UNIVERSIDADE ESTADUAL PAULISTA
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Campus de Araçatuba

HENRIQUE HADAD

PREVENÇÃO DA OSTEONECROSE DOS MAXILARES INDUZIDA POR MEDICAMENTOS COM A UTILIZAÇÃO DA TERAPIA FOTODINÂMICA, BIOMATERIAL E DOXICICLINA. ESTUDO EXPERIMENTAL IN VITRO E IN VIVO

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Tese apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista (Unesp),, para obtenção do Título de Doutor em Odontologia (Área de concentração em Cirurgia e Traumatologia Buco Maxilo Facial).

Orientador: Prof. Dr. Francisley Ávila Souza.

ARAÇATUBA - SP
2023

Catalogação na Publicação (CIP)
Diretoria Técnica de Biblioteca e Documentação – FOA / UNESP

H125d

Hadad, Henrique.
Prevenção da osteonecrose dos maxilares induzida por medicamentos com a utilização da terapia fotodinâmica, biomaterial e doxiciclina. Estudo experimental in vitro e in vivo / Henrique Hadad. - Araçatuba, 2023
178 f. : il. ; tab.

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientador: Prof. Francisley Ávila Souza.

1. Osteonecrose 2. Fotoquimioterapia 3. Doxiciclina 4. Alvéolo dental 5. Materiais biocompatíveis I. T.

Black D7
CDD 617.64

Claudio Hideo Matsumoto – CRB-8/5550

AGRADECIMENTOS ESPECIAIS

Início meus agradecimentos sendo grato ***a Deus***, por tudo que me proporcionou durante esses anos de pós-graduação. Eu sonhei com essa oportunidade, e foram tantos os pedidos para que hoje eu pudesse estar onde estou, e eu fui agraciado por ter essa oportunidade de estudar em Faculdade de excelência como essa. Agradeço a Ele por nunca ter me desamparado, e por sempre me mostrar luz mesmo quando eu não conseguia enxergar nada, agradeço mais ainda por essa Jornada linda que eu passei, por tantas oportunidades que tive e por tantas pessoas que conheci.

Nos agradecimentos do meu Mestrado, eu cito a importância de se acreditar no sonho para que ele se torne realidade. Hoje, completando mais uma etapa na minha carreira acadêmica, venho destacar a importância que a minha família teve para que meu sonho se tornasse realidade. Eu fui muito fortunado de ter na minha base familiar pessoas que primeiro, incentivarem a educação como oportunidade de crescimento e evolução, e também pela sua resiliência em deixar seus amados voarem para crescer.

Nessa jornada, destaco aqui minha avó ***Amélia Chead Hadad de Lima***, que sempre foi uma mulher muito afrente do seu tempo. Filha de libaneses que chegaram ao Brasil sem saberem ler e escrever e mesmo assim a incentivaram a estudar. Meu maior exemplo dentro na educação e no amor, minha avó se graduou pelo Instituto de Biociências - Universidade de São Paulo em 1958, e posteriormente se torna uma das primeiras Professoras de Ciências da minha cidade. Admiro sua trajetória reta e trabalho árduo para criar seus filhos e se manter como eximia Professora. Deixo aqui registrado, minha imensa gratidão por tudo que fez por nossa família, por tudo que fez por mim e pela minha irmã Laura. A minha avó Amélia, agradeço por ser minha fonte de amor inesgotável, por me trazer tranquilidade em tempos difíceis e principalmente por compreender e guiar minha jornada.

A minha mãe ***Maria Amélia Hadad de Lima*** e minha irmã ***Laura Hadad***, por serem uma fonte de amor e alegria. Vocês possuem uma habilidade rara no mundo que é a habilidade social e comunicativa, que contagia as pessoas por onde passa com alegria e simplicidade. É impossível estar ao lado de vocês e não se sentir essa energia que transborda e que ampara tantas pessoas. Obrigado por estarem ao lado, por me amarem tanto.

Aos meus tios ***José Carlos Hadad de Lima***, ***Roberto Hadad de Lima*** e ***Sandra Hadad de Lima***, que são minha fonte inesgotável de amor, carinho e suporte; que foram presentes em todas as etapas da minha vida, que me guiaram e incentivaram nos mentos mais difíceis, e que torcem e vibram com todos as minhas conquistas como se fossem as deles (e são, pois, nada disso seria possível sem a ajuda de cada um de vocês). Obrigado por sempre me conduzirem no melhor caminho, por me oferecerem as melhores oportunidade e educação. Amo vocês e serei eternamente grato! E também aos meus primos ***Carolina***, ***Marco Aurélio***, ***Sarah***, e ***Pedro***, por estarem presente durante essa caminha, na casa da avó Amélia compartilhando amor e felicidade.

Ao ***Professor Dr. Francisley Ávila Souza***, agradeço por me escolher ser parte do grupo em 2018 no mestrado e por confiar no meu potencial. Ser seu orientado é algo único para qualquer pessoa. Muito além dos ensinamentos científicos, publicações, e laboratório, o senhor trás para nosso grupo o ensinamento da vida. Sempre adepto do positivismo, e com uma habilidade social incrível o senhor sempre fez questão de construir um ambiente calmo, respeitoso, alegre e agregador. Minha gratidão pela confiança que me foi dada pelo senhor durantes esses últimos 6 anos, pela amizade que criamos e por todas as oportunidades que o senhor me ofereceu. Estendo também meus agradecimentos a sua esposa ***Heloisa Helena Nímia*** e seus filhos ***Marina*** e ***Eduardo***, que sempre estiveram presente nas nossas comemorações e celebraram nossas conquistas, e sempre me receberam com muito carinho em sua casa.

Quando eu menciono grupo de pesquisa, eu jamais poderia deixar de citar aqui o ***Professor Dr. Paulo Sérgio Perri de Carvalho***, não só pela sua incontestável, história, habilidade e sabedoria na Implantodontia, mas como nosso mentor. O senhor, mesmo que distante, sempre nos guiou nas pesquisas e nos possibilitou inúmeras oportunidades. Deixo meu respeito pela sua excelência, sua dedicação e paixão pela pesquisa. Agradeço também por ter confiado em mim desde meu início da pós-graduação, e por permitir acompanhar sua rotina clínica onde eu aprendi tanto. Ver o senhor ministrar uma aula é algo estonteante tamanha quantidade de ensinamento que o senhor passa.

Ao ***Professor Dr. Juliano Milanezi de Almeida,*** agradeço por toda a parceria com o nosso grupo de pesquisa. Obrigado por dividir seus conhecimentos comigo durante esses anos, e por contribuir com o nosso crescimento.

Ao ***Professor Dr. Ronaldo Célio Mariano,*** agradeço o seu incentivo constante e por todos os ensinamentos. Minha Jornada na cirurgia começou com o senhor, tenho muito carinho e muito respeito pela sua história e dedicação na docência. Obrigado por todas as oportunidades e por todos os caminhos que o senhor me abriu durante a graduação e pós-graduação. Eu disse isso na defesa do Mestrado e vou repedir aqui, ser seu aluno é uma oportunidade única na vida, é poder vivenciar uma vida de ensinamentos. Muito obrigado por todas as oportunidades e por toda orientação.

A ***Professora Dra. Daniela Ponzoni,*** obrigado por todas as oportunidades que senhora me proporcionou, por confiar tanto no meu trabalho. Eu admiro seu jeito de conduzir os trabalhos, sempre calma, organizada e muito centrada. Gostaria de agradecê-la por tudo, e em especial pela sua dedicação clínica da pós-graduação, juntamente com a Profa. Ana Paula, que me permitiram operar muitos casos e melhorar minha habilidade cirúrgica.

Ao ***Professor Dr. Fernando Pozzi Semeguini Guastaldi***, minha eterna gratidão por ter me proporcionado uma das melhores e maiores experiencia da minha vida. A internacionalização durante o doutorado foi uma oportunidade única que só foi possível graças a toda dedicação e disposição do senhor em organizar toda a documentação com escritório internacional durante a pandemia para me receber. Agradeço por todo conhecimento compartilhado, por todas as pessoas que o senhor me apresentou, por acreditar no meu potencial, e por me incentivar a continuar na academia. Estendo meus agradecimentos também a sua esposa ***Rita Souza***, que sempre esteve presente nos momentos de descontração e celebração durante meu período em Boston.

Also, I would like to extend my sincere appreciation to all the friends I made during this experience in Boston, ***Nelson Carvajal***, ***Jessica Burke Pallotta***, ***Nick DeLuca***, ***María Béjar-Scribner***, ***Tina Jones***, ***Kalyn McGuire***, who provided me with incredible moments and an immense exchange of knowledge. In particular, I would like to thank ***Nicolò Rossi***, ***Yanis Berkane***, ***Anet Calisir***, ***David Kostyra***, ***Fernanda Boos Lima*** and ***Thalisa Buehl*** for being so affectionate during this period of Fellowship. My biggest fear during this fellowship was not being able to make friends and being alone, and I never felt alone. Instead, we shared some incredible moments; I learned a lot from you, and I admire each of your journeys. I look forward to meeting you all again, whether in Boston or anywhere else in the world. I also thank ***Dr Mark***

Randolph**, **Dr Robert Redmond, Dr Youssef Jounaidi, Dr Sara Pai**, and **Dr Joseph McCain for giving me opportunities in their respective departments, allowing me to expand my knowledge.

Aos Professores da disciplina de Cirurgia e Traumatologia Buco-Maxilo-Facial, ***Professor Dr. Idelmo Rangel Garcia Junior, Professor Dr. Osvaldo Magro Filho, Professora Dra. Alessandra Marcondes Aranega, Professora Dra. Daniela Ponzoni, Professora Dra. Ana Paula Farnezi Bassi, Professor Dr. Leonardo Perez Faverani***, por me receberem de portas aberta nessa Instituição, por me ensinarem tanto, por acreditarem na educação de excelência e na prática clínica com ética e qualidade. Sinto um orgulho imenso de ter sido aluno nessa casa que formou tantos nomes da Cirurgia e Trauamatologia BucoMaxiloFacial, e foi pioneira na pesquisa dentro da área.

Ao ***Professor Dr. Idelmo Rangel Gárcia-Júnior***, toda minha gratidão pelos ensinamentos durante esses anos de convívio. Obrigado por compartilhar sua história e sua habilidade cirúrgica conosco e nos permitir aprender sempre.

Ao ***Professor Dr. Osvaldo Magro Filho***, obrigado por permitir que pudesse exercer meu Estágio de Docência em sua ala de cirúrgica e por dividir comigo seus conhecimentos desde o primeiro ano da pós-graduação.

A ***Professora Dra. Ana Paula Farnezi Bassi,*** me faltam palavras para agradecer todo o carinho durante todos esses anos. Não sei quantas vezes eu fui à sua sala buscando por uma palavra de conforto e a senhora sempre estava disponível. A senhora é um espelho na docência e eu tenho um carinho e respeito enorme pela senhora. Obrigado!

A ***Professora Dra. Alessandra Marcondes Aranega,*** que me deu a oportunidade de poder conhecer o **CAOE**, e que confiou no meu potencial e me permitiu poder operar e aprender tanto dentro desse centro de ensino. Obrigado por todo carinho e por sempre lembrar de mim nos eventos do grupo PET e por sempre me oferecer oportunidades.

Ao ***Professor Dr. Leonardo Perez Faverani***, uma inspiração na prática cirúrgica e na pesquisa. Agradeço por ter me ensinado tanto durante esses anos, por sempre me incentivar e por todas as oportunidades.

Aos Professores da PG que sempre estiveram com as portas abertas, compartilhando seu conhecimento e laboratórios com a nossa equipe ***Professora Dra. Sandra Helena Penha de***

Oliveira, ***Professor Dr. Juliano Milanezi de Almeida***, ***Professora Dra. Roberta Okamoto.*** *Obrigado pela parceria, pela ajuda e por permitir que seus alunos nos ajudassem no desenvolvimento da pesquisa.*

Aos Colegas dessa Jornada na pós-graduação ***Bruno Coelho Mendes***, ***Breno dos Reis Fernandes***, ***Bruna Junger***, ***Yasmin Comoti Vita Bantin***, ***Vinícius Ferreira Bizelli***, ***Edith Umasi Ramos*** e ***Izabella Sol***, ***Paulo Matheus Honda Tavares***, ***Harrisson Lucho Mamani Valeriano, Nelson Padilha Silva*** por dividir a caminhada, pela amizade e os bons momentos.

A minha amiga ***Laís Kawamata de Jesus***, que foi minha maior parceira nessa pós-graduação desde 2019. Criamos um laço de amizade desde seu início no grupo. Não sabia se seriamos bons amigos porque nessa época você ainda não tomava café e não conversava muito. E eu fui muito abençoado com a sua amizade. Eu sou muito grato por ter tido a oportunidade de conhecer alguém com o seu potencial, talento, organização e principalmente com um coração bom. Obrigado por estar ao meu lado em cada etapa dessa pesquisa e por me aconselhar todos os dias. Durante a pandemia, vivemos um dos momentos mais assustadores do mundo e nós estávamos lá, juntos, terminando as etapas do seu mestrado e começando o meu doutorado. Você foi a pessoa que eu tive a oportunidade de ensinar tudo o que eu aprendi na pós-graduação e seu crescimento foi exponencial. Cada etapa do meu projeto tem sua amizade, sua dedicação, sua ajuda, e principalmente sua parceria. Eu torço que você tenha um futuro lindo e de muitas conquistas, e em especial torço para estarmos juntos um dia, dando aula em uma Universidade. Obrigado pela amizade. Estendo meus agradecimentos a sua mãe **Olga Tyoko** que me recebeu com muito carinho, e que confiou em mim como seu amigo. Agradeço por sempre torcer pelas minhas conquistas e por sempre mandar umas comidinhas gostosas para alegrar nossos dias.

Ao meu amigo e irmão ***Rodrigo Capalbo da Silva***, com quem eu tive a honra e a felicidade de iniciar essa Jornada em 2018. Sou muito grato de ter tido ao meu lado uma pessoa tão receptiva, do bem durante a pós-graduação e que me recebeu em Araçatuba de coração aberto. Eu tenho inúmeros momentos incríveis da nossa amizade durante esses anos e vou guardá-los comigo para sempre. Não bastasse a convivência na pós-graduação fizemos a especialização em Implantodontia juntos e foi mais uma oportunidade de te conhecer melhor. Você é uma pessoa incrível, que sempre esteve presente na minha vida, sempre me incentivou a ser uma pessoa melhor, que me deu todo o incentivo durante a jornada na pós e principalmente

que acreditou no meu potencial. Estendo meus agradecimentos aos seus pais ***Jorge Luís da Silva Vieira*** e ***Erica Cristina Capalbo da Silva*** e seu irmão ***Rafael Capalbo da Silva*** que sempre me receberam com muito carinho na sua casa.

A minha primeira amizade de Araçatuba ***Raquel Parra Barroso da Silva***, obrigado por me acolher com tanto carinho desde os primeiros dias da pós-graduação. Durante esses anos, compartilhamos inúmeros momentos, conquistas, felicidades, experiencias e você sempre estava presente em todos os momentos. Sou muito grato por você ter aberto as portas da sua casa, me apresentado seus pais ***Sr. Jose Aparecido da Silva*** e ***Marli Parra Barroso da Silva***, que são pessoas que admiro e respeito imensamente. Muito além da pós-graduação, nossa amizade se estendeu para a vida. Eu aprendi muito com você e eu te admiro demais. Obrigado por tudo!

A minha amiga ***Luara Teixeira Colombo***, que foi a primeira pessoa que eu conheci dentro do grupo do Professor Francisley. Eu lembro de chegar ao departamento 10A na segunda-feira cedinho e ser recebido por você com tanto carinho. Obrigado por todo suporte que você me deu durante a pós-graduação. Durante minha caminhada na pós-graduação você sempre esteve no meu coração e sempre estará. Tenho muitas saudades de você.

A minha amiga e conterrânea ***Hiskell Francine Fernandes e Oliveira.*** Engraçado como a vida tem um jeito estranho de trazer certas as pessoas para as nossas vidas. Eu te conhecia por nome, mas nunca achei que seriamos amigos. E hoje tenho a imensa felicidade de ter divido esses anos de pós-graduação com você. Eu sou muito grato por ter você na minha vida, por todas as nossas conversas (de horas e horas, sem parar), pela sua amizade e por toda torcida que você tem por mim. Você é uma pessoa incrível e de muita luz, e que me acolheu em Araçatuba e que faz questão de estar presente no dia a dia, sempre convidando para um breve cafezinho, ou tomando um vinho. Estendo meus agradecimentos aos seu país ***Helenice Fernandes e Oliveira*** e ***Amancio de Oliveira***, que sempre me recepcionaram bem e com muito carinho em Araçatuba e em Ipuã.

Ao meu amigo ***Henrique Rinaldi Matheus***, obrigado por compartilhar uma das melhores experiencia da minha vida. Agradeço por toda ajuda e por cada conselho. Você é uma pessoa incrível, com um coração enorme e uma mente extraordinária. Sua dedicação e foco me inspiram a querer ser melhor e poder conviver e aprender com você em Boston foi sensacional. Fico muito feliz que nos aproximamos e podemos criar essa amizade linda, por me ensinar tanto e sempre me incentivar. Obrigado por tudo!

A minha amiga ***Karen Rawen Tonini,*** agradeço por todos os momentos compartilhados dentro e fora da universidade. Por toda a sua ajuda sempre, por dividir os momentos de stress e muito cafezinho, pelas jantinhas sempre deliciosas na sua casa com a Beth nos julgando.

A minha amiga ***Ana Flávia Piquera Santos***, parceira de pós-graduacão durante 6 anos. Obrigado pelos momentos que dividimos no departamento conversando ou pesquisando. Você é uma pessoa incrível, extremamente dedicada e do bem.

A minha amiga ***Maria Eduarda de Freitas Santana Oliveira***, que felicidade a nossa de tê-la no nosso grupo. Obrigado por sempre ser tão dedicada e presente em todas as atividades, por compartilhar sua felicidade e carinho todos os dias. Convivemos por tão pouco tempo, mas você fez questão de estar presente em cada etapa dessa pesquisa no último ano, e muito além da pesquisa você tornou-se uma pessoa querida e que vou levar no meu coração para sempre.

A minha amiga ***Maisa Pereira da Silva***, gratidão pelo nosso tempo de trabalho juntos. Sua dedicação e colaboração foram fundamentais para o crescimento do nosso grupo de pesquisa. Obrigado por ser sempre presente, pelas risadas e por todo conhecimento compartilhado.

A minha primeira orientada de Iniciação Científica, ***Letícia Gabriella de Souza Rodrigues***, agradeço por toda a sua ajuda durante a execução dessa pesquisa. Com toda certeza sem a sua ajuda isso não teria sido possível. Nós começamos essa pesquisa meses antes do início da Pandemia, e como a pesquisa já estava em andamento quando as restrições começaram não tínhamos como parar. Mesmo assim, você sempre esteve presente para ajudar a aplicar a medicação e cuidar dos ratos, comigo e com a Laís. Você é pessoa de coração imenso, que traz felicidade e carisma por onde passa, obrigado por toda ajuda e por carinho nesses anos.

Deixo também registrado gratidão a todas a pessoas que de alguma forma de ajudaram e me incentivaram a estar aqui. Nessa caminhada eu fui muito abençoado de ter pessoas do bem ao meu lado, agradeço a ***Amanda Correa***, que me acolheu 2 anos em sua casa durante minha Faculdade em Franca, a ***Dr. Mariangela Baptistussi***, quem me deu a primeira oportunidade de estágio na odontologia, a ***Dr. Fernanda Gould Nakano***, quem me inspirou a ser Cirurgião-dentista.

Agradeço também aos Professores que de alguma forma me inspiraram a seguir na área acadêmica ***Professora Dra. Luciana Assirati Casemiro, Professora Dra. Soraya Marangoni,***

Professora Dra. Alessandra Campos, Professa Dra. Greciana Bruzi, Professor Dr. Marcelo Taveira Barbosa, ***Professor Dr. João Adolfo Costa Hanemann*** e que me proveram tantos ensinamentos durante a graduação. Em especial, agradeço ao ***Professor Dr. Felipe Fornias Sperandio*** que me possibilitou a primeira bolsa de IC na área de Patologia, e com quem eu tive minha primeira publicação científica. Gratidão imensa por todo cuidado e carinho que recebi de cada um de vocês.

AGRADECIMENTOS

A ***Faculdade de Odontologia de Araçatuba – Universidade Estadual Paulista "Júlio de Mesquita Filho" (FOA/UNESP)*** na pessoa do atual diretor ***Professor Dr. Alberto Carlos Botazzo Delbem***. Agradeço o acolhimento e o conhecimento que foi passado a mim. Essa faculdade representa um centro de excelência em formar de novos profissionais. E nítido a competência e dedicação dos professores e funcionários dessa instituição na formação acadêmica de cada aluno. Minha eterna gratidão a essa Universidade que me fez tão feliz e que me proporcionou grandes amizades e aprendizado.

Ao ***Programa de Pós-Graduação em Odontologia***, da Faculdade de Odontologia de Araçatuba, da Universidade Estadual Paulista "Júlio de Mesquita Filho" com o atual Coordenador ***Professor Dr. Wirley Gonçalves Assunção***.

Aos ***funcionários da Pós-graduação da Faculdade de Odontologia de Araçatuba (FOA/UNESP)*** pela disponibilidade, paciência e gentileza em todas as etapas do mestrado, em especial as Servidoras ***Cristiane Regina Lui Matos*** e ***Valéria de Queiroz Marcondes Zagatto.*** Agradeço o trabalho sempre eficiente por vocês desempenhado.

Aos ***funcionários da Biblioteca da Faculdade de Odontologia de Araçatuba (FOA/UNESP)*** pela prontidão em nos atender.

Aos funcionários do Departamento de Diagnóstico e Cirurgia, ***Renato Gomes de Oliveira, Marco Aurélio de Oliveira Ianner*** **e** ***Paulo Roberto Gratão*** pela prontidão, auxílio e carinho.

À ***Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP),*** pela concessão da Bolsa de Doutorado ***(2019/22657-8)*** durante os anos de pós-graduação. Agradeço por promover o apoio financeiro que me permitiu a realização do doutorado.

À ***Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES)*** - *Código de Financiamento 001*, pela concessão da Bolsa de Internacionalização no programa ***CAPES PrInt (88887.570145/2020-00)*** durante o doutorado, que me permitiu realizar um Fellowship de 12 meses no Massachussets General Hospital (MGH), Harvard School of Dental Medicine (HSDM) em Boston (MA, EUA).

Ao ***Massachusetts General Hospital, Harvard School of Dental Medicine*** na pessoa do Chefe do Programa de Cirurgia e Traumatologia BucoMaxiloFacial, ***Dr Zachary S Peacock***, e em especial ao Diretor do ***Skeletal Biology Research Center, Professor Dr. Fernando Pozzi Semeghini Guastaldi***.

A ***Faculdade de Odontologia de Araraquara – Universidade Estadual Paulista "Júlio de Mesquita Filho" (FOAr/UNESP)*** na pessoa do ***Professor Dr. Elcio Marcantonio*** e da Técnica ***Suleima Ferreira*** por permitirem a utilização do Laboratório de Análise Histopatológica para Cortes não Descalcificados, e da ***Professora Dr. Paula Aboud Barbugli*** pela orientação no Laboratório de Microscopia de Fluorescência Confocal.

Ao ***Laboratório de Multiusuário - Sala de Microtomografia Computadorizada*** na pessoa do ***Professor Dr. Alberto Carlos Botazzo Delbem***.

Ao ***Centro de Assistência Odontológica à Pessoa com Deficiência (CAOE)***, na pessoa da ***Professora Dra. Letícia Helena Theodoro***, da ***Professora Dra. Alessandra Marcondes Aranega***, da Enfermeira ***Juliana Franco de Angelis*** e equipe.

Ao **Laboratório de Endodontia**, coordenado pelo ***Professor Dr. Luciano Tavares Angelo Cintra*** por permitir a utilização do Microscópio Polarizado para análise de birrefringência, e a sua aluna de Doutorado ***Juliana Goto*** pela orientação e assistência.

Ao ***Laboratório para Estudo de Tecidos Mineralizados***, coordenado pela ***Professora Dra. Roberta Okamoto***.

Ao ***Laboratório de Imunofarmacologia de Araçatuba***, coordenado pela ***Professora Dra. Sandra Helena Penha de Oliveira***.

“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.

(Marthin Luther King)

Hadad H. Prevenção da osteonecrose dos maxilares induzida por medicamentos com a utilização da terapia fotodinâmica, biomaterial e doxiciclina. Estudo experimental in vitro e in vivo [tese]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2023.

RESUMO

Os bifosfonatos (BF's) são medicamentos antirreabsortivos empregados no tratamento de desordens esqueléticas, como a osteoporose, porém seu uso prolongado pode induzir a osteonecrose dos maxilares induzida por medicamentos (OMIM), e o tratamento desta condição representa um desafio para odontologia. Esse projeto foi dividido em 4 capítulos, de modo que no Capítulo 1, o objetivo do trabalho foi avaliar os resultados de vários modelos animais de roedores para estudar a OMIM. Após uma busca na literatura, seguindo a estratégia PICO estabelecida 2004 artigos foram localizados e 118 corroboraram com os fatores de inclusão (estudo in vivo em roedores avaliando a extração dentária como um fator de risco para o desenvolvimento da OMIM. Os resultados demonstram diversos estudos que estabeleceram protocolo para indução da OMIM. O ácido zoledrônico (ZA) foi o medicamento mais utilizado, seguido pelo alendronato (ALN). Mesmo quando o ZA não levou ao desenvolvimento de MRONJ, seu efeito comprometeu a homeostase do osso e do tecido mole. A associação de outros fatores de risco (dexametasona, diabetes e doenças inflamatórias locais), além da extração dentária também desempenharam um papel importante no desenvolvimento da OMIM. Além disso, os estudos demonstraram uma relação entre a dose e o desfecho e entre a frequência e a via de administração. Desse modo, conclui-se que o ZA pode causar OMIM em roedores, e que procedimentos cirúrgicos e doenças inflamatórias locais na cavidade oral são elementos-chave no desenvolvimento da OMIM; No Capítulo 2, o objetivo foi avaliar os efeitos de diferentes concentrações de ácido zoledrônico (ZA) durante a diferenciação osteogênica de células-tronco da medula óssea humana (hBMSCs). Para tal, a diferenciação osteogênica das hBMSCs foi conduzida com diferentes concentrações de ZA (0, 0,1, 1,0 e 5,0 μM) durante os primeiros 3 dias. O metabolismo celular foi quantificado em 1, 3, 7 e 14 dias. Aos 7 e 14 dias, foram realizadas as seguintes análises: a) ensaio de nódulo de mineralização, b) LIVE/DEAD™, c) adesão e espalhamento celular, d) atividade da fosfatase alcalina (ALP) e e) análise qPCR para RUNX-2), ALPL e COL1 A1. Os dados foram analisados por ANOVA de 2 vias, seguido pelo teste post hoc de Tukey ($p < 0,05$). O metabolismo celular (3, 7 e 14 dias) ($p < 0,001$), a mineralização (7 e 14 dias) ($p < 0,001$) e a atividade de ALP (14

dias) ($p < 0,001$) foram reduzidos em ZA 5,0 µM quando comparados ao controle (sem ZA). Além disso, a ZA 5,0 µM reduziu a expressão de RUNX2 aos 7 e 14 dias ($p < 0,001$). Desse modo, concluiu-se que a ZA (5,0 µM) pode prejudicar a diferenciação de hBMSC em osteoblastos e interferir em sua fase de mineralização; No Capítulo 3, o objetivo foi avaliar as terapias preventivas para OMIM. Após quatro ciclos de administração de ácido zoledrônico (35µg/kg a cada 15 dias), os ratos *Wistar* tiveram seus molares extraídos e foram organizados em nove grupos de tratamento: grupo de controle negativo (NCG), tratado com solução salina e coágulo sanguíneo no alvéolo; grupo de controle positivo (PCG), com coágulo sanguíneo no alvéolo; BG, biomaterial à base de β-fosfato tricálcico; DG, gel de doxiciclina a 10%; aG, terapia fotodinâmica antimicrobiana; e DBG, aBG, aDG e aDBG, usando terapia combinada. Após 28 dias, o menor volume ósseo (BV/TV) foi registrado em PCG (42,17% ± 2,65) e o maior em aDBG (69,85% ± 6,25) ($p < 0,05$). Os valores mais altos da taxa de aposição mineral diária foram registrados no aDBG (2,64 ± 0,48) e no DBG (2,30 ± 0,37) ($p < 0,001$). Além disso, o aDBG apresentou a maior área de osso neoformado (82,44% ± 2,69) ($p < 0,05$). O osso não vital foi relatado apenas no GPC (37,94 ± 18,70%). Devido ao papel fundamental do biomaterial, a abordagem combinada (aDBG) foi a mais eficaz na prevenção de MRONJ após a extração dentária; No Capítulo 4, o objetivo do estudo foi avaliar o efeito tópico da doxiciclina gel (10%) na prevenção da OMIM. Para isso, ratos *Wistar* (72) foram tratados com 70 µg de ZA/mês por 2 meses e, em seguida, foi realizada a extração do primeiro molar, um grupo recebeu apenas um coágulo no alvéolo (grupo ZA), outro recebeu um gel de doxiciclina a 10% (DOXI), enquanto o grupo controle foi tratado sistemicamente com solução salina (SAL). Após 7, 14 e 28 dias, as amostras foram submetidas a a) análise histomorfométrica, b) imunohistoquímica e c) maturação das fibras de colágeno. Os dados demonstraram que a DOXI apresentou maior área de osso novo quando comparada à ZA em 7 ($p = 0,0058$), 14 e 28 dias ($p < 0,0001$), e a quantidade de osso não vital na ZA (44,17± 10,93%) foi estatisticamente significativa somente aos 28 dias ($p < 0,0001$) quando comparada à SAL e à DOXI. A marcação TRAP foi semelhante para ZA e DOXI, exceto aos 28 dias, quando ZA apresentou marcação leve, enquanto DOXI apresentou marcação moderada. Além disso, a marcação de OCN foi semelhante entre os grupos ZA e DOXI, porém, aos 7 dias, o DOXI apresentou marcação moderada, enquanto o ZA apresentou marcação leve. O grupo DOXI apresentou uma quantidade maior de fibras de coloração amarelo-esverdeada nos dias 14 e 28 ($p = 0,0001$ e $p < 0,0001$, respectivamente), quando comparado ao grupo ZA. Eles

também apresentaram maior maturação das fibras de colágeno (cor vermelho-amarelada) nos dias 14 e 28 ($p = 0,0027$ e $p < 0,0001$, respectivamente). Desse modo, concluiu-se que a doxiciclina parece ser um agente eficaz e seguro na prevenção da OMIM.

Palavras-chave: Osteonecrose. Terapia fotodinâmica. Doxiciclina. Alvéolo Dental. Materiais biocompatíveis.

Hadad H. Doxycycline, beta-tricalcium phosphate and aPDT applied as a preventive method for MRONJ. In vitro and in vivo experimental study [tese]. Araçatuba: Faculdade de Odontologia da Universidade Estadual Paulista; 2023.

ABSTRACT

Bisphosphonates (BP's) are anti-resorptive drugs used in the treatment of skeletal disorders, such as osteoporosis, but their prolonged use may induce osteonecrosis of the jaw (MRONJ), which represents a challenging treatment for dentistry. This thesis were divied in 4 chapters, in this way, Chapter 1 aimed to assess the outcomes of several rodent animal models for studying medication-related osteonecrosis of the jaw (MRONJ). After a search of the databases, 2004 articles were located, and 118 corroborated the inclusion factors (*in vivo* studies in rodents evaluating tooth extraction as a risk factor for the development of MRONJ). Numerous studies attempting to establish an optimal protocol to induce MRONJ were found. Zoledronic acid (ZA) was the most used drug, followed by alendronate (ALN). Even when the ZA did not lead to the development of MRONJ, its effect compromised the homeostasis of the bone and soft tissue. The association of other risk factors (dexamethasone, diabetes, and inflammatory dental disease) besides tooth extraction also played a role in the development of MRONJ. In addition, studies demonstrated a relationship between dose and outcome and between frequency and administration route for MRONJ. Thus, it was concluded that ZA may cause MRONJ in rodents. Surgical procedures and inflammatory dental disease in the oral cavity are key elements in starting MRONJ; Chapter 2 aimed to evaluate the effects of different concentrations of ZA during the osteogenic differentiation of human Bone Marrow Stem Cells (hBMSCs). Thus, osteogenic differentiation of hBMSCs were conducted with different concentrations of ZA (0, 0.1, 1.0, and 5.0μM) for the first 3-days. Cell metabolism was quantified at 1-, 3-, 7-, and 14 days. At 7- and 14-days, the following analyses were performed: a) mineralization nodule assay, b) LIVE/DEAD™, c) cell adhesion and spreading, d) alkaline phosphatase (ALP) activity, and e) qPCR analysis for RUNX-2), ALPL, and COL1 A1. Data were analyzed by ANOVA 2-way, followed by Tukey's post hoc test ($p < 0.05$). Results demonstrated that cell metabolism (3-, 7-, and 14-days) ($p < 0.001$), mineralization (7-, 14-days) ($p < 0.001$), and ALP activity (14-days) ($p < 0.001$) were reduced in ZA 5.0 μM when compared to control (no ZA). Also, ZA 5.0 μM downregulated the expression of RUNX2 at 7- and 14-days ($p < 0.001$). It is possible to conclude that ZA (5.0 μM)

can impair hBMSC differentiation into osteoblasts and interferes with its mineralization phase; Chapter 3 aimed to evaluate the preventive therapies for MRONJ. Following four cycles of zoledronic acid administration, *Wistar* rats had their molar extracted, and were organized into nine treatment groups: negative control group (NCG), treated with saline solution and blood-clot in the alveolus; positive control group (PCG), with blood-clot in the alveolus; BG, β-tricalcium phosphate-based biomaterial; DG, 10% doxycycline gel; aG, antimicrobial photodynamic therapy; and DBG, aBG, aDG, and aDBG, using combination therapy. After 28 days, the lowest bone volume (BV/TV) was reported in PCG (42.17% ± 2.65), and the highest in aDBG (69.85% ± 6.25) ($p < 0.05$). The higher values of daily mineral apposition rate were recorded in aDBG (2.64 ± 0.48) and DBG (2.30 ± 0.37) ($p < 0.001$). Moreover, aDBG presented with the highest neoformed bone area (82.44% ± 2.69) ($p < 0.05$). Non-vital bone was reported only in the PCG (37.94 ± 18.70%). Owing to the key role of the biomaterial, the combination approach (aDBG) was the most effective in preventing MRONJ following tooth extraction; and Chapter 4 aimed to assess the topical effect of doxycycline gel (10%) during bone repair in vivo on the osteonecrosis model. For this purpose, *Wistar* rats (72) were treated with 70 µg of ZA/month for 2 months, then extraction of the first molar was performed, one group received only a clot in the socket (ZA group), one received a 10% doxycycline gel (DOXI), while the control group was treated systemically with saline solution (SAL). After 7, 14, and 28 days, samples were submitted for a) histomorphometric analysis, b) immunohistochemical, and c) collagen fibers maturation. Data demonstrated that DOXI presented higher new bone area when compared to ZA in 7 ($p = 0.0058$), 14, and 28 days ($p < 0.0001$), and the amount of non-vital bone in ZA (44.17± 10.93%) was statistically significant only at 28 days ($p < 0.0001$) when compared to SAL and DOXI. TRAP labelling was similar for ZA and DOXI, except for 28-day when ZA presented mild labelling, while DOXI showed moderate labelling. Also, OCN labelling was similar between the ZA and DOXI groups, however at 7 days DOXI showed moderate labelling, while ZA showed mild labelling. DOXI group presented a higher amount of collagen type 3 (greenish-yellow coloured) fibers on days 14 and 28 ($p = 0.0001$ and $p < 0.0001$, respectively), when compared to ZA. They also showed greater maturation of collagen fibers – type 1 (yellowish-red colour) on days 14 and 28 ($p = 0.0027$ and $p < 0.0001$, respectively). Conclusion: doxycycline seems to be an effective agent in the prevention of osteonecrosis.

Keywords Osteonecrosis. Photodynamic Therapy. Doxycycline. Tooth Socket. Biocompatible materials.

LISTA DE TABELAS

LISTA DE FIGURAS

Figure 19. Representative graph of the percentage area of yellowish-red fibers for each group in the periods of 7, 14, and 28 days. * means $p = 0.0027$, and ** means $p < 0.0001$. 171

LISTA DE ABREVIATURAS E SIGLAS

#	Not reported
%NBA	Percentage of new bone area
1-3M	First, second, and third molar
1M	First molar
2M	Second molar
3M	Third molar
AAOMS	American Association of Oral and Maxillofacial Surgery
aBG	aPDT + biomaterial group
aDBG	aPDT + doxycycline + biomaterial group
aDG	aPDT + doxycycline group
aG	aPDT group
ALN	Alendronate
ALP	Alkaline phosphatase
aPDT	Antimicrobial photodynamic therapy
ARRIVE	Animal Research: Reporting of In Vivo Experiments
Ars	Antiresorptive drugs
ATP	Adenosine triphosphate
BG	Biomaterial group
BPs	Bisphosphonates
BSA	Bovine serum albumin
BTCP	β-tricalcium phosphate
BV/TV	Percentage of bone volume fraction
CD31	Platelet endothelial cell adhesion molecule
COL1A1	Colagen type 1
CT	Control Group
CYP	Cyclophosphamide
DAPI	4′,6-diamidino-2-phenylindole
DBG	Doxycycline + biomaterial group
DG	Doxycycline group
DMEM	Dulbecco’s modified eagle media

DOXI	Doxycycline
DX	Dexamethasone
EP	Experimental periodontitis
EPD	Experimental periapical disease
ES	Extremely Supraphysiologic
F	Female
FPP	Farnesyl-diphosphate synthase
HBMSCs	Human bone marrow stem cells
HGF	Hepatocyte growth factor
IM	Intramuscular
In	Incisor
IP	Intraperitoneal;
IV	Intravenous;
M	Male
M	Months
Md	Mandible
MicroCT	Microcomputed tomography
MRONJ	Medication-Related Osteonecrosis of the Jaw
MWF	Monday, Wednesday, and Friday
Mx	Maxilla
N-BPs	Nitrogen-containing bisphosphonates
NCG	Negative Control group
OCN	Osteocalcin
OP	*Oryzomys palustris* rats
OPG	Osteoprotegerin
P	Physiologic
PAM	Pamidronate
PBS	Phosphate Buffer Solution
PCG	Positive Control group
PGinv	*Porphyromonas gingivalis*
Post-XT	Post-extraction period

Post-XT	Treat Drug treatment applied during post-extraction period
PoTot	Total porosity
Pre-XT	Pre-extraction treatment period
qPCR	Real-time polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa beta ligand
RUNX2	Runt-related transcription factor 2
S	Supraphysiologic
SAL	Saline solution
SC	Subcutaneous
SD	*Sprague Dawley* rats;
SUN	Suninitib
TACE	Factor-α converting enzyme
TbSp/TbN	Separation and number of trabeculae
TbTh	Trabecular bone thickness
TRAP	Tartrate-resistant acid phosphatase
TRICT	Tetramethylrhodamine
VAB	Anti-VEGFA neutralizing antibody
VEGF	Vascular endothelial growth factor
W	Weeks
WA	*Wistar Albinus* rats;
ZA	Zoledronic acid

SUMÁRIO

Capítulo 1

1 CAPÍTULO 1 - Rodents as an animal model for studying tooth extraction-related medication-related osteonecrosis of the jaw: assessment of outcomes*

1.1 ABSTRACT

Objective: To assess the outcomes of several rodent animal models for studying medication-related osteonecrosis of the jaw (MRONJ). **Design**: After a search of the databases, 2004 articles were located, and 118 corroborated the inclusion factors (*in vivo* studies in rodents evaluating tooth extraction as a risk factor for the development of MRONJ). **Results**: Numerous studies attempting to establish an optimal protocol to induce MRONJ were found. Zoledronic acid (ZA) was the most used drug, followed by alendronate (ALN). Even when the ZA did not lead to the development of MRONJ, its effect compromised the homeostasis of the bone and soft tissue. The association of other risk factors (dexamethasone, diabetes, and inflammatory dental disease) besides tooth extraction also played a role in the development of MRONJ. In addition, studies demonstrated a relationship between dose and outcome and between frequency and administration route for MRONJ. **Conclusions**: ZA may cause MRONJ in rodents. Surgical procedures and inflammatory dental disease in the oral cavity are key elements in starting MRONJ.

Keywords: MRONJ, ONJ; Animal models; BRONJ; Bisphosphonates; Rodents.

1.2 INTRODUCTION

Controlling the dosages and frequency allows nitrogen-containing bisphosphonates (such as zoledronate acid [ZA], alendronate [ALN], pamidronate [PAM], etc.) and anti-RANKL antibodies (e.g., denosumab) to be used either for managing hypercalcemia and bone metastases in patients with cancer (Duran et al., 2017; Van Poznak et al., 2017) or for preventing fragility fractures in osteoporotic patients (Yu et al., 2020). The antiresorptive drugs (ARs) include a class of drugs called bisphosphonates, and they are related to the occurrence of a potentially severe adverse event, medication-related osteonecrosis of the jaw (MRONJ). MRONJ has been defined

* Submitted and edited according to the guidelines of *Archives of Oral Biology*. https://www.elsevier.com/journals/archives-of-oral-biology/0003-9969/guide-for-authors

by the American Association of Oral and Maxillofacial Surgery (AAOMS) as the presence of exposed bone in the maxillofacial region for a period longer than 8 weeks in patients with no history of radiation therapy or metastatic disease in the jaws which have been treated with ARs (Ruggiero et al., 2022).

The incidence is higher in patients with cancer who receive antiresorptive drugs (2–5%), the incidence of MRONJ in patients under the use of oral antiresorptive in osteoporotic dosages is not negligible (0.01-0.03%) (Khan et al., 2015; Coropciuc et al., 2023). Besides the systemic impact of antiresorptive drugs on bone metabolism, local oral risk factors such as tooth extraction and major dental surgery during BP therapy, inflammatory/infectious dental disease (e.g., periodontal, and periapical abscess), ill-fitting removable dental prostheses, or possibly dental implants are required to trigger MRONJ (Khan et al., 2015; Hasegawa et al., 2017; Otto et al., 2018; Castillo et al., 2021; Otto et al., 2021; Wan et al., 2020). There is broad consensus that the incidence of MRONJ is higher in patients with cancer who take antiresorptives than in patients with osteoporosis who take antiresorptives and that the ten-fold higher cumulative absorbed dose of antiresorptives in patients with cancer than in patients with osteoporosis seems likely to play a role. However, a complete understanding of how local oral risk factors interact with antiresorptives to cause MRONJ is not yet in place. Importantly, although it is more common at high dosages, a similar progression from health to disease can be expected when comparing MRONJ in cancer and osteoporosis patients (Khan et al., 2015; Wan et al., 2020), thus indicating that these local factors are central players which are fundamental to the occurrence of MRONJ.

The mechanistic interactions between the potent suppression of osteoclast activity and patient-related local risk factors are critical to fully comprehending the pathophysiology of MRONJ while seeking further precise therapeutic approaches for its prevention and treatment. Therefore, an appropriate animal model is a reasonable step for *in vivo* validation. The superiority of large animal models over rodents has been proven in some scenarios (Lunney et al., 2021). However, the interspecies comparison performed by Pilawski et al. (2021) provided accurate information assuring equivalency in the morphology and physiology of pristine alveolar bone when comparing mice, rats, mini-pigs, and humans. Furthermore, Pan et al. (2020) supported the hypothesis that the underlying mechanisms of alveolar bone healing following tooth extraction are histologically equivalent and conserved between mice and mini pigs. Therefore, the inherent

financial benefit and easy manipulation, combined with the non-superiority of pigs over mice, substantiate the use of rodents as a human biomedical model for studying the processes related to socket healing.

The wide variety of drugs, dosages, administration routes, frequency of administration, and presence of local/systemic predisposing factors results in significant heterogeneity among the models used for studying tooth extraction-related MRONJ (Aguirre et al., 2021). Only by knowing what to expect in a specific animal scenario can a pre-clinical experiment accurately mimic equivalent clinical situations, thus providing a basis for translational research. Therefore, this review aims to provide data from distinct rodent models for studying tooth extraction-related MRONJ.

1.3 METHODS

A systematic screening process was used to select the articles to be included, from which the data were collected and shown narratively. The search included articles reporting rodent studies evaluating the development of MRONJ in a tooth extraction model published up to April 1, 2023. Ethical approval was not necessary.

Search criteria

The search was performed in PubMed/MEDLINE, Embase, and Cochrane Library by applying the following strategy: (rodents OR rat OR mice OR mouse) AND (zoledronic acid OR bisphosphonate OR anti-resorptive drugs OR rank ligand antibody OR denosumab) AND (control OR saline solution) AND (osteonecrosis OR osteonecrosis of the jaw OR necrosis OR microtomographic OR microct OR bone volume OR bone parameters OR histologic OR histomorphometry OR immunobiological OR in vivo).

Inclusion and exclusion criteria

The following criteria were applied to assess the eligibility of the studies: *in vivo* studies in rodents evaluating tooth extraction as a risk factor for the development of MRONJ through clinical and complementary imaging (x-ray and micro-tomography), and histopathologic analyses were included. Reviews, clinical studies, technical notes, abstracts, in vitro studies, and studies not published in English were not eligible. Articles lacking proper control groups were not included.

Data extraction

After removing duplicates, two reviewers independently assessed titles and abstracts before evaluating full texts and applying the inclusion and exclusion criteria. A third reviewer was consulted in case of disagreement. The following data were extracted: types of drugs; dose, frequency, and duration; route of administration; animal species, gender, and age; the presence of associated risk factors; surgical site-related characteristics (which tooth, number of extractions, and location [maxilla or mandible]; and the reported outcomes.

1.4 RESULTS

Search results and study characteristics

A total of 2004 articles were identified (1810 from PubMed/MEDLINE, 194 Embase). After removing duplicates, 1568 publications were screened by title and abstract. A total of 67 full texts were selected. Eighteen papers were excluded due to the absence of a control group, 7 due to the lack of a tooth extraction, and 8 due to reference to a previously described model. Hence, 49 articles meeting the inclusion and exclusion criteria were included in this review, and 69 papers were further added after a manual search, totaling 118 articles.

Data demonstrated 73 rat studies (Tables 1-4) and 45 mouse studies (Table 5). The rat studies are divided into three tables for ZA: a) Physiologic Doses (N=14); b) Supraphysiologic Doses (N=23); and c) Extremely Supraphysiologic Doses (N=27); and one Table (#4) (N=12) for ALN rat studies. The ZA rat studies span doses of 25-4500% of the rat ZA oncology dose. The

mouse studies span ZA doses of 25-5200% of the mouse ZA oncology dose. The ALN rat studies span absorbed doses of 0.17-2333% of the putative ALN rat oncology dose, over four orders of magnitude. Pre-extraction treatment covers 0-147 days but focuses on 14-21 days. Post-extraction treatment covers 3-105 days but focuses on 28-42 days.

In the first category (rats), the studies were stratified by the drug (ZA, ALN, or other drugs) and by the dosages of these drugs used by which authors, considering not only the mg/kg of the individual dose as it was administered but also the total number of doses given and the period over which they were administered. Thus, the ZA doses were categorized considering the percentage (%) of rat ZA oncology dose (80µg/kg ZA/monthly IV), according to Aguirre et al. (2021) and Gasser et al. (2008) in physiologic (≤200% of the rat ZA oncologic dose) (Group P), supraphysiologic (>200% to <1000% of the rat ZA oncologic dose) (Group S), and extremely supraphysiologic (≥1000% of the rat ZA oncologic dose) (Group ES) cumulative absorbed dose. For ALN, the percentage of drug used was calculated based on rat osteoporotic dose (120µg/kg/monthly SC) (Seedor et al., 1991). In the second category (mice), each author's ZA percentage was calculated based on the mouse ZA oncology dose, which is ~540µg/kg/month IV (Pozzi et al., 2009; Park et al., 2015; Aguirre et al., 2021).

Thirty-six studies (30%) combined tooth extraction with other risk factors, such as dexamethasone (DX) administration (Sonis et al., 2009; Kikuiri et al., 2010; Bi et al., 2010; Ali-Erdem et al., 2011; Abtahi et al., 2012; Zhao et al., 2012; Berti-Couto et al., 2014; Kuroshima & Yamashita, 2013; Jabbour et al., 2014; Kaibuchi et al., 2016; Yanik et al., 2016; Jung et al., 2021; Mergoni et al., 2019; Tamari et al., 2019; Movahedian-Attar et al., 2020; Adachi et al., 2020; Sanda et al., 2022; Liu et al., 2021; Gao et al., 2021; Yoshioka et al, 2022), senescence in animals (Statkievicz et al., 2018; Biguetti et al., 2019; Paulo et al., 2020; Liu et al., 2021), vitamin D deficiency (Hokugo et al., 2010), repeated trauma (Howie et al., 2015), ovariectomy with estrogen deficiency (Kim et al., 2015), diabetes (Takaoka et al., 2015; Zhang et al., 2015), and inflammatory dental disease [periodontitis (Kim et al., 2018; Soundia et al., 2018; Statkievicz et al., 2018; Ervolino et al., 2022; Williams et al., 2020), periapical lesions (Song et al., 2016; Hadaya et al., 2019; Bolette et al., 2019; Du et al., 2022), and perirradicular disease (Soundia et al., 2016)].

Intraperitoneal, intravenous (tail vein), and subcutaneous were the main routes for the administration of the drugs with oral dosing of alendronate even attempted several times. The following drug administration frequencies were the most used: once/week, twice/week, and daily. Molars, sometimes more than one, were by far the most frequent tooth extracted. Two-thirds came from the maxilla, while one third came from the mandible. The most frequent was the first molar.

The following outcome parameters were used to characterize MRONJ and microscopic osteonecrosis events in the extraction sites: clinical evaluation, histological and immunohistochemical analyses, x-ray, micro-tomography, and biochemical analysis of the blood. The comprehensive findings are reported below.

Experimental models in rats

Zoledronic Acid (ZA)

Physiologic (Group P)

Fourteen studies using doses ≤200% of rat ZA oncology doses were included in this section (Table 1). For convenience, all study data in tables are arranged in order of increasing cumulative absorbed dose rate. Only Guevarra et al. (2015), Marino et al. (2012), Ali-Erdem et al. (2011), Tamari et al. (2019), and Sonis et al. (2009) used doses significantly inferior to the oncologic dose (25%, 33%, 38%, 38%, and 38% of the rat ZA oncology dose, respectively), which is 2-4X the rat ZA osteoporosis dose.

Guevarra et al. (2015) used two IV administrations of 0.02 mg/kg of ZA at 4 weeks intervals and after 4 weeks post-operative it was verified open wound with MRONJ signs in 38% of the sample. Interestingly, authors performed vascular perfusion followed by microCT which demonstrated that ZA decreased connectivity and branching, and a decrease in the ordered pattern of blood vessels when compared to control. Marino et al. (2012) reported that administration of ZA alone (0.02 mg/kg, intravenous) three weeks before and three weeks after tooth extraction led to the exposed bone in 75% and necrotic bone in 100% of rats at 8 weeks after tooth extraction. Their findings supporting MRONJ were both clinical (bone exposure and

sequestration) and histological (presence of empty lacunae, inflammatory infiltration, and bacterial colonization).

Ali-Erdem et al. (2011), using ZA and dexamethasone (ZA 0.075 mg/kg once/week for 3 weeks and dexamethasone 1.0 mg/kg once/week for 3 weeks), noted that 60% of the sample had bone necrosis in ZA group. Histological analysis demonstrated that ZA led to histological bone necrosis, decreased percentage of new bone area, and exacerbated inflammatory process ($p < 0.05$) compared to the control group. The incidence of MRONJ was 60% in the ZA+DX group vs. 32% in the control group ($P<0.015$). Moreover, the combination of ZA+DX (0.075 mg/kg + 1mg/kg, once/week over 11 weeks) therapy increased the incidence of the chronic wound and MRONJ in a rat model (Tamari et al., 2019), characterized by a higher area of necrosis and number of empty lacuna ($p < 0.05$), decreased number of blood vessels and reduction in the VEGF and CD31 expression when compared to control. Similarly, Sonis et al. (2009), using the same protocol, reported that the rats that received ZA+DX remained with non-healed extraction sites, and in general, the lesion was characterized by exposed bone, erythema and rolled edematous borders.

The same dose of ZA (140 µg ZA/Kg in 2 months, 87% of the rat ZA oncologic dose) was used in 4 studies under different designs, which were as follows: *Wistar–Albino* rats (Curra et al., 2016; Cardoso et al., 2019), *Sprague–Dawley* rats with vitamin D deficiency (Hokugo et al., 2010), and *Sprague–Dawley* with diabetes (Takaoka et al., 2015). Curra et al. (2016) reported bone exposure in 40%, osteolysis, loss of integrity of the alveolar walls (micro-tomography), and osteonecrosis events with microbial and inflammatory infiltration (histology) in 100% of the samples. Similarly, Cardoso et al. (2019) showed that 40% of the sample had bone exposure, and 16% had bone exposure and suppuration. MicroCT demonstrated that ZA treatment led to osteolysis, fractures, or loss of socket integrity in all samples. A decrease in VGF expression was also noted.

Although the study by Hokugo et al. (2010) reported delayed socket healing and the presence of necrotic bone and bone sequestrum (micro-tomography) in the ZA group (14.3% of animals), the incidence of these events in animals affected by both ZA and vitamin D deficiency was significantly higher (67%). Similarly, Takaoka et al. (2015) proposed a tooth extraction model related to MRONJ in combination with type 2 diabetes mellitus. The protocol used by

Takaoka et al. (2015) (612.5 µg ZA/Kg in 8.75 months, 87% of the rat ZA oncologic dose) induced substantial damage to the socket (100% of bone exposure [clinically], and 37% of animals with necrotic bone [histologically]) compared to ZA alone, in which necrotic bone tissue was found in only 12.5% of the samples.

Also, osteoid matrix deposition and mild neutrophil infiltration with the same dose (ZA, 0.04 mg/kg weekly, over 8 weeks, IP, 100% of rat ZA oncology dose) were reported by Silva et al. (2015), who observed MRONJ restricted to one sample. Likewise, Elsayed et al. (2018) demonstrated that ZA (0.08mg/kg/week for 13 weeks, IV, 119% of rat ZA oncologic dose) effected gingival repair leading to open wounds, also microCT evidenced bone sequestration, and higher percentage of empty lacuna was notated ($p < 0.0001$) when compared to control. In the same way, Brierly et al. (2019), using two doses of ZA (0.1mg/kg with 3 weeks interval) found typical signs of MRONJ in the histological section, such as nonvital bone, empty lacunae, and reduction in the vascularization without statistical significance.

On the other hand, Biasotto et al. (2010) (using 200% of the rat ZA oncologic dose) found that the weekly intravenous administration of 0.04 mg/kg ZA (for 5 weeks) led to clinical exposure of the bone and expansion of the original defect (post-extraction socket). Micro-tomography identified irregular cortical contour and bone destruction, while non-vital bone and peripheral resorption without inflammatory infiltration were indicated. In addition, Zhu et al. (2019) conducted an experiment using 200% of the rat ZA oncologic dose (0.08mg/kg, once/week for 8 weeks) and demonstrated that MRONJ signs could be clinically found in 25% of the sample, and histologically in 75%. Also, the period of 8 weeks of drug holiday was evaluated in this study and did not alleviate the development of MRONJ but increased the percentage of MRONJ for 44% and 89% respectively.

Supraphysiologic (Group S)

This section includes studies using doses >200% of the rat ZA oncology dose to <1000% of the rat ZA oncology dose (Table 2).

The administration of ZA (0.06 mg/kg/week over 7 weeks, 300% of the rat ZA oncologic dose) led to MRONJ and bone exposition in all samples (Zandi et al., 2016) and/or fistula

associated. In this same study, tooth extraction was performed in different time point (0, 7, 14, 21) from the beginning of the ZA administration, and results demonstrated that after 4-weeks all groups present MRONJ. Likewise, Borke et al. (2015) also using 300% of the rat ZA oncologic dose (0.06 mg/kg/week for 2 weeks), highlighted that after 4 and 8 weeks od tooth extraction, 100% and 75% of the wounds presented exposed bone with incomplete closure in ZA group, also histological was characterized by necrotic bone with empty lacunae, general tissue disorganization, and lymphocytic infiltration. Imada et al. (2019), using the same protocol of Borke et al. (2015), also found exposed bone in 86% and 57% in the animals treated with ZA, after 3 and 8 weeks (respectively). It is also noted that the ZA impaired the volume of new bone formation, and after 8 weeks 86% of the sample were diagnosed with MRONJ.

Although Vidal-Gutiérrez et al. (2017) applied only two doses of 0.06 mg/kg on the 7th and 14th day postoperatively, intramuscular (120 µg ZA/Kg in 0.5 months, 300% of the rat ZA oncologic dose), data demonstrated that ZA led to MRONJ, confirmed by the bone exposure, and a histologically evident ulcerative lesion with bone necrosis, sequestrum, and bacterial colonization, as well as radiographic evidence of osteolytic lesions, extensive destruction, and evident sequestrum. In the same way, Zandi et al. (2015) found that intraperitoneal delivery of 0.06mg/kg/week of ZA for 4 weeks (300 µg ZA/Kg in 1.25 months, 300% of the rat ZA oncologic dose) resulted in clinical bone exposure or fistula in 85% of the samples and histological discontinuity of the epithelium, the presence of inflammatory infiltrate, sequestra, and compromised new bone formation as compared with the control.

Zandi et al. (2017), using the same protocol of ZA used in 2016 (Zandi et al., 2016) but now over 12 weeks, and it was demonstrated bone exposure or fistula in 80% of the extraction sites. Also, histological findings suggest that ZA impaired bone remodeling and epithelial covering, and necrotic bone, inflammatory infiltrate were noted in 83% of the sample.

Howie et al. (2015) and Yang et al. (2015) used the same ZA dose (400% of the rat oncologic dose). However, while the first used a protocol of 1040 µg ZA/Kg in 2 months, the second used 3 different protocols: 320 µg ZA/Kg in 1 month, 2) 560 µg ZA/Kg in 1.75 months, and 3) 1200 µg ZA/Kg in 3.75 months. Howie et al. (2015) (0.080 mg/kg weekly, 13 weeks, intravenous) used a model of repeated trauma (2 independent extractions at distinct time points) in which the ZA group exhibited progressive mucosal dehiscence with bone exposure sites.

Histologically, empty osteocyte lacunae were found in 26.73% of the specimens but with no detectable bacterial colonization in the extraction site. Micro-tomography showed no bone formation within the socket and substantial fragmentation of the alveolar bone extending from the extraction site. Yang et al. (2015) demonstrated that ZA (0.08 mg/kg weekly for 3, 7, and 15 weeks, intravenous) resulted in the clinical presence of necrotic bone in all samples, inflammatory infiltration in most samples (6/8), and soft tissue impairment in some samples (3/8).

Also, the data obtained by Gong et al. (2017) demonstrated that ZA (0.08mg/kg/week over 12 weeks, 400% of the rat oncologic dose) led to 65% and 45% of bone exposure after 4 and 12 weeks (respectively) after tooth extraction; and histological findings demonstrating necrotic bone and mucosal disruption. Janovszky et al. (2015) using 0.08mg/kg/week of ZA over 8 weeks, evidenced histological signs of osteonecrosis, such as the absence of nuclear staining in the osteocytes, necrotic bone, increased inflammatory infiltration, and granulation tissue formation. Sequester formation was present in 60% of the ZA-treated animals. Similarly, Elsayed et al. (2020) using 0.08mg/kg/week of ZA over 13 weeks, showed exposed necrotic bone after 8 weeks of tooth extraction. Although Isaias et al. (2021) have not observed bone exposure using ZA (0.2mg/kg/week over 4 weeks), it demonstrated deleterious effects in bone repair, such as necrotic bone, empty lacunae and increased TNF-α expression.

When combining ovariectomy with ZA administration (1500 µg ZA/Kg in 3.75 months, 500% of the rat oncologic dose), Kim et al. (2015) reported that the incidence of MRONJ was as high as 78%. Histological evidence demonstrated extensive ulcerative lesions accompanied by exposed and necrotic bone with sequestrum, and bacterial colonies were observed. Barba-Recreo et al. (2014) used 900 µg ZA/Kg in 2.25 months, which corresponded to 500% of the rat oncologic dose (applied 0.1 mg/kg, once/week), only 25% of their samples presented osteonecrosis and incomplete epithelial healing, and 50% of the samples showed disrupted alveolar bone (micro-tomography). Also, Silva et al. (2015) demonstrated histological evidence of bone necrosis in all samples ($p = 0.0004$, when compared to the control group), associated with large bone sequesters and intense inflammatory infiltrate.

Kuroshima et al. (2014), using 570% of the ZA oncologic dose (150 µg ZA/Kg in 0.33 months), demonstrated that ZA suppressed bone resorption and retained the necrotic bone within

the socket. They also highlighted the impact of ZA in impairing soft tissue healing and decreasing the number of blood vessels. Additionally, micro-tomography showed the presence of osteolytic lesions, extensive destruction, and disorganization of trabecular patterns with cortical disruption and sequestrum formation. Similarly, dexamethasone plus ZA led to a greater incidence (90%) of open wounds with clinical signs of inflammation and sequestrum (micro-tomography) than ZA alone (62.5%; no bone sequestrum [micro-tomography]) (Jabbour et al., 2014).

Using 750% of the rat ZA oncologic dose, Ferreira et al. (2020) showed that ZA (0.6mg/kg every 4 weeks over 20 weeks) delayed bone remodeling leading to 100% of the sample present bone sequestration, but only 8% with mucosal covering absence. Also, Maahs et al. (2011) (0.6mg/kg every 4 weeks over 12 weeks, 750% of the rat ZA oncologic dose) found a 100% of loss of mucosal integrity combined with osteonecrosis lesions in 80% of their sample. The study by Almeida et al. (2018) using 1800 µg ZA/Kg in 0.9 months, which also corresponded to 750% of the rat ZA oncologic dose, reported a complete absence of epithelialization of the extraction socket and histological findings, indicating a high incidence of osteonecrosis (70%), intense inflammatory infiltrate, and the presence of microbial colonies.

Still using the % above mentioned, Vasconcelos et al. (2012) used 0.6mg/kg of ZA every 4 weeks over 17 weeks and found that the absence of epithelial healing and signs of osteonecrosis were found in 100% of the sample. Similarly, Silveira et al. (2016) using ZA (0.6mg/kg every 4 weeks over 16 weeks) induced osteonecrosis (92% of non-vital bone). Finally, histological data from Kosach et al. (2020) demonstrated that ZA (0.18mg/kg/week over 6 weeks, 900% of rat ZA oncologic dose) led to a necrotic and inflammatory process in bone repair, characterized by the spread of loose fibrous tissue, and an uneven alternation of compact and spongy tissue.

Extremely Supraphysiologic (Group ES)

Studies using doses ≥1000% of rat ZA oncologic doses were included in this section (Table 3). Initially, Vilarinho et al. (2017) reported that 76% of specimens had clinical bone exposure, and 41% had cortical bone destruction under microCT analysis when ZA (0.066 mg/kg 3 times/week, 3 weeks, IP, 100% of rat ZA oncologic dose) was administered. Then, Kolpakova

et al. (2017) used ZA (1080% of the rat oncologic dose) and reported bone sequestration and disrupted cortical walls under microCT analysis. Also, histological section highlighted necrotic lesions, and inflammatory infiltration in the histopathological sections when ZA (0.36 mg/kg once + 0.18 mg/kg weekly, 4 weeks, intravenous) was administered.

Although Poubel et al. (2018a) and Cui et al. (2020) used very similar protocols of ZA (0.125mg/kg twice/week over 4 weeks, and 0.125mg/kg twice/week over 5 weeks, respectively – both 1250% of the rat ZA oncologic dose), Poubel et al. (2018a) did not report clinical signs of MRONJ. Still, it was demonstrated that a moderate to severe inflammatory infiltrate was identified in 63% of the ZA-treated animals, and 75% of the sample showed bone sequestration. However, Cui et al. (2020) evidenced that ZA led to 80% of bone exposure 5 weeks of tooth extraction, a decrease in the percentage of bone volume, and histological findings demonstrated hollow bone and irregular alignment of trabecular bone.

Then, increasing the dose up to 1500% of the rat oncologic dose, Barba-Recreo et al. (2014, 2015) highlighted the impact of the cumulative dose on the expected outcomes. Using 2700 μg ZA/Kg in 2.25 months, they found that 80% of the samples presented osteonecrosis, decreased vascularization, and incomplete epithelial healing (histology), and 100% of the samples displayed disrupted alveolar bone under micro-tomography, different from the data present by them when using 500% of the rat oncologic dose (Table 2). Interestingly, the data presented by Sousa et al. (2022) evidenced not only a high percentage of bone exposure (91%) and moderate inflammatory infiltrate, but an increase in the expression of IL-6, TNF-α and caspase3; histological sections pointed to a decreasing of 52% in the number of viable osteocytes and an increasing of empty lacunae.

Both studies performed by Dayisoylu et al. (2013; 2014) used the same protocol of ZA (0.1mg/kg, MWF, over 8 weeks), corresponding to 1500% of the rat oncologic dose. In these studies, ZA impaired bone repair leading to unhealed exposed bone and pus formation, also minimal inflammatory process around the periodontal area was noted. Similarly, Silva et al. (2015), animals treated with ZA exhibited radiolucent areas, while the histological findings of all animals were compatible with osteonecrosis lesions (bone sequestrum and intense inflammation infiltrate). Enhancing the ZA dose up to 3000% of the rat oncologic dose, Ersan et al. (2014) could not find mucosal disruption or bone exposure. Still, the ZA protocol of 0.2mg/kg, MWF,

over 6 weeks reduced alveolar bone width, and increased osteonecrosis area when compared to control.

Fourteen studies in this category associated ZA effects with another risk factor besides tooth extraction. The factors considered were concomitant use of DX, which were associated with 1000% (Adachi et al., 2020; Kaibuchi et al., 2016; Sanda et al., 2022; Yanik et al., 2016; Liu et al., 2021; Gao et al., 2021) or with 1500% of the ZA rat oncologic dose (Mergoni et al., 2019; Jung et al., 2021); senescence and periodontitis, which were associated with 1500% of the ZA rat oncologic dose, 2100 µg ZA in 1.75 months (Statkievicz et al., 2018); senescence and diabetes, which were associated with 1500% of the ZA rat oncologic dose, 2100 µg ZA in 1.75 months (Ervolino et al., 2022); age, which was associated with 1500% of the ZA rat oncologic dose, 2100 µg ZA in 1.75 months (Paulo et al., 2020); periapical disease, which was associated with 2000% of the ZA rat oncologic dose, 2000 µg ZA in 1.75 months, or 3200 µg ZA in 1.75 months, or 4000 µg ZA in 1.75 months (Hadaya et al., 2019); experimental periodontal disease, which was associated with 2000% of the ZA rat oncologic dose, 3600 µg ZA in 2.25 months (Soundia et al., 2018); and periapical lesions, which were associated with 4500% of the ZA rat oncologic dose, 3600 µg ZA in 1 month (Bolette et al., 2019).

The protocol of ZA+DX (0.066mg/kg + 5 mg/kg three times a week over 4 weeks) was performed by Adachi et al. (2020), Kaibuchi et al. (2016), and Sanda et al. (2022). These 3 studies basically showed ZA+DX led to exposed necrotic bone with incomplete restoration of epithelial continuity in 100% of the sample, and increased area of necrotic bone in these histological findings. Also, Kaibuchi et al. (2016) demonstrated microCT signs of delayed and irregular bone healing, and reduction in the expression of VEGF, HGF, RANKL, and OPG.

Still approaching papers using 1000% of the ZA oncologic dose, Yanik et al. (2016) modified the concentration of ZA+DX (0.1mg/kg + 1mg/kg, twice a week over 7 weeks), however, histological osteonecrosis was not significantly different between groups, and MRONJ or bone exposure was not reported. Also, Liu et al. (2021) demonstrated that ZA+DX (0.2mg/kg + 2mg/kg once week over 8 weeks) delayed soft tissue healing characterized by open wounds and bone exposition. As well, microCT showed signs of bone resorption and absence of bone regeneration for animals treated with ZA, and MRONJ was diagnosed in 83% of the sample. Same for Gao et al. (2021) who used ZA+DX (0.2mg/kg + 5mg/kg once a week over 16 weeks)

and induced classic osteonecrosis signs, such as incomplete mucosal healing and bone exposure in 100% ZA-treated rats. Also, microCT revealed irregularities in alveolar bone and disruption of cortical bone, and histological pointed to almost 90% of necrotic bone with empty lacunae.

The association of ZA+DX (0.1mg/kg+1mg/kg, 3x/week over 9 weeks, 1500% of the ZA oncologic dose) proposed by Mergoni et al. (2019) impaired wound healing but did not led to MRONJ or bone exposition. The same ZA+DX protocol was used by Jung et al. (2021), which demonstrated 50% of bone exposure in ZA+DX-treated animals, and histological signs of necrotic bone, empty lacunae and decreased number of vessels.

Similar results were reported by Statkievicz et al. (2018) (0.1 mg/kg every Monday, Wednesday, and Friday (MWF), 7 weeks, IP) when proposing a model of MRONJ associating ZA administration, periodontitis, and tooth extraction in senile female rats. Their model led to oral bone exposure in some samples (clinically), with histological findings supporting the assumption that ZA significantly impairs post-extraction socket healing by decreasing repair capacity and being associated with MRONJ.

The data from Ervolino et al. (2022) showed that 100% of their sample presented MRONJ with an area of exposed bone. In addition, ZA negatively affected the tissue repair process, presenting histological characteristics consistent with MRONJ-like lesions, a moderate/severe inflammatory response in the entire extension of connective tissue and bone tissue, and an association with more TRAP-positive cells. Likewise, Paulo et al. (2020) found that in the follow-up 21 days after tooth extraction, the ZA group contained 57% of the cases evaluated on the 21st day as presenting a solution of continuity and 28% of those with abscesses and purulent content. In the same period, animals from the ZA group had a lower bone density coefficient when compared to the control group ($p = 0.001$). Histological findings from the ZA group showed alterations in the epithelium, such as a decrease in the thickness and areas of discontinuity of the mucosa associated with the absence of bone tissue in the extraction site. However, immature bone was noted in the most coronal region of the surgical site, surrounded by inflammatory cells and hemorrhagic areas, which suggests osteomyelitis.

Hadaya et al. (2019) reported bone exposure and a lack of socket healing in ZA-treated animals. Their results strongly supported a synergistic contribution of severe dental disease and tooth extraction to MRONJ pathogenesis since experimental periapical disease (EPD) enhanced

osteonecrosis-like lesions, and these were associated with the bacterial presence in areas of osteonecrotic alveolar bone, highlighting the importance of infection in tooth extraction-related MRONJ. Very similar data were presented by Soundia et al. (2018), who demonstrated through microCT that experimental periodontitis (EP) and ZA administration could compromise socket healing and decrease the percentage of bone volume fraction (BV/TV). Histologically, ZA and EP increased osteonecrosis areas, with more empty osteocyte lacunae in alveolar bone associated with marked inflammatory response and increased matrix metallopeptidase 9 and 13 (MMP9 and MMP13) expression. Similarly, Bolette et al. (2019) performed pulpal exposure before the tooth extraction model to study the influence of infection in MRONJ. Data demonstrated that when the combination of mandible and infection was present, MRONJ significantly increased ($p = 0.0074$) compared to maxillae and infection. Histologically, animals with infection and ZA comprised 88.2% of the sample with MRONJ and 50% of the control group (ZA, but no associated infection).

Alendronate (ALN)

Twelve studies used ALN as a potential drug to induce MRONJ (Table 4). The rodent "osteoporosis dose" of ALN, which is 120µg/kg/month/SC (Seedor et al., 1991), was used in this review to classify these studies according to their cumulative doses. This dose completely stops ovariectomy-induced bone loss. It is usually given at 15µg/kg twice weekly, SC. It is equivalent to the ZA dose (8µg/kg/month IV) quoted in Gasser et al. (2008).

Of the 12 studies in this category, only half of them reported MRONJ (Abtahi et al., 2012; Berti-Couto et al., 2014; Conte Neto et al., 2013, 2016; Movahedian-Attar et al., 2020; Gonçalves et al., 2023). First, Conte Neto et al. (2016) demonstrated that using 8000 µg ALN/Kg over 2 months, corresponding to 3333% of the osteoporotic rat dose, led to MRONJ following tooth extraction. Their findings were: clinical bone exposure, necrosis (histology), and radiographic sclerosis of the bone with 1 mg/kg SC, once/week for 8 weeks, applied subcutaneously. Conte Neto et al. (2013) also demonstrated the association of ALN (63000 µg ALN/kg SC in 2.25 months, which is 23333% of the rat osteoporosis dose or 2333% of the rat oncology dose) with necrotic bone. In that model, ALN could induce ONJ-like lesions in rodents. After 28 days, the authors observed bone exposure, partial epithelial coverage, and infection. When examined

histologically, animals treated with ALN presented significantly increased necrotic bone ($p < 0.01$). Similar finds were also reported by Gonçalves et al. (2023), which demonstrated that ALN (1 mg/kg/day over 90 days, 16700% of the osteoporotic rat dose) impaired bone and soft tissues in the post-extraction sockets. Also, the histological analysis pointed to necrotic bone, absence of osteocytes, empty gaps, and lack of epithelial covering.

Noteworthily, Berti-Couto et al. (2014) showed that using a dose of ALN 100x lower (0.05mg/kg once a week, over 16 weeks, 167% of the osteoporotic rat dose) than Gonçalves et al. (2023), it was not able to induce osteonecrosis. However, when a DX (5 mg/kg/day) or 10% D-glucose anhydrous to induce diabetes were associated with ALN administration, the rates of osteonecrosis were 28 and 78%, respectively. Notably, while 2800 µg ALN/Kg in 0.5 months, which corresponded to 4666% of osteoporotic rat dose, did not induce MRONJ, the combination of this same cumulative dose of ALN (0.2 mg/kg daily for 14 days) with DX (1 mg/kg daily for 14 days) led to bone exposure (clinical) and discontinuity of the overlying epithelium, inflammatory infiltration, and distinguishable sequestrum (histology) (Abtahi et al., 2010). Same for Movahedian-Attar et al. (2020) which demonstrated that ALN+DX (0.2mg/kg/day + 1mg/kg/day over 14 days, 4666% of the osteoporotic rat dose) induced open wounds, necrotic exposed bone, and infection.

MRONJ did not occur following tooth extraction in Maahs et al. (2011) most likely because the absorbed dose of ALN was only 1.67% of the ALN osteoporosis dose. Histological findings (percentage of vial bone, the amounts of connective and epithelial tissues, microbial colonies, and inflammatory infiltrate) were similar between groups (test [5 cycles of 0.6 mg/kg ALN every 28 days, oral gavage)] and control). Similarly, Isaias et al. (2021) used several protocols of ALN varying between 2.1-16.7% of the ALN osteoporosis dose, and Mustakim et al. (2022) (167% of the ALN osteoporosis dose) and reported no signs of MRONJ, and histological findings demonstrated extraction sites filled with viable bone and no greater inflammatory signs.

However, Aguirre et al. (2010) (using doses ranging between 100 -1000% of ALN osteoporotic dose) and Altundal & Güvener (2004) (using a SC dose of 5833% of ALN osteoporotic dose) evidenced a decrease in bone formation and vascularity. It can be inferred that these events could contribute to the endurance of ONJ lesions.

Other drugs

This section included only the paper by Soundia et al. (2016). Their study used OPG/Fc antibody (10 mg/kg, twice/week for 8 weeks, IP) in 9-week-old male mice, followed by tooth extraction in the maxillae. It was reported that this dose could led to MRONJ with teeth extraction, especially with a periradicular disease. This protocol showed significant alveolar bone loss and a 70% rate of mucosal defects with exposed bone; histologically, the bone presented more empty osteocyte lacunae and osteonecrotic areas.

Experimental models in mice

This section includes studies designed using mice, ranging from 25-2200% of the ZA mouse oncologic dose. The routes of injection used in the 46 studies are as follows: IV (26), SC (10), and IP (10).

Summary

- At the lowest dose, 25% of the mouse ZA oncology dose, a single study reported no necrotic or exposed bone.
- Nine studies applied ZA doses which approximate 75% of the mouse ZA oncology dose. Though two found necrotic bone at 28d after tooth extraction, after 7-21d pre-treatment plus continued treatment with ZA, two others found no necrotic bone. Five others added either cyclophosphamide or DX to ZA. While all reported necrotic bone, only three noted exposed bone.
- Six studies used ZA doses ranging from 93-173% of the mouse oncology dose. One added DX. All six reported some degree of necrotic bone at 14-112d after tooth extraction, after 7-21d pre-treatment and generally continued ZA treatment, but only three reported exposed bone.
- Twenty-one studies gave a ZA dose equal to 185-225% of the mouse oncology dose. Six reported necrotic bone after 7-21d pre-treatment and 7-56d after tooth extraction with continued treatment. Fifteen others applied cofactors which included periodontitis, periapical lesions, DX, diabetes, or senescence after 7-21d pre-treatment

and saw an increased amount of necrotic bone compared to ZA alone at 7-84 days post-extraction. Exposed bone was reported by 75% of the studies, but often only in 30-50% of the mice.

- Four studies applied a ZA dose equal to 370% of the mouse oncology dose. All reported necrotic bone after 7-21d pre-treatment and 7-21d after tooth extraction and continued treatment. None reported exposed bone.
- Five studies used ZA doses of 740% or more of the mouse oncology dose. Four reported necrotic bone, after 7-21d pre-treatment and 7-21d after tooth extraction and continued treatment. Three reported exposed bone. The one which reported no necrotic bone pre-treated for 7d and only looked at just 5d post-extraction.

Initially, no signs of bone necrosis or exposition were found in the studies designed by Hokugo et al. (2019), which used the lower dose in this section (25% of the ZA mouse oncologic dose). The data demonstrate that there was no loss of mucosal integrity, no swelling area of mucosal tissue, and only 5% of necrotic bone area inside the socket, even increasing the ZA dose up to 70% of the ZA mouse oncologic dose (0.3mg/kg once/week over 3 weeks). Same for Hayano et al. (2020), also using 74 % of ZA mouse oncologic dose (0.05 mg/kg twice a week, over 5 or 7 weeks), however when ZA was associated with cyclophosphamide (CYP, 150 mg/kg) led to 100% bone exposure, with necrotic bone, and empty lacunae. The percentage of collagen fibers and the amount of inflammatory infiltrate were also affected. Similarly, the 700 μg ZA/Kg dose in 1.75 months, used by Kozutsumi et al. (2022) (74% of the ZA mouse oncologic dose) did not lead to clinical evidence of MRONJ. However, decreased epithelial thickness and rete ridge length, increased area of non-vital bone, and increased percentage of bone volume, number, thickness, and separation of trabeculae were observed in the ZA group compared to the control group.

However, data obtained by Sun et al. (2016) demonstrated that ZA (0.5mg/kg once, 70% of the ZA mouse oncologic dose) enhanced the percentage of necrotic bone after 2 and 4 weeks after tooth extraction. Igarashi et al. (2023) showed that the use of ZA (0.05 mg/kg twice a week, over 3 weeks, 74% of the ZA mouse oncologic dose), associated or not to CYP, delayed soft

tissue repair, however, bone exposure was not reported after 2 weeks evaluation. Same for Kuroshima et al. (2021), demonstrated that ZA+CYP (0.05 mg/kg + 150mg/kg twice a week, over 7 weeks, 74% of the ZA mouse oncologic dose) reduced the percentage of living bone, and enhanced necrotic bone, empty lacunae and PMN cells. Also, Kuroshima et al. (2018a, 2018b), using a methodology very similar, demonstrated that was not able to induce MRONJ unless it has been associated with CYP. Likewise, Kuroshima et al. (2013) showed that a combination of ZA + melphalan (MEL) (0.05mg/kg + 7mg/kg once a week, over 7 weeks) led to MRONJ and exposed necrotic bone.

The combination of ZA+DX (0.125mg/kg + 5mg/kg once a week, over 10 weeks, 93% of the ZA mouse oncologic dose) proposed by Yu et al. (2020) showed histological and morphological impacts, such as 78% with necrotic bone as 56% of the sample presented bone exposure. Similarly, Park et al. (2015) showed that ZA (100% of the ZA mouse oncologic dose) exhibited clinically open wounds (50% of the sample), swelling surrounding the wound, and bone exposure. Still, in Hokugo's study (Hokugo et al., 2019), the effect of ZA became even more significant when the doses used were increased even more, 0.5 mg/kg in 0.75 months (124% of ZA mouse oncologic dose) and 0.7 mg/kg in 0.75 months (173% of ZA mouse oncologic dose) since the data demonstrated inflammation in the oral mucosa, and connective tissue, signs of bone necrosis (around 30% of the alveolus compromised by non-vital bone). On the contrary, Kaneko et al. (2023) did not report bone exposure when using 148% of ZA mouse oncologic dose, however, there was an increase in the percentage of necrotic bone and empty lacunae, and these numbers were more evident when the anti-VEGFA neutralizing antibody was associated. Using the same percentage of ZA oncologic dose however in a different protocol (0.1mg/kg twice a week, over 12 weeks), Córdova et al. (2016) showed changes in bone healing, such as exposed and necrotic bone in 21% of the ZA-treated animals.

Using ZA in a dose of 185% of mouse oncologic dose, Zhu et al. (2019), Shen et al. (2022), Zhang et al. (2013), Williams et al. (2014), Kim et al. (2017), and Taniguchi et al. (2020) demonstrated similar results, in which, MRONJ and bone exposure was found in the in the samples. Besides that, the number of empty lacunae and percentage of necrotic bone was higher in ZA-treated mice when compared to the control group (saline solution). Similarly, Yoshioka et al. (2022), using ZA+DX (0.125 mg/kg + 5 mg/kg twice/week over 3 weeks), showed significant

increases in BV/TV, Tb.Th, Tb.N compared to control (saline solution), and a substantial decrease in Tb.Sp. Additionally, a higher percentage of bone necrosis and empty lacunae were found. Also, in a previous study, Kim et al. (2017) using ZA (0.125 mg/kg twice/week over 4 weeks) demonstrated unhealed osteomucosal tissues in tooth-extracted sockets associated with an overexpression of IL-36, which is related to inhibition of collagen expression. Similarly, Zhang et al. (2015) (0.125 mg/kg twice/week, over 4 weeks) demonstrated that ZA resulted in open sockets with impaired mucosal coverage restricted to 37.5% of the samples and delayed bone healing, and when this dose was associated with diabetes these results were more expressive and bone exposure was also reported.

Kikuiri et al. (2010), using ZA + DX (0.125 mg/kg + 5 mg/kg twice/week over 3 or 8 weeks) or ZA isolated (0.125 mg/kg twice/week over 8 weeks), demonstrated an incomplete mucosal healing with open sockets and exposed bone (50% of the sample for ZA+DX and 30% for ZA) in the extraction sockets after 8 weeks. In contrast, the control group presents 100% of the sample healed. Histological findings evidenced increased inflammation, necrotic bone, fibrosis, and absence of epithelium. Likewise, Zhao et al. (2012) demonstrated incomplete mucosal healing and open sockets with 20% of exposed bone in samples treated with ZA (0.125 mg/kg twice/week over 3 weeks) and 45% in samples treated with ZA+DX (0.125 mg/kg + 5 mg/kg twice/week over 3 weeks).

Also, Bi et al. (2010) demonstrated that angiogenesis and bone remodeling were suppressed by ZA (0.125mg/kg twice/week over 6 or 15 weeks) associated with DX (5 mg/kg once a week) induced sclerotic and necrotic bone during bone repair. Then, Kim et al. (2018) associated the ligature with inducing periodontitis in mice receiving ZA (0.125 mg/kg twice/week over 7 weeks) and demonstrated that the periodontal inflammatory conditions exacerbate the amount bone loss, number of empty lacunae, and percentage of necrotic bone. Biguetti et al. (2019), using ZA 0.25 mg/kg weekly for 4 weeks, reported a model of MRONJ in which ZA disturbed the socket healing (although after 21 days, 100% of the animals achieved complete healing); delayed blood clot and debris removal; and increased inflammatory infiltration, ensuing bone sequestrum, and the presence of empty lacunae. Corroborating with these data, Mahmoud et al. (2021), using 1000 µg ZA/Kg in 1 month, showed the reduced quantity and quality of the

inorganic matrix within the alveolar sockets and the presence of osteonecrosis lesions in the ZA group.

Similarly, Song et al. (2016) associated the pulp exposure with inducing periapical disease in mice receiving ZA (0.125 mg/kg + 5 mg/kg twice/week over 6 weeks) and found that periapical periodontitis exacerbates MRONJ. Also, a more recent study by Williams et al. (2020) associated the long-term ligature with inducing periodontitis in mice receiving ZA (0.125 mg/kg twice/week over 6 or 13 weeks). The data showed that the induced inflammation led MRONJ since microCT evidenced bony sequestrum, and histological analysis revealed increased bone necrosis and the number of empty lacunae. Interestingly, this article showed that the duration of inflammation can be associated with the amount of necrotic bone formation in mice receiving ZA.

Du et al. (2022) associated the pulp exposure with inducing periapical disease in mice receiving ZA (0.125 mg/kg + 5 mg/kg twice/week over 9 weeks) and found that this protocol led to MRONJ. Same for Wu et al. (2022) that used ZA (0.125mg/kg twice a week, over 7 or 11 weeks, 216% of ZA mouse oncologic dose) associated with *Porphyromonas gingivalis* and showed MRONJ signs without bone exposure. Using a dose % of ZA mouse oncologic dose, but now associated with DX (5mg/kg), Yoshioka et al. (2022) demonstrated that the percentage of necrotic bone and empty lacunae enhanced however no signs of bone exposure. Hokugo et al. (2019), using a dose of ZA (0.9 mg/kg, once a week, over 3 weeks), which corresponds to 225% % of the ZA mouse oncologic dose, demonstrated abnormal swelling in the mucosal tissue, and inflammatory infiltrate in connective tissue, and signs of bone necrosis (around 30% of the alveolus compromised by non-vital bone).

Thus, using ZA (0.25mg/kg twice a week over 4 weeks), Chen et al. (2021) showed a significant increase in the percentage of necrotic bone and apoptosis markers, microCT also revealed in the bone volume. Then, Soma et al. (2021, 2022) demonstrated that ZA (0.5 mg/kg once/week over 8 weeks, which corresponds to 370% of ZA mouse oncologic dose) could let to 60% of the alveolar socket with non-vital bone, characterized in histological analysis by empty lacunae. Also, Kozutsumi et al. (2022), using 3500 µg ZA/Kg in 1.75 months, which corresponded to 500% of the ZA mouse oncologic dose, highlighted the greater incidence of bone necrosis and a more significant number of empty lacunae ($p<0.01$ and $p<0.001$, respectively), as

well as a lower amount of vital bone ($p<0.05$) in the ZA group as compared with the control. Also, in this same paper, when ZA was increased to 1000% of the ZA mouse oncologic dose (7000 µg ZA/Kg in 1.75 months), it was demonstrated a dose-dependent factor, since this dose significantly impaired bone healing after tooth extraction and increased necrotic bone and number of empty lacunae.

Finally, Zheng et al. (2023) using ZA (0.5mg/kg twice a week over 4 weeks, 740% of ZA mouse oncologic dose) showed necrotic bone exposure, decrease in bone volume (microCT), and absence of epithelium covering. Also, expression of IL-1 and IL-6 were highly elevated in ZA-treated animals. Finally, the data presented by Kobayashi et al. (2010) (0.25 mg/kg daily, 11 days, subcutaneous) demonstrated that ZA (1455% of ZA mouse oncologic dose) delayed wound healing and inhibited osteogenesis and angiogenesis, as evinced by the decreased expression of platelet endothelial cell adhesion molecules. It also affects migration and cell viability, resulting in open sockets prone to bacterial infiltration. Also, Zhao et al. (2023) using ZA (1mg/kg, MWF, over 4 weeks, 2222% of ZA mouse oncologic dose) with periodontitis associated 30% of sample present bone exposure. Also, there was a high number of empty lacunae and necrotic bone.

1.5 DISCUSSION

Despite the numerous studies attempting to establish an optimal protocol to induce MRONJ, there need to be appropriate guidelines for properly designing in vivo experimental models to induce MRONJ. This review emphasizes that osteonecrosis lesions (histological findings) occur in antiresorptive-treated rodents undergoing a tooth extraction, while taking at the minimum, ZA or ALN doses that approximate the oncology doses of the respective drugs. This confirms the utility of these rodent models, as the lowest antiresorptive dose applied was 2-4X greater than the human osteoporosis treatment dose. It is well-known that MRONJ incidence in patients with osteoporosis is above zero, at 0.01-0.03%.

MRONJ, a potentially serious condition which requires the coexistence of systemic and local oral risk factors, is common in patients with cancer (2-5%) and rare in patients with osteoporosis (0.01-0.03%), who take anti-resorptive medications. Accordingly, the most frequent systemic risk factor is anti-resorptive medication. Patients with cancer receive a ten-fold greater

anti-resorptive dose than do patients with osteoporosis. It has previously been established that such profound inhibition of bone resorption exists at oncology doses of anti-resorptives, that further increases in dose cause no further inhibition of bone resorption. Tooth extraction is the major procedural local oral risk factor and inflammatory dental disease is the major medical-condition local oral risk factor identified for MRONJ in humans.

Over 100 studies of tooth extraction-related MRONJ in mice and rats are summarized here. Approximately 20 of these studies were previously reviewed (Poubel et al., 2018b). We review here studies that used zoledronate (ZA) and alendronate (ALN), two widely-available bisphosphonate-class anti-resorptives used in humans. The specific drugs used in humans can be easily applied pre-clinically in rodents. Their pharmacology in both humans and rodents is well-documented, particularly that surrounding the pre-clinical equivalents of the oncology dose in humans. ZA and ALN are excellent representative anti-resorptives. Pre-clinical studies with a second major anti-resorptive class used often in humans, RANKL antibodies, are difficult because the agents used in humans are fully-humanized antibodies which are not efficacious in rats. Thus, the specific RANKL inhibitor drugs used in humans cannot be applied pre-clinically in rodents. When custom-developed rodent-specific RANKL inhibitors have been applied, the results have been similar, but such pre-clinical experiments will continue to be rare, because these rodent-specific RANKL agents are not widely available.

In the current series of studies, a range of anti-resorptive doses which covers more than four orders of magnitude of absorbed dose has been tested. This range includes the doses given to patients with cancer and patients with osteoporosis. The study repetition at key dose levels in mice is good with nine studies using approximately 75% of the mouse ZA oncology dose and 21 studies using approximately twice the mouse ZA oncology dose. Study repetition at key dose levels for the rat is also good with six studies using approximately the rat ZA oncology dose and 14 studies using 2-4X the rat ZA oncology dose.

A large range of pre-extraction treatment time periods that focuses on 21-28d, but includes up to 147 days has been tested. A large range of post-extraction observation periods that focuses on 28-56d, but extends to 105 days has also been used. About two-thirds of studies continued anti-resorptive treatment during the post-extraction observation period.

Though many general histopathologic observations are recorded in these publications (e.g., signs of inflammation, fibrosis, swelling, number of empty lacunae, sequestrum, etc.), the most fundamental endpoints reported in all studies concern: a) exposed bone in the oral cavity; and b) necrotic alveolar bone in and around the tooth extraction socket. Authors monitored exposed bone mainly because it is the hallmark of MRONJ in humans. Early histopathologic studies of human MRONJ cases showed that underlying necrotic bone was generally present in patients with exposed bone. Eight weeks' persistence of exposed bone has been accepted for nearly two decades as a diagnostic marker of MRONJ in humans. Biopsy to formally identify necrotic bone in humans suspected of having MRONJ is now rarely done to avoid negatively altering the course of MRONJ. Although many studies included in this review show that anti-resorptive drugs have an effect on bone repair from an early stage, they alter the expression of growth factors necessary for vascularization, such as VEGF, as well as delay the mucosal coverage by decreasing cells migration and increasing toxicity and impairing bone repair.

The data from these studies generally suggest that exposed bone in rodent studies is found less consistently than necrotic bone in and around the tooth extraction site. Given that exposed bone is only an indirect biomarker for MRONJ in humans, the inconsistent reporting of exposed bone in these studies should not be taken to suggest that rodents area a weak model for tooth extraction-related MRONJ. In fact, the most important diagnostic endpoint used in rodent studies is a thorough histopathologic examination of 4µm thick H&E-stained sections of decalcified bone from both the mesial, distal, buccal, and lingual bony walls surrounding the tooth extraction socket and the interior of the tooth extraction site, looking for fields of contiguous empty osteocyte lacunae in bone tissue that define necrotic bone tissue (Fondi, & Franchi, 2007). These pre-clinical studies, unlike human MRONJ studies, directly document the actual existence of necrotic bone. Quantitative measurements, including "empty lacunae/mm2" and "% of empty lacunae" are used as supportive data for an MRONJ diagnosis in pre-clinical studies.

It is very encouraging that MRONJ was not reported by three studies of up to five months duration that applied ≤25% of the ALN rat osteoporosis dose. Considering the rarity of MRONJ in patients with osteoporosis who take antiresorptives, it is not surprising that MRONJ was not reported by two other studies of up to thirteen weeks duration that applied ≤ 167-1000% of the ALN rat osteoporosis dose.

When ZA doses in the range of the oncology dose in mice and rats (and ALN in rats) were used, MRONJ was occasionally reported. Adding simultaneous systemic risk factors (e.g., dexamethasone, diabetes) or simultaneous local oral risk factors (localized experimental periodontitis, periapical abscess, or P. gingivalis) generally increased the % of cases in which MRONJ was seen in individual study reports. When ZA doses two-fold and more above the ZA oncology dose were applied, MRONJ incidence generally approached 100%, particularly when local oral risk factors were added.

Finding MRONJ in ZA-only groups may imply that surgical trauma to bone that leaves behind traumatically-detached pieces of bone that eventually die, that are resorbed abnormally slowly, is one cause of MRONJ. However, since other local risk factors, such as local infection increase MRONJ incidence, it likely that other local risk factors are also important.

In this review, data obtained in Group P demonstrated that 9 studies used doses approximately 33–200% of the rat oncologic dose, and MRONJ was only not diagnosed by one (Cardoso et al., 2019) of them (Table 1), however, 40% of the sample present bone exposure. Three studies (Sonis et al., 2009; Ali-Erdem et al., 2011; Marino et al., 2012) used less than 80 µg ZA/Kg/month (ZA rat oncology dose), and the researchers were able to report MRONJ and bone exposure (Sonis et al., 2009; Marino et al., 2012). In Group SP (Table 2), all studies using >200% of the rat ZA oncologic dose to <1000% of the rat ZA oncologic dose reported MRONJ, except by Kuroshima et al. (2014). However, ZA's impact on bone resorption, soft tissue healing, and angiogenic potential was highlighted. While the use of supraphysiologic and extremely supraphysiologic ZA doses has proven indispensable during model development, when it was necessary to convincingly prove that anti-resorptive-treated mice and rats could express necrotic bone after tooth extraction, it would be helpful in the future to execute experiments concerning tooth extraction-related MRONJ that focus on models that have both local oral risk factors (trauma and infection) present in the extraction of most human teeth, possibly facilitating the more reliable development of MRONJ while simultaneously using more physiologic doses. Using physiologic ZA doses would increase the likelihood that the animal model did not rely on unknown metabolic pathways affected by ZA to create MRONJ.

Regarding the studies performed in mice (Table 5), it can be highlighted that data obtained from the studies using doses above 125% of ZA mouse oncological are very consistent.

In general, all these studies were able to induce MRONJ, except for Zhao et al. (2012) that used ZA only a 7d pre-treatment period with a 14d post-extraction examination period. This may have been too short to observe MRONJ. However, Zhao et al. (2012) demonstrated that ZA could delay mucosal healing and led to open sockets with 20% of exposed bone in samples treated with ZA and 45% when treated with ZA+DX. Studies with doses ranging from 33% to 100% of ZA mouse oncological dose did not develop MRONJ, except for Kozutsumi et al. (2022) using ZA 0.1 mg/kg twice/week over 7 weeks. It is worth noting that the study by Hokugo et al. (2019) investigated various doses of ZA in mice (0.1, 0.3, 0.5, 0.7, and 0.9 mg/kg once over 3 weeks) and demonstrated that a particular threshold concentration of bioavailable BP dose in the jawbone is necessary to trigger osteonecrosis pathogenesis, which in their model, it was from 0.5 mg/kg.

Although ALN is the most common drug used to treat osteoporosis worldwide (Paiva-Fonseca et al., 2014), epidemiological studies demonstrate that the incidence of MRONJ associated with ALN is low (0.01–0.03%) (Sedghizadeh et al., 2009; Abtahi et al., 2012; Ruggiero et al., 2022). In this review, only 6 studies using ALN reported the occurrence of MRONJ (Table 4). Conte Neto et al. (2013), using 1 mg/kg daily for 8 weeks, and Conte Neto et al. (2016), using 1 mg/kg once/week for 8 weeks, demonstrated bone exposure through clinical findings associated with histological and radiographic changes in the bone tissue. All other ALN studies reported no clinical signs of MRONJ (Altundal & Güvener, 2004; Aguirre et al., 2010; Maahs et al., 2011; Isaias et al., 2021; Mustakim et al., 2022). However, these studies reported either delayed initial healing or decreased bone volume and vascularization (Altundal & Güvener, 2004; Aguirre et al., 2010), reduced number of osteoclasts per measured area (Altundal & Güvener, 2004), or no signs of osteonecrosis lesions (Maahs et al., 2011). The cumulative absorbed ALN dose delivered in these studies was often less than 25% of the osteoporosis dose of ALN (Maahs et al., 2011; Isaias et al., 2021), a dose so low that MRONJ could not reasonably be expected to occur. In another case (Altundal & Güvener, 2004), there was no pre-treatment with ALN, something that does not parallel what happens in humans.

Dexamethasone inhibits the inflammatory process and suppresses the immune system for multiple reasons, including supportive care during cancer treatment (Yennurajalingam & Bruera, 2014; Giles et al., 2018). However, it has already been shown that dexamethasone may lead to side effects on bone metabolism (Liu et al., 2015) though it is not known whether those effects

are expressed in animals receiving oncologic doses of anti-resorptives. It is suggested that the effects of using DX may be significant because of its negative influence on wound healing (Johnston, 1990; Beer et al., 2000; Poetker & Reh, 2010; Harris et al., 2015). The data reported in this review indicated that combination therapy with ALN and dexamethasone (Abtahi et al., 2012) could induce MRONJ (clinically). Aside from delayed healing, ALN alone did not foster clinical changes in the socket. This finding should be emphasized. Similarly, the association of ZA and dexamethasone may be appreciated as a reliable model to study MRONJ (Sonis et al., 2009), as demonstrated in the study by Jabbour et al. (2014). In this research, while ZA alone led to osteonecrosis lesions in 62.5% of the samples, the incidence was as high as 90% when combined with dexamethasone. Similar results in mice were also found (Table 5) when ZA associated with DX (Bi et al., 2010; Kikuiri et al., 2010; Zhao et al., 2012; Yoshioka et al., 2022) led to MRONJ. It must be remembered that the majority of MRONJ cases in humans occur in the absence of glucocorticoid administration. Using DX as a standard way to cause MRONJ in pre-clinical studies introduces a systemic condition that does not occur in most human MRONJ.

It may also be instructive that both dexamethasone and diabetes increase the incidence of MRONJ (Berti-Couto et al., 2014; Takaoka et al., 2015; Zhang et al., 2015). Both are known for delaying wound healing (Jiao et al., 2015; Chen et al., 2022). It is possible that a portion of MRONJ cases result from a failure to heal oral events that would ordinarily resolve promptly. It is indeed possible that the anti-angiogenic properties which have been identified for bisphosphonates by in vitro studies, present themselves as failed wound healing when doses that far exceed the oncology dose are employed.

In consideration of the multifactorial nature of MRONJ, a role in the pathophysiology of the condition ought to be assigned to the trauma caused by surgical procedures in the oral cavity (e.g., tooth extractions), since previous studies (Hokugo et al., 2010; Barba-Recreo et al., 2014) demonstrated that ZA alone does not create an environment sufficient to the spontaneous onset of MRONJ. Also, mimicking the clinical scenario, the presence of infected teeth is an essential factor included in this review and deserves recognition, as the data demonstrated here to highlight how the presence of inflammatory disease can contribute to the progression of MRONJ (Soundia et al., 2016; Hadaya et al., 2019; Soundia et al., 2018; Song et al., 2016; Statkievicz et al., 2018; Kim et al., 2018; Bolette et al., 2019; Ervolino et al., 2022; Du et al., 2022; Williams et al.,

2022). Also noteworthy are the results published by Williams et al. (2020), which show that the duration of the inflammatory process is associated with increased MRONJ in ZA-treated mice.

The role of the drug and dosing interval in triggering osteonecrosis events in the bone is clearly stated in the literature. However, it is unlikely to prompt spontaneous bone sequestration (Khan et al., 2015; Ruggiero et al., 2022). The cumulative effect of duration of anti-resorptive therapy during long-term treatments is more important than the drug itself or the dosing intervals (Thumbigere-Math et al., 2012). This is a potent enabler of MRONJ lesions when associated with other risk factors, such as oral surgical procedures, inflammatory dental disease, diabetes, or trauma (Hasegawa et al., 2017; Otto et al., 2018).

In rats, ZA led to MRONJ regardless of the doses used (physiologic [Sonis et al., 2009; Hokugo et al., 2010; Biasotto et al., 2010; Ali-Erdem et al., 2011; Marino et al., 2012; Takaoka et al., 2015; Silva et al., 2015; Curra et al., 2016], supraphysiologic [Maahs et al., 2011; Barba-Recreo et al., 2014; Jabbour et al., 2014; Howie et al., 2015; Kim et al., 2015; Yang et al., 2015; Zandi et al., 2015; Silva et al., 2015; Vidal-Gutiérrez et al., 2017; Almeida et al., 2018], and extremely supraphysiologic [Bolette et al., 2019; Barba-Recreo et al., 2014; 2015; Silva et al., 2015; Kolpakova et al., 2017; Vilarinho et al., 2017; Hadaya et al., 2019; Soundia et al., 2018; Statkievicz et al., 2018; Paulo et al., 2020; Liu et al., 2021; Ervolino et al., 2022], and when associated with dexamethasone [Sonis et al., 2009; Ali-Erdem et al., 2011; Jabbour et al., 2014]). This is not entirely unexpected, as the lowest dose of ZA tested was 250% of the rat osteoporosis dose of ZA. It is well-known that the incidence of MRONJ in humans treated with osteoporosis doses of anti-resorptives is very low, but definitely above zero, at 0.01-0.03%.

Six studies reported MRONJ when using ALN (Abtahi et al., 2012; Conte Neto et al., 2013; Berti-Couto et al., 2014; Conte Neto et al., 2016; Movahedian-Attar et al., 2020; Gonçalves et al., 2023) and one when using OPG/Fc antibody (Soundia et al., 2016). In mice given 100-370% of the mouse ZA oncology dose, MRONJ was seen in Park et al. (2015), Hokugo et al. (2019), Kaneko et al. (2023), Córdova et al. (2016), Zhu et al. (2019), Shen et al. (2022), Zhang et al. (2013), Williams et al. (2014), Yoshioka et al. (2022), Kim et al. (2017), Kikuiri et al. (2010), Zhao et al. (2012), Taniguchi et al. (2020), Bi et al. (2010), Kim et al. (2018), Biguetti et al. (2019), Mahmoud et al. (2021), Du et al. (2022), Song et al. (2016), Williams et al. (2022), Wu et al. (2022), Chen et al. (2021).

Almost all pre-clinical models included in this review emphasized histopathologic examination that revealed the presence of necrotic bone. Only 3 studies (Basi et al., 2011; Jabbour et al., 2014; Vilarinho et al., 2017) did not report histological findings. In animal models, the standard for MRONJ calls for histopathological detection of fields of adjacent empty lacunae in bone tissue (Fondi & Franchi, 2007) and quantification of the empty lacunae/mm^2 of bone tissue and % of empty osteocyte lacunae. Ideally, the authors would present clinical, histological, and imaging data, which could contribute to MRONJ diagnoses in pre-clinical models.

1.6 CONCLUSION

This review supports the conclusion that ZA can lead to osteonecrosis lesions (histologic) when dose and observation time are adequate in rodents undergoing tooth extraction. This is not surprising because the tested doses of ZA range from 25% of the ZA rat osteoporosis dose to 4500% of the ZA rat oncology dose. The ZA osteoporosis dose causes MRONJ in humans. Combining the local oral risk factor, inflammatory dental disease, with tooth extraction led to a higher incidence of MRONJ. Similarly, combining the systemic risk factor, dexamethasone, with tooth extraction also led to a higher incidence of MRONJ.

Investigators who choose a physiologic dose of ZA for a tooth extraction-related MRONJ rat or mouse model face the likelihood that the incidence of MRONJ in their ZA groups may be only 10-40%. The protocol might call for two IV ZA doses per month, with tooth extraction occurring four weeks after the first dose and necropsy occurring six to eight weeks after tooth extraction. Though this will necessitate relatively large group sizes for MRONJ incidence findings in comparator groups to achieve statistical significance compared to the ZA group, investigators can be reasonably certain that the ZA environment in the experiment occurs in humans with MRONJ.

Investigators who choose a supraphysiologic dose of ZA for a tooth extraction-related MRONJ rat or mouse model can achieve an incidence of MRONJ in their ZA groups of 70-100%. The specific protocol would resemble the above timing. Though this will allow smaller group sizes for any findings about MRONJ incidence in comparator groups to achieve statistical

significance compared to the ZA group, investigators will be less certain that the ZA environment in the experiment is relevant to what occurs in humans with MRONJ.

Investigators who choose a physiologic dose of ZA for a tooth extraction-related MRONJ rat or mouse model could consider adding a relevant local oral risk factor such as inflammatory dental disease, an intervention that would likely increase the incidence of MRONJ in the ZA groups into the 60-80% range. The specific protocol would resemble the above timing. This will allow smaller group sizes for findings about MRONJ incidence in comparator groups to achieve statistical significance compared to the ZA group while maintaining a ZA environment in the experiment that occurs in humans with MRONJ. Given the data presented in this manuscript, this type of experiment is a reasonable to consider.

Declaration

All authors made a significant contribution to this article. Conceptualization: F.P.S.G. and H.H.; Methodology: H.H. and F.A.S.; Formal analysis: H.H.; Investigation: H.H. and H.R.M.; Data curation: H.H.; Writing: H.H. and H.R.M.; Writing, review, and editing: H.H., and F.P.S.G.; Supervision: F.P.S.G., S.I.P., and F.A.S.; Project administration: F.P.S.G. All authors reviewed the manuscript.

Funding

This work was partly supported by the Educational and Research Fund, Department of Oral and Maxillofacial Surgery, Massachusetts General Hospital (Boston, MA).

Conflict of interest

Henrique Hadad, Henrique R. Matheus, Sara I. Pai, Francisley A. Souza, and Fernando P.S. Guastaldi declare that they have no conflict of interest.

Acknowledgments

The São Paulo State Foundation for Research (FAPESP) awarded Dr. Henrique Hadad a Ph.D. scholarship (#2019/22657-8), and the scholarship granted by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), within the scope of the Program CAPES-PrInt; process number #88887.570145/2020-00.

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Table 1 Characteristics of studies evaluating Zoledronic Acid [Physiologic Dose (≤200% of rat ZA oncology dose)].

Author/ Year	Dose	% of rat ZA oncology dose	Route	Animal/ Gender/Age	Risk Factor	Surgical Site	MRONJ	Bone Exposure	Pre-XT (d)	Post-XT (d)	Post-XT treat
Guevarra et al., 2015	0.02 mg/kg-monthly 8wks 40 µg/kg 2m	25	IV	SD/F/RB	No	Md/1M	Yes (38%)	Yes (38%)	28	28, 56, 84	No
Marino et al., 2012	0.02 mg/kg every 21d – 3wks 40 µg/kg 1.5m	33	IV	SD/F/8m	No	Md/1M	Yes	Yes (75%)	21	28, 56	No
Ali-Erdem et al., 2011	0.0075 mg/kg once/wk – 3wks 22.5 µg/kg 0.75 m	38	SC	WA/M/5w	DX	Md Mx/ 1-3M	Yes[1]	No	14	28	No
Tamari et al., 2019	0.0075 mg/kg-1X/wk –11wks 83 µg/kg 2.75m	38	SC	Lewis/F&M /13w	DX	Mx/1M	Yes (52%)	Yes	14	56	Yes
Sonis et al., 2009	0.0075 mg/kg once/wk – 3wks 22.5 µg/kg 0.75 m	38	SC	SD/F/11w	DX	Md Mx/ 1-3M	Yes	Yes[5]	21	14, 28	No
Hokugo et al., 2010	0.035 mg/kg every 2 wks – 11wks 210 µg/kg 2.75 m	87	IV	SD/#/7w	Yes*	Mx/1-3M	Yes[2]	Yes	21	7, 14, 28, 56	Yes
Cardoso et al., 2019	0.035mg/kg every 2 wks – 8 wks 140 µg/kg 2 m	87	IV	WA/M/8w?	No	Mx/In	No	Yes	42	14, 28, 42	No
Curra et al., 2016	0.035 mg/kg every 2 wks - 8 wks 140 µg/kg 2 m	87	IV	WA/M/10w?	No	Mx /In	Yes	Yes	63	14, 28, 42	No
Takaoka et al., 2015	0.035 mg/kg every 2 wks - 29 wks 525 µg/kg 7.5 m	87	IV	SD/M/5w	Yes[γ]	Mx/1-3M	Yes[3]	Yes	147	14, 28, 56	Yes
Silva et al., 2015	3 cycles of 0.04 mg/kg once/wk + 1 cycle 5 wks later 160 µg/kg 2 m	100	IV	WA/M/8w	No	Md/1M	Yes[4]	No	42	28	Yes
Elsayed et al., 2018	0.08 mg/kg/-2X/wk – 13wks 2080 µg/kg 3.25m	119	IV	SD/F/10-12mo	No	Md/1-2M	Yes	Yes	91	35	No
Brierly et al., 2019	0.1 mg/kg- 3wks - 6wks 200 µg/kg 1.5m	166	IV	SD/?/9w	No	Md/1M	Yes	Yes	21	56	No
Biasotto et al., 2010	0.04 mg/kg once/wk - 5 wks 200 µg/kg 1.25 m	200	IV	WA/M/13w[6]	No	Mx/1M	Yes	Yes	14	56	Yes
Zhu et al., 2019	0.08 mg/kg/wk - 8 wks – 16wks 640 µg/kg 4m 0.08 mg/kg/wk - 8 wks – 16wks 640 µg/kg 6m	200 200	IP	SD/F/8mo	Yes[o]	Md/1-3M	Yes (75%) Yes (89%)	No No	56 56+56H	56 56	No No

Rat ZA oncology dose is 0.08mg/kg IV/month (Aguirre et al., 2022).
Column MRONJ: authors' interpretation of their results (*Yes* means treated rats developed osteonecrosis).
Column Risk Factor: * means vitamin D deficiency; [γ] means diabetes associated.
Column MRONJ and Bone Exposure, 1 means 60% of samples had osteonecrosis; 2 means 14% of samples in the VitD+/ZA group had osteonecrosis, while 67% in VitD-/ZA had osteonecrosis; 3 means 17 % of samples in ZA + diabetes group had osteonecrosis and 100% of samples in ZA + diabetes had osteonecrosis and bone exposure; 4means 14% of samples had osteonecrosis; 5 40% of samples had bone exposure; 6 means "skeletally mature"

Abbreviation: ZA, zoledronate; DX, dexamethasone, SC, subcutaneous; IP, intraperitoneal; IV, intravenous; SD, *Sprague Dawley* rats; WA, *Wistar Albinus* rats; M, male; #, not reported; w, wks; m, mos; Mx, maxilla; Md, mandible; 1M, fist molar; 2M, second molar; 3M, third molar; 1-3M, first, second, and third molar; In, incisor; Pre-XT: pre-extraction treatment period; Post-XT: post-extraction period; Post-XT Treat: drug treatment applied during post-extraction period.

Table 2 Overall characteristics of studies evaluating Zoledronic Acid [Supraphysiologic Dose (>200%>1000% of rat ZA oncology dose)].

Author/ Year	Dose	% of rat ZA oncology dose	Route	Animal	Risk Factor	Surgical Site	MRONJ	Bone Exposure	Pre-XT (d)	Post-XT (d)	Post-XT treat
Zandi et al., 2016	0.06 mg/kg- 1X/wk - 7wks 420 µg/kg 1.75m	300	IP	WA/M/10w	No	Md/1M	Yes (100%)	Yes (100%)	0, 7, 14, 21	28	Yes
Borke et al., 2015	0.06 mg/kg-1X/wk 2wks 120 µg/kg 0.5m	300	IV	SD/F/12w	No	Md/1M	Yes (100%)	Yes (100%)	7	28	No
Imada et al., 2019	0.06 mg/kg/wk –2wks 120µg/kg 0.5m	300	IV	SD/F/10w	No	Md/1M	Yes	Yes	14	21 or 56	No
Vidal-Gutiérrez et al., 2017	0.06 mg/kg every 7d – 2 cycles 120 µg/kg 0.5 m	300	IM	WA/M/10w	No	Mx/2M	Yes	Yes	21	14, 28, 42	No
Zandi et al., 2015	0.06 mg/kg once/wk – 5 wks 300 µg/kg 1.25 m	300	IP	WA/M/10w	No	Md/1M	Yes	Yes (85%)	28	56	No
Zandi et al., 2017	0.06 mg/kg- 1X/wk - 12wks 720 µg/kg 3m	300	IP	WA/M/13w	No	Md/1M	Yes (83%)	Yes (80%)	84	56	No
Gong et al., 2017	0.08 mg/kg-1X/wk –12wks 960µg/kg 3m	400	IV	SD/F/9w	No	Md Mx/1M	Yes	Yes (45%)	14	70	Yes
Yang et al., 2015	0.08 mg/kg once/wk – 3 wks 240 µg/kg 0.75 m 0.08 mg/kg once/wk – 7 wks 560 µg/kg 1.75 m 0.08 mg/kg once/wk – 15 wks 1200 µg/kg 3.75 m s	400 400 400	IV	SD/M/9w	No	Mx/1M	Yes	Yes	21 21 21	7 28 84	Yes Yes Yes
Janovszky et al., 2015	0.08 mg/kg- 1X/wk - 8wks 640 µgkg 2m	400	IV	WA/M/8w	No	Md/1-2M	Yes (60%)	Yes (100%)	21	42	Yes
Isaias et al., 2021	0.2 mg/kg/wk - 3 wks +1 wk 800 µg/kg 2.5m	400	IV	WA/M/9w	No	Md/1M	Yes	No	42	28	No
Elsayed et al., 2020	0.08 mg/kg/-2X/wk – 13wks 1040 µg/kg 3.25m	400	IV	SD/F/10-12mo	No	Md/1-2M	Yes	Yes	91	56	No
Howie et al., 2015	0.08 mg/kg once/wk – 13 wks 1040 µg/kg 3.25 m	400	IV	SD/F/11m	Yes #	Md/1-2M	Yes	Yes	91	7, 14, 56	No
Silva et al., 2015	3 cycles of 0.20 mg/kg once/wk + 1 dose 5 wks later 800 µg/kg 2 m	500	IV	WA /M /8w	No	Md /1M	Yes2	No	42	28	Yes
Kim et al., 2015	0.1 mg/kg once/wk – 15 wks 1500 µg/kg 3.75 m	500	SC	SD /F/16w	Yes º	Md/1-3M	Yes1 78%-OVX 47%-Sham	Yes	42	56	Yes
Barba-Recreo et al., 2014	0.1 mg /kg once/wk – 9 wks 900 µg/kg 2.25 m	500	IV	WA /M /8w	No	Mx/1-3M	Yes3	No	56	7	Yes
Kuroshima et al., 2014	0.1 mg/kg every 2 d – 10 d 150 µg/kg 0.33 m	570	SC	SD /# /6w	No	Mx/2M	Yes	No	0	10	Yes

Jabbour et al., 2014	0.13 mg/kg once/wk - 7 wks 910 µg/kg 1.75 m	650	IP	SD /# /32w	DX	Md Mx/1M	Yes	Yes	21	28	Yes
Ferreira et al., 2020	0.6 mg/kg - 4 wks – 20wks 3000 µg/kg 5m	750	IP	WA/M/12w	No	Mx/1-3M	Yes	No	45	105	Yes
Maahs et al., 2011	0.6 mg/kg once/mo - 5 cycles 3000 µg/kg 5 m	750	IP	WA/M/20w	No	Mx/1-3M	Yes	No	45	105	Yes
Almeida et al., 2018	0.6 mg/kg once/mo – 3 cycles 1800 µg/kg 3 m	750	#	WA/M/20w	No	Mx/2-3M	Yes	Yes (100%)	60	15 30	No
Vasconcelos et al., 2012	0.6 mg/kg- 1X/mo - 17wks 3000 µg/kg 5m	750	IP	WA/F/16w	No	Mx/1-3M	Yes (100%)	Yes (100%)	60	42	Yes
Silveira et al., 2016	0.6 mg/kg/mo –4mos 2400µg/kg 4mos	750	IP	WA/F/17w	No	Mx/1-3M	Yes	Yes	60	60	Yes
Kosach et al., 2020	0.18 mg/kg- 1X/wk - 6wks 1080 µg/kg 1.5m	900	IV	WA/M/26w	No	Md/1M	Yes	DNR	42	28	No

Rat ZA oncology dose is 0.08mg/kg IV/month (Aguirre et al., 2022).
Column MRONJ relates to how authors interpret their results (*Yes* means treated rats developed osteonecrosis, *DNR* means do not reported).
In Column Risk Factor, # means repeated trauma associated; º means OVX associated.
In Column MRONJ and Bone Exposure, 1 means 78% of the samples present osteonecrosis; 2 means 14% of the samples present osteonecrosis; 3 means 25% of the samples present osteonecrosis.
Abbreviation: ZA, zoledronate; DX, dexamethasone, SC, subcutaneous; IP, intraperitoneal; IV, intravenous; IM, intramuscular; SD, *Sprague Dawley* rats; WA, *Wistar Albinus* rats; F, female; M, male; #, not reported; w, weeks; m, months; Mx, maxilla; Md, mandible; 1M, fist molar; 2M, second molar; 3M, third molar; 1-3M, first, second, and third molar; Pre-XT: pre-extraction treatment period; Post-XT: post-extraction period; Post-XT Treat: drug treatment applied during post-extraction period.

Table 3 Overall characteristics of studies evaluating Zoledronic Acid [Extremely Supraphysiologic Dose (≥1000% of rat ZA oncology dose)].

Author/ Year	Dose	% of rat ZA oncology dose	Route	Animal	Risk Factor	Surgical Site	MRONJ	Bone Exposure	Pre-XT (d)	Post-XT (d)	Post-XT treat
Vilarinho et al., 2017	0.066 mg/kg 3X/wk - 3 wks 600 µg/kg 0.75 m	1000	IP	WA/F/8w	No	Md/1M	Yes	Yes[3]	21	42	No
Adachi et al., 2020	0.066 mg/kg-3X/wk –4wks 800 µg/kg 1m	1000	SC	WA/F/4w	DX	Mx/1M	Yes	Yes	14	14	Yes
Kaibuchi et al., 2016	0.066 mg/kg-3X/wk –4wks 800 µg/kg 1m	1000	SC	SD/F/4w	DX	Mx/1M	Yes	Yes	14	14	Yes
Sanda et al., 2022	0.066 mg/kg-3X/wk –4wks 800 µg/kg 1m	1000	SC	WA/F/4w	No DX	Mx/1M	No Yes	No Yes	14	28	Yes
Yanik et al., 2016	0.1 mg/kg- 2X/wk - 7wks 1400 µg/kg 1.75m	1000	IP	SD/F/12w	DX	Mx/1M	No	No	49	21, 56, 70	No
Liu et al., 2021	0.2 mg/kg once/wk – 8 wks 1600µg/kg 2 m	1000	IP	SD/F/8w	Yes[o] DX	Md/1M	Yes	Yes	56	56	No
Gao et al., 2021	0.2 mg/kg-1X/wk – 16 wks 3200 µg/kg 4m	1000	IV	SD/M/6-8w	DX	Mx/1M	Yes	Yes	56	5 56	Yes
Kolpakova et al., 2017	0.36 mg/kg once +0.18 mg/kg once/week – 4 wks 1080 µg/kg 1.25 m	1080	IV	WA/M/16	No	Md/1-2M	Yes	No	0	56	Yes
Poubel et al., 2018a	0.125 mg/kg twice/wk – 4 wks 1000 µg/kg 1 m	1250	IP	WA/M/4w	No	Md/1M	No	No	7	28	Yes
Cui et al., 2020	0.125 mg/kg-2X/wk – 5wks 1250 µg/kg 1.25m	1250	IP	WA/M/8w	No	Mx/1M	Yes	Yes	7	28	Yes
Statkievicz et al., 2018	0.1 mg/kg MWF - 7 wks 2100 µg/kg 1.75 m	1500	IP	WA/F/80w	Yes [o ψ]	Md/1M	Yes	Yes	21	28	Yes
Ervolino et al., 2022	0.1 mg/kg MWF – 7 wks 2100 µg/kg 1.75 m	1500	IP	WA/F/80w	Yes [ψ]	Md/1M	Yes	Yes	21	28	Yes
Paulo et al., 2020	0.1 mg/kg MWF – 7 wks 2100 µg/kg 1.75 m	1500	IV	WA/F/17w	Yes [o]	Md/1M	Yes	Yes	28	14, 21	Yes
Barba-Recreo et al., 2014	0.1 mg /kg MWF – 9 wks 2700 µg/kg 2.25 m	1500	IP	WA/M/8w	No	Mx/1-3M	Yes[1]	No	56	7	Yes
Barba-Recreo et al., 2015	0.1 mg /kg MWF – 9 wks 2700 µg/kg 2.25 m	1500	IP	WA/M/8w	No	Mx/1-3M	Yes[2]	No	56	7	Yes
Sousa et al., 2022	0.1 mg/kg-MWF 9wks 2700 µg/kg 2.25m	1500	IP	SD/F/12w	No	Mx/1-3M	Yes (83%)	Yes (91%)	56	21	Yes
Dayisoylu et al., 2014	0.1 mg/kg- 3X/wk - 8wks 2400 µg/kg 2m	1500	IP	SD/F/12w	No	Md/1M	Yes	Yes	56	56	No
Dayisoylu et al., 2013	0.1 mg/kg- 3X/wk - 8wks 2400 µg/kg 2m	1500	IP	SD/F/12w	No	Md/1M	Yes (66%)	Yes (100%)	56	56	No

Mergoni et al., 2019	0.1 mg/kg-3X/wk –9wks 2700μg/kg 2.25m	1500	IP	WA/F/8w	DX	Mx/1M	No*	No	63	8	No
Jung et al., 2021	0.1mg/kg MWF - 14wks 4200 μg/kg 3.5m	1500	IP	SD/F/10-12w	DX	Mx/1-3M	Yes*	Yes (50%)	77	21	Yes
Hadaya et al., 2019	0.2 mg/kg twice/wk – 9 wks 3600 μg/kg 2.25m	2000	IP	W/A/M/8w	No	Md/1-2M	No	No	35	28	Yes
	0.2 mg/kg twice/wk – 9 wks 3600 μg/kg 2.25m	2000			Yes ∆		Yes	Yes	35	28	Yes
Soundia et al., 2018	0.2 mg/kg twice/wk – 9 wks 3600 μg/kg 2.25 m	2000	IP	W/A/M/8w	Yes ψ	Mx/1-2M	Yes	No	35	28	Yes
Silva et al., 2015	3 cycles of 1 mg/kg once/wk +1 dose 5 wks later 4000 μg/kg 2 m	2500	IV	WA/M/8w	No	Md/1M	Yes	No	42	28	Yes
Ersan et al., 2014	0.2 mg/kg-3X/wk –6wks 3600μg/kg 1.5m	3000	IP	SD/F/11w	No	Md/1M	Yes	No	42	28	No
Bolette et al., 2019	0.3 mg/kg 3X/wk – 4 wks 3600 μg/kg 1 m	4500	IP	WA/M/8w	Yes ∆	Mx Md/1M	Yes	Yes	28	56	No

Rat ZA oncology dose is 0.08mg/kg IV/month (Aguirre et al., 2022).

Column MRONJ relates to how authors interpret their results (*Yes* means treated rats developed osteonecrosis).

In Column Risk Factor, ɵ means senescence animal associated; ψ means experimental periodontitis associated; ɣ means diabetes associated; Δ means periapical lesions associated; º means OVX-associated.

In Column MRONJ and Bone Exposure, 1rmeans 80% of the sample present osteonecrosis; 2 means 50% of the sample present osteonecrosis; 3 means 76% of the sample present bone exposure.

Abbreviation: ZA, zoledronate; DX, dexamethasone; MWF Monday, Wednesday, Friday; SC, subcutaneous; IP, intraperitoneal; IV, intravenous; SD, *Sprague Dawley* rats; WA, *Wistar Albinus* rats; Mi, mice; OP, *Oryzomys palustris* rats; F, female; M, male; #, not reported; w, weeks; m, months; Mx, maxillae; Md, mandible; 1M, fist molar; 2M, second molar; 3M, third molar; 1-3M, first, second, and third molar; Pre-XT: pre-extraction treatment period; Post-XT: post-extraction period; Post-XT Treat: drug treatment applied during post-extraction period.

Table 4 Overall characteristics of the studies evaluating Alendronate.

Author/ Year	Dose	% of rat ALN OP dose	Route	Animal	Risk Factor	Surgical Site	MRONJ	Bone Exposure	Pre-XT (d)	Post-XT (d)	Post-XT treat
Maahs et al., 2011	0.05 mg/kg once/wk – 23 wks 11.5 µg/kg 5.75 m	1.7	PO	WA/M/20w	No	Mx/1-3M	No	No	45	105	Yes
Isaias et al., 2021	2.5 mg/kg/wk - 3 wks +1 wk 10000 µg/kg 2.5m	2.1	PO	WA/M/9w	No	Md/1M	No	No	42	28	Yes
	5 mg/kg/wk - 3 wks +1 wk 20000 µg/kg 2.5m	4.2	PO						42	28	Yes
	7.5 mg/kg/wk - 3 wks +1 wk 30000 µg/kg 2.5m	6.3	PO						42	28	Yes
Isaias et al., 2021	2.5 mg/kg/wk - 10 wks 25000 µg/kg 2.5m	8.3	PO	WA/M/9w	No	Md/1M	No	No	42	28	Yes
	5 mg/kg/wk - 10 wks 50000 µg/kg 2.5m	16.7	PO						42	28	Yes
	7.5 mg/kg/wk - 10 wks 75000 µg/kg 2.5m	25	PO						42	28	Yes
Aguirre et al., 2010	0.015 mg/kg twice/wk - 3 wks 90 µg/kg 0.75 m	100	SC	SD/F/10w	No	Md/1M	No	No	21	10, 21, 35, 70	Yes
	0.150 mg/kg twice/wk - 3 wks 900 µg/kg 0.75 m	1000							21		
Mustakim et al.,,2022	1 mg/kg/d – 5X/wk- 3wks 15000 µg/kg 0.75m	167	PO	SD/M/7w	No	Md/1M	No	No	21	21 & 35	No
	1 mg/kg/d – 5X/wk- 8wks 40000 µg/kg 2m	167					No	No	63	21 & 35	No
Berti-Couto et al., 2014	0.05 mg/kg-1X/wk –16wks 800 µg/kg 4m	167	SC	WA/F/20w	No DX DM	Mx/1-3M	No Yes (28%) Yes (78%)	DNR	90	21	Yes
Conte Neto et al., 2016	1 mg/kg once/wk - 8 wks 8000 µg/kg 2 m	3333	SC	WA/M/10w	No	Md/1M	Yes	Yes	60	28	Yes
Abtahi et al., 2012	0.2 mg/kg daily – 2 wks 2800µg/kg 0.5 m	4666	SC?	SD/M/10w	No DX	Mx/1M	No Yes	No Yes	0	14	Yes
Movahedian-Attar et al., 2020	0.2mg/kg-daily –14d 2800 µg/kg 0.5m	4666	SC	WA/M/9-11w	DX	Mx/1-2M	Yes (85%) Con=23%	Yes	0	14	Yes
Altundal & Güvener, 2004	0.25 mg/kg daily – 2 wks 3500µg/kg 0.5m	5833	SC	WA/M/8w	No	Md/1M	No	No	0	14	Yes
	0.25 mg/kg daily – 4 wks 7000 µg/kg 1 m	5833							0	28	
Gonçalves et al., 2023	1 mg/kg/d - 90d 60000 µg/kg 3m	16700	SC	WA/M/10w	No	Md/1M	Yes	No	60	30	No
Conte Neto et al., 2013	1 mg/kg daily – 63 days 63000 µg/kg 2.25 m	23333	SC	WA /M/8w	No	Md/1M	Yes	Yes	60	3	Yes

	1 mg/kg daily – 88 days 88000 μg/kg 3.12 m	23333								28	

Rat ALN osteoporosis dose is 0.120mg/kg SC/month (Seedor et al., 1999).
The column MRONJ relates to how authors interpret their results (*Yes* means treated rats developed osteonecrosis).
Abbreviations: ALN, alendronate; SC, subcutaneous; PO, orally by gavage; OP, osteoporosis; DX, dexamethasone; SD, *Sprague Dawley* rats; WA, *Wistar Albinus* rats; F, female; M, male; #, not reported; w, wks;
Mx, maxillae; Md, mandible; 1M, fist molar; 2M, second molar; 3M, third molar; 1-3M, first, second, and third molar; Pre-XT: pre-extraction treatment period; Post-XT: post-extraction period; Post-XT Treat: drug treatment applied during post-extraction period.

Table 5 Overall characteristics of studies using Zoledronic Acid in mice.

Author/ Year	Drug	% of mouse ZA oncology dose	Route	Animal	Risk Factor	Surgical Site	MRONJ	Bone Exposure	Pre-XT (d)	Post-XT (d)	Post-XT treat
Hokugo et al., 2019	0.1 mg/kg once – 3 wks 100 μg/kg 0.75m	25	IV	F/7-10w	No	Mx/1M	No	No	7	14	No
Sun et al., 2016	0.5 mg/kg/-once 500 μg/kg 1.33m	70	IV	F/7w	No	Mx/1M	Yes (14d, 28d)	Yes (14d, 28d)	10	3, 14, 28	Yes
Hokugo et al., 2019	0.3 mg/kg once – 3 wks 300 μg/kg 0.75m	74	IV	F/7-10w	No	Mx/1M	No	No	7	14	No
Igarashi et al., 2023	0.05 mg/kg-2X/wk –3wks 300μg/kg 0.75m	74	SC	F/6w	CYP	Mx/1M	Yes	No	21	14	Yes
Kuroshima et al., 2021	0.05 mg/kg/-2X/wk – 7wks 700 μg/kg 1.75m	74	SC	F/8w	CYP	Mx/1M	Yes[1]	Yes[1]	21	14, 28	Yes
Hayano et al., 2020	0.05 mg/kg/-2X/wk – 5wks 500 μg/kg 1.25 0.05 mg/kg/-2X/wk – 7wks 700 μg/kg 1.75m	74	SC	F/8w	No +CYP	Mx/1M	No Yes[1]	No Yes[1]	21	14 28	Yes
Kuroshima et al., 2018a	0.05mg/kg 2X/wk - 7 wks 700 μg/kg 1.75m	74	SC	F/8-12w	CYP	Mx/1M	Yes[1]	No	21	28	Yes
Kuroshima et al., 2018b	0.05mg/kg 2X/wk - 7 wks 700 μg/kg 1.75m	74	IV	F/8-12w	CYP	Mx/1M	Yes[1]	No	21	28	Yes
Kozutsumi et al., 2022	0.05 mg/kg 2X/wk - 7 wks 700 μg/kg 1.75 m	74	SC	F/8w	No	Mx /1M	No	No	21	28	Yes
Kuroshima & Yamashita, 2013	0.05mg/kg 2X/wk - 7 wks 700 μg/kg 1.75m	74	SC	F8-12w	No DX MEL	Mx/1M	Yes*	No No Yes (81%)	21	28	Yes
Yu et al., 2020	0.125mg/kg 1X/wk - 10wks 1250 μg/kg 2.5m	93	IV	F/8-10w	DX	Mx/1M	Yes (78%)	Yes (56%)	14	56 112	Yes
Park et al., 2015	0.540 mg/kg once – 4 wks 540 μg/kg 1m	100	IV	F/7wk	No	Mx/1M	Yes	Yes	7	4, 7, 14, 28	No
Hokugo et al., 2019	0.5 mg/kg once – 3 wks 500 μg/kg 0.75m	124	IV	F/7-10w	No	Mx/1M	Yes	No	7	14	No
Kaneko et al., 2023	0.1 mg/kg/-2X/wk – 5wks 1000 μg/kg 1.25m	148	IV	F/8w	VAB	Mx/1M	Yes	No	21	14	Yes
Córdova et al., 2016	0.10 mg/kg/-2X/wk – 12wks 2400 μg/kg 3m	148	IP	M/10w	No	Mx/1M	Yes (21%)	Yes (21%)	21	21 63	Yes
Hokugo et al., 2019	0.7 mg/kg once – 3 wks 700 μg/kg 0.75m	173	IV	F/7-10w	No	Mx/1M	Yes	No	7	14	No
Zhu et al., 2019	0.125 mg/kg-2X/wk – 4wks 1000 μg/kg 1m	185	IV	M/8w	No	Mx/1M	Yes	Yes (40%)	7	7 21	Yes
Shen et al., 2022	0.125 mg/kg-2X/wk – 5wks 1250 μg/kg 1.25m	185	IV	M/8w	No	Mx/1M	Yes	Yes (67%)	7	7 28	Yes

Zhang et al., 2013	0.125 mg/kg-2X/wk – 3wks 750 µg/kg 0.75m	185	IV	F/8-10w	No	Mx/1M	Yes	Yes	7	14	Yes
Williams et al., 2014	0.125mg/kg 2X/wk – 4 wks 1000 µg/kg 1m	185	IV	F/8w	No	Mx/1M	Yes	Yes (60%)	7	21	Yes
Yoshioka et al., 2022	0.125 mg/kg 2X/wk– 3 wks 750 µg/kg 0.75m	185	IV	F/5w	DX	Mx/1M	Yes	No	7	14	Yes
Kim et al., 2017	0.125mg/kg 2X/wk – 3 wks 750 µg/kg 0.75mo 0.125mg/kg 2X/wk – 4 wks 1000 µg/kg 1m	185	IV	F/8w	No	Mx/1M	Yes	Yes (20%)	7 7	14 21	Yes
Zhang et al., 2013	0.125mg/kg 2X/wk – 3 wks 750 µg/kg 0.75m	185	IV	F/8-10w	Yes ɣ	Mx/1M	Yes	No	7	14	Yes
Kikuiri et al., 2010	0.125 mg/kg 2X/wk – 3 wks 750 µg/kg 0.75m 0.125 mg/kg 2X/wk – 8 wks 2000 µg/kg 0.75m	185	IV	F/8-10w	DX	Mx/1M	Yes Yes	Yes(50%) Yes(30%)	7 7	14 49	Yes
Zhao et al., 2012	0.125mg/kg 2X/wk – 3 wks 750 µg/kg 0.75m	185	IV	C3H/HeJ F/8w	DX	Mx/1M	Yes	Yes(45%)	7	14	Yes
Taniguchi et al., 2020	0.125mg/kg/-2X/wk – 3wks 750 µg/kg 0.75m	185	IV	F/6	No	Mx/1M	Yes (11%)	Yes (78%)	14	7	Yes
Bi et al., 2010	0.125mg/kg 2X/wk – 6 wks 1500 µg/kg 1.5m 0.125mg/kg 2X/wk – 15 wks 3750 µg/kg 3.75m	185	IP	8-12w	DX	Mx Md/1M	Yes	No	21 21	21 84	Yes Yes
Kim et al., 2018	0.125mg/kg twice/week – 7 wks 1750 µg/kg 1.75m	185	IV	F/6w	Yes ψ	Mx/1M	Yes	Yes (20%)	28	21	Yes
Biguetti et al., 2019	0.25 mg/kg once/wk – 7 wks 1750 µg/kg 1.75 m	185	IP	129Sv/ F/60w	Yes ɵ	Mx/In	Yes	No	28	7, 21	Yes
Mahmoud et al., 2021	0.25 mg/kg once/wk – 7 wks 1750 µg/kg 1.75 m	185	IP	129Sv/ F/60w	Yes ɵ	Mx /In	Yes	Yes	28	7, 21	Yes
Song et al., 2016	0.125mg/kg 2X/wk – 6 wks 1500 µg/kg 1.5m	185	IV	F/6w	Yes ∆	Mx/1M	Yes	Yes (20%)	28	21	Yes
Williams et al., 2014	0.125mg/kg/-2X/wk – 7wks 1750 µg/kg 1.75m	185	IV	F/6	No Yes ψ	Mx/2M	No Yes	No Yes	28	21	Yes
Williams et al., 2020	0.125mg/kg 2X/wk – 6 wks 1500 µg/kg 1.5m 0.125mg/kg 2X/wk – 13 wks 3250µg/kg 3.25 m	185	IV	F/8w	Yes ψ	Mx/2M	Yes Yes++	Yes Yes++	49x/21 70	21 21	Yes
Du et al., 2022	0.125mg/kg 2X/wk – 9 wks 2250 µg/kg 2.25m	185	IV	F/6w	Yes ∆	Mx/1M	Yes	Yes (20%)	63	21	Yes
Wu et al., 2022	0.125 mg/kg-2X/wk –7wks 1750 µg/kg 1.75m 0.125 mg/kg-2X/wk –11wks 2750 µg/kg 2.75m	216	IP	F/4w	PGinv	Md/1M	Yes (only with PGinv)	No	21	28 56	Yes

Yoshioka et al., 2022	0.125 mg/kg-2X/wk –3wks 750 µg/kg 0.75m	216	IV	F/5w	DX	Mx/1M	Yes	Yes	7	14	Yes
Hokugo et al., 2019	0.9 mg/kg once – 3 wks 900 µg/kg 0.75m	225	IV	F/7-10w	No	Mx/1M	Yes	No	7	14	No
Chen et al., 2021	0.25 mg/kg/-2X/wk – 4wks 2000 µg/kg 1m	370	IP	F/4w	No	Mx/1M	Yes	DNL	7	21	Yes
Soma et al., 2022	0.5 mg/kg once/wk – 8 wks 4000 µg/kg 2m	370	SC	F/8w	No	Md/1-2M	Yes	No	14	42	Yes
Soma et al., 2021	0.5 mg/kg once/wk – 8 wks 4000 µg/kg 2m	370	IP	F/8w	No	Mx/1-2M	Yes	No	14	42	Yes
Kozutsumi et al., 2022	0.25 mg/kg 2X/wk - 7 wks 3500 µg/kg 1.75 m	370	SC	F/8w	No	Mx /1M	Yes	No	21	28	Yes
Zheng et al., 2023	0.5 mg/kg-2X/wk –4wks 4000 µg/kg 1m	740	IP	F/7w	No	Mx/2M	Yes	Yes	14	14	Yes
Kozutsumi et al., 2022	0.5 mg/kg 2X/wk – 7 wks 7000 µg/kg 1.75m	740	SC	F/8w	No	Mx/1M	Yes	Yes	21	28	Yes
Kobayashi et al., 2010	0.25 mg/kg/d – 11d 2750 µg/kg 0.35m	1455	SC	M/6w	No	Mx/1M	No	No	7	5	Yes
Zhao et al., 2023	1 mg/kg/-MWF – 4wks 12000 µg/kg 1m	2222	IP	M/6-8w	ψ SUN ψ+SUN	Mx/2M	Yes No Yes	Yes (30%) Yes (10%) Yes (70%)	14	14	Yes
Zheng et al., 2023	1 mg/kg/ - daily – 4wks 28000 µg/kg 1m	5200	IP	F/6-8w	No	Mx/1M	Yes	No	14	14	Yes

Mouse ZA oncology dose is 0.54mg/kg IV/month (Park et al., 2015).
Mice were strain C57BL/6 unless otherwise noted.
Column MRONJ is related to how authors interpret their results (Yes means some animals developed osteonecrosis).
In the column Risk Factor, ɵ means senescence animal associated; ψ means periodontitis associated; Δ means periapical lesions associated; ɣ means diabetes associated.
In the column MRONJ and Bone Exposure, 1 means osteonecrosis was diagnosed only when ZA was associated with CYP; 2 means 10% of the samples present osteonecrosis and exposure with ZA, and 33% with ZA+DX.
Abbreviations: ZA, zoledronate; DX, dexamethasone, SC, subcutaneous; IP, intraperitoneal; IV, intravenous; SD, Sprague Dawley rats; WA, Wistar Albinus rats; F, female; M, male; CYP- cyclophosphamide; SUN- Suninitib; VAB- anti-VEGFA neutralizing antibody; PGinv- *Porphyromonas gingivalis*.
#, not reported; wk, weeks; m, months; Mx, maxillae; Md, mandible; 1M, fist molar; 2M, second molar; 3M, third molar; 1-3M, first, second, and third molar; Pre-XT: pre-extraction treatment period; Post-XT: post-extraction period; Post-XT Treat: drug treatment applied during post-extraction period.

Capítulo 2

2 CAPÍTULO 2 - Dose-dependent effects of zoledronic acid on the osteogenic differentiation of human bone marrow stem cells (hBMSCs)[†]

2.1 ABSTRACT

Objective: This study aimed to evaluate the effects of different concentrations of Zoledronic acid (ZA) during the osteogenic differentiation of human Bone Marrow Stem Cells (hBMSCs) in vitro. **Materials and methods:** Thus, osteogenic differentiation of hBMSCs were conducted with different concentrations of Zoledronic Acid (ZA) (0, 0.1, 1.0, and 5.0μM) for the first 3-days. Cell metabolism was quantified at 1-, 3-, 7-, and 14 days. At 7- and 14-days, the following analyses were performed: 1) mineralization nodule assay, 2) LIVE/DEAD™, 3) cell adhesion and spreading, 4) alkaline phosphatase (ALP) activity, and 5) qPCR analysis for RUNX-2), ALPL, and COL1 A1. Data were analyzed by ANOVA 2-way, followed by Tukey's post hoc test ($p < 0.05$). **Results:** Cell metabolism (3-, 7-, and 14-days) ($p < 0.001$), mineralization (7-, 14-days) ($p < 0.001$), and ALP activity (14-days) ($p < 0.001$) were reduced in ZA 5.0 μM when compared to control (no ZA). Also, ZA 5.0 μM downregulated the expression of RUNX2 at 7- and 14-days ($p < 0.001$). **Conclusion:** It is possible to conclude that ZA (5.0 μM) can impair hBMSC differentiation into osteoblasts and interferes with its mineralization phase. **Clinical relevance:** Recent studies have shown that bisphosphonates can also impact osteoblasts besides osteoclasts. The comprehension of the mechanisms of action of this drug, and how it acts on the tissues and cells related to the jaw, may help in the better understanding of the MRONJ physiopathology.

Keywords: Zoledronic acid; Medication-related osteonecrosis of the jaw; Human bone marrow stem cells; Osteogenic differentiation.

2.2 INTRODUCTION

Bisphosphonates (BPs), classified as nitrogen-containing or non-nitrogen-containing, act primarily in osteoclasts [1]. Their mechanism of action consists of incorporating nonhydrolyzable analogs of adenosine triphosphate (ATP) [2] or inhibiting farnesyl-diphosphate synthase (FPP)

[†] Published at *Journal of Stomatology and Oral Maxillofacial Surgery*, doi: 10.1016/j.jormas.2023.101479.

and blocking prenylation of small GTPase in the mevalonate pathway of cholesterol synthesis [3]. Studies have shown a concentration-dependent effect of BPs on osteoblasts [4,5] since they can up or down-modulate this cell line depending on the concentrations used. [6-10] Regulation of osteoclastogenic factors by osteoblasts can be modulated by the BPs through inhibition of IL-6 [11], decreasing RANK-L expression in osteoblast-like cells by increasing the expression of osteoprotegerin (OPG) and tumor necrosis by the factor-α converting enzyme (TACE) [11-13].

The pharmacological effects of BPs are effective for managing hypercalcemia, bone metastases, or even preventing skeletal-related events (SRE), such as fractures in osteopenic/osteoporotic patients [14-16]. Besides the significant suppression of the osteoclasts, BPs can impair significantly jawbone remodeling, tissue properties, and extraction healing [17]. The usage of BPs is directly related to microdamage accumulation in the oral cavity, which has been correlated with the pathophysiology of the osteonecrosis [18]. A concerning side effect when BPs are used in high dosages and frequency is the development of medication-related osteonecrosis of the jaw (MRONJ), [19] especially when associated with local risk factors, such as tooth extraction, periodontitis, and surgery [20, 21].

Although the entire pathophysiology of this disease is not completely understood, findings have shown that the use of BPs (mainly zoledronic acid [ZA]) can affect the migration and viability of human umbilical vein endothelial cells (HUVEC), fibroblasts, and osteoblasts [22-24]. In addition, BPs can exert cytotoxic effects on osteoblasts in a dose-dependent manner, [9] which suppresses osteogenesis, by decreasing mineralization capacity and exhibits an anti-angiogenic effect [24-26]. The aim of this study is to evaluate the biological effects of different ZA concentrations during the osteogenic differentiation of hBMSCs in vitro.

2.3 MATERIALS AND METHODS

Cell culture of human Bone Marrow Stem Cells (hBMSCs)

Cells (hBMSCs) were purchased from Lifeline® (Cell Technology, San Diego, CA, USA). The hBMSCs were cultured in basal media consisting of Dulbecco's modified eagle media (DMEM, Invitrogen, USA) supplemented with 10% of fetal bovine serum (FBS, Gibco, USA), 1% of modified eagle media non-essential amino acids solution (MEM NEAA, Gibco, USA), and

1% of antibiotic solution (Sigma-Aldrich, USA) under standard conditions (5% CO2, 95% humidity, and 37°C). The cells were cultured until passage 4 and once 90% confluence was reached, they were plated in 24-well plates at a seeding density of 7 x 104 cells/well (Sigma-Aldrich, USA).

Osteogenic differentiation assay of hBMSCs

The day after plating hBMSCs, osteogenic differentiation was induced by adding 100 nM of dexamethasone (Sigma-Aldrich, USA), 200 μM of ascorbic acid (Sigma-Aldrich, USA), and 10 mM of glycerol 2-phosphate (Sigma-Aldrich, USA) to the basal media by 14 days, with changes every three days [27].

Determination of ZA dose

To select the dose in this study, the authors performed a pilot study. The hBMSCs were seeded in a 24-well plate (7 x 104 cells/well) and incubated with different concentrations of zoledronic acid (Sigma-Aldrich, USA) (CT or 0, 0.1, 1.0, and 5.0 μM) to assess cell viability, and to determine the mineralization deposition.

Quantification of cell metabolism

Cell metabolism was quantified by using alamarBlue™ Reagent (Invitrogen, USA), [28] at 1-, 3-, 7-, 14 days. For this purpose, the media was changed to DMEM without FBS, added by the alamarBlue (10:1). The plates were incubated for 4 hours, protected from light and under standard conditions (5% CO2, 95% humidity, and 37°C). The analysis of fluorescence intensity was performed with a microplate reader (Varioskan™ LUX, Life Technologies Holdings Pte. Ltd, Singapore) at 530 nm for excitation and 590 nm for readings. The mean fluorescence value in the control group on day 1 was set as 100% cell viability to indirectly establish the percentage of cell metabolism for each group and period.

Mineralization nodule assay with Alizarin red

To evaluate mineral deposition, media from the 7- and 14-day plates was aspirated, and the well was rinsed once with phosphate-buffered saline solution (PBS, Sigma-Aldrich, USA). Then, cells were fixed in 4% formaldehyde and incubated at room temperature for 15 minutes, followed by 3 careful rinses with PBS (5 minutes each). 1% alizarin-red solution (pH 4.2; Sigma-Aldrich, USA) was applied for 45 minutes at room temperature, a deionized water wash was performed, and then the cells were dried at 37ºC [29]. For quantitative analysis, the nodules were dissolved in 10% cetylpyridinium chloride (10 mM, pH 7.0 Sigma-Aldrich, USA) for 15 minutes by stirring, and then, absorbance was measured in a microplate reader (Varioskan™ LUX, Life Technologies Holdings Pte. Ltd, Singapore) at 620 nm [30].

Cell viability

LIVE/DEAD™ Cell Imaging Kit (Molecular Probes™, USA) was used to assess the viability of the cells at 7 and 14 days. Samples were incubated for 45 minutes in culture media supplemented with Calcein AM and ethidium homodimer-1 at a 1:1000 concentration, followed by 3 washes with PBS (Sigma-Aldrich, USA), and finally evaluated through Confocal Microscope (FLoid□, Life-Technologies) under 488/515 nm FITC channel (live - green) and 570/602 nm TRITC channel (dead - red).

Cell adhesion and spreading

Samples at 7 and 14 days of culture were fixed in a 4% formaldehyde solution (10 min) and rinsed 3 times with PBS (Sigma-Aldrich, USA). Next, they were permeabilized in 0.1% Triton X (Thermo Fisher Scientific, Waltham, MA, USA) (10 min), followed by PBS washings (3 times) and incubation in Alexa Fluor Phalloidin 555 in BSA 2% (1:40 Life Technologies) for 30 min. Samples were rinsed with PBS and exposed to 1 µL of DAPI (300 µM) in 1000 µL PBS for 5 minutes. Then, samples were washed in PBS and analyzed under Confocal Microscope (FLoid□, Life-Technologies) at 345 nm for DAPI and 455 nm for TRITC.

ALP activity

The ALP activity was assessed at 7 and 14 days. Samples were rinsed once with PBS and lysed with 0.1% sodium lauryl sulfate for 40 minutes. The supernatant was moved to a 96-well plate with thymolphthalein monophosphate (22 mmol/L; pH 10.1 and 37oC). Afterward, ALP activity was measured by an ALP colorimetric kit assay (Abcam, USA) using p-nitrophenyl phosphate (pNPP) as substrate and alkaline phosphatase as standard. The plate was incubated at 25oC for 60 minutes while protected from light. Before measurements, a stop solution was added to each well and then absorbance was read at OD 405nm.

qPCR analysis

Total RNA was extracted directly in the wells at 7 and 14 days using TRIzol Reagent (Invitrogen). Then, RNA (1 μg) was converted into cDNA using an enzyme to perform unbiased cDNA synthesis (SMARTScribe Reverse Transcriptase, Takara, Japan), followed by measurement with NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). Next, qRT-PCR analysis was performed with 1.2 μg of cDNA associated with PowerTrack™ SYBR Green Master Mix (Applied Biosystems™) in a 7500 Fast Real-Time PCR System (Applied Biosystems™). The PCR primers used to assess osteogenic differentiation [31] in this study were: runt-related transcription factor 2 (Runx-2) for assessing the differentiation of hBMSC into osteoblasts; and alkaline phosphatase (ALPL) and collagen type 1 (COL-1) for assessing the mineralization phase of osteogenic differentiation. The sequences of the primers used are presented in Table 1. The relative standard curve method (2^-ΔΔCT) was used to determine the relative mRNA expression, using the GAPDH housekeeping gene.

Statistical analysis

The data was organized and submitted for statistical analysis using Prism (version 8.4.3.). First, a homogeneity test (Shapiro-Wilk) was applied to verify the data distribution. A two-way analysis of variance was performed for all outcome parameters. Tukey's post hoc test was used for multiple comparisons. For all tests, the statistical significance level was set at $p < 0.05$.

2.4 RESULTS

High doses of ZA reduce cell proliferation

As demonstrated in Figure 1, ZA exposure (0.1 and 1.0 µM) did not affect cell viability at 14 days. However, ZA 5.0 µM significantly reduced cell metabolism at 3, 7, and 14 days ($p < 0.001$). Also, differences were observed between 0.1 and 5.0 µM, and 1.0 and 5.0 µM at 3, 7, and 14 days ($p < 0.001$).

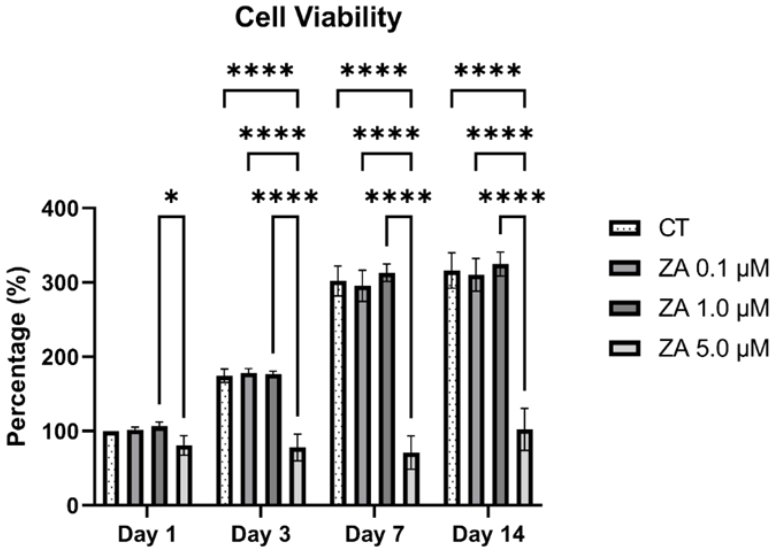

Fig. 1. Graph of cell proliferation at 1, 3, 7, and 14 days **** means $p < 0.0001$; * $p = 0.0353$.

Mineralization nodules assay with Alizarin red

Except for the ZA 5.0 µM group, all groups (CT, ZA 0.1, and 1.0 µM) showed an increase in the amount of mineralization over time (Figure 2). The quantitative analysis demonstrated that exposure to ZA, regardless of the dose, negatively influenced the results when compared to the CT group at 7 days ($p <0.0001$). On the other hand, at 14 days, ZA 0.1 µM showed higher values for mineralization when compared with CT, however, without statistical significance ($p =0.1632$).

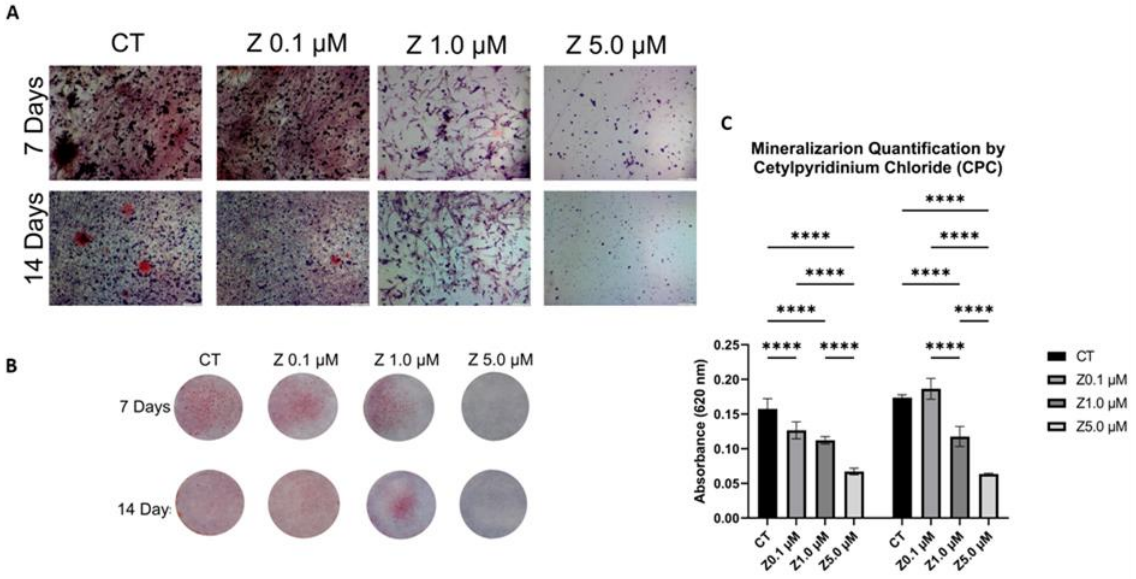

Fig. 2. Representative plot of the mineralization analysis. (A) Histological images at 100x of the cells fixed in formalin and stained with alizarin red at the times of 7 and 14 days for the control groups (CT), and the different concentrations of ZA (01, 10, and 50 µM); (B) Macroscopic images of the wells stained with alizarin red at the different times and concentrations; and (C) Representative graph of the mineralization quantification through cetylpyridinium and analyzed by absorbance at 620 nm **** means p <0.0001.

Doses of 1.0 and 5.0 µM reduce adhesion, spreading, and viability

Fluorescence analysis under confocal microscopy demonstrated that exposure to ZA 1.0 and 5.0 µM compromised adhesion and spreading of cells to the wells (Figure 3), as well as reducing the number of live cells as demonstrated by the LIVE/DEAD™ assay (Figure 4). Interestingly, ZA 0.1 µM at 7 and 14 days led to a higher density of cells.

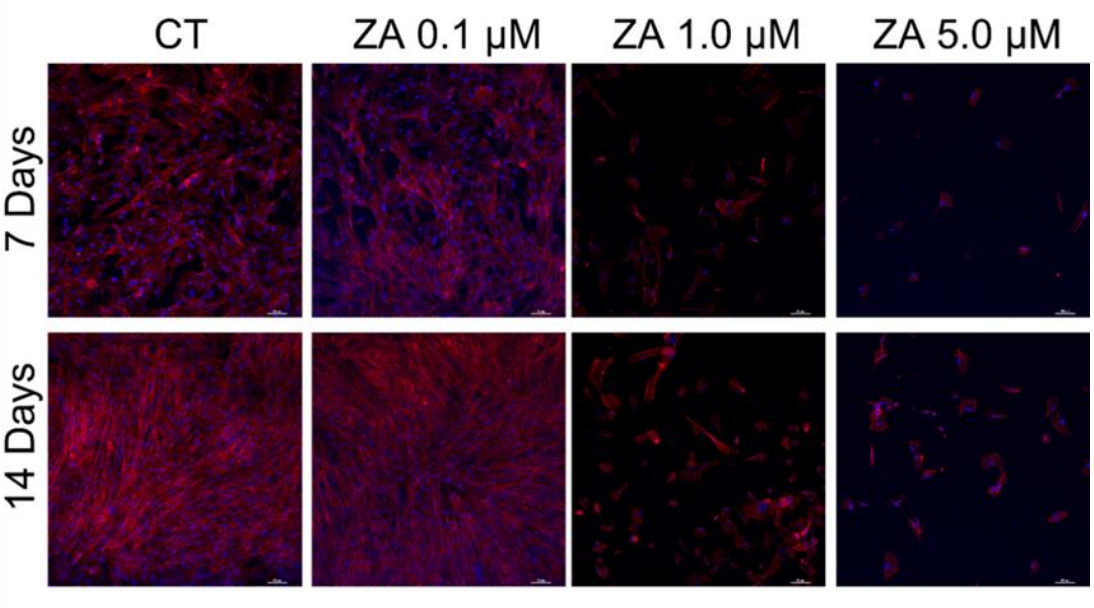

Fig. 3. Confocal microscope images representing adhesion and spreading assay at 7 and 14 days, for the control groups (CT) and the different ZA concentrations (0.1, 10, and 50 µM) Scale bars: 100 µm.

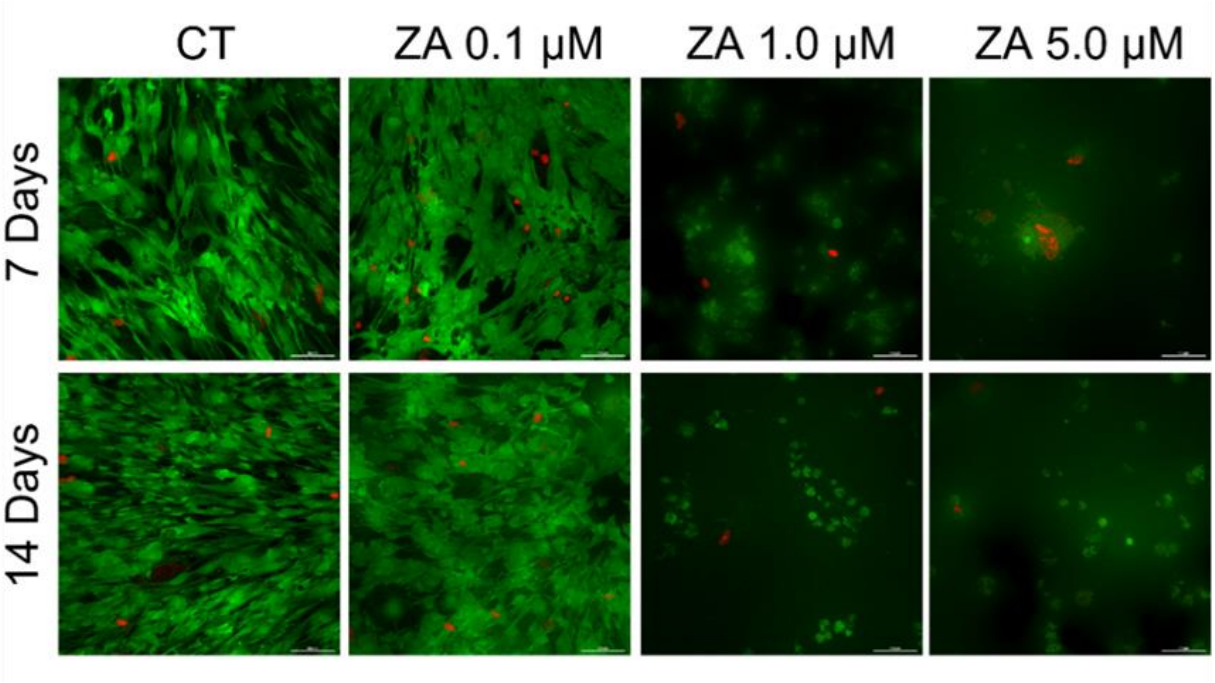

Fig. 4. Confocal microscope images representing LIVE/DEAD assay at 7 and 14 days, for the control groups (CT) and the different ZA concentrations (0.1, 10, and 50 µM) Scale bars: 100 µm.

Doses of 1.0 and 5.0 µM reduce ALP expression

The CT group showed the highest ALP expression at 7 and 14 days ($p < 0.002$), and ZA, regardless of concentration (0.1, 1.0, and 5.0 µM), reduced ALP activity at 7 ($p > 0.05$) and 14 days ($p < 0.001$) when compared to CT group (Figure 5).

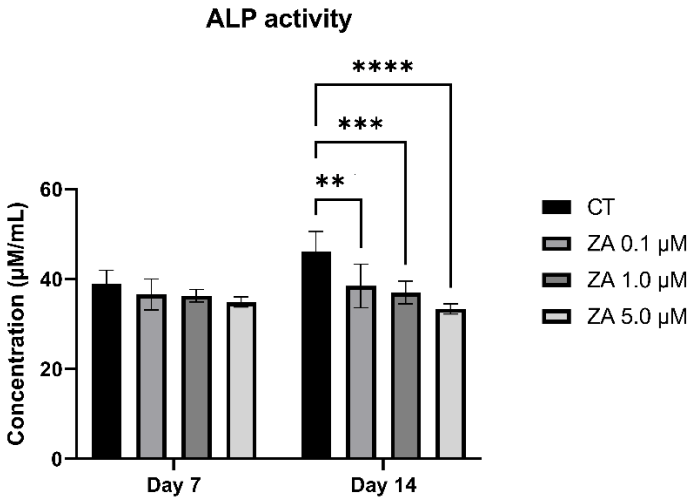

Fig. 5. Graph of ALP activity at 7 and 14 days **** means p <0.0001; *** means p = 0.0002; ** means p = 0.0007.

ZA inhibits hBMSC differentiation into osteoblasts and interferes with the mineralization phase

qPCR data demonstrated that there is a reduction in RUNX2 expression regardless of ZA concentration (0.1, 1.0, and 5.0 µM) at 7 and 14 days (Figure 6), demonstrating that ZA interferes with hBMSC differentiation in pre-osteoblasts and mature osteoblasts. It is noteworthy that the concentration of ZA 5.0 µM interfered with the expression of COL1 A1 and ALPL at 7 and 14 days, although without significant differences ($p > 0.05$) (Figure 7).

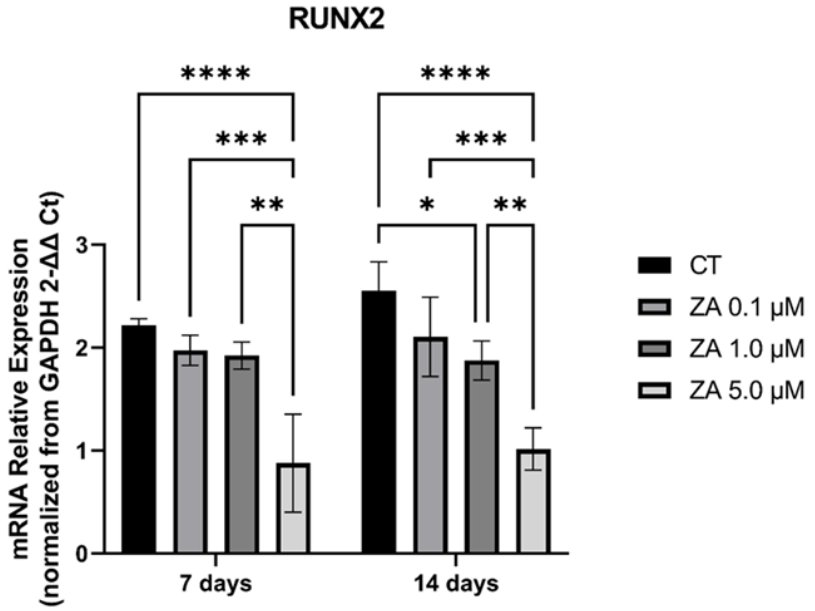

Fig. 6. Representative graph of RUNX2 expression at the 7- and 14-day periods. At 7 days, **** means p <0.0001, *** p = 0.0007, ** p = 0.0011; at 14 days **** means p <0.0001, *** p = 0.0007, ** p = 0.0059, * p = 0.0317.

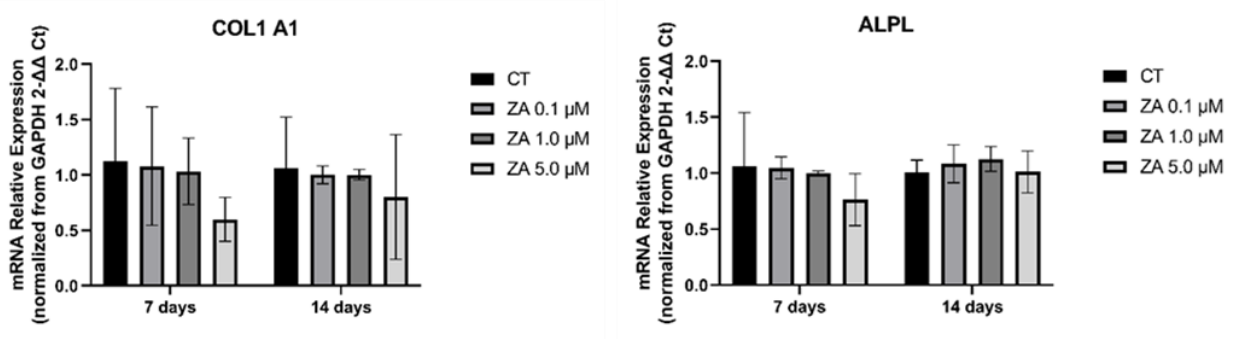

Fig. 7. The graph on the left represents the expression of COL1 A1, and on the right ALPL, at 7 and 14 days.

2.5 DISCUSSION

This study demonstrated the impact of different ZA concentrations on the differentiation process of hBMSCs into osteoblasts. The higher concentrations tested in this study reduced cell

viability, cell proliferation, mineralized matrix deposition, and expression of a transcription factor involved with pre-osteoblasts differentiation into osteoblasts, thus indicating that the capacity of hBMSCs to differentiate towards its osteoblastic lineage was harmed. Previously published data demonstrated that ZA could inhibit the viability and function of osteoblasts in vitro, in a dose-dependent manner [32]. The reduced viability and compromised cytoarchitecture, as confirmed by the LIVE/DEAD™ assay (Figure 4) and by cell adhesion and spreading imaging (Figure 3), were more evident at the 1.0 and 5.0 μM concentrations.

Chronic exposure to ZA above 1.5 mM impairs cell viability and induces apoptosis [33]. It has been demonstrated that ZA (1 and 5 μM) decreased cell viability in MG-63 human osteoblast-like cells and decrease expression of osteocalcin (OCN) and ALP after 14 days [34]. Previous data suggest that ZA negatively affects human hip bone osteoblasts (HOB-c) by decreasing cyclin D1 expression after the 6^{th} day of exposure, possibly resulting in compromised osteoblast proliferation [35]. Interestingly, this study demonstrated that ZA at a significantly low concentration (0.1 μM) stimulates cell adhesion and proliferation (Figure 4), characterized by a hormetic phenomenon. In general, hormesis refers to a beneficial or stimulating effect caused by low doses of substances that would normally be toxic at higher doses [36].

RUNX2 is a transcription factor that plays a central role in the differentiation and maturation of osteoblasts, and thus impacts bone formation and growth [37]. Specifically, it regulates the differentiation of mesenchymal stem cells (MSCs) into pre-osteoblasts, which continue to mature into osteoblasts [38]. Results from this study demonstrated that ZA, regardless of the concentration, reduced the expression of RUNX2 at 14 days (Figure 6). Hence, it can be inferred that although lower dosages of ZA do not affect (or improve [0.1 μM]) metabolism and viability, the hBMSCs exposed to ZA failed to differentiate into osteoblasts and to deposit minerals, as confirmed by the mineralization assay with alizarin red (Figure 2).

The activity of ALP and expression of COL1 A1 and OCN are characteristics of mature osteoblasts [39]. In the present study, it was demonstrated that ZA can also disrupt the maturation phase of the osteogenic differentiation, as confirmed by the downregulated expression of COL1 A1 and ALPL by ZA 5.0 μM at 7 and 14 days (Figure 7). Also, corroborating with this data, the ALP activity assay, a method routinely used for measuring the osteogenic differentiation process

and bone formation, [40] demonstrated that ZA, regardless of concentration, reduced ALP activity in both periods of analysis (Figure 5).

The benefits of using BPs for the treatment of hypercalcemia, bone metastases, or prevention of fragility fractures in osteopenic/osteoporotic patients [14-16] is irrefutable. As previously mentioned, although the most prominent effect of BPs is osteoclast inhibition, the results from this experiment support previously published studies [32,33] while demonstrating that osteogenic differentiation towards the osteoblastic lineage is negatively affected by ZA and possibly jeopardizes the capacity of the bone to repair [41]. Thus, the bone healing process is characterized by constantly bone matrix deposition, altering bone homeostasis. In this way, bone tissue became extremely mineralized and poorly vascularized, which may favor the development and progression of MRONJ.

There are substantial pieces of evidence regarding MRONJ and rising interest in investigations on the pathophysiology of this disease. As demonstrated in this study and by recent findings, [42] ZA increases cell apoptosis while reducing migration and proliferation. This leads to prolonged wound healing, chronic inflammation, and altered bone remodeling, directly affecting the progression of MRONJ [43]. The development of more precise preventive or therapeutic options for MRONJ will rely on a better understanding of its pathophysiology and the drug's mechanism of action that studies evaluating the impact of ZA in non-osteoclast cells may be incorporated to help improve treatment outcomes.

Although the rationale for testing 0, 0.1, 1.0, and 5.0 μM of ZA is based on clinical findings in humans with MRONJ (range from 0.4 to 4.5 μM) [44], as for any other in vitro experiment, the findings of our research need to be reproduced in vivo prior to translational development into clinical practice. We encourage future studies exploring the behavior of osteoblasts in animal models of alveolar bone repair.

2.6 CONCLUSION

Within the limitations of this in vitro study, it can be concluded that ZA, especially in the concentration of 5 μM, negatively affects the differentiation of hBMSCs towards the osteoblastic lineage by reducing cell proliferation, compromising adhesion, and spreading, and

downregulating RUNX2 expression, which culminates in reduced ALP activity and downregulated expression of ALPL and COL1 A1 (markers of later phases of osteogenesis).

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Capítulo 3

3 CAPÍTULO 3 - Beta Tricalcium Phosphate, either alone or in combination with antimicrobial photodynamic therapy or doxycycline, prevents medication-related osteonecrosis of the jaw‡

3.1 ABSTRACT

Surgical trauma in those under a prolonged use of bisphosphonates, can lead to mediation-related osteonecrosis of the jaw (MRONJ). This study aimed to evaluate the preventive therapies for MRONJ. Following four cycles of zoledronic acid administration, *Wistar* rats had their molar extracted, and were organized into nine treatment groups: negative control group (NCG), treated with saline solution and blood-clot in the alveolus; positive control group (PCG), with blood-clot in the alveolus; BG, β-tricalcium phosphate-based biomaterial; DG, 10% doxycycline gel; aG, antimicrobial photodynamic therapy; and DBG, aBG, aDG, and aDBG, using combination therapy. After 28 days, the lowest bone volume (BV/TV) was reported in PCG (42.17% ± 2.65), and the highest in aDBG (69.85% ± 6.25) ($p < 0.05$). The higher values of daily mineral apposition rate were recorded in aDBG (2.64 ± 0.48) and DBG (2.30 ± 0.37) ($p < 0.001$). Moreover, aDBG presented with the highest neoformed bone area (82.44% ± 2.69) ($p < 0.05$). Non-vital bone was reported only in the PCG (37.94 ± 18.70%). Owing to the key role of the biomaterial, the combination approach (aDBG) was the most effective in preventing MRONJ following tooth extraction.

Keywords: Biomaterials; Medication-related osteonecrosis of the jaws; Photodynamic therapy; Prevention; Tooth socket; Wound healing

3.2 INTRODUCTION

Anti-resorptive drugs (ARs), including anti-RANKL antibodies inhibit osteoclastic activity, and are thus commonly used to prevent hypercalcemia or bone metastases in patients with cancer[1,2], and fractures in osteopenic/osteoporotic patients[3]. However, the prolonged use of these medications is a risk factor for medication-related osteonecrosis of the jaw (MRONJ)[4-7].

‡ Published at *Scientific Reports*, doi: 10.1038/s41598-022-20128-4.

The prevalence of MRONJ is higher with the use of high bisphosphonate (BP) doses, especially of nitrogen-containing bisphosphonates (N-BPs), as they are more resistant to enzymatic degradation[8,9].

According to a recent by the American Association of Oral and Maxillofacial Surgeons (AAOMS) position paper[9], MRONJ is defined as an area of exposure in the maxillofacial region, persistent for >8 weeks, that can be diagnosed in patients undergoing to antiresorptive therapy alone or in combination with immune modulators or antiangiogenic medications, without prior exposure to radiotherapy in the head and neck region[10-14]. Despite its advantages and benefits in the treatment of skeletal disorders, prolonged use of BPs can lead to the development of MRONJ[13,15,16].

There is currently no universal protocol for the treatment of MRONJ. Treatment can range from conservative therapy to surgical intervention, depending on the disease stage. Several patients undergo conservative treatment, including oral health maintenance, infection removal, and antibiotic use[17]. Numerous published reports have described the success of antimicrobial photodynamic therapy (aPDT) in clinically treating this condition[18-22].

Thus, studies exploring the prevention of MRONJ have emerged[23-25]. Recent *in vitro* studies have demonstrated the use of calcium phosphate ceramics in the reduction or elimination of zoledronic acid (ZOL) toxicity in fibroblasts[26]. In antimicrobial photodynamic therapy (aPDT), a low-power laser biostimulates the osteoblasts, even during ZOL treatment[27], and reduces the number of bacterial colonies with a photosensitizing agent[24]. Moreover, satisfactory results were observed with doxycycline-loaded collagen sponge placed over the alveolar bone, in rats undergoing antiresorptive drug treatment[28].

Thus, this study aimed to comparatively evaluate the preventive effects of locally applied β-tricalcium phosphate (BTCP) ceramics, aPDT, and doxycycline, alone or in combination, on the development of MRONJ, in the alveolar bone of rats undergoing ZOL therapy.

3.3 METHODS

This study was approved by the Ethics Committee on Animal Experimentation (CEUA) of São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil (#0810-2018), following the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Institute of Laboratory Animal Resources [U.S.])[29].

Animals

Seventy-two 3-month-old male Wistar rats (*Rattus norvegicus albinus*) (weight, 300–350 g), obtained from the UNESP facility were group-housed in cages (four animals per box), and acclimatized to a temperature of 24 ± 2°C, with controlled light cycle (12/12 h), and free access to water and feed. To determine the sample size, a power test was performed at a significance level of 5% (with a standard deviation of 2%), $\alpha = 0.05$, and 80% power. Thus, eight rats ($n = 8$) were required per group.

ZOL application

All animals were treated with Zoledronate (ZOL) (Zometa®, Novartis Biosciences SA, São Paulo, Brazil), except for the animals in the negative control group (NCG), which received only 0.1 mL of saline solution (0.9% NaCl), using the same application protocol. Following an experimental model proposed by Curra et al.[30], the animals received four intravenous (IV) applications of 0.035 mg/kg of ZOL, dissolved in 0.1 mL of vehicle (0.9% NaCl), through the tail vein, at 15-day intervals. One week after the fourth ZOL application, the mandibular right first molars of the animals were extracted, and locally treated. ZOL injections were continued until euthanasia.

Tooth extraction and experimental groups

All animals had the mandibular right first molars extracted [23] on the 52nd day of ZOL treatment initiation (corresponding to 7 days after the fourth ZOL application). The surgical procedure was performed under anesthesia via an intraperitoneal injection (IP) of 90 mg/kg of ketamine hydrochloride (Vetaset, Fort Dodge Animal Health Ltd., São Paulo, Brazil) and 10 mg/kg of xylazine hydrochloride (Dopaser, Laboratórios Calier do Brasil Ltda, São Paulo, Brazil). After sedation, antisepsis was performed with degerming and topical povidone-iodine (10% PVP-I; Riodeine, Rioquímica, São José do Rio Preto, Brazil) application, followed by sterile draping. The animals were placed in the dorsal decubitus position on a customized operating table, and syndesmotomy was carefully performed using a delicate detacher (Molt Descolador no. 9, Quinelato, São Paulo, Brazil), followed by luxation and extraction of the tooth. The animals were randomly divided into nine treatment groups (www.randomization.com) by a blinded assessor, unrelated to the study.

- **Negative Control group (NCG):** Treated systemically with NaCl and locally left empty, except for the blood clots in the alveolus.
- **Positive Control group (PCG):** Treated systemically with ZOL and locally left empty, except for the blood clots in the alveolus.
- **Biomaterial group (BG):** The alveolus in the mandibular right first molar was filled with approximately 0.03 cc of BTCP paste (Graftys HBS, Latin American Solutions [LAS], Brazil).
- **Doxycycline group (DG):** The alveolus in the mandibular right first molar was filled with approximately 0.03 cc of 10% doxycycline gel.
- **aPDT group (aG):** The alveolus in the mandibular right first molar was irrigated for 1 min with 1 mL of neutral methylene blue photosensitizer, followed by a diode laser of indium gallium aluminum phosphide at the red wavelength (660 ± 10 nm), with a spot size of 0.0283 mm, 35 mW, in continuous mode, with a 2.1 J/point for 60 s, according to the model proposed by Ervolino et al.[24]

- **Doxycycline + biomaterial group (DBG):** The alveolus in the mandibular right first molar was filled with approximately 0.03 cc of mixture in a proportion of 50% each (10% doxycycline gel and B-tricalcium phosphate paste, 1:1) (Graftys HBS, LAS, Brazil).

- **aPDT + biomaterial group (aBG):** The alveolus in the mandibular right first molar received aPDT (as described above) and was subsequently filled with approximately 0.03 cc of BTCP paste (Graftys HBS, LAS, Brazil).

- **aPDT + doxycycline group (aDG):** The alveolus in the mandibular right first molar received aPDT (as described above) and was subsequently filled with approximately 0.03 cc of 10% doxycycline gel.

- **aPDT + doxycycline + biomaterial group (aDBG):** The alveolus in the mandibular right first molar received aPDT (as described above) and was subsequently filled with approximately 0.03 cc of mixture in a proportion of 50% each (10% doxycycline gel and BTCP paste, 1:1) (Graftys HBS, LAS, Brazil).

Post-extraction, the soft tissue was detached from the extraction site, and sutured with 4-0 polyglactin 910 (Vicryl, Ethicon, Johnson Prod., São José dos Campos, Brazil), to assist in the clot maintenance, treatment as previously randomized, and primary closure of the alveolus.

Fluorochrome application

Fifteen days after molar extraction, the fluorochrome calcein was administered intramuscularly (20 mg/kg) to each animal, and repeated after 10 days, using alizarin red (30 mg/kg)[31-33].

Euthanasia

All animals were euthanized 80 days after ZOL application (28 days after molar extraction) with a lethal dose of sodium thiopental (150 mg/kg; Thiopentax, Cristália Ltda, Itapira, SP, Brazil) and IV administration of 2% injectable lidocaine hydrochloride (VETOne®;

Pasadena, ID, USA). The right hemimandibles of all animals were collected and analyzed for the following analysis (Figure 1).

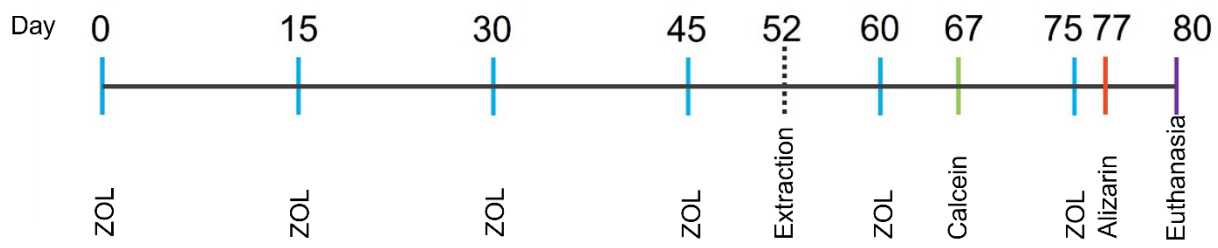

Figure 1. Flowchart of the work phases for mineralized tissue analysis, mentioned in days. (Blue lines, ZOL application; dotted line, extraction, and treatment of alveolus; green line, calcein application; red line, alizarin application; purple line, euthanasia, and collection of right hemimandible).

Microcomputed tomography analysis

The hemimandibles of the animals from the nine experimental groups were fixed in a 10% buffered formalin solution (Analytical Reagents, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP, Brazil) for 24 h, followed by washing under running water for 24 h. After fixation, the samples were stored in 70% alcohol for microcomputed tomography (micro-CT) analysis.

Micro-CT analysis was performed according to the methodology described by Bouxsein et al. (2010), using a SkyScan microtomograph (SkyScan 1272 Bruker MicroCT, Aatselaar, Belgium, 2003)[34]. The samples were scanned in 6-µm thick sections (90 kV and 111 µA), with a 0.5 mm Al + 0.038 mm Cu filter, and a 0.5-mm rotation step, at a pixel size of 2,016 × 1,344 µm, and acquisition time of 52 min. The distal root of the mandibular first molar was established as the region of interest (ROI), and a circular ROI size of 200 was used for all the specimens. The X-ray projection images of the samples were stored and reconstructed, and the area of interest was determined using the NRecon software (version 1.6.6.0; SkyScan, 2011), with the following modes: smoothing, 1; correction of artifact rings, 8; correction, 24% beam hardening, and image conversion, ranging from 0.0 to 0.14. The images were reconstructed in three planes (transverse, longitudinal, and sagittal), using the DataViewer software (version 1.4.4, 64-bit; SkyScan). The

percentage of bone volume (BV/TV), trabecular bone thickness (TbTh), separation and number of trabeculae (TbSp and TbN), and total porosity (PoTot) were evaluated using the CT-Analyser software (version 1.12.4.0; 2003-11SkyScan, 2012 Bruker MicroCT), and subsequently reconstructed in three dimensions (3D), using the CTVox software (version 2.7; SkyScan).

Laboratory processing

After micro-CT analysis, the hemimandibles of the nine experimental groups were dehydrated using increasing concentrations of alcohol (70%, 90%, and 100%), and subsequently immersed at different concentrations (70/30, 50/50, 30/70) of Technovit® light-curing resin (Heraeus Kulzer GmbH, Wehrheim, Germany), in a mixture with 100% alcohol solution. The samples were stabilized on a glass plate with composite resin, such that the long axis of the distal alveolus of the mandibular first molar was parallel to the ground. Subsequently, the Technovit resin was incorporated, light-cured, Exakt microtome-cut (Apparatebau GmbH, Hamburg, Germany), calcified, and ground using an Exakt system sander, until the center of the mesial root of the mandibular right second molar was located (standardized to establish the center of the alveolus of the first molar). Longitudinal sections of approximately 50-μm thickness, were obtained in the alveolar region (in the mesiodistal plane).

Laser confocal microscopy

These sections were captured using a Leica CTR 4000 CS SPE confocal laser microscope (Leica Microsystems, Heidelberg, Germany) with a 10× at the Confocal Fluorescence Microscopy Laboratory of the São Paulo State University (UNESP), School of Dentistry, Araraquara, Brazil. Thus, images of the fluorochromes calcein and alizarin red (old/new bone) were obtained separately, and finally reconstructed with an overlap, to assess bone turnover through the mineral apposition rate (MAR). The area of fluorochrome precipitation (calcein/alizarin) was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). MAR was determined using the value obtained on tracing five measurements extending from the outer margin of calcein towards the outer margin of alizarin, and dividing it by 10, which represents the time interval between the two injections[35].

Histomorphometric analysis

After confocal microscopy analysis, all the slides were stained with Stevenel's blue and acid fuchsin. Images of the sections, obtained at 1.6× and 40× magnifications, were analyzed under an optical light microscope (Diastar, Leica Reichert and Jung Products, Germany), and captured using an attached digital camera (Leica Microsystems DFC-300-FX, Germany), with 1.3 MP resolution. Histomorphometric analyses were performed using the ImageLab 2000 analysis software (version 2.4, Image Laboratory Inc., Belmont, MA, USA). The measurements obtained in micrometers (μm), were presented after conversion as percentage of the neoformed bone area (%NBA), connective tissue, and necrotic bone area (non-vital bone).

Statistical analysis

Data were analyzed using SigmaPlot (version 12.0; Exakt Graph and Data Analysis, USA). After performing a normality test (Shapiro–Wilk test) to determine the data distribution, a one-way analysis of variance test (ANOVA) was performed to verify the difference between the means of the groups, followed by Tukey's post hoc test for multiple comparisons.

3.4 RESULTS

Micro-CT analysis

Bone volume percentage (BV/TV)

The NCG had an average BV/TV of 54.01% ± 2.60, whereas the PCG had an average BV/TV of 42.17% ± 2.65 ($p = 0.193$). Notably, all treatment groups exhibited a higher BV/TV than that of PCG (Figure 2A). The aDBG had the highest BV/TV (69.85% ± 6.25), which was significantly different from that of NCG, PCG, aG, and aDG ($p = 0.034$, $p < 0.001$, $p < 0.001$ and, $p = 0.003$, respectively). However, no difference was observed when compared to BG, DG, aDG, and DBG ($p = 0.071$, $p = 0.092$, $p = 0.941$, and $p = 0.939$, respectively). The aDG and DBG

presented with similar BV/TV values (64.62% ± 0.13 and 64.69% ± 4.40, respectively), significantly different from that of PCG, aG, and aBG ($p = 0.002$, 0.005, and 0.034, respectively).

Total porosity (PoTot)

The NCG had an average PoTot of 45.98% ± 2.60, whereas the PCG presented the highest value of 57.82% ± 2.65 ($p = 0.193$), which was significantly different from that of aDBG, aDG, and DBG ($p < 0.001$, $p = 0.001$, and $p = 0.002$, respectively), followed by aG with 55.31% ± 1.67, which was also significantly different when compared to aDBG, aDG, and DBG ($p < 0.001$, $p = 0.005$, and $p = 0.005$, respectively) (Figure 2B).

Trabeculae thickness (Tb.Th)

The NCG presented with a mean TbTh of 0.138 ± 0.0074 mm, whereas PCG had a mean TbTh of 0.115 mm ± 0.016 ($p = 0.792$). A significant difference ($p = 0.043$) was noted between DG (0.163 ± 0.015 mm) and aG (0.111 ± 0.0070 mm) (Figure 2C).

Number of trabeculae (TbN)

A significant difference ($p < 0.05$) was observed between aDG (5.53 ± 5.3 mm) and PCG (3.68 ± 3.48 mm) (Figure 2D).

Trabecular separation (TbSp)

In contrast to BV/TV, the aDBG, aDG, and DBG, which presented with the highest bone volume, in this analysis, demonstrated the lowest TbSp (0.177 ± 0.05 mm, 0.176 ± 0.03 mm, and 0.243 ± 0.02 mm, respectively); however, all groups remained within the general average (Figure 2E).

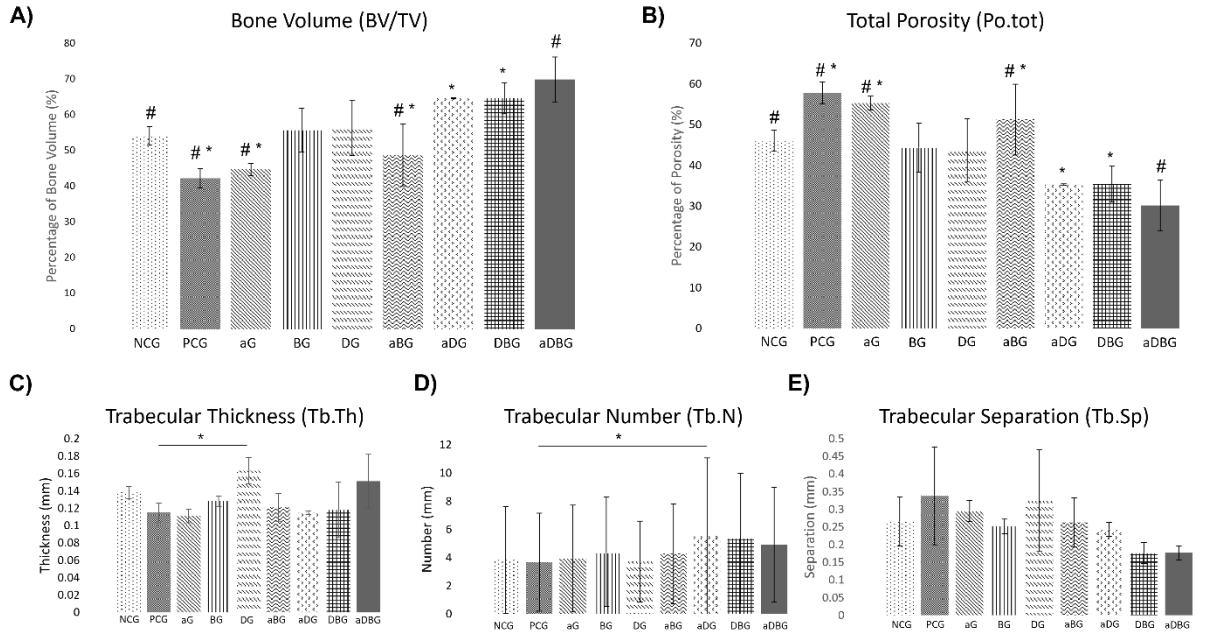

Figure 2. Representative images of the quantitative analysis of bone quality parameters obtained through micro-CT. A) Representative image of the average percentage of bone volume in all groups ($p < 0.05$, represented by #, when compared to aDBG; *, when compared to aDG and DBG); B) Image representing the average percentage of general porosity in all groups ($p < 0.05$, represented by #, when compared to aDBG; *, when compared to aDG and DBG); C) Image representing the mean thickness of the bone trabeculae (in mm) in all groups (*, $p < 0.05$); D) Image representing the mean number of trabeculae per mm in all groups ($p > 0.05$); E) Image of the separation of trabeculae per mm in all groups ($p > 0.05$).

Qualitative analysis of the alveolus

Micro-CT images (Figure 3) revealed a normal alveolar healing process, with mature, trabecular bone formation in the apical and middle thirds, thus maintaining the alveolar crest in the NCG. Conversely, in the PCG, bone sequestrum prevented the assessment of alveolar characteristics. Osteonecrosis was prevented in all the treatment groups, thus facilitating alveolar healing, and maintaining the alveolar anatomy. Furthermore, the alveolar volume was maintained in the BG, DG, and combination therapy groups (aDG, DBG, aBG, and aDBG), with aDBG exhibiting the most satisfactory results.

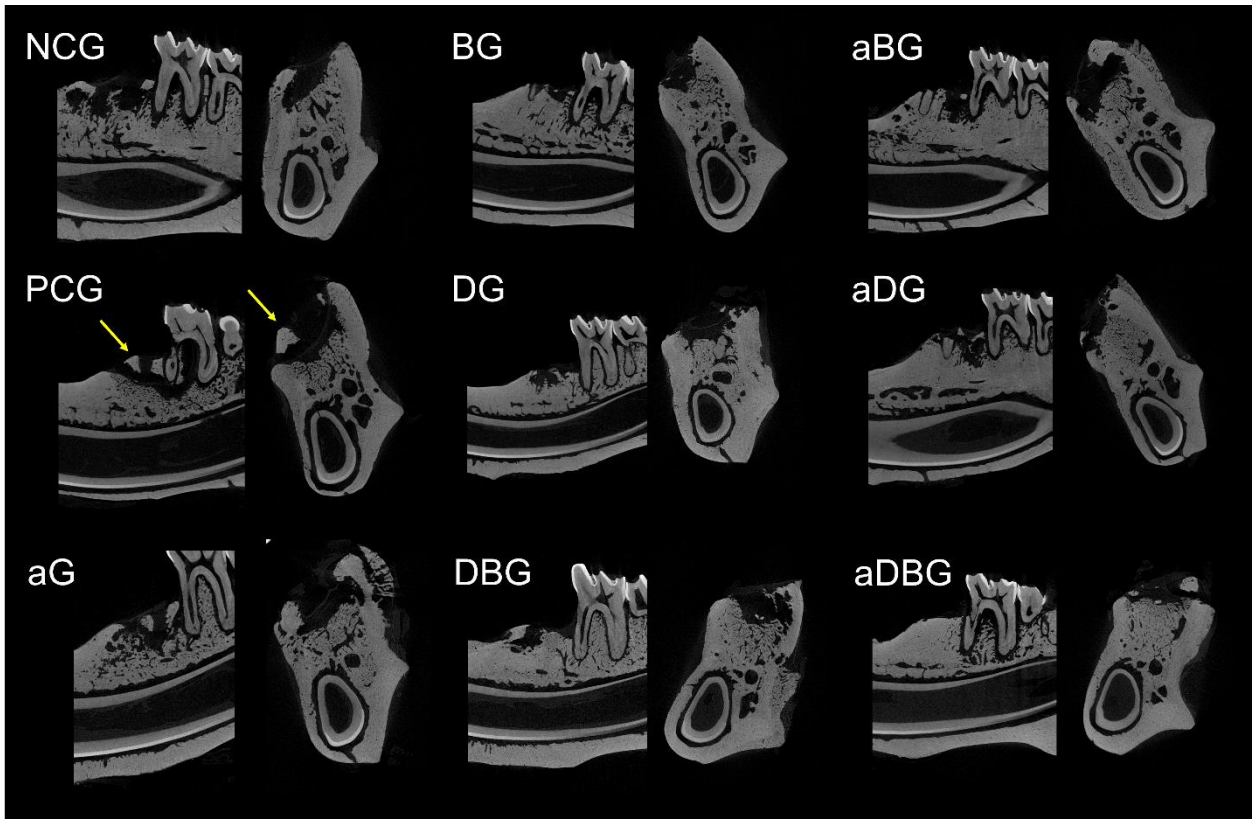

Figure 3. Representative images from the micro-CT. Each group is identified by its initials, followed by the sagittal and coronal section in the alveolar region of the distal root of the mandibular first molar. Yellow arrows demonstrate bone sequestrum.

Laser confocal microscopy

Mineral Apposition Rate

The aDBG had the highest daily MAR (2.64 ± 0.48), when compared to the other groups ($p < 0.001$), except for DBG (2.30 ± 0.37, $p = 0.359$). All groups had higher MAR values, when compared to NCG and PCG ($p < 0.05$) (Figure 4).

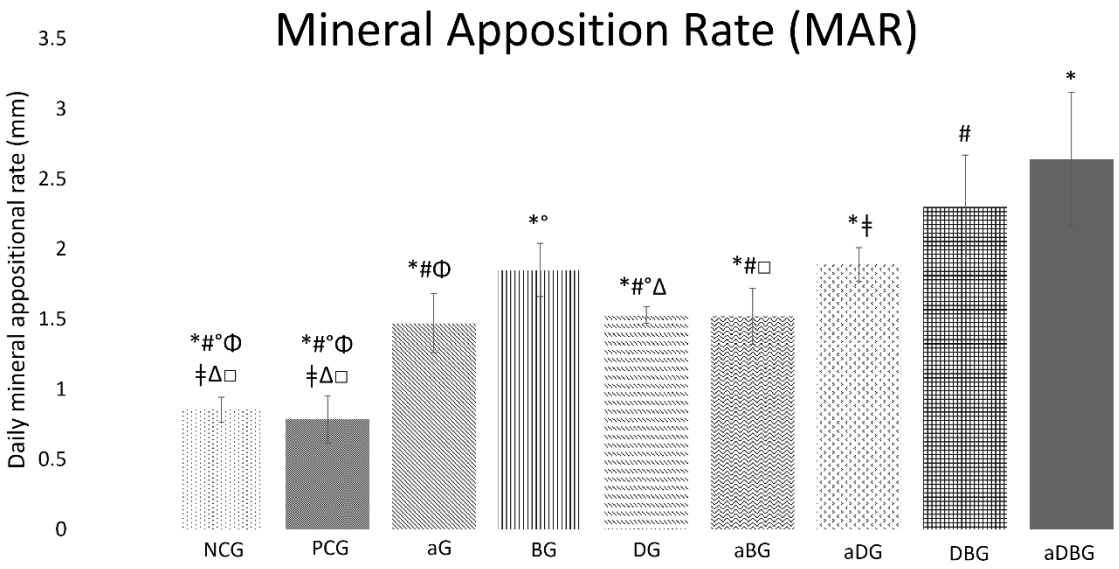

Figure 4. Graph representing the daily group averages of bone mineralization ($p < 0.001$, represented by *, when compared to aDBG; #, when compared to DBG; ǂ, when compared to aDG; °, when compared to BG; $p < 0.05$, represented by Δ, when compared to DG; □, when compared to aBG; Φ, when compared to aG).

Qualitative analysis

Calcium precipitation in the organic matrix with calcein and alizarin, was observed as a fluorescent line in the images. In this study, the fluorochromes were injected 15 (calcein) and 25 (alizarin) days after molar extraction. It stands out that, a green line (calcein) marked the older bone, whereas a red line (alizarin) marked the new bone. Concomitant observation of the images helped differentiate the amount of old and new bone (Figure 5).

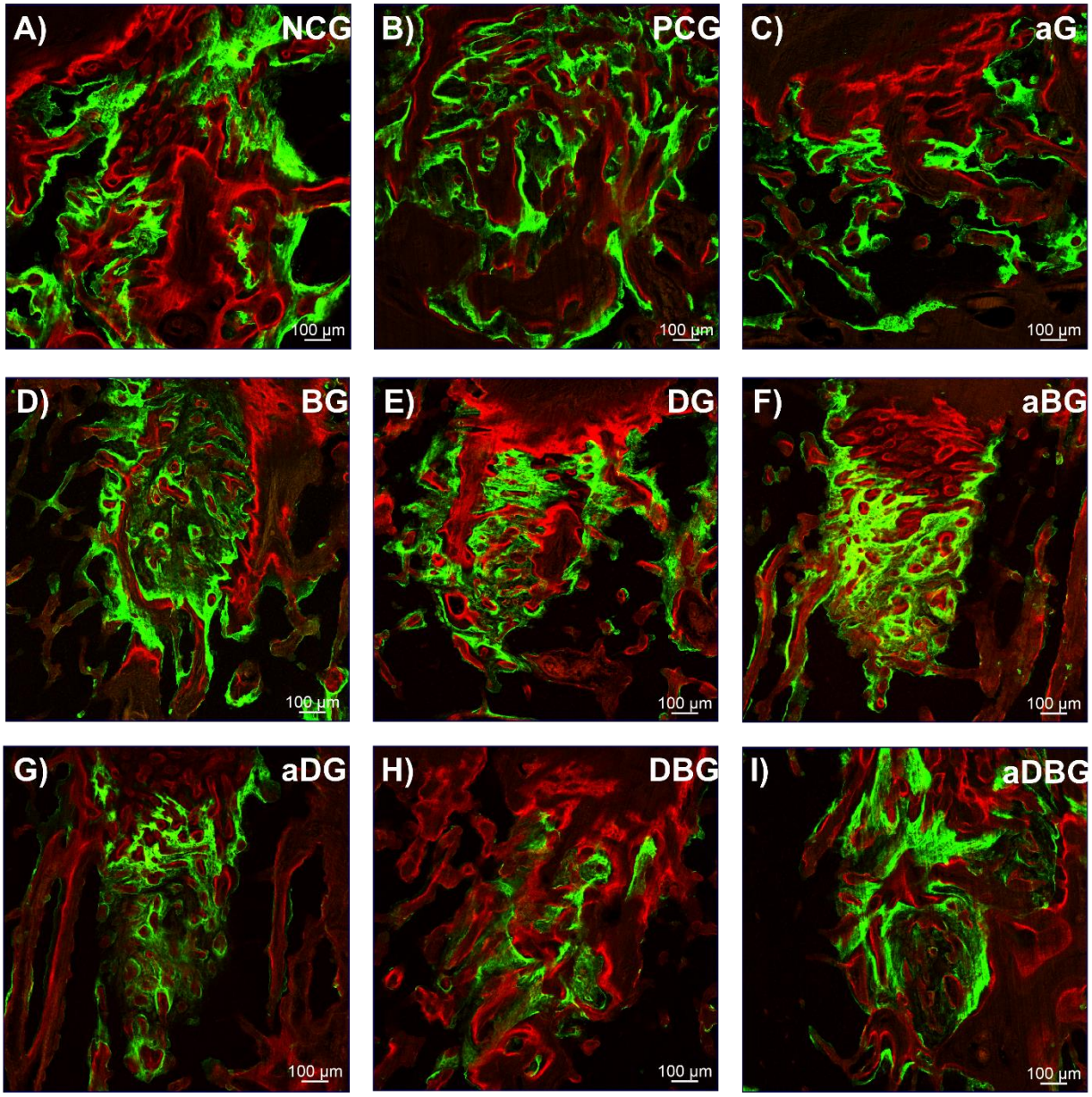

Figure 5. Images representing the dynamics of mineral apposition in the tissue as observed through a confocal laser microscope (10×) with calcein fluorochrome (stained in green) and alizarin fluorochrome (stained in red).

Histomorphometric analysis

Qualitative analysis

Analysis revealed that bone formation occurred through a continuous remodeling process involving resorption of old bone and subsequent formation of new bone by osteoblastic action (Figures 6 and 7). The presence of mature cortical bone (Figure 6A), along with concentric lamellae, and osteocytes (Figure 7A) were indicative of the alveolar bone healing in the apical and middle thirds, in the NCG. However, irregularities were observed on the surface of the bony trabeculae in PCG, with the absence of continuity of the cortical layer, and bone fragment loosening, characterizing the bony sequestrum (necrotic bone) (Figure 6B). At higher magnification, osteocytes and empty Haversian canals were observed (Figure 7B).

Similarly, the isolated treatments (aG, BG, and DG) allowed bone neoformation in the middle and apical thirds, with foci of vital bone in the connective tissue (Figure 6C, D, and E, respectively), with a greater amount of bone area in the BG. It can be highlighted that the BG and DG had more trabecular bone (Figure 7D and E), whereas the aG (Figure 7C) demonstrated less bone formation.

The combination therapies proposed in this study (aBG, aDG, DBG, and aDBG) were effective in the bone-healing process. Bone neoformation was noted in the apical and middle thirds of the alveolus, in addition to the cervical-third portion, in the DBG and aDBG. aBG (Figure 6F) and aDG (Figure 6G) demonstrated more trabecular bone tissue, with several islands of surrounding connective tissue (Figure 7F and G, respectively), whereas DBG (Figure 6H) and aDBG (Figure 6I) showed more organized bone tissue and less connective tissue (Figure 7 H and I, respectively).

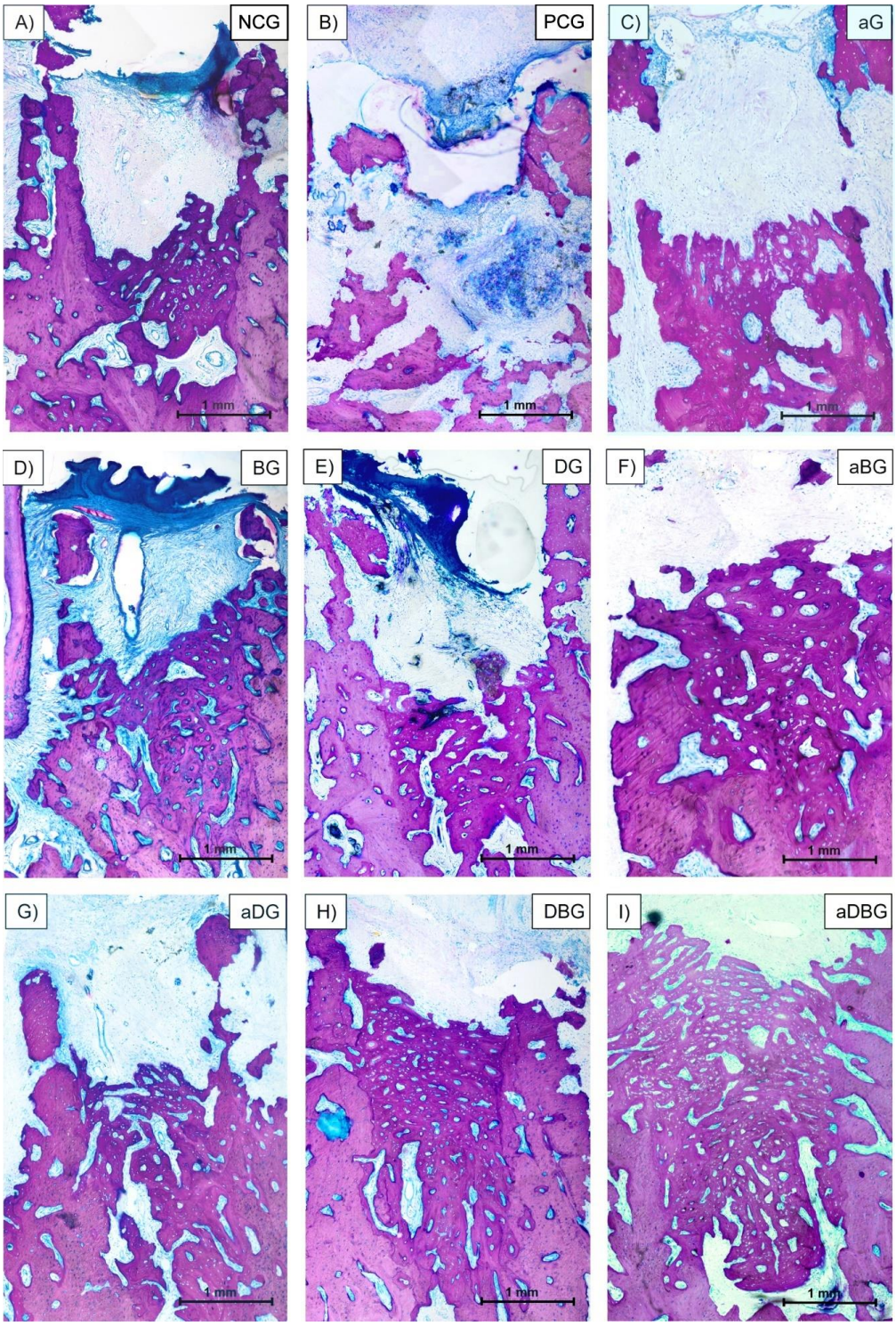

Figure 6. Representative photomicrographs of sections, stained in Stevenel's blue and acidic fuchsin at 1.6× magnification (blue, connective tissue; pink, mineralized tissue). Image (A) represents the NCG group, (B) PCG, (C) aG, (D) BG, (E) DG, (F) aBG, (G) aDG, (H) DBG, and (I) aDBG.

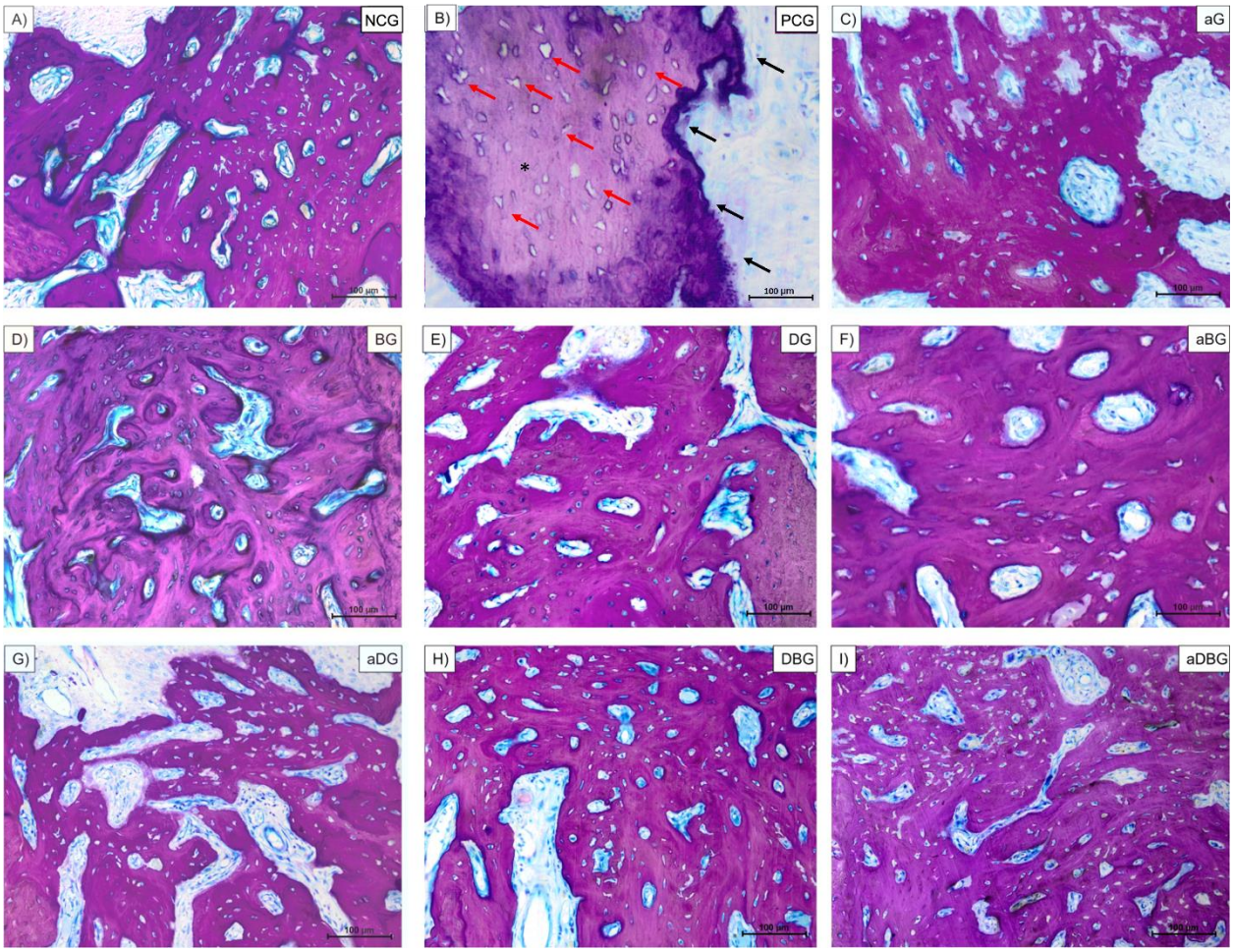

Figure 7. Representative photomicrographs of the sections stained in Stevenel's blue and acidic fuchsin at 40× magnification (blue, connective tissue; pink, mineralized tissue). Image (A) represents the NCG group, (B) PCG, (C) aG, (D) BG, (E) DG, (F) aBG, (G) aDG, (H) DBG, and (I) aDBG. (*, bone fragment in the middle of connective tissue; red arrows, empty lacunae without osteocytes; black arrows, irregularities in bone surface).

Bone dynamics

The dynamics of the tissues present within the distal alveolus of the extracted tooth were quantified and organized into (1) %NBA, (2) connective tissue, and (3) necrotic bone (non-vital bone) (Figure 8); their quantifications and p values are presented in Table 1.

Groups	% NBA	*p* value	% Connective Tissue	*p* value
PCG	11.15% ± 7.45		47.78% ± 20.73	
NCG	47.16% ± 4.03	< 0.001	51.36% ± 4.03	> 0.05
aG	35.48% ± 6.58	0.007	64.52% ± 6.58	> 0.05
BG	57.13% ± 5.89	< 0.001	42.86% ± 5.89	> 0.05
DG	41.64% ± 11.46	< 0.001	58.35% ± 11.46	> 0.05
aBG	52.08% ± 10.70	< 0.001	47.92% ± 10.70	> 0.05
aDG	55.90 % ± 13.78	< 0.001	44.10% ± 13.78	> 0.05
DBG	60.82% ± 4.37	< 0.001	39.17% ± 4.37	> 0.05
aDBG	82.44% ± 2.69	< 0.001	17.59% ± 2.69	> 0.05

Table 1. Summarization of the % NBA and % connective tissue values with theirs respectively significances differences when compared to PCG.

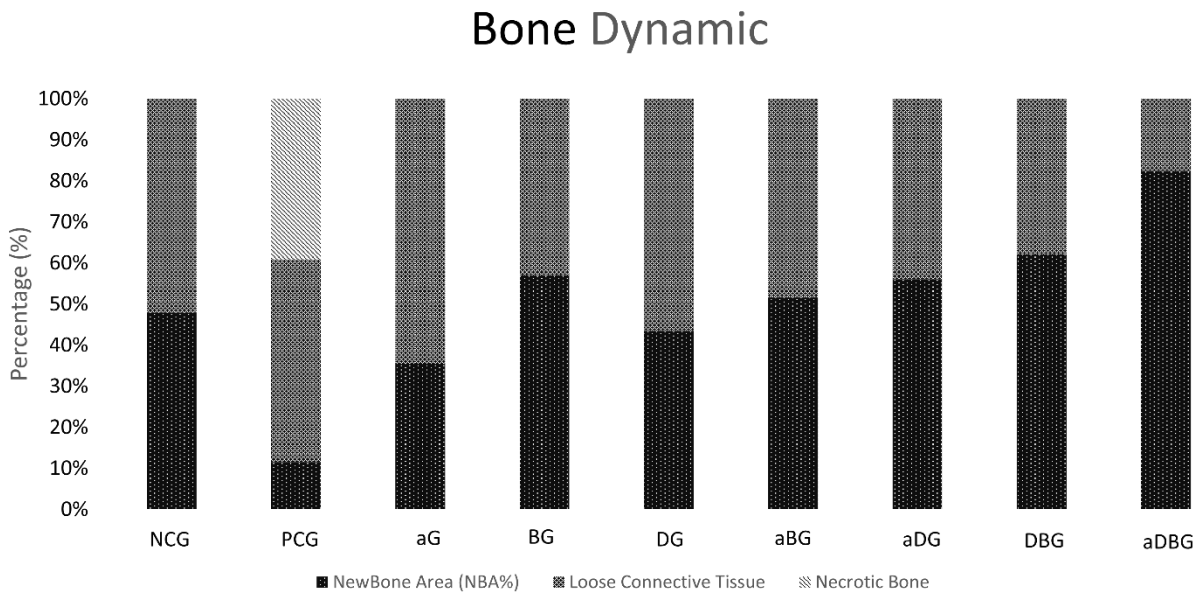

Figure 8. Graph representing the dynamics/composition of the sections, according to each group (in percentage). The components evaluated were neoformed bone area (pink), connective tissue (blue), and necrotic tissue or non-vital bone (gray).

Percentage of new bone area (%NBA)

The NCG had a %NBA of 47.16% ± 4.03, whereas the PCG had the lowest %NBA (11.15% ± 7.45; $p < 0.001$) (Figure 9). The aDBG had the highest %NBA (82.44% ± 2.69) ($p < 0.05$), followed by the DBG (60.82% ± 4.37). The combination therapies aBG and aDG showed satisfactory results for %NBA (52.08% ± 10.70 and 55.90 % ± 13.78, respectively), which were different from those of the PCG ($p < 0.001$). Furthermore, the %NBA of aDG was superior to that of aG ($p = 0.037$). Among the isolated treatment groups, the BG showed the best results (57.13% ± 5.89), with significant differences from that of aG ($p = 0.022$) and PCG ($p = 0.001$). The DG and aG showed %NBA of 41.64% ± 11.46 and 35.48% ± 6.58, respectively, which were both superior to that of the PCG ($p < 0.001$ and $p = 0.007$, respectively).

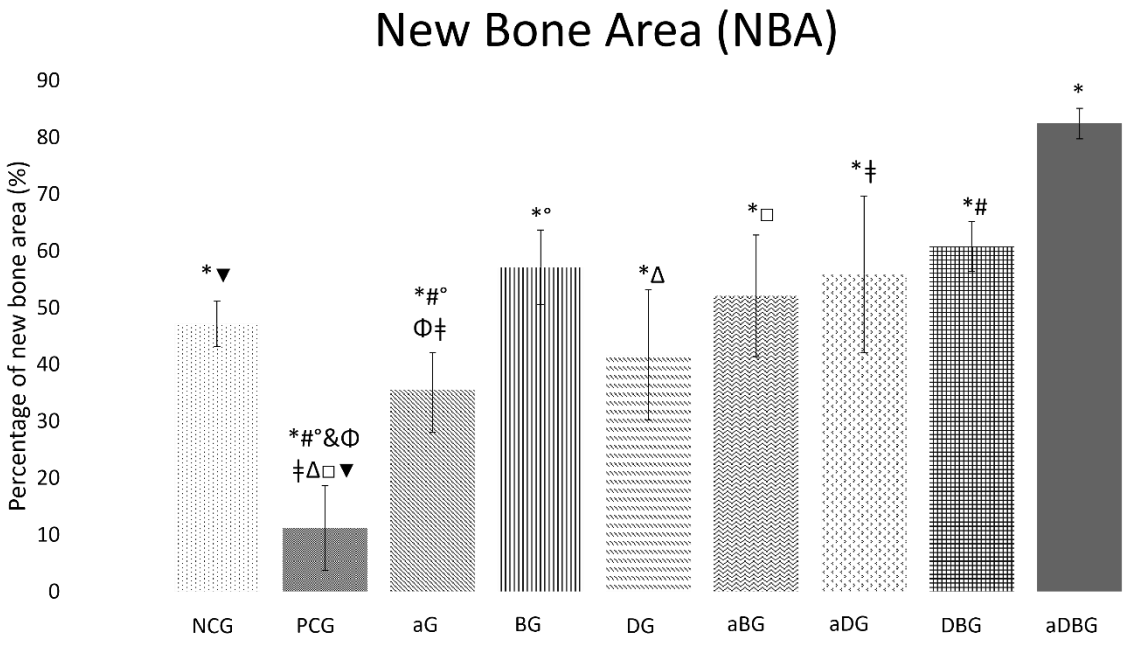

Figure 9. Graph representing the group-wise average percentage of the neoformed bone area ($p < 0.05$, represented by *, when compared to aDBG; #, when compared to DBG; ǂ, when compared to aDG; °, when compared to BG; □, when compared to aBG; Δ, when compared to DG; Φ, when compared aG; ▼, when compared to PCG).

Percentage of connective tissue

The NCG presented with 51.36% ± 4.03 of connective tissue, whereas the PCG presented with a smaller amount (47.78% ± 20.73; p = 0.688) (Figure 10). In contrast to the %NBA, the aDBG had the lowest mean percentage of connective tissue (17.59% ± 2.69), especially when compared to that of the aG and DG (both $p < 0.05$). For the DBG, aDG, and aBG, the percentages of connective tissue were 39.17% ± 4.37, 44.10% ± 13.78, and 47.92% ± 10.70, respectively ($p > 0.05$). The BG had the lowest mean connective tissue among the isolated treatment groups (42.86% ± 5.89), followed by the DG (58.35% ± 11.46) and aG (64.52% ± 6.58) ($p > 0.05$).

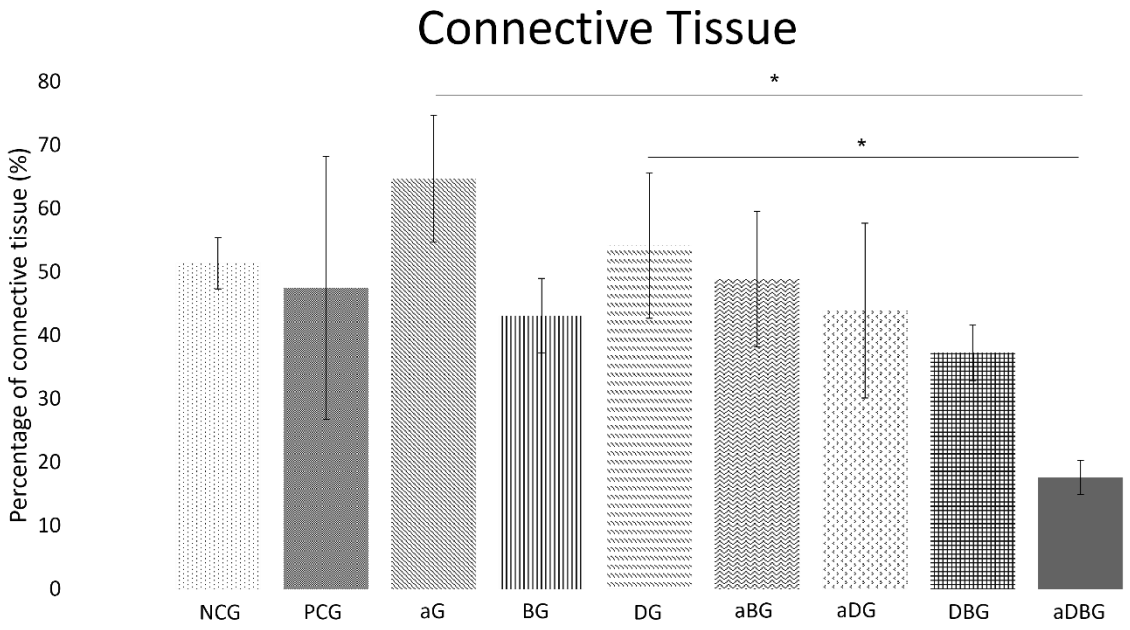

Figure 10. Graph representing the group-wise average percentage of connective tissue area. (*, $p < 0.05$) when compared to aDBG).

Percentage of necrotic bone (non-vital bone)

Necrotic bone was not observed in the samples from any of the groups, except for those from PCG (37.94% ± 18.70).

3.5 DISCUSSION

In this study, we hypothesized that local therapies would help prevent MRONJ, thus favoring the bone-healing process. The therapies used here, either alone or in combination, facilitated the formation of vital and organized bone tissue (Figures 6 and 7). The aDBG, DBG, and aDG exhibited the best results, as demonstrated by the BV/TV through micro-CT analyses (Figure 2), MAR (Figure 4), bone dynamics (Figure 8), and %NBA (Figure 9); however, the PCG presented 37.94 ± 18.70% of non-vital bone (Figure 8), embedded in a loose connective tissue stroma (Figure 6B and 7B).

Precipitation analysis (MAR) represents the amount of bone formed after calcium precipitation in the collagen-bone matrix; owing to the fluorochromes' property of binding to calcium, the amount of fluorescence represents the amount of bone formed[32,33,36,37]. It is noteworthy that BG exhibited the highest response among the therapies applied in isolation (Figure 4), and among all treatments, the highest MAR was observed in the aDBG ($p < 0.05$). It is believed that the osteoconductive potential of the BTCP-based biomaterial could induce and guide bone formation, providing good results as demonstrated in this research. Furthermore, the aDBG group conglomerated the beneficial effects of each therapy, as demonstrated by MAR.

Excessive suppression of osteoclasts is believed to impair bone turnover, and bone trauma prevents tissue healing, resulting in bone necrosis[16]. Moreover, some BPs, such as ZOL, have antiangiogenic effects that decrease the expression of endothelial growth factors in the circulation, leading to areas of ischemia, facilitating MRONJ development[38,39]. Unfortunately, in this study, the vascularization potential was not assessed; however, it was demonstrated that the use of BPs resulted in the formation of non-vital bone fragments (Figure 7B), characterizing bone sequestrum (Figure 3). The use of ZOL allows the tissues to present an intense daily MAR (Figure 4), reflecting an altered bone function[40,41].

The presence and viability of fibroblasts and endothelial cells are essential for alveolar bone healing[42-44]. ZOL can inhibit osteoclast formation, alter cell migration, suppress angiogenesis and osteogenesis, thus generating toxicity in these cells[45-51]. Inhibition of these cells encourages the formation of a more dense, cortical bone, instead of a vascularized tissue (Figure 6B), thus increasing the likelihood of bone sequestration associated with inflammation, and consequentially, local infection[52,53], as demonstrated in the PCG (Figure 7B).

Animal models have been used to demonstrate the repercussions of BPs on bone, such as an increase in bone density and a decrease in the spaces between the trabeculae, causing a decrease in the nutritive channels of the bone tissue[30, 54-56]. In the present study, the region corresponding to the distal alveolus of the first molar was evaluated, and no suggestive results of an increased bone density were found (TbTh, p = 0.792; TbN, p = 1.901; TbSp, p = 0.199; and PoTot, p = 0.109). However, in the micro-CT analysis using the NRecon tool in the PCG, areas of bone irregularities, sequestration, and absence of bone within the alveolus were observed, as demonstrated in previously published studies on animals treated with ZOL[20,57-61].

Recently, an *in vitro* study demonstrated that the use of calcium phosphate-based synthetic ceramics in a fibroblast culture exposed to ZOL therapy reduced or prevented toxicity[26], thus favoring alveolar bone healing, as observed in the present study in BG, DBG, and aBG, as %NBA (Figure 9) and BV/TV (Figure 2D). Ceramics-based biomaterials composed of BTCP are highly biocompatible and bioactive, in addition to being osteoinductive[62-64], and are thus indicated for filling bone cavities[65]. Studies have shown beneficial effects on cell growth, and these biomaterials can be considered as good candidates for bone tissue engineering applications[66,67], in addition to demonstrating ectopic bone formation even in large animal models, with a rapid bone formation pattern as that of autogenous bone[68]. The use of ceramic biomaterials based on BTCP[69] or borate bioactive glass[70] can help prevent MRONJ and enhance vital bone tissue formation.

The multi-session aPDT in newly extracted alveoli of senile rats treated with ZOL, has prevented MRONJ[24], as demonstrated in this study (Figure 3). Biomodulation with a low-power laser has biostimulatory effects on osteoblasts, even when treated with ZOL[27]; the association with a photosensitizing agent (methylene blue) reduces the bacterial load[71], thus preventing MRONJ, as observed in this study in aG (Figure 7C).

Infection control strategies, ranging from topical to systemic antibiotics, are essential in the treatment of MRONJ[17]. Several antimicrobial combinations serve as adjuvant therapies. Doxycycline, a structural isomer of tetracycline, has antibacterial, anti-inflammatory, and anti-collagenase activities; its anti-osteoclastogenic potential is essential for bone healing[72-75]. The application of a collagen sponge with doxycycline to the alveolus, after treatment with ZOL, showed positive effects on the reduction of necrotic bone tissue in rats[28]. Similarly, in this study,

the use of 10% DG prevented MRONJ (Figure 3), and contributed to the maintenance of bone volume (Figures 6E and 7E).

Further, the combination of local treatments showed good results by their synergistic effect, thus satisfying the primary objective of this study to prevent osteonecrosis (Figure 8). Even though the isolated use of aPDT (aG) has not demonstrated large amounts of bone in %NBA analysis, the authors believe that its antimicrobial and biostimulatory potentials are essential for the prevention of osteonecrosis, and when associated with doxycycline (aDG) and a biomaterial (aBG), a greater amount of bone volume was observed in %NBA analysis. The use of 10% DG alone showed good results in repairing critical bone defects in rat calvaria[76]. It is believed that the anti-osteoclastogenic effect of doxycycline allows the biomaterial to maintain its osteoconductive function. Thus, the combination of the three local methods (aDBG) prevented MRONJ (Figure 3), favoring alveolar bone healing (Figures 9I and 10I) with higher %NBA, BV/TV, and MAR.

The mechanisms of alveolar bone healing are similar among species[77], and rodent models are ideal for assessment as there is no evidence of superiority for the larger animal models in representing human bone biology[78]. Given the methodology and results demonstrated in this study, we believe that the use of a low-dose concentration of ZOL in this study (0.035 mg/kg) could be a limiting factor; it is similar to that used for preventing osteoporosis[30,79], unlike the high-dose concentrations used in cancer treatment[23,24]. In addition, it can be noted that the choice for male rats could be a limitation in this research, since in translational clinical application, women are more likely to develop osteoporosis, owing to menopause and estrogen deficiency. Further, they pose a higher risk of developing osteonecrosis with the use of antiresorptive drugs to prevent bone loss. Therefore, future studies are necessary to evaluate different concentrations of ZOL, as well as methodologies for the evaluation of demineralized tissues such as immunohistochemical analysis and real-time polymerase chain reaction.

3.6 CONCLUSION

This research reveals that MRONJ induced by the ZOL protocol (0.035 mg/kg) is counteracted by the use of beta tricalcium phosphate (BTCP), either alone or in combination, by

guiding alveolar bone healing. While the use of doxycycline and aPDT demonstrated positive effects on MRONJ prevention, the combination of all three therapies proved satisfactory.

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Capitulo 4

4 CAPÍTULO 4 - The role topical application of 10% doxycycline gel in the prevention of medication-related osteonecrosis of the jaw[§]

4.1 ABSTRACT

Prolonged use of bisphosphonates can lead to mediation-related osteonecrosis of the jaw (MRONJ). This study aimed to assess the effect of doxycycline to prevent osteonecrosis of the jaw. For this purpose, *Wistar* rats (72) were treated with 70 µg of ZA/month for 2 months, then extraction of the first molar was performed, one group received only a clot in the socket (ZA group), one received a 10% doxycycline gel (DOXI), while the control group was treated systemically with saline solution (SAL). After 7, 14, and 28 days, samples were submitted for 1) histomorphometric analysis, 2) immunohistochemical, and 3) collagen fibers maturation. Data demonstrated that DOXI presented higher new bone area when compared to ZA in 7 ($p = 0.0058$), 14, and 28 days ($p < 0.0001$), and the amount of non-vital bone in ZA (44.17± 10.93%) was statistically significant only at 28 days ($p < 0.0001$) when compared to SAL and DOXI. TRAP labelling was similar for ZA and DOXI, except for 28-day when ZA presented mild labelling, while DOXI showed moderate labelling. Also, OCN labelling was similar between the ZA and DOXI groups, however at 7 days DOXI showed moderate labelling, while ZA showed mild labelling. DOXI group presented a higher amount of collagen type 3 (greenish-yellow coloured fibers) on days 14 and 28 ($p = 0.0001$ and $p < 0.0001$, respectively), when compared to ZA. They also showed greater maturation of collagen fibers – collagen type 1 (yellowish-red colour) on days 14 and 28 ($p = 0.0027$ and $p < 0.0001$, respectively). Conclusion: doxycycline seems to be an effective agent in the prevention of osteonecrosis.

Keywords: MRONJ; antiresorptive drugs; bisphosphonates; bone repair; doxycycline.

[§] Edited according to the guidelines of *Journal of Dental Research*. https://journals.sagepub.com/author-instructions/JDR

4.2 INTRODUCTION

The use of antiresorptive drugs such as bisphosphonates, in particular, zoledronic acid can lead to a condition known as medication-related osteonecrosis of the jaw (MRONJ) (Otto et al. 2018; Kuroshima et al. 2022). According to the American Association of Oral and Maxillofacial Surgery (AAOMS) MRONJ has been defined as the presence of exposed bone in the maxillofacial region for a period longer than 8 weeks in patients with no history of radiation therapy or metastatic disease in the jaws which have been treated with ARs (Ruggiero et al., 2022). Even though it has a low incidence (0.01-0.03% for patients treating osteoporosis, and 2-5% in patients undergoing cancer treatment) (Khan et al. 2015; Coropciuc et al. 2023), its outcome can impact patients' quality of life (Cassia Tornier et al. 2021).

Although the pathophysiology of this disease remains unknown, the literature shows that some factors may be directly related to the progression of this disease, such as the suppression of osteoclastic activity (Russell et al. 2008), by incorporating non-hydrolysable analogs of adenosine triphosphate (ATP) (Frith et al. 2001) or inhibiting peroxisomal enzyme farnesyl diphosphate (FPPS) synthase and blocking prenylation of small GTPase in the mevalonate pathway of cholesterol synthesis (Reszka and Rodan 2004) which reduces bone resorption and lead to an extremely corticalized bone tissue with poor nutrition.

It is also known that this drug has an antiangiogenic effect (Fournier et al. 2002; Allen and Burr 2009; Stresing et al. 2011; Lang et al. 2016) which would further reduce the vascularization of the tissue, making it more susceptible to the progression of necrosis. Drug toxicity can also be an important factor as it affects cell migration and viability, preventing the alveolus from being covered by soft tissue from the newly operated areas, and delaying repair (Reid et al. 2007). These drugs can also affect fibroblast and osteoblast activity impairing bone formation (Walter et al. 2011; Hadad et al. 2023).

Recently, there has been a growing number of publications exploring methodologies for preventing MRONJ. These methodologies propose to improve vascularization, reduce toxic effects, stimulate bone formation or even control local contamination (Ervolino et al. 2019; Sharma et al. 2021; Hadad et al. 2023). It is worth mentioning that the use of doxycycline loaded in a sponge proposed by Çapar et al. (2016) reduced the percentage of necrotic bone in the group treated with zoledronic acid. Doxycycline is a structural isomer of tetracycline, which presents an

antibacterial, anti-inflammatory, and anticollagenase action (Bonnetblanc 2002). Also, this drug can inhibit osteoclastogenesis, which favors the bone repair process (Lucateli et al. 2018). Thus, this study aimed to investigate the application of 10% doxycycline gel in the alveolus of rats treated with zoledronic acid after tooth extraction, and to evaluate bone quality and collagen fiber formation.

4.3 METHODS

This study was approved by the Ethics Committee on Animal Experimentation (CEUA) of the São Paulo State University (UNESP), School of Dentistry, Araçatuba, Brazil (#0810-2018), following ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and in according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Institute of Laboratory Animal Resources (U.S.) (Kilkenny et al. 2010).

Animals

The sample size was established based on a previously published paper (Hadad et al. 2023). Thus, eight rats (n = 8) were required per group. In total, seventy-two 3-month-old male *Wistar rats* (Rattus norvegicus albinus) (weight, 300–350 g) obtained from the UNESP Facility were housed in collective cages (four animals per box) with a temperature of 24 ± 2°C, controlled light cycle (12/12 h), and free access to water and feed.

Osteonecrosis model

In this study, the administration of zoledronic acid followed by tooth extraction was used to establish the osteonecrosis model. First, all animals were treated with ZA (Zometa®, Novartis Biosciences SA, São Paulo, Brazil), except for the animals in the control group (SAL), which received only 0.1 mL of saline solution (0.9% NaCl) using the same application protocol.

A dose of 35 µg/kg of ZA was administered intravenously (tail vein), every 15 days for 2 months (Curra et al., 2016). Then, one week after the last ZA application, the animals had their

mandibular right first molars extracted (Statkievicz et al. 2018), and local treatment was performed. After extraction, the animals were continuously medicated until euthanasia, according to the protocol described above.

The surgical procedure was performed under anesthesia through an intraperitoneal injection (IP) of 90 mg/kg of ketamine hydrochloride (Vetaset, Fort Dodge Animal Health Ltd., São Paulo, Brazil) and 10 mg/kg of xylazine hydrochloride (Dopaser, Laboratórios Calier do Brasil Ltda, São Paulo, Brazil). In the sequence, antisepsis was performed with degerming and topical povidone-iodine (10% PVPI, Riodeine, Rioquímica, São José do Rio Preto, Brazil), followed by the apposition of sterile drapes. The animals were placed in dorsal decubitus position on a customized operating table, and gingival insertion syndesmotomy was carefully performed using a delicate detacher (Molt Descolador no. 9, Quinelato, São Paulo, Brazil) followed by dislocation and extraction of the tooth. The animals were randomly divided into 3 groups (on the website www.randomization.com) by a blinded staff external to the study, according to the local treatment received: **Control group (SAL)**, treated systemically with NaCl and locally with blood clots filling the socket; **Zoledronic Acid group (ZA)**, treated systemically with ZA and locally with blood clots filling the socket; **Doxycycline group (DOXI)**, treated systemically with ZA and locally with approximately 0.03 cc of 10% doxycycline gel filling the socket. Then, after 7, 14 and 28 days, the animals were euthanized, the samples (jaws) were collected, and submitted to laboratory processing according to the analysis.

Histological and histomorphometric analysis

The mandibles were fixed in 10% buffered formalin (Reagentes Analíticos, Dinâmica Odonto-Hospitalar Ltda, Catanduva, SP - Brazil) for 24 hours, followed by demineralization in a solution of ethylenediamine tetraacetic acid (EDTA) and 20% sodium hydroxide for 10 weeks, The samples were then dehydrated in a sequence of alcohols and diaphanized with xylene. Finally, the samples were embedded in paraffin.

Subsequently, 5 µm-thick sagittal sections were acquired using a microtome (RM2235, Leica Biosystems Nussloch GmbH 2017). Four slides from each sample were separated for hematoxylin and eosin (HE) staining, four for picrosirius staining, and another six for

immunohistochemistry reactions. The images of the HE-stained slides were obtained using an optical microscope (Leica DM4000 B LED, Heerbruggm Switzerland) with the aid of Carls Zeiss objective lenses (2.5X0.07, 10X, 20X and 40X0. 65) coupled to an image capture camera and connected to a computer (Configuration: Intel Core i5® with Windows 7® operating system and image processing and analysis software (AxioVision 4.9.1®0, Carl Zeiss by Imaging Associates ltd, Jena, Deutschland).

For the quantitative analysis, the following outcomes were assessed: area of intact bone tissue, area of necrotic bone tissue, connective tissue, and blood vessels. The analysis was carried out on the region of interest, which corresponds to the area of the distal alveolus corresponding to the first molar in the anteroposterior direction.

Immunohistochemical analysis

For immunohistochemical analysis, the indirect immunoperoxidase technique was used, using polyclonal primary antibodies, produced in goats, against OCN and TRAP and as a secondary antibody, biotinylated anti-goat antibody, produced in donkeys (Jackson Immunoresearch Laboratories). The signal was amplified using the Avidin/Biotin system (Vector Laboratories) and the reaction was developed using the Diaminobenzidine kit (Dako). At the end of the reactions, counterstaining was performed with Meyer's Hematoxylin.

Scores were assigned (ordinal qualitative analysis) referring to the extent of immunolabeling observed in the area of interest, where score 1 represents mild labelling, with approximately 25% of the area of interest positively labelled for the antibody under analysis; score 2 represents moderate labelling, with approximately 50% of the area of interest positively labelled for the antibody under analysis and finally score 3, representing intense labelling, with approximately 75% of the area of interest positively labelled for the antibody.

The scores were assigned by a previously calibrated researcher for the immunolabeling in question and the kappa test was performed on the assigned scores, which were considered satisfactory with a correspondence level of 0.8.

Picrosirius Red Staining and Collagen maturity

The sections were stained with Picrosirius-red (PSR) and then analyzed under a polarized light microscope to assess the organization and quantity of collagen fibers, considering the intensity of the birefringence of the collagen matrix under polarized light microscopy (Oliveira et al. 2022) Images were acquired in 400x magnification followed by the analysis using the LAS program (Leica LAS, Leica Microsystems) to asses each type of collagen fibers. The software recognized the greenish-yellow fibers as immature and thin, and the yellowish-red fibers were mature and tick, then the program automatically calculated the area of each fiber.

Statistical analysis

Data were analyzed using SigmaPlot (version 12.0; Exakt Graph and Data Analysis, USA). After performing a normality test (Shapiro–Wilk test) to determine the data distribution, a two-way analysis of variance test (ANOVA) was performed to verify the difference between the means of the groups, followed by Tukey's post hoc test for multiple comparisons.

4.4 RESULTS

Histomorphometric analysis

At 7 days, in the SAL group (Figure 1), repair occurred normally, characterized by primary bone neoformation in the apical region, with very irregular collagen fibers and fine bone trabeculae. In the middle third, there was dense connective tissue and collagen fibers concentrated in the lateral region of the alveolus, as well as the presence of granulation tissue that extended to the cervical portion, characterized by moderate vascularization and a discreet mononuclear inflammatory infiltrate, with no epithelial covering. In the ZA group (Figure 2), primary bone neoformation was also observed in the apical and middle thirds of the alveolus. However, fewer collagen fibers were seen distributed throughout the connective tissue. Granulation tissue extended throughout the three-thirds of the alveolus, with a moderate mononuclear inflammatory infiltrate. In the SAL and ZA groups, the bone crests were absent from the nucleus, suggesting an initial process of bone remodelling. In the DOXI group (Figure

3), there was primary bone neoformation completing the apical and middle thirds, overlaid by well-organized dense connective tissue with collagen fibers and blood vessels, filling the entire length of the alveolus. There was a slight inflammatory infiltrate which extended into the connective tissue present in the cervical third, highlighting the presence of thin epithelial tissue which completely recovered the extraction site.

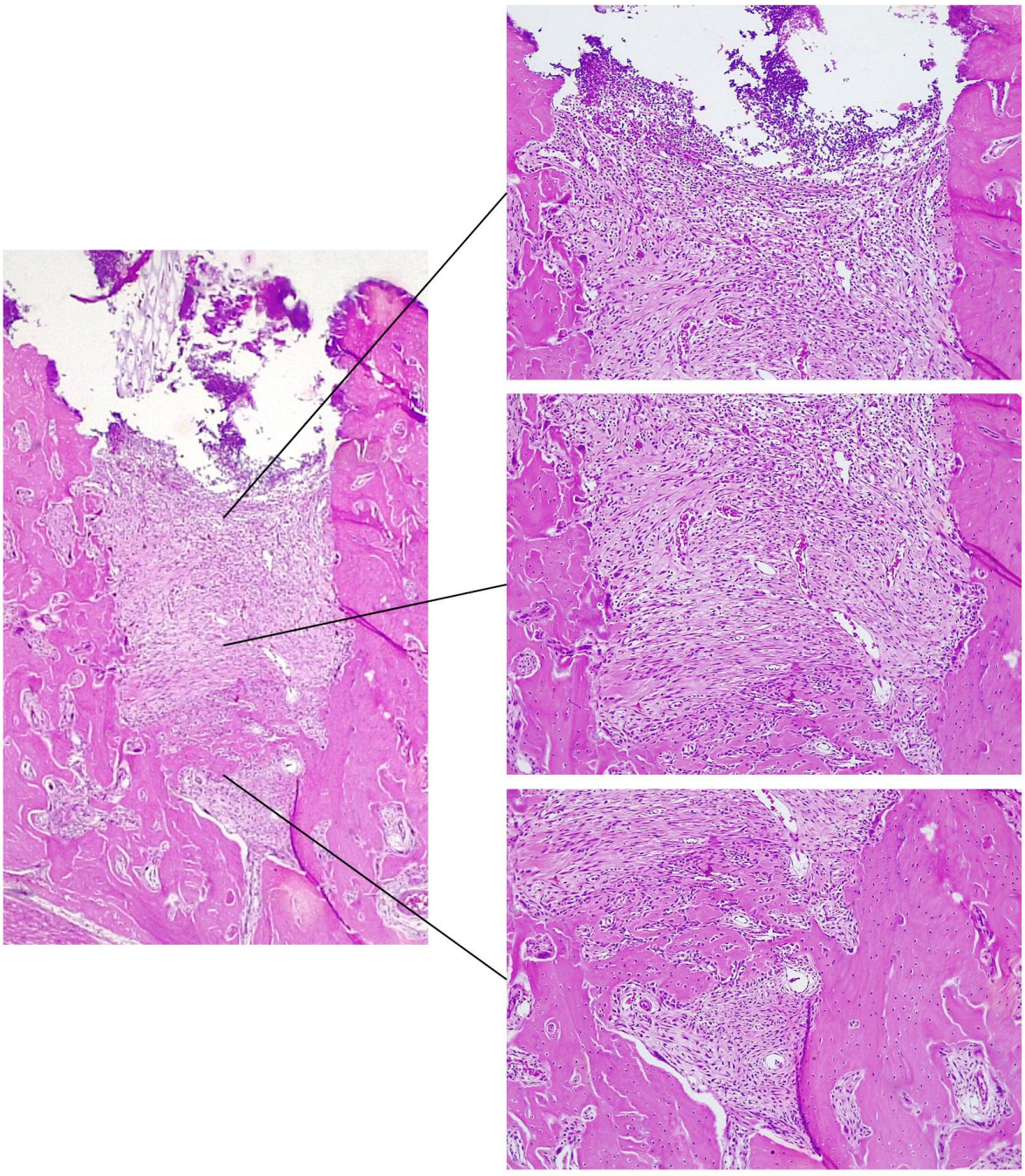

Figure 1. Representative histological images of the SAL group at 7 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

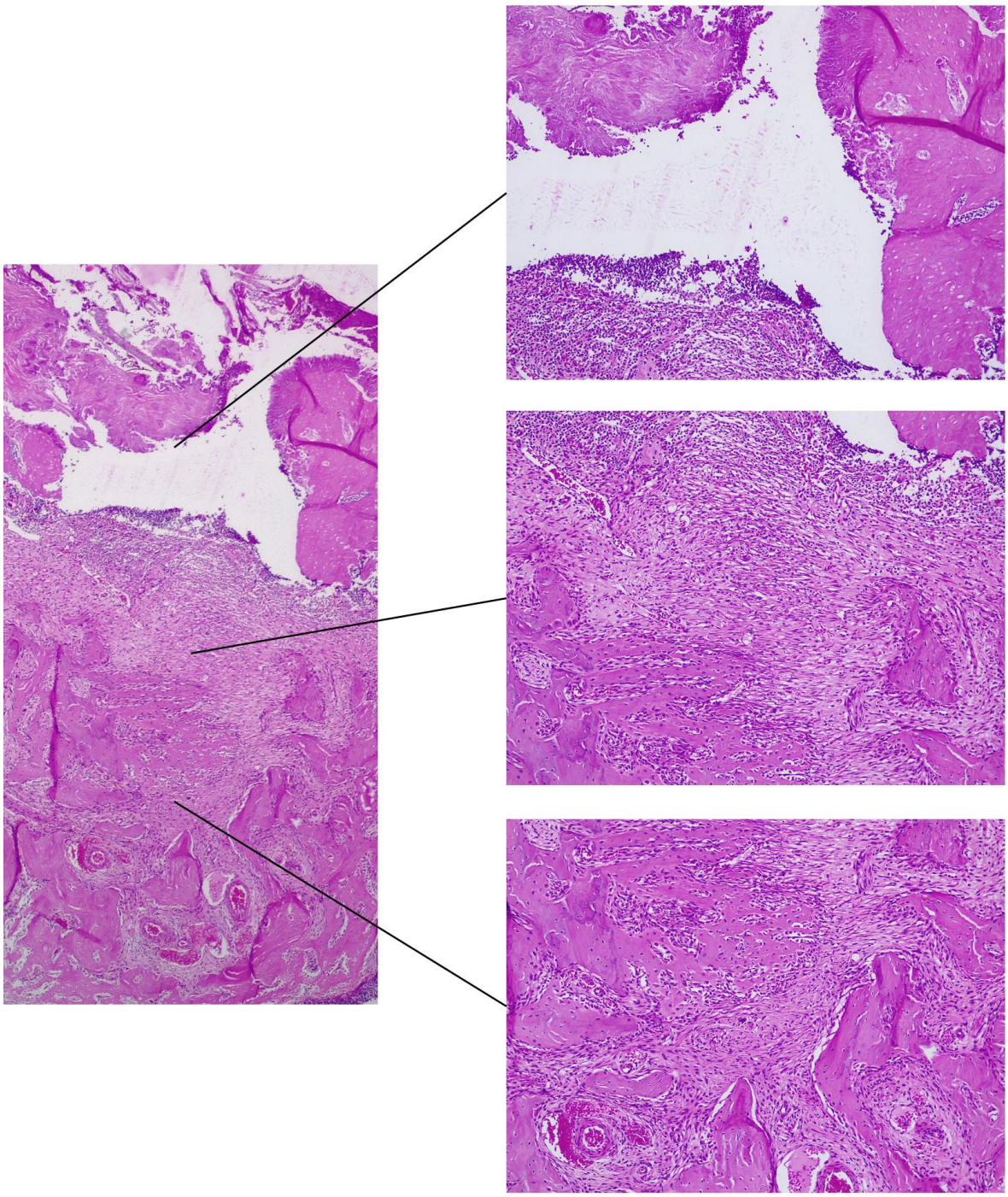

Figure 2. Representative histological images of the ZA group at 7 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

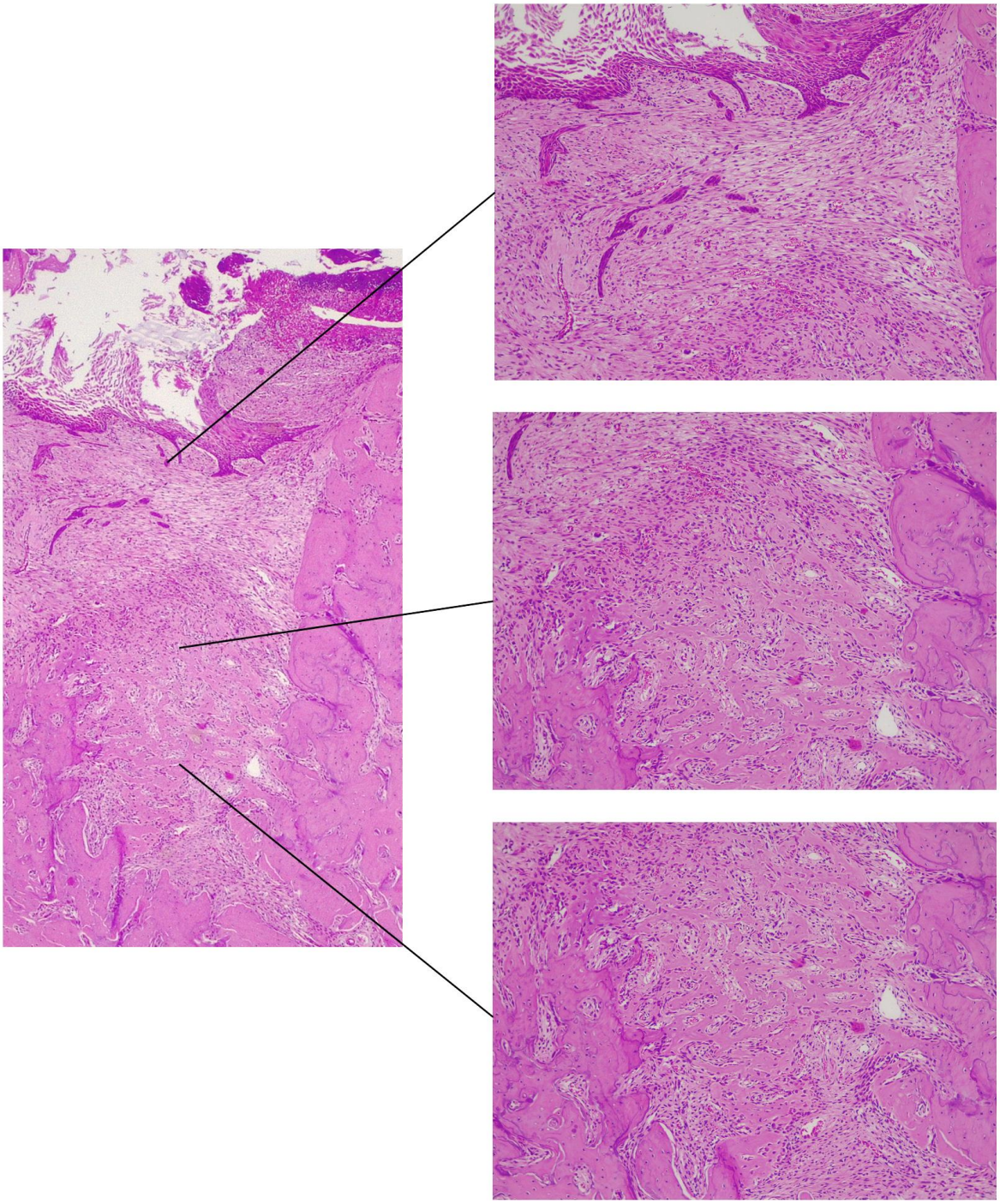

Figure 3. Representative histological images of the DOXI group at 7 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

At 14 days, the SAL group (Figure 4) showed greater bone neoformation compared to 7 days, which extended to the apical and middle thirds, with thicker bone trabeculae and lamellar organization. In the middle and cervical thirds, there was dense, organized connective tissue rich in collagen fibers, which filled the socket. There was a slight mononuclear inflammatory infiltrate in the cervical portion and the presence of blood vessels, covered by a thin layer of epithelium. The height of the alveolar ridges was reduced by the alveolar remodelling process, and some areas of resorption could be seen in the cervical third. In contrast, the ZA group (Figure 5) showed bone neoformation concentrated in the apical third of the alveolus, interspersed with dense connective tissue. It also shows regions of disorganized connective tissue in the middle third, with an intense mononuclear inflammatory infiltrate in the middle third that extends to the cervical third. The cervical third shows slightly disorganized and loose connective tissue, covered by a thin layer of epithelium. Although the connective tissue completely covers the alveolus, there are bone fragments without vitality from the alveolar crests. In the experimental group (DOXI) (Figure 6), there was well-organized neoformed bone tissue in the apical and middle thirds, with thick trabeculae and little distance between the trabeculae. Dense connective tissue, slight vascularization and a large number of collagen fibers extending to the cervical third, with no inflammatory infiltrate. A moderate layer of keratinized epithelial tissue was observed, occluding the entire surgical wound, and bone ridges undergoing bone remodelling.

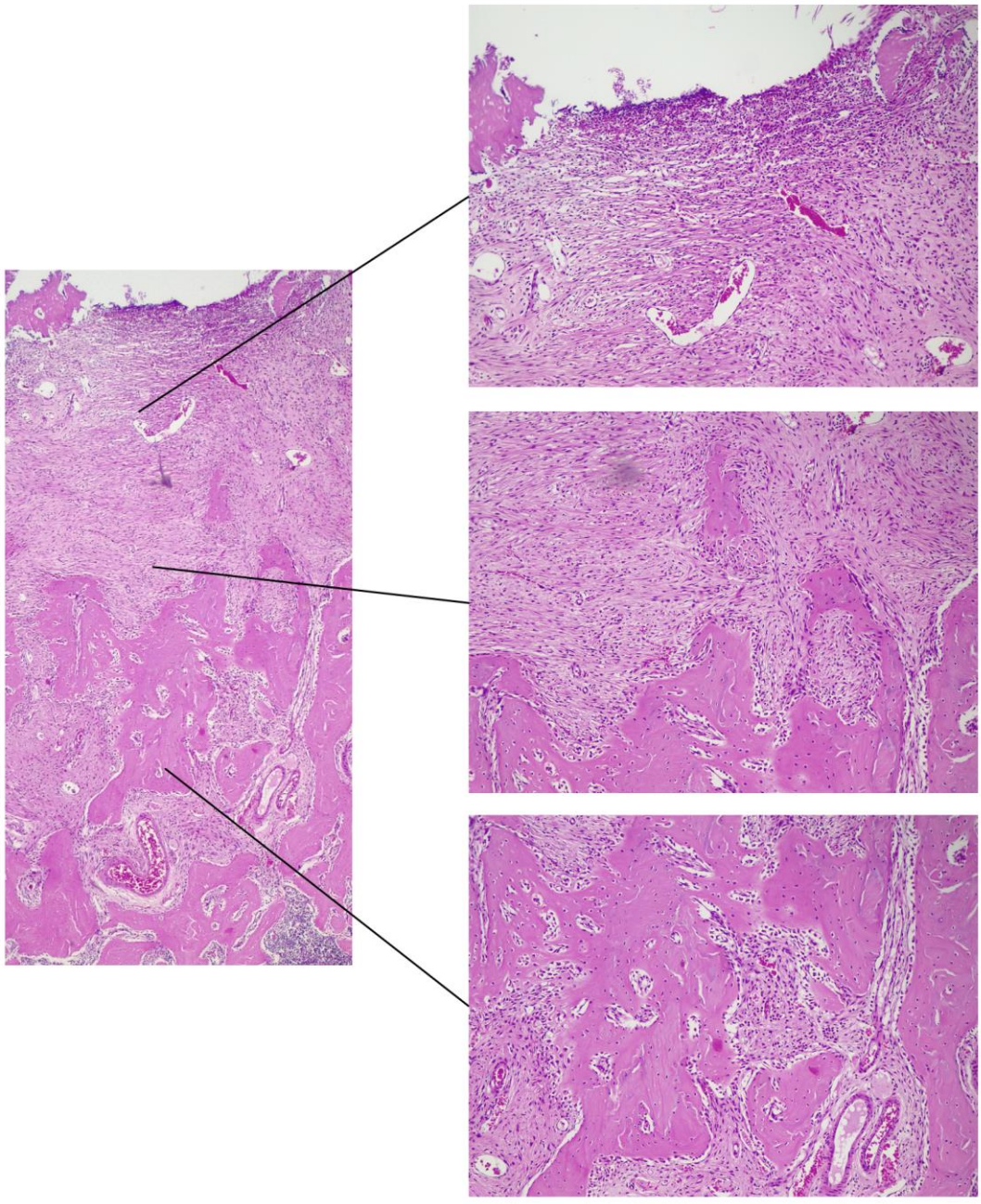

Figure 4. Representative histological images of the SAL group at 14 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

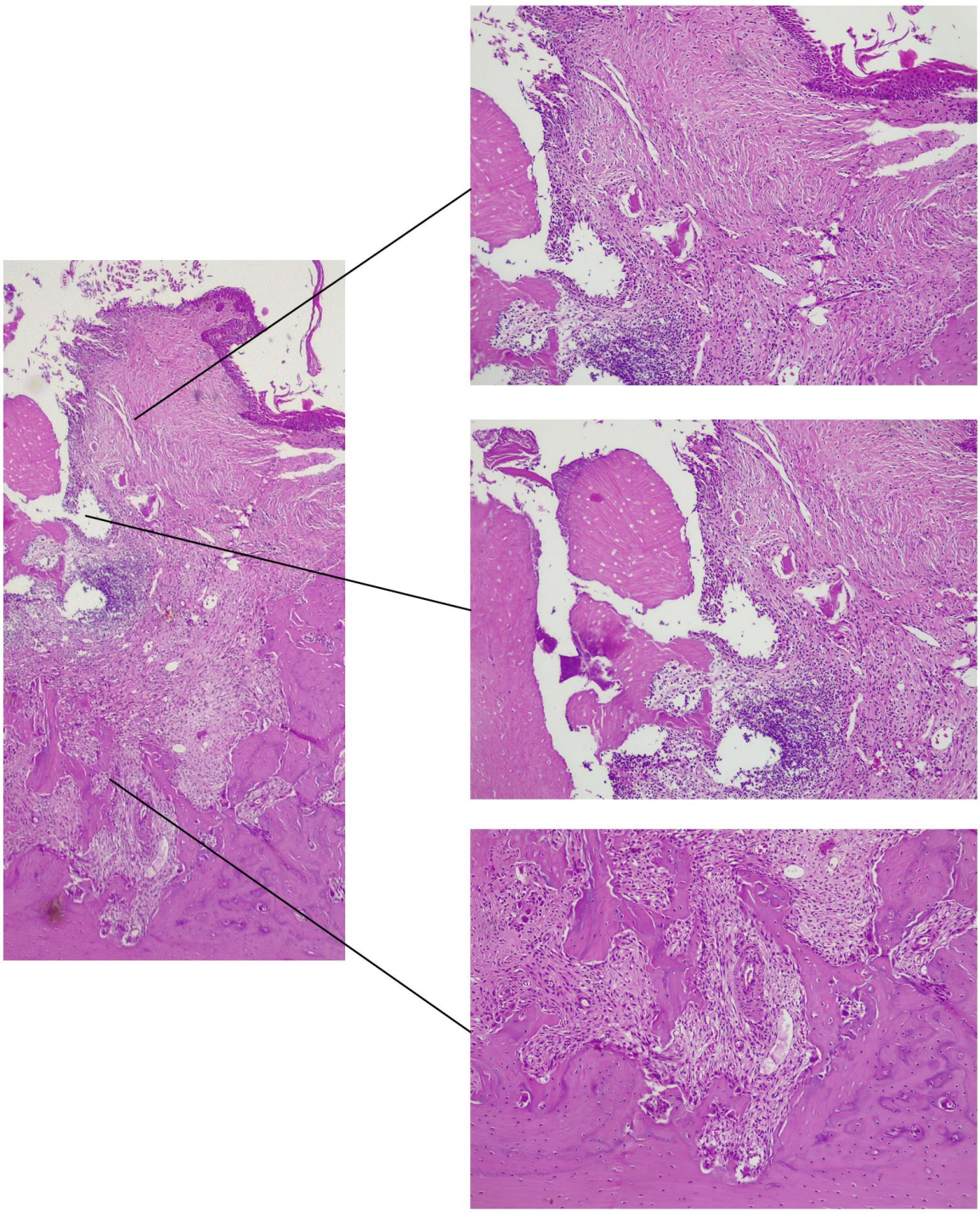

Figure 5. Representative histological images of the ZA group at 14 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

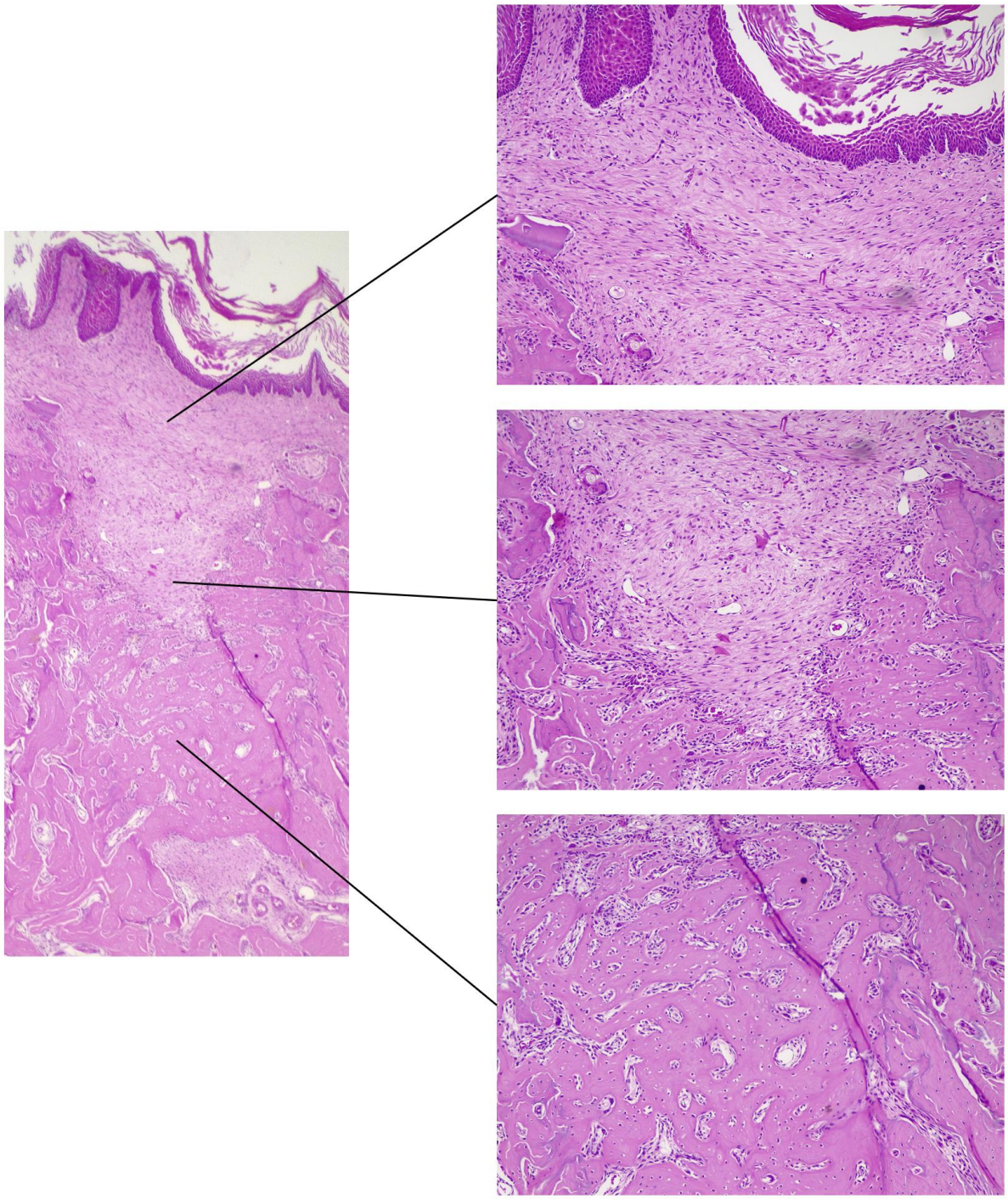

Figure 6. Representative histological images of the DOXI group at 14 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

Finally, at 28 days, the SAL group (Figure 7) had a completely repaired socket covered with a layer of keratinized epithelium. It was also observed that, although bone volume was not maintained in this group, the repair process occurred without delay, allowing the formation of bone tissue with thick and evenly distributed trabeculae. The ZA group (Figure 8), on the other hand, showed delayed bone repair, with a moderate to severe inflammatory infiltrate extending into the connective tissue and/or bone tissue, absence of epithelium covering the post-operative alveolus, and extremely disorganized connective tissue. The bone tissue, when present, was presented as loose fragments, characteristic of bone sequestration, with the absence of nuclei in the bone lacunae, as well as bone irregularities at the margins. In the DOXI group (Figure 9), the bone tissue formed in the apical and middle thirds was organized and mature, with the presence of very thick lamellae and trabeculae, with discrete spaces between the trabeculae. There was well-organized connective tissue in the cervical third with plenty of collagen fibers, no inflammatory infiltrate and a moderate layer of epithelial tissue completely covering the socket.

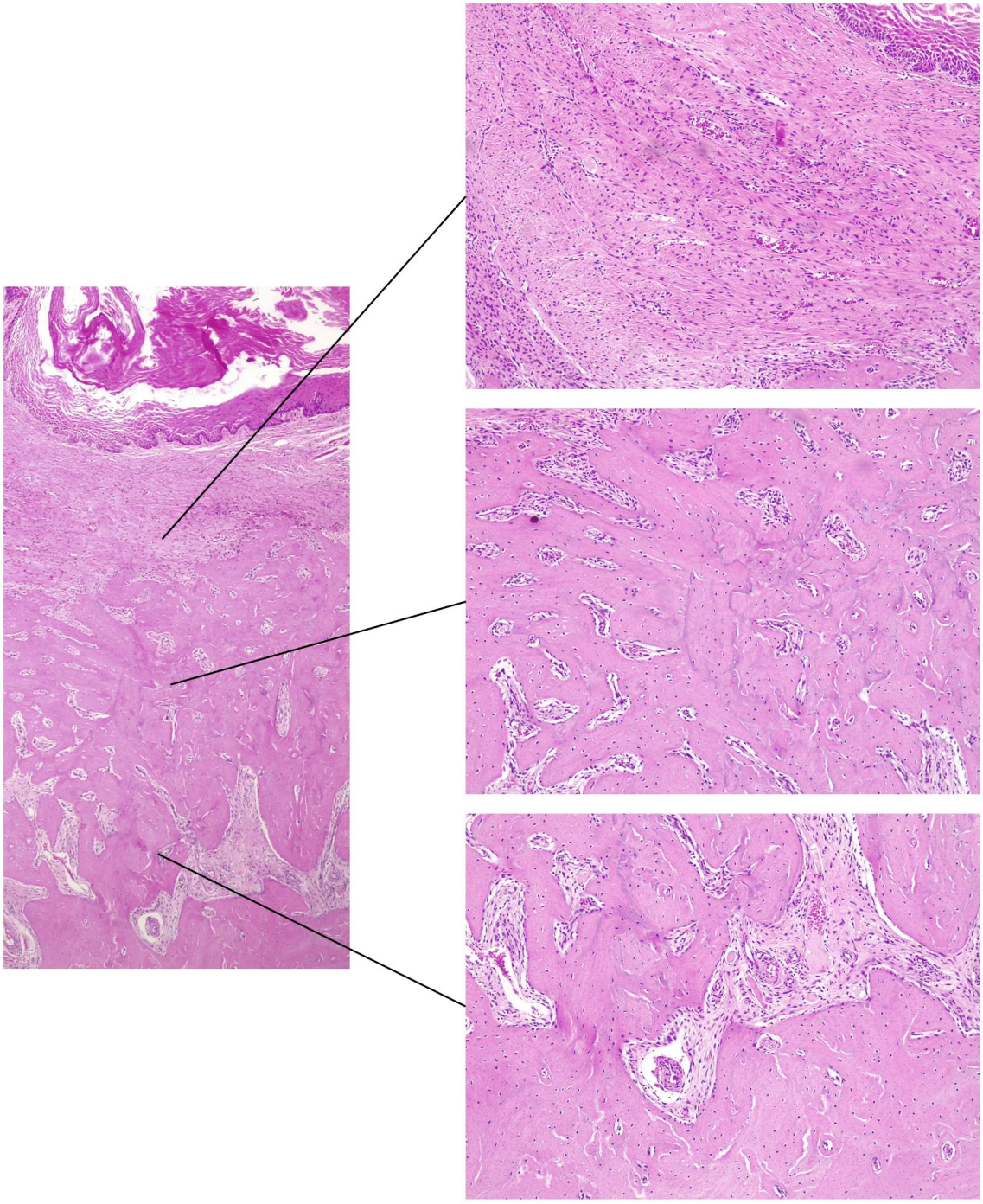

Figure 7. Representative histological images of the SAL group at 28 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

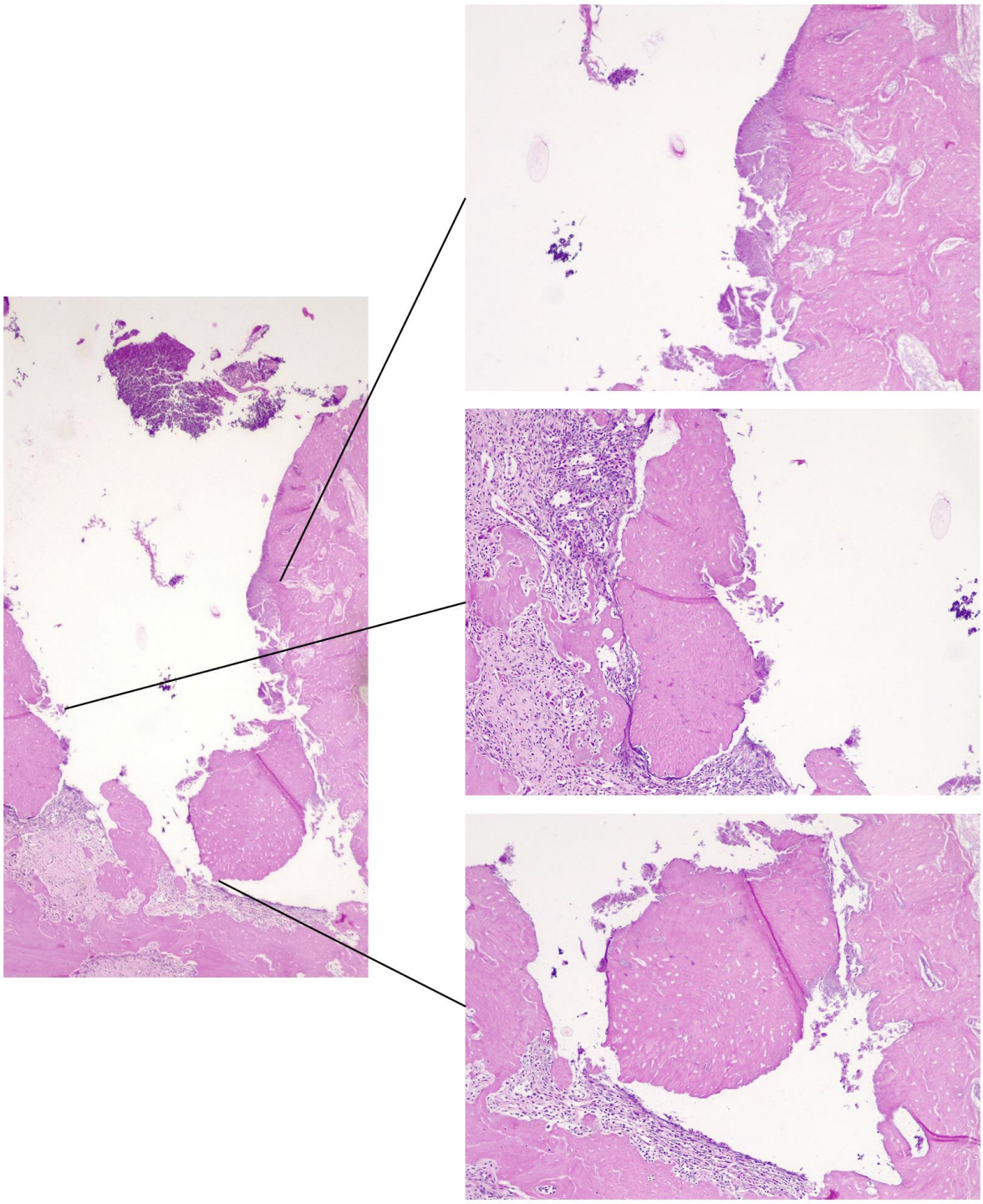

Figure 8. Representative histological images of the ZA group at 28 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

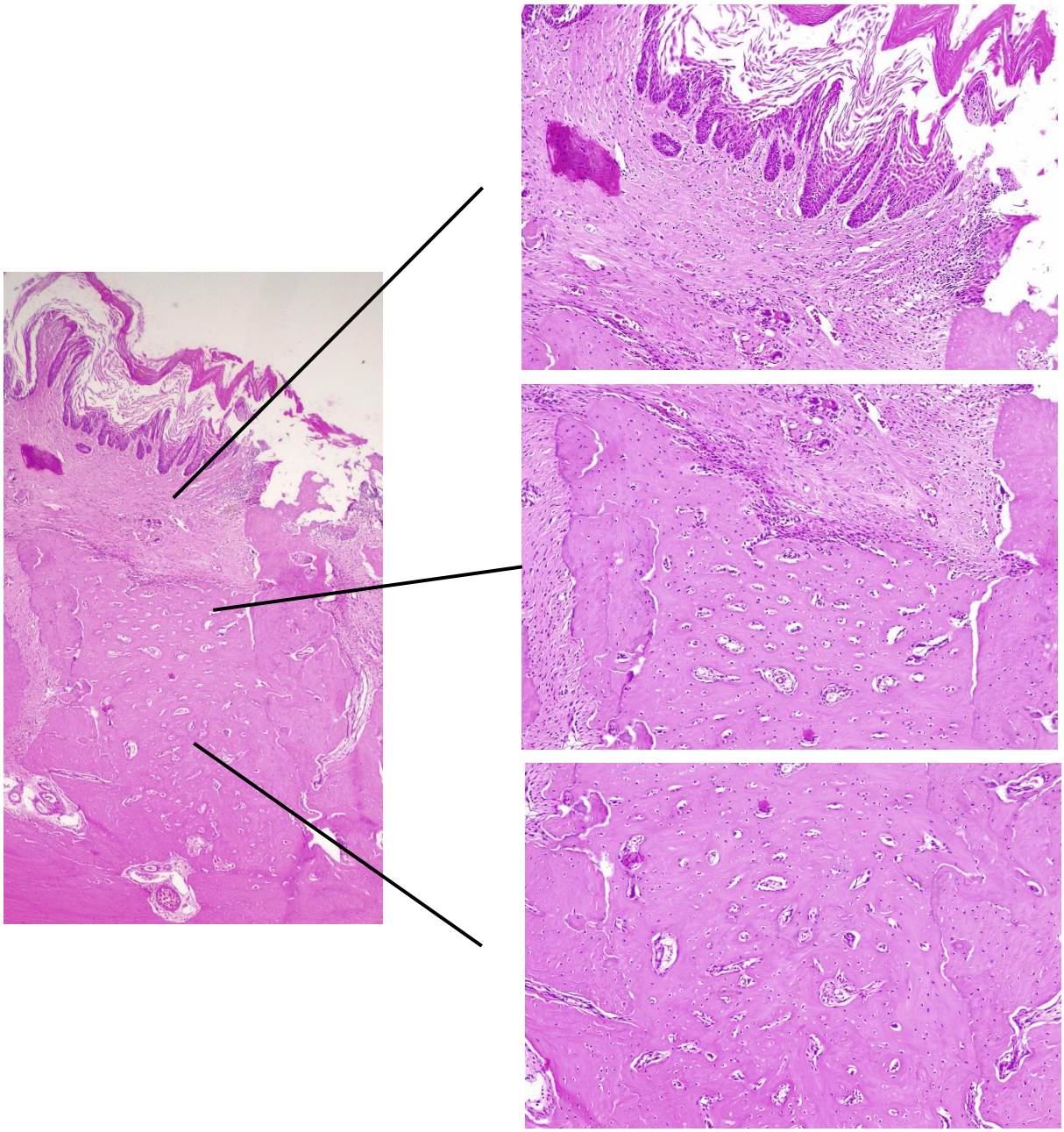

Figure 9. Representative histological images of the DOXI group at 28 days, stained in hematoxylin and eosin (HE). On the left side the complete image of the alveolus was obtained with a 2.5x objective, followed by the respective images of the cervical, middle, and apical thirds obtained in a 100x magnification.

The percentage of vital bone (%VB) was enhanced over time for all groups except by ZA in 28 days (Figure 10). At 7 days, group SAL and DOXI present similar amount %VB (34.01% ± 1.64% and 37.84% ± 4.01%, respectively, $p = 0.0059$), and ZA showed27.09% ± 9.75% ($p > 0.05$). At 14 days, there was a significant increase in the %VB of DOXI group (60.12% ± 3.43%) when compared to SAL (36.29% ± 4.78%, $p < 0.0001$) and ZA (30.01% ± 6.85%, $p < 0.0001$). Also, at 28 days, DOXI remained as highest %VB (65.83% ± 2.81%), when compared to SAL (40.48% ± 2.56%, $p < 0.0001$) and ZA (18.92% ± 5.22%, $p < 0.0001$).

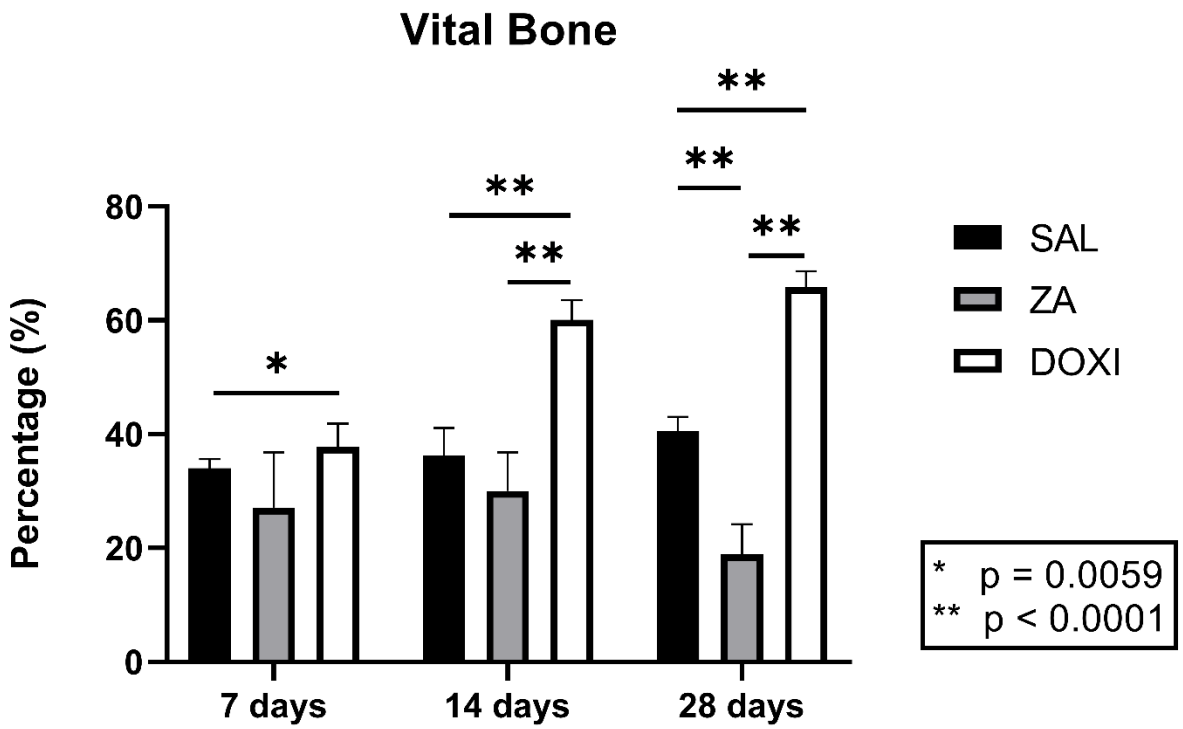

Figure 10. Representative graph of the percentage of vital bone area for each group in the periods of 7, 14, and 28 days.

Meanwhile, the percentage of non-vital bone (%N-VB) was indeed notable in the ZA group (Figure 11). At 7 days, only SAL and ZA presented %N-VB (1.16% ± 0.67% and 2.59% ± 1.56%, respectively, $p > 0.05$). At 14 and 28 days, %N-VB was noted only in ZA group (4.29% ± 2.11% and 44.17% ± 10.93%, respectively). However only at 28 days it was observed a statistical difference between ZA and SAL, and ZA and DOXI (both, $p < 0.0001$).

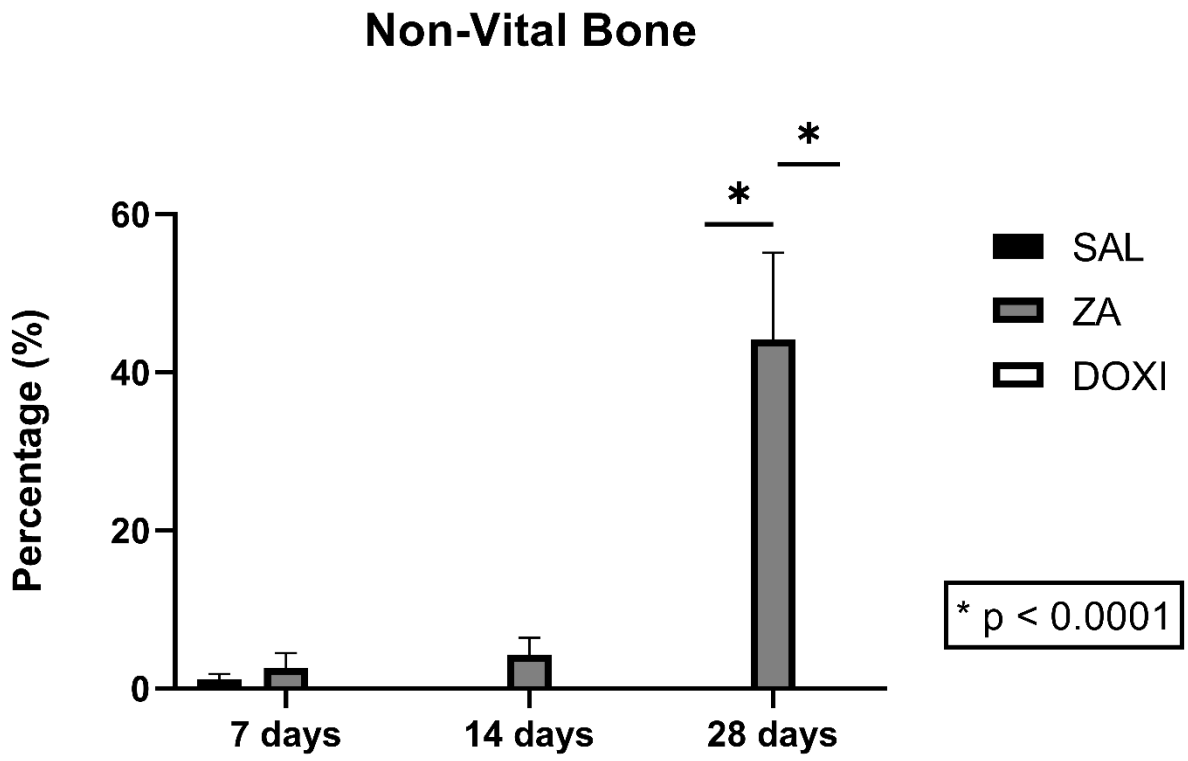

Figure 11. Representative graph of the percentage of non- vital bone area for each group in the periods of 7, 14, and 28 days.

At 7 days, the highest percentage of connective tissue (%CT) was found in the ZA group (64.76% ± 9.29%) followed by SAL and DOXI (59.89% ± 3.15% and 55.95% ± 6.35%, respectively, $p > 0.05$) (Figure 12). At 14 days, DOXI demonstrates the lowest %CT (35.71% ± 3.76) when compared to SAL (55.12% ± 4.23%, $p < 0.0001$) and ZA (59.54% ± 5.36, $p < 0.0001$). At 28 days, SAL presents the highest amount of lowest %CT (54.68% ± 2.46%) when compared to ZA and DOXI (29.24% ± 11.43% and 30.03% ± 2.35%, respectively, $p < 0.0001$).

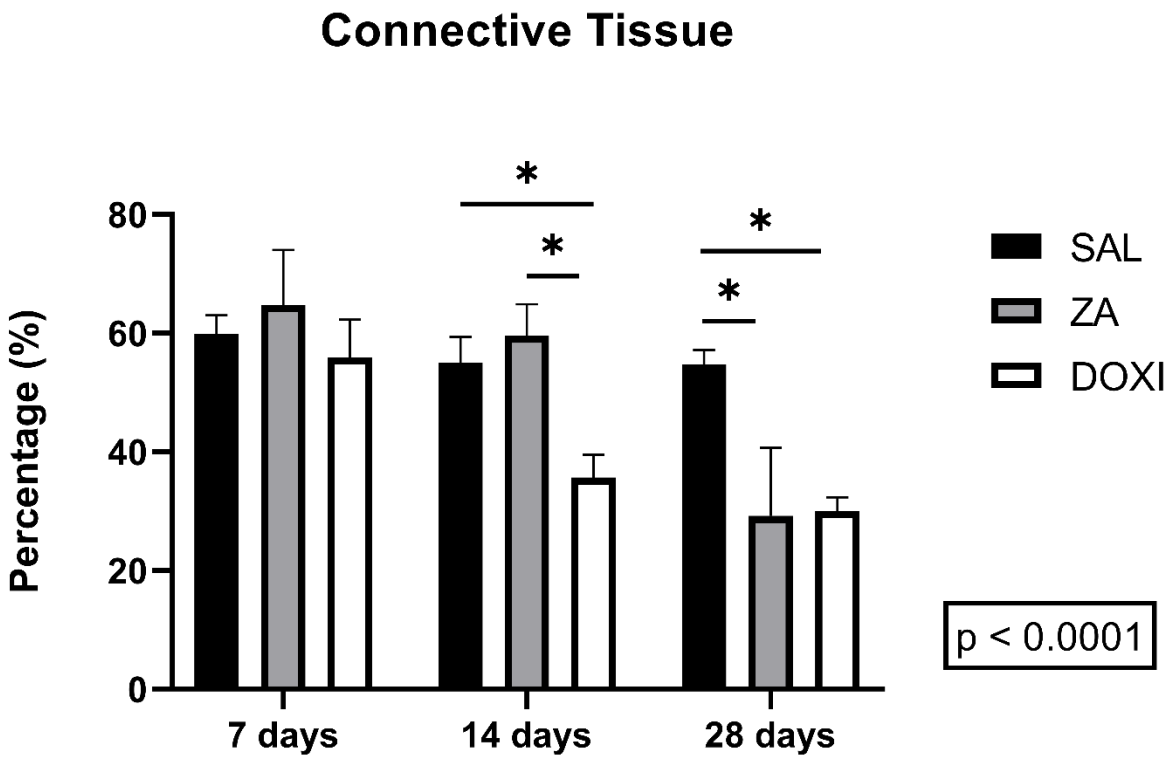

Figure 12. Representative graph of the percentage of connective tissue for each group in the periods of 7, 14, and 28 days.

Finally, the percentage of vessels (%VS) was also quantified (Figure 13). Data demonstrated that at 7 days, group SAL present highest %VS (7.90% ± 2.30%), however without statistical differences between ZA (6.28% ± 2.01%) and DOXI (7.01% ± 1.48%). At 14 days, a slight reduction in the vascularization was noted for all groups (5.93% ± 2.02%, 5.54% ± 1.15%, 4.16% ± 0.40% for SAL, ZA, and DOXI respectively). At 28 days, %VS in the ZA group was considerably reduced (1.16% ± 0.16) when compared to SAL (4.83% ± 1.37%, p = 0.0016) and DOXI (4.60% ±0.89%, p = 0.0030).

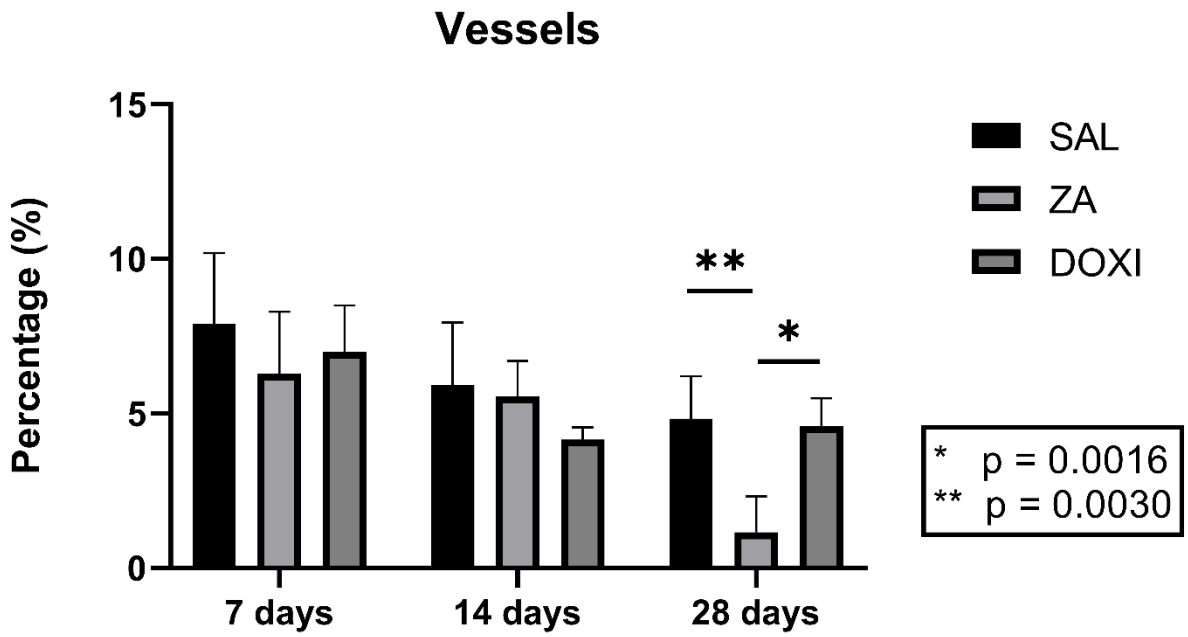

Figure 13. Representative graph of the percentage of vessels for each group in the periods of 7, 14, and 28 days.

Immunohistochemical analysis

The scores applied for each marker according to the group and period were summarized in Table 1. OCN is a marker of bone mineralization and the markings observed in the cytoplasm of cells of the osteoblastic lineage were considered positive, as were the markings present in the non-mineralized and mineralized extracellular matrix, the latter representing the neoformed bone tissue in the region of interest. TRAP is a marker of osteoclast activity, and this enzyme is present in the brush border membrane of osteoclasts in the resorptive space.

Table 1. Scores of OCN and TRAP immunolabelling according to each group and period of analysis.

	SAL			ZA			DOXI		
	7 days	14 days	28 days	7 days	14 days	28 days	7 days	14 days	28 days
OCN	+++/+++	+++/+++	+/+++	+/+++	++/+++	+/+++	++/+++	++/+++	++/+++
TRAP	++/+++	++/+++	+/+++	+/+++	+/+++	+/+++	+/+++	+/+++	++/+++

In this Table: +/+++ means mild labelling, ++/+++ means moderate labelling, and +++/+++ means intense labelling.

In the SAL group (Figure 14), at 7 days, OCN was expressed moderately to intensely in cells and in the non-mineralized extracellular matrix. Incipient areas of bone tissue in formation were also observed, with positive markings for osteocalcin. At 14 days, moderate to intense marking was observed in cells, non-mineralized extracellular matrix and mineralized extracellular matrix in the newly formed bone tissue. At 28 days, there was a slight positive marking for cells and non-mineralized and mineralized extracellular matrix. In this group, TRAP showed moderate labelling at 7 days, mild to moderate at 14 days and mild at 28 days.

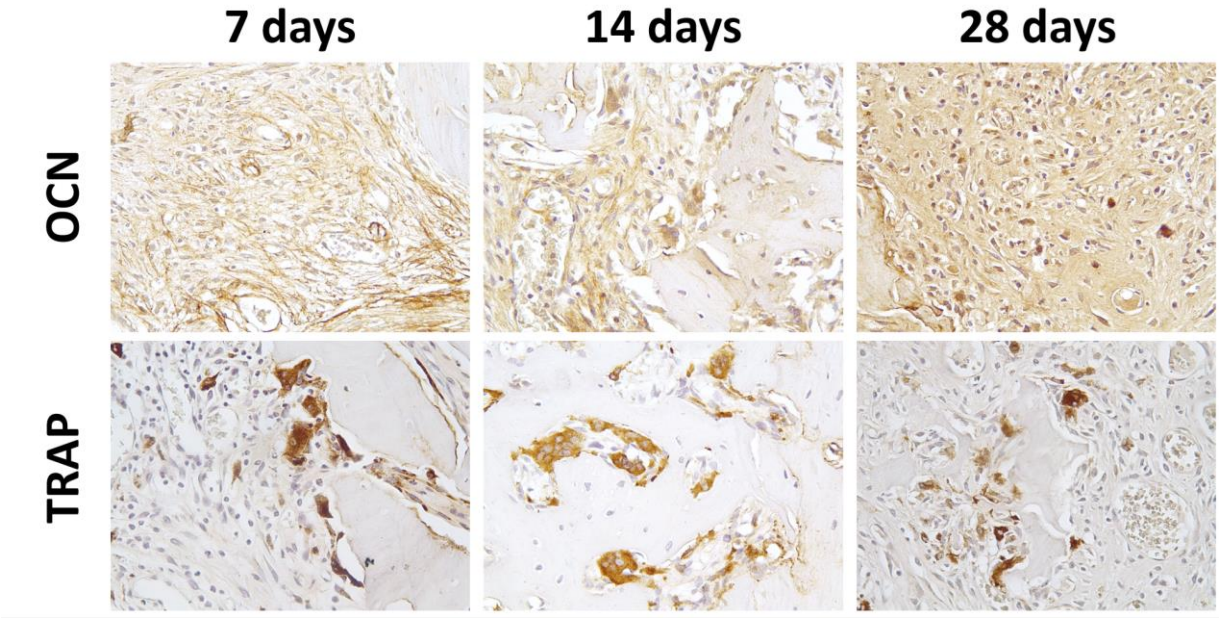

Figure 14. Representative images of the immunolabelling for OCN and TRAP of the SAL group, at 7, 14 and 28 days, in a magnification of 400x.

In the ZA group (Figure 15), mild OCN labelling was observed in cells present in the region of interest at 7 days. At 14 days, moderate marking was observed in cells and in the non-mineralized and mineralized extracellular matrix in the region of interest. At 28 days, mild labelling was observed in the region of interest. However, TRAP labelling was mild at all periods.

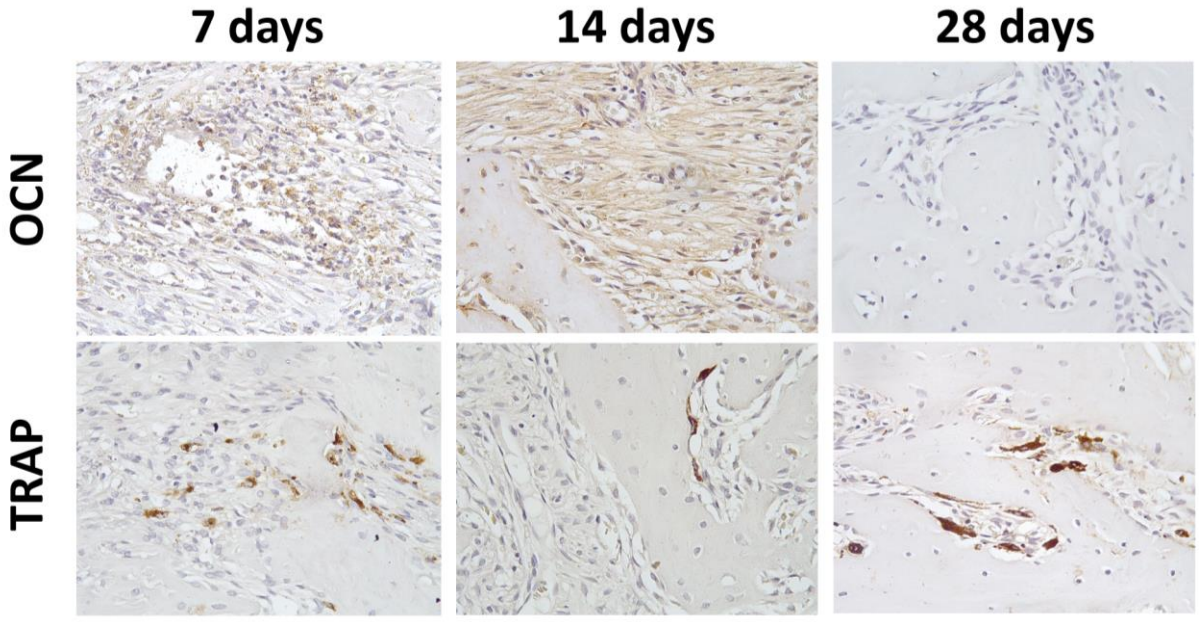

Figure 15. Representative images of the immunolabelling for OCN and TRAP of the ZA group, at 7, 14 and 28 days, in a magnification of 400x.

In the DOXI group (Figure 16), at 7 days OCN showed moderate marking in cells and in the non-mineralized extracellular matrix. There were also incipient areas of bone tissue in formation, positive for osteocalcin. Similarly, at 14 days there was moderate to intense staining in cells and in the non-mineralized and mineralized extracellular matrix. At 28 days, moderate expression in cells and in the non-mineralized extracellular matrix. In this group, TRAP expression was mild at 7 days, mild to moderate at 14 days and mild at 28 days.

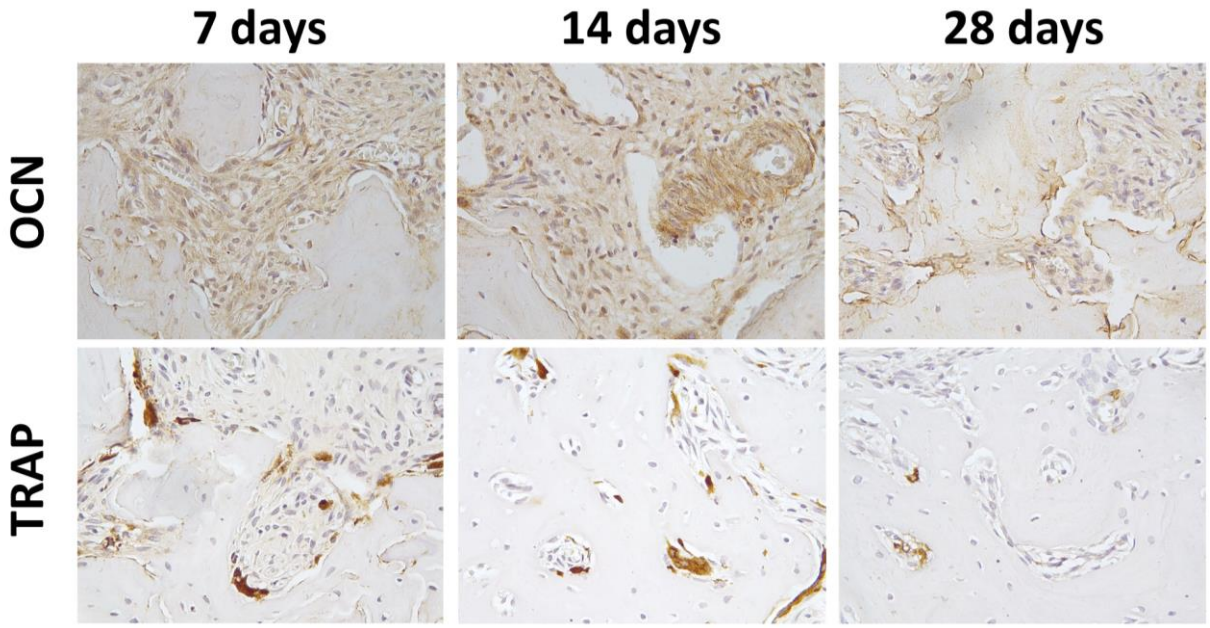

Figure 16. Representative images of the immunolabelling for OCN and TRAP of the DOXI group, at 7, 14 and 28 days, in a magnification of 400x.

Picrosirius Red Staining and Collagen maturity

The distribution and organization of the fibers can be observed in Figure 17, which enables comparisons to be made between the groups and in relation to the periods. Greenish-yellow fibers are related to immature and thin fibers while yellowish-red fibers are associated with mature and thick fibers.

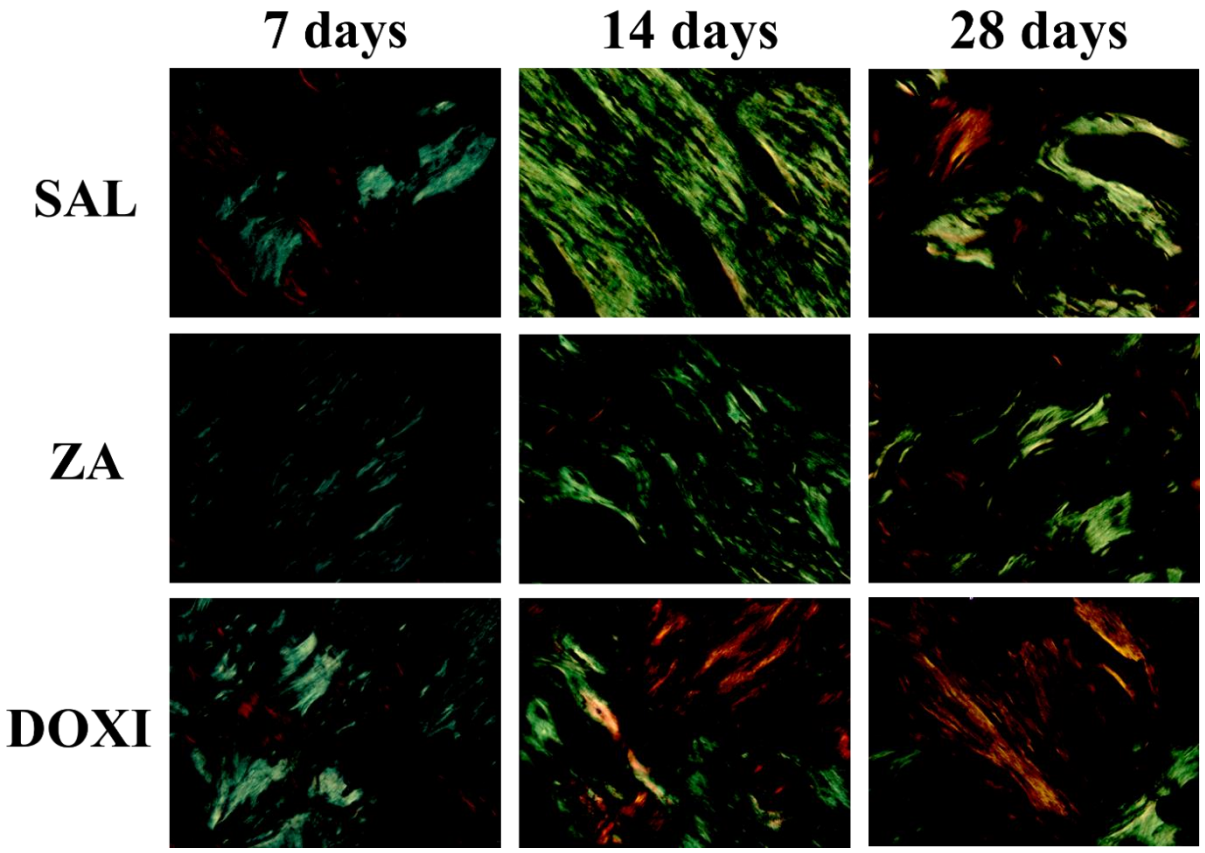

Figure 17. Representative images obtained in a polarized microscope evidencing immature fibers in greenish-yellow and mature fibers in yellowish-red for each group in the periods of 7, 14, and 28 days, in a magnification of 400x.

The percentage of fiber area in the greenish-yellow colour (Figure 18) at 7 days for SAL was 3.90% ± 1.3%, while ZA was 2.51% ± 1.06% and DOXI 4.95% ± 1.90%, but there was no difference between the groups. At 14 days, ZA showed the lowest number of fibers (3.50% ± 0.46%) when compared to SAL (12.69% ± 3.59%, $p < 0.0001$) and DOXI (11.19% ± 2.27%, $p = 0.0001$). At 28 days, DOXI had the highest percentage of fibers (16.16% ± 4.93%) compared to SAL (8.43% ± 2.80%, $p = 0.0001$) and ZA (4.77% ± 2.34%, $p < 0.0001$).

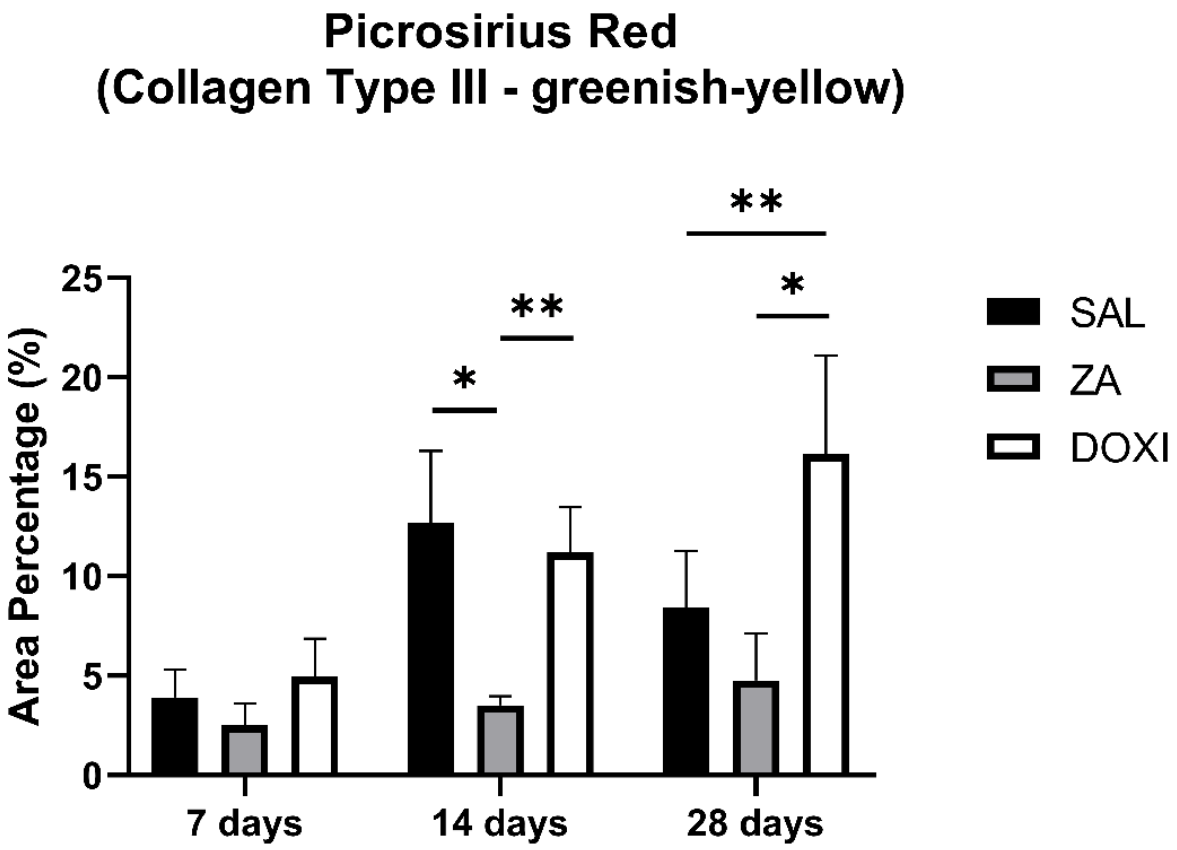

Figure 18. Representative graph of the percentage area of greenish-yellow fibers for each group in the periods of 7, 14, and 28 days. * means $p < 0.0001$, and ** means $p = 0.0001$.

The highest percentage of fiber area in the yellowish-red colour (Figure 19) at 7 days were noted in SAL (2.10% ± 0.25%), while ZA presented 0.35 ± 0.25, and DOXI 1.72% ± 0.55%, however without statistical differences. At 14 days, SAL presented 4.50% ± 2.1% of fibers however differences were noticed just when DOXI (8.29% ± 3.69%) and ZA (0.88% ± 0.59%) when compared each other ($p = 0.0027$). Finally, at 28 days, DOXI had the highest percentage of fibers (21.06% ± 8.48%) compared to SAL (3.24% ± 1.58%, $p < 0.0001$) and ZA (2.03% ± 1.47%, $p < 0.0001$).

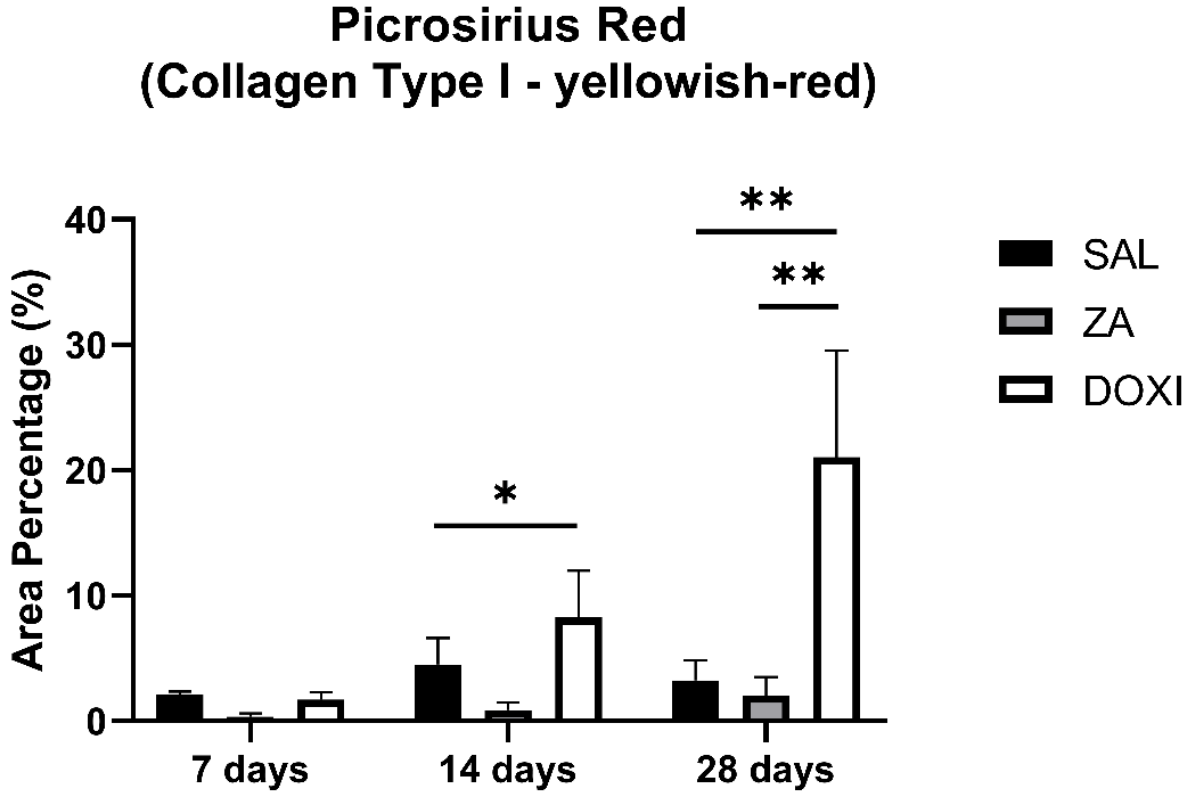

Figure 19. Representative graph of the percentage area of yellowish-red fibers for each group in the periods of 7, 14, and 28 days. * means $p = 0.0027$, and ** means $p < 0.0001$.

4.5 DISCUSSION

This study demonstrates the effectiveness of 10% doxycycline gel to prevent MRONJ in a rodent model treated with zoledronic acid followed by tooth extraction (Figure 9 and 10). From the earliest period, vascularization (Figure 12) and connective tissue (Figure 13) organization were maintained, leading to bone repair at 28 days. Although the amount of bone in the DOXI group was greater than in the SAL group, it was observed that DOXI showed the same repair pattern as SAL, such as - mature and lamellar bone, epithelium present, presence of collagen fibers and absence of inflammation. This study also demonstrated and characterized the MRONJ through the proposed experimental model in group ZA (through acid zoledronic administration

followed by tooth extraction), characterized by a delayed in the healing, irregularity in bone contour, low vascularization, empty lacunes, inflammatory infiltrate and bone sequestration.

Another interesting fact observed in this study refers to the amount of collagen fibers in the DOXI group, which was maintained during the periods of 7- and 14-day at the same level as the SAL. In the 28-day period, DOXI showed a greater amount when compared to SAL and ZA, regardless of whether greenish-yellow or yellowish-red fibers ($p < 0.0001$). It is already known that zoledronic acid can affect the amount and disposition of collagen fibers (Chang et al. 2021). It is supposed that since doxycycline has anticollagenase action (Bonnetblanc 2002), this factor helped to control the degradation of the fibers against the toxic effect of zoledronic acid, as well as allowing the collagen maturation from the 14 days onwards (Figure 17). The presence of collagen fibers is an important factor during bone repair to guarantee bone healing and strength (Nair et al. 2022). Also, disruption or alteration in collagen fibers are related to bone fragility and are observed in many diseases (Zieba et al. 2020).

Since BP's, such as zoledronic acid, are directly related to the suppression of osteoclastic activity (Russell et al. 2008) it is expected that the reaction of TRAP in immunolabeling would be reduced, as demonstrated in this study (Table 1), in which ZA group demonstrated mild labelling over all periods analyzed. However, while SAL presented moderate immunolabeling at 7 and 14 days, DOXI showed mild activity, which can be explained by the fact that doxycycline also has an anticlastogenic effect (Lucateli et al. 2018), thereby reducing TRAP immunolabeling. In contrast, OCN is a marker of osteoblastic activity during bone formation and previous studies have shown that the use of zoledronic acid impacts the differentiation of osteoblasts and interferes with its mineralization phase (Hadad et al., 2022). In this study, the ZA group demonstrated mild immunolabeling, while SAL and DOXI evidenced intense and moderate immunolabeling, respectively (Table 1).

Although the benefit of antiresorptive medications in controlling diseases such as osteoporosis, and hypercalcemia, or even in preventing bone metastases is unquestionable (Duran et al. 2017; Van Poznak et al. 2017; Yu et al. 2020) the occurrence of MRONJ affects patients' quality of life, albeit with a low incidence (0.01 - 5%) (Khan et al. 2015; Coropciuc et al. 2023). Thus, the literature is full of studies that try to somehow modulate the deleterious effects that

these medications have on bone, thus preventing the development of MRONJ (Ervolino et al. 2019; Sharma et al. 2021; Hadad et al. 2022).

In this context, searching for new options, recent results have shown that the application of a sponge with doxycycline assisted to reduce non-vital bone in animals treated with zoledronic acid (Capar et al. 2016). In addition, studies show that doxycycline can induce bone repair, or even accelerate it (Limirio et al. 2016; Gomes et al. 2017; Lucateli et al. 2018). The application of doxycycline in gel inside a freshly operated alveolus had not been evaluated until now, considering the osteonecrosis model. The data obtained in this study strongly suggest benefits and advantages in using this drug. Doxycycline contributed to the maintenance and organization of the connective tissue, vascularization, and bone neoformation throughout the period evaluated (7, 14, and 28 days), enabling bone repair without bone exposure or signs of necrosis (Figures 9, 10, and 11).

It should also be noted that another factor that may have contributed to the results of this study was the antimicrobial control caused by doxycycline (Warner et al. 2022). Although this aspect was not explored in this study, the literature shows that the presence of local infection can play a fundamental role in the progression of MRONJ (He et al. 2020; Otto et al. 2021). There are studies linking the presence of bacteria and MRONJ, mainly *Actnomyces* which are regularly found in exposed bones (Naik and Russo 2009). The presence of previous diseases, such as periodontitis, at the site to be operated on, also contributes to the progression of MRONJ, since the bacteria present in periodontitis disease are also found in MRONJ (Panya et al. 2017), such as *Porphyromonas, Lactobacillus, Tannerella, Prevotella, Actinomyces, Treponema, Streptococcus,* and *Fusobacterium*.

The search for new therapies is prompting new methodologies, drugs and studies that can be applied to prevent osteonecrosis. The use of doxycycline in the form of a gel is easy to apply clinically, low cost and provides excellent results. New studies need to be carried out in order to answer existing gaps, such as on the microbiological control of doxycycline in this osteonecrosis model, and modulations of the osteogenesis and angiogenesis pathways. It is also important to apply this methodology in scenarios that mimic clinical problems, such as patients with periodontal disease or metabolic alterations such as diabetes.

4.6 CONCLUSION

The data showed that the gel of doxycycline 10% was effective to prevent MRONJ occurrence, leading to a healed socked with vital mature bone.

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