Universidade Estadual De Campinas
Faculdade De Odontologia De Piracicaba

Jennifer Santos Pereira

Caracterização metalômica de lesões periapicais induzidas: estudo *in vivo*

Metallomic characterization of induced periapical lesions: an in vivo study

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2025

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Clínica Odontológica, na Área de Endodontia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Clinical Dentistry, in Endodontics area.

Orientadora: Profª. Dra. Marina Angélica Marciano da Silva
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Resumo

Objetivo: A periodontite apical é uma inflamação crônica associada a infecções endodônticas e diretamente regulada pela resposta imune do hospedeiro. Os metais são essenciais para o metabolismo e desempenham um papel crucial nas funções biológicas. No entanto, o perfil metalográfico diferencial entre a periodontite apical e a condição óssea saudável ainda não foi explorado. **Metodologia:** Este estudo incluiu 76 primeiros molares inferiores (de 38 ratos Wistar), comparando lesões periapicais induzidas e controles (sham). A indução das lesões periapicais foi realizada por meio da exposição pulpar dos dentes, permitindo o desenvolvimento da infecção. Após 40 dias, os animais foram reponderados, eutanasiados e suas hemimandíbulas analisadas por radiografia periapical, análise histológica, microtomografia computadorizada (μ-CT), microscopia de fluorescência de raios X (μ-XRF), microscopia eletrônica de varredura com espectroscopia de energia dispersiva (SEM/EDS), espectrometria de emissão óptica com plasma indutivamente acoplado (ICP-OES) e espectrometria de massa com plasma indutivamente acoplado (ICP-MS). Foram analisados dez metais essenciais ao metabolismo (sódio, potássio, magnésio, cálcio, ferro, manganês, cobalto, cobre, zinco e molibdênio). O nível de significância adotado foi de 5%. **Resultados:** A análise radiográfica confirmou a indução das lesões periapicais, sem diferença no peso dos animais entre as condições ($p > 0,05$). Histologicamente, as lesões periapicais apresentaram infiltrado inflamatório intenso, células com grânulos citoplasmáticos azulados, reabsorção alveolar e escores variando de moderado a intenso. A análise por μ-CT da lesão induzida revelou uma diferença significativa no volume da região periapical (12,74 mm^3, $p = 0,0017$). O SEM/EDS apresentou sensibilidade limitada para os elementos químicos investigados; entretanto, o μ-XRF identificou intensidades reduzidas para cálcio e zinco e aumentadas para ferro. As análises por ICP-MS e ICP-OES identificaram concentrações aumentadas de sódio ($p = 0,0137$), potássio ($p = 0,0005$), cálcio ($p = 0,0059$), magnésio ($p = 0,0004$), ferro ($p < 0,001$), ferro-56 ($p = 0,0078$), ferro-57 ($p = 0,0315$) e manganês ($p < 0,001$) na lesão periapical induzida, sugerindo um impacto direto na homeostase mineral decorrente dessa patologia. **Conclusões:** Este estudo demonstrou diferenças nos níveis de diversos elementos essenciais entre as condições com lesões periapicais e os controles saudáveis.

Palavras-chave: Endodontia, lesão periapical, metalômica, metal.

Abstract

Aim: Apical periodontitis is a chronic inflammation associated with endodontic infections and directly regulated by the host immune response. Metals are essential to the metabolism, playing a crucial role in biological functions. However, the differential metallographic profile between apical periodontitis and its healthy bone condition has not yet been explored. **Methodology:** This study included 76 lower first molars (from 38 Wistar rats) where induced periapical lesions and controls (sham) were compared. Periapical lesion induction was performed by pulp exposure of these teeth, allowing spontaneous infection development. After 40 days, the animals were reweighed, euthanised, and their hemimandibles analysed by periapical radiography, histological analysis, micro-computed tomography (μ-CT), X-ray fluorescence microscopy (μ-XRF), scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS), inductively coupled plasma optical emission spectrometry (ICP-OES), and inductively coupled plasma mass spectrometry (ICP-MS). Ten essential metals for metabolism were analysed (sodium, potassium, magnesium, calcium, iron, manganese, cobalt, copper, zinc, and molybdenum). The analyses observed a significance level of 5%. **Results:** Radiographic analysis confirmed the induction of periapical lesions, without difference in animal weight between the conditions ($p > 0.05$). Histologically, the periapical lesions showed intense inflammatory infiltrate, cells with bluish cytoplasmic granules, alveolar resorption, and scores ranging from moderate to intense. The μ-CT analysis of the induced lesion revealed a significant difference in the periapical region volume (12.74 mm^3, $p = 0.0017$). SEM/EDS showed limited sensitivity for the investigated chemical elements; however, μ-XRF identified diminished intensities for calcium and zinc and increased intensities for iron. ICP-MS and ICP-OES identified increased concentrations of sodium ($p = 0.0137$), potassium ($p = 0.0005$), calcium (0.0059), magnesium ($p = 0.0004$), iron ($p < 0.001$), 56iron ($p = 0.0078$), 57iron ($p = 0.0315$), and manganese ($p < 0.001$) within the induced periapical lesion, suggesting a direct impact on mineral homeostasis following this pathology. **Conclusions:** These findings indicate, for the first time, changes in the mineral profile in periapical lesions compared with healthy periapices highlighting the importance to investigate therapeutic or diagnostic approaches aiming to restore the mineral homeostasis disrupted by this pathological condition. Future metallic-content smart technology for both endodontic materials and diagnostic tools are desirable.

Keywords: Endodontics, periapical lesion, metallomics, metal

Sumário

1. INTRODUÇÃO

A periodontite periapical é uma doença inflamatória crônica, causada por infecção endodôntica, e o seu desenvolvimento é regulado pela resposta imune e inflamatória do hospedeiro (1–3). As comunidades microbianas endodônticas presentes na periodontite apical foram amplamente investigadas tanto em lesões primárias (4) quanto persistentes após o tratamento endodôntico (5). Assim, essas lesões são tratadas clinicamente através de um tratamento ou retratamento endodôntico que desinfecta, promove o selamento do canal radicular através da obturação e permite a reabilitação por promover o selamento da coroa dental (6). Os resultados a longo prazo do tratamento endodôntico são influenciados tanto pela natureza da interação hospedeiro/infecção (fatores inerentes ao paciente) quanto pela eficácia operatória do tratamento e manutenção da integridade do dente tratado para resistir à reversão da infecção (7).

Na tentativa de se descrever o mecanismo dessa condição patológica, estudos anteriores consideraram o papel da polarização dos macrófagos em lesões periapicais associada à infecção (8); o papel das endotoxinas bacterianas na etiologia da lesão periapical, incluindo mecanismos moleculares envolvidos no seu reconhecimento e na ativação celular (9); a potencial ação supressora das citocinas com implicações na intensidade da reação inflamatória e na extensão da perda óssea alveolar que resulta em lesão periapical (10,11); e a potencial influência da dieta no desenvolvimento de lesões periapicais (12).

Diversos eventos histológicos e etiopatogênicos ocorrem para o reparo de lesões periapicais após a terapia endodôntica (13) que incluem mecanismos de remodelação óssea para o reparo de periodontite apical crônica após a terapia endodôntica (14) e o processo de reparo que ocorre paralelamente à presença de lesão periapical exercido por células indiferenciadas (*stem cells*) (15). No entanto, mesmo considerando os esforços dos relatos anteriores no intuito de se estabelecer o mecanismo dessa patologia, nenhum estudo anterior considerou potenciais diferenças entre o perfil metalográfico da periodontite apical comparativamente ao estado de saúde analisando os níveis de elementos químicos metálicos essenciais ao metabolismo.

As ciências 'ômicas' são de fundamental importância considerando uma abordagem holística na definição de um modelo de doença (16). A metalômica compreende a análise da composição inorgânica de células, tecidos ou fluidos biológicos e determina possíveis alterações nos níveis de elementos químicos devido ao aparecimento de uma doença (17,18). O corpo humano precisa de cerca de vinte elementos essenciais para um correto metabolismo e, dentre eles, dez (sódio, potássio, magnésio, cálcio, ferro, manganês, cobalto, cobre, zinco e molibdênio) são elementos químicos metálicos (19,20). O comportamento catalítico de metais no metabolismo quando resulta na formação de radicais hidroxila reativos e estresse oxidativo pode causar danos ao DNA, proteínas e membranas (21). Dessa forma, o estabelecimento do perfil metalográfico da periodontite apical potencialmente contribuiria para um entendimento mais amplo das variações relacionadas aos elementos químicos essenciais para essa condição patológica endodôntica. Além disso, a compreensão do perfil metalográfico dessa patologia poderia alicerçar a futura investigação de marcadores e etapas operatórias que contribuiriam para um desfecho mais previsível do tratamento endodôntico.

O metaloma está envolvido em uma variedade de processos vitais, incluindo equilíbrio de carga e atividade elétrica, estrutura e conformação, sinalização, equilíbrio ácido-base, transferência de elétrons, armazenamento de energia e catalisação redox, além de contribuir para a biomineralização (22). Quando se realiza a combinação da investigação do metaloma com outros fenômenos biológicos, como o metaboloma e proteoma, é possível identificar redes de interações específicas nos órgãos em diferentes campos e estudos. Estudos têm demonstrado, que a concentração de ferro e a composição de isótopos de cobre estão relacionadas a indicadores de saúde metabólica, como percentual de gordura corporal e capacidade máxima de corrida, bem como processos bioquímicos como a adipogênese (22).

Além disso, em um estudo envolvendo a caracterização metalômica de amostras de plasma de crianças e adolescentes com obesidade e resistência à insulina, observou-se uma correlação significativa entre essas alterações multi-elementares e as complicações metabólicas comumente associadas à obesidade infantil. Especificamente, constatou-se que a presença de desequilíbrios nos elementos metálicos estava intimamente ligada ao comprometimento do metabolismo de carboidratos e lipídios, que é mediado pela insulina (23). Vale ressaltar também que o potássio é um elemento que ao ocorrer um distúrbio na sua homeostase pode provocar o desenvolvimento de várias doenças crônicas, incluindo hipertensão, doenças cardiovasculares, diabetes, condições neurodegenerativas e de saúde óssea. Nesse contexto, a composição isotópica do potássio tem se mostrado um biomarcador promissor para avaliar a saúde e o estado metabólico dos indivíduos (24).

Estudos anteriores (25–27) justificam a validade de adoção do modelo animal (*Wistar rat*) aqui proposto para a caracterização metalômica de lesões periapicais induzidas. É fundamental estabelecer a caracterização metalográfica inicial (sem outros fatores associados) comparativa entre as condições com e sem lesão periapical para servir de alicerce para futuras análises. Vale ressaltar a importância do presente estudo no sentido de esclarecer futuras correlações com estudos microbiológicos, especialmente considerando a questão de o porquê alguns indivíduos apresentam lesão periapical e outros não, mesmo com tratamentos endodônticos similarmente insatisfatórios (28). Além disso, estudos anteriores em periodontia buscaram estabelecer correlações tanto entre marcadores locais e sistêmicos quanto demonstrando alterações metalômicas em comparação entre as condições de saúde e doença (29–32) o que demonstra a preocupação dessa especialidade em investigar esse tipo de correlação.

2. ARTIGO: CARACTERIZAÇÃO METALÔMICA DE LESÕES PERIAPICAIS INDUZIDAS – ESTUDO IN VIVO

A presente dissertação foi formatada seguindo as normas do método alternativo de apresentação (Elaboração e Normalização de Teses e Dissertações da FOP/Unicamp, 2015 e atualização de 2023) incluindo um artigo.

Artigo foi submetido ao periódico *International Endodontic Journal* com título Caracterização metalômica de lesões periapicais induzidas: estudo *in vivo* (Anexo 2).

Introduction

Apical periodontitis is a chronic inflammatory disease caused by endodontic infection, and its development is regulated by the host's immune and inflammatory response (Gomes and Herrera 2018; Nair 2004; Sasaki et al. 2016). The initial phase of this pathology is characterised by an inflammatory response mediated by pro-inflammatory macrophages, whereas in its resolution phase, macrophages and regulatory T cells aim to promote tissue repair. Moreover, symptomatic periapical lesions are associated with elevated levels of pro-inflammatory cytokines and factors such as RANK-L, which induce bone resorption, and matrix metalloproteinases (MMPs), which contribute to tissue destruction. Host immune regulation and the production of reactive oxygen species (ROS) also appear to play a crucial role in the progression and exacerbation of periapical lesions (Hussein and Kishen 2022).

Endodontic microbial communities present in apical periodontitis have been extensively investigated in both primary lesions (Buonavoglia et al. 2023) and those persisting after endodontic treatment (Gomes et al. 2021). Clinically, these lesions are managed by endodontic treatment or retreatment, aiming to disinfect the root canal, achieve proper sealing through obturation, and allowing the rehabilitation of the dental structure (Karamifar 2020). Long-term outcomes of endodontic treatment are influenced by the nature of the host-infection interaction, the procedural effectiveness of the treatment, and the maintenance of the treated tooth's integrity to support the reversal of the pathological process (Gulabivala and Ng 2023).

The success rate of primary endodontic treatment in teeth with pulp necrosis and asymptomatic apical periodontitis is 81.1% (Da Silva et al. 2023). Non-surgical primary endodontic treatments have a success rate of 85–94% (Sjögren et al. 1997). In the case of retreatments, non-surgical approaches show a success rate of 74–82% (Sundqvist et al. 1998; De Chevigny et al. 2008), while endodontic microsurgery achieves a 94% success rate (Serefoglu et al. 2021) Retreatment of symptomatic mandibular first molars with periapical lesions has shown an 88% success rate (Serefoglu et al. 2021). This suggests that the predictability of periapical lesion treatment has not yet been fully achieved and that factors beyond anatomical and microbiological aspects need further investigation. Moreover, although significant advancements have been made in incorporating innovative technologies into endodontic armamentarium, challenges still remain to be fully overcome.

Mechanisms of apical periodontitis have been described, considering: the role of macrophage polarization in infection-associated periapical lesions (Song et al. 2022); the involvement of bacterial endotoxins in the aetiology of periapical lesions, including molecular mechanisms involved in their recognition and cellular activation (Lucisano et al. 2014); the potential suppressive action of cytokines, which impacts the intensity of the inflammatory response and the extent of alveolar bone loss leading to periapical lesions (Braz-Silva et al. 2019; Menezes et al. 2008); and the possible influence of diet on the development of periapical lesions (Tibúrcio-Machado et al. 2021). Additionally, various histological and pathogenic events related to the repair of periapical lesions following endodontic therapy have been described (García et al. 2007), including bone remodelling mechanisms involved in the repair of chronic apical periodontitis after endodontic treatment (Luo et al. 2022) and the concurrent processes influenced by undifferentiated cells (stem cells) in the presence of periapical lesions (Lyu et al. 2022).

Despite previous advances in elucidating the mechanisms of apical periodontitis, no prior research has analysed the metallographic differences between healthy and apical periodontitis-affected tissues. Evidences that the incorporation of metals into endodontic materials may modulate inflammation, stimulate bone repair, and improve treatment predictability (Wu et al. 2020; Huang et al. 2021; Silingardi et al. 2024) are available. However, opening new therapeutic possibilities, such as the development of medications or endodontic materials enriched with essential metals could contribute to maintaining and/or influencing the homeostasis, creating a favourable environment for tissue regeneration and repair.

Metallomics is a branch of omics sciences that investigates the metallographic profile in biological systems (Roverso et al. 2023; Yasuda et al. 2020). The human body requires approximately twenty essential elements for proper metabolism, ten of which (sodium, potassium, magnesium, calcium, iron, manganese, cobalt, copper, zinc and molybdenum) are metallic chemical elements (Jomova et al. 2022; Zoroddu et al. 2019). It is known that the catalytic behaviour of essential metabolic metals can lead to the formation of reactive hydroxyl radicals and oxidative stress, potentially causing damage to DNA, protein synthesis, and membranes (Pizzino et al. 2017).

Changes in essential elements within biological tissues have been identified in pathological conditions (Bjorklund et al. 2018; Doroszkiewicz et al. 2023; Takeda 2003; Andrews 2002), but evidence of this phenomenon in apical periodontitis is still lacking. Recent studies have shown that trace metal analysis in tissues can provide accurate information, allowing the identification of specific patterns associated with disease etiology (Stelling et al. 2019). For example, studies on thyroid tissues have shown that each thyroid disease exhibits a unique profile of metals, such as arsenic, lead, cadmium, copper, zinc, and selenium, in both healthy and pathologically altered tissues (Stojsavljević et al. 2019). Altered intracellular zinc levels impair antioxidant and stress responses, promoting endothelial damage and inflammation, which may worsen cardiovascular diseases like ischemia/reperfusion by disrupting metal homeostasis (Smith et al. 2023). Preeclampsia, a placental disorder with uteroplacental hypoperfusion, is linked to lower magnesium, calcium, iron, copper, zinc, and selenium levels. Increased selenium may reduce risk, suggesting a link between metal imbalances and the disease (Hao et al. 2024).

This study aims to establish, for the first time, the differential metallomic profile between healthy and lesion-affected periapical tissues. Identifying specific metal patterns could improve the understanding of disease pathogenesis and unlock new perspectives for advancements in diagnosis, prognosis, and more effective therapeutic strategies. The null hypothesis posits that there are no elemental differences between healthy and lesioned periapical bone.

Material and methods

This in vivo study was performed in accordance with the Preferred Reporting Items for Animal Studies (PRIASE) 2021 guidelines (Nagendrababu et al. 2020) (Figure 1), and following ethical guidelines approved by the Animal Use Ethics Committee (Protocol CEUA: 6219-1/2023).

Sample size calculation

The sample size was determined using G*Power 3.0 software (Kavoli et al. 2017), considering a statistical power of 0.80, a 5% significance level, and a medium effect size. The calculation resulted in 40 male Rattus norvegicus, Albinus lineage, Wistar strain (Supplementary table 1) of which two first mandibular molars, totalling 80 experimental units (n = 80), were desired.

Figure S1 (Supplementary material) shows the experimental design here further described.

Animals

The animals were housed in an animal facility throughout the experimental period under a controlled room temperature of 22±1°C and a 12-hour light-dark cycle (lights on from 7:00 am to 7:00 pm). From birth until day 56 of life, the animals were kept in collective cages, having *ad libitum* access to water and food throughout the study.

AIM/HYPOTHESIS

This study aimed to investigate the metallomic characterization of periapical lesions using an animal model

ETHICAL APPROVAL AND COMPLIANCE WITH ANIMAL WELFARE STANDARDS

UNICAMP, Ethics Committee on the Use of Animals, Protocol: 6219-1/2023

AGE, GENDER, TYPE OF ANIMAL

3-week old, male, White Albino rats

SAMPLES

The left and right mandibular molar

INTERVENTION / CONTROL GROUPS

During the immediate postoperative period, one animal from each experimental condition did not survive anesthesia administration, leading to their exclusion from the study. As a result, the initial sample of 40 animals was reduced to 38, with 19 animals per conditions.

OUTCOMES ASSESSED

The differential metallographic profile between the health and periapical disease states.

METHODS AND WHO ASSESSES OUTCOMES

Periapical radiography, histological analysis, micro-computed tomography (μ-CT), X-ray fluorescence microscopy (μ-XRF), scanning electron microscopy with energy dispersive spectroscopy (SEM/EDS), inductively coupled plasma optical emission spectrometry (ICP-OES), and inductively coupled plasma mass spectrometry (ICP-MS).

RESULTS WITH STATISTICAL ANALYSES

No significant differences were observed in the animal weight ($p > 0.05$). However, the periapical region volume showed a statistically significant difference of 12.74 mm^3 ($p = 0.0017$).

Histological analysis revealed a significant inflammatory infiltrate in the molars with induced periapical lesions, with scores ranging from moderate to severe, showing a statistically significant difference ($p < 0.0001$).

Regarding trace element concentrations in tissues with periapical lesions and in the negative control (Sham), the results indicated significant differences in the levels of: Manganese (Mn), Iron-56 (Fe), Iron-57 (Fe), Copper-63 (Cu), Copper-65 (Cu), Sodium (Na), Potassium (K), Calcium (Ca), and Magnesium (Mg). In contrast, no significant differences were observed in the levels of Molybdenum-96 (Mo), Molybdenum-98 (Mo), Cobalt-59 (Co), Zinc-66 (Zn), Zinc-68 (Zn), Zinc-64 (Zn), and Zinc-67 (Zn) between conditions.

ADVERSE EVENTS

Two animals were lost during anesthesia and the division altered to the animals (n=38) were divided into two conditions: 1)Control (n=19) 2) Test -periapical lesions (n=19)

CONCLUSION(S)

These findings indicate, for the first time, changes in the mineral profile in periapical lesions and highlight the importance of investigating therapeutic or diagnostic approaches aiming to restore the mineral homeostasis disrupted by this pathological condition. Future metallic-content smart technology for both endodontic materials and diagnostic tools are desirable.

FUNDING DETAILS

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CONFLICTS OF INTERSET

The authors deny no conflict of interest

Figure 1: PRIASE 2021 flowchart illustrating the steps involved in conducting the present study.

Induction of periapical lesion in lower molars

At 56 days of age, animals were weighed and anaesthetised using a combination of Ketamine (90 mg/kg) (Vet Brands Int, Miramar, Fl, USA) and Xylazine (10 mg/kg) (AnaSed®, Akorn Animal Health, United States) via intramuscular injection. After anaesthesia, the animals were carefully positioned on an operating table to allow for containment and mouth opening, providing access to the occlusal surface of the lower molars (Chicarelli et al. 2021; Kavoli et al. 2017). The mesial sulcus of the first lower molar was determined as the drilling point, as the root with a larger canal volume is anatomically located just beneath this structure.

In sequence, pulp exposure was performed bilaterally (Metzger et al. 2002) using a 1/2 spherical bur (EARC4, Dentsply Tulsa Dental Specialities, Oklahoma, United States), mounted on a high-speed turbine. The bur was introduced to a depth of approximately 1 mm, avoiding furcation damage, with confirmation of pulp exposure by light probing and visual inspection of pulpal bleeding. To induce bacterial contamination, potentially leading to periapical lesion formation, teeth were left without sealing for a period of 40 days.

For analgesia, subcutaneous administration of metamizole sodium (100 mg/kg) (Neo-Melubrina®, Sanofi, México) was performed immediately after the procedure, and it was also diluted in drinking water at a ratio of 200 ml of water to 0.2 ml of metamizole sodium. For the sham controls, a simulated intervention was performed, which included immobilization, anesthesia and administration of metamizole sodium, but without pulp exposure procedure.

In the immediate postoperative period, one animal from each methodological condition did not survive the anaesthesia application, leading to its exclusion from the study.

Euthanasia of animals and sample preparation

After 40 days, all animals (92 days-old) were reweighed using an electronic scale with an accuracy of 10^2 g. Following this, the animals were administered a triple overdose of anaesthetic, and ventral access up to the heart was performed to ensure euthanasia and samples collection containing the induced periapical lesions (n = 38) and control shams (n = 38).

For analyses, the mandibles were carefully removed, dissected, and divided into hemimandibles using blunt-end scissors and a #15 scalpel blade (Bard-Parker, Dickinson & Co., Franklin Lakes, USA). Samples were stored according to the specific methodological requirements. The experimental protocol for sampling and storage is shown in Figure S2 (Supplementary material).

Analysis of periapical lesion area

Digital periapical radiographs were taken of all the hemimandibles (n = 76) using a radiology device (Dexis® Titanium, Dexis, United States). The hemimandibles were positioned perpendicular along the long axis of the tooth, with a focal-film distance of 6 cm, 70 kVp and 7 mA parameters, and an exposure time of 0.3 seconds. The ImageJ software (version 1.54, National Institutes of Health, Washington, DC, USA) was used to measure the area suggestive of the induced periapical lesion.

For each image, the image calibration tool (set scale) was initially applied by entering the dimensions of the radiographic sensor (43 mm x 31 mm). Then, using the freehand selections tool, the radiolucent area at the apices of the first lower molar roots was manually outlined. The same operator took the measurement, and the area was obtained. When the image of the induced periapical lesion displayed fusion between the roots, a single area measurement was calculated, and when the lesions were separate, the area was measured individually for each root and these measurements were summed.

Histological analysis

Histological analysis was conducted (n = 9 per group) in bone tissue blocks cut into a quadrangular shape, with the strategic removal of the condyles and incisor tooth to optimize processing. The specimens were placed in labelled cassettes and washed for two hours under running water.

Over 80 days, the samples were immersed in 17% ethylenediaminetetraacetic acid (EDTA) with daily solution changes aiming for decalcification. Afterward, these were embedded in paraffin and sectioned at 5 μm thickness using a Leica RM 2155 microtome (Nussloch, Germany). The sections were then mounted on glass slides and stained with haematoxylin and eosin (H&E). Representative digital images of the periapical region, including bone tissue, dental root, and soft tissue, were obtained using a Zeiss Axioskop II microscope (Switzerland), equipped with a Sony CCD IRIS RGB DXC-151A camera (Tokyo, Japan) and Kontron KS300® software (München, Germany). Images were captured at 4x, 10x, and 40x magnification for each histological section.

The intensity of periapical inflammation was evaluated using a scoring system. Manual counting of inflammatory cells was performed in six quadrants around the root apex, by a single calibrated operator. The intensity of the inflammatory infiltrate was classified based on the mean cell count as follows (Liu et al. 2012; Aranha et al. 2013):

- Absent (0 to few cells) – Score 1;
- Mild (<25 cells) – Score 2;
- Moderate (25–125 cells) – Score 3;
- Severe (>125 cells) – Score 4;

Micro-computed tomography (μ-CT) analysis of lesion volume

The hemimandibles (n = 9 per group) were dissected and stored at -20°C before analysis by μ-CT. Scanning was performed using the SkyScan 1174 (Bruker, Kontich, Belgium) with a 0.5 mm aluminium filter, a pixel size of 6.46 μm, 360° rotation, and a 1.0° step, with a total scanning time of 26 minutes, adjusted to a voltage of 55 kV and a current of 800 μA. Raw images were reconstructed using the NRecon software (Bruker, Kontich, Belgium) with a smoothing filter of 1%, beam hardening correction of 0%, ring artifact reduction of 1%, and a grayscale dataset of 0.000–0.091. Subsequently, using the Data Viewer software (SkyScan, Version 1.4.4, 64-bit), images were oriented and standardised in the three anatomical planes: transverse, longitudinal, and sagittal.

After reconstruction, the region of interest was defined using the CTAn software (v1.6.6.0, Bruker, Belgium) to include the bone resorption cavities of the entire sample under the first lower molars. The roots of the lower molars and the mandibular canals were excluded from the region of interest. This analysis was performed to quantify the periapical lesion volume (in mm^3). The grayscale threshold was determined using a density histogram, generating a binary image with black and white pixels. For segmentation, a density histogram ranging from 21 to 255 was used to select bone tissue. Manual selection of the region of interest was performed in all image-sets in the axial view, starting from the first image where all root apices of the left mandibular first molar were visible and ending twenty slices after the lesion disappeared (Figure S3 - Supplementary material). For the calculation of 2D areas and 3D volumes, the original grayscale images were processed with a Gaussian filter for noise reduction and an automatic segmentation threshold (Bouxsein et al. 2010).

Fluorescence microscopy analysis (μ-XRF)

For μ-XRF and SEM/EDS (n = 10 per group), accesses to the periapical region were achieved through controlled wear using a Zekrya bur, ensuring uniform tissue exposure. In the periapical lesions, the wear was extended until the complete exposure of the lesion. A benchtop μ-XRF system (Orbis PC EDAX, USA) equipped with a Rh anode operating at 45 kV and 200 μA was used. The equipment was configured to operate with a capillary optic of 30 μm.

Detection was performed using a 30 mm² silicon drift detector (140 eV FWHM at the 5.9 keV Mn-Kα line). The pixels produced by the Orbis Vision software were linearly interpolated and mapped using Origin Lab 2016 software (Origin, Northampton, MA, USA).

Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDS)

To perform a confirmatory analysis of the metallic content present in the periapical region, the same prepared samples were analysed using SEM/EDS. These were mounted on metal stubs and carbon coated. Photomicrographs were obtained in secondary electron mode using a scanning electron microscope (JEOL, JSM-IT300, Akishima, Tokyo, Japan). Elemental mapping was conducted using the EDS line scan tool to determine the distribution of the elements of interest along the tooth–periapical region interface.

Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectroscopy (ICP-OES)

Elemental quantification was performed using two spectrometry methods adapted to sensitivity requirements, including ICP-OES (n=8) and ICP-MS (n=9). Bone samples containing both the first mandibular molar and the periapical region with a weight between 15 and 22 mg, were analysed. The samples were individually digested in Teflon rotors using an acid digestion process with 6 mL of 20% P.A. nitric acid (HNO_3), redistilled by sub-boiling, and 2 mL of 30% hydrogen peroxide (Sigma Aldrich - Merck, Germany). The containers were secured in the microwave rotor, and digestion was performed (Milestone, Shelton, CT, USA) under heating conditions: 1000 W power, 20 bar pressure, with a time/temperature cycle of 5 minutes at 160 ºC, 2 minutes at 160 ºC, 5 minutes at 170 ºC, and 15 minutes at 170 ºC. After cooling to 60 °C, the fully digested content was transferred to 15 mL Falcon™ tubes (Sigma-Aldrich, Thermo Fisher, USA). Reagent blanks (without samples) were processed in each cycle for quality control.

For analysis, the samples were further transferred to new Falcon tubes (Sigma-Aldrich, Thermo Fisher, USA) and diluted 1:10 (v/v) with ultrapure water (18 MΩ·cm resistivity) from a Milli-Q purification system (Millipore Sigma, Bedford, MA, USA). To ensure efficient removal of suspended solid particles, the samples were filtered using a 0.22 µm hydrophobic polyvinylidene fluoride syringe filter (Biocentrix, USA). Given the analysed elements, different isotopes were selected to achieve low detection limits. Quantification was performed using an ICP-MS spectrometer (PlasmaQuant MS Elite, Analytik Jena, Jena, Germany) with an internal standard solution containing indium (In) (ICP-grade, Merck, Darmstadt, Germany), antimony

(Sb) (ICP-grade, Merck, Darmstadt, Germany), and tin (Sn) (ICP-grade, Chem Lab NV, Zedelgem, Belgium). Internal standards ranging from 0 to 50 ppb for molybdenum, cobalt, copper, and manganese, and from 0 to 300 ppb for zinc and iron, were analysed alongside the samples.

Operational conditions for the elemental analysis performed using both spectrometers are presented in Supplementary Table 2 and 3. Calibration curves were constructed using multi-element standards (1000 ± 3 µg/mL) diluted in 1% nitric acid (HPS, High Purity Standards, North Charleston, SC, USA). Additionally, standard laboratory rodent chow and wood shavings (bedding material) were collected and analysed via ICP-OES for all the studied metals.

Statistical analysis

Data were analysed using GraphPad 10.1.1 (323). Normality was assessed using the Shapiro-Wilk test. Depending on normality, comparisons between conditions were performed using either the t-test or the Mann-Whitney test. A significance level of $p < 0.05$ was considered statistically significant. Graphical representations of statistical differences followed a specific convention: a bilaterally closed bar indicated a normal data distribution; a unilaterally closed bar signified that one dataset was normal (closed bar) while the other was non-normal (open bar); and a bilaterally open bar denoted that both datasets were non-normal.

Results

Body weight assessment

Comparative analysis between animal conditions showed a normal distribution both initially ($p = 0.4556$) and after periapical lesion induction ($p = 0.1904$) (Figure S4 - Supplementary material).

Radiographic analysis

The digital periapical radiographs (Figure S5 - Supplementary material) demonstrate the presence of induced periapical lesions. These lesions are notably characterised by well-defined radiolucent areas surrounding the dental roots. In contrast, the sham control radiographs exhibit a continuous trabecular bone, and no evidence of bone rarefaction around the roots of the first mandibular molars.

Histological analysis

The histological sections of the molars from the sham controls showed no pulp or periapical tissue alterations (Figure 2).

In molars with induced periapical lesions, the histological sections revealed an intense inflammatory infiltrate with neutrophils. Additionally, disorganisation of the periodontal support structures was observed, in the mesial roots. A dense accumulation of polymorphonuclear leukocytes was also noted, with cells containing bluish cytoplasmic granules, areas of bone resorption, and the presence of blood vessels. The observed characteristics are consistent with chronic periapical abscess formation. In the distal roots, inflammatory cells were sparsely distributed and restricted to the vicinity of the periapical foramen (Figure 3).

Teeth with induced periapical lesions did not have any samples classified with a score of 0. Three samples had a score of 1 (mild reaction), none had a score of 2 (moderate reaction), and six samples received a score of 3 (intense reaction), with a median score of 1.5. In the sham controls, the inflammatory infiltrate was classified as absent or with few inflammatory cells (score 0), with all nine samples receiving a score of 0. Statistical analysis showed a significant difference between conditions ($p = 0.0001$), highlighting the association between induced periapical lesions and a more intense inflammatory infiltrate.

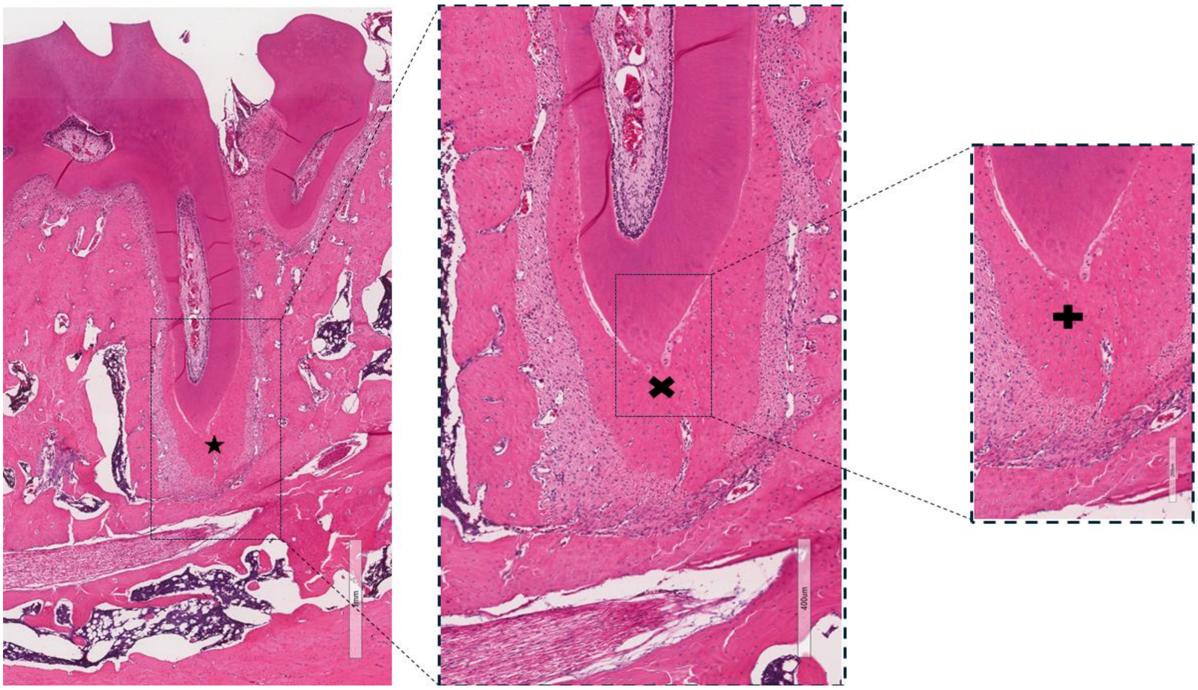

Figure 2: Histological image of bone tissue and dental structure under microscopic analysis, stained with hematoxylin and eosin (HE). The left image (scale: 1 mm) provides an overview of the tissue, highlighting the region of interest with a rectangle and marked by an asterisk (*). The intermediate magnification in the center (scale: 400 µm) allows for a more detailed view of the cellular and structural characteristics of the delimited area, indicated by an 'x'. The right image (scale: 200 µm) shows a higher magnification of the marked region, revealing specific details of the bone matrix and cellularity, indicated by the '+' symbol. Scale bars are located at the bottom right of each image.

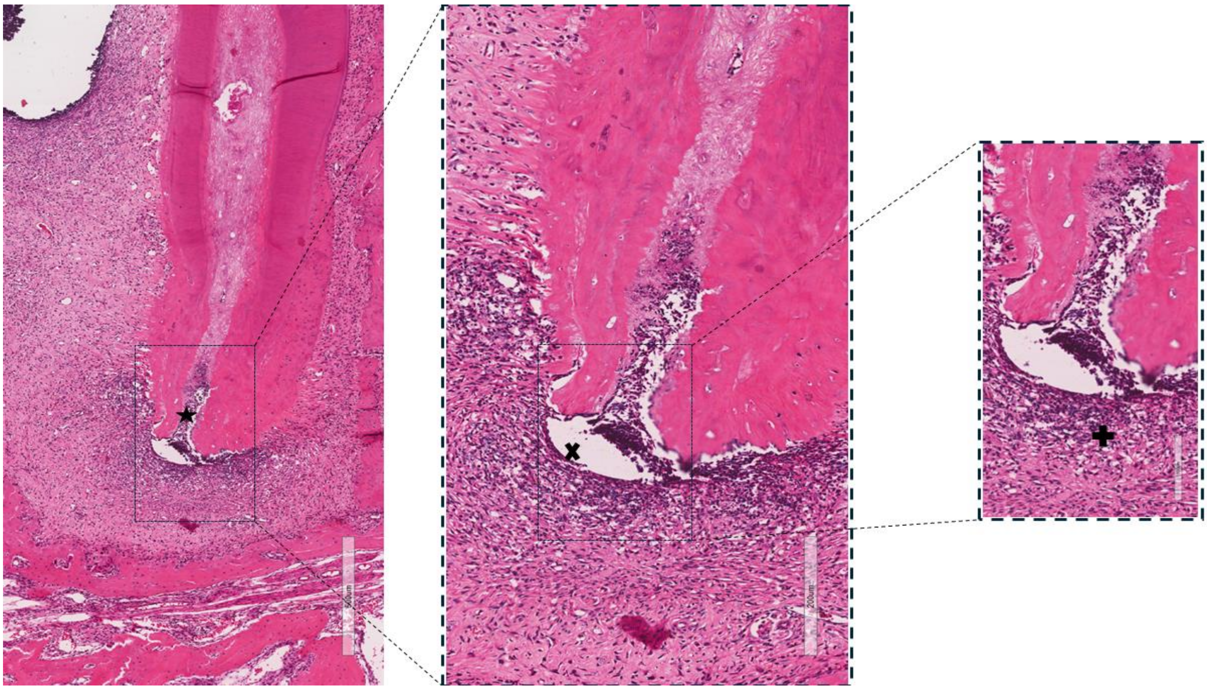

Figure 3 – Light photomicrographs of the mesial root of the first mandibular molar with an induced periapical lesion. Images were obtained using 2×, 5×, and 20× objectives, with scale bars of 500 µm, 200 µm, and 100 µm in the left, middle, and right panels, respectively. (*) Pulp necrosis. (X) Presence of a large abscess area, accompanied by resorption of bone, cementum, and dentin. (+) Extensive periapical inflammatory infiltrate. The images highlight the morphological alterations resulting from inflammation and tissue destruction, emphasizing cellular organization and the interaction between the inflammatory infiltrate and mineralized tissues. Slides were stained with hematoxylin and eosin (H&E).

Micro-computed tomography (µ-CT)

This analysis indicated a statistically significant difference in the mean volume of the induced periapical region, measuring 12.74 mm³ ($p = 0.0017$). Figure 4 presents comparative images between conditions in sagittal, coronal, and axial sections.

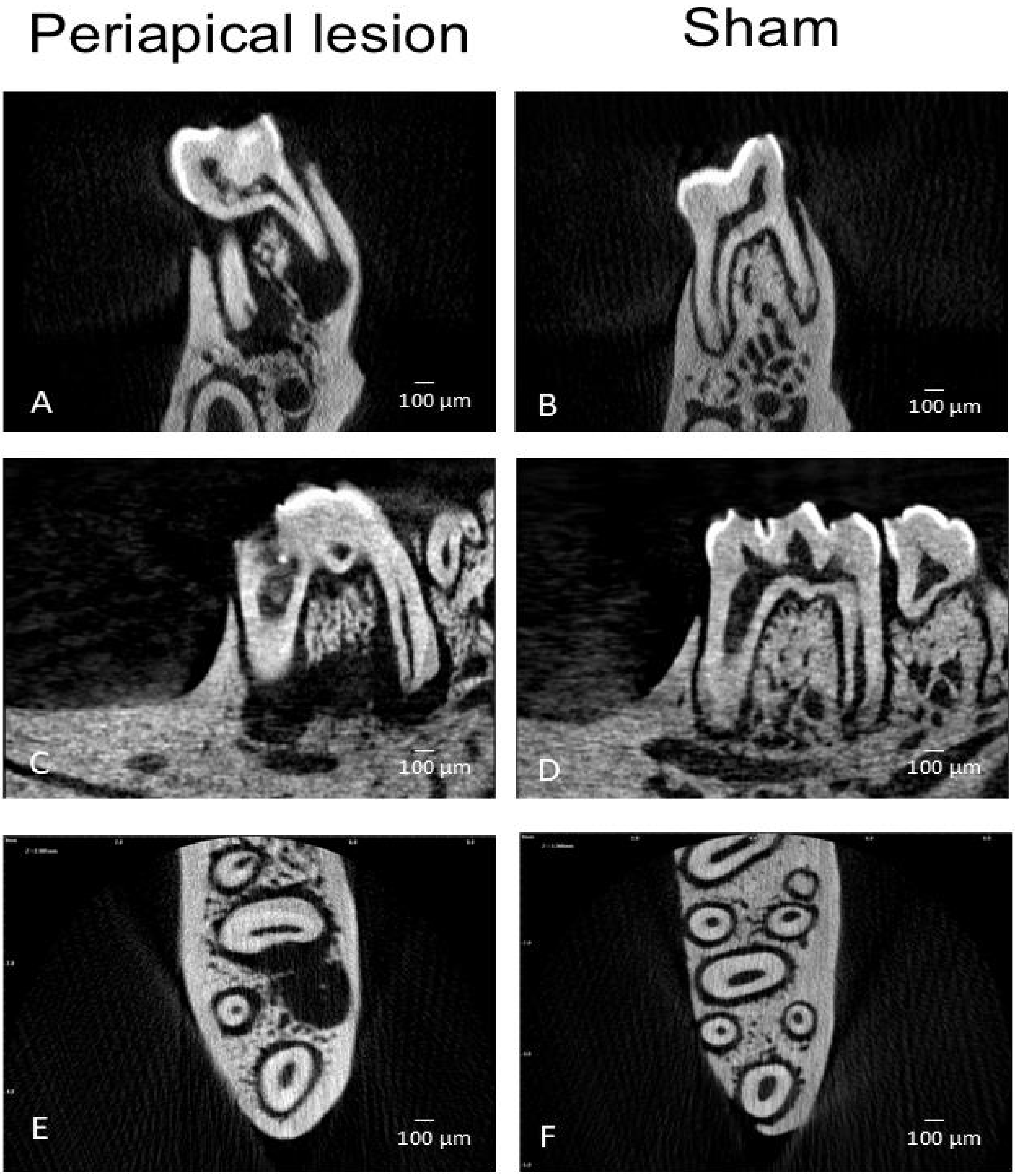

Figure 4. Micro-computed tomography (µ-CT) images of rat molars with induced periapical lesions (periapical lesion) and controls (sham). (A-B) Sagittal sections highlighting dental and bone morphology. In the induced periapical lesions, the extent and location of the lesion are observed, identified by a hypodense image in the apical region. In contrast, in the sham controls, the bone structure remains preserved. (C-D) Coronal sections focusing on the mesial region of the first mandibular molar, which was accessed for periapical lesion induction. In the induced periapical lesions, a hypodense shadow is noted in the furcation region, suggesting bone resorption. (E-F) Axial sections demonstrating, in the induced periapical lesions, a widening of the periodontal ligament space, accompanied by a hypodense image corresponding to the periapical lesion. In the sham controls, no loss of continuity is observed along the periodontal ligament space. Scale bars represent 100 µm.

Scanning electron microscopy / energy dispersive spectroscopy (SEM/EDS)

This analysis demonstrated morphological differences and variations in elemental composition (Figure 5). In the induced periapical lesions (A), areas of bone resorption, irregularities, and lacunae were observed, suggesting structural loss. In contrast, the sham controls (B) exhibited a preserved surface without pathological defects.

Elemental analysis (C and D) revealed a reduction in calcium and phosphorus concentrations in the periapical lesion, indicating demineralisation. Conversely, in the sham controls, the homogeneous distribution of these elements suggests the preservation of the bone mineral matrix. Additionally, variations in magnesium and sodium levels may reflect changes in the mineralisation of the affected tissue.

However, this analysis provided limited results for a comprehensive characterisation of the elements of interest, due to the lower sensitivity of the method. For this reason, it was essential to complement the findings with more robust techniques, such as µ-XRF and ICP-OES/ICP-MS-based methods, which offered greater accuracy and coverage in the identification and quantification of the investigated chemical elements.

Fluorescence microscopy (µ-XRF)

In the elemental mapping analysis using µ-XRF, the elements sodium, potassium, magnesium, calcium, iron, manganese, cobalt, copper, zinc, and molybdenum were investigated. However, only three metallic elements (calcium, iron and zinc) were consistently detected in all samples, along with phosphorus, a non-metallic element included in this analysis (highlighted in Figure 6 and Figure 7).

Phosphorus, an essential component of calcium phosphate, is associated with calcium and plays a crucial role in mineral homeostasis. Its analysis – not initially aimed – was relevant for understanding alterations in elements related to bone health and mineralization, as well as evaluating interactions between metallic and non-metallic elements in the context of lesions such as apical periodontitis.

Calcium exhibited low intensity in regions near the mesial root and the periapical lesion, whereas in the sham controls, it showed higher intensity and homogeneous distribution throughout the analysed region. Iron had lower intensity in the sham controls but showed variable intensity in the induced periapical lesions. Zinc was detected with higher intensity in the sham controls, while showing lower intensity in the lesion regions. Phosphorus also exhibited significant variations, with higher intensity in the sham controls and reduced levels in periapical lesion regions.

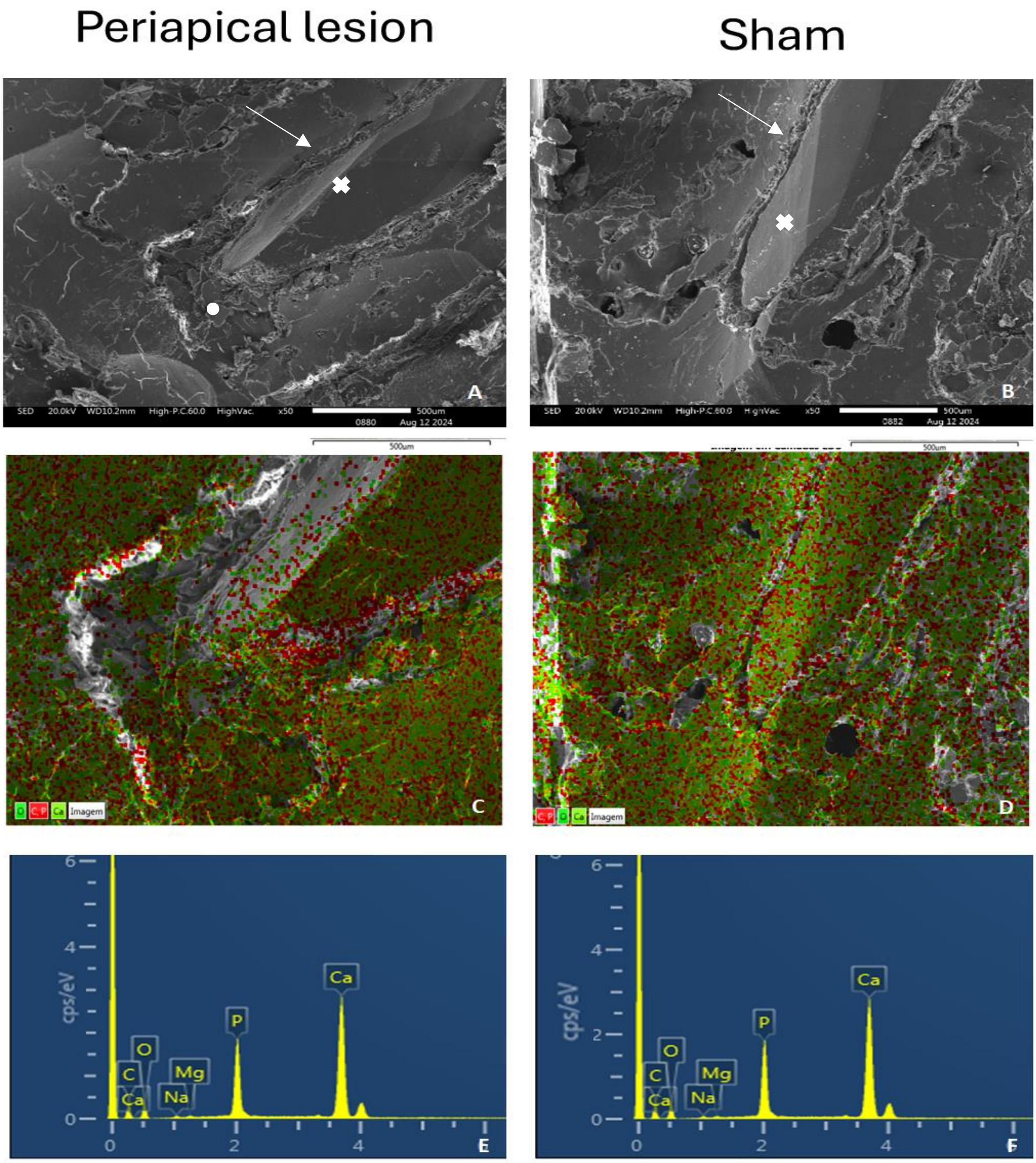

Figure 5: SEM/EDS analysis comparing periapical bone tissue between the periapical lesion and sham controls. (A-B) SEM images highlighting the microstructure of the bone tissue in a tooth with an induced periapical lesion, where the arrow indicates the periodontal ligament, the white dot indicates the periapical lesion, and the white "X" indicates the periapical root (A). In contrast, the sham controls (B) show preserved tissue, while areas of decalcification are evident in the induced periapical lesions. The scale bar represents 500 µm. (C-D) EDS elemental distribution maps, illustrating the distribution of calcium (Ca, red), phosphorus (P, green), and carbon (C, white) in teeth with periapical lesions (C) and sham controls (D). This analysis confirms the absence of calcium in specific areas within the periapical lesions. (E-F) EDS spectra highlighting the elemental composition of the samples, indicating the presence of oxygen (O), carbon (C), calcium (Ca), phosphorus (P), magnesium (Mg), and sodium (Na) in both conditions.

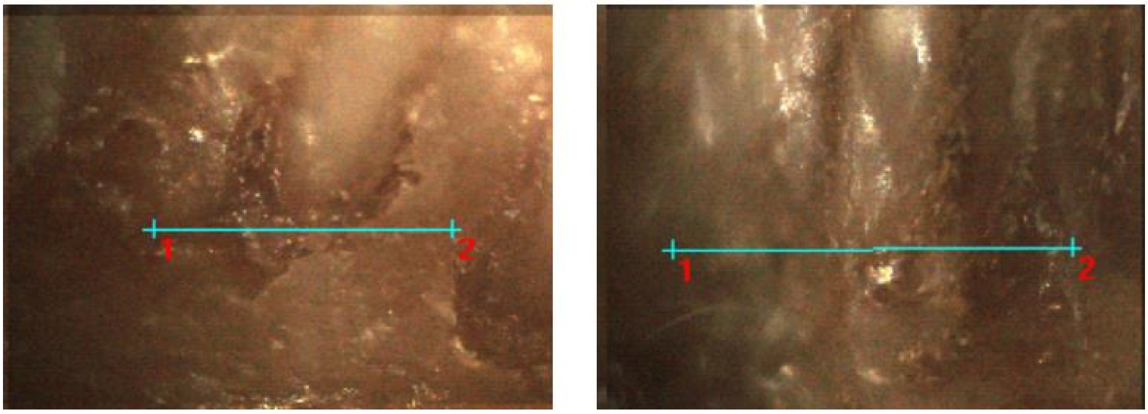

Figure 6: Highlight of the microscopic images ROI of hemimandibles from animals with induced periapical lesions and controls (Sham), analysed by μ-XRF. In the upper image, the induced periapical lesion (a) and the control (Sham) (b) highlight the periapical region where the linear scan was performed, indicated by a blue line (1.3 mm) with markings at the starting point (1) and the endpoint (2).

Inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES)

The concentrations of sodium, potassium, calcium and magnesium were measured using ICP-OES (Figure 8), while iron, manganese, cobalt, copper, zinc, and molybdenum were measured by ICP-MS (Figure 9) after acid digestion.

The elemental concentration analysis by ICP-OES revealed statistically significant differences between the periapical lesion and sham controls for sodium, potassium, calcium and magnesium. Sodium showed significantly higher concentrations in the periapical lesions (approximately 7,500,000 ng/g) compared to the sham controls (approximately 6,000,000 ng/g) with $p = 0.0137$. Similarly, potassium levels were higher in the periapical lesions (1,000,000 ng/g) compared to the sham controls (600,000 ng/g) with $p = 0.0005$. Calcium also exhibited higher concentrations in the periapical lesions (300,000 ng/g) compared to the sham controls (250,000 ng/g) with $p = 0.0059$. Finally, magnesium showed a significant increase in the periapical lesions (6,000,000 ng/g) compared to the sham controls (4,500,000 ng/g) with $p = 0.0004$.

The elemental concentration analysis by ICP-MS revealed statistically significant differences ($p < 0.05$) in the iron isotopes between the sham controls and induced periapical lesions, with higher concentrations in the latter. Similarly, manganese and copper levels were significantly higher in the periapical lesions ($p < 0.0001$). However, no significant differences were observed for the zinc, cobalt, and molybdenum isotopes ($p > 0.05$). In general, a higher metallic content was observed within periapical lesions when compared with sham controls, regardless the metal analysed.

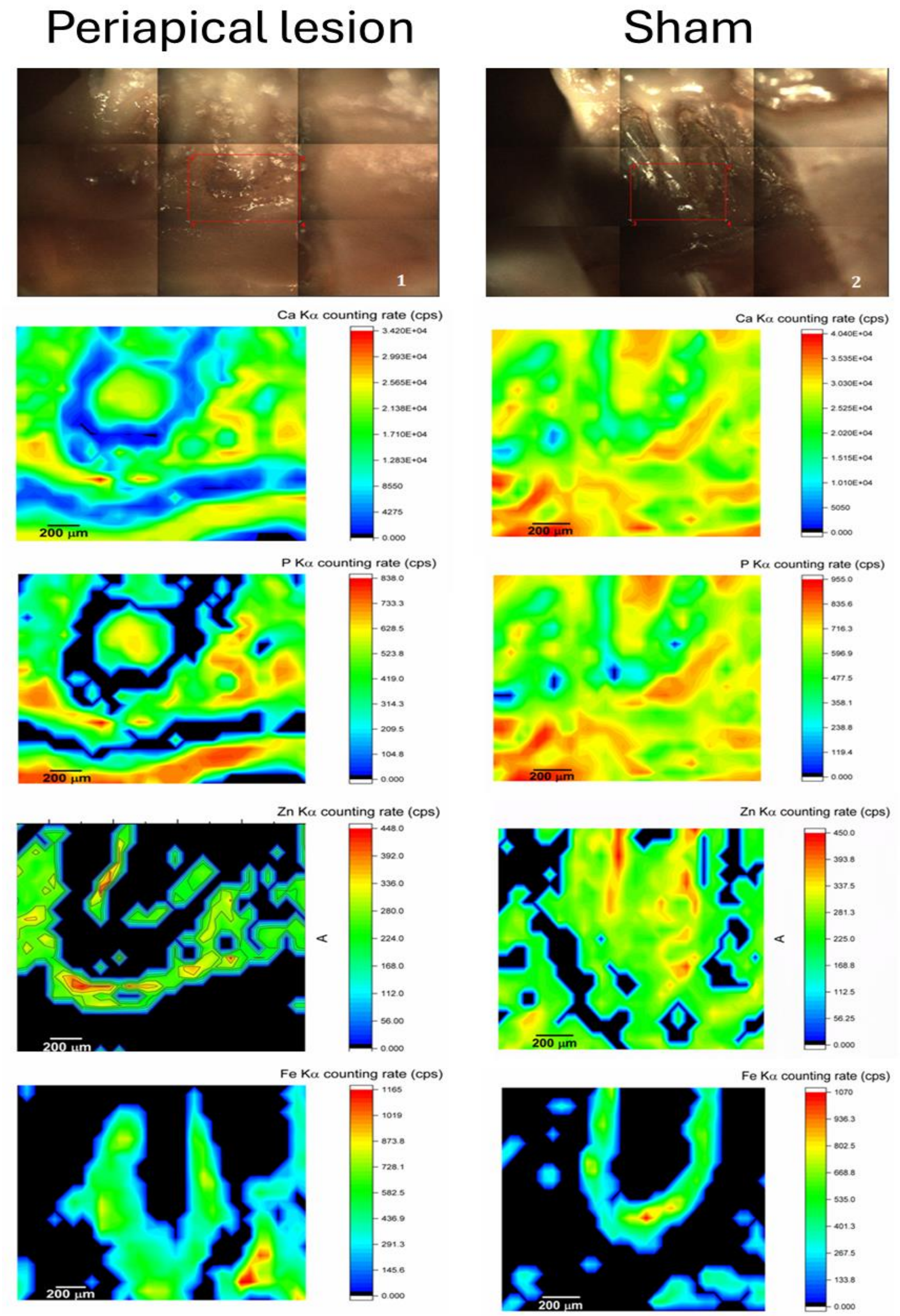

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Figure 7: Comparative analysis between the periapical lesion and control (sham) conditions using µ-XRF elemental distribution maps. Optical image of the analysed area in both conditions (1 - Periapical Lesion, 2 - Sham).

The calcium (Ca Kα) map demonstrates a reduction in calcium intensity in the induced periapical lesions, indicating mineral loss, in contrast to the more homogeneous distribution in the sham controls. The phosphorus (P Kα) map corroborates this lower concentration in the periapical lesion, reflecting demineralization, while the concentration is maintained in the sham controls. The zinc (Zn Kα) map shows a more irregular distribution in the periapical lesion, while the sham controls exhibit a more uniform distribution. Finally, the iron (Fe Kα) map displays higher signal intensity in the induced periapical lesions, while in the sham controls, its levels are less intense.

Analysis of feed and wood shavings

The feed showed detectable concentrations of sodium (34,730 ng/g), magnesium (54,086.67 ng/g), potassium (170,033.33 ng/g), calcium (82,103.33 ng/g), iron (3,061.33 ng/g), copper (20 ng/g), and zinc (673 ng/g). In contrast, the wood shavings showed no detectable concentrations of these elements, except for sodium (7 ng/g).

It is noteworthy that all animals were kept under the same housekeeping conditions.

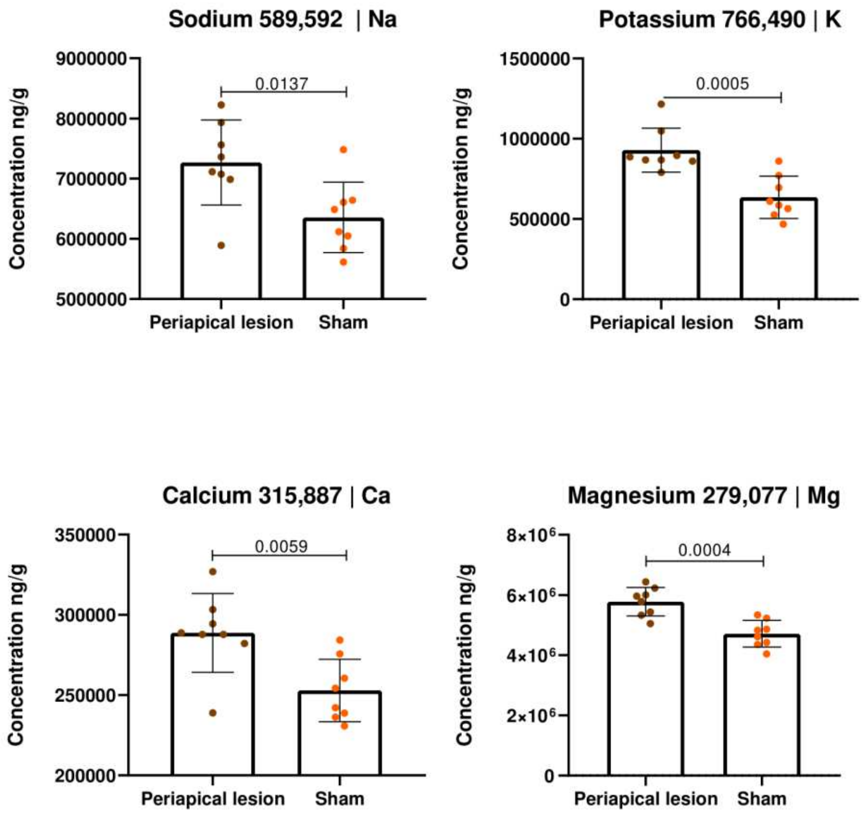

Figure 8: Graphical representation of the ICP-OES analysis, showing the concentrations of essential elements (in ng/g) in tissues with periapical lesions compared to the control (sham). For these elements, significant higher concentrations were observed within the periapical lesions.

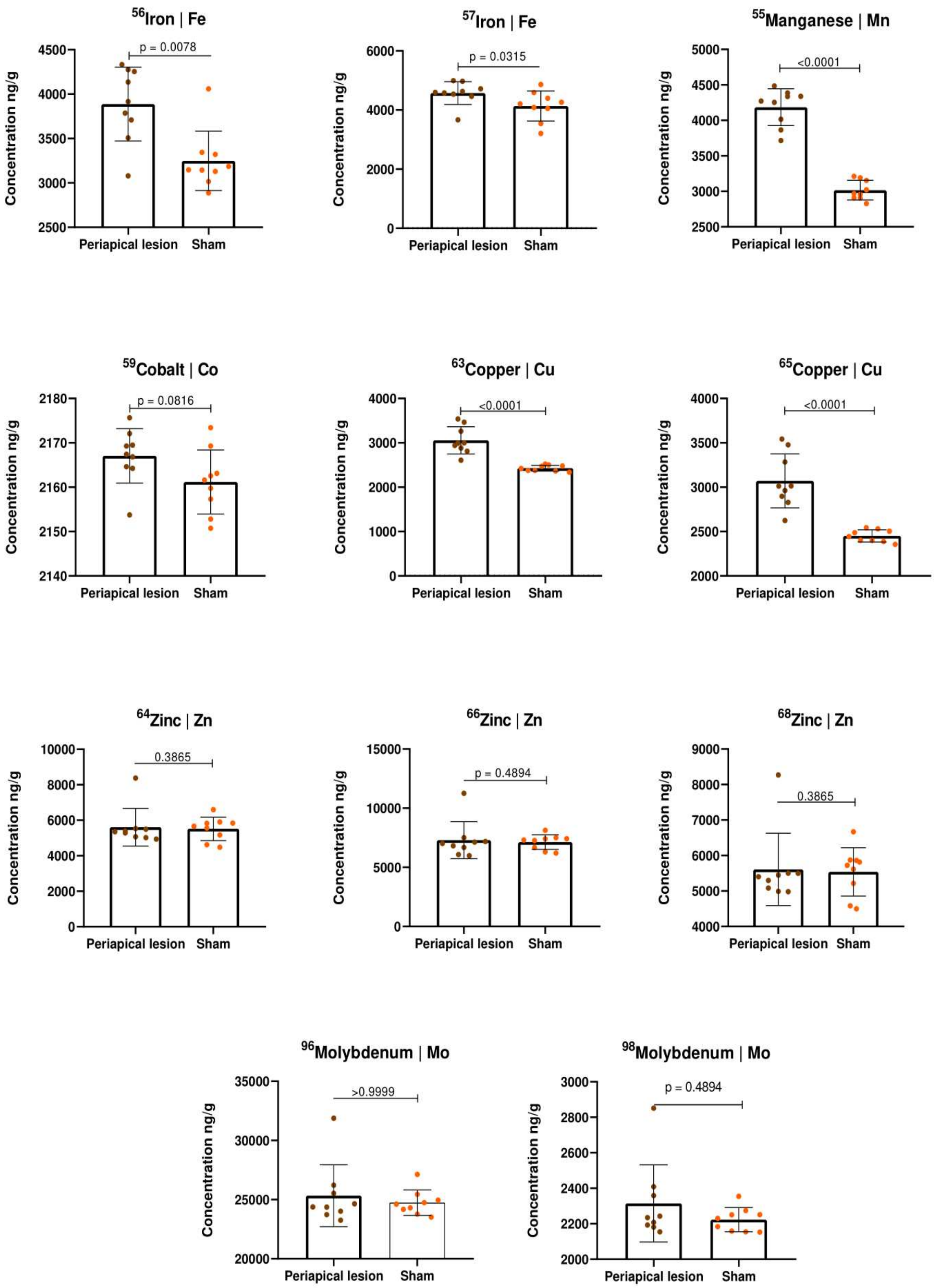

Figure 9: Graphical representation of the ICP-MS analysis showing the concentrations (ng/g) of different elements in tissues with periapical lesions compared to the control (sham). No differences were observed between the isotopes in terms of statistical significance.

Discussion

This study aimed to determine the metallographic profile of induced periapical lesions in an animal model, comparing it to healthy periapical bone tissue. The findings here reported indicate, for the first time, significant changes in the mineral profile in periapical lesions compared with healthy periapices; thus, the initial null hypothesis could be fully rejected. In general, a higher metallic content was observed within periapical lesions when compared with sham controls, regardless the metal analysed. Mineral homeostasis is essential for maintaining bone tissue integrity, and changes here observed in the concentrations of its elements may be linked to chronic inflammatory processes, such as apical periodontitis.

The experimental induction of periapical lesions can be performed using various methods, including pulp exposure (Brilhante Wolle et al. 2012; Liu et al. 2012) or stimulation with lipopolysaccharides and other endodontic pathogens (Aranha et al. 2013; Fukada et al. 2008). In this study, pulp exposure to the oral environment during the experimental period was chosen, aiming to simulate a clinical condition of microbial contamination. This methodology was able to promote periapical lesions, inducing an inflammatory reaction similar to that observed in other studies (Almeida et al. 2024; Anan et al. 1993; Silva et al. 2011; Zhang and Peng 2005). For the induction of apical periodontitis lesions, the first lower molar was selected due to its anatomical similarity to human teeth (Dammaschke 2010) and the biological progression of the inflammatory response in rats (Moretton et al. 2000) within the experimental timeline here proposed.

The analysis of body weight showed that bilateral pulp necrosis and periapical lesion did not significantly affect this criterion ($p > 0.05$). This suggests that the local inflammation did not cause a significant systemic impact on the animals' weight. A possible recommendation of unilateral induction to avoid influencing animal weight could be refuted by the present study since a bilateral induction was here performed. A previous study (Şehirli et al. 2024) indicated apical bone resorption following induction solely on pulp chambers of the right mandibular first molar also with an increase in periapical radiolucency, as here observed bilaterally.

Periapical bone resorption was here analysed using different methods, such as histological evaluation, digital radiography, and μ-CT, following methodological approach as previous studies (Rittling et al. 2010; Sun et al. 2017; Teixeira et al. 2011). Additionally, the present study conducted observations under SEM, which revealed the periapical lesion and allowed for sample mapping through EDS. This methodological addition was important to validate the expected calcium deficiency in the periapical lesion area and, along with phosphorus, demonstrated the absence of bone tissue observed in periapical lesions. However, the further addition of μ-XRF and ICPs were essential to deepen the elemental analysis of metals here desired.

The area analysis allowed for the identification of visible differences between the left and right sides of the periapical lesions. However, conventional radiographs often underestimate the actual size of periapical lesions (Ferreira et al. 2006), as they do not allow for an accurate assessment of the lesion's extent. Periapical lesions only become evident when there is involvement of the cortical plate or junctional trabeculae. This finding is consistent with previous studies (Attaelmanan et al. 2000; Hamachi et al. 1995; Yoo et al. 2023).

The μ-CT analysis allowed for the identification of the average lesion volume of 12.74 ± 3.02 mm^3 (standard deviation), aligning with previous reports that observed volumes of 12.15 mm^3 after 4 weeks. This expansion occurs more rapidly during the first 15 days (active phase) and slows down thereafter (chronic phase) (Stashenko and Yu 1989; Okiji et al. 1994; Wang and Stashenko 1991; Wang et al. 2009; Yoneda et al. 2017). The decrease in periapical lesion volume after 4 weeks reinforces the hypothesis of a modulation of the host immune response against bacterial infection during this period (Yoneda et al. 2017).

The experimental period of 40 days was here chosen to ensure that the induced periapical lesions reached a sufficiently advanced stage, allowing for a clearer detection of pathological alterations. This period was ideal for observing changes in the periapical structure here observed and ensure that the lesions were well-developed for analysis, consistently with previous findings (Brasil et al. 2017; Armada-Dias et al. 2006; Brasil et al. 2021).

In the histological sections of the molars from the sham controls, the analyses did not reveal significant alterations in the pulp and periapical tissues, highlighting healthy and well-preserved structures. The integrity of these tissues suggests the absence of harmful stimuli or inflammatory processes, establishing a crucial baseline for comparison with animals with induced periapical lesions. In contrast, in the molars with induced periapical lesions, the histological findings revealed an intense inflammatory response, particularly concentrated in the apical region of the root. This process could be entirely attributed to pulp exposure, which allowed bacterial colonisation and proliferation within the pulp tissue, followed by its necrosis and subsequent infection of the periapical tissues. Bacterial infection triggers neutrophil activation, leading to a massive migration of these cells to the apical foramen (Yamasaki et al. 2008). In this study, dense accumulations of polymorphonuclear leukocytes containing bluish cytoplasmic granules associated with blood vessels were identified. These findings align with previous reports describing the formation of periapical abscesses three weeks after pulp exposure (Matsui et al. 2011).

In this area of bone tissue resorption, the further analyses (SEM/EDS, μ-XRF, ICP-OES, and ICP-MS) were performed, revealing significant differences in the metallographic profile when comparing healthy bone tissue with that affected by apical periodontitis. This indicates that the changes were not limited to decalcification altering the calcium metallographic profile. Therefore, the null hypothesis was rejected, indicating significant differences in the levels of other chemical elements between bone conditions with and without periapical lesions.

Previous studies emphasizes the fundamental role of essential metals in regulating physiological processes, ensuring the balance of vital functions in the body (Costa et al. 2024; Baj et al. 2023). Imbalances in the homeostasis of these elements can induce cellular stress and promote disease development (Baj et al. 2023; Cannas et al. 2020; Ceko et al. 2016; Himoto and Masaki 2020; Kamińska et al. 2021; Xu et al. 2023). However, as this is the first study to comparatively investigate, with an endodontic focus, the composition and distribution of these metals, no comparisons with other studies are available.

Given the complexity of identifying and quantifying these elements, this study utilised ICP-MS and ICP-OES techniques to determine their concentrations between conditions. Among the analysed metallic elements, seven of them (sodium, potassium, magnesium, calcium, iron, manganese, and copper) showed significant differences between conditions, all with higher concentrations in the periapical lesions. The homeostasis of healthy bone tissue is maintained essentially by osteoblasts, osteoclasts, and osteocytes, as well as by an organic matrix (mainly type I collagen) and inorganic minerals (apatites), primarily hydroxyapatite (Boivin and Meunier 2003; Henmi et al. 2017). This complex composition grants bone tissue its dynamic properties, allowing constant metabolic interactions between the chemical elements within the extracellular fluid and the apatite crystals that form the mineral matrix (Boivin and Meunier 2003). Not surprisingly, elemental exchanges involving magnesium, zinc, and strontium for calcium, as well as carbonate for phosphate, have been associated with normal bone metabolism (Maciejewska et al. 2014).

Intuitively, a reduction in calcium concentration in the periapical lesion is expected, as bone resorption associated with the inflammatory process tends to result in mineral loss. Calcium depletion in the cellular environment has been associated with a significant reduction in the mineralization rate (Bellows et al. 1991). The μ-XRF analysis corroborated this expectation, demonstrating a lower calcium signal intensity in the affected periapical region compared to the healthy condition, where the signal was significantly stronger. The same pattern was observed for phosphorus, which showed a reduced or nearly absent signal in the lesion, while remaining elevated in the healthy condition. However, the results obtained by ICP-

OES indicated a significantly higher total calcium concentration in the induced periapical lesions compared to the healthy condition. This apparent contradiction can be partially explained by the differences in the measurement principles of the techniques. ICP-OES quantifies the total concentration of dissolved calcium in the sample without distinguishing its spatial distribution (bone matrix, extracellular phase, or protein-associated fraction) (Szymczycha-Madeja et al. 2021), whereas μ-XRF provides a surface mapping of up to 20 μm of the element's location (Maciejewska et al. 2014). Therefore, it is not possible to obtain the depth distribution of these elements using these techniques alone.

Further discussion on the calcium content can be stablished based on suggestions that bone regeneration is a complex process involving inflammation, induction, and remodelling, intrinsically regulated by the substantial interaction between innate immune cells and bone cells (Jeong et al. 2025). Calcium is recruited to the bone defect site through a process in which osteoblasts are attracted to the lesion area by chemical signals, leading them to migrate and deposit minerals, effectively initiating bone regeneration. While inflammation is an essential part of the bone regeneration process, a chronic or persistently dysregulated inflammatory response in inflammatory alveolar disease is detrimental to the surrounding tissues (Hussein and Kishen 2022). This process can be further enhanced by the use of biomaterials such as calcium phosphate ceramics, which serve as a scaffold for calcium deposition and cell adhesion, essentially mimicking the natural bone matrix (Amini et al. 2012).

Additionally, the relationship between calcium and phosphorus must be considered. Phosphorus, in the form of phosphate, is known to inhibit both active cellular resorption and bone mineral dissolution (Raisz 1969). However, in this study, its concentration was found to be reduced in the periapical lesion, which may suggest that, under certain pathological conditions, this inhibition of bone resorption does not occur effectively. This decrease in phosphate availability may be associated with an imbalance in local bone metabolism (Tenenbaum et al. 1989), favouring disorganised calcium deposition or serving as a response to the inflammatory process (Sigel et al. 2013). Thus, the findings of this study indicate that, despite the characteristic bone resorption of periapical lesions, the higher calcium concentration detected by ICP-OES may reflect the presence of reactive calcification, remnants of mineralised matrix, or even an initial bone repair process, representing an attempt by the body to prevent the progression of the periapical lesion.

Although iron concentration was significantly higher in the induced periapical lesion conditions compared to the sham control, its distribution, as observed in the μ-XRF mapping, was limited to the apex of the periapical region and the adjacent bone tissue. This localised distribution was not observed in the healthy bones. In the ICP-MS analysis, the concentrations of the isotopes ^{56}iron e ^{57}iron were investigated, as they are the most abundant, while ^{54}iron is considered potentially radioactive (Dauphas and Rouxel 2006). The results revealed statistically significant differences between the experimental conditions, and may be related to the essential role of iron in collagen biosynthesis (Beattie and Avenell 1992), in the formation of the bone matrix (Maciejewska et al. 2014), and its significant influence on bone mineral density (Medeiros et al. 2002).

Both manganese and copper were elements that showed an increased concentration in the induced periapical lesions. Copper has two predominant and stable isotopes (Lauwens et al. 2018), while manganese has only one stable isotope (Moreira et al. 2024). Both metals possess antioxidant properties, which may be associated with an increased need for these metals to neutralize free radicals and protect cells (Chellan and Sadler 2015; Lowe et al. 2002). Copper's effects are closely related to iron homeostasis and the regulation of reactive oxygen species (Yu et al. 2024). Manganese, on the other hand, plays a crucial role in the synthesis of mucopolysaccharides (Saltman and Strause 1993), and its deficiency is associated with impairments that delay the osteogenesis process (Cashman and Flynn 1998). This element also appears to play a role in the regulation of bone remodelling, as its absence has been previously correlated with elevated extracellular concentrations of calcium, phosphate, and alkaline phosphatase (Bergstrom 1954; Friedman et al. 1987).

Both sodium and potassium showed increased concentrations with significant differences between conditions, highlighting the potential impact of local inflammatory lesions on the concentration of these elements in bone tissue. This finding supports a previous study (Starke et al. 2012) that describes how potassium and sodium play a crucial role in bone health, modulating the activity of osteoblasts and osteoclasts through potassium and sodium channels present in the cell membranes of these cells. The presence and proper functioning of these channels are essential for the homeostasis of the bone microenvironment, as they regulate the intracellular chemical balance, which is crucial for bone formation and resorption processes (Singh and Kushwaha 2024). Thus, potassium and sodium not only act as essential electrolytes for systemic functions (Sigel et al. 2013), but also play a specific role in bone health (Bergstrom 1954).

Magnesium is an essential cofactor in many biochemical reactions that occur in bone (De Baaij et al. 2015), especially in the formation and maintenance of hydroxyapatite (the main mineral form of bone), as it helps stabilise hydroxyapatite crystals and, therefore, is important for the structural integrity of bone (Salimi et al. 1985). The results showed a statistically significant difference with an increase in magnesium concentration when comparing animals with induced periapical lesions to the control (sham). This alteration may be related to the magnesium regulatory role in inflammation, as altered levels of this mineral influence the release of pro-inflammatory cytokines and modulate macrophage activity, which are essential cells in the immune response to the infectious process (Maier et al. 2021).

Although zinc did not show a statistically significant difference between the healthy and diseased states, it has been reported that this metal plays a role in the growth, development, and maintenance of healthy bones by promoting osteoblast activity, inhibiting osteoclast function, and stimulating the synthesis of bone proteins, which results in increased bone mass and growth (Bouglé et al. 2004; Fang et al. 2024; Yamaguchi et al. 2000). The μ-XRF analysis (by mappings) revealed that the distribution of zinc is not homogeneous in both conditions, health and disease. A greater number of areas with reddish coloration, indicative of elevated zinc concentrations, were observed in the sham control compared to the induced periapical lesions. However, the ICP-MS analysis did not reveal statistically significant differences in zinc concentrations, even when considering the different isotopes of this element (Cloquet et al. 2008). This pattern suggests that, in the absence of lesions, zinc may be more evenly distributed and present in higher concentrations in specific regions, reflecting its role in balancing bone metabolism and maintaining the integrity of healthy tissue (Yamaguchi 2010). On the other hand, in the induced periapical lesions, the reduction in areas of high concentration may be associated with increased osteoclastic activity or the redistribution of the metal in response to the inflammatory and infectious process (Ceylan et al. 2021).

The potential advancements provided by the incorporation of strontium, cobalt, and manganese into the composition of calcium phosphate bone cements are already established (Bernhardt et al. 2017; Cummings et al. 2017; Wu et al. 2020). Zinc-releasing ceramics have been investigated due to the combination of their osteoinductive and immunomodulatory properties, which act synergistically (Huang et al. 2021). These strategies can enrich these materials with the biological properties specific to the elements, while modulating their physicochemical characteristics, a modification that could promote greater viability and activity of human mesenchymal stem cells and stimulate angiogenesis, highlighting the biological benefits associated with ionic modification (Montesi et al. 2017; Tadier et al. 2012; Thormann et al. 2013).

Although this study has limitations due to the use of an animal model to translate its results to humans, it provides novel preliminary data that can be deepened in clinical studies. A more detailed understanding of these element interactions in health and disease conditions may open new perspectives for the development of therapeutic strategies based on the role of the metallic elements investigated here in bone formation and repair processes. Among the potential approaches, mineral replacement or chelation targeting the periapical microenvironment and/or local interventions that regulate the levels of these metals to control or modulate inflammation and oxidative stress would be highly desired. After a thorough establishment of the metallographic profile, and with further studies, it could be possible to develop enriched endodontic sealers and/or materials with the ability to benefit periapical repairing processes.

Conclusion

This study demonstrated differences in the levels of various essential elements between conditions with periapical lesions and healthy controls. Significant changes were observed in calcium, iron, manganese, copper, sodium, potassium, and magnesium levels, indicating that periapical lesions can directly affect the mineral homeostasis of periapical bone tissue. These findings emphasize the importance of investigating the mineral profile in bone lesions to gain a deeper understanding of the mechanisms involved in their pathophysiology and potentially aid in developing targeted therapeutic strategies.

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3. CONCLUSÃO

Os achados deste estudo evidenciam alterações marcantes nos níveis de cálcio, ferro, manganês, cobre, sódio, potássio e magnésio, indicando um impacto direto da lesão periapical na homeostase mineral do osso alveolar. A caracterização metalográfica e estrutural, por meio de múltiplas metodologias, permitiu uma compreensão mais aprofundada da fisiopatologia da doença, contribuindo para o avanço do conhecimento e o desenvolvimento de estratégias terapêuticas para a preservação da integridade óssea periapical.

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Todos os documentos a seguir são referentes ao Apêndice da dissertação e material suplementar do artigo submetido à revista.

Apêndices

Apêndice 1: Supplementary material 1 – Sample size calculation

Supplementary table 1 – Description of the reference methodologies used for sample size calculation, unit, estimated standard deviation, estimated minimum difference to be detected and estimated number of animals.

Reference method	Unit	Estimated standard deviation	Estimated minimum detectable difference	N estimated / Observations
Histological Analysis (Marciano et al., 2016)	Inflammatory score (0 to 3)	0.5 average score	1	10
ICP-MS of relevant chemical elements adapted from literature studies (Grassin-Delyle et al., 2019; Marciano et al., 2023)	Mass fractions (ng/g)	10 ng/g	20 ng/g	10
Weight variation of animals (Marciano et al., 2023)	Grams (g)	31.8 g (obtained from all animals before the procedure)	18 g	118 animals in total; however, reducible (3Rs) if normally distributed at baseline since this is not the main outcome of the study.

Apêndice 2: Supplementary material 2 – Experimental study design

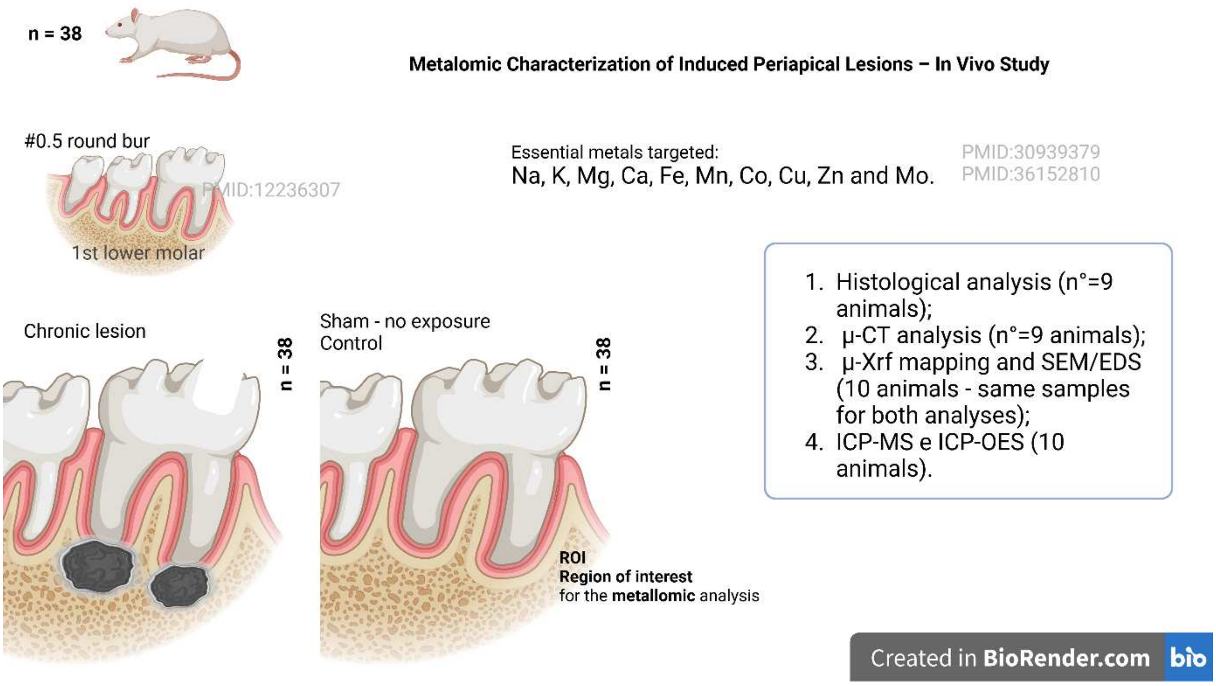

Figure S1: Experimental Scheme: Half of the animals underwent periapical lesion induction for 40 days, following previously proposed methodologies (Metzger et al., 2002; Gomes et al., 2019). For the sham control, identical procedures were performed, except for the lesion induction cavity in the first lower molars. During the immediate postoperative period, one animal designated for each experimental condition did not survive anaesthesia administration, leading to their exclusion from the study. As a result, the initial sample of 40 animals was reduced to 38, with 19 animals per group. After the 40-day induction period, all animals were euthanised, and different methodologies, including periapical radiography, X-ray Fluorescence Microscopy, histology, μ-CT, scanning electron microscopy with energy dispersive spectroscopy, ICP-MS, and ICP-OES, were conducted to establish the metallographic profile, focusing on the ten chemical elements considered essential for metabolism: sodium, potassium, magnesium, calcium, iron, manganese, cobalt, copper, zinc, and molybdenum, comparing the conditions with and without lesion in the region-of-interest (ROI).

Apêndice 3: Supplementary material 3 - Experimental sampling and storage scheme

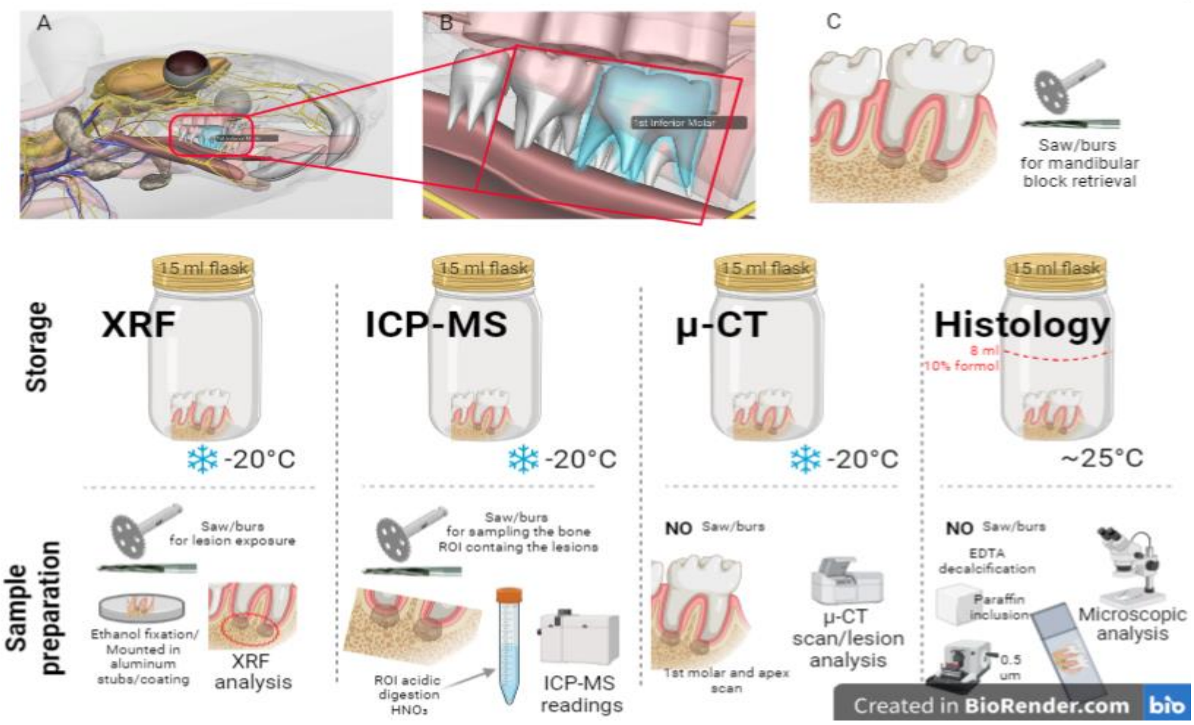

Figure S2: Experimental sampling and storage scheme: (A, B) Tissue that was removed from the animal's mandible (C) using blunt-ended scissors. Each sample was stored individually in 15 mL containers, following the specific methodology employed. The samples intended for analysis by µ-XRF, ICP-MS, and µ-CT were frozen at -20°C until processing. For histological analysis, the specimens were immersed in 8 mL of 10% formalin and stored protected from light at controlled room temperature. Before performing any method, all hemimandibles were subjected to digital radiographs. The preparation for µ-XRF involved exposing the lesion region, keeping it attached to the dental element, fixation, and dehydration in an increasing ethanol gradient, followed by mounting on stubs. For SEM/EDS analysis, carbon coating was applied for lesion analysis. For ICP-MS and ICP-OES analysis, the bone sample was separated from the dental element and digested in nitric acid under a microwave-activated pressure system to allow for the reading of the chemical elements of interest. For µ-CT analysis, no additional sectioning was performed, and the specimens were scanned, reconstructed, and analysed using specific software. For histological analysis, decalcification, paraffin embedding was performed to enable the use of a microtome (slices of 0.5 µm), which were then mounted and stained with H&E for analysis under an optical microscope.

Apêndice 4: Supplementary material 4 – μ-CT analysis

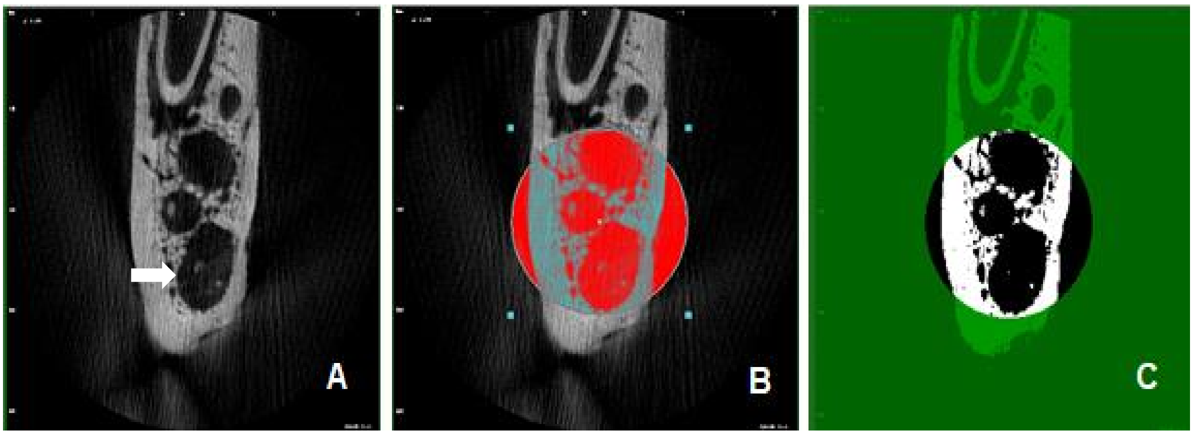

Figure S3: (A) μ-CT image of the region of the first lower molar, axial view, with white arrow indicating the periapical lesion site adjacent to the mesial root. (B) Delimitation of the region of interest containing the induced periapical lesion (red circle). (C) Selected region of interest for analysis of the volume of the induced periapical lesion.

Apêndice 5: Supplementary material 5 - Operational conditions for ICPs analysis

Supplementary table 2: Settings and flow parameters for ICP-MS analysis

Category	Parameter	Value
Flows (L/min)	Plasma Flow	9.0
	Auxiliary Flow	1.5
	Protection Gas Flow	0.0
	Nebulizer Flow	1.03
Torch Configuration	Sampling Depth (mm)	5.0
Operation	RF Power (kW)	1.35
	Pump Rate (rpm)	25
	Stabilization Time (s)	15
Skimmer and Gases	Skimmer Gas Source	He
	Skimmer Flow (mL/min)	80
	Nitrox - Flow (mL/min)	0
	Skimmer - Polarization	0.5

Supplementary table 3. Operational conditions of ICP-OES

Parameter	Valor
Power	1.450 W
Plasma Gas Flow	Not applicable
Auxiliary Gas Flow	0.5 L/min
Nebulizer Gas Flow	0.75 L/min
Nebulization Chamber	Cyclonic

Apêndice 6: Supplementary material 6 – Weight assessment

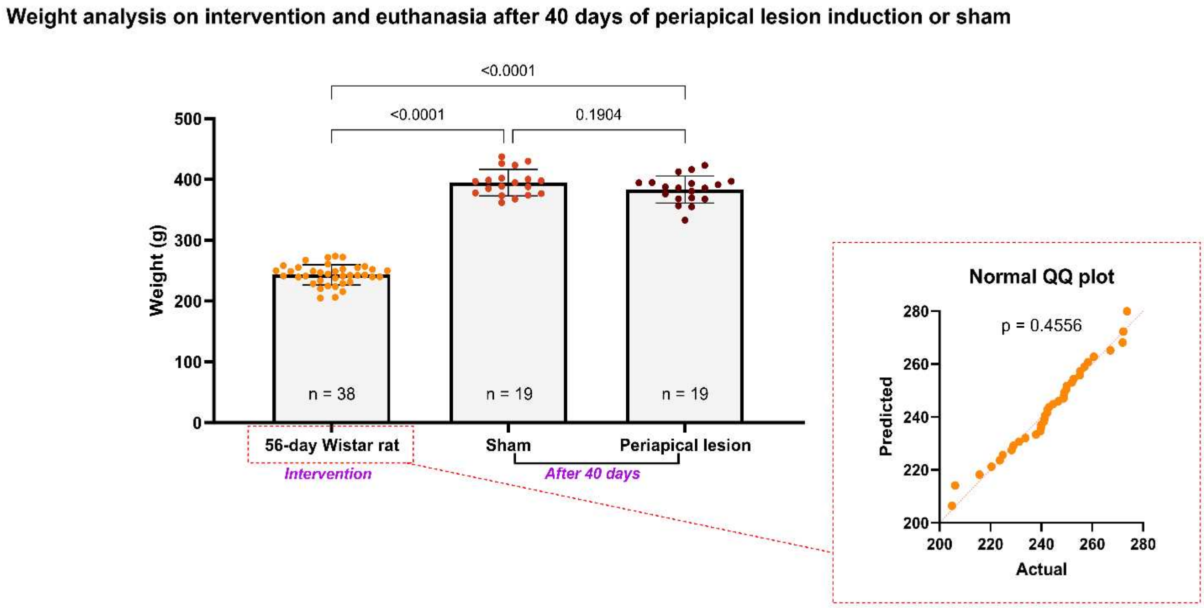

Figure S4: During the immediate postoperative period, one animal designated for each experimental condition did not survive anaesthesia administration, leading to their exclusion from the study. As a result, the initial sample of 40 animals was reduced to 38, with 19 animals per condition. The initial body weight of these 38 animals, recorded on the day of lesion induction (or its sham), followed a normal distribution, as indicated in the Normal QQ plot representation ($p = 0.4556$). After the 40-day experimental period, similar ($p = 0.1904$) body weight was observed between the two experimental conditions.

Apêndice 7: Supplementary material 7 – Radiographic images from all molars' periapical region

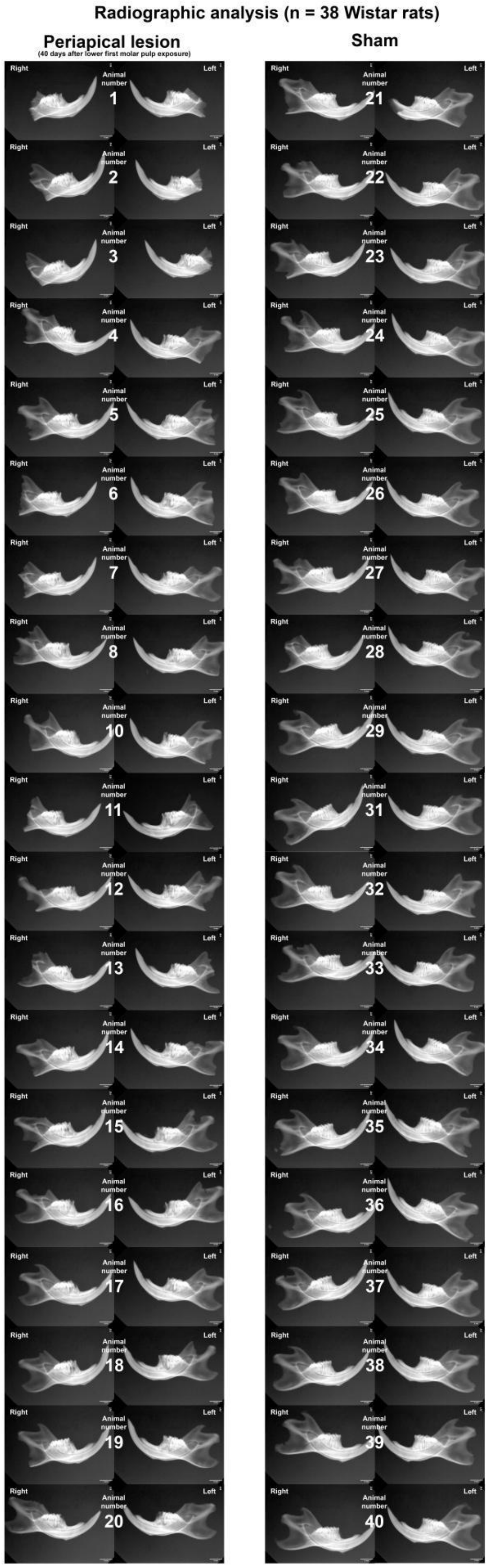

Figure S5: Periapical radiographs of Wistar rat (n = 38) hemimandibles (n = 76) obtained for the evaluation of bone structure within the two experimental conditions. The periapical lesions (left) underwent pulp exposure of the first lower molar for 40 days, leading to the induction of periapical lesions as suggested by the images; while sham controls (right) received no pulp exposure. Images were acquired using a high-resolution digital radiographic (SPECTRO 70X model and SOPRO – FIT Digital Sensor Software, Brazil) system with standardised exposure parameters to ensure comparability. Differences in radiopacity and bone continuity between conditions can be observed, allowing the identification of bone resorption areas associated with the periapical inflammatory process.

Anexos

Anexo 1 – Comitê de ética

CERTIFICADO CEUA nº 118/2023

C E R T I F I C A D O

Certificamos que a proposta intitulada **Caracterização metalômica de lesões periapicais induzidas**, registrada com o nº **6219-1/2023**, sob a responsabilidade de **Prof. Dr. Marina Angélica Marciano da Silva e Lauter Eston Pelepenko Teixeira**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, 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), tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP**, em reunião de **19/04/2023**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	01/06/2023 a 01/10/2025
Vigência da autorização para manipulação animal:	19/04/2023 a 01/10/2025
Espécie / linhagem/ raça:	Rato heterogênico / HanUnib:WH
No. de animais:	20
Idade/Peso:	9.00 Semanas / 300.00 Gramas
Sexo:	20 Machos
Espécie / linhagem/ raça:	Rato heterogênico / HanUnib:WH
No. de animais:	20
Idade/Peso:	9.00 Semanas / 300.00 Gramas
Sexo:	20 Machos
Origem:	CEMIB/UNICAMP (Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório - UNICAMP)
Biotério onde serão mantidos os animais:	Biotério da Faculdade de Odontologia de Piracicaba, FOP/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização a junto ao **IBAMA**, **SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, **20 de junho de 2023**.

Profa. Dra. Cinthia Baú Betim Cazarin
Vice-presidente da CEUA/Unicamp

Eduardo Villaverde Haszler
Secretário Executivo

IMPORTANTE: [illegible]

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Anexo 2 - Comprovante de submissão artigo - *International Endodontic Journal*

INTERNATIONAL ENDODONTIC JOURNAL
The official journal of the British Endodontic Society and the European Society of Endodontology

Original Article

Metallomic characterisation of induced periapical lesions – in vivo study

Submission ID	2505d455-2263-4717-9954-46c30f3105ec
Submission Version	Initial Submission
PDF Generation	27 Feb 2025 21:18:42 EST by Atypon ReX

Files for peer review

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Name	**Type of File**	**Size**	**Page**
IEJ _ metallomic.docx	Anonymized Main Document - MS Word	8.4 MB	Page 2
Supplementary.docx	Supplementary Material for Review	16.0 MB	Page 48

Anexo 3 - Relatório final de similaridade

Caracterização metalômica de lesões periapicais induzidas: estudo in vivo

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