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

RENAN DAL FABBRO

Efeito do consumo de vinho tinto no desenvolvimento da periodontite apical induzida em ratos

Araçatuba

2021

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

Orientador: Prof. Tit. João Eduardo Gomes Filho

Araçatuba

2021

Catalogação na Publicação (CIP)

Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

Fabbro, Renan Dal.

F113e Efeito do consumo de vinho tinto no desenvolvimento da periodontite apical induzida em ratos / Renan Dal Fabbro. - Araçatuba, 2021

55 f. : il. ; tab.

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

Orientador: Prof. João Eduardo Gomes Filho

1. Periodontite periapical 2. Quercetina 3. Resveratrol 4. Vinho 5. Polifenóis I. T.

Black D24

CDD 617.67

Claudio Hideo Matsumoto – CRB-8/5550

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Dedicatória

Aos meus pais,

Reinaldo e Rosa Cristina,

Pelo amor incondicional, força, dedicação, e pela imensa contribuição para que eu pudesse realizar meus sonhos. Por sempre me apoiarem em todas as decisões que tomo em minha vida. Mais do que a educação formal que vocês me ofereceram e que sempre se esforçaram para que fosse a melhor, a formação humana foi o que de mais importante vocês fizeram por mim. Eu só posso retribuir tentando ser o melhor filho que pais como vocês merecem ter. Obrigado pelo maior presente que vocês puderam me dar, a vida!

À minha irmã,

Rafaela,

Que mesmo fisicamente distante sempre esteve presente na minha formação, torcendo e vibrando com as minhas conquistas. Tivemos tantas brigas quando crianças que certamente ajudaram intensificar nossa amizade depois de adultos. Minha vida não seria completa sem a sua presença!

Aos meus avós,

Cecília, Dante (in memorian), Leonice (in memorian) e Marino (in memorian)

Fazer parte da família que vocês construíram com tanto empenho é algo indescritível. Embora três de vocês não estejam mais presentes entre nós, sei que continuam de olho em mim aí de cima. De vocês guardo as melhores memórias, os melhores ensinamentos e recordo como foi importante ter vocês em minha vida. Vocês foram uma verdadeira lição de sabedoria para mim e terei sempre um grande orgulho de levar vossos DNA adiante.

À minha esposa,

Letícia,

Que no momento desta defesa já será minha esposa, a pessoa que compartilho todos os detalhes da minha vida desde novembro de 2012. Esteve sempre ao meu lado nos momentos fáceis e difíceis da vida. Agradeço do fundo do meu coração todos os dias pelo privilégio de ter você ao meu lado. Você é uma companheira carinhosa, engraçada, linda e leal. Seu abraço me dá o conforto que preciso para me sentir mais tranquilo. Tenho muito orgulho do que você tem conquistado e prometo que lutarei intensamente pela nossa felicidade. Nossa história está apenas começando, tenho certeza de que ficaremos juntos para sempre!

Agradecimentos

À Universidade Estadual Paulista "Júlio de Mesquita Filho", na pessoa do diretor da Faculdade de Odontologia de Araçatuba **Prof. Titular Glauco Issamu Miyahara** e do vice-diretor **Prof. Titular Alberto Carlos Botazzo Delbem**.

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

À **Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP)**, por ter concedido a minha bolsa de doutorado (Processo nº2017/27219-3) e por ter fomentado meu maior sonho, que era o de fazer pesquisa no exterior, por meio da bolsa estágio de pesquisa no exterior (Processo nº2019/05148-2).

Ao meu orientador **Professor Titular João Eduardo Gomes Filho**, por ter me aceitado como aluno de Iniciação Científica lá em meados de 2012 e aberto as portas da pesquisa científica naquele momento. Por sempre ter me apoiado e me incentivado nos momentos difíceis, reconsiderando as solicitações à FAPESP inúmeras vezes, por toda a paciência e ajuda que sempre teve comigo e demais orientados. Sou eternamente grato pelos ensinamentos, pelas oportunidades e conhecimentos repassados durante todo esse tempo. Espero um dia poder retribuir tudo.

Ao **Prof. Adj. Dr. Luciano Tavares Ângelo Cintra**, pela generosidade em compartilhar seus ensinamentos durante toda a graduação e pós-graduação. Por ter

me aceitado no início do doutorado como seu orientado por um breve período. Pela sua dedicação e excelente didática com as aulas. Pelo carinho com que comanda a pós-graduação e sua pronta disposição em nos ajudar com as correções de artigos científicos.

Ao **Prof. Assist. Dr. Gustavo Sivieri de Araújo**, pela amizade iniciada durante seu pós-doutorado em 2012, quando comecei minha iniciação científica com uma parte do seu projeto, e que permanece até hoje. Pela paciência e educação com que trata não só a mim, mas todos os alunos do departamento. Agradeço também por ter aceitado participar da banca do meu EGQ de doutorado e desta defesa de tese.

Aos **Profs. Dr. Mauro Juvenal Nery** e **Dra. Carla Renata Sipert**, por serem peças importantes durante minha graduação, sendo meus primeiros professores de endodontia no longínquo 2012, tendo me feito apaixonar pela especialidade à primeira vista. A alegria e paciência com que transmitiram seus ensinamentos a mim e aos alunos são memórias e exemplos que levarei para minha carreira profissional. Agradeço também à **Profª. Carla** pelo pronto aceite ao meu convite para ser banca desta defesa de tese, tendo eu o privilégio e a satisfação em poder contar com as suas correções e observações.

Aos demais docentes da disciplina de Endodontia da Faculdade de Odontologia de Araçatuba – UNESP, **Prof. Adj. Dr. Eloi Dezan Junior**, **Prof. Adj. Dr. Rogério de Castilho Jacinto** e **Prof. Dr. José Arlindo Otoboni Filho**, pela amizade construída, pelas inúmeras oportunidades de monitoria oferecidas, aulas, experiências clínicas e apoio compartilhados durante minha formação.

À colega de pós-graduação e **Profª. Dra. Francine Benetti,** por todo o auxílio e disponibilidade em ajudar incansavelmente seus colegas no laboratório, e como professora pelo pronto aceite em colaborar com a arguição da minha defesa de tese de doutorado. Fran, você é uma pessoa incrível, por onde você passa deixa sua marca e cativa a todos positivamente.

Ao **Prof. Assist. Dr. Edilson Ervolino**, pela educação e disponibilidade em utilizar seus laboratórios e materiais para análise imunohistoquímica realizada neste trabalho. Sua competência e reconhecimento por outros professores é uma inspiração para mim.

Ao **Prof. Assist. Dr. Antônio Hernandes Chaves Neto**, obrigado por sempre manter o seu laboratório disponível e de portas abertas para mim durante a análise bioquímica, pela consideração e por sempre estar disposto em me ajudar.

To **Prof. Hajime Sasaki**, thank you for hosting me during the sandwich PhD for a year into your laboratory at the University of Michigan. Even with cultural differences and some difficulties in living together, I will be eternally grateful for the opportunity to make my dream come true.

À amiga de pós-graduação e **Profª. Dra. Mariane Maffei Azuma**, pessoa fundamental durante todo o meu doutorado, principalmente durante o doutorado sanduíche na Universidade de Michigan. Obrigado pela ajuda na conexão com o Prof. Sasaki enquanto eu estava no Brasil e por todo o apoio nos Estados Unidos, juntamente com seu marido **Alex Presse**, ambos foram fundamentais para minha estadia no exterior, nas mais diversas adversidades, principalmente durante a pandemia. No

laboratório sei que fomos fundamentais um para o outro superar os perrengues e contratempos. Me inspiro demais na sua caminhada e espero um dia alcançar as suas conquistas. Tenho certeza de que sua carreira será brilhante onde quer que esteja, e desejo toda a sorte do mundo na sua nova jornada!

Ao colega de pós-graduação e **Prof. Dr. Leopoldo Cosme Silva** pela parceria de sucesso durante o doutorado. Obrigado por ser o melhor amigo que eu como pós-graduando poderia ter! Sempre ajudando nos experimentos, no delineamento de projetos e na escrita de artigos. Fizemos uma parceria de sucesso que com certeza tem muito pela frente ainda. Sei que a nossa amizade é verdadeira, e para todas as amizades verdadeiras o tempo nunca passa, as distâncias nunca existem, não importa onde a gente sempre estaremos próximos.

Aos pais da Letícia, **Lúcia** e **Marcos**, por serem minha segunda família, por toda prestatividade e carinho comigo. À **Bruna**, **Guilherme** e minha afilhada **Maria Clara**, pela amizade, apoio e alegria que este pequeno ser provoca em nós.

Aos meus amigos de graduação e pós, companheiros de república, **Dr. Luis Felipe Pupim** e **Dr. Ronaldo Cruz**, pela vida em harmonia e alegria que tínhamos em casa, onde tornaram-se verdadeiros irmãos aqui em Araçatuba, os quais levarei por toda a vida.

Aos meus amigos de graduação e pós **Hiskell** e **Rodrigo** pelo tempo que passamos juntos compartilhando experiências, dificuldades, sonhos, desejos e infinitas conversas nos grupos de mensagem. Kell, caminhamos juntos desde 2010 nesta longa jornada universitária, muito obrigado pela sua amizade, ela foi fundamental para eu

estar hoje aqui! Tenho certeza de que suas carreiras serão de muito sucesso!

Aos funcionários do Departamento de Odontologia Preventiva e Restauradora da Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" - **Peterson Moura**, **Carlos Suetake** e **Jorge Luis Trevelim** pela amizade, paciência, e toda ajuda necessária a mim prestada durante o doutorado.

Aos funcionários da Seção Técnica de Graduação e Pós-Graduação da Faculdade de Odontologia de Araçatuba - UNESP, **Valéria Queiroz Marcondes Zagatto**, **Lílian Sayuri Mada** e **Cristiane Regina Lui Matos**, pela eficiência e profissionalismo.

Finalizo meus agradecimentos endereçando ao grande grupo de amigos que fiz desde o início da Iniciação Científica em 2012 que, direta ou indiretamente, me ajudaram de alguma maneira para que este dia fosse possível: **Amanda Andolfatto**, **Ana Maria Vasques**, **Ana Claudia Rodrigues**, **Ana Paula Ribeiro**, **Carlos Bueno**, **Carolina de Barros**, **Caroline Loureiro**, **Christine Men Martins**, **Cristiane Cantiga**, **Diego Valentim**, **Flávia Plazza**, **Flávio Duarte**, **Henrique Banci**, **Índia Queiroz**, **Isabela Prado**, **Karina Caiaffa**, **Letícia Citelli**, **Loiane Massunari**, **Marina Carminatti**, **Marina Cury**, **Marjorie Gallinari**, **Nathália Machado**, **Paulo Tobias**, **Pedro Henrique Chaves** e **Vanessa Marques**. Obrigado por deixarem os meus dias mais alegres e por compartilharem o conhecimento de vocês. Tenho imenso orgulho e honra em ter trabalhado com pessoas talentosas com tantas qualidades como vocês!

Epígrafe

"Hard work beats talent when talent doesn't work hard."

Tim Notke

Dal-Fabbro, R. **Efeito do consumo de vinho tinto no desenvolvimento da periodontite apical induzida em ratos**. 55 f. 2021. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2021.

RESUMO

Objetivo: Avaliar o efeito do consumo de vinho tinto ou de seus polifenóis nos processos de inflamação / reabsorção associados à periodontite periapical (PP) em ratos. Metodologia: Trinta e dois ratos Wistar com 3 meses de idade tiveram a periodontite periapical induzida nos quatro primeiros molares, dispostos em quatro grupos: controle (C) - ratos com periodontite periapical; vinho (W) - ratos com PP recebendo 4,28 mL/kg de vinho tinto; resveratrol + quercetina (R+Q) - ratos com PP recebendo 4,28 mL/kg de solução contendo 1,00 mg/L de quercetina e 0,86 mg/L de resveratrol; e álcool (ALC) - ratos com PP recebendo a mesma dose alcoólica contida no vinho. Os tratamentos por gavagem foram administrados diariamente, do início ao 45º dia. No 15º dia a PP foi induzida e no 45º dia os animais foram eutanasiados. Foram realizadas análises histológicas, imunohistoquímica para RANKL, OPG, TRAP, IL-10, TNF-α e IL-1β e por microtomografia computadorizada nas mandíbulas. O teste de Kruskal-Wallis com Dunn's foi realizado para dados não paramétricos e o teste ANOVA com Tukey's para dados paramétricos, $p < 0,05$. Resultados: A mediana do escore do processo inflamatório foi menor no grupo R + Q (1) em comparação aos grupos C (2) e ALC (3), e não diferente do grupo W (1.5). Embora os grupos W e R+Q tenham apresentado menor pontuação para RANKL, TNF-α e IL-1β, não foram encontradas diferenças em relação aos grupos C e ALC. A marcação imunológica para OPG foi maior no grupo R+Q em comparação com todos os grupos; o mesmo observado para IL-10, sendo diferente dos grupos C e ALC. O grupo R+Q apresentou a menor contagem de células TRAP, seguido pelo

grupo W, ambos inferiores aos grupos C e ALC, que apresentaram os piores resultados. O menor valor de reabsorção óssea foi no grupo R+Q (0,50 mm^3 ± 0,21 mm^3), inferior ao grupo C (0,88 mm^3 ± 0,10 mm^3). O grupo W (0,60 mm^3 ± 0,25 mm^3) e o grupo R+Q apresentaram menor reabsorção óssea em comparação com o grupo ALC (0,97 mm^3 ± 0,22 mm^3). Conclusão: A administração de vinho tinto reduziu a inflamação oriunda da PP, a marcação TRAP e a reabsorção óssea periapical em comparação ao ALC; a administração de resveratrol-quercetina reduziu o processo inflamatório da PP, a reabsorção óssea periapical e alterou a expressão de OPG, IL-10 e TRAP em comparação aos grupos C e ALC.

Palavras-chave: Periodontite Periapical; Vinho; Resveratrol; Quercetina; Polifenóis

Dal-Fabbro, R. **Effect of red wine consumption on the induced apical periodontitis development in rats**. 55 f. 2021. Tese (Doutorado) - Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2021.

ABSTRACT

Aim: To evaluate the effect of red wine consumption or its polyphenols on the inflammation/resorption processes associated with periapical periodontitis (PP) in rats. Methodology: Thirty-two 3-month-old Wistar rats had the PP induced in the four first molars, arranged into four groups: control (C) - rats with PP; wine (W) - rats with PP receiving 4.28 mL/kg of red wine; resveratrol+quercetin (R+Q) - rats with PP receiving 4.28 mL/kg of solution containing 1.00 mg/L of quercetin and 0.86 mg/L of resveratrol; and alcohol (ALC) - rats with PP receiving the alcoholic dose contained in the wine. The oral gavage treatments were daily administered, from day 0 to day 45th. At the 15th day the PP was induced, and at the 45th day the animals were euthanized; histological, immunohistochemical (RANKL, OPG, TRAP, IL-10, TNF-α and IL-1β), and micro-computed tomography analysis were performed in the jaws. The Kruskal-Wallis with Dunn's test was performed for nonparametric data, and the ANOVA with Tukey's test for parametric data, $p<0.05$. Results: The median score of inflammatory process was statistically lower in the R+Q group (1) compared to the C (2) and ALC (3) groups, and not different from the W (1.5) group. Although the W and R+Q groups had a lower score for RANKL, TNF-α and IL-1β, no differences were found compared to C and ALC. The immunolabelling for OPG was higher in the R+Q group compared to all groups; the same observed for IL-10, being different from groups C and ALC. The R+Q group had the lowest TRAP cell count, followed by the W group, both inferior to C and ALC groups, which presented the worst results. The lowest bone resorption value was in the R+Q group

($0.50mm^3 \pm 0.21mm^3$), statically lower than the C group ($0.88mm^3 \pm 0.10mm^3$). The W group ($0.60\ mm^3 \pm 0.25\ mm^3$) and R+Q group showed less bone resorption compared to the ALC group ($0.97mm^3 \pm 0.22mm^3$). Conclusion: Red wine administration led to lowers PP inflammation, TRAP marking, and periapical bone resorption compared to ALC; resveratrol-quercetin administration reduced the PP inflammatory processes, periapical bone resorption, and altered the OPG, IL-10, and TRAP expression compared to C and ALC groups.

Keywords: Periapical Periodontitis; Wine; Resveratrol; Quercetin; Polyphenols

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LISTA DE ABREVIATURAS

COX	Cyclooxygenase
DAB	3,3′-di-aminobenzidine tetrahydrochloride
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-related kinase
ESI-MS	Electrospray ionization/mass spectrometry
HPLC/DAD	High-performance liquid chromatographic/diode array detector
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
LDL	Low-density lipoprotein
LOX	Lipoxygenase
NF-κB	Nuclear factor kappa B
NOS	Nitric oxide synthase
OPG	Osteoprotegerin
PP	Periapical periodontitis
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
SIRT1	Silent mating type information regulation 2 homolog 1
TGFB	Transforming growth factor beta
TNF-α	Tumor necrosis factor *alpha*
TRAP	Tartrate-resistant acid phosphatase
μCT	Micro-computed tomography

SUMÁRIO

EFFECT OF RED WINE OR ITS POLYPHENOLS ON INDUCED PERIAPICAL PERIODONTITIS IN RATS

Artigo submetido e em revisão 1 no periódico International Endodontic Journal em 02/07/2021 (Anexo B)

INTRODUCTION

Red wine is a popular worldwide drink culturally known as beneficial for the body when ingested in adequate amounts (Artero et al. 2015). It is mainly composed of water, ethanol, glycerol, polysaccharides, different acids, and phenolic compounds (Snopek et al. 2018). The good properties of red wine such as the cardioprotective potential, the inhibition of the low-density lipoprotein (LDL) oxidation, and endothelial dysfunction prevention occur at the expense of wine polyphenols, especially resveratrol, anthocyanins, and catechins, which are the most effective wine antioxidants (Haseeb et al. 2017). The phenolic compounds of wine can be divided into flavonoids and non-flavonoids, and the precise content of each one is dependent on elements, such as the grape variety, and manufacturing technique; but it is a fact that red wine contains 10-fold more phenolic compounds than white wine (Waterhouse 2002). In addition to the polyphenols, the low alcohol content is pointed out as one of the responsible for the beneficial effect that the beverage can exert (Golan et al. 2019). In general, the beneficial effect of regular and moderate wine consumption is obtained with approximately 150 ml/day for women and 300 ml/day for men, as defined by previous studies and by the Dietary Guidelines for Americans, 2020-2025 (Rotondo et al. 2001, Pavlidou et al. 2018, United States. Department of Health and Human Services. et al. 2020).

Resveratrol (3,5,4'-trihydroxystilbene) is a nonflavonoid polyphenol present in red wine and in foods that are found commonly in the human diet, such as strawberry, blueberry, mulberry, grapes, grape juice, peanuts, and dark chocolate (Das & Das 2007). It started to gain salience in 1992 when it was postulated to explain some of the cardioprotective effects of red wine consumption, called "French Paradox", which described the inverse relationship between coronary heart disease mortality and the predominantly red wine consumption seen in France (Renaud & de Lorgeril 1992). Since then, the substance has been extensively investigated in the health field, showing benefits regarding cancer prevention, neuroprotection, cardiovascular disease, ischaemic injuries, anti-aging, enhance stress resistance and extend the lifespans of various organisms (Rauf

et al. 2018, Galiniak *et al.* 2019, Li *et al.* 2019). Specifically in bone tissue, the non-flavonoid resveratrol acts by inducing SIRT1 and regulating RUNX2, inducing in this way the osteoblasts differentiation. It also suppresses the NF-κB activation, leading to lesser differentiation and osteoclastic activity (Manna *et al.* 2000, Pandey *et al.* 2018).

In addition to resveratrol, the flavonoid quercetin (3, 3', 4', 5, 7-pentahydroxyflavone) is also found in red wine, as well as in vegetables, fruits, and teas, being one of the most prominent dietary antioxidants in health research (Boots *et al.* 2008). Its properties have been gaining notoriety due to antioxidant, anti-inflammatory, anti-tumor, metabolic regulation, and neuroprotective activities (Li *et al.* 2016, Khan *et al.* 2019, Reyes-Farias & Carrasco-Pozo 2019, Wong *et al.* 2020). In bone tissue, this flavonoid has been reported to decrease osteoclastogenesis via inhibition of the activating receptor for nuclear factor-kB ligand (RANKL), involved in osteoclastic differentiation (Wattel *et al.* 2004), in addition to the ability to directly induce apoptosis of mature osteoclasts (Wattel *et al.* 2003). Quercetin is also believed to activate osteoblasts, either by activating the TGF beta signaling pathway, p38 mitogen-activated protein kinases, and WNT/β-Catenin pathways, thus regulating bone metabolism (Yamaguchi & Weitzmann 2011, Casado-Díaz *et al.* 2016, Pandey *et al.* 2018). These studies demonstrate that quercetin has great potential to be used as a bone health supplement.

Studies specifically evaluating the influences of red wine on the inflammatory response and bone tissue are important, but so far scarce. These investigations fall on the epidemiological studies on alcohol consumption, and only few of them specifiy the type of alcoholic beverage. The wine is proposed to act more beneficially than other beverages, due to the richest phenolic components allied to a low alcohol content, which would facilitate the absorption of these phenolic compounds (Kutlesa & Budimir Mrsic 2016). To date, a single study has evaluated the effect of red wine and its major components on periodontitis and showed that animals exposed to red wine presented a lower occurrence of spontaneous periodontitis, and lower levels of TNF-α and C-reactive protein (Wagner *et al.* 2019).

Periapical periodontitis (PP) is the product resulting from persistent bacterial contamination of the root canal system in the face of combat led by the host's immune system, characterized by an inflammation of periradicular tissues (Kakehashi *et al.* 1965). When the dental pulp becomes

infected, bacteria and their byproducts evoke nonspecific inflammatory responses, as well as specific immunological reactions, leading to the destruction of bone by osteoclasts and resorption of dental hard tissues (cementum and dentin) by multinucleated cells designated as odontoclasts (Nair 1997, Liapatas *et al.* 2003). Red wine and the isolated polyphenols (resveratrol and quercetin) have been established to alter the functioning of bone tissue and the immune system, both crucial elements for the development of PP (Das & Das 2007, Wong & Rabie 2008b, Wong & Rabie 2008a, Pervaiz & Holme 2009, Li *et al.* 2016, Esteban-Fernández *et al.* 2017, Wong *et al.* 2020).

Considering the evidence above and that no study evaluating the effects of wine or the polyphenols consumption on the development of PP has been found, this study aimed to evaluate the effect of red wine or a polyphenols solution consumption on PP induced in rats. Thus, the null hypothesis tested in this study was that exposure to wine or resveratrol associated with quercetin does not alter the size and severity of PP.

MATERIAL AND METHODS

Animals

Thirty-two three-month-old adults male Wistar rats *(Rattus norvegicus albinus)*, weighing between 250-300g, were used. The animals were maintained in a temperature-controlled environment (22 °C ± 1 °C, 70% humidity) with a 12h light-dark cycle and ad libitum access to water and food. All experimental protocols were approved by the Institutional Ethics Committee on Animal Use (00154-2019) of Universidade Estadual Paulista, São Paulo, Brazil. The general health condition was weekly evaluated. The animals were randomly arranged into four groups (n=8): Control (C) - rats with periapical periodontitis; Wine (W) - rats with periapical periodontitis receiving wine; Resveratrol + Quercetin (R+Q) - rats with periapical periodontitis receiving a solution with resveratrol and quercetin; and Alcohol (ALC) - rats with periapical periodontitis receiving an alcoholic solution. All the treatments were conducted through oral gavage for forty-five days, starting fifteen days before induction of periapical periodontitis, and extending for thirty more days after induction of injury (Figure 1).

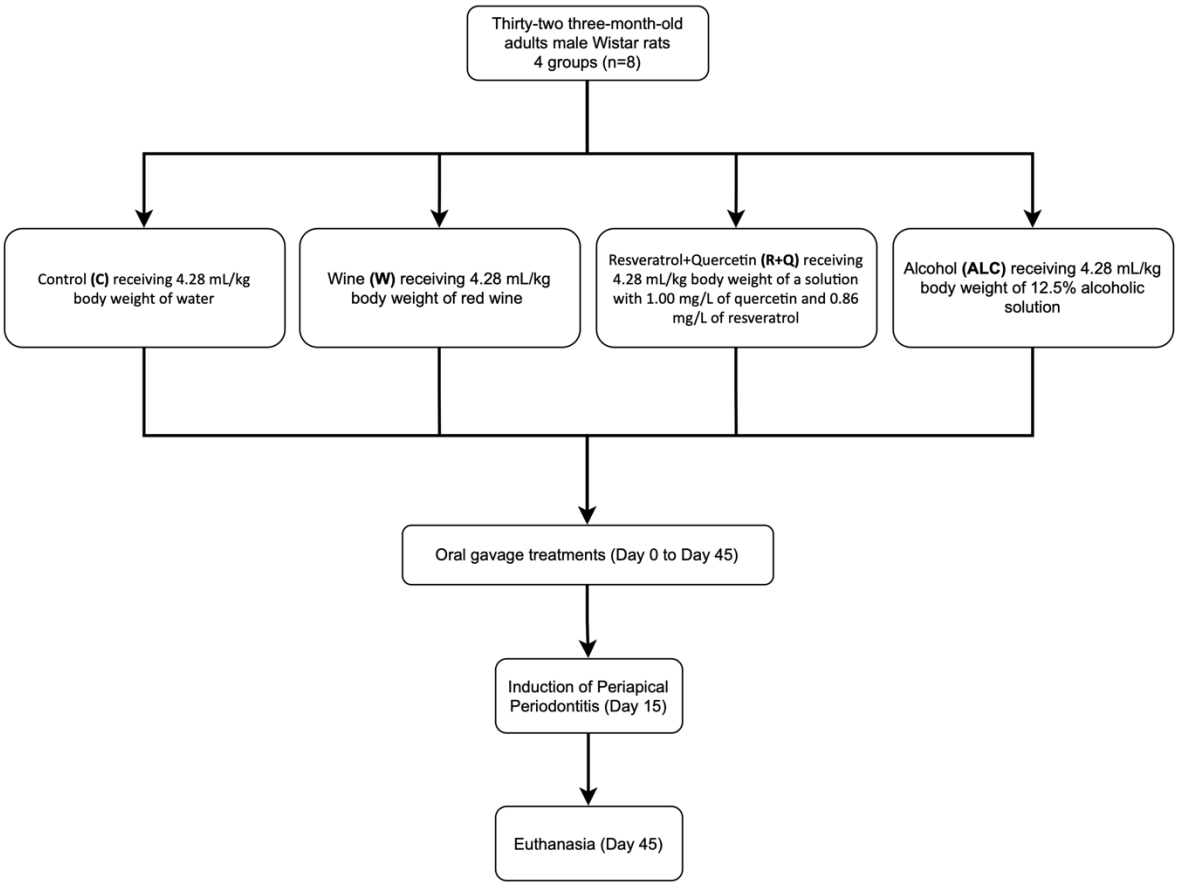

FIGURE 1 - Flowchart showing the experimental stages and their order fulfillment.

Sample size calculation

The sample size was estimated based on the parameters used in previous studies (Cintra *et al.* 2014). Using an alpha error of 0.05% and 95% power to recognize a significant difference, a minimum of seven animals per group was considered necessary. Taking into consideration possible complications that could occur during the study, one more animal was added in each group, resulting in eight rats per group, summing thirty-two animals.

Quantification of the phenolic compounds present in the red wine

The total phenolic compounds present in the red wine Miolo Seleção ® (Miolo Wine Group Vitivinicultura S.A, Campanha, RS, Brazi) were determined using a high-performance liquid chromatographic/diode array detector (HPLC/DAD) coupled with electrospray ionization/mass

spectrometry (ESI-MS). For quantification, the wine sample was diluted three times with a solution of water acidified with 0.2% formic acid and filtered using a nylon filter with 0.45 μm porosity. The quantification of phenolic compounds was performed using a high-performance liquid chromatograph (Shimadzu LC-20T, Shimadzu Corporation, Tokyo, Japan), equipped with a diode array detector, degassing system, autosampler, and oven column. Chromatographic separation was performed on a reverse-phase column (C18, 150 mm x 4.6 mm, particle size 3.5 μm (Agilent Technology, Santa Clara, CA, United States). The mobile phase consisted of water acidified with 0.2% formic acid (v/v) (mobile phase A) and acetonitrile (mobile phase B). For chromatographic separation, the gradient elution used was composed of 0 min 100% A, 10 min 88% A, 15 min 45% A, 17 min 65% A, 23 min 100% A, 30 min 100% A. The temperature of the column was maintained at 40 ° C, the sample injection volume was 10 μL, with a flow rate of 0.5 mL/min. The wavelengths used were 306 nm for t-resveratrol and 360 nm for quercetin. Quantification was performed by external calibration, with seven concentrations equidistant from the analytical standards (Nixdorf & Hermosin-Gutierrez 2010, Lago-Vanzela *et al.* 2011a, Lago-Vanzela *et al.* 2011b). The concentrations of resveratrol (0.86 ± 0.02 mg / L) and quercetin (1.00 ± 0.02 mg / L) found in the analyzed red wine were used to prepare the solution administered to the R+Q group.

Oral gavage treatments

The animals from the Control group (C) received water at 4.28 mL/kg body weight through gavage to mimic the same procedure suffered from the other animals. The animals from the Wine group (W) received 4.28 mL/kg body weight of red wine (Miolo Seleção ®, Wine Group Vitivinicultura S.A) (Schmatz *et al.* 2013). For the animals from Resveratrol + Quercetin group (R+Q) a solution with 1.00 mg/L of quercetin and 0.86 mg/L of resveratrol was prepared and administered in the same volume to red wine (4.28 mL/kg body weight). The animals from the Alcohol group (ALC) received 4.28 mL/kg body weight of an alcoholic solution containing 12.5% alcohol by volume (the same amount of alcohol present in the wine administered to group W), prepared by diluting absolute ethyl alcohol in water (Dal-Fabbro *et al.* 2019b).

Induction of periapical periodontitis

Fifteen days after the oral gavage has been started as previously described, rats were subjected to dental pulp exposure (Astolphi *et al.* 2013, Cosme-Silva *et al.* 2019). Briefly, rats were

anesthetized with 87 $mg.kg^{-1}$ ketamine (Dopalen, Ceva Santé Animale Ltda, Paulínia, SP, Brazil) and 13 $mg.kg^{-1}$ xylazine (Xilazina, Vencofarma do Brasil Ltda, Londrina, PR, Brazil) by intramuscular injection and placed on a jaw-retraction board. All four first molars had the dental pulps surgically exposed using a no. 1/4 dental round bur (Jet Carbide, Kavo Kerr Group, Orange, CA, USA) using an electric handpiece. The exposure size consisted in approximately equivalent to the diameter of the bur. The access cavity was left open to the oral cavity after removal of the pulp tissue using an endodontic file, allowing contamination of root canals with oral commensal microorganisms. Rats were killed on day 30 after pulp exposure.

Sample collection

The animals were killed with an overdose of anaesthetic solution (Thiopentax, Cristalia Produtos Quimicos Farmaceuticos Ltda., São Paulo, Brazil). After, the right and left side jaws were removed, stored in a 4% buffered formaldehyde solution, and subsequently sent for microtomographic and histological/immunohistological analysis respectively.

Histological/Immunohistochemical analysis

Fixed left side semi-jaws were decalcified in 10% EDTA solution, embedded in paraffin, and sectioned at 6 μm thickness following a general histology protocol. Haematoxylin and Eosin (H&E) staining were used for the analysis of the inflammatory profile and condition of the periapical region of the first mandibular molar. Histopathological analysis was conducted following the guidelines of quality of inflammation and the cellularity pattern of dental and periodontal tissues to score the inflammatory infiltrate as follows: low (0 to few inflammatory cells, score = 0), mild (<25 cells, score = 1), moderate (25– 125 cells, score = 2) and severe (>125 cells, score = 3) (Cintra *et al.* 2016). The immunohistochemical analysis was performed using the immunoperoxidase technique as previously described (Matheus *et al.* 2020). Concisely, histological sections were arranged into six batches, incubated with one of the following primary antibodies 1:100 diluted: Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL) (Goat anti-RANKL - SC7627; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Osteoprotegerin (OPG) (Rabbit anti-OPG - SC11383; Santa Cruz Biotechnology), Tumor Necrosis Factor – Alpha (TNF-α) (Goat anti-TNF-α SC1348, Santa Cruz Biotechnology), Interleukin 1-beta (IL-1β) (Rabbit - orb101745 Biorbyt, San Francisco, CA, USA), Interleukin 10 (IL-10) (Rabbit - orb221323,

Biorbyt), and Tartrate-Resistant Acid Phosphatase (TRAP) (Goat anti-TRAP - SC30832; Santa Cruz Biotechnology). In sequence, biotinylated secondary universal antibodies were applied to the slices for 2 hours followed by a streptavidin–horseradish peroxidase conjugate for 2 hours (Universal Dako-Labeled Streptavidin-Biotin Kit; Dako Laboratories, Carpinteria, CA). Lastly, the slices received the chromogen 3,3'-di-aminobenzidine tetrahydrochloride (DAB chromogen Kit - Dako Laboratories). The negative controls contained sections following the same protocol described above, excluding the application of the primary antibody. Semiquantitative immunolabeling analyses of RANKL, OPG, TNF-α, IL-1β, and IL-10 through the designation of the ensuing score system: 0 = absence of immunoreactive (IR) cells; 1 = low immunolabeling of both IR cells and extracellular matrix; approximately one-quarter of the field; 2 = moderate immunolabeling of both IR cells and extracellular matrix; approximately one half of the field and 3 = high immunolabeling of both IR cells and extracellular matrix; approximately three-quarters of the field (Azuma *et al.* 2017). The TRAP analysis was performed as follows, first surrounding the entire perimeter of bone resorption resulting from periapical periodontitis, followed by counting the positive multinucleated TRAP cells. Lastly, the ratio between TRAP cells and perimeter size was obtained (Gomes-Filho *et al.* 2015). All the analyses were performed by a single-certified histologist using a light microscope (DM 4000 B; Leica) and a colour camera (DFC 500; Leica, Wetzlar, Germany) blinded regarding the groups.

Micro-computed tomography

Fixed right side semi-jaws were scanned as previously described using a cone beam-type tomograph (Bruker SkyScan 1272, Aartselaar, Belgium) using the following parameters: 50 kV, 800 μA, 1º rotation step, 7000 ms exposure, 3 frame averages, 11 a pixel image and 13 a pixel camera size (Cosme-Silva *et al.* 2020). In brief, the images were reconstructed using the software NRecon (SkyScan, Belgium) and the alveolar bone volume loss were calculated using the CTAn software (SkyScan, Belgium) and expressed in cubic millimeter. The region of interest (ROI) comprised the empty space of periradicular resorption area, containing the root canal and apical foramen, starting from the first transversal cut showing a beginning in the resorption of distal root periapical bone, continued through the apex of the root, ending at the last slice where the empty space was seen.

Statistical analysis

The data were analyzed using GraphPad Prism 8 software (La Jolla, CA, USA). After the test of normality, the Kruskal-Wallis with Dunn's multiple comparisons test was performed for nonparametric data, and the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was performed for parametric data. The level of significance was 5%.

RESULTS

Histological analysis

The inflammatory scores and the representative haematoxylin–eosin-stained sections are shown in Figures 2 and 3. All the animals were submitted to pulp exposure surgically and, consequently, showed pulp necrosis with an inflammatory infiltrate in the periapical region consisted mainly of neutrophils and mononuclear cells, concomitantly with bone destruction, confirming the effectiveness of the method thirty days after pulp exposure. The C group presented moderated levels of inflammation, attributed a median of score 2, significantly greater than that presented by the R+Q group, which showed a mild inflammatory process in the periapical region, with a median score of 1, $p<0.05$. There was no statistical difference when we compared the W group (median of scores 1.5) with Control and neither with R+Q, $p>0.05$. Moreover, the ALC group evidenced the highest median score of inflammation (3), significantly higher than the inflammatory process found in groups W and R+Q groups, $p<0.05$.

Immunohistochemical analysis

The immunoreactivity pattern for IL-10, RANKL, OPG, TNF-α, IL-1β, and TRAP are described below and in Figures 2 and 4. The R+Q group presented moderate immunoreaction for IL-10 expression (median of score 2), being significantly higher than that presented by groups C and ALC (median of score 1), $p<0.05$; although the W group presented the same median as the R+Q group, the statistical analysis showed no differences when compared to the other groups. With regard to OPG, all groups showed mild immunoreaction (score 1), except for the R+Q group, which showed a significantly higher expression of this osteoclastogenesis inhibitory factor (score 2), when compared with the other three groups, $p<0.05$. Although the groups W and R+Q had fewer

immunoreactive cells for RANKL, TNF-α and IL-1β, these differences were not significant, $p>0.05$. The TRAP immunolabelling technique applied was specific to identify osteoclasts. The W and R+Q groups had a significantly lower load of TRAP-positive multinucleated cells per mm of the perimeter in the periapical region, 2.44 ± 0.53 and 1.88 ± 0.36, respectively, when compared to groups C (3.07 ± 0.35) and ALC (3.95 ± .048), $p<0.05$. The ALC was significantly more TRAP inductive than the C group, $p<0.05$. No difference was found when comparing W and R+Q groups.

Micro-computed tomography analysis

The micro-CT slices and data are shown in Figures 2 and 3. All animals subjected to pulp exposure showed increased periapical hypodense areas on microtomography by day 30, proving the development of periapical periodontitis. The R+Q group had bone resorption mean of 0.50 mm^3 ± 0.21 mm^3, significantly lower than the bone resorption volume presented by groups C (0.88 mm^3 ± 0.10 mm^3) and ALC (0.97 mm^3 ± 0.22 mm^3), $p<0.05$. The W group showed a volume of bone resorption of (0.60 mm^3 ± 0.25 mm^3), significantly lower than that resorbed in the ALC group, $p<0.05$, however without significant differences when compared to groups C and R+Q.

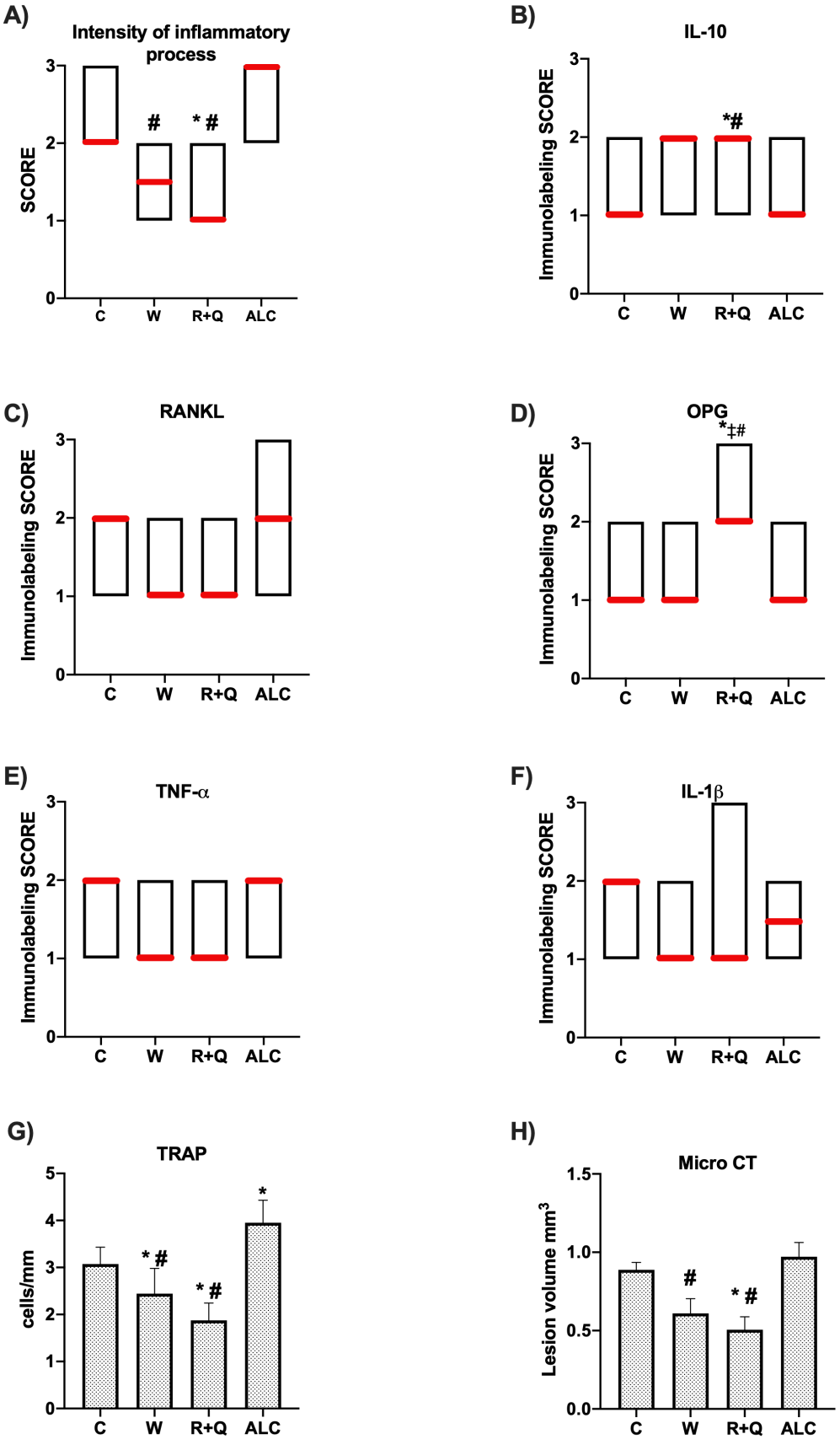
A)
Intensity of inflammatory process
SCORE
0
1
2
3
#
* #
C
W
R+Q
ALC
B)
IL-10
Immunolabeling SCORE
*#
C)
RANKL
D)
OPG
*‡#
E)
TNF-α
F)
IL-1β
G)
TRAP
cells/mm
*
H)
Micro CT
Lesion volume mm3
0.0
0.5
1.0
1.5
4
5

FIGURE 2 - A) Chart showing scores for the intensity of inflammatory process; red line indicates the median of each group. B) Chart showing scores for the IL-10 immunolabeling; red line indicates the median of each group. C) Chart showing scores for the RANKL immunolabeling; red line indicates the median of each group. No significant differences were present between the groups. D) Chart showing scores for the OPG immunolabeling; red line indicates the median of each group. E) Chart showing scores for the TNF-α immunolabeling; red line indicates the median of each group. No significant differences were present between the groups. F) Chart showing scores for the IL-1β immunolabeling; red line indicates the median of each group. No significant differences were present between the groups. G) Bar graph for positive TRAP cells per millimeter perimeter of periapical periodontitis showing mean and standard deviation for each group. H) Bar graph for lesion volume showing mean and standard deviation for each group. Symbols: *: $p<0.05$ vs. Control; ‡: $p<0.05$ vs. Wine; #: $p<0.05$ vs. Alcohol.

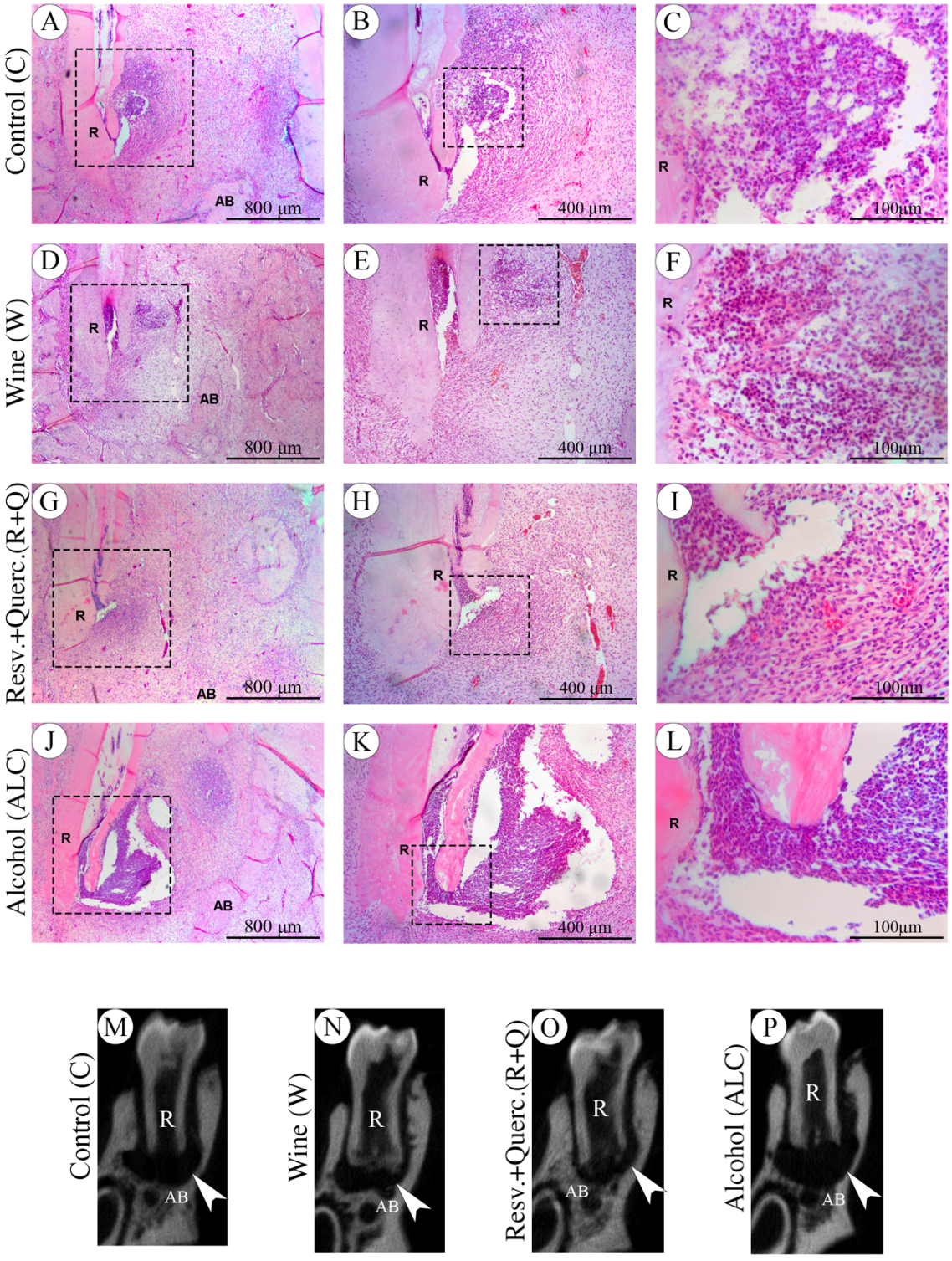
Control (C)
Wine (W)
Resv.+Querc.(R+Q)
Alcohol (ALC)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
R
AB
800 µm
400 µm
100µm

FIGURE 3 - Photomicrographs showing histological aspects of periapical regions around the foraminal opening of the distal root (R) of the lower first molar (A-L). Moderate inflammatory process can be observed in the Control group (A, B, and C at 50x, 100x, and 400x magnification, respectively). Mild to moderate inflammatory process can be observed in the Wine group (D, E, and F at 50x, 100x, and 400x magnification, respectively). Mild inflammatory process can be observed in the Resveratrol+Quercetin group (G, H, and I at 50x, 100x, and 400x magnification, respectively). And severe inflammatory infiltrate is observed in the Alcohol group (J, K, and L at 50x, 100x, and 400x magnification, respectively). Haematoxylin and eosin staining. Rectangles indicate the enlarged area in the next magnification. (A, D, G, J) Scale bars: 800 μm, (B, E, H, K) Scale bars: 400 μm, and (C, F, I, L) Scale bars: 100 μm. Micro-computed tomography images (μCT) of periapical periodontitis in distal root (R) sections of the mandibular first molars (M, N, O, P). Note a diminished bone resorption on microtomography in the resveratrol+quercetin group. White arrowheads points to the area of the periapical periodontitis surrounded by radiopaque alveolar bone (AB).

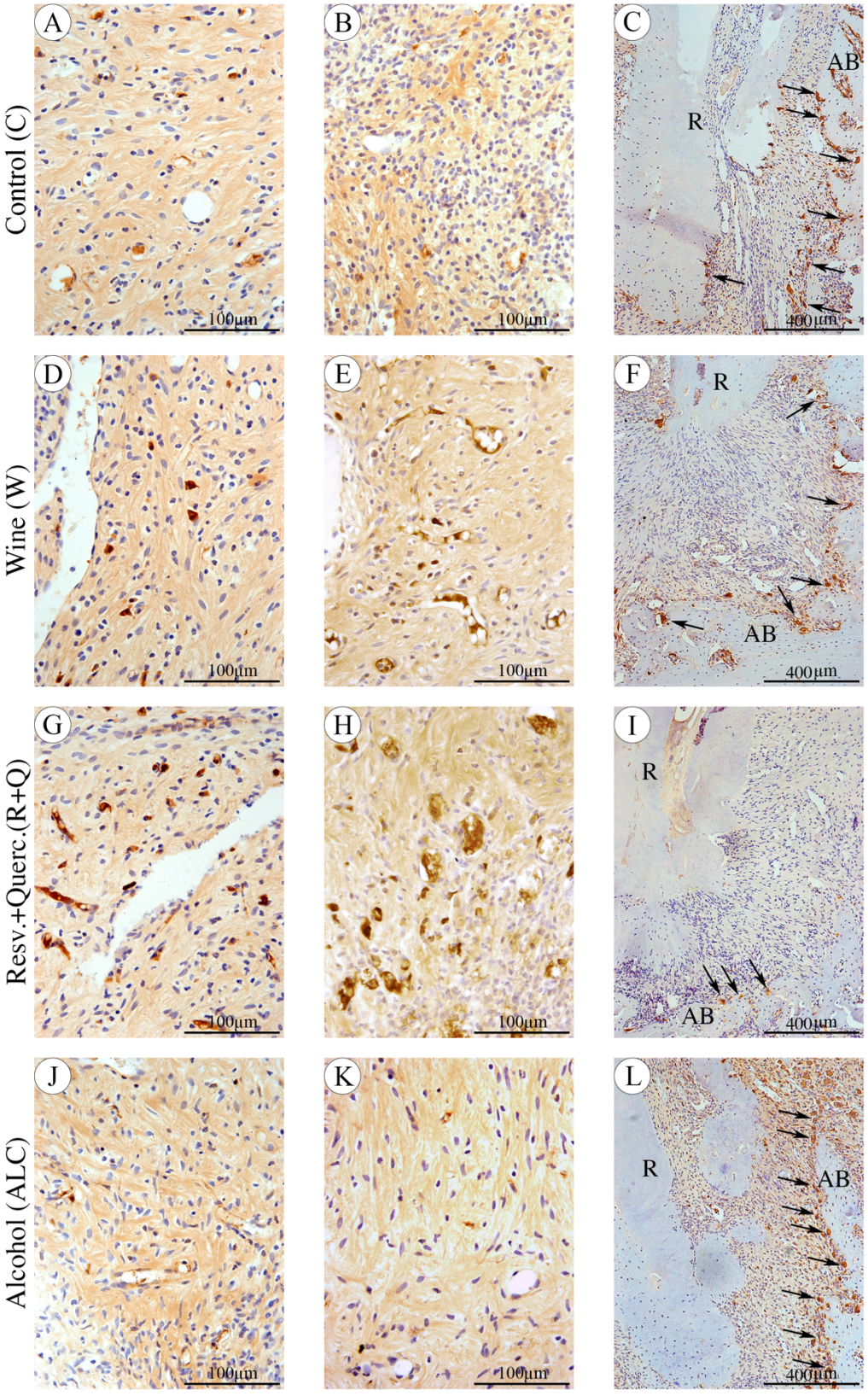
Control (C)
Wine (W)
Resv.+Querc.(R+Q)
Alcohol (ALC)
A
B
C
D
E
F
G
H
I
J
K
L
R
AB
100µm
400µm

FIGURE 4 - Photomicrographs showing the immunostaining for OPG (A, D, G, J), IL-10 (B, E, H, K), TRAP (C, F, I, L) in periapical periodontitis around the foraminal opening of the distal root of the lower first molar in Control group (A, B, C), Wine group (D, E, F), Resveratrol+Quercetin group (G, H, I), and Alcohol group (J, K, L). Counterstaining: Harris haematoxylin. Original magnification for OPG and IL-10: 400X with scale bars: 100 μm, and 100X for TRAP with scale bars: 400 μm. Regarding OPG and IL-10 note a large amount of immunoexpression of these proteins in the R+Q group. For TRAP, black arrowheads points to osteoclastic cells reabsorbing the alveolar bone (AB) located below the opening of the apical foramen. Note a reduced TRAP immunolabeled cells in the R+Q group.

DISCUSSION

Red wine or its polyphenols compounds are consumed by practically the entire world population, regardless of the source. The present study is the first one evaluating the effect of red wine consumption or the association between resveratrol and quercetin through oral gavage on the development of periapical periodontitis in rats, which revealed a beneficial effect of isolated consumption of these polyphenols on the inflammatory process magnitude and bone resorption associated with periapical periodontitis, and the red wine consumption led to less osteoclastic marking than the control, rejecting the null hypothesis. Although light to moderate wine intake seems to have some beneficial effects, there is still a long way to go before definitive recommendations on wine intake can be made (Minzer *et al.* 2020). Furthermore, excessive alcohol consumption represents a public health concern (Schuckit 2009).

The administration model via oral gavage in rats is widely used, since it allows the precise control of the compound amount to be ingested by the animal, differently from the free availability in the water source (Venturini *et al.* 2010, Correa *et al.* 2018). Also, in human studies, the polyphenols investigated may come from countless types of food. Regarding wine consumption, is difficult to determine and remain constant throughout the experimental time, since they are based on the collaboration of the patient regarding the protocol imposed for prospective studies and the accuracy of the answers provided in cases of retrospective studies. The 4.28 mL/kg of body weight dosage of red wine given to the animal in the present study was used based on the general recommendation of consumption of 300 mL/day of red wine for humans weighing seventy kilograms (Rotondo *et al.* 2001, Pavlidou *et al.* 2018). In order to have a homogeneous comparison, the wine used in our investigation was first subjected to the quantification of the polyphenols of interest, through an established methodology (HPLC / DAD / ESI-MS) (Nixdorf & Hermosin-Gutierrez 2010, Lago-Vanzela *et al.* 2011a, Lago-Vanzela *et al.* 2011b). This quantification allowed the group receiving the two associated polyphenols to receive the exact same amount that would be present in the wine dose administered to animals in group W.

The infection induced model through the exposure of the rat's pulp molars was chosen due to the similarity of periapical response to pulp exposure as those seen in humans, once the bacterial infection from the oral environment infects the pulp tissue leading to pulp necrosis, resulting in PP

(Yamasaki *et al.* 1994). The thirtieth-day post-lesion induction is considered an adequate time to assess the extent of the inflammatory process and bone resorption resulting from the injury, once the plateau stage of development has already been reached (Yamasaki *et al.* 1994).

The histological analysis showed an inflammatory process associated with PP of lesser extent and intensity in the R+Q group when compared to the other groups. This finding is in agreement with those already published in the literature proving the anti-inflammatory activity of the two polyphenols. Resveratrol, for instance, protects from inflammation by acting at different phases of inflammation; this anti-inflammatory activity may be performed through the inhibition of both cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) mediated pro-inflammatory signaling, suppression of pro-inflammatory mediator production, acting in nuclear transcription factor-kB (NFkB) in macrophage inhibition, suppressing interleukin-6 release and interleukin-8, which plays an important role in inflammation as it recruits leucocytes to the lesion area (Das & Das 2007). Quercetin, in turn, downregulates nitric oxide synthase (NOS) expression, inhibit matrix metalloproteinases, inhibits the production of inflammation-producing enzymes (COX) and lipoxygenase (LOX), blocks TNF-α, and prevents it from directly activating extracellular signal-related kinase (ERK) and (NF-kB), which are potent inducers of inflammatory gene expression and protein secretion (Li *et al.* 2016).

The alcoholic solution in the 12.5% concentration administered alone presented the worst results, thus excluding the hypothesis that low alcoholic levels could be one of the factors responsible for the beneficial effects of red wine consumption. Results that agree with those already published in the literature, which showed a deleterious effect of 15% and 20% alcohol concentration in PP (Dal-Fabbro *et al.* 2019a, Dal-Fabbro *et al.* 2019b). Although the red wine given in the present study has the same alcohol concentration, the inflammatory infiltrate was significantly lower when comparing these two groups. A possible explanation is that the nonalcoholic compounds present in the red wine, such as polyphenols, may reversed the harmful effect evoked by the alcoholic concentration present in the red wine (de Lorimier 2000).

Even though in the present study no significant reduction in IL-1β and TNF-α levels was achieved through red wine consumption or polyphenols therapy, a propensity to a higher inhibition of these cytokine was observed in these two groups. However, numerous studies confirmed that

resveratrol and quercetin can suppresses the TNF-α release (Takada *et al.* 2004, Lee & Moon 2005, Yuan *et al.* 2018, Lee *et al.* 2019, Chen *et al.* 2020, Wang *et al.* 2020). TNF-α is a proinflammatory cytokine released by macrophages and presents vital role in periodontitis mediated bone loss by inducing the expression of mediators that amplify or sustain the inflammatory response such as prostaglandins and matrix metalloproteinases (Graves & Cochran 2003). Also, IL-1 and TNF-α acts synergistically enhancing bone resorption, and TNF play a critical role in to prepare the innate host response to defend against bacteria, except when overstimulated, which can cause significant collateral damage (Graves & Cochran 2003). Moreover, the pro-inflammatory cytokine IL-1β, primarily secreted by macrophage, is a key regulator of host responses to microbial infection, being frequently found in elevated levels in persistent periapical periodontitis (Yang *et al.* 2018). IL-1β is a potent stimulator of periodontal tissue breakdown, and its properties includes promotion of bone resorption and production of tissue-degrading proteinases (Cheng *et al.* 2020). A possible explanation for the lack of significance in the reduction found in our study may be due the dosage of resveratrol and quercetin administered, since they were lower than those applied by other studies that showed the effectiveness in reducing this interleukin (Napimoga *et al.* 2013, Ribeiro *et al.* 2017).

Moreover, the supplementation with the associated resveratrol and quercetin caused the elevated release of IL-10 in a significant way. Red wine, in turn, also increased the production of IL-10, but without significance, probably due to the presence of alcohol in the drink, hindering the beneficial effects of polyphenols (Gavala et al. 2015). IL-10 is a pleiotropic cytokine with potent anti-inflammatory ability that suppresses both immunoproliferative and inflammatory responses, regulates B-cell proliferation and differentiation, and downregulates vast processes such as the release of proinflammatory cytokines and chemokines, such as IL-1, IL-6, and TNF-α, the production of nitric oxide, and collagenase (Armstrong *et al.* 1996, Sun *et al.* 2019). Moreover, IL-10 affects osteoclast precursors, and inhibits osteoclast activation and has been regarded as a key regulator of bone homeostasis, in homeostatic and inflammatory conditions, once the IL-10 lack in animals leaded to increased femur and alveolar bone loss (Cheng *et al.* 2020). Besides that, Il-10 is recognized as an important suppressor factor for periodontal disease and apical periodontitis development in vivo (Sasaki *et al.* 2000, Sasaki *et al.* 2004). The molecular mechanism of bone loss prevention evoked by the IL-10 is based on the upregulation in OPG expression and downregulation expression of the RANKL (Cheng *et al.* 2020).

The bone tissue is a dynamic complex that constantly undergoes renovation and repair (or remodeling). The cells responsible for this process are osteoblasts, which secrete new bone, and osteoclasts that remove the old ones. Normally, the fine balance between these two cells is in harmony, so there is no increase or loss of bone mass. The control of bone metabolism is a key factor in obtaining a reduction in bone resorption in inflammatory diseases (Hadjidakis & Androulakis 2006). Therefore, the use of substances that are capable of interfering positively in inflammatory processes that lead to bone resorption should be considered.

A number of signaling pathways maintain the activities of osteoblasts and osteoclasts; one of the most important and frequently targeted as a new treatment strategy in bone related-disease conditions is the RANK/RANKL/OPG system (Silva & Branco 2011). The OPG/RANKL ratio is considered an important information to assess the cellular state of bone tissue, since the OPG is an osteoprotective protein, acting by binding to RANKL preventing it from binding to RANK and sequencing osteoclastic formation (Silva & Branco 2011). In the present study, as the OPG-RANKL pathway was shifted towards OPG in group R+Q, due to a higher expression of this protein and no differences in RANKL, less formation of bone resorptive cells occurred. This data is confirmed by the decreased number of positive TRAP multinucleated cells (osteoclasts) per millimeter of PP perimeter in the same group. Reduced periapical bone destruction was evidenced by the μCT analysis in the R+Q group reinforcing the correlation between reduced TRAP-positive cells found on immunohistochemistry, displaying attenuated bone loss due to the lower osteoclast activities in the PP region when compared to control. Red wine consumption also led to less TRAP multinucleated cells when compared to control, but this finding did not reflect in a smaller volume lesion analyzed by μCT. Previous studies reinforce the action of resveratrol and quercetin on OPG (Ribeiro et al. 2017, Ge et al. 2020). In addition to this pathway, others not evaluated in the present study, but already published in other areas, may be related to this diminished bone resorption presented by the phenolic group (Wattel et al. 2004, He et al. 2010). The group receiving alcohol alone showed the highest number of TRAP-positive cells per millimeter of the PP perimeter, confirming the deleterious effect of alcohol consumption on this marker in the PP induced in rats (Dal-Fabbro et al. 2019a, Dal-Fabbro et al. 2019b). Interestingly, when comparing this group with wine, the latter one had a significantly lower cell count, leading us to believe that the polyphenols present in the drink counterbalanced the deleterious effect of alcohol.

A prospective cohort study found that intake of wine is inversely associated with clinical attachment loss in men (Kongstad *et al.* 2008). Another study in southern Brazilian adults showed evidence of a beneficial effect of wine on periodontal status (Susin *et al.* 2015). Previous investigations in ligature-induced periodontitis in animals pointed to promising paths regarding the use of the polyphenols present in the red wine. Firstly, continuous administration of resveratrol decreased periodontal breakdown induced experimentally in rats (Casati *et al.* 2013). Resveratrol administered via oral gavage to rats caused a significant reduction in inflammation-mediated destruction of periodontal soft tissues and bone (Correa *et al.* 2017). When given by subcutaneous injection in the same lesion model, it protected rats from periodontal tissue damage by inhibiting inflammatory responses and by stimulating antioxidant defense systems (Bhattarai *et al.* 2016). The same results observed when administered freely in drinking water (Tamaki *et al.* 2014). Moreover, resveratrol had a positive influence in decreasing periodontal breakdown during smoking in rats (Ribeiro *et al.* 2017). Similar to resveratrol, quercetin exhibited protective effects in bacterial-induced periodontitis, reducing the alveolar bone loss by mechanisms involving the reduction of pro-inflammatory cytokine production and down-regulation of the osteoclastogenic cytokine RANKL (Napimoga *et al.* 2013). In addition, it reduced alveolar bone loss in ligature-induced periodontitis by increasing osteoblastic activity, decreasing osteoclastic activity, apoptosis, and inflammation (Taskan & Gevrek 2020). These data, concomitantly with the findings in our study, spotlight a promising approach to inhibit the development of bone loss during periapical periodontitis development.

Considering the high prevalence of PP throughout life, combined with frequent intake of red wine and the polyphenols through other sources, the present study offers some insights regarding the mechanisms of how these compounds may affect the periapical periodontitis which had not been addressed in the endodontic literature. However, due to some limitations such as the use of an animal model, the resveratrol and quercetin dosage, the ingestion frequency, and time of administration treatment before the periapical injury induction, the results cannot be extrapolated, and therefore, more studies are encouraged considering these relevant parameters.

CONCLUSION

Red wine administration led to lowers PP inflammation, TRAP marking, and periapical bone resorption compared to ALC; resveratrol-quercetin administration reduced the PP inflammatory processes, periapical bone resorption, and altered the OPG, IL-10, and TRAP expression compared to C and ALC groups.

DISCLOSURE STATEMENT

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

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ANEXO A - Comitê de Ética no Uso de Animais (CEUA)

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

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

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Efeito do consumo de vinho tinto no desenvolvimento da periodontite apical induzida em ratos"**, Processo FOA nº 00154-2019, sob responsabilidade de João Eduardo Gomes Filho apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 15 de Março de 2019.

VALIDADE DESTE CERTIFICADO: 31 de Dezembro de 2019.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 31 de Janeiro de 2020.

CERTIFICATE

We certify that the study entitled **"Effect of red wine consumption on the induced apical periodontitis development in rats"**, Protocol FOA nº 00154-2019, under the supervision of João Eduardo Gomes Filho presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on March 15, 2019.

VALIDITY OF THIS CERTIFICATE: December 31, 2019.
DATE OF SUBMISSION OF THE FINAL REPORT: January 31, 2020.

Prof. Ass. Dr. Leonardo Perez Faverani
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

ANEXO B - Comprovante de submissão da revisão 1 no International Endodontic Journal em 02/07/2021

Submission Confirmation

Thank you for your revision

Submitted to	International Endodontic Journal
Manuscript ID	IEJ-21-00267.R1
Title	Effect of red wine or its polyphenols on induced periapical periodontitis in rats
Authors	Dal Fabbro, Renan Cosme-Silva, Leopoldo Oliveira, Fernanda Capalbo, Letícia Plazza, Flávia Ervolino, Edilson Cintra, Luciano Gomes-Filho, João
Date Submitted	02-Jul-2021

ANEXO C - Author Guidelines for Publishing in the International Endodontic Journal

GENERAL

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

MANUSCRIPT FORMAT AND STRUCTURE

Format

Language: The language of publication is English. It is preferred that manuscript is professionally edited.

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

Abbreviations: International Endodontic Journal adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. When non-standard terms appearing 3 or more times in the manuscript are to be abbreviated, they should be written out completely in the text when first used with the abbreviation in parenthesis.

Structure

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

Abstract for Original Scientific Articles should be no more than 350 words giving details of what was done using the following structure:

Aim: Give a clear statement of the main aim of the study and the main hypothesis tested, if any.

Methodology: Describe the methods adopted including, as appropriate, the design of the study, the setting, entry requirements for subjects, use of materials, outcome measures and statistical tests.

Results: Give the main results of the study, including the outcome of any statistical analysis.

Conclusions: State the primary conclusions of the study and their implications. Suggest areas for further research, if appropriate.

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

Introduction: should be focused, outlining the historical or logical origins of the study and gaps in knowledge. Exhaustive literature reviews are not appropriate. It should close with the explicit statement of the specific aims of the investigation, or hypothesis to be tested.

Material and Methods: must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced.

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

All studies using human or animal subjects should include an explicit statement in the Material and Methods section identifying the review and ethics committee approval for each study, if applicable. Editors reserve the right to reject papers if there is doubt as to whether appropriate procedures have been used.

(iii) Suppliers: Suppliers of materials should be named and their location (Company, town/city, state, country) included.

Results: should present the observations with minimal reference to earlier literature or to possible interpretations. Data should not be duplicated in Tables and Figures.

Discussion: may usefully start with a brief summary of the major findings, but repetition of parts of the abstract or of the results section should be avoided. The Discussion section should progress with a review of the methodology before discussing the results in light of previous work in the field. The Discussion

should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

Conclusion: should contain a summary of the findings.

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

References

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

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

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

Examples of correct forms of reference follow:

Standard journal article

Bergenholtz G, Nagaoka S, Jontell M (1991) Class II antigen-expressing cells in experimentally induced pulpitis. *International Endodontic Journal* **24**, 8-14.

Corporate author

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

Journal supplement

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

Books and other monographs

Personal author(s)

Gutmann J, Harrison JW (1991) *Surgical Endodontics*, 1st edn Boston, MA, USA: Blackwell Scientific Publications.

Chapter in a book

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

Published proceedings paper

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

URLs

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

Smith A (1999) Select committee report into social care in the community [WWW document]. URL http://www.dhss.gov.uk/reports/report015285.html
[accessed on 7 November 2003]

Tables, Figures and Figure Legends

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

Figures: All figures should be planned to fit within either 1 column width (8.0 cm), 1.5 column widths (13.0 cm) or 2 column widths (17.0 cm), and must be suitable for photocopy reproduction from the printed version of the manuscript. Lettering on figures should be in a clear, sans serif typeface (e.g. Helvetica); if possible, the same typeface should be used for all figures in a paper. After reduction for publication, upper-case text and numbers should be at least 1.5-2.0 mm high (10 point Helvetica). After reduction, symbols should be at

least 2.0-3.0 mm high (10 point). All half-tone photographs should be submitted at final reproduction size. In general, multi-part figures should be arranged as they would appear in the final version. Reduction to the scale that will be used on the page is not necessary, but any special requirements (such as the separation distance of stereo pairs) should be clearly specified.

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

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

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

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

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