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

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Aim: High heterogeneity in ligature and repair models might reflect different disease status and preclude comparisons. 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. **Methods:** Forty-eight male Wistar rats were included. The negative control group (n=8) received no ligature, while experimental groups (Ligature and Repair; n=40) received a cotton ligature around the lower first molars, bilaterally. After 7d (baseline), ligatures were either maintained or removed for another 28 or 56 days (n=8/group). Hemi-mandibles were collected for radiographic, histological and stereometric analyses. Serum and gingival tissues were collected for the analysis of inflammatory markers using multiplex. **Results:** Mean bone loss progressively increased in ligated rats ($p<0.05$). Ligature placement increased the percentage of inflammatory cells at all timepoints and blood vessels at 7 and 35 days, while reducing fibroblasts and extracellular matrix ($p<0.05$). After 56 days of ligature removal, radiographic bone loss increased compared to 28 days ($p<0.05$). Ligature removal significantly decreased the percentage of inflammatory cells and blood vessels, while increasing fibroblasts and extracellular matrix only at 56 days. Immunological assay revealed significant time-dependent alterations in serum and gingival markers during disease progression and repair. **Conclusion:** Ligatures led to cumulative alveolar bone loss and heightened pro-inflammatory cytokines, with distinct inflammatory profiles over time. During repair, despite reducing inflammatory cells and cytokines, bone loss increased in the long term. Our data can assist in designing future experiments and clarify previously published findings.

Keywords: Periodontitis. Alveolar bone loss. Inflammation. Rats.

Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with a dysbiotic biofilm and characterized by progressive destruction of the tooth-supporting tissues[1]. 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 considered to be one of the most prevalent oral diseases worldwide[2]. Periodontitis develops in several phases: primary colonization by 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[3].

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[4].

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[5]. 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[6-8]. 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[9].

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. Studies using 5, 7, 14, 28, and 35 days to longer periods such as three months have been reported to induce periodontitis[10-14]. After ligature removal, repair is expected to occur[15], generally between 3 to 30 days after the ligature removal[16-19]. On the other hand, some studies suggest that bone loss can increase after ligature removal[20,21]. Furthermore, comprehensive immunoinflammatory characterization of how repair occurs in rodents in the long term is not available.

Considering these conflicting results in the literature, we hypothesized that different timepoints could reflect distinct periodontal and systemic inflammatory statuses. 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.

Materials and methods

Animals

Forty-eight male adult Wistar rats (*Rattus norvegicus albinus*), aged approximately eight weeks, were kept in cages (n=2-3/cage) 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. All experimental protocols were approved by the Positivo University Institutional Ethics Committee for Animal Experimentation (protocol #7.1/2021) and carried out following the Brazilian Society of Science in Laboratory Animals (SBCAL) and the 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.

Induction of experimental periodontal bone loss and periodontal repair

After seven days of acclimatization, animals were randomized into three major parallel groups: Negative Control (n=8), Ligature (n=24), and Repair (n=16), using computer-generated numbers. 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[22] 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), as this would be the worst-case scenario (aging animal). Cages were cleaned three times a week, when all animals underwent visual inspection for oral bleeding or injuries, low mobility, modified behavior or other signs suggesting suffering, in which cases a humane endpoint was planned. During the allocation and conduction of the experiment three investigators were aware of animals' allocation and blinding (PATR, HKT and FBR). A graphic representation of the study design can be found in Figure 1.

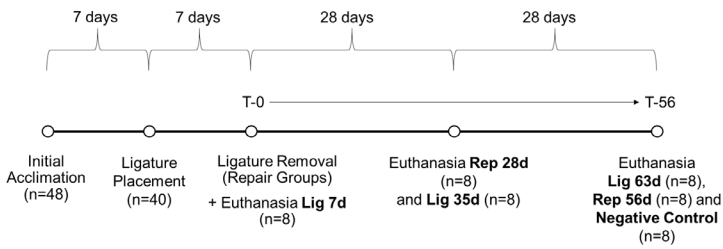

Figure 1. Study timeline and experimental procedures.

Euthanasia and sample collection

Animals were euthanized by anesthetic overdose. Blood samples were collected through cardiac puncture and centrifuged for 10min at 3,000 rpm to obtain serum,

which was kept at -80°C until used for the analyses of inflammatory markers, growth factors and acid phosphatase. Left hemi-mandibles were used to collect mucogingival tissues around the first molars, which were instantly frozen in dry ice and kept at -80°C for subsequent analysis of inflammatory markers and growth factors. The right hemi-mandibles were stored in 10% formalin for 48h, then washed in running tap water and kept in 70% ethanol for radiographic analysis and, subsequently, further histological processing.

Radiographic analysis

The right hemi-mandibles 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. Standardization was optimized using the device's settings for digital sensor for infants in periapical technique, resulting in 190ms of exposure. The distance was standardized using the sensor holder, in which the X-ray tubehead was then positioned, resulting in a distance of 7 cm between the sensor and the tubehead. 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 and blinded examiner (JVSR) under similar environmental conditions, and the mean values were used for statistical analysis. An intraclass correlation coefficient was calculated a posteriori and resulted in 0.9445 (95%CI: 0.9194-0.963).

Histological processing

After radiographic analysis, 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 5 µm thickness. Four histological slides (Slides 1-4) with three semi-serial sections (Sections A-C) were made per animal and stained with Hematoxylin and Eosin. Two standardized slides from each group were selected (Slides 2 and 4), and one standardized section of each slide (Section B) was photographed using software (Cell^F 2008, Olympus Soft Imaging Solutions Gmbh, Germany) and then submitted for evaluation by a single trained and blinded evaluator (JVSR). 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[23]. 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%).

Inflammatory profile analysis

Serum and gingival tissue samples were used for inflammatory profile analysis (n=8/group). Gingival tissues were macerated for protein extraction in tissue protein extraction reagent (T-PER lysis buffer, Thermo Fisher Scientific Inc., Waltham, MA USA) containing protease inhibitor (SIGMAFAST, Sigma-Aldrich Co., Saint Louis, MO, USA). After centrifugation, both serum and gingival samples were evaluated using multiplex (MILIPLEX Rat Cytokine/ Chemokine Magnetic Bead Panel, Merck KGaA, Darmstadt, Germany), according to the manufacturer's instructions. The following cytokines were analyzed in both serum and gingival tissues: Tumor Necrosis Factor-Alpha (TNF-α), Interleukin (IL)-1β, IL-4, IL-6, IL-10, Interferon-gamma (IFN-γ) and Vascular Endothelial Growth Factor (VEGF). Epidermal Growth Factor (EGF) was analyzed only in gingiva due to a manufacturer's problem. Total protein was quantified in each gingival sample using the DC Protein Assay protocol (Bio-Rad Laboratories, Inc., Dr. Hercules, CA, USA) and used for data normalization.

Acid Phosphatase Assay

Acid Phosphatase (ACP) was analyzed in serum samples using a colorimetric kit (Sigma-Aldrich, MAK446, Saint Louis, MO, USA) according to the manufacturer's instructions. Acid Phosphatase absorbance was read using a microplate reader (Versamax Tunable Microplate Reader, Molecular Devices Corporation, Sunnyvale, California, USA) at 405 nm, and was calculated according to the formula provided by the manufacturer, and the result was expressed in U/L.

Statistical analysis

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%. 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 software (GraphPad Prism version 10.0.0 for Windows, GraphPad Software, Boston, MA, USA, https://www.graphpad.com). The significance level was set at $p<0.05$.

Results

No animals or ligatures were lost in this experiment and visual inspections indicated no adverse reactions. The presence of ligature significantly induced alveolar bone loss. In the mesial root, only 35 and 63 days showed a statistically significant increase compared to the Negative Control ($p<0.001$). Distal analysis showed that although alveolar bone loss increased over time compared with the Negative Control ($p<0.001$), there was a peak at 28 days post-baseline (Lig 35d). 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, at 56 days of repair (Rep 56d), bone loss was significantly increased when compared with Repair 28d in the mesial root (p<0.001) and mean values (p<0.05) (Figure 2).

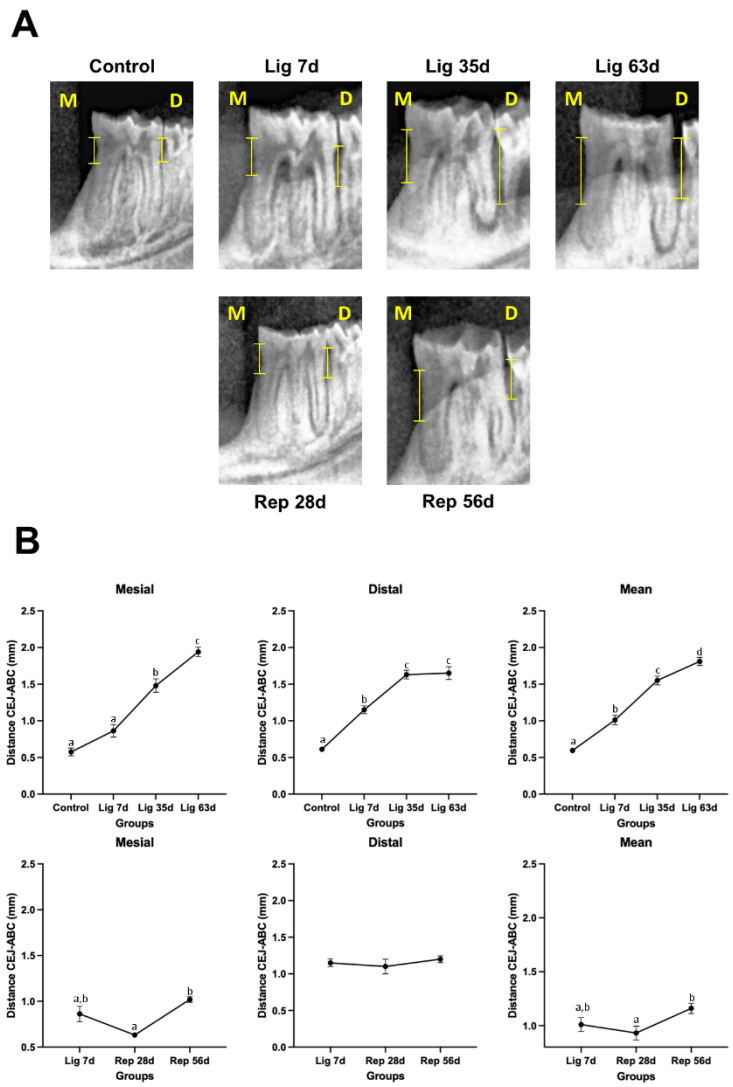

Figure 2. Estimation of bone loss using radiographic analysis. A) Representative images of each experimental group in the mesial (M) and distal (D) roots. B) Linear measurement (in millimeters) between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC) on the mesial, distal, and mean [(M+D)/2] of both aspects. Results are 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 (ANOVA; p<0.05).

Stereometric analysis showed increased inflammatory infiltrate in the ligature groups compared to the negative control, accompanied by a decreased fibroblast count ($p<0.001$). Blood vessels increased at Lig 7d, followed by a progressive decrease ($p<0.001$). Extracellular matrix decreased in all ligature groups compared to the control ($p<0.001$) (Figure 3).

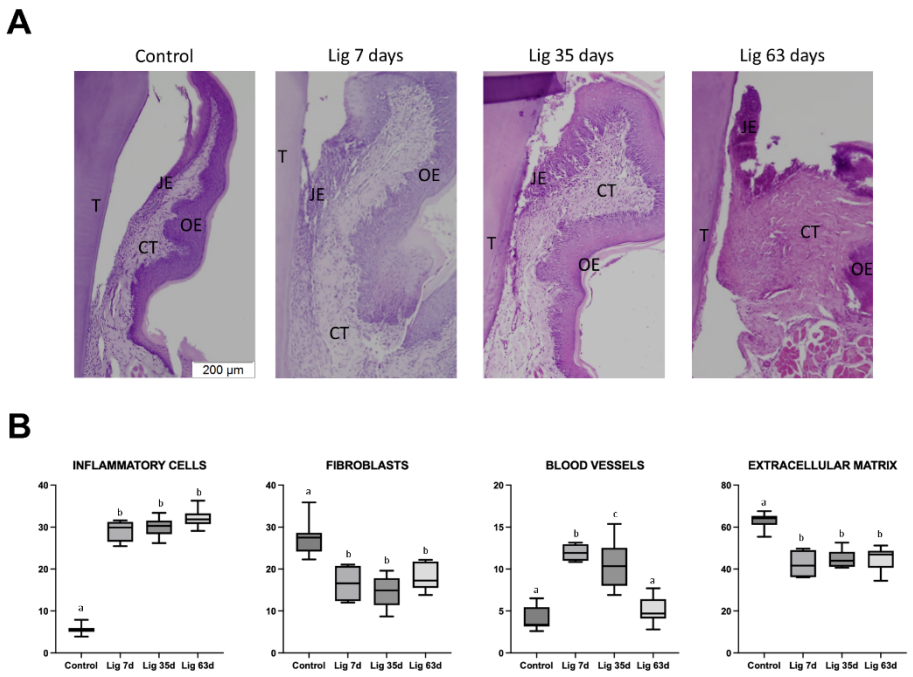

Figure 3. Histological analysis of the ligature groups. A) Representative histological images stained with hematoxylin and eosin at 20x magnification; B) BoxPlot graph of: inflammatory cells (%), fibroblasts (%), blood vessels (%), extracellular matrix (%). OE—oral epithelium; JE—junctional epithelium;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

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, and Rep 56 showed a further reduction compared to 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 Rep 56d was significantly increased when compared to Lig 7d ($p<0.01$) (Figure 4).

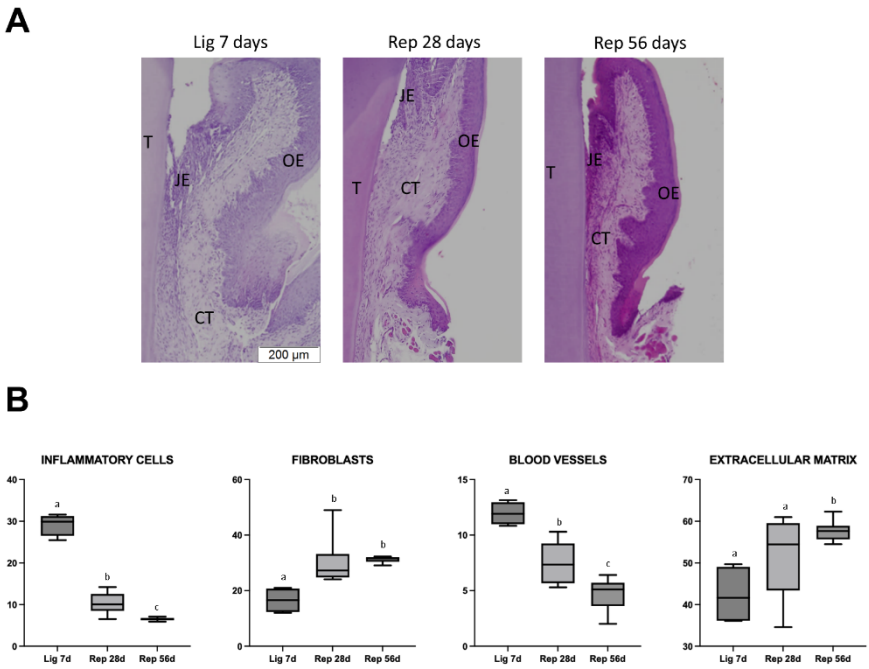

Figure 4. Histological analysis of the repair groups. A) Representative histological images stained with hematoxylin and eosin at 20x magnification; B) BoxPlot graph of: inflammatory cells (%), fibroblasts (%), blood vessels (%), extracellular matrix (%). OE—oral epithelium; JE—junctional epithelium; CT—connective tissue; T—tooth; Y-axis: percentage of each cellular component; X-axis: different time points evaluated. Different letters indicate statistically significant differences between groups (ANOVA or Kruskal-Wallis; $p<0.05$).

In the analyzed inflammatory profile, despite great intragroup variability, ligature groups exhibited elevated serum levels of TNF-α during early-stage periodontitis ($p<0.01$), while VEGF levels were fluctuating ($p<0.05$). Gingival tissue demonstrated elevated IL-1β and IL-4 levels in Lig 7d ($p<0.01$), while TNF-α was significantly reduced at 35d compared to Lig 7d and 63d groups ($p<0.05$) (Figure 5). IL-6 and IFN-γ were not detected in the samples.

In the serum of repair groups, IL-1β ($p<0.05$), IL-10 ($p<0.01$) and VEGF ($p<0.05$) were significantly reduced at 56d when compared to Rep 28d. Gingival tissue in the repair groups showed reduced IL-1-β and VEGF at both time points compared to the Lig 7d group ($p<0.01$), while IL-4 ($p<0.01$), IL-10 ($p<0.01$) and EGF ($p<0.01$) were only significantly reduced at 56 days (Figure 6). IL-6 and IFN-γ were not detected in the samples.

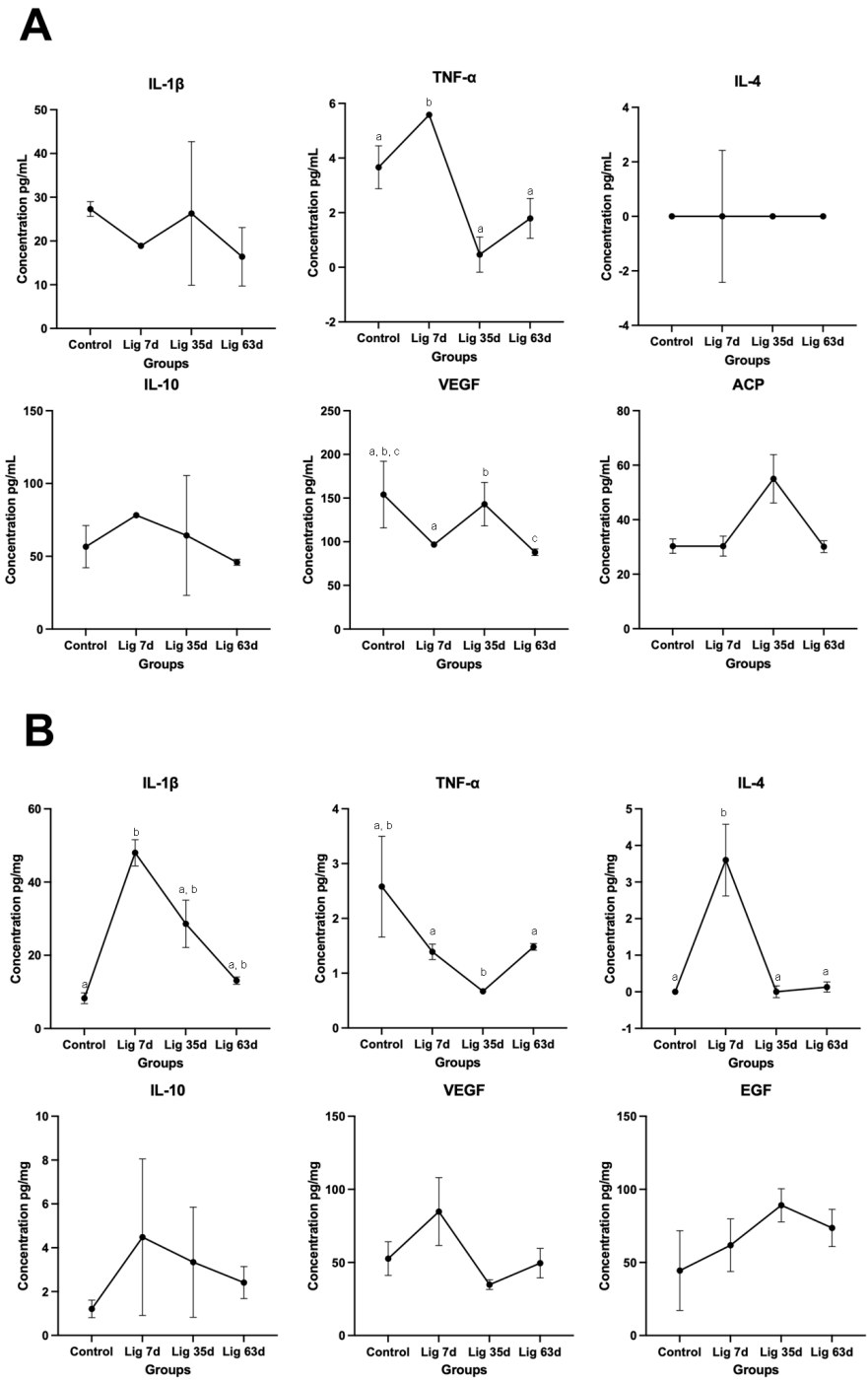

Figure 5. Immuno-inflammatory profile of the ligature groups. A) Serum analyses. B) Gingival tissue analyses. Results expressed as median and standard error of the mean. Different letters indicate statistically significant differences between groups (Kruskal-Wallis; $p<0.05$).

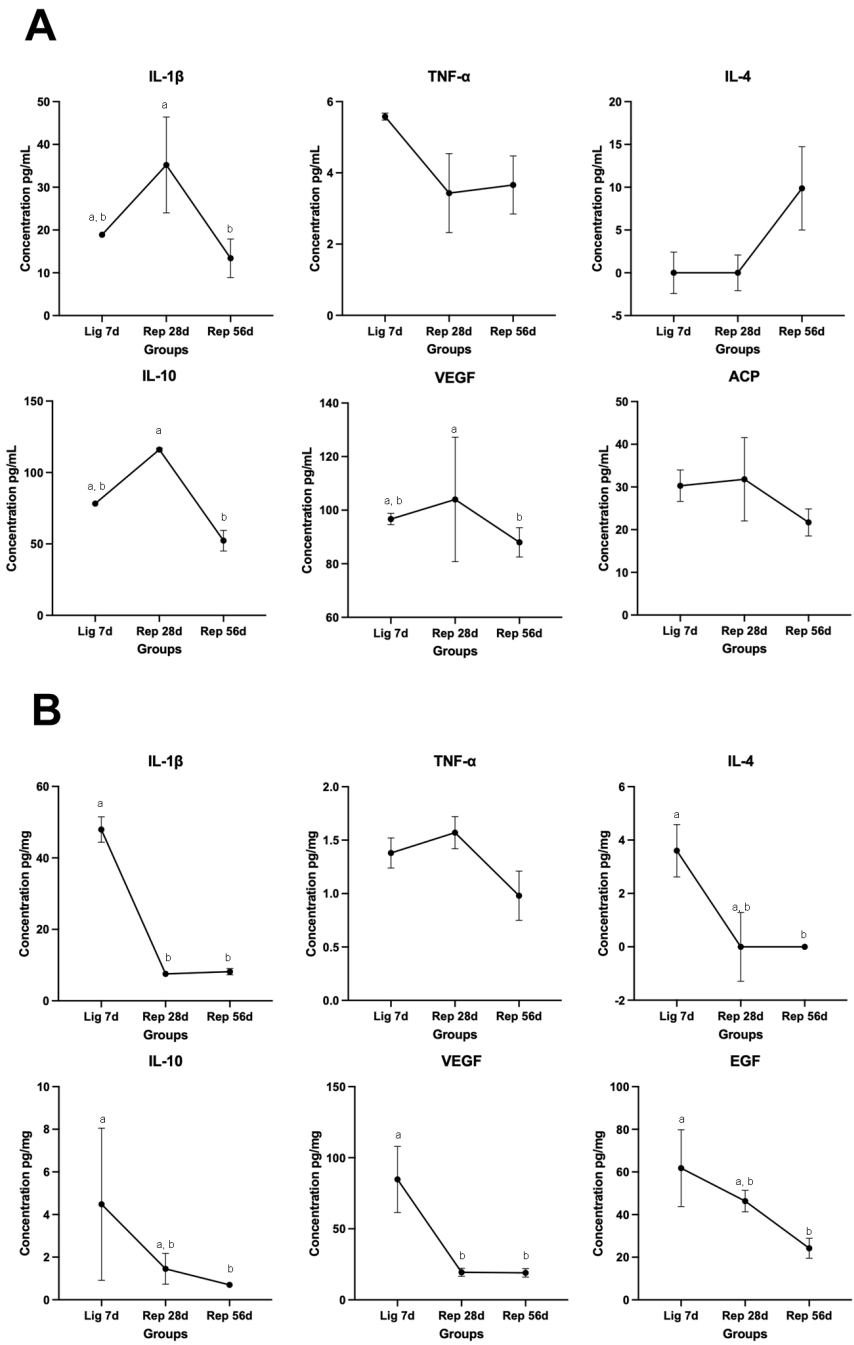

Figure 6. Immuno-inflammatory profile of the repair groups. A) Serum analyses. B) Gingival tissue analyses. Results expressed as median and standard error of the mean. Different letters indicate statistically significant differences between groups (Kruskal-Wallis; $p<0.05$).

Discussion

In this study, we analyzed the progression of periodontitis and 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 unstable 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 compared to 28 days of repair, 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 methods 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[24]. From image acquisition to analysis, this 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[23,25].

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[26-29]. In fact, if our hypothesis was simply that 7 days induced significant bone loss compared to a control group, our results would support that information (Student's t-test; $p=0.014$).

A body of evidence suggests that ligature-induced periodontitis tends to stabilize after a certain period. De Molon et al.[26] (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. Similarly, Wu et al.[30] (2020) described that despite 28 days of ligature-induced periodontitis compared to control, bone loss stabilized after 14 days. 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 molars; and 2nd molar only), ligature characteristics, and knot position. For instance, the study by De Molon et al.[26] (2018) used a sterilized silk thread around the cervix of the first and second maxillary molars in an "8" shape, knotted at the palatal surface of the second molar, while we used a cotton thread around the mandibular first molar knotted at the mesial aspect.

We hypothesized that the periodontium would undergo periodontal repair following ligature removal. In fact, histological analysis showed a clear progression toward resolution of inflammation. However, bone loss increased significantly at 56 days after ligature removal compared to 28 days of repair ($p<0.05$), but not compared to Ligature

7d (baseline). 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[15,22,31,32]. On the other hand, some results suggest an increase in alveolar bone loss following 7, 14, and 30 days of ligature removal[20,21,27].

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. 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[33]. Additionally, it must be noted that signaling for bone resorption, which involves a complex pathway related to receptor activator of nuclear factor kappa B (RANK), its ligand (RANKL) and osteoprotegerin (OPG), occurs later in the inflammatory process and is a time-depending event. For this reason, a previous study from our group reported distinct time points for histology and inflammatory markers: 3 days after ligature removal, to reflect early resolution phase when there is a burst in cytokine release; 14 days, to detect serum markers related to bone metabolism; and 28 days to reflect bone change[22]. In this study, however, we chose to simplify the protocol to analyze 28 and 56 days of repair - this choice, however, may be the reason why bone loss and the inflammatory profile at 56 days of repair seem not to match.

Our findings align closely with prior research on ligature-induced periodontitis[11,12,26]. Consistent with these studies, we observed an initial increase in pro-inflammatory cytokines, such as TNF-α in serum and gingival IL-1β, immediately following ligature placement, indicative of early local and systemic inflammatory response. Furthermore, our findings of serum TNF-α levels peaking at Lig 7d followed by a subsequent decrease over time, as well as the oscillatory pattern of cytokines and growth factors, support the dynamic nature of cytokine regulation during periodontitis progression[11,12]. Additionally, our observation of elevated gingival IL-4 levels shortly after ligature placement underscores the complex interplay between pro-inflammatory and anti-inflammatory cytokines during the disease process.

Comparisons of our histological and inflammatory profile findings following ligature removal are dampened by a lack of standardization in time points. We observed a non-significant increase in serum IL-1β at 28 days after ligature removal. This initial increase is consistent with the heightened levels of these cytokines reported by previous studies[20,27]. In our study, IL-1β, IL-10, and VEGF presented an initial non-significant 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 overall decreased IL-1β, IL-4, IL-10, VEGF, and EGF levels at both 28- and 56 days post-repair, which aligns with the findings[16] 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. 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[34].

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 markers. 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[35].

However, our study also has limitations that should be disclosed. Firstly, while multiplex analysis is a valuable, cost-effective and time-saving tool compared to ELISA, technical constraints such as assay sensitivity for multiple targets and the need for result validation could introduce variability and affect the interpretation of results. Additionally, as in any other scientific study, our sample size was calculated for the primary endpoint - radiographic bone loss. Therefore, considering the great intragroup variability in other analyses, results should be interpreted accordingly. In this study, we used the times of 7-, 28- and 56-days post-baseline to evaluate alveolar bone loss; however, in the literature, there are several different time points and methodological aspects (such as the agent for disease induction; ligature type; location; animal strains; bone loss analysis area – furcation or interdental; among others) which preclude further comparisons with previous studies. Also, it should be pointed out that our study only used male rats, and extrapolation to female rats should be avoided. Nevertheless, our study provides important data on the progression and repair of ligature-induced periodontitis that may be useful in future study designs.

In conclusion, ligature-induced periodontitis led to time-dependent alveolar bone loss. Inflammatory cells and pro-inflammatory cytokines increased in ligated animals, but with distinct inflammatory signatures across timepoints. During repair, despite reducing inflammatory cells and cytokines, alveolar bone loss increased after 56 days of ligature removal. Our data can assist in designing future experiments and clarify previously published findings.

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Conflicting Interest Statement

All authors declare that there is no conflict of interest.

Data Availability Statement

Data used in this manuscript are available from the corresponding author upon reasonable request.

Author Contribution

João Victor Schoemberger Roth: Investigation, Methodology, Software, Writing - original draft. **Priscila Alves Teixeira Ribas:** Conceptualization, Investigation, Methodology. **Henrique Kenji Takarada:** Methodology, Investigation. **Mariana Ortelan Borges:** Investigation, Methodology. **Flávia Braga Reitmeyer:** Conceptualization, Investigation. **João César Zielak:** Conceptualization, Funding acquisition, Investigation. **Joao Paulo Steffens:** Conceptualization, Data Curation, Methodology, Supervision and Writing- review & editing. All authors actively revised and approved the final version of the manuscript.

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