UNIVERSIDADE FEDERAL DO PARANÁ

JOÃO VICTOR SCHOEMBERGER ROTH

CARACTERIZAÇÃO HISTOLÓGICA E DO PERFIL INFLAMATÓRIO LOCAL E SISTÊMICO DA PROGRESSÃO E REPARO PERIODONTAL APÓS LIGADURA EM RATOS

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia, Setor de Ciências da Saúde da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Odontologia.

Orientador: Prof. Dr. João Paulo Steffens

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ODONTOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **JOÃO VICTOR SCHOEMBERGER ROTH** intitulada: **CARACTERIZAÇÃO HISTOLÓGICA E DO PERFIL INFLAMATÓRIO LOCAL E SISTÊMICO DA PROGRESSÃO E REPARO PERIODONTAL APÓS LIGADURA EM RATOS**, sob orientação do Prof. Dr. JOÃO PAULO STEFFENS, que após terem inquirido o aluno e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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RESUMO

A periodontite induzida por ligadura é um dos diversos métodos possíveis para se estudar a progressão e o reparo dos tecidos periodontais em modelos animais. Compreender como fatores temporais, como a presença da ligadura e o tempo de reparo, influenciam o perfil imunológico e a perda óssea é crucial para se compreender a etiopatogênese da doença. Este estudo teve como objetivo caracterizar o perfil imuno-inflamatório sistêmico e gengival durante a progressão e reparo da periodontite induzida por ligadura em modelo animal. Um total de 48 ratos Wistar machos foram incluídos, sendo oito animais no grupo Controle (nenhum procedimento foi realizado e com eutanásia ao final do experimento). Os 40 animais designados para os grupos experimentais Ligadura e Reparo foram submetidos a inserção de uma ligadura de algodão ao redor dos primeiros molares inferiores bilateralmente, e mantida por 7 dias. Depois disso, em T-0 (baseline) as ligaduras foram mantidas ou removidas por mais 28 ou 56 dias (n=8/grupo). Totalizando 7, 35 e 63 dias para os grupos ligadura. Após a eutanásia, hemimandíbulas, sangue e tecidos gengivais foram coletados. As hemimandíbulas do lado direito foram submetidas inicialmente ao exame radiográfico periapical e em seguida ao processamento histológico para análise estereométrica. Soro e tecido gengival ao redor dos primeiros molares das hemimandíbulas do lado esquerdo foram submetidos ao ensaio multiplex para analisar interleucina (IL)-1β, fator de necrose tumoral (TNF)-α, interferon (IFN)-γ, IL-4, IL-6, IL-10, fator de crescimento endotelial vascular (VEGF) e fator de crescimento epidermal (EGF). Amostras de soro foram submetidas também a análise colorimétrica da fosfatase ácida. A perda óssea foi maior nos animais que mantiveram a ligadura com o passar do tempo ($p<0,05$). Já nos animais do grupo reparo, em 56 dias após baseline observa-se um aumento na perda óssea alveolar ($p<0,05$). A ligadura aumentou significativamente as células inflamatórias e reduziu os fibroblastos, enquanto os vasos sanguíneos aumentaram apenas em 7 e 35 dias. A remoção da ligadura diminuiu significativamente as células inflamatórias e os vasos sanguíneos, com aumento de fibroblastos ($p<0,05$). Os animais do grupo ligadura apresentaram, no ensaio imunológico de soro e tecido gengival, aumento de TNF-α e IL-1β e diminuição de IL-4 e VEGF em diferentes tempos ($p<0,05$). Nos grupos reparo, IL-1β, IL-4, IL-10, EGF e VEGF apresentaram diminuição do padrão dependente do tempo ($p<0,05$). O VEGF no soro demonstrou nível aumentado durante o reparo de 28 dias com aumento no reparo de 56 dias ($p<0,05$). A periodontite induzida por ligadura aumentou a perda óssea de maneira dependente do tempo, com aumento de células inflamatórias, vasos sanguíneos e citocinas pró-inflamatórias, como IL-1β e TNF-α. Após a remoção da ligadura, o reparo periodontal não diminuiu a perda óssea alveolar. Porém, após 56 dias, houve redução de células inflamatórias e citocinas pró-inflamatórias, apesar do aumento da perda óssea.

Palavras-chave: periodontite; perda do osso alveolar; ratos wistar; inflamação.

ABSTRACT

Ligature-induced periodontitis is one of several methods used to study the progression and repair of periodontal tissues in animal models. Understanding how temporal factors, such as the presence of the ligature and repair time, influence the immunological profile and bone loss is crucial for comprehending the etiopathogenesis of the disease. This study aimed to characterize the systemic and gingival immuno-inflammatory profile during the progression and repair of ligature-induced periodontitis in an animal model. A total of 48 male Wistar rats were included, with eight animals in the Control group (no procedures were performed and euthanized at the end of the experiment). The 40 animals designated for the Ligature and Repair experimental groups underwent the insertion of a cotton ligature around the lower first molars bilaterally, which was maintained for 7 days. Subsequently, at T-0 (baseline), the ligatures were either maintained or removed for an additional 28 or 56 days (n=8/group). Totaling 7, 35, and 63 days for the ligature groups. After euthanasia, hemimandibles, blood, and gingival tissues were collected. The right-side hemimandibles were initially subjected to periapical radiographic examination and then to histological processing for stereometric analysis. Serum and gingival tissue around the first molars of the left-side hemimandibles were subjected to multiplex assays to analyze interleukin (IL)-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-4, IL-6, IL-10, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF). Serum samples were also subjected to a colorimetric analysis of acid phosphatase. Bone loss was greater in animals that maintained the ligature over time ($p<0.05$). In the repair group, an increase in alveolar bone loss was observed 56 days after baseline ($p<0.05$). The ligature significantly increased inflammatory cells and reduced fibroblasts, while blood vessels increased only in 7 and 35 days. Ligature removal significantly decreased inflammatory cells and blood vessels, with an increase in fibroblasts ($p<0.05$). The ligature group animals showed, in the immunological assay of serum and gingival tissue, an increase in TNF-α and IL-1β and a decrease in IL-4 and VEGF at different times ($p<0.05$). In the repair groups, IL-1β, IL-4, IL-10, EGF, and VEGF showed a time-dependent decrease pattern ($p<0.05$). Serum VEGF levels demonstrated an increase during the 28-day repair period, with a further increase at 56 days ($p<0.05$). Ligature-induced periodontitis increased bone loss in a time-dependent manner, with an increase in inflammatory cells, blood vessels, and pro-inflammatory cytokines such as IL-1β and TNF-α. After ligature removal, periodontal repair did not decrease alveolar bone loss. However, after 56 days, there was a reduction in inflammatory cells and pro-inflammatory cytokines despite the increased bone loss.

Key-Words: periodontitis, alveolar bone loss, inflammation, rats.

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INTRODUÇÃO

A periodontite é uma doença inflamatória crônica multifatorial associada ao biofilme disbiótico e caracterizada pela destruição progressiva do aparelho de suporte dentário. Suas principais características incluem a perda de suporte do tecido periodontal, manifestada através da perda de inserção clínica e perda óssea alveolar avaliada radiograficamente, presença de bolsas periodontais e sangramento gengival (PAPAPANOU; SANZ; BUDUNELI; DIETRICH *et al.*, 2018). Além disso, as doenças periodontais também resultam em um estado pró-inflamatório sistêmico, que está relacionado com diversas doenças como: doenças cardiovasculares, doenças cardiometabólicas (DCM), problemas de saúde mental, doenças autoimunes e até mesmo com condições musculares, como a força de preensão manual (ROTH; GUARENGHI; FERRO; VALENGA *et al.*, 2024; ZEMEDIKUN; CHANDAN; RAINDI; RAJGOR *et al.*, 2021). **(ANEXO 1)**

Um estudo epidemiológico recente demonstrou que em uma população idosa entre 65 e 74 anos cerca de 82% dos indivíduos apresentava periodontite, além de ser considerada como uma das causas mais frequentes de perda dentária (NAZIR; AL-ANSARI; AL-KHALIFA; ALHAREKY *et al.*, 2020). É importante explorar a etiopatogenia da periodontite, mas os estudos clínicos enfrentam limitações devido à complexidade da doença, que pode envolver interações entre genes, comportamento e o biofilme dentário. Além disso, questões éticas e a diferença na predisposição dos indivíduos humanos à progressão da periodontite são um desafio.(YOON; JUNG; YOO; LEE *et al.*, 2023).

Na progressão da periodontite, a microbiota periodontopatogênica, principalmente bactérias anaeróbias gram-negativas como *Porphyromonas gingivalis*, causam danos aos tecidos através de fatores de virulência únicos, levando à inflamação e ao aumento de mediadores pró-inflamatórios (MOISEEV; DONSKOV; DUBROVIN; KULYUKINA *et al.*, 2023). A todo momento autores estão explorando novas abordagens para melhor compreender e desenvolver novas opções de tratamento. Para isso a investigação em animais de laboratório se faz necessária. Encontrar um modelo animal ideal é um desafio devido aos

fatores complexos envolvidos na progressão da periodontite (MOISEEV; DONSKOV; DUBROVIN; KULYUKINA *et al.*, 2023).

Nas últimas décadas, uma variedade de modelos de periodontite em roedores foi estabelecida e aplicada com eficácia para a exploração do desenvolvimento da doença (GRAVES; FINE; TENG; VAN DYKE *et al.*, 2008). Dentre eles, a manipulação de uma dieta com alta ingestão de carboidratos, a injeção de lipopolissacarídeos bacterianos (LPS) diretamente no sulco gengival ou a colocação de uma ligadura com fio de nylon ou algodão foram relatados como modelos bem-sucedidos de periodontite (DUMITRESCU; ABD-EL-ALEEM; MORALES-AZA; DONALDSON, 2004; TAKADA; YOSHINARI; SUGIISHI; KAWASE *et al.*, 2004).

Durante a indução da periodontite experimental com uso da ligadura, o biofilme se acumula ao redor da ligadura, o que leva à destruição do tecido periodontal em um período curto de tempo (ABE; HAJISHENGALLIS, 2013). A administração oral de bactérias e a injeção local de lipopolissacarídeos (LPS) ou *P. gingivalis* também são métodos comumente utilizados para estabelecer a periodontite (ROJAS; GARCÍA; POLANCO; GONZÁLEZ-OSUNA *et al.*, 2021). No entanto, estes dois métodos levam mais tempo (cerca de quatro até seis semanas) para produzir uma perda significativa de tecido periodontal em relação à ligadura (7 a 14 dias) (HART; SHAFFER; AKILESH; BROWN *et al.*, 2004). Portanto, o modelo de periodontite induzida por ligadura possui uma fácil aplicação com excelentes resultados em um curto período. O uso da ligadura também pode estar associado a fatores bacterianos, inoculados localmente no fio de algodão, uma injeção sistêmica de LPS ou *P. gingivalis* ou uma ligadura com gavagem oral de *P. gingivalis* (LIN; NIIMI; OHSUGI; TSUCHIYA *et al.*, 2021).

Atualmente os estudos apresentam heterogeneidade em relação ao tempo considerado ideal para o estabelecimento da doença, e após sua remoção o tempo considerado ideal para promover o reparo. Estudos usando 5, 7, 14, 28, 35 dias e até mesmo o período de 3 meses já foram descritos na literatura (BOSTAN; YEMENOGLU; KOSE; AKYILDIZ *et al.*, 2024; LEE; KIM; TRANG; LEE *et al.*, 2023; MENDES; SORDI; OLCHANHESKI; MACHADO *et al.*, 2014; WU; YAN; WU; CHEN *et al.*, 2023; ZHANG; TAN; LUO; JIA, 2023). Após a

remoção da ligadura, espera-se que ocorra o reparo (SPOLIDORIO; LUCAS; STEFFENS; DA SILVA *et al.*, 2014), geralmente entre o período de 3 até 30 dias após a remoção da ligadura (COIMBRA; STEFFENS; ROSSA; GRAVES *et al.*, 2014; GUIMARAES-STABILI; DE AQUINO; DE ALMEIDA CURYLOFO; TASSO *et al.*, 2019; ZUZA; GARCIA; THEODORO; ERVOLINO *et al.*, 2018; ÖNGÖZ DEDE; BOZKURT DOĞAN; BALLI; DURMUŞLAR *et al.*, 2021). Entretanto, alguns estudos também apontam que após a remoção da ligadura, o processo de reparo ósseo pode não ocorrer, levando a progressão da perda óssea alveolar (GARCIA; MIESSI; ESGALHA DA ROCHA; GOMES *et al.*, 2022; GARCIA; ROCHA; GOMES; MIESSI *et al.*, 2023). Além disso, uma caracterização imuno-inflamatória abrangente de como ocorre o reparo em roedores a longo prazo não está disponível.

OBJETIVOS

Objetivo Geral

O objetivo deste trabalho foi avaliar e caracterizar as alterações locais e sistêmicas decorrentes da periodontite induzida por ligadura e o subsequente reparo periodontal após a sua remoção em ratos Wistar.

Objetivos Específicos

1. Avaliar a perda óssea radiográfica dos grupos experimentais.
2. Avaliar através de análise estereométrica o tecido gengival para o perfil inflamatório característico de cada grupo experimental.
3. Avaliar através de ensaio multiplex as citocinas e fatores de crescimento envolvidas no processo de progressão da periodontite e reparo periodontal.

ARTIGO – ARCHIVES OF ORAL BIOLOGY

Local and systemic characterization of inflammatory profile in long-term ligature-induced periodontitis and repair in rats.

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Abstract

Objective: This study aimed to characterize time-dependent alveolar bone loss, histological changes, and immunoinflammatory profiles in rats during ligature-induced periodontitis progression and repair in the long term.

Design: Forty-eight male Wistar rats were included. The negative control group (n=8) received no ligature and was euthanized at the end of the experiment. In experimental groups (Ligature and Repair; n=40), rats received a cotton ligature around the lower first molars bilaterally, which was kept for seven days before baseline. After this period, in baseline, ligatures were either maintained or removed for another 28 or 56 days (n=8/group). Hemi-mandibles, blood, and gingival tissues were collected for analyses.

Results: Bone loss was higher in ligated animals over time ($p<0.05$). Ligature placement significantly increased inflammatory cells and reduced fibroblasts, with blood vessel increases only at 7 and 35 days. After 56 days of ligature removal, alveolar bone loss increased ($p<0.05$). Ligature removal decreased inflammatory cells and blood vessels and increased fibroblasts ($p<0.05$). Immunological assays showed increased TNF-α and IL-1β and decreased IL-4 and VEGF in ligated animals ($p<0.05$). In repair groups, IL-1β, IL-4, IL-10, EGF, and VEGF decreased over time ($p<0.05$).

Conclusion: Ligature-induced periodontitis led to time-dependent alveolar bone loss. Inflammatory cells and pro-inflammatory cytokines increased in ligated animals. During repair, despite reducing inflammatory cells and cytokines, alveolar bone loss increased after 56 days of ligature removal.

Key-Words: Periodontitis, Alveolar Bone Loss, Inflammation, Rats.

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Author Guidelines Archives of Oral Biology **(Annex 2)**

1. Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with a dysbiotic biofilm and characterized by progressive destruction of the tooth support tissues (Papapanou et al., 2018). The presence of periodontal pocket, gingival bleeding, and bone attachment loss, as observed by clinical or radiographic analyses, can be described as the main signs. It is also described as one of the most prevalent oral diseases worldwide (Nazir et al., 2020). Periodontitis develops in stages and includes several phases: primary colonization by pathologic bacterial agents, formation and maturation of microbial biofilm, invasion of periodontal tissues by oral microbiota and their metabolites, induction of exaggerated host immune response, and destruction of periodontal tissues with secondary changes in the dentoalveolar complex (Marchesan et al., 2018).

Despite the need to understand the etiopathogenesis of periodontitis, clinical studies face limitations due to the complexity of the disease, which can involve interactions between genes, behavioral aspects, comorbidities, and dental plaque. Also, ethical issues and differences in human individuals' predisposition to periodontitis progression increase the challenge in the clinical setting (Yoon et al., 2023).

In the past few decades, a variety of periodontitis models in rodents have been established and effectively applied to the exploration of the mechanism of periodontitis and the efficacy of new treatments (Graves et al., 2008). Among them, manipulating a diet with high carbohydrate intake, injecting bacterial lipopolysaccharides (LPS) directly into the gingival sulcus, or placing a ligature with nylon, cotton, or orthodontic thread, which acts as a factor that facilitates the accumulation of periodontopathogens, have been reported as successful periodontitis models (Dimitriu et al., 2021; Dumitrescu et al., 2004; Takada et al., 2004). In fact, rodents are widely used for experimental modeling of periodontitis and have several advantages: small size, low cost, known age and genetic background, controlled microbiota, and ease of care and handling; they are characterized by the development of the highly reproducible inflammatory process in the periodontium (Moiseev et al., 2023).

A great source of heterogeneity among studies evaluating the progression or repair of ligature-induced periodontitis is the time carried out for the disease development and, after ligature removal, the time expected to restore health. Studies using 5, 7, 14, 28, and 35 days to longer periods such as three months have been reported to induce periodontitis (Bostan et al., 2024; Lee et al., 2023; Mendes et al., 2014; Wu et al., 2023; Zhang et al., 2023). After ligature removal, repair is expected to occur (Spolidorio et al., 2014), generally between 3 to 30 days after the ligature removal (Coimbra et al., 2014; Guimaraes-Stabili et al., 2019; Zuza et al., 2018; Öngöz Dede et al., 2021). However, some studies

suggest that bone loss can increase after ligature removal (Garcia et al., 2022; Garcia et al., 2023). Furthermore, comprehensive immunoinflammatory characterization of how repair occurs in rodents in the long term is not available.

Therefore, this study aimed to characterize time-dependent alveolar bone loss, histological changes, and systemic and local immunoinflammatory profile in rats during ligature-induced periodontitis progression and repair.

2. Materials and methods

2.1 Animals

Sample size calculation was carried out based on alveolar bone loss, for an intergroup difference of ten points with a standard deviation of seven points, eight animals per group were needed, establishing alpha at 5% and a power of 80%. For that, forty-eight male adult Wistar rats (*Rattus norvegicus albinus*), aged approximately eight weeks, were kept in cages with similar conditions (temperature 22±2°C, humidity 45±15%, and light cycles 12/12-hour light/dark). Food and water were available ad libitum. After seven days of acclimatization, animals were randomized into three groups: Negative Control (n=8), Ligature (n=24), and Repair (n=16), using computer-generated numbers (Figure 1). All experimental protocols were approved by the Positivo University Institutional Ethics Committee for Animal Experimentation (protocol #7.1/2021) **(Annex 3)** and carried out following the Brazilian Society of Science in Laboratory Animals (SBCAL) and National Council for the Control of Animal Experimentation (CONCEA). This study was developed and reported following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) standards.

2.2 Induction of experimental periodontal bone loss and periodontal repair

On day -7 (7 days before baseline), the animals in the ligature and repair groups were anesthetized with ketamine (0.08mL/100g) and xylazine (0.04mL/100g) and received a cotton ligature (#30) around the lower first molars bilaterally. Animals in the ligature groups were euthanized at baseline (n=8) or maintained the ligatures for another 28 (n=8) or 56 (n=8) days after baseline (total, 35 or 63 days, respectively). Animals in the repair group had their ligatures removed at baseline to promote periodontal repair (Steffens et al., 2018) and were euthanized after 28 (n=8) or 56 days (n=8). Control animals received no ligature and were euthanized at the end of the experiment (n=8).

2.3 Euthanasia and sample collection

At baseline, 28 and 56 days after, animals were euthanized by anesthetic overdose. Blood samples were collected through cardiac puncture for serum analyses of inflammatory markers and growing factors. Mucogingival tissues around the first molars on the left side were removed and kept at -80°C for subsequent analysis of inflammatory markers and growing factors. The

anatomical pieces of the right side were stored for radiographic and histological analysis.

2.4 Radiographic analysis

Eight right hemi-mandibles per group were submitted to radiographic examination to analyze radiographic bone loss. A dental X-ray device (xDent x70 device, XDENT Dental Equipment, Ribeirão Preto/ SP, Brazil) was used to perform the radiographic shots, with standardization of distance, exposure time, and positioning of the samples. Digital software (Saevo Digital Image Software, Version 2.0.0.20, Ribeirão Preto/ SP, Brazil) was used to capture and analyze all images. Measurements were taken in millimeters from the Cementoenamel Junction (CEJ) to the Alveolar Bone Crest (ABC) from the mesial and distal root of the lower first molars. Measurements were performed three times by a trained examiner under similar environmental conditions, and the mean values were reported.

2.5 Histological processing

After radiographic processing, five hemi-mandibles from each group were decalcified in an aqueous solution containing formic acid and formalin for 30 days. Histological processing was carried out with sections of 0.5 µm thickness. Four histological slides with three semi-serial sections were made per animal and stained with Hematoxylin and Eosin. To carry out the analysis, two slides were selected, and one section of each was photographed using software (Cell^F 2008, Olympus Soft Imaging Solutions GmbH. Coermühle 2, 48157 Nord, Germany) and then submitted for evaluation by a single trained evaluator. For stereometric analysis, a 1.000µm x 500µm grid was positioned vertically over the connective tissue in the histological images at 20x magnification, with the lower edge of the grid in contact with the bone tissue and the side edge in contact with the tooth. The grid consisted of a 19x9 format totaling 200 points of interest, as previously described (Steffens et al., 2015). Structures in intersection points were identified as ''fibroblasts'', "inflammatory cells'', "blood vessels'', "extracellular matrix" or "outside region of interest''. Intersections reported outside the zone of interest were discarded, and the remaining points were considered the total number of possible points (100%).

2.6 Inflammatory profile analysis

Serum and gingival tissue samples were used for inflammatory profile analysis. Gingival tissues were macerated for protein extraction in tissue protein extraction reagent (T-PER™ lysis buffer Thermo Fisher Scientific Inc., 168 Third Avenue. Waltham, MA USA), containing protease inhibitor (SIGMAFAST™- Sigma-Aldrich Co. 3050 Spruce Street, Saint Louis, MO 63103, USA). After centrifugation, both serum and gingival samples were evaluated using multiplex (MILIPLEX Rat Cytokine/ Chemokine Magnetic Bead Panel, 2021 Merck KGaA,

Darmstadt, Germany), according to the manufacturer's instructions. The following cytokines were analyzed: Tumor Necrosis Factor-Alpha (TNF-α), Interleukin (IL)-1β, IL-4, IL-6, IL-10, Interferon-gamma (IFN-γ), Epidermal Growth Factor (EGF), and Vascular Endothelial Growth Factor (VEGF). In the gingival tissue, total protein was quantified in each sample using the DC Protein Assay protocol (Bio-Rad Laboratories, Inc. 2000 Alfred Nobel Dr. Hercules, CA, USA) for data normalization.

2.7 Acid Phosphatase Assay

For the serum samples collected, Acid Phosphatase (ACP) was analyzed using a colorimetric Acid Phosphatase Assay Kit (Sigma-Aldrich®, MAK446, 3050 Spruce Street, Saint Louis, MO 63103, USA) according to the manufacturer's instructions. Acid Phosphatase activity was calculated according to the formula provided by the manufacturer, and the result was expressed in U/L.

2.8 Statistical analysis

Homogeneity of variance and normal distribution were checked using Levene and Shapiro-Wilk tests, respectively. If both criteria were met, one-way ANOVA and post-hoc Tukey tests were performed. Otherwise, the non-parametric Kruskal–Wallis and Dunn's post-test was performed. Alternatively, Mann–Whitney and unpaired Student's t-test were performed for pairwise comparisons. Values were expressed as median or mean and standard error of the mean. All tests were performed using free software (Jamovi version 2.4.11.0 for Windows 10, https://www.jamovi.org). All graphics were made using paid software (GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, https://www.graphpad.com). The significance level was set at $p<0.05$.

3. Results

No animals or ligatures were lost in this experiment. The presence of ligature significantly induced alveolar bone loss. In the mesial root, all groups showed a statistically significant increase compared to the Negative Control ($p<0.001$). The alveolar bone loss increased in a time-dependent manner over the 63 days ($p<0.001$). Distal analysis showed that although alveolar bone loss increased over time compared with the Negative Control ($p<0.001$), there was no increase in bone loss at 28 days post-baseline (Lig 35d) and 56 days post-baseline (Lig 63d). When mean values were analyzed from mesial and distal measurements, alveolar bone loss increased over time with a statistical difference among all groups ($p<0.001$). In the periodontal repair groups, in Repair 56 (Rep 56d), bone loss was significantly increased when compared with Ligature 7 days (Lig 7d) in the mesial root ($p<0.001$) and mean values ($p<0.05$) (Figure 2).

Stereometric analysis showed increased inflammatory infiltrate in the ligature groups compared to the negative control, accompanied by a decreased fibroblast

count as the disease progressed ($p<0.001$). Blood vessels increased at Lig 7d, followed by a progressive decrease ($p<0.001$). Extracellular matrix analysis indicated a decrease in all ligature groups compared to the control ($p<0.001$) (Figure 3). In the repair group, inflammatory infiltrate decreased following ligature removal ($p<0.001$). Both Repair 28 days (Rep 28d) and Rep 56d groups exhibited a significant decrease compared to Lig 7d, with Rep 56d showing a statistically more significant reduction than Rep 28d ($p<0.001$). Fibroblasts increased in both repair groups ($p<0.01$). Blood vessels decreased during repair, with Rep 28d and Rep 56d significantly differing from each other ($p<0.01$) and from Lig 7d ($p<0.001$). Extracellular matrix content in the repair groups significantly increased when comparing Lig 7d to Rep 56d ($p<0.01$) (Figure 4).

In the analyzed inflammatory profile, ligature groups exhibited elevated serum levels of TNF-α during early-stage periodontitis ($p<0.01$), with VEGF levels decreasing in Lig 7d, subsequently increasing in Lig 35d and then decreasing again in Lig 63d ($p<0.05$). Gingival tissue demonstrated elevated IL-1β levels in Lig 7d ($p<0.01$), decreased TNF-α compared to the control group ($p<0.05$), and reduced levels of IL-4 in Lig 7d ($p<0.001$) (Figure 5). In the serum repair groups, IL-1β ($p<0.05$), IL-10 ($p<0.01$), and VEGF increased at Rep 28d, followed by a decrease at Rep 56d compared to the Lig 7d group ($p<0.05$). Gingival tissue in the repair groups showed reduced IL-1 β ($p<0.01$), IL-4 ($p<0.01$), IL-10 ($p<0.01$), VEGF ($p<0.01$), and EGF ($p<0.01$) compared to the Lig 7d group ($p<0.05$) (Figure 6). Acid Phosphatase Assay had no statistically significant difference in ligature ($p:0.067$) and repair groups ($p:0.134$). In the ligature groups, an increase was observed in Lig 7d and Lig 35d compared to the negative control, with a decrease in Lig 63d. In the repair groups, there was an increase in Rep 28d and a decrease in Rep 56d compared to Lig 7d (Figures 5 and 6).

4. Discussion

In this study, we analyzed the progression of periodontitis and its repair using radiographic, histological, and immunological analyses. Briefly, ligature maintenance led to alveolar bone loss over 63 days and increased inflammation in the connective tissue, accompanied by the expression of pro-inflammatory cytokines and growth factors in serum and gingival tissue. On the other hand, repair led to a subtle increase in alveolar bone loss 56 days after ligature removal, with decreased inflammatory cells and pro-inflammatory cytokines in serum and gingival tissue.

Our primary outcome was alveolar bone loss, measured as the distance between the CEJ and ABC. There are various methodologies described in the literature, with the most common being morphometric, histological, and radiographic analyses. Radiographic analysis using a digital sensor and software provides greater accuracy and ease of standardization compared to traditional X-ray procedures (Teeuw et al., 2009). From image acquisition to analysis, the entire

process is straightforward. While histological and morphometric analyses are equally reliable, they require additional steps for sample preparation. Micro-CT imaging is highly sensitive, providing detailed three-dimensional images capable of measuring linear structures and volumetrically. However, it is a costly method, and three-dimensional analysis may dilute ligature's fine localized impact on bone level (Li & Amar, 2007; Steffens et al., 2015).

In this study, ligature placement significantly increased alveolar bone loss over different periods (35 and 63 days) ($p<0.05$). However, a multigroup comparison model using ANOVA showed no difference between Lig 7d and the Negative Control in the mesial root, although several studies have evidenced that 7 to 14 days are sufficient to promote alveolar bone breakdown (de Molon et al., 2018; de Paiva Gonçalves et al., 2018; Machado et al., 2014; Wichienrat et al., 2024). A two-group comparison using Student's t-test in our study showed a significant difference between Control and Lig 7d ($p=0.014$; data not shown). Ligature-induced periodontitis tends to stabilize after a certain period. De Molon et al. (2018) proposed that in the initial periods, such as 7 to 14 days, an acute inflammatory response gradually decreases and transitions into a resolution phase after 15 to 21 days (de Molon et al., 2018). Similarly, Wu et al. (2020) described that despite 28 days of ligature-induced periodontitis compared to control, bone loss stabilized after 14 days (Wu et al., 2020). However, in this study, when the mean values of mesial and distal roots were combined, bone loss was not stabilized over the different time periods evaluated ($p<0.05$). Possible explanations include using different teeth locations (mandibular and maxillary) or positioning (1st molar; 1st and 2nd molar and, 2nd molar only), ligature characteristics, and knot position.

We hypothesized that the periodontium would undergo periodontal repair, characterized by decreased bone loss, following ligature removal. However, bone loss increased significantly in the 56 days after ligature removal ($p<0.05$). Conflicting data are found in the literature. On the one hand, periodontal repair led to a decrease in alveolar bone loss after 7, 15, and 30 days (Coimbra et al., 2011; Oliveira et al., 2016; Spolidorio et al., 2014; Steffens et al., 2018). On the other hand, some results suggest an increase in alveolar bone loss following 7, 14, and 30 days of ligature removal (Garcia et al., 2022; Garcia et al., 2023; Wichienrat et al., 2024). Although this study did not aim to evaluate the microbiota, we speculate that the late progression of periodontitis after ligature removal may be due to changes in oral and systemic microbiota composition and not based on the inflammatory profile changes after ligature placement or removal (de Mello-Neto et al., 2023; Lee et al., 2024; Xing et al., 2022). In fact, studies have reported bone tissue resorption associated with dysbiosis in gut microbiota and ligature placement, which is recognized as a critical factor in dysbiosis induction and maintenance (Chen et al., 2017).

To understand the mechanisms involved in the progression of ligature-induced periodontitis and periodontal repair following its removal, our findings align closely with prior research on ligature-induced periodontitis (Bostan et al., 2024; de Molon et al., 2018; Zhang et al., 2023). Consistent with these studies, we observed an initial increase in pro-inflammatory cytokines TNF-α and IL-1β immediately following ligature placement, indicative of early inflammatory response in serum and gingival tissues. This initial surge in cytokines corresponds with the heightened receptor activator of nuclear factor kappa beta/osteoprotegerin (RANKL/OPG) ratio reported by de Molon et al. (2018), suggesting early osteoclastogenic activity. Furthermore, our findings of TNF-α levels peaking at baseline (Lig 7d) followed by a subsequent decrease over time in serum, as well as the oscillatory pattern of VEGF levels in gingival tissues, support the dynamic nature of cytokine regulation during periodontitis progression (Bostan et al., 2024; Zhang et al., 2023). Additionally, our observation of elevated IL-4 levels shortly after ligature placement underscores the complex interplay between pro-inflammatory and anti-inflammatory cytokines during the disease process.

After ligature removal, our study findings match the existing literature on cytokine dynamics in periodontitis. Like observations by Spolidorio et al. (2014), we noted significant increases in pro-inflammatory cytokines IL-1β and TNF-α immediately following ligature removal, indicating a robust inflammatory response (Spolidorio et al., 2014). This initial increase is consistent with the heightened levels of these cytokines reported by (Wichienrat et al., 2024). In our study, IL-1β, IL-10, and VEGF presented an initial increase in serum at 28 days post-ligature removal, followed by a decrease after 56 days, mirroring the pattern of initial inflammation followed by resolution. In gingival tissues, we observed decreases in IL-1β, IL-4, IL-10, VEGF, and EGF at both 28- and 56 days post-repair, which aligns with the findings (Öngöz Dede et al., 2021) regarding impaired anti-inflammatory regulation and subsequent decrease in pro-inflammatory cytokines. Dynamic cytokine interactions act modulating Th1 and Th2 immune responses and in the progression of periodontitis. Th1 cytokines like IL-1β and TNF-α initiate an inflammatory cascade against periodontal infection, while Th2 cytokines, such as IL-4, regulate inflammation and promote tissue healing (Gaffen & Hajishengallis, 2008). The shift from increased pro-inflammatory cytokines (IL-1β and TNF-α) to basal levels of anti-inflammatory cytokines (IL-4 and IL-10) during the repair phase indicates a transition toward a healing-oriented Th2 response (Landén et al., 2016).

To the best of our knowledge, this is the first comprehensive study evaluating different time protocols for ligature-induced periodontitis progression and repair using a panel of inflammatory factors. Animal models are indispensable when human studies are impractical. They provide results into therapeutic effects and interactions with specific physiological environments and aid in decision-making

for human clinical research. Rodent models offer advantages such as extensive knowledge of the immune system, access to genetically engineered strains, and ethical considerations compared to human studies (Acqua et al., 2022).

However, our study also has limitations that should be disclosed. Firstly, while multiplex analysis is valuable, technical constraints such as assay sensitivity and the need for result validation could introduce variability and affect the interpretation of results. Also, inflammatory markers were secondary outcomes; therefore, the sample size may be underestimated. The method used to measure alveolar bone loss also can be a limitation; in this study, we used periapical X-ray shots. Even though micro-CT analysis could evaluate all sites and bone volume, the results could dilute the ligature effect in different sites. In this study, we used the times of 7 days and 28- and 56-days post-baseline to evaluate alveolar bone loss; however, in literature, there are several different times, which also can be a limitation when compared with previous studies. Another point that could be a limitation when compared with other studies is that because of gender differences, our results may not be compared with studies using female Wistar rats. Thus, while acknowledging these, animal models remain indispensable in advancing our understanding of periodontal diseases (Marchesan et al., 2018).

5. Conclusion

Ligature-induced periodontitis increased bone loss in a time-dependent manner, with an increase in inflammatory cells, blood vessels, and pro-inflammatory cytokines such as IL-1β and TNF-α. After ligature removal, periodontal repair did not decrease alveolar bone loss after 56 days of ligature removal. However, after 56 days, there was a reduction in inflammatory cells and pro-inflammatory cytokines despite increased bone loss.

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Credit authorship contribution statement

João Victor Schoemberger Roth: Investigation, Methodology, Software, Writing-original draft. Priscila Alves Teixeira Ribas: Conceptualization, Investigation,

Methodology. Henrique Kenji Takarada: Conceptualization, Methodology, Investigation. Mariana Ortelan Borges: Investigation, Methodology. Flávia Braga Reitmeyer: Conceptualization, Investigation. Joao César Zielak: Conceptualization, Funding acquisition, Investigation. João Paulo Steffens: Conceptualization, Data Curation, Methodology, Supervision and Writing- review & editing.

Declaration of Competing Interest

All authors of this research (João Victor Schoemberger Roth, Priscila Alves Teixeira Ribas, Henrique Kenji Takarada, Mariana Ortelan Borges, Flávia Braga Reitmeyer, João César Zielak and João Paulo Steffens) declare no conflict of interest.

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Figures

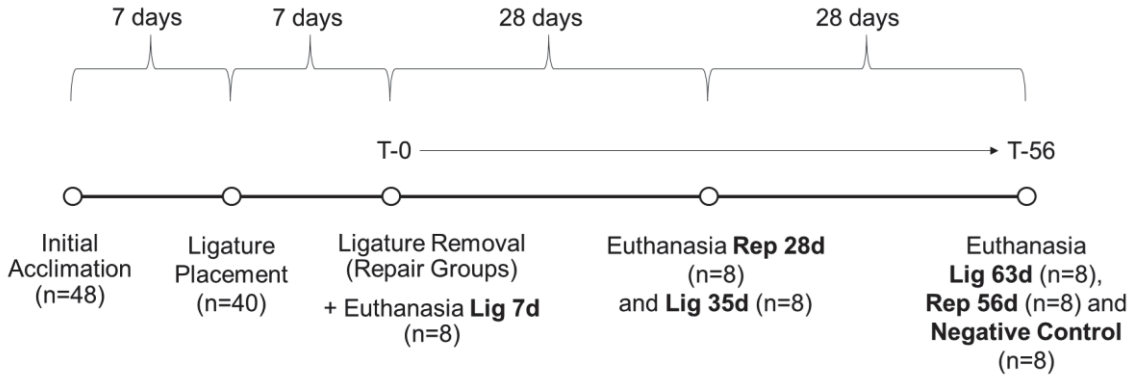

Figure 1. Study timeline. Division of animals within the different experimental groups.

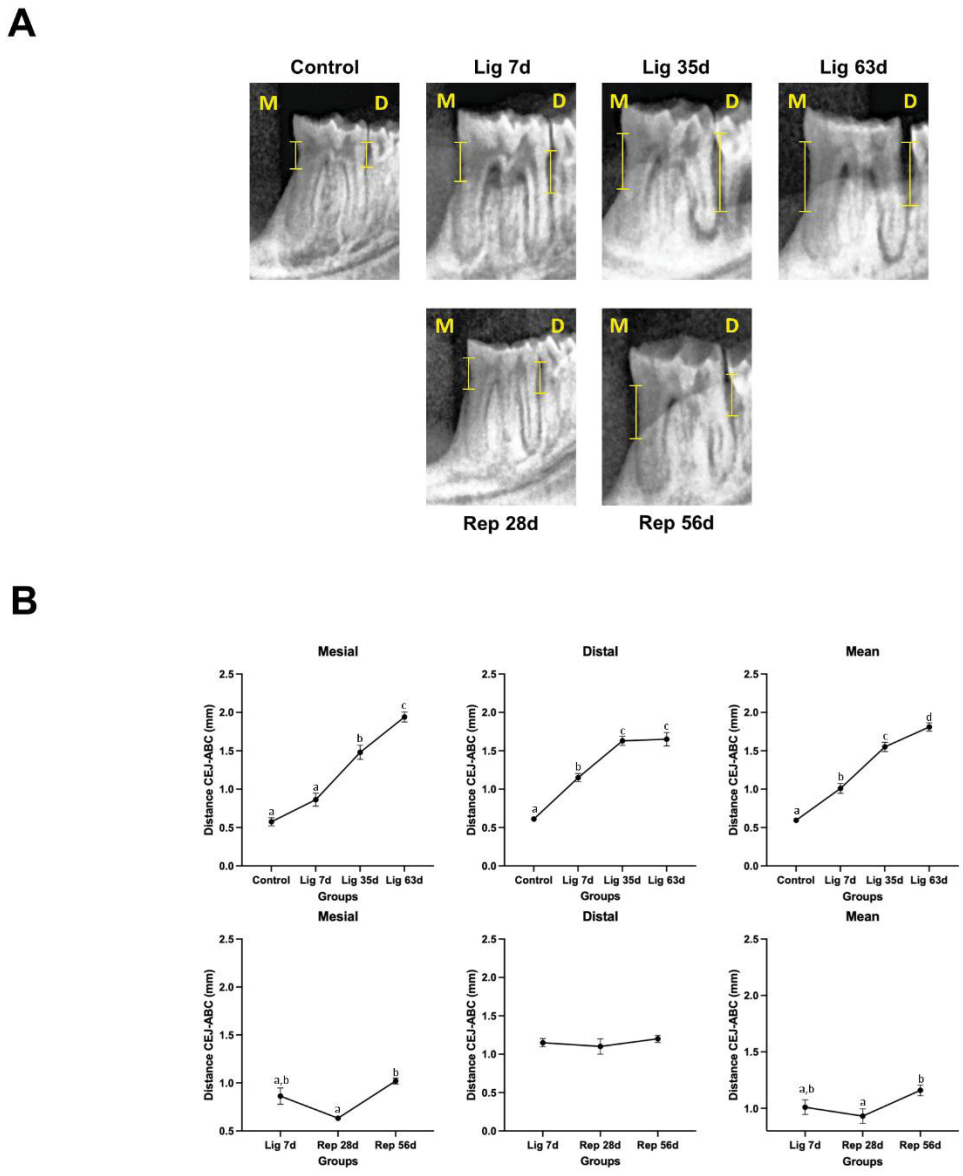

Figure 2. Estimation of bone loss using radiographic analysis. A) Representative images of each experimental group in the mesial root. B) Linear measurement in millimeters between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) on the mesial, distal, and average surfaces of both lower first molars. Results expressed as mean and standard error of the mean. M: Mesial; D: Distal; Measuring bar: The top edge is in contact with the CEJ, and the lower edge is in contact with the ABC. Different letters indicate statistically significant differences between groups ($p<0.05$).

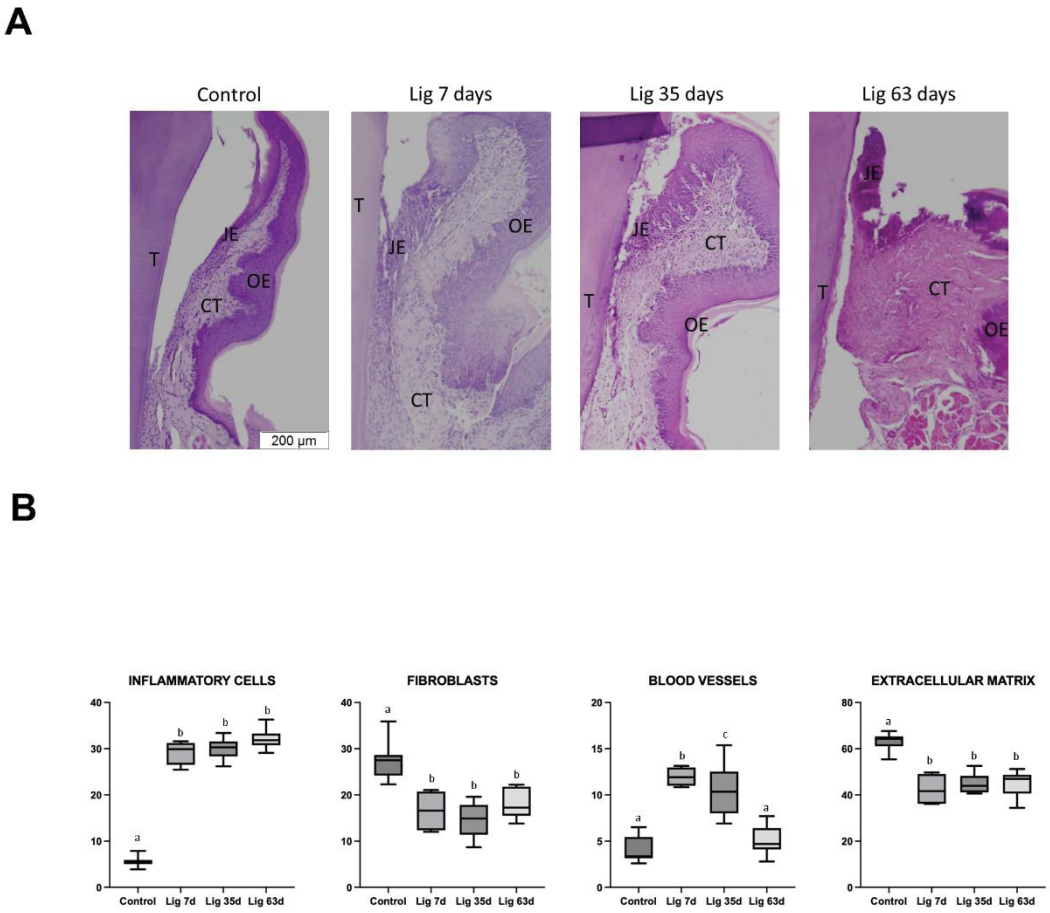

Figure 3. Histological analysis of the ligature groups. A) Representative histological images stained with hematoxylin and eosin at 20x magnification; B) BoxPlot graph of: inflammatory cells, fibroblasts, blood vessels, extracellular matrix. OE—oral epithelium; JE—junctional epithelium; CT—connective tissue; T—tooth. Different letters indicate statistically significant differences between groups ($p<0.05$).

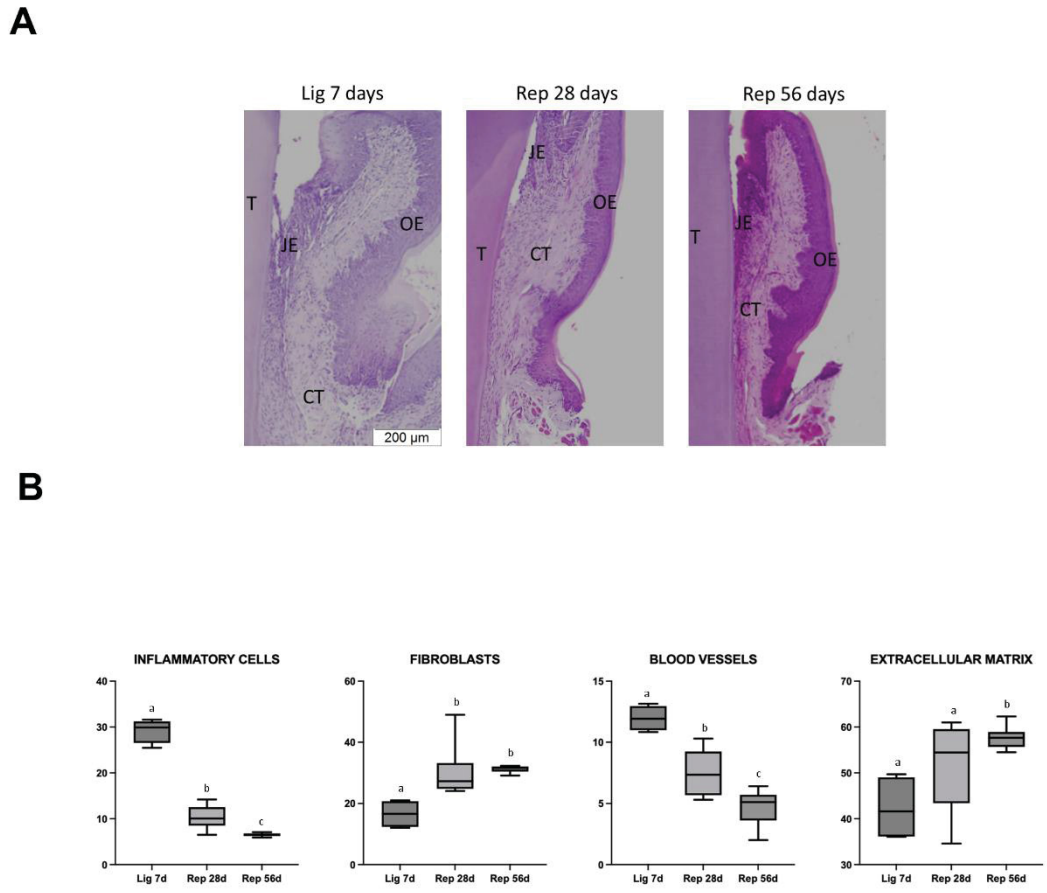

Figure 4. Histological analysis of the repair groups. A) Representative histological images stained with hematoxylin and eosin at 20x magnification; B) BoxPlot graph of: inflammatory cells, fibroblasts, blood vessels, extracellular matrix. OE—oral epithelium; JE—junctional epithelium; CT—connective tissue; T—tooth. Different letters indicate statistically significant differences between groups ($p<0.05$).

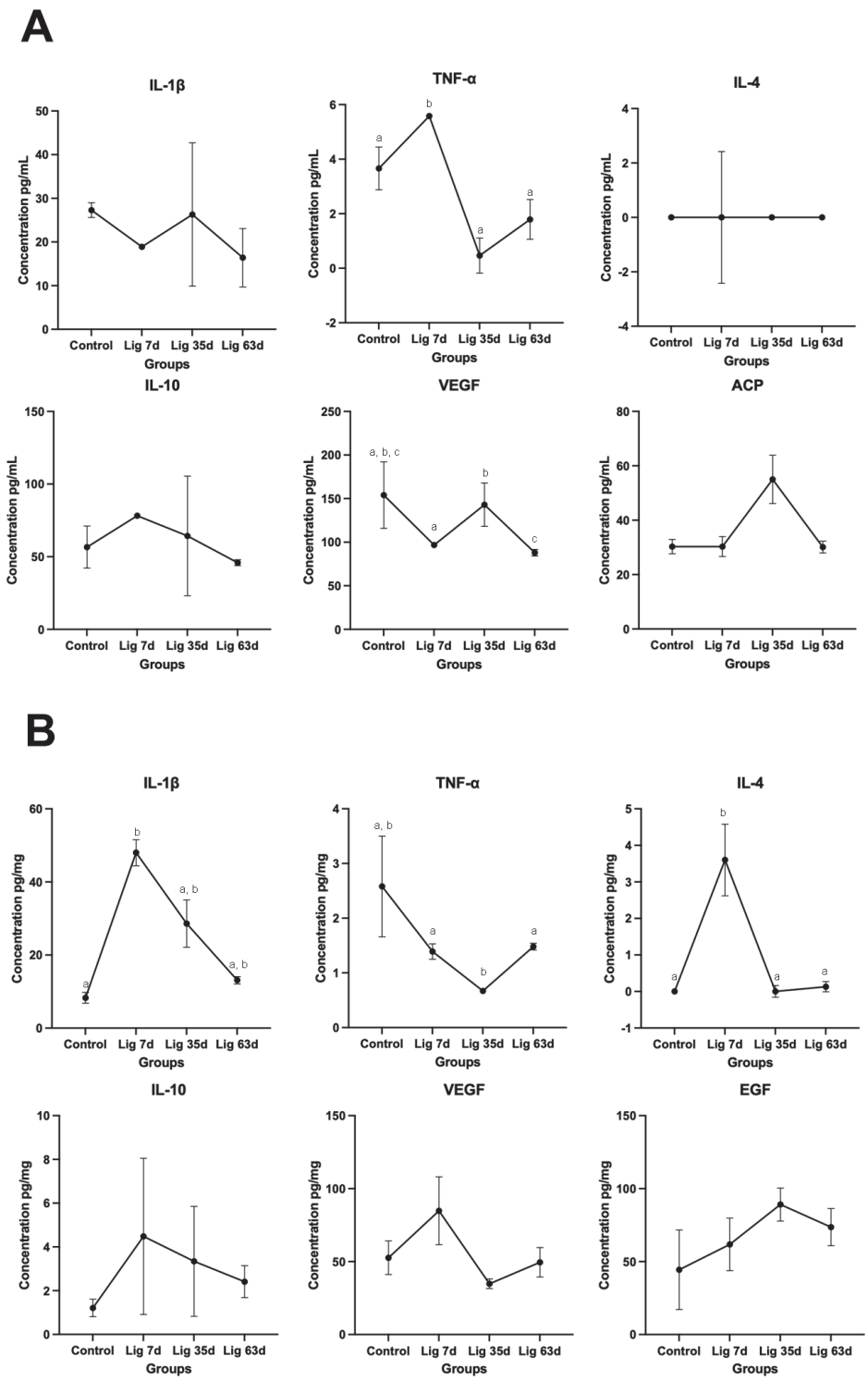

Figure 5. Immuno-inflammatory profile of the ligature groups. A) Serum analyses. B) Gingival tissue analyses. Results expressed as median and standard error of the mean. Different letters indicate statistically significant differences between groups ($p<0.05$).

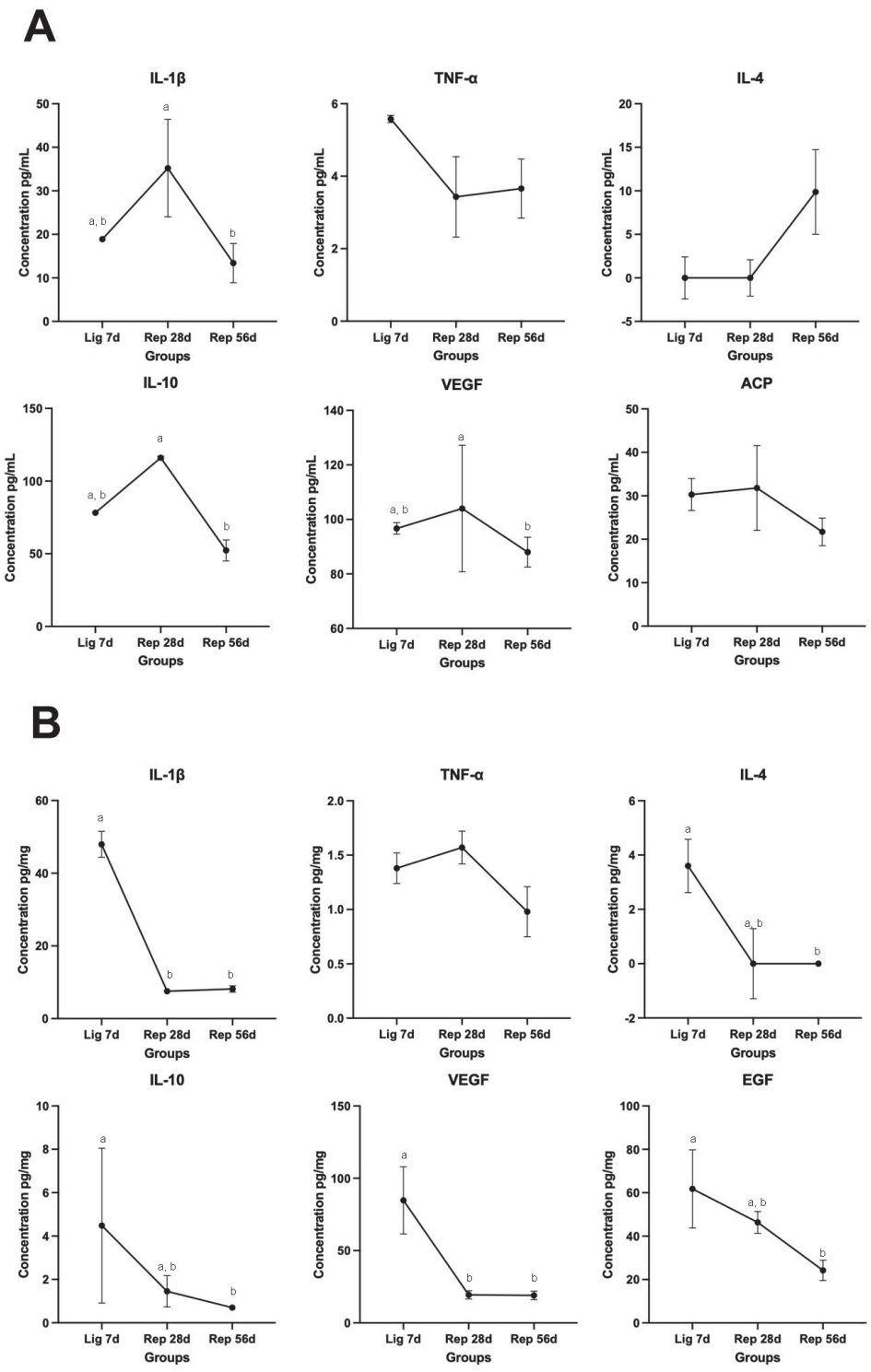

Figure 6. Immuno-inflammatory profile of the repair groups. A) Serum analyses. B) Gingival tissue analyses. Results expressed as median and standard error of the mean. Different letters indicate statistically significant differences between groups ($p<0.05$).

CONSIDERAÇÕES FINAIS

A perda óssea alveolar aumentou conforme o tempo de manutenção da ligadura durante o período de 63 dias. Houve um aumento de células inflamatórias, vasos sanguíneos e citocinas pró-inflamatórias, como IL-1β e TNF-α. Após a remoção da ligadura, a perda óssea alveolar diminuiu em 28 dias, porém após 56 dias observou-se uma progressão. Ocorreu a redução de células inflamatórias e citocinas pró-inflamatórias. Este estudo sugere que o tempo de manutenção e remoção da ligadura deve ser considerado ao interpretar resultados de periodontite induzida por ligadura em ratos, pois diferentes tempos resultarão em perfis imuno-inflamatórios distintos.

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ANEXO 1 – ARTIGO CLINICAL ORAL INVESTIGATIONS 2024

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RESEARCH

Gingival bleeding as a predictor of handgrip strength—an observational study and a pilot randomized clinical trial

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Abstract

Objective The aim of this study was to (i) assess the association between self-reported periodontal disease and gingival bleeding as predictors of handgrip strength (HGS) in the elderly and (ii) evaluate the impact of baseline periodontal clinical parameters on the improvement of HGS in trained or non-trained treated periodontitis patients.

Methods For (i), cross-sectional data from the Brazilian Longitudinal Study of Aging were retrieved and association between HGS (dependent variable) and self-reported gingival bleeding, periodontal disease, and missing teeth was analyzed using multiple linear regressions. For (ii), a pilot study was conducted with 17 patients randomly allocated to two groups—physical training or non-training—and followed for 45 days after subgingival instrumentation. Clinical parameters and HGS were recorded before and after treatment.

Results The observational study showed a significant association between HGS and tooth loss, edentulism and gingival bleeding. The clinical trial showed that baseline bleeding on probing, but not other parameters, was associated with delta HGS.

Conclusion Taken together, our findings suggest that gingival bleeding could act as a predictor of handgrip strength and its improvement after non-surgical periodontal therapy.

Clinical relevance Gingival bleeding, either as self-perceived or clinically detected, may impact handgrip strength, an important marker of muscle frailty and mortality.

Keywords Muscle strength · Periodontal diseases · Aging · Exercise

Introduction

Periodontitis is a noncommunicable periodontal disease associated with a dysbiotic biofilm that affects the supporting tissues of the teeth, in which the host response plays a very important role. Its main features include clinical attachment loss, radiographic bone loss, periodontal pockets, and gingival bleeding [1]. As a chronic inflammatory disease, there is an increase in the local and systemic production of pro-inflammatory markers such as C-reactive protein (CRP), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α [2]. Thus, periodontitis-related burden may contribute to "inflammaging," a term coined to represent the increase in the inflammatory state observed in older individuals [3]. Also, periodontitis is considered to be the leading cause of tooth loss in older adults [4].

Handgrip strength (HGS) test measures the strength of the hands and forearms in order to reflect the physical health state of individuals, including systemic and oral health conditions [5]. It is natural and expected that there is a decrease in the physical strength of the individual with increasing age [6]. Additionally, inflammatory states may contribute to HGS decrease [7, 8], which has been associated with depression, cognitive function, suicidal thoughts, mobility limitations, falls, cardiovascular and pulmonary problems, diabetes,

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kidney dysfunction, osteoporosis-related factors, morbidity, and even mortality [9]. There is also a significant association with oral health status, number of teeth, masticatory force, mastication ability, and maximum mastication force [10]. Additionally, physical exercise and highly active individuals have been associated with increased HGS [5, 11].

However, the direction of the association between HGS and inflammatory diseases is still unclear. Isometric handgrip training may significantly reduce blood pressure [12, 13], and there is evidence to support a causal association between low HGS and cardiovascular diseases [14]. On the other hand, it is possible that the low-grade chronic inflammatory burden caused by periodontitis could impact overall muscle strength resulting in decreased HGS. Therefore, the objective of this study was (i) to assess the association between self-reported periodontal disease and gingival bleeding as predictors of HGS in the elderly and (ii) to evaluate the impact of baseline periodontal clinical parameters on the improvement of HGS in trained or non-trained treated periodontitis patients.

Materials and methods

Observational study

Cross-sectional data from the Brazilian Longitudinal Study of Aging 2015–2016 (ELSI-BRAZIL, 1st wave) was retrieved. The general objective of this study is to investigate the dynamics of aging in the Brazilian population and its determinants, as well as the demand for this population to health and social systems. Sampling strategies were designed to be representative of the noninstitutionalized population aged 50 years or older within the eligible age range. Briefly, it combines stratification of primary sampling units (municipalities), census tracts, and households. More information on sampling design can be found elsewhere [15]. A total of 9412 individuals were included. The protocol was approved by the Ethics Committee of FIOCRUZ (No. 34649814.3.0000.5091), and informed consents were signed by all participants. This manuscript was prepared following the STROBE guidelines [16].

The questionnaire was divided into two stages, including household and individual questions. For the present study, HGS was considered to be the dependent variable. Three attempts were made using a dynamometer (described in the questionnaire as mf27, mf28, and mf29). Periodontal disease, gingival bleeding, and number of missing teeth, were independent variables (predictors). The following questions were used, respectively: "Has a dentist ever told you that you have/had gum disease?," "Do your gums currently bleed?," "Now think of your upper/lower teeth. How many natural teeth do you have in the upper/lower part of your mouth?."

Model building was carried out using the purposeful approach [17], considering age, sex, skin color, education level, history of physical activity, frequency of vegetable or alcohol consumption, smoking, diabetes, history of dental visits, body mass index (BMI), and self-perception of oral health as potential confounding factors. The final adjusted model included sex, age, skin color, education level, diabetes, last dental visit, BMI, and self-perception of oral health. Individuals with missing data for any of those variables were excluded.

Simple and multiple linear regressions were performed, and coefficient and p values are shown in each table. Sensitivity analyses were conducted excluding edentulous patients from the analysis of periodontal disease and bleeding. All analyses were performed using Stata 13.0 software (Stata Corp., College Station, Texas, USA). Statistical significance was set at 5%.

Pilot randomized clinical trial

This pilot parallel-design blinded randomized clinical trial was carried out between 2019 and 2020 at a university setting in Curitiba, Brazil. All patients signed informed consents. The protocol was approved by the Institutional Ethics Committee under No. 02621018.4.0000.0102 and registered at the Brazilian Registry of Clinical Trials (ReBEC No. RBR-5jxh6c). It was performed according to the Helsinki declaration and reported according to CONSORT guidelines [18].

A total of 120 patients were screened for eligibility, from which 25 were randomized. Inclusion criteria were having at least 20 teeth excluding third molars with at least two non-adjacent sites with probing depth (PD) $\geq$ 4 mm, bleeding on probing (BoP) and clinical attachment loss (CAL); being 30–50 years old; non-practitioners of physical exercise for at least 1 year (and without contraindication for it); having a body mass index (BMI) ranging from 18.5 to 29.9 (kg/m^2); and having a smartphone. Exclusion criteria are history of periodontal therapy within the previous 6 months; smokers; having systemic disease; and making use of any other medication that could influence the inflammatory, immunological, microbiological, and clinical conditions of the periodontium.

All participants were randomly assigned to two groups using computer-generated random numbers and followed for 45 days after subgingival instrumentation (SI). At baseline, the non-trained group received standard non-surgical periodontal treatment within 48 h, including SI using curettes. The trained group was asked to complete a 7-min circuit training workout using only bodyweight resistance 45 days prior to baseline [19, 20]. A free smartphone application (Seven, 2018, Perigee AB, Malmö, Sweden) was used to guide the subjects through the exercises. The exercise circuit included pushups, abdominal crunches, squats, plank, high knees,

lunges, and side plank. The circuit was repeated twice. Exercises were performed for 30 s followed by 10-s rest. Subjects received a video tutorial to ensure proper performance. Print screens of the app-generated reports were requested by the investigators weekly to ensure compliance. Then, at baseline, they received the same treatment as the non-trained group and were followed for another 45 days, continuing their workout routine.

A previously calibrated and trained researcher (GGG, Kappa ± 1 mm = 0.97) performed a whole mouth clinical examination at baseline and 45 days, assessing dichotomic plaque index (PI) at four sites per tooth and PD, BoP, and CAL at six sites per tooth.

HGS was measured using a calibrated dynamometer (Instrutherm DM-90, São Paulo, SP, Brazil). In each dental visit (baseline and 45 days), individuals were requested to squeeze the dynamometer as tightly as possible using their dominant hand, while the highest value was automatically registered. Three attempts were made, with 5-s interval, and the highest value was used for the analyses.

All data were submitted to statistical analyses. Intragroup comparisons at baseline were performed using Fisher's exact test for proportions and *t* test or Mann–Whitney test for numerical variables, depending on normal distribution and homogeneity of variances. The intra- and intergroup differences in HGS were assessed using paired *t* test and *t* test, respectively. Then, linear regressions were used to evaluate baseline periodontal parameters, including mean PD, CAL, BoP, and plaque index, as predictors of the dependent variable delta HGS. Association of delta HGS with possible confounding factors, including sex, age, and BMI were also assessed using Pearson correlation test. All analyses were performed using software (Jamovi version 2.3), and statistical significance was set at a p value < 5%.

Results

Observational study

From the initial sample of 9412 individuals, 539 were excluded due to missing data of the dependent variable, 406 due to missing data of the independent variables, and 605 due to missing data of the other variables used for modelling. The final sample consisted of 7862 participants, with an average age of 63.1 ± 9.8 years and 55.98% of women. Self-reported periodontal diseases accounted for 10.8% of the sample, while 7.49% reported gingival bleeding. Table 1 contains all sociodemographic data from the analyzed population.

A significant association was observed between edentulism and tooth loss and HGS ($p < 0.01$) using simple regression. When the model was adjusted for potential confounding factors, gingival bleeding was also significantly associated with HGS ($p < 0.05$). The coefficients of simple and multiple regressions are shown in Table 2.

For sensitivity analysis, we excluded edentulous patients from the fully adjusted model, but no relevant changes in results were detected (Table 3).

Table 1 Sociodemographic characteristics of the analyzed population ($n = 7862$)

Variable	Mean (± SD) or frequency (%)
Age (mean, years)	63.06 ± 9.75
Sex (female, %)	55.98%
Skin color (%)	
White	40.12%
Black	9.49%
Other	50.39%
Education (%)	
Less than high school	74.14%
High school	19.63%
College or higher	6.23%
Diabetes (presence, %)	15.89%
Last dental appointment (less than one year, %)	32.05%
BMI (%)	
Low/reference	31%
Overweight	39.72%
Obesity	29.28%
Self-perception of oral health (good/very good, %)	55.23%
Periodontal disease (presence, %)	10.80%
Gingival bleeding	
Yes (%)	7.49%
Tooth loss (mean, *n*)	21.06 ± 10.77
0 (%)	1.64%
32 (%)	30.98%
Hand grip strength (mean, kg)	27.11 ± 10.2

BMI, body mass index

Pilot randomized clinical trial

From the 25 randomized patients, eight were lost during follow-up ($n = 4$/group). A total of 17 patients completed the study ($n = 9$ in non-trained group and $n = 8$ in trained group). The majority of the sample was comprised of women with mean age of approximately 42 years in both groups. There was no statistically significant difference between the groups for any sociodemographic data. Baseline periodontal parameters, however, showed statistically significant differences between groups for PD and plaque score (Table 4).

After periodontal treatment, there was no intra- or intergroup statistically significant difference in HGS ($p > 0.05$). Hence, data from both groups were combined in order to test if baseline clinical periodontal parameters could predict the

Table 2 Coefficients of simple and multiple regressions using self-reported periodontal disease, gingival bleeding, and tooth loss as independent variables and handgrip strength as the dependent variable

	Simple regression		Multiple regression*	
	Coefficient	*p* value	Coefficient	*p* value
Periodontal disease (yes)	0.39 (−0.34–1.11)	0.295	−0.27 (−0.77–0.23)	0.286
Gingival bleeding				
Yes	−0.51 (−1.36–0.33)	0.235	−0.68 (−1.27–−0.09)	**0.025**
Edentulous	−5.21 (−5.69–−4.73)	**< 0.001**	−0.63 (−1.02–−0.25)	**0.001**
Tooth loss	−0.25 (−0.27–−0.23)	**< 0.001**	−0.05 (−0.06–−0.03)	**< 0.001**

*Adjusted for sex, age, skin color, education level, diabetes, last dental visit, BMI, and self-perceived oral health

Bold numbers indicate statistical significance

Table 3 Sensitivity analyses excluding edentulous patients from the fully adjusted model (Adjusted for sex, age, skin color, education level, diabetes, last dental visit, BMI, and self-perceived oral health) ($n = 5426$)

	Coefficient	*p* value
Periodontal disease (yes)	−0.44 (−1.03 – 0.15)	0.148
Gingival bleeding (yes)	−0.71 (−1.33 –−0.09)	**0.024**
Tooth loss	−0.04 (−0.07 –−0.02)	**< 0.001**

Bold numbers indicate statistical significance

Table 4 Baseline sociodemographic and periodontal condition data of patients in the non-trained and trained groups

Variable	Non-trained (*n* = 9)	Trained (*n* = 8)	*p* value (test)
Age (years)	41.2 ± 6.7	42.1 ± 7.9	NS (*t* test)
Sex (%, *n*)			
Female	55.5 (5)	62.5 (5)	NS (Fisher)
Skin color (%, *n*)			
White	77.7 (7)	87.5 (7)	NS (Fisher)
Non-white	22.2 (2)	12.5 (1)	
BMI (kg/m^2)	25.2 ± 3.2	23.1 ± 3.3	NS (*t* test)
PD (mm)	2.38 ± 0.3	2.08 ± 0.4	**0.04** (MW)
CAL (mm)	2.36 ± 0.6	2.35 ± 0.9	NS (MW)
BoP (%)	17 ± 6.4	11 ± 5.1	NS (*t* test)
Plaque (%)	54.6 ± 15	31.4 ± 5.6	**< 0.001** (*t* test)
HGS (kg)	28.1 ± 6.7	28.6 ± 11.4	NS (*t* test)

BMI, body mass index; *PD*, probing depth; *CAL*, clinical attachment loss; *BoP*, bleeding on probing; *HGS*, handgrip strength; *MW*, Mann–Whitney test

Bold numbers indicate statistical significance

change in HGS after treatment (delta HGS). From all parameters tested, only baseline BoP was significantly associated with delta HGS ($r^2 = 0.277$; *F* test, 5.76; $p = 0.03$). Since HGS was not significantly associated with sex, BMI, or age in this study, models were not adjusted. The linear regression representations for all periodontal parameters can be found in Fig. 1.

Discussion

Several observational association studies have been published under the topic of HGS and oral health [10, 21–24], but the direction of the association remains to be determined. Here, we combine evidence from a large national representative study of the elderly and a pilot randomized clinical trial to support that gingival bleeding (whether as self-reported or professionally detected) could serve as a predictor for HGS or its improvement after periodontal treatment.

Studies that analyzed HGS and periodontal diseases mostly used databases from population studies, whether cross-sectional [5, 24–28], or cohort [22, 29], and found either an inverse significant association or no association. However, gingival bleeding is not frequently reported, while the community periodontal index is generally used to classify periodontitis as mild, moderate, or severe [26, 27]. Additionally, some studies dichotomize health and disease, and gingivitis is included as health since disease is characterized by attachment loss [24, 28].

Our study specifically focused on HGS as a consequence of periodontal inflammation. Although the observational study does not allow us to assess the direction of the association, our complementary pilot clinical trial suggests that this direction is plausible. In fact, infectious inflammatory diseases lead to an increase in the levels of circulating cytokines, chemokines, and acute phase proteins [30]. Higher levels of circulating inflammatory markers were significantly associated with lower skeletal muscle strength and muscle mass in a recent systematic review [31]. Circulating concentrations of CRP are an independent predictor of HGS and are also associated with inflammatory diseases such as periodontitis [25]. In vitro studies have shown that TNF-α is a key endocrine stimulus for depressing specific force of muscle fiber, which can lead to muscle atrophy [32]. Furthermore, prolonged exposure to IL-6 has been shown to facilitate muscle atrophy by blunting muscle anabolism and energy homeostasis and may also directly mediate muscle catabolism [31, 33, 34].

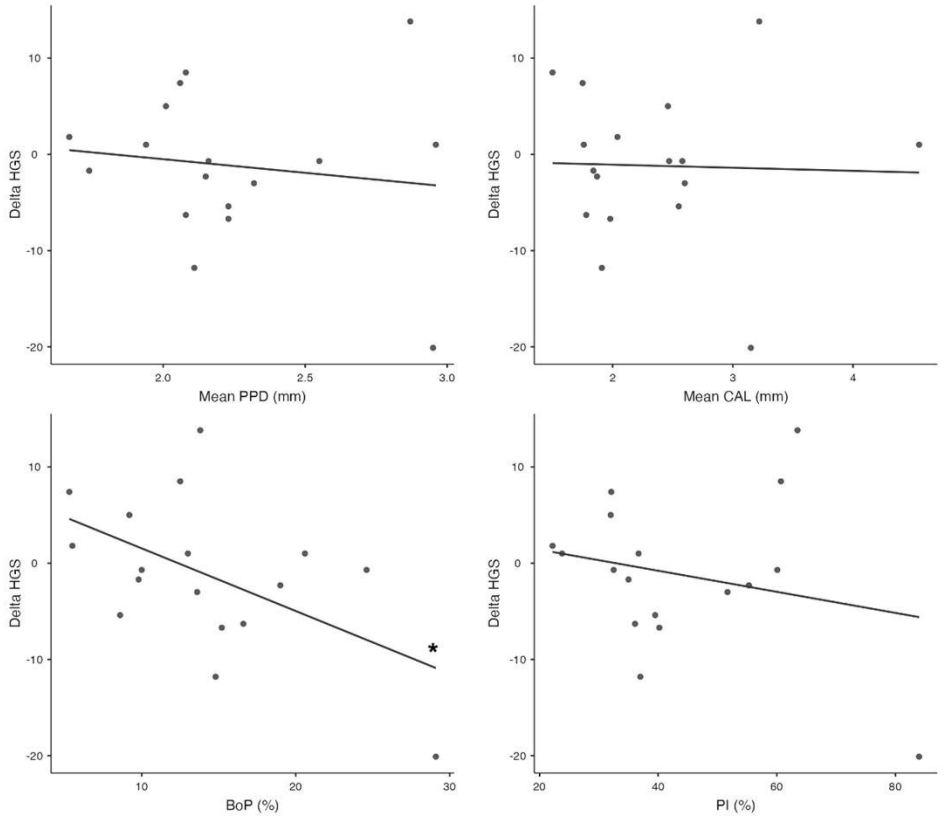

Fig. 1 Representation of the linear regression analysis using periodontal clinical parameters probing pocket depth (PPD), clinical attachment level (CAL), bleeding on probing (BoP), and plaque index (PI) as predictors of handgrip strength before and after periodontal treatment (delta HGS). *Statistically significant ($p < 0.05$)

We had hypothesized that trained individuals would have better responses to HGS improvement than non-trained participants. However, our results showed no statistically significant difference between HGS in trained and non-trained individuals. Previous studies have shown that physical exercise may significantly improve HGS, including after a 6-week protocol as reported here [35, 36], although the magnitude of this effect can be debatable [37, 38]. Hence, it is possible that either our literature-based follow-up of 45 days was not enough to reflect HGS improvement or that the workout routine used in this study was not suitable to provide those changes. However, it is important to notice that individuals who had been training for 45 days had lower plaque and PD, either as a direct benefit of exercising or as a consequence of participating in a clinical trial (Hawthorne effect).

Based on the significant associations between HGS and several chronic diseases, and the ease to obtain HGS values, the latter has been suggested as an early detection test for people at risk, allowing early interventions [39, 40]. HGS levels tend to reach their peak around the age of 35 and decrease from the age of 40, with a magnitude of 1.3 kg in women and 1.5 kg in men every 5 years [40]. Therefore, the difference of 0.63 kg found in our study between edentulous and dentate individuals may be considered clinically relevant.

To our knowledge, this is the first study to comprehensively assess the relationship between HGS and periodontal diseases using multiple methods and age ranges. Among the limitations of this study, we can cite the fact that periodontal disease and gingival bleeding were self-reported. Of course, low access to clinicians and illiteracy may bias the patients' awareness of periodontal disease, but self-reported gingival bleeding may have an interesting practical implication. Additionally, our pilot clinical trial supports the plausibility tested in our study, but it is still underpowered for definite conclusions. Unfortunately, recruiting participants for physical exercise interventions in our public university setting is not an easy task due to the socioeconomic characteristics of this population. Therefore, additional longitudinal studies and clinical trials should be performed.

Conclusion

Taken together, our findings suggest that gingival bleeding could act as a predictor of handgrip strength and its improvement after non-surgical periodontal therapy.

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Author contribution All authors have made substantial contributions to the conception and design of the study. JR was involved in data interpretation and drafting the manuscript. GG, RF, and HM were involved in data collection and data analysis of the pilot study. AH and RP were involved in the observational study data collection and analysis. JS guided the entire work. All authors agreed on the final version of the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

Ethical approval This study was approved by the Institutional Ethics Committee under No. 02621018.4.0000.0102.

Informed consent Informed consent was obtained from all individual participants included in the study.

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ANEXO 2 – NORMAS PARA PUBLICAÇÃO ARCHIVES OF ORAL BIOLOGY

Writing and Formatting

- **File format**

✓ We ask you to provide editable source files for your entire submission (including figures, tables and text graphics). Some guidelines:
✓ Save files in an editable format, using the extension .doc/.docx for Word files and .tex for LaTeX files. A PDF is not an acceptable source file.
✓ Lay out text in a single-column format.
✓ Use spell-check and grammar-check functions to avoid errors.
✓ We advise you to read our Step-by-step guide to publishing with Elsevier.

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✓ You are required to include the following details in the title page information:
✓ Article title. Article titles should be concise and informative. Please avoid abbreviations and formulae, where possible, unless they are established and widely understood, e.g., DNA).
✓ Author names. Provide the given name(s) and family name(s) of each author. The order of authors should match the order in the submission system. Carefully check that all names are accurately spelled. If needed, you can add your name between parentheses in your own script after the English transliteration.
✓ Affiliations. Add affiliation addresses, referring to where the work was carried out, below the author names. Indicate affiliations using a lower-case superscript letter immediately after the author's name and in front of the corresponding address. Ensure that you provide the full postal address of each affiliation, including the country name and, if available, the email address of each author.
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✓ Avoid references. If any are essential to include, ensure that you cite the author(s) and year(s).
✓ Avoid non-standard or uncommon abbreviations. If any are essential to include, ensure they are defined within your abstract at first mention.

- **Structured abstract**

✓ A structured abstract, by means of appropriate headings, should provide the context or background for your research. Some guidelines:
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✓ Include your main findings, providing specific effect sizes and their statistical significance, if possible, and your principal conclusions.

- ✓ Emphasize new and important aspects of your study or observations.

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- ✓ We encourage you to view example article highlights and read about the benefits of their inclusion.

- **Acknowledgements**

- ✓ Include any individuals who provided you with help during your research, such as help with language, writing or proof reading, in the acknowledgements section. Acknowledgements should be placed in a separate section which appears directly before the reference list. Do not include acknowledgements on your title page, as a footnote to your title, or anywhere else in your article other than in the separate acknowledgements section.

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- ✓ Authors must disclose any funding sources who provided financial support for the conduct of the research and/or preparation of the article. The role of sponsors, if any, should be declared in relation to the study design, collection, analysis and interpretation of data, writing of the report and decision to submit the article for publication. If funding sources had no such involvement this should be stated in your submission.
- ✓ List funding sources in this standard way to facilitate compliance to funder's requirements:
- ✓ Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa].
- ✓ It is not necessary to include detailed descriptions on the program or type of grants, scholarships and awards. When funding is from a block grant or other resources available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.
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- ✓ This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

- **Appendices**

- ✓ We ask you to use the following format for appendices:
- ✓ Identify individual appendices within your article using the format: A, B, etc.
- ✓ Give separate numbering to formulae and equations within appendices using formats such as Eq. (A.1), Eq. (A.2), etc. and in subsequent appendices, Eq. (B.1), Eq. (B. 2) etc. In a similar way, give separate numbering to tables and figures using formats such as Table A.1; Fig. A.1, etc.

- **References**

- ✓ References within text

- ✓ Any references cited within your article should also be present in your reference list and vice versa. Some guidelines:
- ✓ References cited in your abstract must be given in full.
- ✓ We recommend that you do not include unpublished results and personal communications in your reference list, though you may mention them in the text of your article.
- ✓ Any unpublished results and personal communications included in your reference list must follow the standard reference style of the journal. In substitution of the publication date add "unpublished results" or "personal communication."
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- ✓ DOI link example (for an article not yet in an issue):
- ✓ VanDecar J.C., Russo R.M., James D.E., Ambeh W.B., Franke M. (2003). Aseismic continuation of the Lesser Antilles slab beneath northeastern Venezuela. Journal of Geophysical Research, https://doi.org/10.1029/2001JB000884.
  - **Reference style**
- ✓ Citations in the text should follow the referencing style used by the American Psychological Association. You are referred to the Publication Manual of the American Psychological Association, Seventh Edition (2020) ISBN 978-1-4338-3215-4.
- ✓ The reference list should be arranged alphabetically and then chronologically. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

* https://www.sciencedirect.com/journal/archives-of-oral-biology/publish/guide-for-authors
* Link para acessar as normas para submissão na íntegra.

ANEXO 3 – PARECER DE APROVAÇÃO DO COMITE DE ÉTICA

COMITÊ DE ÉTICA EM PESQUISA NO USO DE ANIMAIS DA UNIVERSIDADE POSITIVO – CEUA/UP

PARECER: 7.1/2021

PARECER CONSUBSTANCIADO

IDENTIFICAÇÃO
IMPACTO DO EXERCÍCIO FÍSICO SOBRE O REPARO PERIODONTAL – ESTUDO *IN VIVO* SOBRE O MOMENTO DA ATIVIDADE NO TRATAMENTO PERIODONTAL

RESPONSÁVEL
JOÃO CÉSAR ZIELAK

COLABORADORES
JOÃO PAULO STEFFENS TATIANA MIRANDA DELIBERADOR

INSTITUIÇÃO DO PESQUISADOR
FACULDADE ILAPEO UP – UNIVERSIDADE POSITIVO UNIVERSIDADE FEDERAL DO PARANÁ - UFPR

OBJETIVO

Parecer: 7.1/2021

COMITÊ DE ÉTICA EM PESQUISA NO USO DE ANIMAIS DA UNIVERSIDADE POSITIVO – CEUA/UP

Curitiba – PR, 20 de maio de 2021

CERTIFICADO

Certificamos que o projeto **''Impacto do exercício físico sobre o reparo periodontal – Estudo *in vivo* sobre o momento da atividade no tratamento periodontal.'',** sob a responsabilidade de **JOÃO CÉSAR ZIELAK,** que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), 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, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi **APROVADO** pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DA UNIVERSIDADE POSITIVO – CEUA/UP.

- **Finalidade:( ) Ensino (X) Pesquisa Científica ( ) Pós-Graduação**
- **Espécie/linhagem/raça:** *Rattus norvegicus, albinus, Wistar*
- **Nº de animais:** 80
- **Origem**: Biotério da Universidade Positivo

Thais Andrade Costa Casagrande
Coordenadora - CEUA/UP