

Juliana Simeão Borges

Efeitos da radiação ionizante no reparo ósseo alveolar e dos tecidos periodontais associada a doença periodontal: estudos pré-clínicos in vivo

Effects of ionizing radiation on alveolar bone repair and periodontal tissues associated with periodontal disease: preclinical in vivo studies

Tese apresentada à Faculdade de Odontologia da Universidade Federal de Uberlândia, como requisito parcial para obtenção do Título de Doutor em Odontologia na Área de Concentração de Clínica Odontológica Integrada.

Uberlândia, 2023

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Uberlândia, 2023



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***“A persistência é o caminho do êxito.”
(Charles Chaplin)***

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RESUMO

A radioterapia (RT) é um dos tratamentos para pacientes com câncer de cabeça e pescoço. No entanto, efeitos adversos são observados nos tecidos ósseo e periodontal. A extração dentária é considerada um fator de risco para osteorradionecrose e pode resultar em cicatrização tecidual retardada e doença periodontal tende a piorar com o tratamento oncológico. Estudos em modelo animal associando metodologias como a microtomografia computadorizada (micro-CT) vem crescendo e podem contribuir para elucidar os processos que ocorrem nos tecidos ósseo e periodontal em condição irradiada. No entanto, questões relacionadas a sua aplicação e a definições de parâmetros necessitam de maiores investigações. Esse estudo foi dividido em 3 objetivos específicos; *objetivo específico 1*: avaliar, por micro-CT, a influência do alvéolo de extração (raiz distal ou lingual) e o tipo de definição da região de interesse (ROI) (manual ou pré-definida) na avaliação do reparo alveolar após extração dentária. O estudo demonstrou que o alvéolo e o tipo de ROI selecionado podem influenciar nos resultados morfométricos; *objetivo específico 2*, a aplicação da microCT em associação a outras metodologias, foi utilizada para investigar os efeitos dose-resposta da radiação ionizante no reparo ósseo alveolar e na resistência óssea após exodontia. O estudo demonstrou por meio da análise microtomográfica e histomorfométrica, que a RT afeta o reparo alveolar e que doses de 30Gy são recomendadas em estudo em ratos; *objetivo específico 3*, aplicação da micro-CT, em associação a outras metodologias, foi utilizada para avaliar os efeitos da radiação ionizante no reparo periodontal em modelo de rato com periodontite induzida por ligadura. A RT modulou a extensão da perda óssea alveolar na periodontite induzida em ratos e a longo prazo níveis aumentados de reabsorção óssea podem ser observados mesmo após o tratamento da doença periodontal.

PALAVRAS-CHAVE: Reparo alveolar; Doença Periodontal; Microtomografia por Raio-X;

ABSTRACT

Radiotherapy (RT) is one of the treatments for patients with head and neck cancer. However, adverse effects are observed in bone and periodontal tissue. Tooth extraction is considered a risk factor for osteoradionecrosis and can result in delayed tissue healing. In addition, periodontal disease tends to worsen with cancer treatment. Animal model studies associating methodologies such as computed microtomography (micro-CT) have been growing and may contribute to elucidate the processes that occur in bone and periodontal tissue in the irradiated condition. However, questions related to its application and parameter definitions need further investigation. The objective 1 of this study was to evaluate the influence of the extraction socket (distal or lingual root) and the type of region of interest (ROI) definition (manual or predefined) on the assessment of alveolar repair following tooth extraction using micro-CT. The study demonstrated that the socket and the type of ROI selected can significantly influence the morphometric results and should be well established in the study. In objective 2, the application of micro-CT in association with other methodologies, was used to investigate dose-response effects of ionizing radiation on alveolar bone repair and bone strength after tooth extraction. The study demonstrated, through microtomographic and histomorphometric analysis, that RT affects alveolar bone repair and that doses of 30Gy are recommended in study models in rats. Finally, in objective 3, the application of micro-CT, in association with other methodologies, was used to assess the effects of ionizing radiation on periodontal repair after treatment of experimental periodontitis in rats. Ionizing radiation modulated the extension of alveolar bone loss. In the long term (20d), RT increased alveolar bone loss in an experimental periodontitis rat model, even after treatment of periodontal disease.

KEYWORDS: Alveolar repair; Periodontal disease; X-Ray Microtomography

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

A radioterapia (RT) é uma das modalidades terapêuticas para tratamento do câncer em região de cabeça e pescoço, consistindo na utilização de radiação ionizante sobre a região do tumor, sendo também indicada em associação com a quimioterapia ou como tratamento paliativo para tumores em estágio avançado (Schiegnitz *et al.*, 2014). No entanto, a impossibilidade de isolamento da massa tumoral durante a aplicação da RT, faz com que a toxicidade produzida pela radiação afete não só as células tumorais, como também os tecidos saudáveis, acarretando em efeitos deletérios indesejáveis (Khan., 2003; Schuurhuis *et al.*, 2018).

O manejo de pacientes irradiados representa um desafio para os profissionais de saúde, inclusive na área odontológica. Vários efeitos secundários relacionados à RT podem ocorrer na cavidade oral, incluindo perda progressiva de inserção periodontal, desenvolvimento de cárie, comprometimento do reparo ósseo e osteorradionecrose (ORN) (Schuurhuis *et al.*, 2018). Esses efeitos orais afetam negativamente a qualidade de vida e devem ser consideradas antes, durante e após a RT (Köse *et al.*, 2017; Rodrigues *et al.*, 2018; Schuurhuis *et al.*, 2018; Irie *et al.*, 2018).

O tempo entre a primeira sessão de RT e o início das alterações, bem como a severidade dessas, parece ser dose-dependente. Os efeitos deletérios da radiação ionizante (IR) no osso podem afetar o volume, a composição e a microarquitetura óssea, levando a uma maior predisposição a fraturas devido à diminuição da densidade óssea (Tchanque-Fossuo *et al.*, 2011; Soares *et al.*, 2019; Mendes *et al.*, 2020; Soares *et al.*, 2020; Borges *et al.*, 2021), resultando em modificações da rede de canais ósseos (Rabelo *et al.*, 2010; Borges *et al.*, 2021). Os componentes da matriz são danificados (Bartlow *et al.*, 2018), diminuindo a vascularização e reduzindo o número e a atividade das células osteoblásticas, afetando a neoformação de tecido ósseo (Sønstevoid *et al.*, 2015; Mendes *et al.*, 2020),

A extração dentária durante o período pós-RT é considerada um fator de risco para ORN e pode resultar em cicatrização tecidual retardada (Wang *et al.*, 2017); portanto, a indicação antes da terapia de RT é preferível (Köse *et al.*, 2017; Irie

et al., 2018). Além disso, pacientes irradiados na região de cabeça e pescoço apresentam risco aumentado para doença periodontal, pois geralmente está associada à hipossalivação e modificação do microbioma oral. Além disso, a periodontite em adultos tende a piorar com o tratamento oncológico (Sroussi *et al.*, 2017). O efeito da radiação na saúde periodontal é dose-dependente e está associado a má saúde periodontal antes do início da radioterapia (Köse *et al.*, 2017; Irie *et al.*, 2018).

O uso de modelo animal pode elucidar pontos importantes no processo de reparo dos tecidos frente a uma condição irradiada. Com isso, estudos pré-clínicos permitem observar diversos fatores a serem investigados, além de permitir análises que não são possíveis em ensaios clínicos (Higham & Faithfull., 2015).

1.1. Processo de reparo ósseo alveolar

O tecido ósseo, é uma forma especializada de tecido conjuntivo constituído por uma fase mineral ou inorgânica relacionada à uma matriz orgânica. A fase inorgânica é composta, essencialmente, por cristais de fosfato de cálcio sob a forma de hidroxiapatita, enquanto a fase orgânica é composta por colágeno tipo I, proteoglicanas e proteínas não colágenas (Hassumi *et al.*, 2018). A combinação entre os componentes minerais e os componentes orgânicos confere ao tecido ósseo rigidez com boa maleabilidade, tornando-o resistente às demandas funcionais (Clarke., 2008).

Apesar do seu aspecto aparentemente inerte e estático, os ossos são altamente dinâmicos, onde as células ósseas conduzem os mecanismos moleculares e celulares envolvidos na cicatrização óssea (Kim *et al.*, 2011). Essas células atuam em conjunto por meio de sinalização de moléculas para manter o “turnover ósseo”. Os mecanismos de desenvolvimento e manutenção do osso ocorrem constantemente, pois fatores locais como estimulação mecânica e fatores sistêmicos podem interferir neste processo (Hassumi *et al.*, 2018). Em condições ideais, o turnover ósseo é equilibrado pela síntese e reabsorção óssea, permitindo a manutenção da massa óssea e garantindo os níveis de minerais (Schoppet *et al.*, 2002). Assim, a caracterização do processo

dinâmico do osso para substituir um dente extraído é um tema de especial interesse na Odontologia.

O reparo alveolar após extração dentária tem sido analisado em muitas condições experimentais e clínicas e já é bem estabelecido na literatura. Um modelo clássico utilizado para estudar o reparo ósseo é através da extração de molares em ratos (Hassumi *et al.*, 2018). Com isso é possível descrever a cicatrização alveolar em três fases distintas: primeiro a inflamatória, com formação de coágulos e proliferação de células do tecido conjuntivo; segundo a fibroblástica, com formação e cicatrização do tecido conjuntivo; por fim, a fase de ossificação que se completa por volta de 28 dias com o alvéolo quase totalmente preenchido com osso novo (*woven bone*), correspondendo a 64 dias em humanos (Okamoto & de Russo., 1973; Mueller *et al.*, 1991). A mineralização ocorre progressivamente para formação de um tecido ósseo primário. Nesta etapa, numerosas trabéculas de osso imaturo se formam e substituem a maior parte do tecido de granulação precoce. Podem ser observadas no defeito, portanto, trabéculas ósseas e espaços fibrovasculares (Mueller *et al.*, 1991).

Além disso, algumas condições como osteoporose, diabetes não controlada, hipertensão, uso de medicações/drogas como bifosfonatos, pacientes que são submetidos à radioterapia têm sido associadas ao comprometimento do metabolismo ósseo (Chen *et al.*, 2013; Koth *et al.*, 2021). Assim, é essencial determinar o processo de reparo alveolar em condições não fisiológicas, a fim de fornecer as alterações morfométricas e celulares que influenciam na cicatrização óssea favorável (Kim *et al.*, 2011; Hassumi *et al.*, 2018).

1.2. Efeito da radiação ionizante no processo de reparo

Alterações no tecido ósseo decorrente da RT, tais como, apoptose celular, reabsorção trabecular, alterações corticais, maior fragilidade óssea, têm sido demonstradas em estudos com pré-clínicos *in vivo* (Wong *et al.*, 2008; Rabelo *et al.*, 2010; Limirio *et al.*, 2019; Mendes *et al.*, 2020; Borges *et al.*, 2021). Os efeitos da radiação no reparo incluem diminuição da atividade osteoblástica e morte de osteócitos (Dhakal *et al.*, 2011). A presença de lacunas vazias e diminuição de

osteócitos afetam negativamente a mecanotransdução, a mecanosensibilização, retardam o reparo e aumentam a formação de osso imaturo (Sønstevoid *et al.*, 2015). Os danos aos osteoblastos causam diminuição da produção de matriz óssea e reabsorção sem oposição pelos osteoclastos (Barth *et al.*, 2011). Logo após a irradiação, há um aumento precoce de osteoclastos que reabsorvem o osso trabecular neoformado (Baselet *et al.*, 2019). Essas alterações podem comprometer consideravelmente o reparo ósseo do tecido irradiado fibrótico (Bartlow *et al.*, 2018; Mendes *et al.*, 2020).

A osteoclastogênese no osso irradiado ocorre de forma alterada. O aumento das citocinas pró-inflamatórias no tecido ósseo submetido à RT contribui para uma maior diferenciação das células osteoclásticas e diminuição na expressão de osteoblastos. Com isso, a formação de uma nova matriz óssea é prejudicada, com consequente reposição da medula óssea vermelha pela medula óssea amarela, a qual possui pobre vascularização (Da Cruz Vegian *et al.*, 2020).

Com os recentes avanços nas técnicas de RT, os efeitos indesejados da irradiação permanecem focalizados na região de irradiação (Gooi *et al.*, 2016). Novas tecnologias de planejamento da RT, conhecidas como radioterapia conformacional tridimensional (convencional 3D) e radioterapia de intensidade modulada (IMRT), têm aumentado a eficácia do tratamento e diminuído os efeitos nos tecidos circunvizinhos por entregarem a dose de radiação mais diretamente na massa tumoral (Nguyen *et al.*, 2019). No entanto, os pacientes ainda sofrem de reações da mucosa, mandíbula ou glândulas salivares, entre as quais um efeito adverso específico é a ORN da mandíbula (Marx.,1983).

A caracterização do tecido ósseo irradiado pode contribuir na compreensão de quais componentes biológicos são especificadamente afetados e como as diferentes doses de irradiação contribuem nas alterações de suas propriedades moleculares. Assim, o modelo animal é de extrema importância para avaliar os achados histopatológicos do osso irradiado (Nguyen *et al.*, 2019). Diferentes doses de radiação são utilizadas nos poucos estudos que envolvem radiação de cabeça e pescoço em animais. Portanto, observa-se que não há um protocolo bem determinado da dose de radiação para estudos em animais bem

como nenhum estudo avaliou essas doses no processo de reparo alveolar. Assim, é necessário avaliar o efeito dessas doses no organismo animal (Elsaadany *et al.*, 2020).

1.3. Doença Periodontal

Outro fator de grande importância clínica na Odontologia é a doença periodontal (DP). É uma condição inflamatória crônica causada pela colonização de biofilme subgengival na superfície do dente, podendo afetar tecido gengival, ligamento periodontal, e reabsorção óssea (Kinane *et al.*, 2017; Pereira *et al.*, 2020). As bactérias periodontais desempenham um papel importante na etiologia da doença; no entanto, a progressão avançada da destruição do tecido periodontal depende da resposta imune do hospedeiro (Zambrano *et al.*, 2018), e é modulada por múltiplos fatores, incluindo genética, tabagismo, saúde geral, variáveis sociais e dieta (Akpınar *et al.*, 2017). O desenvolvimento da DP é caracterizada por vias moleculares chave. Como resultado do início dessas vias, as proteinases produzidas pelo hospedeiro são ativadas e, portanto, ocorre a destruição das fibras do ligamento periodontal, bem como a migração apical do epitélio juncional, o que cria um ambiente favorável para migração do biofilme para a região apical ao longo das superfícies radiculares (Tonetti *et al.*, 2018).

A inflamação causada pela doença é caracterizada por níveis aumentados de citocinas inflamatórias (Liu *et al.*, 2010), levando à gengivite, formação de bolsa periodontal e reabsorção óssea alveolar até a perda dentária (Ximénez-Fyvie *et al.*, 2000). As bactérias periodontopatogênicas são o principal agente causador da destruição dos tecidos periodontais de suporte. A gravidade da DP é proporcional à extensão da destruição tecidual causada pela resposta ampliada do hospedeiro suscetível, provavelmente influenciada por fatores de risco ambientais, adquiridos ou genéticos (Borba *et al.*, 2019). O tratamento convencional para periodontite baseia-se na remoção mecânica da placa bacteriana das bolsas periodontais por raspagem e alisamento radicular, que são considerados os procedimentos convencionais e essenciais em terapia periodontal (Ebersole *et al.*, 2014) que podem ser associados a adjuvantes.

O uso de modelos animais em estudos de progressão, prevenção e tratamento da periodontite tem aumentado substancialmente nos últimos anos, devido às semelhanças anatômicas e histológicas com o desenvolvimento da doença em humanos. Um modelo fácil e rápido para induzir a doença periodontal consiste em colocar uma ligadura de seda em torno de incisivos ou molares. A inoculação bacteriana e a ligadura colocada aumentam a retenção de placa e produzem lesões no tecido gengival, o que provoca uma resposta inflamatória notável. Essas mudanças resultam na quebra do tecido periodontal e permitem a fácil transferência de patógenos para a corrente sanguínea (Ionel *et al.* 2017; de Molon *et al.*, 2018).

1.4. Efeito da radiação ionizante na doença periodontal

O tecido periodontal é sensível à irradiação, e alterações morfológicas e histológicas são observadas após a RT (Ammajan *et al.*, 2013). Os efeitos diretos e indiretos da radioterapia de alta dose no periodonto resultam em diminuição da vascularização e celularidade da membrana periodontal e aumento do risco de perda de inserção periodontal (Epstein *et al.*, 1998). Além disso, a hipovascularização no osso alveolar está relacionada à endarterite causada pela radioterapia de cabeça e pescoço e resulta em risco aumentado para o desenvolvimento de ORN, principalmente em pacientes com periodontite (Irie *et al.*, 2018). É relatado que a radiação ionizante gera radicais livres nos tecidos irradiados. Esses radicais livres causam dano tecidual por uma variedade de mecanismos diferentes, incluindo peroxidação lipídica, dano ao DNA e estimulação de citocinas pró-inflamatórias liberadas por monócitos e macrófagos (Köse *et al.*, 2017).

A perda óssea periodontal pode ser acentuada em indivíduos pós-tratamento radioterápico na região dos maxilares, principalmente naqueles com doença periodontal pré-existente (Schuurhuis *et al.*, 2018). Essa alteração parece ser mais relacionada à própria ação deletéria da radiação no osso do que pela mudança na microbiota da placa dentária, já que essa última tende a normalizar após um ano do término da RT (Al-Nawas & Grötz, 2006).

A prevalência de periodontite em adultos é frequente e tende a piorar com o tratamento oncológico (Irie *et al.*, 2018). Não se sabe ao certo como o tecido periodontal se comporta quando a fração de alta dose é usada. Estudos experimentais têm sido conduzidos para determinar o mecanismo de destruição causado na progressão da doença periodontal, para prevenir a destruição, ou para determinar as formas de compensar as consequências desta destruição (Aydinyurt *et al.*, 2021). No entanto, poucos esforços têm sido realizados para avaliar a destruição causada pela radiação ionizante em situações após o tratamento periodontal.

O manejo de pacientes irradiados representa um desafio para os profissionais de saúde. Com isso, estudos pré-clínicos em animais permitem análises que não são possíveis em ensaios clínicos. Dessa forma, a associação de metodologias como ensaios mecânicos, microtomografia computadorizada (micro-CT) e histomorfometria contribuem enormemente para o avanço do conhecimento dos efeitos da irradiação (Koontz *et al.*; 2017).

1.5. Microtomografia computadorizada na análise do tecido ósseo

A micro-CT evoluiu para o padrão-ouro na avaliação da microarquitetura óssea em modelos de roedores, complementando e, em alguns aspectos, superando as capacidades da histomorfometria tradicional, produzindo dados bidimensionais (2D) e tridimensionais (3D) (Chavez *et al.*, 2021). Por meio do escaneamento, reconstrução e análise por meio softwares, é possível visualizar com precisão a estrutura óssea em três dimensões, juntamente com numerosos parâmetros estruturais (Chatterjee *et al.*, 2017). No entanto, os parâmetros adotados em micro-CT devem estar bem estabelecidos para que não induza vieses nos resultados.

Determinadas variáveis associadas à obtenção das imagens, processamento, e análise dos dados podem afetar os resultados morfológicos (Christiansen., 2016). Dessa forma, o entendimento destas etapas é necessário para correta aplicação e interpretação dos dados. Apesar da vasta literatura que aborda o uso da microtomografia computadorizada (Bouxsein *et al.*, 2010), algumas questões relacionadas a sua aplicação na Odontologia e, também, à

definições de parâmetros, ainda carecem de investigações para melhor entendimento dos efeitos destes parâmetros, relacionados à de aquisição da imagem, processamento e análise, nos resultados morfométricos obtidos.

2. OBJETIVOS

Fornecer informações relevantes à literatura referentes ao efeito da radiação ionizante no reparo ósseo alveolar e dos tecidos periodontais associado a doença periodontal em ratos, bem como estabelecer critérios para definição do modelo de estudo e dos parâmetros de análise através do uso da micro-CT para análise do tecido ósseo em associação a outras metodologias.

2.2 Objetivos específicos

Objetivo específico 1: avaliar a influência do alvéolo de extração (raiz distal ou lingual) e o tipo de definição da região de interesse (ROI) (manual ou pré-definida) na avaliação do reparo alveolar após extração dentária usando microtomografia computadorizada (micro-CT).

Objetivo específico 2: avaliar o efeito dose-resposta da radiação ionizante no reparo ósseo alveolar e na resistência óssea após 14 dias de exodontia dos molares de ratos, por meio de micro-CT e análises histológica e histomorfométrica

Objetivo específico 3: avaliar os efeitos da radiação ionizante no reparo periodontal em modelo de rato com periodontite induzida por ligadura, por meio de micro-CT e análises histológica e histomorfométrica

3. CAPÍTULOS

3.1. Capítulo 1:

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Definition of the Region of Interest for the Assessment of Alveolar Bone Repair Using Micro-computed Tomography

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Abstract

The objective of this study was to evaluate the influence of the extraction socket (distal or lingual root) and the type of region of interest (ROI) definition (manual or predefined) on the assessment of alveolar repair following tooth extraction using micro-computed tomography (micro-CT). The software package used for scanning, reconstruction, reorientation, and analysis of images (NRecon[®], DataViewer[®], CT-Analyzer[®]) was acquired through Bruker <<https://www.bruker.com>>. The sample comprised the micro-CT volumes of seven Wistar rat mandibles, in which the right first molar was extracted. The reconstructed images were analyzed using the extraction sockets, i.e., the distal and intermediate lingual root and the method of ROI definition: manual (MA), central round (CR), and peripheral round (PR). The bone volume fraction (BV/TV) values obtained were analyzed by two-way ANOVA with Tukey's post hoc test ($\alpha=5\%$). The distal extraction socket resulted in significantly lower BV/TV values than the intermediate lingual socket for MA ($P=0.001$), CR ($P<0.001$), and PR ($P<0.001$). Regarding the ROI, when evaluating the distal extraction socket, the BV/TV was significantly higher ($P<0.001$) for MA than for CR and PR, with a lower BV/TV for CR. However, no significant difference was observed for MA ($P=0.855$), CR ($P=0.769$), or PR ($P=0.453$) in the intermediate lingual extraction socket. The bone neoformation outcome (BV/TV) for alveolar bone repair after tooth extraction is significantly influenced by the ROI and the extraction socket. Using the predefined method with a standardized ROI in the central region of the distal extraction socket resulted in the assessment of bone volume, demonstrating the most critical region of the bone neoformation process.

Keywords Microcomputed tomography · Region of interest · Alveolar bone · Tooth extraction · Bone repair

Introduction

The alveolar bone is characterized by the continuous and rapid remodeling of tissues in response to stimuli [1–3]. Several clinical procedures, including rehabilitative therapies, depend on the socket-healing process after tooth extraction, and its understanding in preclinical studies is crucial. Therefore, the

characterization of the dynamic process of bone replacement of an extracted tooth and improvement of alveolar bone regenerative strategies is a topic of special interest in dentistry [4–6].

Micro-computed tomography (micro-CT) has quickly become a standard tool in the assessment of bone microarchitecture in animal models as a complementary alternative to traditional histomorphometry [7–12]. The nondestructive method, short turnaround time, and volumetric analyses are appealing in the analysis of the microarchitecture of native and newly formed bone using morphometric parameters (2D and 3D) and mineral density [7, 9, 13, 14]. In addition, the same sample can later be prepared for histological analysis [7]. As the use of micro-CT has expanded, the analyses have grown to encompass complex structures such as the dentoalveolar complex and have been widely used to study alveolar bone remodeling [12, 15–17]. However, some variables and selected parameters in micro-CT may affect morphological outcomes [18].

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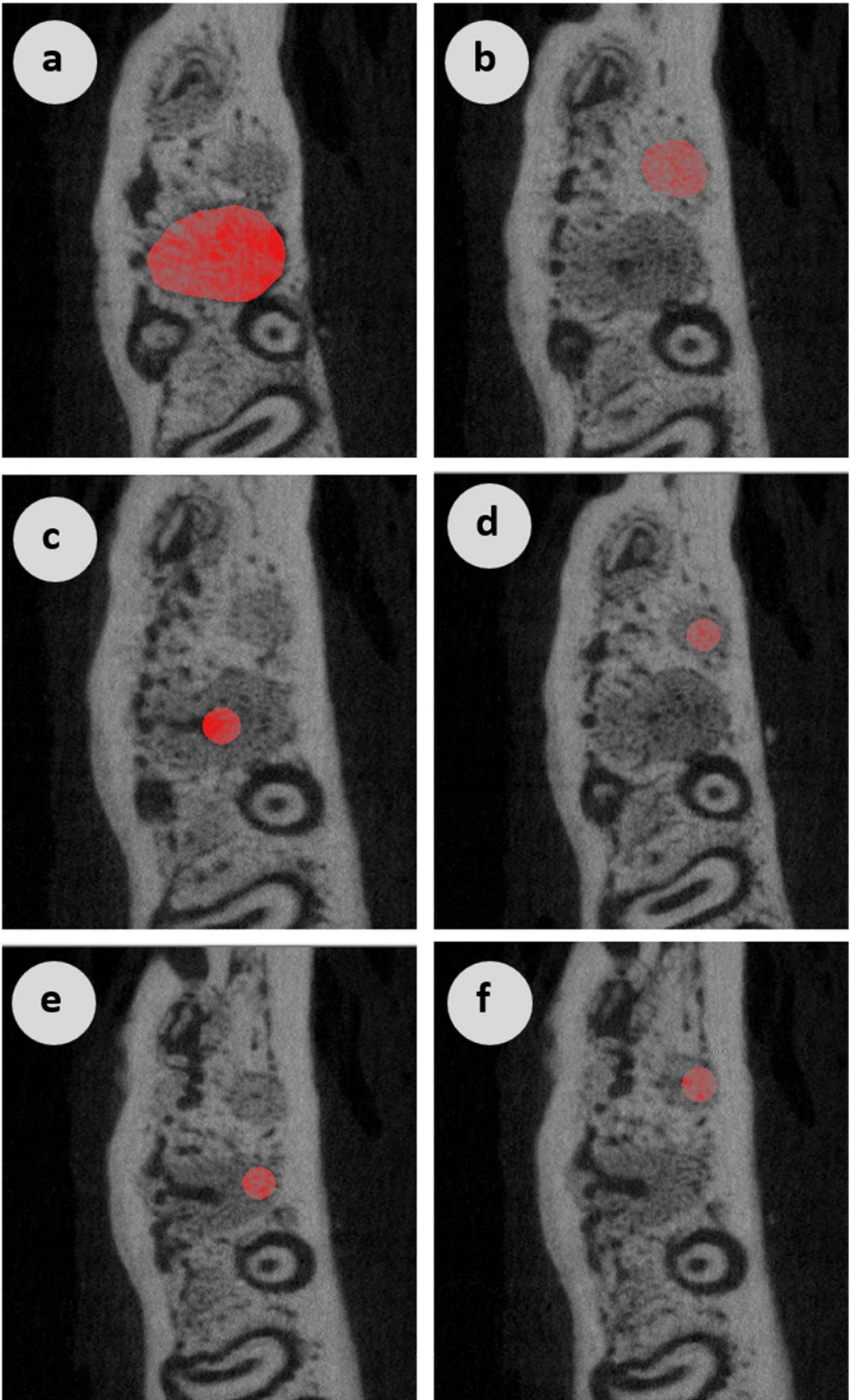


Fig. 1 Demonstration of the region of interest (ROI) delimitation in the analyzed extraction socket. **a** Manual ROI and distal extraction socket of the lower first molar (M1). **b** Manual ROI and intermediate lingual extraction socket of the M1. **c** Central round and distal extraction socket of the M1. **d** Central round and intermediate lingual extraction socket of the M1. **e** Peripheral round and distal extraction socket of the M1. **f** Peripheral round and intermediate lingual extraction socket of the M1

One analysis parameter that influences the quality and the accuracy of the results obtained from micro-CT is the region of interest (ROI) definition. Ideally, the ROI should be delimited based on experimental questions to be answered, and should be used with caution as it can bias results and provide imprecise values [12, 19, 20]. This process can be performed manually [15, 21] or in an automatic/semi-automatic fashion [11, 22, 23]. Different ROI formats (e.g., rectangular, polygonal, round, cubic, and ring) can be used as representative samples in bone neoformation studies [12, 24]. Another factor considered in the alveolar repair analysis is the location of the ROI, i.e., the extraction socket used for analysis. Several studies have evaluated the distal extraction socket [21, 24] and the mesial extraction socket [23, 25], showing that the densities of the den-toalveolar tissues and the amount of neoformed bone vary by location and the size of the socket, which makes it difficult to directly compare the results of bone microarchitecture [12, 24].

Despite current advances, there is limited available information regarding standardization of important parameters, including the ROI definition, in the analysis and reporting of data on the assessment of alveolar repair using micro-CT in preclinical studies. Literature on protocols for rodent jawbones is scarce, and the current methodology is not informative or sufficiently detailed to allow reproducible quantitative analysis. This makes it difficult to understand how the analyses were performed and if the findings can be broadly compared to those of other models and studies [12, 24], which may compromise the scientific impact of studies [20]. To our knowledge, no study has directly compared the analysis of different extraction sockets and ROIs formats in the same study when assessing the outcome of alveolar bone repair using micro-CT. Thus, this study aimed to answer the following question: What region should be analyzed in the assessment of alveolar bone repair using micro-CT? To this end, we evaluated the influence of the extraction socket (distal or lingual root) and the type of ROI definition (manual or predefined) on the assessment of alveolar repair following tooth extraction using micro-CT. The null hypothesis was that the type of ROI and the analysis region would not influence the outcome of micro-CT analysis.

Material and Methods

This study was conducted in accordance with the ARRIVE guidelines for preclinical studies and the normative guidelines of the National Council for Animal Control and Experimentation.

The sample consisted of micro-CT images of the mandible of seven male Wistar rats that were acquired for previously approved research by the Research Ethics Committee of the institution Federal University of Uberlândia (CEUA protocol 013/19).

Mandibular right first molar (M1) extractions were performed using luxator hollembach Sculptor No. 3 (Golgran, São Paulo, São Paulo, Brazil) and micro-forceps No. 5 (Golgran, São Paulo, São Paulo, Brazil) to avoid fracture of the roots following established protocols [5]. The animals were euthanized 14 days after tooth extraction by an intraperitoneal overdose of thiopental (150 mg/kg). The hemimandibles were fixed in 4% paraformaldehyde solution and 10% 0.1 M phosphate buffer (pH 7.4) for 48 h and scanned using a micro-CT (Skyscan 1176, Bruker, Kontich, Belgium) with the following acquisition settings: 65 kV, 385 μ A, isotropic voxel size of 9 μ m, 1-mm aluminum filter, 0.5° rotation step, 3 frames per rotation degree; 180° rotation, and 55-min scan time. Each mandible was fixed with wax on the scanning platform to ensure a standardized position with the long axis vertical to the horizontal plane, wrapped in wet paper to maintain the moisture during the scanning procedure. After scanning, projection images were reconstructed into cross-sections using Hierarchical (InstaRecon®) reconstruction software (Windows 7 or Vista, 64 bit) engine for NRecon (version 1.6.6.0, Bruker, Kontich, Belgium). The hierarchical reconstruction software is based on unique (non-Feldkamp) reconstruction algorithms [26].

The images were reconstructed using the following settings: 40% beam hardening correction, 0 smoothing, and 12-ring artifact reduction. Reconstructions were performed in the mandibular first and second molar regions.

DataViewer® software (version 1.5.1.2, Bruker, Kontich, Belgium) was used to adjust the images of the hemimandibles to standardize positioning to obtain a better setting of the distal and lingual tooth sockets of the extracted mandibular first molar. The images in the axial-transverse plane with standard orientation were exported to the CT-Analyzer® software (version 1.14.4.1, SkyScan, Bruker, Belgium), and only sections including the distal and lingual extraction sockets of the extracted mandibular first molar were selected to delineate the ROI showing the alveolar socket healing. There was a total of 80 sections along the cervical, middle, and apical thirds of the socket for all samples.

The reconstructed images were analyzed using the extraction sockets of M1, i.e., distal (D) and intermediate lingual (IL) root, and the ROI, i.e., manual (MA), central round (CR), and peripheral round (PR). Segmentation of the ROI in the D and IL extraction sockets was individually performed for each format. In MA, a personalized ROI was acquired manually using a computer mouse to delimit the extraction socket with respect to its contours (Fig. 1a, b). In CR, a standardized 0.5-mm round predefined ROI was selected and centrally positioned in the extraction

socket of the M1 (Fig. 1c, d). In the same manner, for the analysis of PR, a standardized 0.5-mm round ROI was positioned in the region close to the buccal surface in the extraction socket (Fig. 1e, f). The entire region was defined by interpolation of the ROIs for every 10 slices.

After ROI delimitation, a global threshold was used for all samples. A fixed threshold of minimum 71 and maximum 255 (71, 255) based on the grayscale level was visually defined, by analyzing the density of the histogram and through the mean of the automatic threshold values (Otsu method) [27] obtained from 10 samples from the present study, in an attempt to overcome the limitations related to low-resolution and nonhomogeneous samples that affect global thresholding [28]. The images analyzed in this study were scanned using a micro-CT (Skyscan 1176, Bruker, Kontich, Belgium) and the private access software package used for scanning, reconstruction, reorientation, and analysis of images (NRecon®, DataViewer®, CT-Analyzer®) were acquired through Bruker <<https://www.bruker.com>>.

All procedures were performed by a single well-trained operator who performed the personalized (manual) and predefined ROI segmentation for all samples. Three-dimensional analysis of the ROI was performed to examine the features of the bone microarchitecture [7], with the bone volume fraction (BV/TV, %) as the selected parameter.

Statistical analysis was performed using *SigmaPlot*® software (SigmaPlot v13.1; Systat Software Inc., San Jose, CA, USA). The influence of the extraction socket (distal or lingual root) and the type of ROI definition (manual or predefined) on BV/TV values was assessed using two-way analysis of variance (ANOVA) with Tukey's post hoc test, with a significance level of $\alpha=0.05$.

Results

The BV/TV means and standard deviations are presented in Table 1. Two-way ANOVA showed that BV/TV values were significantly influenced by the type of ROI ($P<0.001$), analysis region ($P<0.001$), and the interaction between these factors ($P<0.001$).

Regarding the analyzed extraction socket, the distal socket resulted in significantly lower BV/TV values than the IL socket for MA ($P=0.001$), CR ($P<0.001$), and PR ($P<0.001$). For the ROI, the BV/TV was significantly higher ($P<0.001$) for MA (75.11 ± 6.69) than for CR (65.31 ± 5.16) and PR, with a lower BV/TV for CR (55.96 ± 7.35) when evaluating the distal socket. However, no significant difference was observed between MA (91.38 ± 4.32 , $P=0.855$), CR (92.12 ± 5.49 , $P=0.769$), and PR (93.08 ± 4.16 , $P=0.453$) in the IL socket.

Discussion

The parameters used in micro-CT analysis may directly influence the final characteristics of the image [29] and, consequently, the morphometric outcomes [20]. This study evaluated the influence of analysis parameters, including the ROI and the selected extraction socket of micro-CT images on the evaluation of alveolar bone repair after M1 tooth extraction in rats. The amount of neoformed bone (BV/TV) was significantly influenced by the ROI shape, evaluated extraction socket, and interaction between these factors. Thus, the null hypothesis that these variables would not influence the outcome of micro-CT analysis was rejected.

Several preclinical models have been used to evaluate the bone repair process in surgically created defects, such as in the femur/tibia [30–33], and extraction sockets in mandibles [21, 23, 25, 34, 35]. Reports on the part of extraction sockets selected for the ROI are conflicting. Alveolar repair has been analyzed in different extraction sockets (for example, distal [21, 24] and mesial [23, 25] extraction sockets). Moreover, different ROI formats have been found in the literature, including manually delineated ROIs [15, 21] and those with predefined shapes [11, 22, 36]. However, the method used for ROI delimitation can affect the morphometric results of micro-CT [20, 37]. Further, it has been demonstrated that changing the position of the ROI can result in a 12–37% variation in the BV value [38]. Thus, a direct comparison of the study results is not recommended.

In our study, a round standardized shape was evaluated for the determination of the ROI, and changes in its position resulted in significant differences in BV/TV values.

Table 1 Mean and standard deviation of BV/TV (%) values of the bone morphometric parameters analyzed in the alveolar repair area by micro-CT

Region of interest Extraction socket	Manual ROI	Central round ROI	Peripheral round ROI
Distal M1	75.11 ± 6.69 ^{Ab}	55.96 ± 7.35 ^{Cb}	65.31 ± 5.16 ^{Bb}
Intermediate lingual M1	91.38 ± 4.32 ^{Aa}	92.12 ± 5.49 ^{Aa}	93.08 ± 4.16 ^{Aa}

Different letters indicate statistically significant differences verified by Tukey test ($P<0.05$). Uppercase letters are used for comparing region of interest (manual, central round, peripheral round), lowercase letters are used for comparing extraction socket (distal and intermediate lingual root of the lower first molar (M1))

Less bone neoformation was detected in the CR ROI than in the peripheral ROI. In addition, higher BV/TV values were observed for the manual ROI in the distal root M1. Bone healing is a centripetal process, which occurs as a result of bone formation originating on the socket walls and remains subsequently confluent until all the socket extensions are filled [36, 39, 40]. This process explains the results in the present study, demonstrating a critical area of bone repair in the central region of the socket, with the presence of hypomineralized bone (osteoid) [41]. Additionally, ROIs manually delineated to quantify the bone neoformation process may include regions that are not part of the ROI (e.g., cortical bone or cancellous bone outside the extraction socket) as the healing area might not be well defined. Thus, inaccurate BV/TV values may have been obtained using this method. However, standardized ROIs used as a representative sample can avoid biased results in the evaluation of bone repair [6, 12, 24], considering that the ROI must be chosen stringently and consistently across samples to minimize selection bias [12]. Thus, the use of standardized ROIs compatible with the critical region of the socket throughout the analyses is strongly recommended.

Manual ROIs allow more accurate sizes of the region of interest to be analyzed; however, they have some disadvantages such as the inclusion of undesired areas; segmentation is manual which is time-consuming, in addition to depending more on the operator experience. For predefined ROIs, the segmentation is automatic, reproducible, and quick and can be indicated for regions of interest with regular shapes, being a limitation its use for region of interest when there are irregular borders [7, 9].

Although bone neoformation in the extraction socket is a well-known process [42, 43], large bone defects (critical defects) represent major problems in the bone-healing process [44], as bone grafts are necessary for reconstruction. In the present study, the BV/TV values were not affected by the ROI format for the IL socket. Moreover, a significantly higher amount of new bone formation was observed in the IL socket than in the D socket, regardless of the ROI shape, demonstrating more bone formation with almost complete socket healing. This result was expected because of the difference in the root diameter. IL sockets are smaller when compared to the D socket, and consequently, the bone repair process occurs faster [44].

It is important to previously determine all the crucial parameters to answer the purpose of the study, since they will directly affect the results of a survey [9]. Another important aspect of micro-CT analysis that must be taken into consideration is the size of the socket (i.e., the root to be analyzed). For studies that aim the evaluation of therapies to increase bone quality or quantity [45] or conditions that affect the bone repair process such as radiotherapy [33, 46], diabetes [32] and osteoporosis [17], evaluation of larger

diameter sockets is strongly recommended so that the effect of such therapies or conditions can be properly evaluated in regions with critical-sized defects. Thus, critical-sized defects that do not allow spontaneous bone regeneration/healing [47–49] are indicated for studies on bone neoformation/reconstruction [44]. In the present study, the distal extraction M1 socket was not completely replaced by bone in the evaluated period (15 days), demonstrating a critical repair region, differently of the smaller diameter socket (IL) which was completely repaired by bone.

During the experimental period, the extraction socket repair was completed within 28 days in healthy rats [6, 50, 51]. Our study results are in accordance with the normal course of alveolar bone repair in rats after tooth extraction, with the bone formation phase observed at 14 days.

Most experimental reports lack details of parameters applied for micro-CT image acquisition and analysis [24, 52]. Regarding the ROI definition, few studies have clearly reported how the ROI was defined. The study method should provide descriptions of the size and location of the ROI because the results can be affected by these parameters. Furthermore, it may be useful to provide a representative sample to illustrate the selected ROI and demonstrate the region being analyzed [7, 12]. Precise protocols are mandatory for analyzing the significance of the results, as they can be biased if the procedures are not standardized, as the system is extremely user-sensitive [24]. We aimed to provide optimal parameters for analysis 3D microstructure in micro-CT images on the assessment of alveolar repair following tooth extraction, in order to standardize the ROI definition method, as well as the appropriate positioning of the volumes of interest to be analyzed. However, we examined the trabecular structure in just one atomical area of the jawbone, in the region of the mandibular first molar, which can be considered a limitation of this study. Evaluation in other regions of the rodent jawbone should be performed.

The images in this study were scanned and analyzed using a micro-CT model/data package Bruker. However, there are other models and brands of scanners and software package. All parameters used in this study for scanning and reconstruction of images can be extrapolated to other systems provided that steps to ensure the compatibility between them are performed. Also, the analysis methods evaluated in this study can also be extrapolated to other software like open source. In a study by the research group, we used two different software. The samples were scanned with a Scanco system and the Bruker software system was used in the reconstruction and analysis [53]. This compatibility method might be useful, since the researchers have the possibility to analyze the volumes in a different software from scanning or reconstruction.

The most commonly used reconstruction algorithms in CT and micro-CT are filtered back projection (FBP)–based

[54] and iterative algorithm [55]. A good option is a hierarchical back-projection algorithm. This accelerates the process by dividing the volume into smaller sections, which require fewer projections for reconstruction [26]. This is especially effective with larger datasets, in vivo studies, and very high spatial resolution. The InstaRecon® engine for NRecon developed by Bruker used in the present study uses this type of hierarchical reconstruction algorithm. Using patented algorithms based on unique (non-Feldkamp) reconstruction, reconstruction can be performed many times faster using just one PC than a whole cluster. InstaRecon is compatible with all Bruker SkyScan micro-CT system, and can be used to upgrade existing installed instruments [26, 54]. Hierarchical reconstruction algorithm provides speedups of 10× to > 100× (higher for large image formats) compared to GPU-accelerated reconstruction. All speed-ups made algorithmically and quickly increase compared to standard (Feldkamp) algorithm with increasing dataset sizes [55, 56].

In recent years, various regularized iterative reconstruction algorithms have been proposed [57–59], such as the total variation-based minimization method [60] which can suppress the artifacts and noise when the projection data are acquired within a few-views scanning mode. Iterative and statistical reconstruction algorithm can provide notably better image quality than FBP when the number of projections available for reconstruction is limited and/or the projections are very noisy (e.g., low-dose scanning) [55, 56]. Reduction in the number of views translates directly to reduced radiation dose to the animal, which is useful in an ex vivo study design, where radiation dose might be a problem. Furthermore, it is often impractical to acquire projections with regular-angular sampling in dynamic imaging applications, resulting in reconstruction artifacts when FBP is used [55–57]. Iterative algorithms such as the algebraic reconstruction technique and the simultaneous algebraic reconstruction technique present an alternative to FBP with the potential for robust reconstruction given less than ideal projection data, when the projection data are complete [56]. In the present study, a hierarchical reconstruction algorithm was used with the application of beam hardening correction and ring artifact reduction filters to correct reconstruction artifacts, resulting in an effective reconstruction.

Image segmentation is critical in separating the mineralized and non-mineralized tissues for quantitative analysis [9]. The features used for image classification may be local, global, or both [61]. The protocol applied in the present study made use of global thresholding, which employs a fixed range of greyscales, who was able to achieve the desired image filtration, since the region of interest comprises trabecular bone [24]. Image segmentation algorithms are employed to classify the images based on color, texture, and shape. Presently, image segmentation and processing,

as well as the detection of structures and object comparison, can be done automatically through a dataset using deep learning-based techniques that have been showing promising results [61, 62].

Conclusion

Within the limitations of this preclinical study, the following conclusions can be drawn:

1. Differences in ROI shapes and positions in the extraction socket affect the morphometric results of micro-CT.
2. The bone neoformation outcome (BV/TV) for alveolar bone repair after tooth extraction is significantly influenced by the ROI (manually drawn or predefined shape) and the extraction socket (distal or lingual root).
3. The predefined method with a standardized ROI in the central region of the distal extraction socket resulted in the assessment of bone volume, demonstrating the most critical region of the bone neoformation process.

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Author Contribution PBFS, RSN, and JSB participated in the conception and study design. JSB and VCC performed the experiment. MSI and JSB contributed to the interpretation of the experimental results and statistical analysis. JSB, VCC, and MSI wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Availability of Data and Material Data will be made available upon request to authors.

Code Availability Not applicable.

Declarations

Ethics Approval This study was approved by the Bioethics Committee for Animal Experimentation (#013/19) at Federal University of Uberlândia, Brazil, and conducted in accordance with the ARRIVE guidelines for preclinical studies and the normative guidelines of the National Council for Animal Control and Experimentation (CONCEA).

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3.2 Capítulo 2

Artigo submetido e em revisão na *Clinical Oral Investigations*

Effects of different doses of ionizing radiation on alveolar bone repair in post-extraction tooth socket: an experimental study in rats

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Declarations

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethics approval: This study was approved by the Ethics Committee on Animals Use of Federal University of Uberlandia (CEUA 013/19). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Informed consent: For this type of study, formed consent is not required.

Author contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juliana Simeão Borges, Isabella Santos Paula and Nayara Teixeira de Araújo. Writing - review and editing: Juliana Simeão Borges, Priscilla Barbosa Ferreira Soares, Carlos José Soares. Supervision: Priscilla Barbosa Ferreira Soares. All authors read and approved the final manuscript.

ABSTRACT

Objective: This study aimed to evaluate the dose-response effects of ionizing radiation (IR) on alveolar bone repair and bone strength after tooth extraction.

Materials and Methods: Mandibular first molars of 28 male Wistar rats were extracted. After 7 days, the animals were randomly divided into four groups (n = 7) according to single-dose irradiation: Nlr: Control group; Ir15, irradiated at 15 Gy; Ir20, irradiated at 20 Gy; and Ir30, irradiated at 30 Gy. The tooth extraction sites were subjected to micro-computed tomography (micro-CT), histological, histomorphometric, and biomechanical analyses 14 days after extraction. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test ($\alpha = 0.05$).

Results: Micro-CT analysis revealed that IR led to lower values of bone volume fraction (44.1 ± 8.3 , $P < 0.001$) and bone volume (0.68 ± 0.08 , $P < 0.001$) for the Ir30 group compared to the control group. A significantly lower amount of newly formed bone was observed in the Ir30 ($P = 0.005$) than in the Ir15 group. The histomorphometric and micro-CT results were in agreement. Intermediately and densely packed collagen were predominant in all the groups. IR30 cells showed a lower percentage of densely packed collagen than control cells. No significant differences were found in the biomechanical parameters.

Conclusion: IR affects alveolar bone repair. A dose of 30 Gy reduced the bone healing process owing to a smaller amount of newly formed bone and a lower percentage of densely packed collagen. Therefore, a dose of 30 Gy can be used to successfully establish an animal model of an irradiated mandible that mimics the irradiated clinical conditions.

Clinical relevance: Radiotherapy can lead to severe side effects and tooth extraction is a major risk factor. A proper understanding of the pathological mechanisms of radiation in alveolar bone repair requires the establishment of a suitable animal model of clinical conditions.

keyword: Ionizing radiation, X-ray microtomography, tooth extraction, alveolar bone.

INTRODUCTION

Radiotherapy (RT) is an important treatment for head and neck cancer [1]. However, ionizing radiation adversely affects healthy tissues, resulting in oral complications, such as caries, periodontal disease, infections, and osteoradionecrosis (ORN) [2, 3]. These oral complications negatively affect the quality of life and must be considered before, during, and after RT [3, 4].

The deleterious effects of ionizing radiation (IR) may affect bone volume, composition, and microarchitecture, leading to a greater predisposition to fractures because of decreased bone density [5 – 9]. The components of the matrix are damaged [10], decreasing vascularity and reducing the number and activity of osteoblastic cells. This combination of negative factors hinders bone regeneration [7, 11, 12], resulting in modifications of the network of bone channels [9, 13]. Tooth extraction during the post-RT period is considered a risk factor for ORN and can result in delayed tissue healing [4]; therefore, indication before RT therapy is preferable [2].

Management of irradiated patients remains a challenge for professionals, including dental professionals. Establishing a suitable animal model for RT studies that mimics clinical conditions that can be used to investigate the mechanism and develop novel therapeutic strategies remains a challenge [14]. Most studies involving RT and bone healing have focused on long bones, such as the femur and tibia [6 – 9]. However, this model is limited to long bones, and thus is difficult to compare with the mandible [15]. Additionally, osteoblasts behave differently from osteoblasts derived from long bones in terms of proliferation, mineralization, and angiogenesis [16].

Alveolar repair in post-extraction tooth sockets is a model for preclinical studies that is well established in the literature [17]. However, the effects of post-extraction teeth on the irradiated condition have not been widely described, and the literature does not provide scientific evidence regarding the appropriate protocol and radiation dose [18]. Therefore, this study aimed to evaluate the dose-response effects of ionizing radiation on alveolar bone repair and bone strength after tooth extraction. The null hypothesis was that the ionizing radiation dose would not affect alveolar bone repair after tooth extraction.

MATERIALS & METHODS

Animals

This study was approved by the Research Ethics Committee (CEUA 013/19) of the Federal University of Uberlandia, and all procedures were in accordance with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and conformed to the guidelines of Animal Research: Reporting of *In Vivo* Experiments (ARRIVE). The sample was composed of 28 male rats (*Rattus norvegicus*) with a mean weight of 300–350 g at the beginning of the experiment. The animals were housed in standard microisolators (4 per cage) at 22 °C with a 12-h light/dark cycle (lights on at 7:00 am and off at 7:00 pm). During the experiments, a standard diet of rat chow (Labina, Purina®, Paulínia, SP, Brazil) and filtered water was provided ad libitum.

Surgery - Tooth extraction

The bilateral mandibular first molars (M1) were carefully extracted after an intraperitoneal combining 100 mg/kg of 10% ketamine hydrochloride (Ketamina Agener®; Agener União Ltda, São Paulo, SP, Brazil) and 10 mg/kg 2% xylazine hydrochloride (Rompum ® Bayer SA, São Paulo, SP, Brazil). The animals were placed in a holder to keep their mouth open, and extractions were performed using luxator hollemback Sculptor No. 3 (Duflex®, S.S. White, Rio de Janeiro, RJ, Brazil) and micro-forceps (Duflex®, S.S. White, Rio de Janeiro, RJ, Brazil) to avoid fracture of the roots [3, 19]. A carbide spherical drill was inserted at a low speed into the extraction sites to remove bone fragments in the socket. Bleeding was controlled by local compression and the sockets were filled with clots, covered with mobilized gingival tissue, and sutured with a 4-0 polyglactin suture thread (Johnson & Johnson, São José dos Campos, SP, Brazil).

The animals were identified on the tail and randomly separated using the Microsoft Office Excel software (Microsoft, Redmond, WA, USA) into four experimental groups (n = 7) according to single-dose irradiation: Nir, control group, non-irradiated; Ir15, irradiated at 15 Gy; Ir20, irradiated at 20 Gy; and Ir30, irradiated at 30 Gy.

Ionizing radiation

At 7 days after tooth extraction, the animals of groups Ir15, Ir20 and Ir30 received a single dose of ionizing radiation according to the allocated group in the mandible region using a linear accelerator 6MeV (Varian 600-C © Varian Medical System Inc Palo Alto, California, USA) [20] with a source-skin distance of 60 cm and the field size was 15 × 15 mm [9]. The animals were subjected to general anesthesia, positioned with only their mandible in the radiation field, and immobilized with adhesive tape. A wax bolus measuring 1.5 cm was placed over the mandible to increase the thickness of soft tissues and concentrate the maximal dose on the bone.

Animals were euthanized 14 days after tooth extraction using an intraperitoneal overdose of thiopental (150 mg/kg). For histological observations (n = 7), the right hemimandibles were fixed in 4% paraformaldehyde solution and 10% 0.1 M phosphate buffer (pH 7.4) for 48 h, preserved in 70% alcohol for micro-CT scanning (n = 7), decalcified in 4% ethylenediaminetetraacetic acid (EDTA) for 10 weeks, and embedded in paraffin. The left hemimandibles were removed and covered with moist gauze containing phosphate-buffered solution and stored in plastic tubes at -20 °C until the time of analysis by biomechanical test (n = 7). All analyses were performed in the distal socket of M1 because this is the socket with the largest diameter.

Micro-CT analysis

To evaluate alveolar socket repair, the samples were scanned using a micro-CT (Skyscan 1176, Bruker, Kontich, Belgium) with the following acquisition settings: 65 kV, 385 µA, isotropic voxel size of 9 µm, 1-mm aluminum filter, 0.5 °rotation step, three frames per rotation degree; 180° rotation, and 55-min scan time. After scanning, the images were imported into NRecon Reconstruction software (version 1.6.6.0, Bruker, Kontich, Belgium) for grayscale reconstruction. Images were reconstructed using the following settings: 40% beam-hardening correction, 0 smoothing, and 12 ring artifact reduction.

The DataViewer ® software (version 1.5.1.2, Bruker, Kontich, Belgium) was used to adjust the images of the hemimandibles in order to get better

positioning of the distal tooth socket. The images in the coronal plane with standard orientation were exported to the CT-Analyzer® software (version 1.14.4.1, SkyScan, Bruker, Belgium), and only sections including the distal extraction sockets were selected to delineate the region of interest (ROI) showing alveolar repair. A total of 80 slices along of the socket and a circular predefined shape rectangular area of interest (ROI) measuring 1.7 mm, from the top of the defect (alveolar crest) to the apical end of the root socket and between the mesial and distal alveolar bone surfaces were selected. A global threshold was used for all 71–255 samples, defined based on grayscale. All analyses were performed by a single well-trained operator. Micro-CT images that revealed root remnants in the socket were excluded from the analysis. Percent bone volume (BV/TV; %), bone volume (BV; mm³), total volume (TV; mm³), trabecular thickness (Tb.Th; mm), trabecular number (Tb.N; 1/mm), and trabecular separation (Tb.Sp; mm) were analyzed.

Histologic and Histomorphometric analysis

Hematoxylin and eosin (H&E) were used for qualitative histological analysis, and Masson's trichrome and Picrosirius Red were used for histomorphometric analysis of histological sections of 5 µm in the buccolingual direction of the alveolar portion. Sections were captured with a digital slide scanner at a magnification of 20× (ScanScope AT Turbo® Scanner, Leica Biosystems, Nussloch, Germany). The intensity and extent of the inflammatory response, the cellular and structural patterns of bone and connective tissue, and the amount of newly formed bone were evaluated in nine histological sections per group using H&E staining.

To quantify bone matrix neoformation, 5× magnification images were used. A ROI with dimensions of 1210 × 850 pixels was defined using the ImageJ software (1.51k, Wayne Rasband, National Institutes of Health, USA). The ImageJ aleatorily generated a 'grid,' providing an area per point of 1,000 pixels² for each image. The grid was represented by intersecting lines at a distance between neighboring points of 40 µm. Using a manual counting method, each point was tallied according to its corresponding morphological structure and

categorized as bone (%) or soft tissue (%). Its distal limit consisted of a line parallel to the coronary dentin and the root surface of the lower left second molar, extending 4 mm mesially. Its coronary limit consisted of a line parallel to the gingival margin-cement boundary of the lower left second molar, extended 4 mm apically [19]. Intra-observer calibration was performed by evaluating ten samples at two different time points (7 days apart). High correlation coefficient ($r > 0.8$) was obtained prior to the analysis.

Analysis of the maturation level of collagen fibers under polarized microscopy was performed in four equal areas in the distal extraction socket region stained with 0.1% Picrosirius Red. Images were captured using a polarized light microscope (Nikon Eclipse Ti-S) with a 20× objective lens. As the collagen packing density changed, the birefringence hue shifted from green to red (shorter to longer wavelengths). In ImageJ, color thresholds were determined by individual pixel selection using the tool Color Threshold, allowing the characterization of each pixel as: orange-red staining, representing densely packed collagen (collagen type I); yellow greenish staining, representing intermediately packed collagen or loosely packed (collagen type III) [7, 21]. The results were calculated by counting the number of pixels stained between bins with intensity thresholds set to 8-bit hue values (0–22 for red/orange, 23–49 for yellow, and 50–115 for green, modified from Smith and Barton [22]).

Biomechanical Testing

The left hemimandible from each animal was used in a three-point bending test to evaluate flexural bone strength using a universal testing machine (ElectroPuls E3000; Instron, Norwood, MA, USA). The specimen was placed perpendicular to the lingual side upward on two supports, with a span distance of 10 mm. The load was applied at the first molar midpoint (Figure 1) at a constant speed of 1 mm/min until fracture. The maximum load to failure (N) and ultimate stress (MPa) were determined.

Statistical analysis

Data were tested for normal distribution (Shapiro–Wilk test) and equality of variances (Levene's test), followed by parametric statistical tests. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used, with a significance level of $\alpha=0.05$. Statistical analyses were performed using the SigmaPlot® software (SigmaPlot v13.1; Systat Software Inc., San Jose, CA, USA).

RESULTS

Both irradiation protocols and surgical procedures were well tolerated. The adverse effects observed after irradiation were weight loss and localized alopecia, mainly in animals in the Ir20 and Ir30 groups, which showed severe alopecia.

The micro-CT results are shown in Figure 2. At 14 days after tooth extraction, BV/TV values were significantly different ($P = 0.003$) between the Nir (64.5 $\pm$ 9.5) and Ir30 irradiated groups, with less bone neoformation for Ir30 (44.1 $\pm$ 8.3). Among irradiated groups, there was less new bone formation ($P = 0.005$) in Ir30 compared to Ir15 (62.4 $\pm$ 7.5), but similar to Ir20 (53.4 $\pm$ 7.2). The BV and Tb.N values were significantly different ($P < 0.001$) among the Nir, Ir20 and Ir30 irradiated groups. Among the irradiated groups, there was a significant difference between the Ir15 and Ir30 groups with less bone volume and fewer trabeculae in the Ir30 group. There was no significant difference in the Tb.Th ($P = 0.523$) values between the groups. TV values were similar in all groups ($P = 0.129$), demonstrating that the delimited area of the socket for analysis was equivalent. The Tb.Sp values were significantly higher ($P < 0.001$) in the Ir20 (0.28 $\pm$ 0.06) and Ir30 (0.33 $\pm$ 0.05) groups than in the Nir group (0.15 $\pm$ 0.05). Among the irradiated groups, more widely spaced trabeculae are observed in the Ir20 and Ir30 groups than in the Ir15 group (0.18 $\pm$ 0.03). The microarchitecture of the alveolar socket repair for each group is shown in Figure 3. In the control group, the socket was completely filled with newly formed bone 14 days after tooth extraction. In the irradiated groups, the socket was not completely filled, and

alveolar socket healing was clearly delayed by ionizing radiation at Ir20 and Ir30, 14 days after tooth extraction.

The H&E histological results are shown in Figure 4. All groups had new trabecular bone in the alveolar socket 14 days after tooth extraction. In the Nlr group, mature trabecular bone filled the entire alveolar socket. Many osteocytes were observed throughout the trabecular bone. The Ir15 group showed similar bone repair as the control group. However, fewer active osteocytes were found in the trabecular bone and were localized predominantly at the perimeter of the bone tissue. The Ir20 group showed less newly generated bone and more connective tissue than the Nlr and Ir15 groups. Some active osteoblasts and osteocytes in the trabecular bone were associated with an acellular bone fragment in the extraction socket. The Ir30 group exhibited poorly structured tissues with some trabecular bones. Significant alveolar socket repair involves fibroblasts and connective tissues. Reorganization of collagen fibers at the center of the socket and migration of multinucleated cells from the periphery to the center were observed in the Ir30 group; however, these characteristics were not observed in the other groups.

The results of quantification of bone matrix neoformation are shown in Table 1 and Figure 5. A significant difference was revealed between the Nlr (67.9 ± 5.9) and irradiated groups ($P < 0.001$). Among the irradiated groups, a lesser amount of new bone was observed for the Ir30 (40.3 ± 6.5) compared to the Ir15 (59.5 ± 5.2) and Ir20 (53.9 ± 7.3) groups. No statistically significant differences were observed between the Ir15 and Ir20 groups.

The results of the collagen packing evaluation are shown in Figure 6. All groups had predominantly intermediately (yellow) and densely packed collagen (red). A small fraction of the collagen area ($< 1\%$) was occupied by loosely packed collagen (green) in all groups. The proportions of densely packed and intermediately packed collagen were similar ($P > 0.05$) for the Nlr group (62.5 ± 8.9) compared to the Ir15 (62.3 ± 9.0) and Ir20 (60.85 ± 9.28) groups. However, RT resulted in a decreased amount of densely packed collagen in the Ir30 group compared to that in the control group ($P < 0.05$).

The mean and standard deviation of the maximum load to failure (N) and ultimate stress (MPa) are listed in Table 2. No significant differences were found among the groups for maximum load to failure ($P = 0.314$) and ultimate stress ($P = 0.365$).

DISCUSSION

This study was established and characterized by our research group based on the experimental questions generated in previous studies [6 – 9]. This study aimed to determine the dose that would correlate with RT treatment to identify measurable differences between radiated and non-radiated bone formation. Tooth extraction and subsequent irradiation were performed to mimic the common clinical conditions indicated for patients undergoing head and neck RT. Moreover, stabilizing oral health before RT is crucial, as dental extractions or invasive interventions are risk factors for mandibular ORN [2, 23]. Different radiation doses were used to understand the effects of radiation on alveolar bone repair following tooth extraction in rats. This experimental model can help assess which single dose of IR should be applied in preclinical RT studies and whether rat mandibular first molar extraction is a suitable experimental model to study alveolar repair.

RT is largely used for therapeutic purposes; however, its application often results in deleterious effects [24]. In the present study, we observed that RT negatively affected the alveolar repair process after tooth extraction, rejecting the null hypothesis. High doses of radiation (30 Gy) resulted in the greatest damage to the bone. Alterations in the healing process are caused by changes in the woven bone microstructure, with a decrease in bone volume, number of trabeculae, and mature collagen fibers in the root socket. These aspects were confirmed by the micro-CT and histomorphometric results of the present study. Both methodologies confirmed a delay in the bone-healing process in the early stages after tooth extraction. In contrast, the lower IR dose of 15 Gy was similar to that of the control group, demonstrating that this radiation dose was relatively low enough to cause irradiation sequelae in the rat experimental model. This was also demonstrated in a previous study, where changes in bone mineral content

and reduction in blood flow were only observed after single doses higher than 20 Gy [26, 26].

A dose-dependent decrease in osteoblast proliferative capacity has been demonstrated, suggesting a reversible or permanent change in cell cycle distribution [23, 27]. High irradiation doses may impair the formation of the extracellular matrix and alter bone structure [28]. Previous studies examining the effects of radiation on the murine mandible have demonstrated consistent biomechanical, histological, and radiological changes that correlate with the radiation dose used [5]. In our study, significant changes in bone volume, mineral quality, and mineral maturity were observed in irradiated groups.

Alterations in bone strength seem to be more related to changes in trabecular morphology than to changes in the cortical morphology of irradiated bone [3, 29, 30]. Nevertheless, the influence of radiation on the mechanical properties of bone is still not fully understood, and conflicting results have been reported [29, 31, 32]. In the present study, no significant differences were observed among the biomechanical parameters tested. The IR dose had no effect on the maximum load to failure or ultimate stress of the bone tissue. This behavior may be related to the relatively low compared with the mandibular ORN models. Studies have demonstrated a significant effect of doses > 60 Gy on biomechanical parameters in animal models of mandibular ORN [15, 31, 33].

Collagen provides a scaffold on which hydroxyapatite crystals can mineralize, thus contributing to bone strength and structural integrity [34]. Type I collagen has been reported to enhance osteoblastic differentiation and bone formation through the osteoblast differentiation into mature osteoblasts with collagen fibril synthesis [35, 36], playing an important role in the regulation of osteogenesis. The present study demonstrated that the birefringence observed in the Ir30 group reflected the predominance of collagen fibers with a lower tissue maturation level (collagen type III) compared with the Nlr group, thus affecting the beginning of the bone mineralization process [35].

Histological changes in the mandibular bone tissue of irradiated rats have been observed at lower doses of radiation [3, 11, 37]. Interestingly, in the present study, only higher doses (20–30 Gy) caused significant delays in alveolar bone

repair 14 days after tooth extraction. This result can be attributed to different factors, such as variability in the methodologies used, the type of animal, the extraction socket analyzed (size and area), the evaluation period, and the method of analysis. An important factor to consider is the RT administration protocol. In the present study, external radiation was used, similar to the standard protocol for head and neck radiotherapy [6, 11, 38, 39]. The mandible was chosen for irradiation because it was possible to stabilize the animal on the skull, and the eye region could be excluded from the radiation field because a high dose could induce early mortality [37].

Several studies have demonstrated the impact of single dose versus fractionated radiation and irradiation times [23, 40]. Fractionated irradiation is preferred over a single dose to better mimic human therapeutic irradiation [41]. However, its implementation is complicated by the need for repeated anesthesia and the stress caused by animal transportation, which increases the probability of the premature loss of experimental specimens [5, 15, 20, 39, 42].

IR therapy generally involves a fractionated regimen of 1.8–2 Gy per fraction, with five daily doses per week, resulting in 65–70 Gy, which is equivalent to 30 Gy in a single dose [5]. RT triggers a sequence of events that are responsible for the delay in the alveolar repair process compared to the non-irradiated group. The exacerbated inflammatory response of alveolar repair process is responsible for the impairment of collagen fiber organization, as well as the normal mineralization process, resulting in poor-quality bone healing after tooth extraction.

During alveolar bone healing, the bone presents mature tissue morphology with increased trabecular thickness and, consequently, a decrease in trabecular space [17]. This was also observed in the Nlr group. The Ir20 and Ir30 irradiated groups presented fewer bone trabeculae and, consequently, greater trabecular separation. Low bone formation during alveolar healing suggests that the coupling of osteoblast and osteoclast functions during bone remodeling is affected by RT [43].

This study had some limitations. Our follow-up period was limited to 14 days after tooth extraction. Evaluation of long-term dose-dependent effects is

necessary. The present study investigated the effect of RT on alveolar bone healing using an experimental animal model; however, the results should be carefully interpreted because the effects of RT on alveolar bone healing in human patients could differ from these results. However, this study consistently contributes important findings regarding the radiation dose used in designing experiments to study the effects of RT using animal models. The single dose of 30 Gy, even presenting the same limitations to direct translation for the human clinical perspective, is certain that the effect caused by RT is presented, and can then be used for testing different therapeutic preventive protocols. Clinical studies are needed to establish preventive protocols for patients treated with RT who require oral bone surgery, such as tooth extraction [19].

CONCLUSION

Within the limitations of study design this study, it could be concluded that:

- Ionizing radiation has shown that bone alveolar healing after tooth extraction in rat mandibles is dose dependent.
- A single dose of 30 Gy caused a significant delay in the bone healing process, resulting in a lower amount of newly formed bone (BV/TV) and a lower percentage of densely packed collagen. However, the dose and experimental period used in the present study did not affect bone strength as evaluated by the biomechanical assay.
- A single dose of 30 Gy can be successfully used in an animal model to study an irradiated mandible that mimics the irradiated clinical condition.

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Figure captions

Fig. 1 Biomechanical test. A: Schematic view of three-point bending test of the rat mandible in material-testing machine. B: Mandible was placed with placed lingual side upward on 2 vertical bars with a distance of 10 mm. The central loading point was aligned at the first molar midpoint

Fig. 2 Mean and standard deviation values of the bone morphometric parameters analyzed in the alveolar bone repair area by micro-CT. Different letters indicate statistically significant differences verified by Tukey test ($p < 0.05$) for comparison of each parameter between the groups. Nlr: Non-irradiated group; Ir15: Irradiated with a dose of 15Gy; Ir20: Irradiated with a dose of 20Gy; Ir30: Irradiated with a dose of 30Gy

Fig. 3 Region of repair in the alveolar socket at 14 days after tooth extraction. A: delimitation of a predefined circular ROI. B: Nlr group. C: Ir15- irradiated with a dose of 15Gy. D: Ir20 - irradiated with a dose of 20Gy. E: Ir30- irradiated with a dose of 30Gy

Fig. 4 Histological aspects of tooth extraction site and adjacent area (H&E stained). Approximated view shows the woven bone formed in the central portion of the socket. Thinner trabeculae with moderate amount of connective tissue between them were observed in Ir20 and Ir30 groups. Abbreviations and symbols: organized bone trabeculae (##); connective tissue (++); osteoblasts on the periphery of the bone (black arrows); bone fragment (§); empty osteocyte lacunae (red arrows); fine trabecula (circle); connective tissue/inflammatory infiltrate (**); unorganized bone trabeculae (&); osteocyte being embedded (red rectangle). Original magnification: 10× and 20×

Fig. 5 Histological aspects of tooth extraction site and adjacent area (Masson's trichrome stained). Bone formation was significantly increased in Nlr group and the percentage of soft tissue was higher in irradiated groups, with less newly formed bone for Ir30. Abbreviations and symbols: new bone formation (##); connective tissue/inflammatory (&); extraction socket (red rectangle). Original magnification: 5× and 20×

Fig. 6 Quantification analysis of collagen levels (mean±SD). A: polarized photomicrograph (PicroSirius Red stained) of the woven bone. B: red pixels

selected (white area) by color threshold. C: yellow pixels selected (white area). D: green pixels selected (white area). E: normalized proportion of green, yellow and red birefringent collagen bundles in Nlr and irradiated groups. (Different letters indicate statistically significant differences verified by Tukey test ($p < 0.05$) between non-irradiated and irradiated groups). Nlr: Non-irradiated group; Ir15: Irradiated with a dose of 15Gy; Ir20: Irradiated with a dose of 20Gy; Ir30: Irradiated with a dose of 30Gy

Tables

Table 1. Mean and standard deviation (SD) of percentage of newly formed bone and soft tissue.

Groups	Newly formed bone (%)	Soft tissue (%)
Nlr	67.9 ± 5.9 ^A	32.1 ± 5.9 ^C
Ir15	59.5 ± 5.2 ^B	40.5 ± 5.2 ^B
Ir20	53.9 ± 7.3 ^B	46.1 ± 7.3 ^B
Ir30	40.3 ± 6.5 ^C	59.7 ± 6.5 ^A

Nlr: Non-irradiated group; Ir15: Irradiated with a dose of 15Gy; Ir20: Irradiated with a dose of 20Gy; Ir30: Irradiated with a dose of 30Gy. Different letters in the same column are significantly different, according to ANOVA and the Tukey test ($P < 0.05$).

Table 2. Mean and standard deviation (SD) of load failure (N) and ultimate stress (MPa).

Groups	Load failure (N)	Ultimate stress (MPa)
Nlr	124.2 ± 12.8 ^A	231.5 ± 24.7 ^A
Ir15	107.4 ± 13.7 ^A	201.4 ± 25.6 ^A
Ir20	110.6 ± 21.4 ^A	207.3 ± 40.2 ^A
Ir30	108.0 ± 18.5 ^A	202.9 ± 34.6 ^A

Nlr: Non-irradiated group; Ir15: Irradiated with a dose of 15Gy; Ir20: Irradiated with a dose of 20Gy; Ir30: Irradiated with a dose of 30Gy. Different letters in the same column are significantly different, according to ANOVA and the Tukey test ($P < 0.05$).

Figures

Fig.1

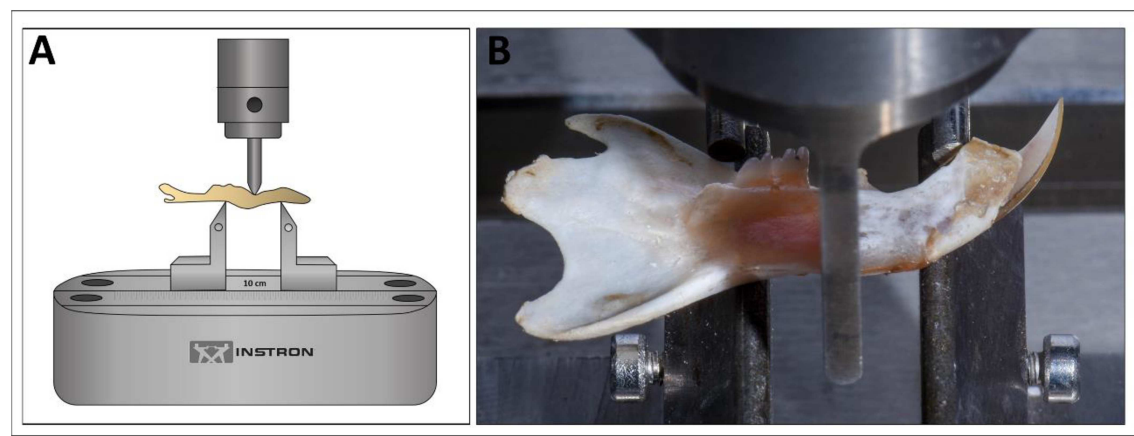


Fig.2

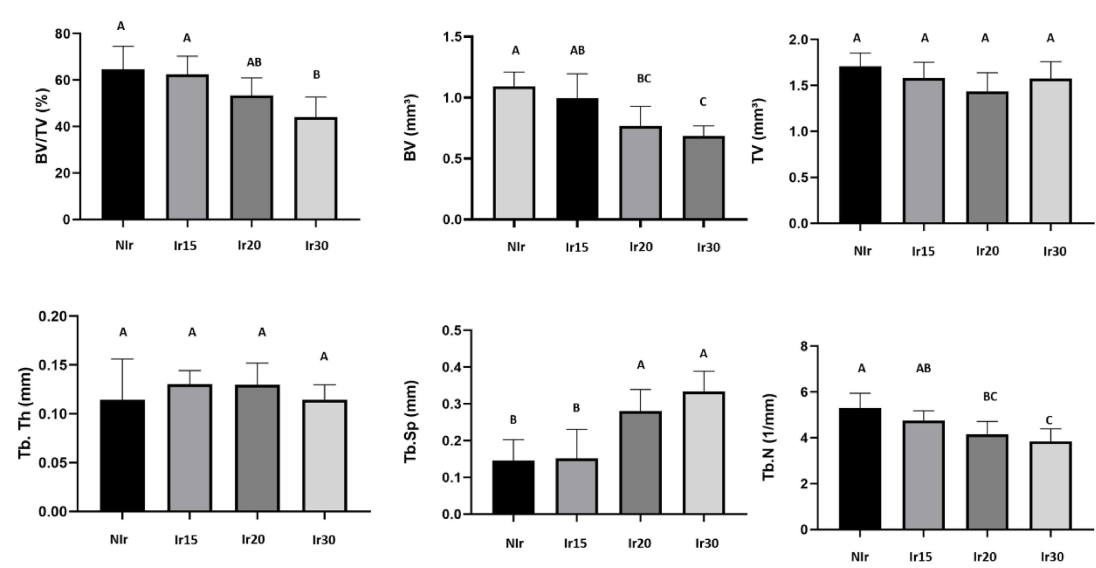


Fig.3

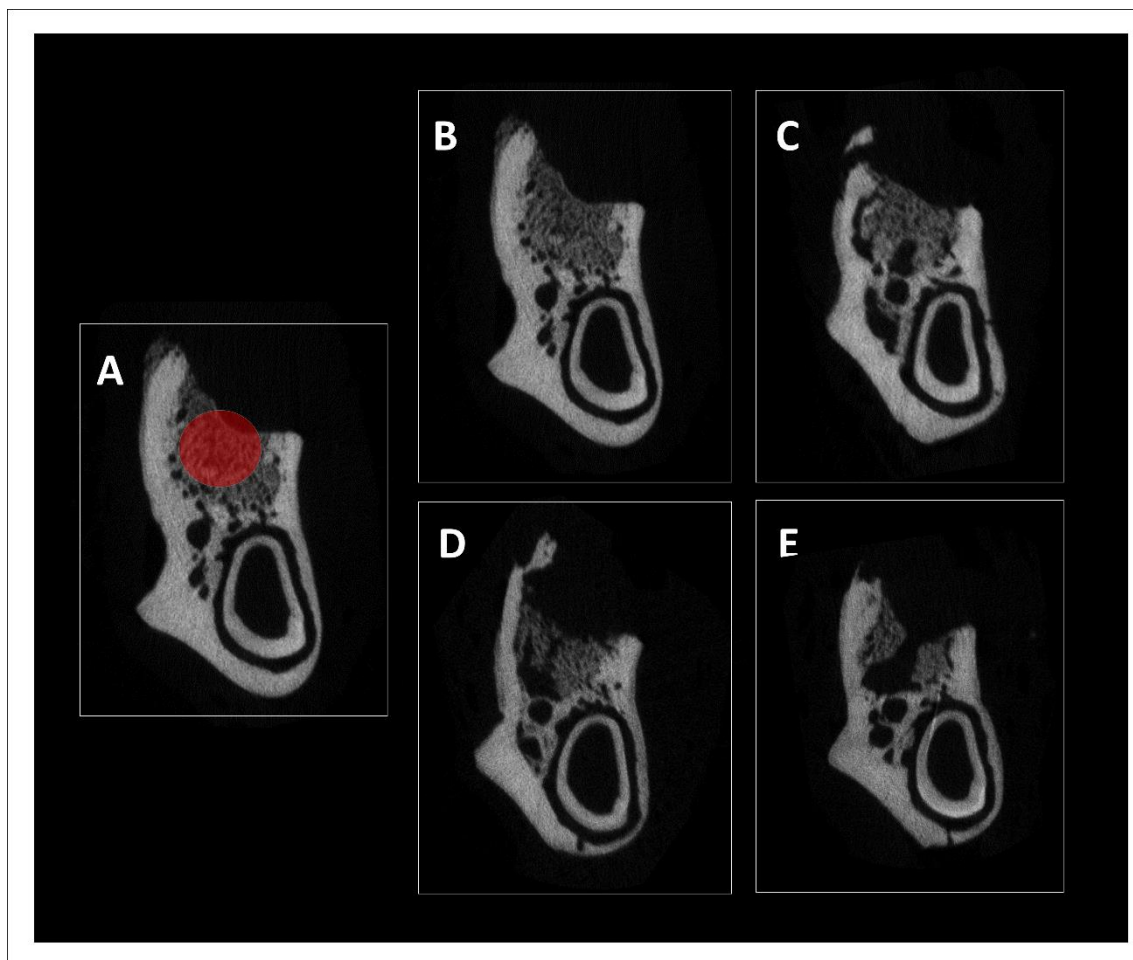


Fig.4

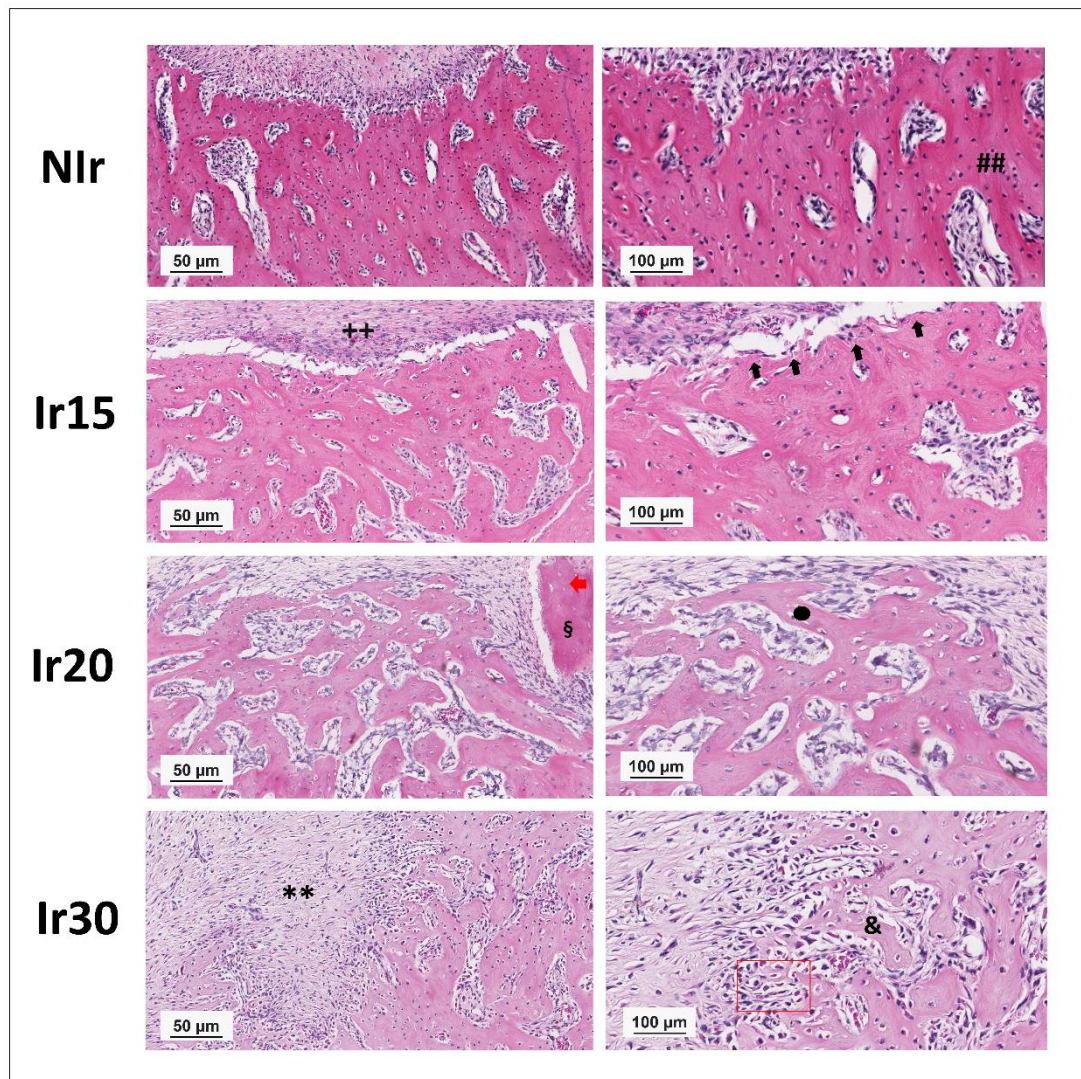


Fig.5

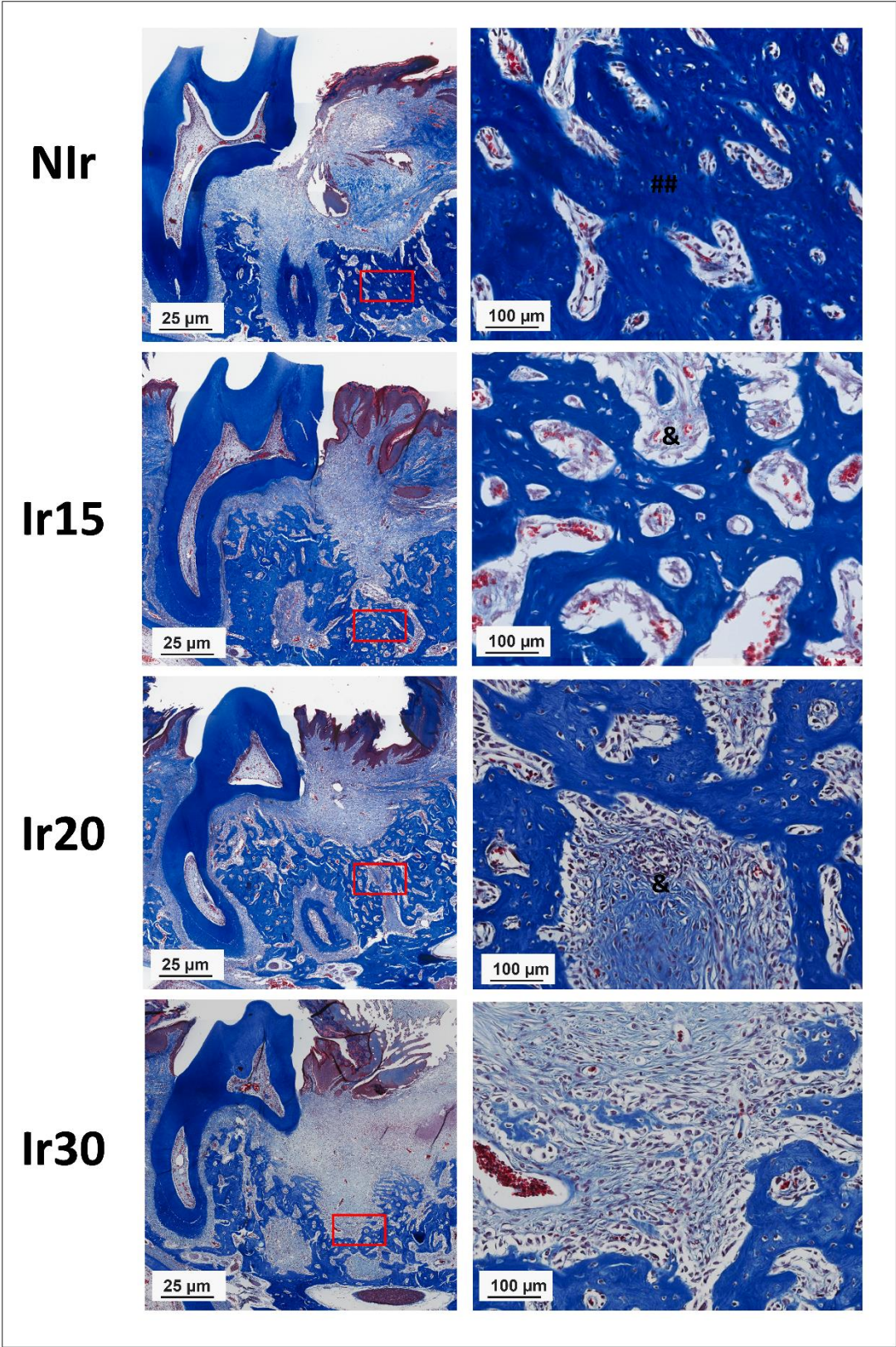
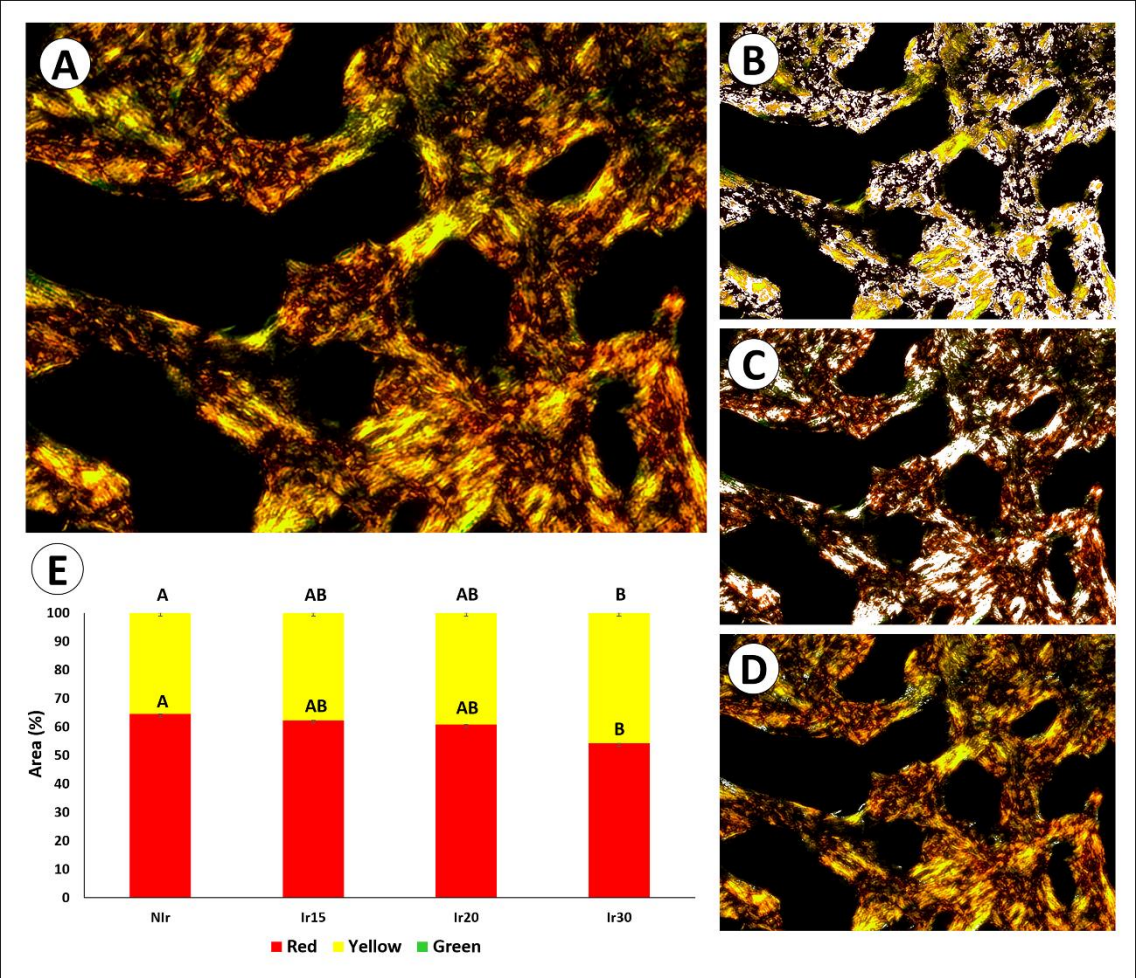


Fig.6



3.3 Capítulo 3

Artigo que será submetido ao periódico *Journal Periodontal Research*.

Effects of ionizing radiation after experimental periodontitis treatment in rats: A morphometric and histopathologic Study

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Conflict of interest: The authors declare that there is no conflict of interest.

Ethical approval: This study was approved by the Ethics Committee on Animals Use of Federal University of Uberlandia (CEUA 103/19). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

ABSTRACT

Background: The aim of this study was to investigate the effects of ionizing radiation (IR) on periodontal repair after treatment of experimental periodontitis in rats. **Methods:** Twenty-eight male Wistar rats were randomly divided to 4 groups (n=7): control group (nPDnIr): non-ligated and nonirradiated; irradiated group (nPDIr): non-ligated and irradiated; periodontal disease group (PDnIr): ligated and nonirradiated; periodontal disease + irradiation group (PDIr): ligated and irradiated. Experimental periodontitis was silk ligatures-induced into the second mandibular molars for 7 days, the contralateral side served as a control, non-ligated. The ligatures were removed and the animals of the irradiated group received a single dose (30Gy) of ionizing radiation at 7 and 20 days after removal of the ligature. After 7d of irradiation, the rats were sacrificed. Micro-computed tomography (micro-CT) was used to measure alveolar bone loss and hematoxylin and eosin staining for histological analysis. Statistical analysis was performed using the Shapiro–Wilk test, ANOVA and Tukey's test for normal data and Kruskal-Wallis and Dunn's test for non-parametric data ($p < 0.05$).

Results: The linear analyzes (alveolar bone loss), the experimental periodontitis groups showed higher values of distance between the cemento-enamel junction (CEJ) to bone crest (BC) regardless of the evaluated region and experimental period used ($p < 0.05$). In addition, the animals submitted to radiotherapy also showed higher values for CEJ-BC than the groups that did not undergo radiotherapy, regardless of the experimental period ($p < 0.05$). Bone volume fraction data were lower for the PDIr at 20 days group. The histological analysis, RT and PD significantly influenced in inflammation and fibrosis ($p < 0.05$).

Conclusions: Ionizing radiation modulated the extension of alveolar bone loss. In the long term (20 days), IR increased alveolar bone loss in an experimental periodontitis rat model, even after treatment of periodontal disease. Ionizing irradiation upregulates the inflammation and this effect may be related with the impairment of the periodontal repair.

Keywords: Ionizing radiation; X-Ray Microtomography; Experimental periodontitis; Inflammation; Alveolar bone loss; Periodontal disease

INTRODUCTION

Periodontal disease (PD) is a chronic inflammatory condition caused by the colonization of highly complex subgingival biofilm on the tooth surfaces, which may affect gingival tissue, periodontal ligament, cementum, and alveolar bone [1,2]. Pathogenesis is considered a dysbiotic process resulting from an imbalance between periodontopathogens microorganisms of the biofilm and the host responses [3,4], and is modulated through multiple factors including genetics, smoking, general health, social variables, and nutritional status [5]. The pathophysiology of periodontitis is characterized by key molecular pathways. The proteinases produced by the host are activated, therefore, destruction of the periodontal ligament fibrils, as well as apical migration of the junctional epithelium, occur, which creates a favorable environment for the bacterial biofilm to spread along the root surfaces [6, 7].

The conventional treatment for periodontitis relies on the mechanical removal of bacterial plaque from the periodontal pockets by scaling and root planning, which are considered the conventional and essential procedures in periodontal therapy [8,9]. Several factors increase the risk of periodontal diseases. These risk factors, modifiable and non-modifiable, contribute toward the clinical significance of periodontal diseases, such as cigarette smoking; medications such as steroids and anti-epilepsy, cancer therapy drugs; systemic diseases such as diabetes mellitus, cardiovascular disease, osteoporosis and others [10].

According to the International Agency for Research on Cancer, the worldwide incidence of cancer is expected to reach 27.5 million by 2040 [11]. In head and neck cancer, adjuvant radiotherapy (RT) is often a critical component of treatment [12,13]. The delivery of that radiation is associated with devastating collateral effects on surrounding healthy tissues. These complications still persist despite advancements in administration protocols and technological advances in radiation oncology [14].

Several secondary effects related to RT can occur in the oral cavity, including progressive periodontal attachment loss, dental, soft tissue necrosis, and osteoradionecrosis (ORN) [15]. Patients who receive radiation in the head

and neck region have increased risk for periodontal disease, which may be associated with hyposalivation and oral microbiome modification [16,17], especially in patients with periodontitis [18]. The prevalence of PD in adults is frequent and is likely to get worse with oncology treatment. The effect of RT on the periodontium seems to depend on the dose and is associated with periodontal problems before radiotherapy initiation, therefore periodontal infection should be eliminated in the pre-radiotherapy period [17,18].

Numerous experimental studies have been conducted to determine the mechanism of destruction caused by periodontal disease, to prevent destruction, or to determine the ways to compensate for the consequences of this destruction [4, 7, 8, 19]. However, few efforts have been carried to assess the destruction caused by ionizing radiation (IR) in situations after periodontal treatment [18]. It is not known for sure how periodontium behaves when high dose fraction is used. Alterations in the cellularity, vascularity, and the repair potential of the periodontium has been shown, however the literature is scarce [17]. Therefore, the aim of this study was to investigate the effects of ionizing radiation on periodontal repair after treatment of experimental periodontitis in rats. The present study was designed to evaluate whether a single dose of ionizing radiation (30 Gy) influence the periodontal repair after ligature removal in animal model of periodontitis. The null hypothesis is that RT does not affect periodontal repair after treatment of experimental periodontitis in rats.

Materials and methods

Animals

The experimental protocol was approved by the Institution's Ethics Committee on the Use of Animals (CEUA 103/19) of the institution, according to the recommendations of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The animals were conditioned in plastic boxes in groups of 4 in a controlled-environment room (temperature, 23 °C ± 3 °C; humidity, 25%; light/dark cycle, 12/12 h), with free access to rat food and water *ad libitum*. The study included 28 male rats (*Rattus norvegicus*, *albinus*, Wistar), aged approximately 9 weeks and weighing 275–325 g.

Ligation-induced experimental periodontitis

Experimental periodontitis was induced at the beginning of the experiment by the accumulation of bacterial biofilm through the ligature in the second lower molar [20]. The animals were submitted to intramuscularly anesthesia combining of 0.1 mL of ketamine hydrochloride (Ketamina Agener®; Agener União Ltda, São Paulo, SP, Brazil), along with 0.05 mL of xylazine hydrochloride (Rompum® Bayer SA, São Paulo, SP, Brazil) per 100 g of body weight. Ligatures (3–0 silk suture, Ethicon, Johnson & Johnson, Somerville, New Jersey) were placed submarginally [18, 21] around the second mandibular molar on the right side, in the most cervical position possible, by two nodes on the buccal side.

To facilitate ligature placement, a slightly separation in the interproximal area with the aid of a periodontal probe (Hu-Friedy, Chicago, Illinois) was performed. In all animals, the ligature was inspected every other day and repositioned if necessary, to maintain the ligature during the entire experimental period. For the ligation conference the animals were anesthetized with an inhalation method using Isoforine® (Cristália Prod. Quím. Farm. Ltda). Ligatures were maintained in position in order to allow biofilm accumulation during the 7-day of periodontal disease experimental induction [20, 22]. The second molars on the left side were left no-ligated so that they could be used as controls (periodontally healthy, nPDnIr).

Periodontal treatment

Periodontal treatment was performed under general anesthesia with the removal of the ligatures after 7 days. Subsequent to the development of the experimental periodontitis, ligatures were removed, and rats were randomized into four groups: Control group (nPDnIr), rats were non-ligated and nonirradiated; Irradiated group (nPDIr), rats were non-ligated and irradiated; Periodontal disease group (PDnIr), rats were ligated and nonirradiated; Periodontal disease + irradiated group (PDIr), rats were ligated and irradiated;

In addition, the groups were evaluated at two evaluation periods, with 7 and 20 days after periodontitis treatment (ligation removal) (n=7 each group)

(Figure 1). Removal of the ligatures allows investigation of the resolution of inflammation and the healing response [23].

Ionizing radiation

After 7 days (nDPIr7d; DPIr7d) and 20 days (nDPIr20d; DPIr20d) of PD treatment by removing the ligature, the animals in the irradiated group received a single dose of 30 Gy of ionizing radiation in the mandible bilaterally through a medical linear accelerator 6MeV (Varian 600-C © Varian Medical System Inc Palo Alto, California, USA) with a source-skin distance of 60 cm and the field size was 15 x 15 mm [24]. The animals were submitted to general anesthesia, positioned with only their mandible region in the radiation field and immobilized with adhesive tape. A wax bolus measuring 1,5 cm was placed over the mandible to increase the thickness of the soft tissues and concentrate the maximal dose on bone. The radiation dose was selected based on previous studies by the research group. Animals of nonirradiated groups were also anesthetized (sham method).

Sample collection

After 7 days of radiotherapy procedure, all animals were submitted to euthanasia by intraperitoneal overdose of thiopental (150 mg/kg). The mandible from each animal was dissected and then hemisected. The hemimandibles were removed and immediately fixed in 4% phosphate-buffered paraformaldehyde solution during 48h and were then kept in ethanol (70%). The samples were first scanned using X-ray microcomputed tomography (micro-CT) for morphometric analysis of alveolar bone loss and subsequently decalcified in 4% EDTA for 8 weeks, under agitation at room temperature, dehydrated with graded ethanol and embedded in paraffin. Longitudinal histological sections of 5 µm were obtained in the bucco-lingual direction (frontal plane), always in the long axis of the tooth and stained with hematoxylin and eosin (H&E) for histological analysis.

Microcomputed tomography (micro-CT) analysis

The samples were scanned using microcomputed tomography Skyscan 1272 microcomputed tomography (Bruker, Kontich, Belgium). Mandibles were scanned at 12- μ m resolution (X-ray source 80 kV, 125 μ A) using an aluminum filter (1 mm of thickness) and exposure of 400 ms with an angular increment of 0.4° and averaging of 2 frames. The reconstructions were performed using the software nRecon (version 1.6.10.1, SkyScan, Bruker, Belgium), following the parameters of smoothing (0), ring artifacts reduction (4) and beam-hardening (0%) for all samples. Three-dimensional (3D) volume viewing and analysis software (DataViewer, CT-volume and CT-analyser, SkyScan, Bruker, USA) were used to visualize and quantify 2D data. All images were reoriented to standard angles to view cemento-enamel junction and root apex in the same slices using Dataviewer software (Skyscan, Bruker-microCT).

The alveolar bone loss (ABL) was evaluated by single experienced operator, and the measurements were determined of distance between the cemento-enamel junction (CEJ) to bone crest (BC) [25, 26] in the mesial and distal root of the mandibular second molar teeth and the furcation ceiling (FC) to bone crest (BC) in the furcation region (Figure 2A,B) using software DataViewer (SkyScan, Bruker, USA). Mean values of measurements in millimeters were calculated for statistical analysis. The measurements were performed after intraexaminer calibration by evaluating 10 images. The examiner took the linear measurements twice within 48 hours.

For volumetric analysis, a standardized region of interest of was defined of 1,400 $\times$ 500 $\times$ 540 μ m (length $\times$ height $\times$ thickness), including the mesial, distal region and furcation of the second molar (Figure 2C) positioned on the images according to anatomical landmarks and a standardized gray-scale (63-255) value was used to visualize mineralized tissues only. Bone volume fraction (BV/TV in %), which means the percentage of the ROI filled with bone volume was calculated using CT Analyzer software (version 1.12.4.0 Bruker microCT, Skyscan, Belgium) by a trained examiner who was not aware of the experimental conditions of each sample.

Histomorphometric analysis

The hematoxylin and eosin-stained sections were scanned in a ScanScope AT Turbo® Scanner (LeicaBiosystems, Nussloch, Germany) in the 20× objective and semiquantitative histopathological scoring was performed. A total of 6 sections per specimen (42 sections for each experimental condition, from 7 different animals) were evaluated.

The Inflammatory cell infiltration (ICI), vascularization, hyperemia, Howship lacunae and fibrosis, in infrapapillary region and periodontal ligament were analyzed in interproximal (M-D) and furcation region of second molar by semiquantitative histopathological scoring as follows: according to the number of blood vessels, number of lacunae Howship (counted in cementum region) and number of vessels with hyperemia with score 0 = absent, score 1 = 1–5, score 2 = 6–9, score 3 = 10≤; according to Inflammatory reaction intensity; score 0 = none, score 1 = mild, score 2 = moderate, score 3 = extensive [7]. The fibrotic component was scored as no fibrosis (0), slightly visible fibrosis (1), or dense fibrosis (2) [27].

The total number of empty osteocytic lacunae in the alveolar bone in the interproximal (M-D) and furcation region were measured. Moreover, ORN was considered present when the bone presented loss of more than 5 contiguous osteocytes with confluent areas of empty gaps [28].

Statistical analysis

The Jamovi software (Version 1.6, software obtained free of charge from <https://www.jamovi.org>.) was used to carry out the statistical analysis of this study. The effect of the independent variables of this study (Induction of experimental periodontitis, irradiation and experimental period) on the study outcomes (alveolar bone loss, BV/TV% and empty osteocytic lacunae count) were evaluated using the Three-way Anova complemented by the Tukey test to compare the groups ($p < 0.05$). The results are expressed as mean $\pm$ standard deviation or percentage.

Kruskal-Wallis test was used for non-parametric data for the histological scores, followed by Dunn's test, when significant differences were detected ($p < 0.05$). The values were summarized by highest value expressed.

Results

Micro-CT analysis, it was found that the presence of periodontal disease, radiotherapy and the experimental period significantly influence the CEJ-BC data. Regarding data from linear analyzes (alveolar bone loss), groups with PD showed higher values of CEJ-BC regardless of the evaluated region and evaluation periods ($p < 0.05$). The animals submitted to RT also showed higher values for these parameters than the groups that did not undergo radiotherapy (nIrr), regardless of the experimental period ($p < 0.05$), except in the furcation region. In the proximal and furcation region, there was a significant difference ($p < 0.05$) in relation to the experimental period for the group with irradiated periodontal disease (PD Irr), with higher values of bone loss for the period 20 days (Table 1; Figure 3).

BV/TV% data were influenced by radiotherapy and periodontal disease induction. Regarding the BV/TV% data, it was verified that in the proximal regions, the PD Irr group presented lower values in both evaluation periods than the control- nPD nIrr group in both evaluation periods ($p < 0.05$) and that in the PD nIrr group at 20 days ($p < 0.05$). Additionally, it was found that the nPD Irr group had lower BV/TV% values in the proximal region than the control- nPD nIrr group in the 20-day period ($p < 0.05$). Regarding the values of BV/TV% in the furcation region, it was verified that the PD Irr 20d group presented the lowest values of this parameter than the other groups ($p < 0.05$) (Table 2).

The H&E histological results are shown in Figure 4. In the nPD nIrr and nPD Irr groups, regardless of experimental period, subepithelial connective tissue appeared normal. Periodontal ligament fibrous connective tissue appeared normal. The inflammatory infiltrate was absent or very few inflammatory cells were observed in a completely organized connective tissue. Howship lacunae were not observed on the surface of the cementum. In the PD nIrr group, regardless of experimental period, a greater degeneration of gingival epithelium

and inflammatory cell infiltration into subepithelial connective tissue were observed. Small Howship lacunae were observed on the surface of the cementum with zones of osteoclastic activity of different sizes toward the periodontal ligament. In the PDlr group, regardless of experimental period, an increased bone remodeling. A moderate inflammatory cell infiltration into the gingival epithelium, subepithelial connective tissue and periodontal ligament was observed and small Howship lacunae were observed on the cementum surface toward the periodontal ligament. No visual differences were observed between 7- and 20-days groups in all groups.

For the results of the semi-quantitative measurements, no statistically significant difference was observed among groups in terms of mesial region vascularization, distal region vascularization, hyperemia and Howship lacunae values ($p > 0.05$) (Table 3, 4). A significant increase in inflammation was observed in the PDlr group at 7 and 20 days ($p < 0.05$). For inflammation values in the mesial region, nPDnIr group at 7 and 20 days was significantly different ($p = 0,000092$) when compared to the PDnIr at 7 days, with higher values for PDnIr at 7 days. In the distal region, nPDnIr group at 7 and 20 days was different from PDlr at 20d, with higher inflammation values for PDlr ($p = 0.00015$). In the furcation region, nPDnIr group at 7 days, was significantly different when compared to the PDlr group at 7 and 20 days ($p = 0,000069$). Significant differences were also observed for the nPDnIr group at 20 days compared to the PDlr group at 7 days (Table 5).

There was no significant difference between groups for empty osteocytic lacunae count ($p > 0.05$) (Table 6). The presence of osteocytes within the osteocytic lacunae of the alveolar bone with absence of osteonecrotic areas were observed between groups, except for PDlr group at 20 days, that presented loss of more than 5 contiguous osteocytes with confluent areas of empty gaps in images of 3 animals in the group (Figure 5).

Discussion

Experimental animal models of periodontitis have provided important insights into the disease pathogenesis [20, 29, 30]. Among the various methods

employed to mimic periodontitis in animals, the most commonly used model is ligature-induction [20]. The use of a ligature throughout the cervical region of teeth leads to accumulation of biofilm, bacterial colonization resulting in apical epithelial migration and bone loss, symptoms similar to those observed in a clinical situation. The connective tissue and bone loss predictably occur over a period of 7–15 days in rats [20, 31]. In the present study, inflammatory cells infiltrated into the connective tissue was observed histologically in rat with experimental periodontitis. In addition, micro-CT results showed an increased CEJ-BC distance in the PD groups compared with the control group (nPD). Other studies also found gingival inflammation and alveolar bone resorption 7 days after ligation [20, 31, 32]. The results of the present study indicate that experimental periodontitis model was successfully established.

The periodontium is sensitive to irradiation, and morphologic and histologic changes are observed after RT [18, 33]. The effects of high-dose RT on the periodontium result in decreased vascularity and cellularity of the periodontal membrane and increased risk of periodontal attachment loss [17, 34]. Hypovascularity in the alveolar bone is related to endarteritis caused by head and neck radiotherapy and results in increased risk for the development of osteoradionecrosis, especially in patients with periodontitis [17, 18]. These effects can lead to periodontal disease (PD) progression, causing greater alveolar bone loss and greater risk for ORN [17].

This study evaluated the role of irradiation in the alveolar bone loss. The animals submitted to RT showed higher values for bone loss than the groups that did not undergo RT (nIr), regardless of the experimental period. Moreover, the lowest bone mineralization (BV/TV) mean value was observed in interproximal and furcation region for the PDIr group at 20 days when compared to the other experimental and control groups. The results of this study corroborate with other findings, that RT increased periodontal attachment level and alveolar bone loss in irradiated rats with experimental periodontitis [18]. This can show the unwanted long-term effects caused by irradiation in the periodontium after PD treatment. Moreover, it is possible to observe the irradiation effects in bone remodeling. This may represent a potential negative effect on tissue repair or turnover [35] in

experimentally induced periodontal disease in rats even after treatment with ligature removal. Thus, the null hypothesis that RT does not affect periodontal repair after treatment of experimental periodontitis in rats was rejected.

Moreover, no significant difference was observed for data obtained from BV/TV in the furcation region in the evaluated groups and their respective control at 7 and 20 days, except for the PDlr20d group. This result provides a comprehensive reading of the entire second molar region. The induction and treatment of PD in the evaluated experimental period, may have been sufficient to improvement of damage caused to bone tissue in furcation region [36].

Regarding the inflammation values, a similar pattern prevailed in all groups and periods evaluated for the mesial, distal and furcation region, with significant increase in inflammation for groups with experimental periodontitis - PDlr at 7 and 20 days and PDnlr at 7 days compared to control groups nPDnlr at 7 and 20 days. This is related with the stimulation of osteoclastogenesis, increasing the resorptive capacity of mature osteoclasts and the number of osteoclasts in bone tissue [37], also confirmed by the higher values of CEJ-BC for these groups, observed in the micro-CT analysis in the present study.

Evaluation of the effects of radiation on osteogenic cells demonstrated significant decreases in osteocytes, increased empty lacunae in the murine mandible [38]. In our study, there was no significant difference between groups for empty osteocytic lacunae count, demonstrating absence of ORN. Furthermore, the amount of osteocytes in bone tissue appeared normal between groups. High doses of RT were related to the development of ORN in sockets after tooth extraction [39, 40]. In fact, this event seems to be more related to bone repair after tooth extraction than associated with the treatment of periodontitis in an irradiated animal model.

Ionizing radiation leads to a decrease in cellularity and vascularity and resorption lacunae are seen in bone tissue [41, 42]. In our study, no difference was found for vessel counts in the interproximal region; vessels with hyperemia and howship lacunae. The divergent results may be attributed to variation in the experimental models used and the experimental period adopted [18].

However, animal models of a human disease have limitations. Molars in rats are similar in anatomic configuration and structure to humans, but are smaller, so it was difficult to perform any sort of periodontal treatment [27, 42], such as scaling and root planning. This procedure performed on the animal can lead to destruction of the gingival tissue and consequently affect histological processing [44]. A further limitation to the experimental model used is that in small animals, there may be the possibility of mechanical trauma by the ligatures, which contributes to alveolar bone loss [20, 31]. Regardless of this condition, biofilm accumulation and bacterial colonization is a major factor in the induction of bone loss [23], observed by the increase in ICI in the PD groups in this study.

It has been suggested that there is apparently an increased risk of alveolar bone loss in those individuals who are being treated for periodontal disease and were submitted to RT. Clinically, this reinforces the need for rigorous dental care for these patients. Despite the findings, it is important to emphasize that this is an animal study and its results cannot be directly applied to humans. More studies are needed to confirm these findings and improve the experimental model. In addition, molecular and immunohistochemical analyses can be important to understand the mechanisms involved in the development of RT associated with periodontal disease [28]. This study may form the basis for future research developing novel systemic or local host-modulation therapies, in addition to periodontal treatment, in order to prevent periodontal tissue damage in irradiated patients.

Conclusion

Within its limitations, this study showed that the

- Ionizing radiation modulated the extension of alveolar bone loss in periodontitis induced in rats.
- In the long term (20d), ionizing radiation increased alveolar bone loss in an experimental periodontitis rat model, even after treatment of periodontal disease.
- Ionizing irradiation upregulates the inflammation and this effect may be related with the impairment of the periodontal repair.

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Legend of figures

Fig 1. Fluxogram of the experiment. nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

Fig 2. Reconstructed 3D micro-CT images. A. Linear measurements of alveolar bone loss (ABL) determined of distance between the cemento-enamel junction (CEJ) to bone crest (BC) in the mesial and distal root of the mandibular second molar teeth and the furcation ceiling (FC) to bone crest (BC) in the furcation region. (Green line: JCE, red line: CO, yellow line: distance (mm)); coronal plane measurements. B. Linear measurements in the sagittal plane. C. Coronal view, a rectangular region was selected as the ROI to evaluate the ratio of the bone volume fraction (BV/TV).

Fig 3. Alveolar bone loss in the mandible in the region of the mandibular second molar observed by micro-CT. The groups induced to experimental periodontitis and ionizing radiation showed higher values of CEJ-BC compared to the other experimental groups ($p < 0.05$).

Fig 4. Histological analysis of the periodontal tissue of the left mandibular molar (H&E stained). The histological images of nPDnIr and nPDIr groups at 7 and 20 days showing an organized connective tissue, the inflammatory infiltrate was absent or very few inflammatory cells and howship lacunae were not observed on the surface of the cementum. In the PDnIr group at 7 and 20 days, a greater degeneration of gingival epithelium and inflammatory cell infiltration were observed. Howship lacunae were observed on the surface of the cementum. In the PDIr group at 7 and 20 days, an increased bone remodeling, a moderate inflammatory cell infiltration into was observed and Howship lacunae were observed on the cementum surface. Abbreviation and Symbols: ab: alveolar bone, pdl: periodontal ligament; CT: connective tissue; M2: mandibular second molar; *: infiltrate inflammatory; black arrows: howship lacunae. Magnification: 4 ×.

Fig 5. Histological aspects of presence of osteocytes within the osteocytic lacunae of the alveolar bone in region furcation (H&E stained). The presence of osteocytes within the lacunae were observed between groups, except for PDlr group at 20 days that presented loss of more than 5 contiguous osteocytes with confluent areas of empty gaps in images of 3 animals in the group, shown by red arrows. Magnification: 15×.

Figures

Fig 1

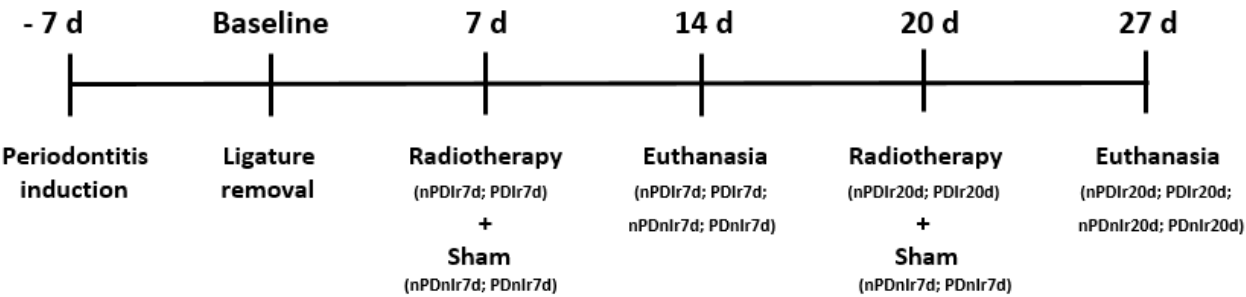


Fig 2

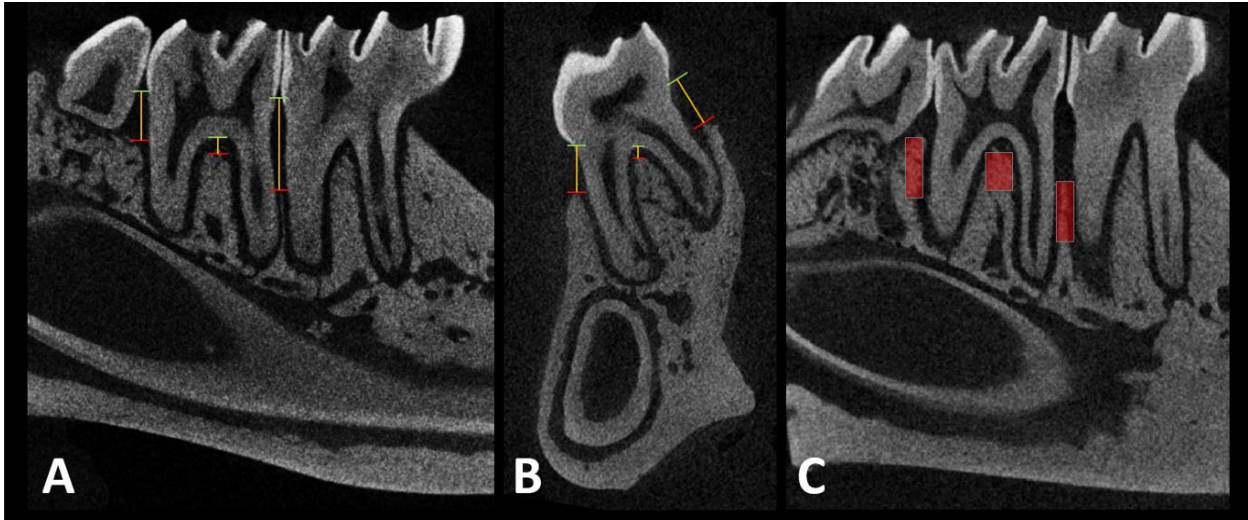


Fig 3

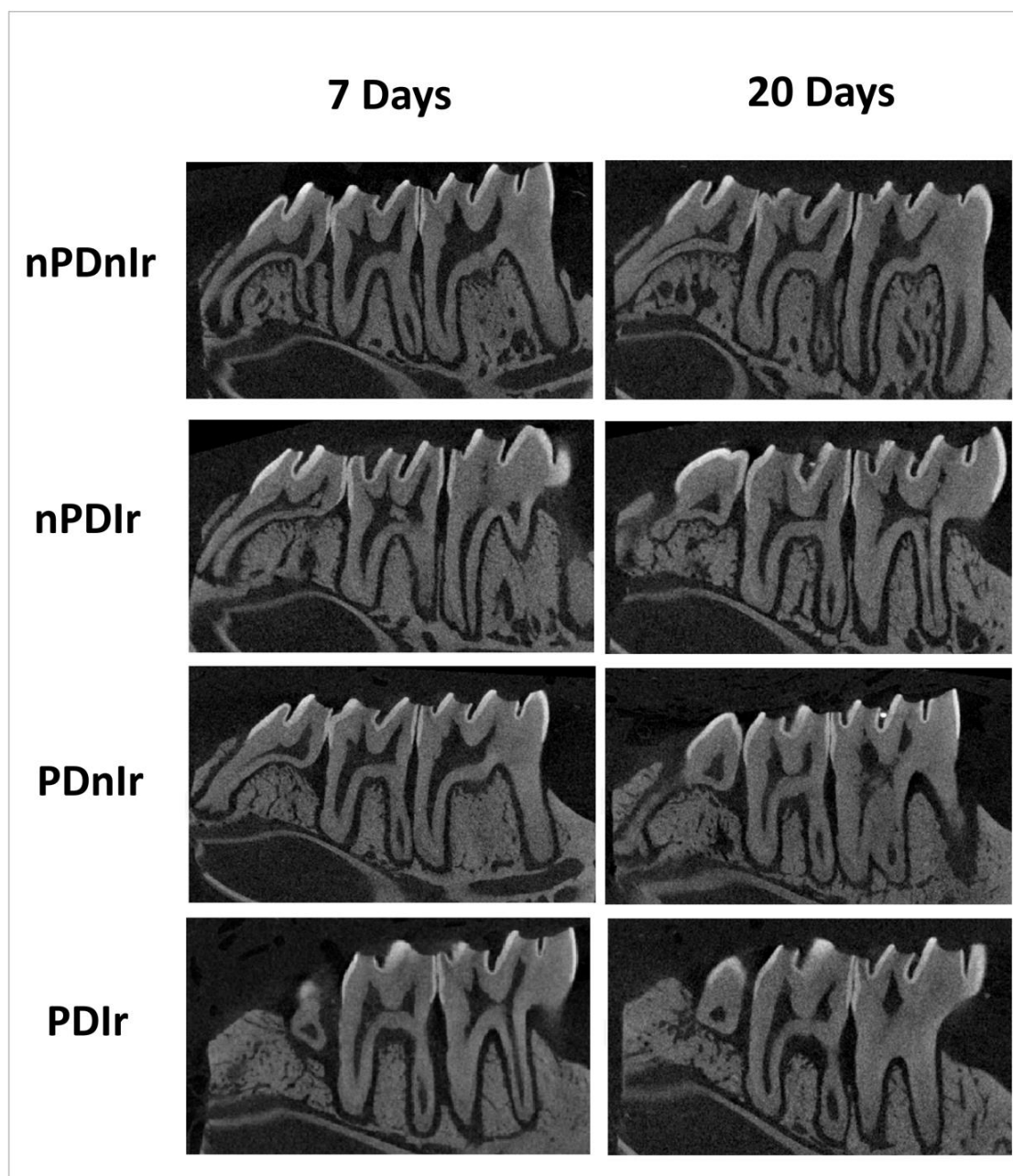


Fig 4

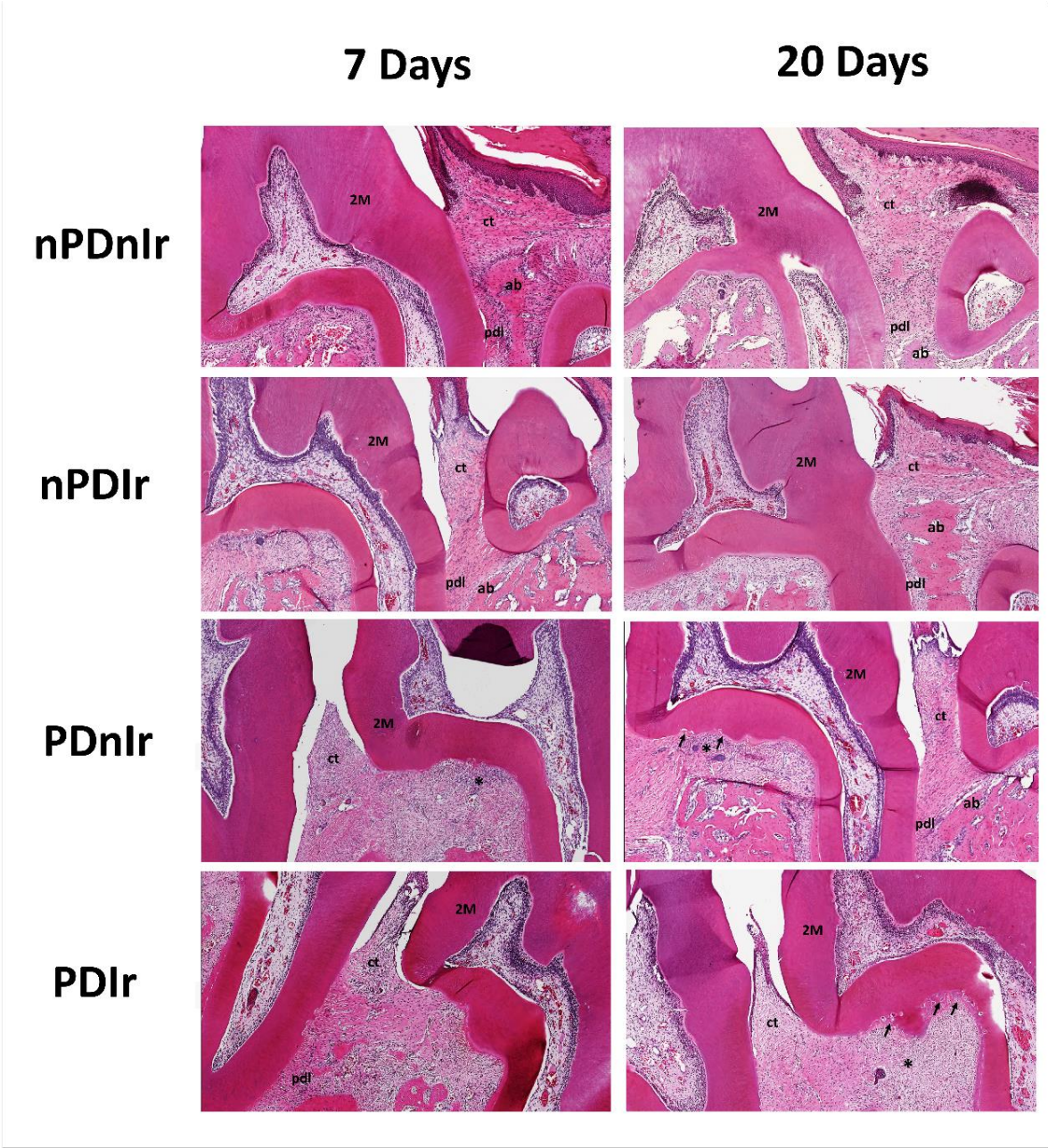
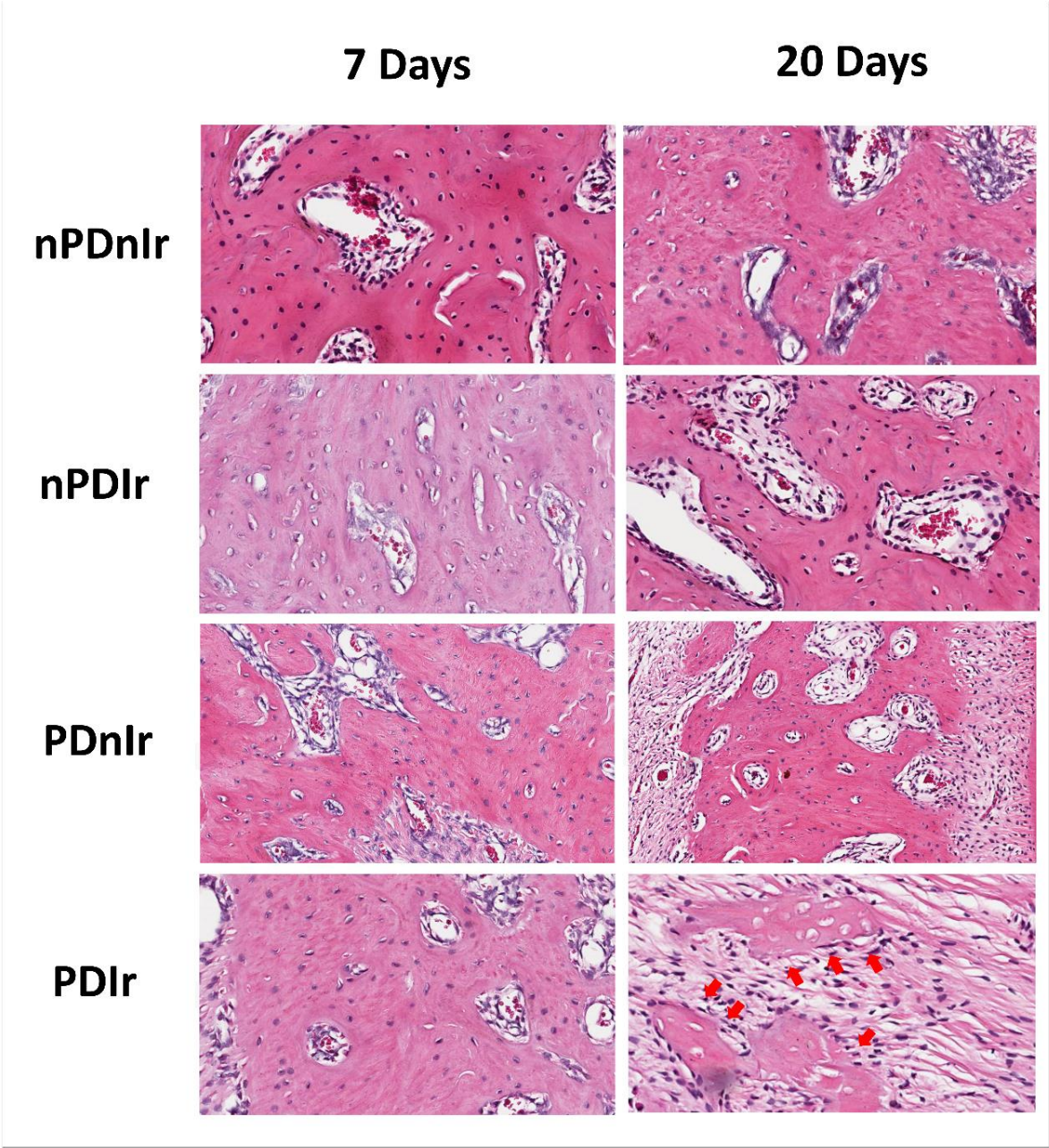


Fig 5



Tables

Table 1: Mean data and standard deviation of linear measurements by micro-CT performed in all groups and experimental periods.

Group	Experimental period	CEJ-BC Interproximal	CEJ-BC Faces livres	FC - BC
nPDnIr	7d	0.60 ± 0.08^a	0.47 ± 0.04^a	0.15 ± 0.05^a
	20d	0.41 ± 0.04^a	0.54 ± 0.04^a	0.13 ± 0.02^a
nPDIr	7d	1.02 ± 0.16^b	0.58 ± 0.06^b	0.18 ± 0.05^a
	20d	0.71 ± 0.13^b	0.53 ± 0.07^b	0.12 ± 0.02^a
PDnIr	7d	1.27 ± 0.11^c	1.00 ± 0.13^c	0.39 ± 0.14^b
	20d	1.17 ± 0.15^c	0.94 ± 0.12^c	0.30 ± 0.09^b
PDIr	7d	1.28 ± 0.18^c	1.11 ± 0.18^d	0.36 ± 0.11^b
	20d	1.64 ± 0.30^d	1.58 ± 0.33^d	1.01 ± 0.04^c

Different letters represent different levels of statistically significant differences. Three-way Anova complemented by Tukey. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days). Abbreviations: cemento-enamel junction (CEJ); bone crest (BC); furcation ceiling (FC).

Table 2: Mean and standard deviation data of BV/TV% values by micro-CT.

Group	Experimental period	BV/TV% Interproximal	BV/TV% Furcation
nPDnIr	7d	72.73 ± 1.77 ^a	79.56 ± 4.36 ^a
	20d	75.45 ± 8.01 ^a	79.70 ± 7.01 ^a
nPDIr	7d	66.96 ± 7.15 ^{a,b}	72.79 ± 10.10 ^a
	20d	62.67 ± 8.69 ^{b,c}	71.30 ± 6.17 ^a
PDnIr	7d	64.00 ± 6.03 ^{a,b}	69.12 ± 5.94 ^a
	20d	72.94 ± 6.10 ^{a,b}	72.02 ± 11.41 ^a
PDIr	7d	60.27 ± 9.31 ^b	68.91 ± 8.56 ^a
	20d	52.26 ± 9.59 ^c	48.46 ± 16.12 ^b

Different letters represent different levels of statistically significant differences. Three-way Anova complemented by Tukey. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

Table 3: Histopathological observations per groups.

Groups/ scores	Experimental period	Vascularization mesial					Vascularization distal					Vascularization furcation				
		0	1	2	3		0	1	2	3		0	1	2	3	
nPDnIr	7d	0	1	4	2	a	0	1	5	1	a	0	0	0	7	a
	20d	0	0	3	4	a	0	0	4	3	a	0	0	0	7	a
nPDIr	7d	0	0	6	1	a	0	0	6	1	a	0	0	3	4	a,b
	20d	0	0	5	2	a	0	1	4	2	a	0	0	6	1	b
PDnIr	7d	0	0	2	5	a	0	0	3	4	a	0	0	1	6	a
	20d	0	1	2	4	a	0	0	5	2	a	0	0	0	7	a
PDIr	7d	0	0	6	1	a	0	0	5	2	a	0	0	0	7	a
	20d	0	0	3	4	a	0	1	4	2	a	0	0	2	5	a,b

Different letters represent different levels of statistically significant differences. Kruskal-Wallis complemented by Dunn's. Abbreviations score 0 = absent, score 1 = 1–5, score 2 = 6–9, score 3 = 10≤. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

Table 4: Histopathological observations per groups.

Groups/ scores	Experimental period	Hyperemia					Howship lacunae					Fibrosis		
		0	1	2	3		0	1	2	3		0	1	2
nPDnIr	7d	5	1	1	0	a	7	0	0	0	a	0	1	2
	20d	6	1	0	0	a	6	1	0	0	a	7	0	0
nPDIr	7d	4	3	0	0	a	3	4	0	0	a	7	0	0
	20d	4	3	0	0	a	4	3	0	0	a	5	2	0
PDnIr	7d	4	3	0	0	a	4	3	0	0	a	0	6	0
	20d	5	2	0	0	a	3	4	0	0	a	1	6	0
PDIr	7d	2	5	0	0	a	2	5	0	0	a	5	2	0
	20d	2	5	0	0	a	2	5	0	0	a	5	2	0

Different letters represent different levels of statistically significant differences. Kruskal-Wallis complemented by Dunn's. Abbreviations: Hyperemia and Howship lacunae: score 0 = absent, score 1 = 1–5, score 2 = 6–9, score 3 = 10≤. Fibrosis: score 0 = no fibrosis, score 1 = slightly visible fibrosis, score 2 = dense fibrosis. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

Table 5: Histopathological observations per groups.

Groups/scores	Experimental period	Inflammation mesial					Inflammation distal					Inflammation furcation				
		0	1	2	3		0	1	2	3		0	1	2	3	
nPDnIr	7d	6	1	0	0	a,c	6	1	0	0	a,b,c	6	1	0	0	a
	20d	7	0	0	0	a	7	0	0	0	a	4	3	0	0	a,c
nPDIr	7d	4	2	1	0	a,b,c	3	3	1	0	a,b,c,d	1	6	0	0	a,b,c
	20d	4	3	0	0	a,b,c	3	3	1	0	a,b,c,d	3	3	1	0	a,b,c
PDnIr	7d	0	3	4	0	b,c	6	1	0	0	b,c,d	7	0	0	0	a,b,c
	20d	2	5	0	0	a,b,c	2	5	0	0	a,b,c,d	2	4	1	0	a,b,c
PDIr	7d	1	6	0	0	a,b,c	0	6	1	0	c	0	1	4	2	b
	20d	0	6	1	0	c	0	4	2	1	c,d	0	3	3	1	c

Different letters represent different levels of statistically significant differences. Kruskal-Wallis complemented by Dunn's. Abbreviations: score 0 = none, score 1 = mild, score 2 = moderate, score 3 = extensive. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

Table 6: Mean and standard deviation data of empty osteocytic lacunae count.

Group	Experimental period	Osteocytic lacunae number
nPDnIr	7d	4.0 ± 0.8 ^a
	20d	4.2 ± 0.8 ^a
nPDIr	7d	4.1 ± 0.9 ^a
	20d	4.2 ± 0.8 ^a
PDnIr	7d	4.2 ± 0.8 ^a
	20d	4.1 ± 0.9 ^a
PDIr	7d	3.9 ± 0.7 ^a
	20d	4.5 ± 0.7 ^a

Different letters represent different levels of statistically significant differences. Three-way Anova complemented by Tukey, $p < 0.05$. Three-way Anova complemented by Tukey. Groups: nPDnIr: control group (non-ligated and nonirradiated at 7 and 20 days); nPDIr: irradiated group (non-ligated and irradiated at 7 and 20 days); PDnIr - periodontal disease group (ligated and nonirradiated at 7 and 20 days); PDIr: periodontal disease + irradiated group (ligated and irradiated at 7 and 20 days).

4. CONCLUSÕES

Com base nas metodologias utilizada e nos resultados obtidos destes estudos, pode-se concluir que:

- A neoformação óssea no reparo alveolar após a extração dentária é influenciada pelo formato da ROI e pelo alvéolo de extração selecionado.
- O método predefinido com ROI padronizada para avaliação do volume ósseo na região central do alvéolo distal mostrou ser a região mais crítica do processo de neoformação óssea.
- A radiação ionizante mostrou que a cicatrização óssea alveolar após extração dentária em mandíbulas de ratos é dose dependente, com atraso no processo de cicatrização óssea e menor porcentagem de colágeno com dose única de 30 Gy.
- A radiação ionizante modulou a extensão da perda óssea alveolar na periodontite induzida em ratos em peridodo de 20 dias, mesmo após o tratamento da doença periodontal.
- A irradiação ionizante regula positivamente a inflamação e este efeito pode estar relacionado com o comprometimento do reparo periodontal.

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¹ De acordo com a Norma da FOUFU, baseado nas Normas de Vancouver. Abreviaturas dos periódicos com conformidade com Medline (Pubmed).

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ANEXOS

COMITÉ DE ÉTICA



Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –



CERTIFICADO

Certificamos que o projeto intitulado “Efeito da radioterapia na progressão do reparo ósseo e de sítios pós-extração dentária”, protocolo nº 013/19, sob a responsabilidade de **Priscilla Barbosa Ferreira Soares** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião **10 de maio de 2019**.

(We certify that the project entitled “Efeito da radioterapia na progressão do reparo ósseo e de sítios pós-extração dentária”, protocol 013/19, under the responsibility of Priscilla Barbosa Ferreira Soares - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of May 10th, 2019).

Vigência do Projeto	Início: 01/07/2019 Término: 01/11/2022
Espécie / Linhagem / Grupos Taxonômicos	Rato heterogênico Wistar
Número de animais	112
Peso / Idade	300g/ 90 dias
Sexo	Macho
Origem / Local	Rede de Biotérios de Roedores da UFU – REBIR-UFU
Local onde serão mantidos os animais:	Rede de Biotérios de Roedores da UFU – REBIR-UFU

Uberlândia, 04 de Junho de 2019.

Prof. Dr. Lúcio Vilela Carneiro Girão

UNIVERSIDADE FEDERAL DE UBERLÂNDIA

Comissão de Ética na Utilização de Animais /UFU

Coordenador da CEUA

Portaria Nº 542 DE 10 DE MAIO DE 2019



CERTIFICADO

Certificamos que o projeto intitulado “Efeito da radioterapia sobre o reparo dos tecidos periodontais após tratamento da doença periodontal experimental.”, protocolo nº **103/19**, sob a responsabilidade de **Priscilla Barbosa Ferreira Soares** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **APROVADA** pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião **08 de Julho de 2020**.

(We certify that the project entitled “Efeito da radioterapia sobre o reparo dos tecidos periodontais após tratamento da doença periodontal experimental.” protocol **103/19**, under the responsibility of **Priscilla Barbosa Ferreira Soares** - involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of July 08th, 2020).

Vigência do Projeto	Início: 01/01/2021 Término: 01/11/2022
Espécie / Linhagem / Grupos Taxonômicos	Ratos Wistar
Número de animais	60
Peso / Idade	300g / 90 dias
Sexo	Macho
Origem / Local	REBIR – UFU
Local onde serão mantidos os animais:	REBIR – UFU

Uberlândia, 19 de Outubro de 2020.

Prof. Dr. Luiz Fernando Moreira Izidoro
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenador da CEUA