α, IL-6 and IL-17 levels in rat's blood

Cytokines (pg/mL)	Groups (Mean ± SD)*			
	C	C-O	AP	AP-O
TNF-α	22.773 ± 8.72[a]	34.980 ± 10.55[ac]	53.904 ± 38.24[b]	50.902 ± 18.51[bc]
IL-6	3343.319 ± 1001.31[a]	4640.422 ± 2272.46[a]	6771.952 ± 2882.27[b]	4991.736± 1850.15[a]
IL-17	130.07 ± 123.82[a]	102.83 ± 118.88[a]	120.47 ± 116.75[a]	126.69 ± 98.54[a]

*Different letters indicate significant statistical differences in rows ($p < 0.05$).

Histologic findings

No inflammation was noted in the periapical regions of the C and C-O groups (Figures 1A, 1E, 1B, 1F). However, in the AP and AP-O groups, the pulp showed signs of necrosis 30 days after pulp exposure. AP was established and restricted exclusively to the periapical regions of the AP and AP-O groups (Figures 1C, 1G, 1D, 1H). Histolopathological analysis showed that periapical inflammatory infiltrate was more intense and larger in the AP group compared with the C, C-O, and AP-O groups ($p < 0.05$) (Table 3). In addition, the area between the alveolar bone and root apex was larger in the AP and AP-O groups compared with the C and C-O groups ($p < 0.05$). Nevertheless, AP was more evident in the AP group compared with the AP-O group ($p < 0.05$) (Table 3).

Table 3 - Scores and median of the histological findings.

Intensity of inflammatory Infiltration	Groups			
	C	C-O	AP	AP-O
1 - Absent	10/10	10/10	0/10	0/10
2 - Mild	0/10	0/10	0/10	4/10
3 - Moderate	0/10	0/10	4/10	6/10
4 – Severe	0/10	0/10	6/10	0/10
Median*	1^{a}	1^{a}	4^{b}	3^{c}
AP ($x10^4$ μm^2 ± standard deviation*)	$12.8±0.95^{a}$	$12.68±1.30^{a}$	$128.24±12.05^{b}$	$46.06±21.43^{c}$

*Different letters indicate significant statistical differences in rows ($p < 0.05$).

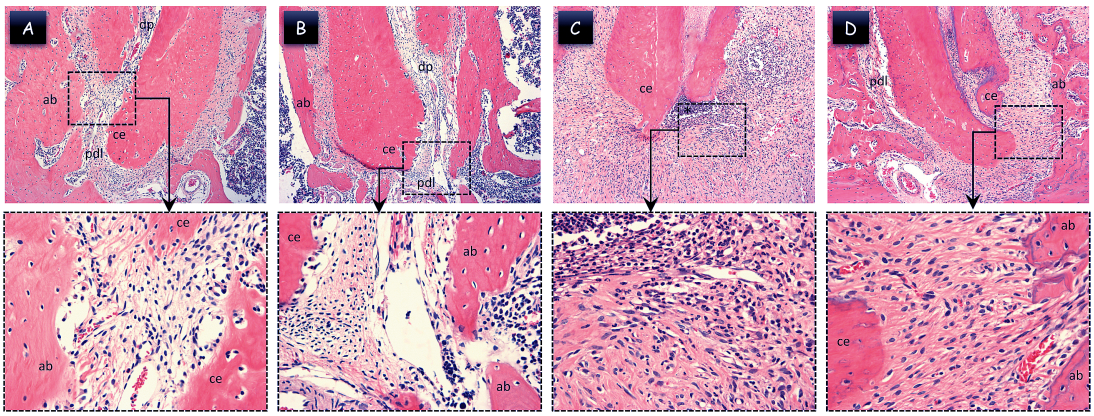

Figure 1. Histologic images of apical periodontitis lesions at 30 days after pulp exposure (H&E staining). (A) Group C. Periapical region is free of inflammatory infiltrate (original magnification, x100 and x400, respectively). (B) Group C-O. Periapical region is free of inflammatory infiltrate (original magnification, x100 and x400, respectively). (C) Group AP. Intense inflammatory infiltration can be observed in the periapical area (original magnification, x100 and x400, respectively). (D) Group AP-O. Decreased inflammatory infiltration can be observed in the periapical area compared to AP group (original magnification, x100 and x400, respectively). dp, dental pulp; pld, periodontal ligament; ce, cementum; ab, alveolar bone. *Site of inflammatory cells.

Discussion

In the present study, four AP foci increased inflammatory cells and proinflammatory cytokines in the blood. However, the systemic administration of ω-3 PUFAs ameliorated, in parts, the systemic alterations. The pathogenic bone resorption induced in the AP group via pulp exposure was significantly reduced when ω-3 PUFAs were administered systemically. These findings are in accordance with a previous study that demonstrated that ω-3 PUFAs can decrease the bone resorption in rats with AP, thus decreasing osteoclastogenesis and increasing osteoblastogenesis [22]. Thus, we can observe that the systemic effects resulting from AP may be proportional with the severity and intensity of pathological bone resorption.

A total blood analysis (hemogram) was used to evaluate the effects of ω-3 PUFAs on the inflammatory cells of rats with four AP foci. Hemogram is frequently used to assess the presence of infection or inflammation and to determine whether periodontal infections alter the hematologic parameters, such as the differential counts of white blood cells, red blood cells, and/or platelets [29]. We found that the four AP foci increased the number of leukocytes, as lymphocytes, monocytes, and eosinophils. These findings corroborate the idea that the systemic effects of AP may be positively correlated with the amount of inflammation, as a previous study showed that the presence of one AP focus did not alter inflammatory cells in blood but only when it was associated with periodontal disease [6]. In addition, ω-3 PUFA systemic administration decreased the total number of lymphocytes in rats with AP. This possibly happened because the AP bone resorption area was lower in rats treated with ω-3 PUFAs, which decreased the number of systemic inflammatory cells. Another hyphotheis is that ω-3 PUFAs exerted direct effects on the inflammatory cells in blood, as evidence exists that ω-3 PUFAs can alter lymphocyte proliferation by altering the plasma membrane topography, thus perturbing the downstream signal required for T cell proliferation [30]. On the other hand, ω-3 PUFA systemic administration did not alter the number of eosinophils and monocytes in rat's blood. Moreover, the C-O group showed, unexpectedly, higher amounts of lymphocytes, monocytes, and eosinophils when compared with the C group. Further studies should be performed to

analyze the effects of ω-3 PUFAs on eosinophils and monocytes during inflammation, as well as their effects on inflammatory cells in healthy individuals. Differences in the neutrophil number among the groups were not found. This may be because after 30 days, AP showed signs of chronic inflammation in rats, represented by the predominance of mononuclear cells and lower levels of neutrophils, as observed in this study and others [6,22], as neutrophils are the first line of defense.

Regarding the systemic alterations resulting from AP, changes in the expression of proinflammatory mediators is one of the major consequences that should be highlighted. Although proinflammatory mediators are produced to protect host organisms from pathogenic agents, an exacerbated inflammatory response may cause injuries to the healthy tissues [31]. For example, an increase in proinflammatory mediators in response to periodontal diseases enhances the pathogenesis of autoimmune diseases, such as diabetes, lupus, and rheumatoid arthritis [32]. This study showed that the presence of four AP foci increased the levels of proinflammatory cytokines TNFα and IL-6 in rat blood, which corroborates previous studies [8,33]. Therefore, the supplementation with ω-3 PUFAs decreased the levels of IL-6 in rat's blood. In agreement with our previous findings regarding inflammatory cells, it seems that systemic alterations reflected, proportionally, the local inflammatory condition related to AP.

Although it was reported that ω-3 PUFAs can inhibit the production of TNF-α by preventing nuclear factor kappa B (NF-κB) activation [34,35], TNF-α levels did not decrease in the blood of the AP-O group compared with the AP group. It seems that other cells as monocytes and eosinophils may continously produce TNF-α, considering that the supplementation with ω-3 PUFAs did not alter these cells. No differences were found in IL-17 among the groups, which corroborates a previous study that showed that AP did not increase IL-17 in rat blood but only when associated with periodontal disease [7]. Considering the results, ω-3 PUFAs showed positive results to control inflammation. However, further studies should be performed to better elucidate the effects of ω-3 PUFAs on the proinflammatory mediators produced systemically in response to AP.

In summary, our results indicated that systemic supplementation with ω-3 PUFAs can ameliorate the systemic alterations stemming from AP, decreasing the number of total leukocytes and lymphocytes as well as the expression of proinflammatory cytokine IL-6. This study lays the groundwork for further investigations to better understand the role of ω-3 PUFAs in reestablishing the body's homeostasis in indivuals with AP.

Acknowledgments

This study was supported by the São Paulo Research Foundation, FAPESP (2014/0050).

Compliance with Ethical Standards

Conflict of Interest

The authors have no conflict of interest to declare.

Ethical approval

This article does not contain any studies with human participants performed by any authors. The animal study was approved by the Institutional Ethics Committee (CEUA 2014-00550) of Universidade Estadual Paulista, São Paulo, Brazil, and conducted in accordance with ethical standards.

Informed consent

For this type of study, formal consent is not required.

References

1. Lima SM, Grisi DC, Kogawa EM, et al. Diabetes mellitus and inflammatory pulpal and periapical disease: a review. Int Endod J. 2013;46:700-9.

2. Segura-Egea JJ, Martín-González J, Castellanos-Cosano L. Endodontic medicine: connections between apical periodontitis and systemic diseases. Int Endod J 2015;48:933-51.

3. Sasaki H, Hirai K, Martins CM, et al. Interrelationship Between Periapical Lesion and Systemic Metabolic Disorders. Curr Pharm Des 2016;22:2204-15.

4. Tibúrcio-Machado CD, Bello MC, Maier J, et al. Influence of Diabetes in the Development of Apical Periodontitis: A Critical Literature Review of Human Studies. J Endod 2017;43:370-6.

5. Cintra LT, da Silva Facundo AC, Azuma MM, et al. Pulpal and periodontal diseases increase triglyceride levels in diabetic rats. Clin Oral Investig 2013;17:1595-9.

6. Cintra LT, da Silva Facundo AC, Prieto AK, et al. Blood profile and histology in oral infections associated with diabetes. J Endod 2014;40: 1139-44.

7. Cintra LT, Samuel RO, Azuma MM, et al. Apical periodontitis and periodontal disease increase serum IL-17 levels in normoglycemic and diabetic rats. Clin Oral Investig 2014;18: 2123-8.

8. Cintra LT, Samuel RO, Azuma MM, et al. Multiple Apical Periodontitis Influences Serum Levels of Cytokines and Nitric Oxide. J Endod 2016;42:747-51.

9. Liljestrand JM, Mäntylä P, Paju S, et al. Association of Endodontic Lesions with Coronary Artery Disease. J Dent Res 2016;95:1358-65.

10. Sánchez-Domínguez B, López-López J, Jané-Salas E, et al. Glycated hemoglobin levels and prevalence of apical periodontitis in type 2 diabetic patients. J Endod 2015;41:601-6.

11. Castellanos-Cosano L, Machuca-Portillo G, Sanchez-Domınguez B et al. High prevalence of apical periodontitis amongst patients with inherited coagulation disorders.Haemophilia 2013;19:110–5.

12. Castellanos-Cosano L, Machuca-Portillo G, Sanchez-Dominguez B et al. Prevalence of apical periodontitis and frequency of root canal treatment in liver transplant candidates. Medicina Oral, Patologıa Oral y Cirugıa Bucal 2013;18:e773–9.

13. Marotta PS, Fontes TV, Armada L, et al. Type 2 diabetes mellitus and the prevalence of apical periodontitis and endodontic treatment in an adult Brazilian population. J Endod 2012; 38:297-00.

14. Marçal JR, Samuel RO, Fernandes D, et al. T-helper cell type 17/regulatory T-cell immunoregulatory balance in human radicular cysts and periapical granulomas. J Endod 2010; 36:995-9.

15. Liu Y, H Yin, Zhao M, et al. TLR2 and TLR4 in Autoimmune Diseases: a Comprehensive Review. Clin Rev Allergy 2014; 47: 136-47.

16. Araujo-Pires AC, Francisconi CF, Biguetti CC, et al. Simultaneous analysis of T helper subsets (Th1, Th2, Th9, Th17, Th22, Tfh, Tr1 and Tregs) markers expression in periapical lesions reveals multiple cytokine clusters accountable for lesions activity and inactivity status. J Appl Oral Sci 2014;22:336-46.

17. Azuma MM, Samuel RO, Gomes-Filho JE, et al. The role of IL-6 on apical periodontitis: a systematic review. Int Endod J 2014;47:615-21.

18. Vardar S, Buduneli E, Türkoğlu O, et al. Therapeutic versus prophylactic plus therapeutic administration of omega3 fatty acid on endotoxin-inducedperiodontitis in rats. J Periodontol 2004;75:1640-6.

19. Deore GD, Gurav AN, Patil R, et al. Omega 3 fatty acids as a host modulator in chronic periodontitis patients: a randomised, double-blind, palcebo-controlled, clinical trial. J Periodontal Implant Sci 2014;44:25-32.

20. Araghizadeh N, Paknejad M, Alaeddini M, et al. The efficacy and prophylactic characteristics of omega-3 fatty acids in experimental gingivitis in rats. Iran J Basic Med Sci 2014;17:87-92.

21. El Khouli AM, El-Gendy EA. Efficacy of omega-3 in treatment of recurrent aphthous stomatitis and improvement of quality of life: arandomized, double-blind, placebo-controlled study. Oral Surg Oral Med Oral Pathol Oral Radiol 2014;117:191-6.

22. Azuma MM, Gomes-Filho JE, Ervolino E, et al. Omega 3 Fatty Acids Reduce Bone Resorption While Promoting Bone Generation in Rat Apical Periodontitis. J Endod 2017; S0099-2399: 30011-0.

23. Alam SQ, Bergens BM, Alam BS. Arachidonic acid, prostaglandin E2 and leukotriene C4 levels in gingival and submandibular salivary glands of rats fed diets containing n-3 fatty acids. Lipids 1991;26:895-00.

24. Blok WL, Vogels MT, Curfs JH, et al. Dietary fish-oil supplementation in experimental Gram-negative infection and in cerebral malaria in mice. J Infect Dis 1992; 165:898-903.

25. Fernandes G, Venkataraman J. Role of omega-3 fatty acids in health and disease. Nutr Res 1993;13:19-45.

26. Cintra LT, Samuel RO, Facundo AC, Prieto AK, Sumida DH, Bomfim SR, Souza JC, Dezan-Júnior E, Gomes-Filho JE. Relationships between oral infections and blood glucose concentrations or HbA1c levels in normal and diabetic rats. Int Endod J. 2014;47:228-37

27. Cintra LT, Samuel RO, Prieto AK, Sumida DH, Dezan-Júnior E, Gomes-Filho JE. Oral health, diabetes, and body weight. Arch Oral Biol. 2017;73:94-99.

28. Gomes-Filho JE, Wayama MT, Dornelles RC, Ervolino E, Coclete GA, Duarte PC, Yamanri GH, Lodi CS, Dezan-Júnior E, Cintra LT. Effect of raloxifene on periapical lesions in ovariectomized rats. J Endod. 2015;41:671-5.

29. Lopez R, Loos BG, Baelum V. Hematological features in adolescents with periodontitis. Clin Oral Invest 2012;16:1209–16.

30. Hou TY, Barhoumi R, Fan YY, et al. n-3 polyunsaturated fatty acids suppress CD4(+) T cell proliferation by altering phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2] organization. Biochim Biophys Acta. 2016;1858:85-96.

31. Bian Z, Guo Y, Ha B, et al. Regulation of the inflammatory response: enhancing neutrophil infiltration under chronic inflammatory conditions. J Immunol 2012;188:844–53

32. Jeffcoat MK, Jeffcoat RL, Gladowski PA, et al. Impact of periodontal therapy on general health: evidence from insurance data for five systemic conditions. Am J Prev Med 2014;47:166–74.

33. Astolphi RD, Curbete MM, Colombo NH. Periapical lesions decrease insulin signal and cause insulin resistance. J Endod 2013;39:648-52.

34. Novak TE, Babcock TA, Jho DH, et al. NF-kB inhibition by ω-3 fatty acids modulates LPS-stimulated macrophage TNF- α-transcription. AJP-Lung 2003;284:84-89.

35. Vedin I, Cederholm T, Freund Levi Y et al. Effects of docosahexaenoic acid-rich n-3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegaAD study. AM J Clini Nutr 2008;87:1616-22.

VI. Conclusão

Conclusões

1) A suplementação alimentar com os ácidos graxos ômega-3 promove a diminuição do infiltrado inflamatório e número de osteoclastos, bem como o aumento do número de osteoblastos, com consequente diminuição de perda óssea dos tecidos periapicais, de ratos com infecção endodôntica.

2) A suplementação alimentar com os ácidos graxos ômega-3 promove a diminuição da expressão das citocinas pró-inflamatórias fator de necrose tumoral alfa, interleucina-6, interleucina-1 beta, interleucina-17 e o aumento da citocina anti-inflamatória interleucina-10, dos tecidos periapicais de ratos com infecção endodôntica.

3) A presença de quatro focos de periodontite apical, em ratos, é capaz de aumentar os níveis séricos das células leucócitos, linfócitos, monócitos e eosinófilos e dos mediadores pró-inflamatórios fator de necrose tumoral alfa e interleucina-6.

4) A suplementação alimentar com os ácidos graxos ômega-3 promove a dimuição das células inflamatórias linfócitos e leucócitos, bem como da citocina pró-inflamatória interleucina-6 no sangue de ratos com infecção endodôntica.

5) A suplementação alimentar com os ácidos graxos ômega-3 promove o aumento sérico das células inflamatórias linfócitos, eosinófilos e monócitos, bem como da citotina pró-finalamtória fator de necrose tumoral alfa, em ratos ratos sem infecção endodôntica.

VII- REFERÊNCIAS

Referências

1. Al-Zahrani MS1, Abozor BM, Zawawi KH. The relationship between periapical lesions and the serum levels of glycosylated hemoglobin and C-reactive protein in type 2 diabetic patients. Saudi Med J. 2017 Jan;38(1):36-40.

2. Araghizadeh N, Paknejad M, Alaeddini M, Minaii B, Abdollahi M, Khorasanie R. The efficacy and prophylactic characteristics of omega-3 fatty acids in experimental gingivitis in rats. Iran J Basic Med Sci. 2014; 17:87–92.

3. American Association of Endodontists; 2012. Focal Infection Theory. Available from www.aae.org/uploadedfiles/publications_and_research/guidelines_and_position_statements/focalinfection.pdf [Cited: 29th June, 2015]

4. Ariza-Ariza R, Mestanza-Peralta M, Cardiel MH. Omega- 3 fatty acids in rheumatoid arthritis: An overview. Semin Arthritis Rheum 1998; 27:366-370.

5. Astolphi RD, Curbete MM, Colombo NH, Shirakashi DJ, Chiba FY, Prieto AK, Cintra LT, Bomfim SR,Ervolino E, Sumida DH. Periapical lesions decrease insulin signal and cause insulin resistance. J Endod. 2013;39(5):648-52.

6. Astolphi RD, Curbete MM, Chiba FY, Cintra LT, Ervolino E, da Mota MS, Antoniali C, Garbin CA, Sumida DH. Periapical Lesions Decrease Insulin Signaling in Rat Skeletal Muscle. J Endod. 2015;41:1305-10.

7. Bain JL, Lester SR, Henry WD, Pongetti JL, Blackman ME, Johnson RB. Association between maternal periapical lesions and brain inflammation in rat pups. Arch Oral Biol. 2013;58:266-71.

8. Bendyk A1, Marino V, Zilm PS, Howe P, Bartold PM. Effect of dietary omega-3 polyunsaturated fatty acids on experimental periodontitis in the mouse. J Periodontal Res. 2009;44:211-6.

9. Berlin-Broner Y, Febbraio M, Levin L. Association between apical periodontitis and cardiovascular diseases: a systematic review of the literature. Int Endod J. 2016 Oct 21.

10. Billings F. Focal infections: its broader application in the etiology of disease. JAMA 1914; 63:899-903.

11. Bittiner SB, Cartwright I, Tucker WFG, Blehen SS. A double-blind, randomized, placebo-controlled trial of fish in psoriasis. Lancet 1988;3:378-380.

12. Blok WL, Vogels MT, Curfs JH, Eling WM, Buurman WA, van der Meer JW. Dietary fish-oil supplementation in experimental Gram-negative infection and in cerebral malaria in mice. J Infect Dis. 1992; 165:898-903.

13. Calder PC. Polyunsaturated fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids 2006;75:197–202

14. Cintra LT, da Silva Facundo AC, Azuma MM, Sumida DH, Astolphi RD, Bomfim SR, Narciso LG, Gomes-Filho JE. Pulpal and periodontal diseases increase triglyceride levels in diabetic rats. Clin Oral Investig. 2013;17:1595-9. (a)

15. Cintra LT, Facundo ACS, Valentim D, Prieto AKC, Silva CO, Sumida DH, Bomfim SRM, Dezan-Junior E, Gomes-Filho JE. Effect of Oral Infections on Serum Creatinine Levels in Diabetic Rats. Int J Diabet Vasc Dis Res. 2013; 1:1-6.2 (b).

16. Cintra LT, da Silva Facundo AC, Prieto AK, Sumida DH, Narciso LG, Mogami Bomfim SR, Oliveira e Silva C, Dezan-Júnior E, Gomes-Filho JE. Blood profile and histology in oral infections associated with diabetes. J Endod. 2014;40:1139-44.

17. Cintra LT, Samuel RO, Azuma MM, Ribeiro CP, Narciso LG, de Lima VM, Sumida DH, Coclete GA, Dezan-Júnior E, Gomes-Filho JE. Apical periodontitis and periodontal disease increase serum IL-17 levels in normoglycemic and diabetic rats. Clin Oral Investig. 2014 Dec;18(9):2123-8. (a)

18. Cintra LT, Samuel RO, Azuma MM, de Queiróz AO, Ervolino E, Sumida DH, de Lima VM, Gomes-Filho JE. Multiple Apical Periodontitis Influences Serum Levels of Cytokines and Nitric Oxide. J Endod. 2016;42:747-51.

19. Cintra LT, Samuel RO, Facundo AC, Prieto AK, Sumida DH, Bomfim SR, Souza JC, Dezan-Júnior E,Gomes-Filho JE. Relationships between oral infections and blood glucose concentrations or HbA1c levels in normal and diabetic rats. Int Endod J. 2014;47:228-37. (b)

20. Colić M, Gazivoda D, Vucević D, Vasilijić S, Rudolf R, Lukić A. Proinflammatory and immunoregulatory mechanisms in periapical lesion. Mol Immunol. 2009; 47:101-13.

21. Costa TH, de Figueiredo Neto JA, de Oliveira AE, Lopes e Maia Mde F, de Almeida AL. Association between chronic apical periodontitis and coronary artery disease. J Endod. 2014;40:164-7.

22. Deore GD, Gurav AN, Patil R, Shete AR, Naiktari RS, Inamdar SP. Omega 3 fatty acids as a host modulator in chronic periodontitis patients: a randomised, double-blind, palcebo-controlled, clinical trial. J Periodontal Implant Sci. 2014;44:25-32.

23. DiNicolantonio JJ, Niazi AK, McCarty MF, O'Keefe JH, Meier P, Lavie CJ. Omega-3s and cardiovascular health. Ochsner J. 2014;14:399-412.

24. El Khouli AM, El-Gendy EA. Efficacy of omega 3 in treatment of recurrent aphthous stomatitis and improvement of quality of life: arandomized, double-blind, placebo-controlled study. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014; 117:191-6.

25. Elwakeel NM, Hazaa HH.Effect of omega 3 fatty acids plus low-dose aspirin on both clinical and biochemical profiles of patients with chronic periodontitis and type 2 diabetes: a randomized double blind placebo-controlled study. J Periodontal Res. 2015; 50:721-9.

26. Fan R, Sun B, Zhang CF, Lü YL, Xuan W, Wang QQ, Yin XZ. Receptor activator of nuclear factor kappa B ligand and osteoprotegerin expression in chronic apical periodontitis: possible association with inflammatory cells. Chin Med J (Engl) 2011;124:2162-6.

27. Farsi PF, Djazayery A, Eshraghian MR, Koohdani F, Saboor-Yaraghi AA, Derakhshanian H, Zarei M, Javanbakht MH, Djalali M. Effects of supplementation with omega-3 on insulin sensitivity and non-esterified free fatty acid (NEFA) in type 2 diabetic patients. Arq Bras Endocrinol Metabol. 2014; 58:335-40.

28. Fernandes G, Venkataraman J. Role of omega-3 fatty acids in health and disease. Nutr Res 1993; 13:19-45.

29. Högström M1, Nordström P, Nordström A. n-3 Fatty acids are positively associated with peak bone mineral density and bone accrual in healthy men: the no2 study. Am J Clin Nutr. 2007 Mar;85(3):803-7.

30. Inchingolo F, Marrelli M, Annibali S, Cristalli MP, Dipalma G, Inchingolo AD, Palladino A, Inchingolo AM, Gargari M, Tatullo M. Influence of endodontic treatment on systemic oxidative stress. Int J Med Sci. 2013; 11:1-6.

31. Jain AP, Aggarwal KK, Zhang PY. Omega-3 fatty acids and cardiovascular disease. Eur Rev Med Pharmacol. 19:441-5, 2015.

32. Kawashima N, Okiji T, Kosaka T, Suda H. Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: a quantitative immunohistochemical study. J Endod. 1996; 22:311–16.

33. Kesavalu L, Bakthavatchalu V, Rahman MM, Su J, Raghu B, Dawson D, Fernandes G, Ebersole JL. Omega3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in Porphyromonasgingivalis-induced experimental periodontal disease. Oral Microbiol Immunol. 2007; 22:232-9.

34. Kesavalu L, Vasudevan B, Raghu B, Browning E, Dawson D, Novak JM, Correll MC, Steffen MJ, Bhattacharya A, Fernandes G, Ebersole JL. Omega-3 fatty acid effect on alveolar bone loss in rats. J Dent Res. 2006;85:648-52.

35. Lee YH, Bae SC, Song GG. Omega-3 polyunsaturated fatty acids and the treatment of rheumatoid arthritis: a meta-analysis. Arch Med Res. 2012; 43:356-62.

36. Liapatas S, Nakou M, Rontogianni D. Inflammatory infiltrate of chronic periradicular lesions: an immunohistochemical study. Int Endod J. 2003;36:464-71.

37. Liljestrand JM, Mäntylä P, Paju S, Buhlin K, Kopra KA, Persson GR, Hernandez M, Nieminen MS,Sinisalo J, Tjäderhane L, Pussinen PJ. Association of Endodontic Lesions with Coronary Artery Disease. J Dent Res. 2016;95:1358-1365.

38. Massaro M, Habib A, Lubrano L, Del Turco S, Lazzerini G, Bourcier T, Weksler BB, De Caterina R. The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKC epsilon inhibition. Proc Natl Acad Sci U S A. 2006;103:15184-9.

39. Maggio M, Artoni A, Lauretani F, Borghi L, Nouvenne A, Valenti G, Ceda GP. The impact of omega-3 fatty acids on osteoporosis. Curr Pharm Des. 2009;15:4157-64.

40. Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, Taubert K, Newburger JM, Gornik HL, Gewitz MH, Wilson WR, Smith SC Jr, Baddour LM. Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association?: a scientific statement from the American Heart Association. Circulation. 2012; 125:2520-44.

41. Miles EA, Calder PC. Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. Br J Nutr. 2012;107 Suppl 2:S171-84.

42. Miller W. The human mouth as a focus of infection. Dental Cosmos 1891; 33:689-713

43. Mukaro VR, Costabile M, Murphy KJ, Hii CS, Howe PR, Ferrante A. Leukocyte numbers and function in subjects eating n-3 enriched foods: selective depression of natural killer cell levels. Arthritis Res Ther. 2008;10:R57.

44. Newman HN. Focal infection. J Dent Res 1996; 75: 1912-9.

45. Pallasch TJ, Wahl MJ. Focal infection: new age or ancient history? Endod Top 2003; 4:32-45.

46. Pereira RF, Cintra LTA, Tessarin GWL, Chiba FY, de Lima Coutinho Mattera MS, Scaramele NF, Tsosura TVS, Ervolino E, de Oliveira SHP, Sumida DH. Periapical Lesions Increase Macrophage Infiltration and Inflammatory Signaling in Muscle Tissue of Rats. J Endod. 2017;43:982-988.

47. Pereira RF, de Oliveira da Mota MS, de Lima Coutinho Mattera MS, Tsosura TV, Chiba FY, Garbin CA, Ervolino E, Cintra LT, Okamoto MM, Machado UF, Sumida DH. Periapical lesions decrease Akt serine phosphorylation and plasma membrane GLUT4 content in rat skeletal muscle. Clin Oral Investig. 2016;20:1625-30.

48. Prieto AKC, Gomes-Filho JE, Azuma MM, Sivieri-Araujo G, Narciso LG, Souza JC, Ciarlini PC, Cintra LTA. Influence of apical periodontitis on stress oxidative parameters in diabetic rats. J Endod 2017. In press

49. Sánchez-Domínguez B, López-López J, Jané-Salas E, Castellanos-Cosano L, Velasco-Ortega E, Segura-Egea JJ. Glycated hemoglobin levels and prevalence of apical periodontitis in type 2 diabetic patients. J Endod. 2015;41:601-6.

50. Silva JA, Ferrucci DL, Peroni LA, Abrahão PG, Salamene AF, Rossa-Junior C, Carvalho HF, Stach-Machado DR. Sequential IL-23 and IL-17 and increased Mmp8 and Mmp14 expression characterize the progression of an experimental model of periodontal disease in type 1 diabetes. J Cell Physiol. 2012; 227:2441-50.

51. Silva MJ1, Kajiya M, AlShwaimi E, Sasaki H, Hong J, Ok P, Rezende TM, Pagonis TC, White RR, Paster BJ, Stashenko P, Kawai T. Bacteria-reactive immune response may induce RANKL-expressing T cells in the mouse periapical bone loss lesion. J Endod. 2012;38:346-50.

52. Siqueira JF Jr, Rocas IN. Bacterial pathogenesis and mediators in apical periodontitis. Braz Dent J 2007; 18: 267-80.

53. Vardar S, Buduneli E, Türkoğlu O, Berdeli AH, Baylas H, Başkesen A, Atilla G. Therapeutic versus prophylactic plus therapeutic administration of omega3 fatty acid on endotoxin-inducedperiodontitis in rats. J Periodontol. 2004;75:1640-6.

54. Vardar-Sengül S, Buduneli N, Buduneli E, Kardeşler L, Baylas H, Atilla G, Lappin D, Kinane DF. Dietary supplementation of omega-3 fatty acid and circulating levels of interleukin-1beta, osteocalcin, and C-reactive protein in rats. J Periodontol. 2006; 77:814-20.

55. Xiong H, Wei L, Peng B. Immunohistochemical localization of IL-17 in induced rat periapical lesions. J Endod 2009;35:216-20.

VIII. Anexos

1. Material e Métodos utilizados

1.1. Animais

Foram utilizados 40 ratos machos (Rattus albinus, Wistar), pesando aproximadamente 250g, provenientes do biotério da Faculdade de Odontologia de Araçatuba- UNESP. Os animais foram mantidos em mini-isoladores (Alesco-Monte Mor, São Paulo, Brasil) com temperatura entre 22 e 24ºC com ciclo de luz controlada (12 horas claro e 12 horas escuro) e em gaiolas coletivas, quatro ratos por gaiola, alimentados durante todo o período experimental com dieta sólida e água “ad libitum”, exceto nas primeiras 24 horas após intervenção. Os procedimentos experimentais propostos neste estudo foram aprovados pelo comitê de conduta e ética no uso de animais em experimentação (CEUA- 2014-00550-UNESP).

1.2. Drogas empregadas

Para anestesia dos animais foi utilizado, via intra-muscular, um sedativo a base de xilazina (Dopasere, Calier S.A - Barcelona, Espanha- 10mg/kg) e um anestésico à base de cloridrato de ketamina a 5% (Vetanarcol, Konig S.A.- Avellaneda, Argentina- 25mg/kg).

1.3. Divisão em grupos

Os ratos foram divididos nos seguintes grupos:

- C: ratos sem suplementação alimentar com ômega-3 e periodontite apical induzida;

- C-O: ratos suplementados com ômega-3 e sem periodontite apical induzida;

- PA: ratos com periodontite apical induzida;

-PA-O: ratos com periodontite apical induzida e suplementados com ômega-3.

1.4. Suplementação alimentar com ômega-3

Os ratos (grupos C-O e PA-O) receberam, por meio de gavage, o ácido graxo ômega-3, na proporção de 40mg/kg (60% EPA + 40%DHA),

durante 15 dias antes da exposição pulpar e durante 30 dias depois da exposição pulpar, totalizando 45 dias de suplementação.

1.5. Indução da periodontite apical

Para a indução da periodontite apical, os ratos foram anestesiados, via intra-muscular, com uma associação do sedativo Dopaser, à base de xilazina (relaxante muscular, analgésico e sedativo), na dosagem de 10mg/kg de peso corporal e o anestésico Vetanarcol, à base de cloridrato de ketamina a 5% na dosagem de 25mg/kg de peso corporal. As polpas dos primeiros e segundos molares superiores e inferiores do lado direito foram expostas, por meio de uma broca em aço carbono (Broca Ln Long Neck- Maillefer, Dentsply) dotada de uma esfera na extremidade com 0,1mm de diâmetro. Desta forma, todas as exposições pulpares foram padronizadas com 0,1mm de diâmetro e as polpas ficaram expostas na cavidade bucal por um período de 30 dias, momento do sacrifício.

1.6. Coleta de sangue

Aos 30 dias pós-operatórios os animais foram novamente anestesiados e foi realizada a coleta de 5ml de sangue por meio de punção cardíaca. Posteriormente, os animais foram mortos por meio de sobredose anestésica. Com as amostras sanguíneas, foram analisados os parâmetros: leucócitos, neutrófilos, linfócitos, monócitos e eosinófilos. As amostras sanguíneas para IL-17, TNF-α, IL-6, osteocalcina foram colocadas em heparina (10U/ml), na sequência, centrifugadas e armazenadas imediatamente a -80ºC.

1.7. Coleta das maxilas e mandíbulas

Após a eutanásia, toda a pele correspondente à face direita do animal foi removida e foram realizados dois cortes, realizados com tesoura, no ângulo da boca, separando a maxila da mandíbula. Foi realizada uma incisão, com lâmina de bisturi intercambiável número 15, em profundidade no palato do animal, com localização no plano sagital mediano acompanhando a sutura intermaxilar, separando a maxila esquerda da direita. Separadas as maxilas, com o auxílio de uma tesoura, foi realizado outro corte com localização tangente a face distal dos molares superiores direito e esquerdo, possibilitando

a obtenção da hemimaxila direita, contendo os dentes posteriores, objeto do estudo. A hemimandíbula direita, contendo os dentes posteriores, também foi separada para o estudo.

1.8 Forma de análise dos resultados

1.8.1. Análise histológica e histométrica das lesões periapicais

Para a análise histológica foram empregadas 5 lâminas com 3 cortes teciduais cada, preparadas e coradas com hematoxilina e eosina (Lillie, 1954). A análise histológica foi utilizada para a caracterização do perfil inflamatório das lesões periapicais (Cintra et al. 2013c). Os resultados foram expostos por meio de duas análises, sendo uma descritiva e outra quantitativa. As lâminas contendo os cortes representativos de cada espécime foram avaliadas sob a microscopia óptica e utilizadas na análise descritiva. A análise descritiva consistiu da descrição dos fenômenos histopatológicos, procurando caracterizá-los globalmente em função das variáveis experimentais (Cintra et al. 2013c).

A análise quantitativa foi realizada por meio da atribuição de escores, graduando as magnitudes dos fenômenos histopatológicos de forma dissociada e da análise histométrica das estruturas do periodonto apical (Cintra et al. 2013c). As variáveis estudas foram: infiltrado inflamatório quando à sua intensidade e extensão; presença de reabsorções radiculares e perda de estrutura óssea periapical. Foram atribuídos escores para os critérios intensidade e extensão do infiltrado inflamatório e para a presença de reabsorções radiculares. Para a perda de estrutura óssea foi realizada a mensuração linear e por área, respectivamente, empregando programa de imagens específico (Leica QWin Plus - Leica Microsystems, Nussloch – Germany).

Os critérios considerados para cada análise foram:

- **Intensidade do infiltrado inflamatório**

A intensidade do processo inflamatório foi analisada em conformidade com o número médio aproximado de células inflamatórias presentes em diferentes campos de um mesmo espécime, examinados em aumento de 400x junto ao periápice dentário **(Quadro A).**

Quadro A – Escores para o critério intensidade do infiltrado inflamatório

Escore 1 Células inflamatórias ausentes ou em número desprezível;
Escore 2 Infiltrado inflamatório discreto (menos de 10 células por campo);
Escore 3 Infiltrado inflamatório moderado (entre 10 e 25 células por campo);
Escore 4 Infiltrado inflamatório intenso (mais que 25 células por campo).

- **Extensão do infiltrado inflamatório**

A extensão do infiltrado inflamatório foi estabelecida em função da alteração do tecido periapical o **(Quadro B).**

Quadro B – Escores para o critério extensão do infiltrado inflamatório

Escore 1 Células inflamatórias ausentes ou em número desprezível;
Escore 2 Células inflamatórias ocupando extensão de até 300μm do ápice radicular;
Escore 3 Células inflamatórias ocupando extensão de até 600μm do ápice radicular;
Escore 4 Células inflamatórias ocupando extensão maior que 600μm do ápice radicular.

- **Perda de estrutura óssea**

Foram selecionados cortes representativos de cada espécime dos grupos para a verificação se há diferença na perda óssea de ratos que fizeram ou não o uso de ômega-3. Foi empregado o software Leica QWin Plus (Leica Microsystems, Nussloch – Germany) e os valores foram expressos em micrometro quadrado (μm2) obtidos em medidas de área.

1.3.2. Imunoistoquímica para a detecção de TRAP, OCN, TNF-α, IL-6, IL-1β, IL-17 e IL-10 nos tecidos periapicais

Os cortes histológicos foram desparafinizados em xilol e hidratados em série decrescente de etanol. A recuperação antigênica foi realizada através da imersão das lâminas histológicas em tampão Diva Decloaker® (Biocare Medical, CA, USA), em câmara pressurizada Decloaking Chamber® (Biocare Medical, CA, USA), a 95°C, por 10 minutos. Ao término de cada uma das etapas da reação imunoistoquímica foram efetuadas lavagens em tampão fosfato salino (PBS) 0,1M, pH 7,4. Os cortes histológicos foram imersos em 3% de peróxido de hidrogênio por 1 hora e em 1% de soro albumina bovino por 12 horas, para o bloqueio da peroxidase endógena e bloqueio dos sítios inespecíficos, respectivamente. As lâminas histológicas contendo amostras de todos os grupos experimentais foram divididas em seis lotes, e cada lote foi submetido à incubação com seus respectivos anticorpos primários. Os anticorpos primários foram diluídos em PBS acrescido de 0,1% Triton X-100 (PBS-TX), durante 24 horas, em câmara úmida. Nas etapas subsequentes foi empregado o Universal Dako Labeled (HRP) Streptavidin-Biotin Kit® (Dako Laboratories, CA, USA). As secções histológicas foram incubadas no anticorpo secundário biotinilado, durante 2 horas, e posteriormente tratadas com estreptavidina conjugada com a peroxidase da raiz forte (HRP), por 1 hora. Na revelação, foi empregado como cromógeno o 3,3'- tetracloridrato de diaminobenzidina (DAB chromogen Kit®, Dako Laboratories, CA, USA) e em seguida a contracoloração foi realizada com hematoxilina de Harris.

1.9. Hemograma

Para a realização do hemograma, foi utilizado o analisador automático ABX (ABX Micros ABC Vet - Horiba ABX Diagnostics, Montpellier, France). Foram observados os seguintes parâmetros: Hemáceas (106/µL), Volume Globular (%), Hemoglobina (g/dL), VCM (fL), CHCM (%), Leucócitos (10^3/µL), Neutrófilos (%), Linfócito (%), Monócito (%), Eosinófilo (%) e Basófilo (%).

Foi empregado o kit ABX VetPack que é composto por 3 reagentes:

O ABX VET Pack, R3 é uma solução salina e tampão eletrolítica que permite a diluição e a preparação da amostra de sangue para análise. O fluxo dos sistemas hidráulicos do aparelho é garantido pela presença de surfactante não iônico. A ação eletrolítica aceita a contagem das células por impedância. Este reagente diferencia populações morfológicas de leucócitos e é utilizado nos ciclos de enxágue e limpeza dos sistemas hidráulicos do instrumento.

O ABX VET Pack, R2 decompõe a membrana celular das hemáceas. Pela adição do agente surfactante, a hemoglobina é libertada. Todo o ferro heme é oxidado e os complexos resultantes são quantificados por espectrofotometria a um comprimento de onda de 530 nm. O detergente presente na solução também diferencia populações morfológicas de leucócitos.

ABX VET Pack, R1 utiliza a ação combinada de uma enzima proteolítica com um detergente para eliminar os resíduos de proteína e evitar que os tubos hidráulicos fiquem obstruídos e/ou bloqueiem o fluxo. Também é usado para decompor os acúmulos de proteína nas aberturas e câmaras de contagem.

1.10. Quantificação das citocinas pró-inflamatórias pelo método ELISA

Inicialmente foram pipetados os anticorpos de captura (1:1000; BD Biosciences, CA, USA) de cada uma das citocinas em suas respectivas placas. A placa permaneu em overnight na geladeira por 16 horas. No dia seguinte, todos os reagentes foram colocados à temperatura ambiente antes de proceder o experimento. A placa foi, então, lavada e aspirada por 4 vezes com solução tampão (PBS) associada a Tween 20 (Sigma, MO, USA). Foram adicionados então 200ul de solução tampão associada à albumina por poço. A placa permaneceu incubada e selada por 1 hora à temperatura ambiente. Em seguida, ela foi lavada novamente com PBS+Tween 20 (Sigma, MO, USA) e aspirada por 4 vezes.

Foram pipetados, então, 100µl do recombinante, e foi realizada a diluição seriada para fazer a curva padrão. Foram pipetadas 50µl das amostras nos poços apropriados. Novamente foi realizado o selamento da placa e a incubação das amostras à temperatura ambiente por 2 horas. A aspiração e lavagem foram realizadas novamente da mesma forma já descrita. Foram adicionados 100µl de anticorpo secundário (1:1000; BD Biosciences, CA, USA) em cada poço, a placa de vedação foi colocada para incubação da amostra por 1 hora à temperatura ambiente. Novamente foi realizada a aspiração e lavagem. Então, foram adicionados 100µl de reagente enzimático diluído (ExtrAvidin®−Peroxidase, Sigma, MO, USA) em cada poço. Foi feito o selamento da placa e a incubação por 30 minutos à temperatura ambiente. A aspiração e lavagem nesta etapa foi feita 7 vezes os poços foram mergulhados por um minuto em tampão de lavagem. Posteriormente, foram adicionados 100µl de solução de substrato TMB (BD Biosciences, CA, USA) em cada poço. A incubação foi realizada sem o selador de placa por trinta minutos à temperatura ambiente no escuro. Por fim, foram adicionados 50µl de solução de parada (H2SO4) em cada poço. A leitura foi realizada em espectofotômetro ajustado para o comprimento de onda de 450nm.

1.11. Análise estatística

A análise estatística foi realizada por meio do programa SigmaPlot 12.0™ (Chicago, IL, USA). A verificação da distribuição normal das variáveis contínuas quantitativas foi feita pelos testes de Kolmogorov-Smirnov e Shapiro-Wilk. As variáveis que apresentarem distribuição normal foram expressas em média e ± desvio padrão e aquelas que apresentaram distribuição não normal foram expressas em mediana com valor mínimo e máximo e percentis.

Para os valores quantitativos das análises que seguiram uma distribuição normal, foi aplicado o teste ANOVA, enquanto que para comparações múltiplas dos resultados entre os grupos estudados, foi utilizado o teste de Tukey.

Para as análises histológicas em que foram atribuídos escores e para os valores quantitativos das análises que não seguiram uma distribuição normal, foi aplicado o teste de Kruskal-Wallis, e quando observou-se alguma diferença significante, foi realizado o cruzamento entre os grupos, pelo teste de comparações múltiplas de Dunn (Dunn, 1958). Também foi empregado o teste de Mann Whitney (Siegel et al., 1956) para comparação entre dois grupos isoladamente.

Os resultados foram considerados estatisticamente significativos quando a probabilidade foi menor que 5% ($p<0,05$).

2. Normas para publicação nos periódicos

2.1. Journal of Endodontics

Guidelines for Publishing Papers in the JOE

1. **General Points on Composition**
    1. Authors are strongly encouraged to analyze their final draft with both software (*e.g.*, spelling and grammar programs) and colleagues who have expertise in English grammar. References listed at the end of this section provide a more extensive review of rules of English grammar and guidelines for writing a scientific article. Always remember that clarity is the most important feature of scientific writing. Scientific articles must be clear and precise in their content and concise in their delivery since their purpose is to inform the reader. The Editor reserves the right to edit all manuscripts or to reject those manuscripts that lack clarity or precision, or have unacceptable grammar or syntax. The following list represents common errors in manuscripts submitted to the *JOE*:
    2. The paragraph is the ideal unit of organization. Paragraphs typically start with an introductory sentence that is followed by sentences that describe additional detail or examples. The last sentence of the paragraph provides conclusions and forms a transition to the next paragraph. Common problems include one-sentence paragraphs, sentences that do not develop the theme of the paragraph (see also section “c” below), or sentences with little to no transition within a paragraph.
    3. Keep to the point. The subject of the sentence should support the subject of the paragraph. For example, the introduction of authors’ names in a sentence changes the subject and lengthens the text. In a paragraph on sodium hypochlorite, the sentence, “In 1983, Langeland et al., reported that sodium hypochlorite acts as a lubricating factor during instrumentation and helps to flush debris from the root canals” can be edited to: “Sodium hypochlorite acts as a lubricant during instrumentation and as a vehicle for flushing the generated debris (Langeland et al., 1983)." In this example,

the paragraph's subject is sodium hypochlorite and sentences should focus on this subject.

4. Sentences are stronger when written in the active voice, *i.e.*, the subject performs the action. Passive sentences are identified by the use of passive verbs such as "was," "were," "could," etc. For example: "Dexamethasone was found in this study to be a factor that was associated with reduced inflammation," can be edited to: "Our results demonstrated that dexamethasone reduced inflammation." Sentences written in a direct and active voice are generally more powerful and shorter than sentences written in the passive voice.
5. Reduce verbiage. Short sentences are easier to understand. The inclusion of unnecessary words is often associated with the use of a passive voice, a lack of focus or run-on sentences. This is not to imply that all sentences need be short or even the same length. Indeed, variation in sentence structure and length often helps to maintain reader interest. However, make all words count. A more formal way of stating this point is that the use of subordinate clauses adds variety and information when constructing a paragraph. (This section was written deliberately with sentences of varying length to illustrate this point.)
6. Use parallel construction to express related ideas. For example, the sentence, "Formerly, endodontics was taught by hand instrumentation, while now rotary instrumentation is the common method," can be edited to "Formerly, endodontics was taught using hand instrumentation; now it is commonly taught using rotary instrumentation." The use of parallel construction in sentences simply means that similar ideas are expressed in similar ways, and this helps the reader recognize that the ideas are related.
7. Keep modifying phrases close to the word that they modify. This is a common problem in complex sentences that may confuse the reader. For example, the statement, "Accordingly, when conclusions are drawn from the results of this study, caution must be used," can be edited to "Caution must be used when conclusions are drawn from the results of this study."

8. To summarize these points, effective sentences are clear and precise, and often are short, simple and focused on one key point that supports the paragraph's theme.
9. Authors should be aware that the *JOE* uses iThenticate, plagiarism detection software, to assure originality and integrity of material published in the *Journal*. The use of copied sentences, even when present within quotation marks, is highly discouraged. Instead, the information of the original research should be expressed by new manuscript author's own words, and a proper citation given at the end of the sentence. Plagiarism will not be tolerated and manuscripts will be rejected, or papers withdrawn after publication based on unethical actions by the authors. In addition, authors may be sanctioned for future publication.

2. **Organization of Original Research Manuscripts**

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

1. **Title Page:** The title should describe the major emphasis of the paper. It should be as short as possible without loss of clarity. Remember that the title is your advertising billboard—it represents your major opportunity to solicit readers to spend the time to read your paper. It is best not to use abbreviations in the title since this may lead to imprecise coding by electronic citation programs such as PubMed (*e.g.*, use "sodium hypochlorite" rather than NaOCl). The author list must conform to published standards on authorship (see authorship criteria in the Uniform Requirements for Manuscripts Submitted to Biomedical Journals at *www.icmje.org*). The manuscript title, name and address (including email) of one author designated as the corresponding author. This author will be responsible for editing proofs and ordering reprints when applicable. The contribution of each author should also be highlighted in the cover letter.
2. **Abstract:** The abstract should concisely describe the purpose of the study, the hypothesis, methods, major findings and conclusions. The

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

3. **Introduction:** The introduction should briefly review the pertinent literature in order to identify the gap in knowledge that the study is intended to address and the limitations of previous studies in the area. The purpose of the study, the tested hypothesis and its scope should be clearly described. Authors should realize that this section of the paper is their primary opportunity to establish communication with the diverse readership of the *JOE*. Readers who are not expert in the topic of the manuscript are likely to skip the paper if the introduction fails to succinctly summarize the gap in knowledge that the study addresses. It is important to note that many successful manuscripts require no more than a few paragraphs to accomplish these goals. Therefore, authors should refrain from performing extensive review or the literature, and discussing the results of the study in this section.
4. **Materials and Methods:** The objective of the materials and methods section is to permit other investigators to repeat your experiments. The four components to this section are the detailed description of the materials used and their components, the experimental design, the procedures employed, and the statistical tests used to analyze the results. The vast majority of manuscripts should cite prior studies using similar methods and succinctly describe the essential aspects used in the present study. Thus, the reader should still be able to understand the method used in the experimental approach and concentration of the main reagents (*e.g.*, antibodies, drugs, etc.) even when citing a previously published method. The inclusion of a “methods figure” will be rejected unless the procedure is novel and requires an illustration for comprehension. If the method is novel, then the authors should carefully describe the method and include validation experiments. If the study utilized a **commercial product**, the manuscript must state that they either

followed manufacturer's protocol *or* specify any changes made to the protocol. If the study used an***in vitro*** **model** to simulate a clinical outcome, the authors must describe experiments made to validate the model, or previous literature that proved the clinical relevance of the model. Studies on **humans** must conform to the Helsinki Declaration of 1975 and state that the institutional IRB/equivalent committee(s) approved the protocol and that informed consent was obtained after the risks and benefits of participation were described to the subjects or patients recruited. Studies involving **animals** must state that the institutional animal care and use committee approved the protocol. The statistical analysis section should describe which tests were used to analyze which dependent measures; p-values should be specified. Additional details may include randomization scheme, stratification (if any), power analysis as a basis for sample size computation, drop-outs from clinical trials, the effects of important confounding variables, and bivariate versus multivariate analysis.

5. **Results:** Only experimental results are appropriate in this section (*i.e.*, neither methods, discussion, nor conclusions should be in this section). Include only those data that are critical for the study, as defined by the aim(s). Do not include all available data without justification; any repetitive findings will be rejected from publication. All Figures, Charts and Tables should be described in their order of numbering with a brief description of the major findings. Author may consider the use of supplemental figures, tables or video clips that will be published online. Supplemental material is often used to provide additional information or control experiments that support the results section (*e.g.*, microarray data).
6. **Figures:** There are two general types of figures. The first type of figures includes photographs, radiographs or micrographs. Include only essential figures, and even if essential, the use of composite figures containing several panels of photographs is encouraged. For example, most photo-, radio- or micrographs take up one column-width, or about 185 mm wide X 185 mm tall. If instead, you construct a two columns-width figure (*i.e.*, about 175 mm wide X 125 mm high when published in the *JOE*), you would be able to place about 12 panels of photomicrographs (or

radiographs, etc.) as an array of four columns across and three rows down (with each panel about 40 X 40 mm). This will require some editing to emphasize the most important feature of each photomicrograph, but it greatly increases the total number of illustrations that you can present in your paper. Remember that each panel must be clearly identified with a letter (*e.g.*, "A," "B," etc.), in order for the reader to understand each individual panel. Several nice examples of composite figures are seen in recent articles by Jeger et al (J Endod 2012;38:884–888); Olivieri et al., (J Endod 2012;38:1007 1011); Tsai et al (J Endod 2012;38:965–970). Please note that color figures may be published at no cost to the authors and authors are encouraged to use color to enhance the value of the illustration. Please note that a multipanel, composite figure only counts as one figure when considering the total number of figures in a manuscript (see section 3, below, for maximum number of allowable figures). The second type of figures are graphs (*i.e.*, line drawings including bar graphs) that plot a dependent measure (on the Y axis) as a function of an independent measure (usually plotted on the X axis). Examples include a graph depicting pain scores over time, etc. Graphs should be used when the overall trend of the results are more important than the exact numerical values of the results. For example, a graph is a convenient way of reporting that an ibuprofen-treated group reported less pain than a placebo group over the first 24 hours, but was the same as the placebo group for the next 96 hours. In this case, the trend of the results is the primary finding; the actual pain scores are not as critical as the relative differences between the NSAID and placebo groups.

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

% NaOCl	N/Group	% Inhibition of Growth
0.001	5	0
0.003	5	0
0.01	5	0
0.03	5	0
0.1	5	100
0.3	5	100
1	5	100
3	5	100

8. Instead, the results could simply state that there was no inhibition of growth from 0.001-0.03% NaOCl, and a 100% inhibition of growth from 0.03-3% NaOCl (N=5/group). Similarly, if the results are not significant, then it is probably not necessary to include the results in either a table or as a figure. These and many other suggestions on figure and table construction are described in additional detail in Day (1998).

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

2. **Acknowledgments:** All authors must affirm that they have no financial affiliation (*e.g.*, employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such

arrangements existed in the past three years. Any other potential conflict of interest should be disclosed. Any author for whom this statement is not true must append a paragraph to the manuscript that fully discloses any financial or other interest that poses a conflict. Likewise the sources and correct attributions of all other grants, contracts or donations that funded the study must be disclosed

3. **References:** The reference style follows Index Medicus and can be easily learned from reading past issues of the *JOE*. The *JOE* uses the Vancouver reference style, which can be found in most citation management software products. Citations are placed in parentheses at the end of a sentence or at the end of a clause that requires a literature citation. Do not use superscript for references. Original reports are limited to 35 references. There are no limits in the number of references for review articles.

2. **Manuscripts Category Classifications and Requirements**

Manuscripts submitted to the *JOE* must fall into one of the following categories. The abstracts for all these categories would have a maximum word count of 250 words:

1. CONSORT Randomized Clinical Trial-Manuscripts in this category must strictly adhere to the Consolidated Standards of Reporting Trials-CONSORT- minimum guidelines for the publication of randomized clinical trials. These guidelines can be found at *www.consort-statement.org/*. These manuscripts have a limit of 3,500 words, [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
2. Review Article-Manuscripts in this category are either narrative articles, or systematic reviews/meta-analyses. Case report/Clinical Technique articles even when followed by extensive review of the literature will should be categorized as “Case Report/Clinical Technique”. These manuscripts have a limit of 3,500 words, [including abstract, introduction, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.

3. Clinical Research (*e.g.*, prospective or retrospective studies on patients or patient records, or research on biopsies, excluding the use of human teeth for technique studies). These manuscripts have a limit of 3,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures and 4 tables*.
4. Basic Research Biology (animal or culture studies on biological research on physiology, development, stem cell differentiation, inflammation or pathology). Manuscripts that have a primary focus on biology should be submitted in this category while manuscripts that have a primary focus on materials should be submitted in the Basic Research Technology category. For example, a study on cytotoxicity of a material should be submitted in the Basic Research Technology category, even if it was performed in animals with histological analyses. These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or 4 tables*.
5. Basic Research Technology (Manuscripts submitted in this category focus primarily on research related to techniques and materials used, or with potential clinical use, in endodontics). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 3 figures and tables *.
6. Case Report/Clinical Technique (*e.g.*, report of an unusual clinical case or the use of cutting-edge technology in a clinical case). These manuscripts have a limit of 2,500 words [including abstract, introduction, materials and methods, results, discussion and acknowledgments; excluding figure legends and references]. In addition, there is a limit of a total of 4 figures or tables*.

* Figures, if submitted as multipanel figures must not exceed 1 page length. Manuscripts submitted with more than the allowed number of figures or tables will require approval of the *JOE* Editor or associate editors. If you are

not sure whether your manuscript falls within one of the categories above, or would like to request preapproval for submission of additional figures please contact the Editor by email at*jendodontics@uthscsa.edu*.

Importantly, adhering to the general writing methods described in these guidelines (and in the resources listed below) will help to reduce the size of the manuscript while maintaining its focus and significance. Authors are encouraged to focus on only the essential aspects of the study and to avoid inclusion of extraneous text and figures. The Editor may reject manuscripts that exceed these limitations.

Available Resources:

Strunk W, White EB. The Elements of Style. Allyn & Bacon, 4th ed, 2000, ISBN 020530902X.

Day R. How to Write and Publish a Scientific Paper. Oryx Press, 5th ed. 1998. ISBN1-57356-164-9.

Woods G. English Grammar for Dummies. Hungry Minds:NY, 2001 (an entertaining review of grammar).

Alley M. The Craft of Scientific Writing. Springer, 3rd edition 1996 SBN 0-387-94766-3.

Alley M. The Craft of Editing. Springer, 2000 SBN 0-387-98964-1.

2.2. **Normas para publicação no periódico Clinical Oral Investigations**

Guidelines for Publishing Papers in the CLOI

Types of papers
Papers may be submitted for the following sections:

Original articles
Invited reviews
Short communications
Letters to the editor

It is the general policy of this journal not to accept case reports and pilot studies.

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

Permissions

Authors wishing to include figures, tables, or text passages that have already been published elsewhere are required to obtain permission from the copyright owner(s) for both the print and online format and to include evidence that such permission has been granted when submitting their papers. Any material received without such evidence will be assumed to originate from the authors.

Online Submission

Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen. The Springer Author Academy is a set of comprehensive online training pages mainly geared towards first-time authors. At this point, more than 50 pages offer advice to authors on how to write and publish a journal article.

Text Formatting
Manuscripts should be submitted in Word. Use a normal, plain font (e.g., 10-point Times Roman) for text. Use italics for emphasis. Use the automatic page numbering function to number the pages. Do not use field functions. Use tab stops or other commands for indents, not the space bar. Use the table function, not spreadsheets, to make tables. Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions). Manuscripts with mathematical content can also be submitted in LaTeX. LaTeX macro package (zip, 182 kB) Headings Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables. Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols. Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

References

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:
1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list. The entries in the list should be numbered consecutively.

Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in

prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted: Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California
Always use the standard abbreviation of a journal’s name according to the ISSN List of Title Word Abbreviations, see ISSN.org LTWA.
If you are unsure, please use the full journal title.
For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.
EndNote style (zip, 2 kB)
Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer’s LaTeX macro package.

Tables

All tables are to be numbered using Arabic numerals. Tables should always be cited in text in consecutive numerical order. For each table, please supply a table caption (title) explaining the components of the table. Identify any previously published material by giving the original source in the form of a reference at the end of the table caption. Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

Artwork and illustrations guidelines

Electronic Figure Submission Supply all figures electronically. Indicate what graphics program was used to create the artwork. For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MSOffice files are also acceptable. Vector graphics containing fonts must have the fonts embedded in the files. Name your figure files with "Fig" and the figure number, e.g., Fig1.eps. Definition: Black and white graphic with no shading. Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size. All lines should be at least 0.1 mm (0.3 pt) wide. Scanned line drawings and line drawings in bitmap format should have a minimum resolution of 1200 dpi. Vector graphics containing fonts must have the fonts embedded in the files.

Halftone Art

Definition: Photographs, drawings, or paintings with fine shading, etc. If any magnification is used in the photographs, indicate this by using scale bars within the figures themselves. Halftones should have a minimum resolution of 300 dpi. Definition: a combination of halftone and line art, e.g., halftones containing line drawing, extensive lettering, color diagrams, etc. Combination artwork should have a minimum resolution of 600 dpi.

Color Art

Color art is free of charge for online publication. If black and white will be shown in the print version, make sure that the main information will still be visible. Many colors are not distinguishable from one another when converted to black and white. A simple way to check this is to make a xerographic copy to see if the necessary distinctions between the different colors are still apparent. If the figures will be printed in black and white, do not refer to color in the captions. Color illustrations should be submitted as RGB (8 bits per channel).

Figure Lettering

To add lettering, it is best to use Helvetica or Arial (sans serif fonts). Keep lettering consistently sized throughout your final-sized artwork, usually about 2–3 mm (8–12 pt). Variance of type size within an illustration should be minimal, e.g., do not use 8- pt type on an axis and 20-pt type for the axis label. Avoid effects such as shading, outline letters, etc. Do not include titles or captions within your illustrations.

Figure Numbering

All figures are to be numbered using Arabic numerals. Figures should always be cited in text in consecutive numerical order. Figure parts should be denoted by lowercase letters (a, b, c, etc.). If an appendix appears in your article and it contains one or more figures, continue the consecutive numbering of the main text. Do not number the appendix figures, "A1, A2, A3, etc." Figures in online appendices (Electronic Supplementary Material) should, however, be numbered separately.

Figure Captions

Each figure should have a concise caption describing accurately what the figure depicts. Include the captions in the text file of the manuscript, not in the figure file. Figure captions begin with the term Fig. in bold type, followed by the figure number, also in bold type. No punctuation is to be included after the number, nor is any punctuation to be placed at the end of the caption. Identify all elements found in the figure in the figure caption; and use boxes, circles, etc., as coordinate points in graphs. Identify previously published material by giving the original source in the form of a reference citation at the end of the figure caption.

Figure Placement and Size

Figures should be submitted separately from the text, if possible. When preparing your figures, size figures to fit in the column width.
For most journals the figures should be 39 mm, 84 mm, 129 mm, or 174 mm wide and not higher than 234 mm. For books and book-sized journals, the figures should be 80 mm or 122 mm wide and not higher than 198 mm.

Permissions

If you include figures that have already been published elsewhere, you must obtain permission from the copyright owner(s) for both the print and online format. Please be aware that some publishers do not grant electronic rights for free and that Springer will not be able to refund any costs that may have occurred to receive these permissions. In such cases, material from other sources should be used.

Accessibility

In order to give people of all abilities and disabilities access to the content of your figures, please make sure that All figures have descriptive captions (blind users could then use a text-to-speech software or a text-to-Braille hardware) Patterns are used instead of or in addition to colors for conveying information (colorblind users would then be able to distinguish the visual elements) Any figure lettering has a contrast ratio of at least 4.5:1

Integrity of research and reporting

Ethical standards

Manuscripts submitted for publication must contain a statement to the effect that all human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. It should also be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. These statements should be added in a separate section before the reference list. If

these statements are not applicable, authors should state: The manuscript does not contain clinical studies or patient data. The editors reserve the right to reject manuscripts that do not comply with the above-mentioned requirements. The author will be held responsible for false statements or failure to fulfill the above-mentioned requirements

Conflict of interest

Authors must indicate whether or not they have a financial relationship with the organization that sponsored the research. They should also state that they have full control of all primary data and that they agree to allow the journal to review their data if requested. Therefore the manuscript must be accompanied by the “Conflict of Interest Disclosure Form”. To download this form, please follow the hyperlink on the right. The manuscript must also be accompanied by the “Authorship & Disclosure Form”. To download this form, please follow the hyperlink on the right.

3. Comitê de ética

unesp

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

cea unesp araçatuba

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 **"Influência local e sistêmica da suplementação alimentar com o ácido graxo ômega-3 em ratos com infecção endodôntica"**, Processo FOA nº 2014-00550, 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 26 de Junho de 2014.

VALIDADE DESTE CERTIFICADO: 01 de Novembro de 2017.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 01 de Dezembro de 2017.

CERTIFICATE

We certify that the study entitled **"Local and systemic influence of dietary supplementation with ômega-3 in rats with endodontic infection "**, Protocol FOA nº 2014-00550, 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 June 26, 2014.

VALIDITY OF THIS CERTIFICATE: November 01, 2017.
DATE OF SUBMISSION OF THE FINAL REPORT: December 01, 2017.

Prof. Dr. Edilson Ervolino
Coordenador da CEUA
CEUA 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