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ORIGINAL ARTICLE

Experimental model of periodontitis and rheumatoid arthritis potentiates the deleterious effects on functional capacity, leukocyte migration, synovial and periodontal tissues in *Wistar* rats

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ABSTRACT

Objective: This study aimed to evaluate whether ligature-induced periodontitis and rheumatoid arthritis (RA) potentiate the deleterious effects on functional capacity, periodontal and synovial tissues, leukocyte migration, and interleukin 17 (IL-17) levels, and to investigate the repercussions of single Freund's Complete Adjuvant (FCA) injection associated with periodontitis.

Materials and methods: Fifty-one male *Wistar* rats were randomised into six groups: control (CG, $n = 8$), RA (RAG, $n = 9$), periodontitis (PG, $n = 9$), periodontitis and RA (PRAG, $n = 9$), periodontitis and intradermal injection (PIDG, $n = 9$), and periodontitis and intra-articular injection (PIAG, $n = 7$). The animals underwent ligature placement and one or two injections with FCA to induce RA. Motor disability, nociceptive threshold, joint edema, and muscle strength were assessed, and the animals were euthanized on day 30. Synovial fluid, hemimandibles, and knee joints were collected.

Results: PRAG showed no reduction of edema or improvement of muscle strength, whereas it showed most significant changes in leukocyte migration, morphological analyses of the synovial membrane (SM), and radiographic and histometric analyses of the jaw. The PIAG showed some alterations, though not permanent.

Conclusion: Ligature-induced periodontitis and RA induced by two FCA injections accentuated the deleterious effects on functional capacity, leukocyte migration, synovial and periodontal tissues.

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Ligature; experimental arthritis; inflammation; Freund's adjuvant

Introduction

Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque and characterised by progressive destruction of the dental attachment apparatus [1]. Specific host-microbe interactions occur during this immune and inflammatory reaction [2].

Bacteria in the dysbiotic plaque produce lipopolysaccharides (LPS) that trigger the release of pro-inflammatory cytokines [3]. The main components involved in this process are interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 17 (IL-17), tumour necrosis factor alpha (TNF-α), prostaglandin E2 (PGE2) matrix metalloproteinases (MMPs) such as those that contribute to alveolar bone resorption and impaired periodontal structure, and C-reactive protein (CRP), which serves as a marker of systemic inflammation [4–7].

Once these biomarkers are present and activated in periodontitis, they can significantly contribute to the systemic inflammatory response of the host. This response is due to the systemic dissemination of bacteria and inflammatory mediators through the vascular system [8].

Evidence supports the association between periodontitis and the development of certain inflammatory and systemic processes such as cardiovascular disease, diabetes mellitus, obesity, and changes during pregnancy [9]. Rheumatoid arthritis (RA) has also been included in these associated disorders [10].

RA is an autoimmune and progressive chronic inflammatory disease that manifests in synovial joints [11]. It is a symmetric polyarthritis that involves peripheral biarticular joints and periarticular structures [12]. In addition to chronic, bilateral, and symmetrical polyarthritis, joint inflammation and pain can lead to deformities, instabilities, and progressive destruction of the synovial joints [13]. Thus, deleterious effects on physical mobility and functional capacity compromise the life expectancy of these patients [14].

Synovitis affects the SM, which is the site of primary inflammation characterised by cell hyperplasia and an intense inflammatory process, with migration of inflammatory cells to the tissue, vasodilation, increased vascular permeability, and

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consequently, edema [12,15]. Joint exudate is a cardinal sign of inflammation related to pain and decreased function, along with leukocyte filtration in the intracapsular region [16]. Different cell types are recruited, such as B and T cells, leading to the production of pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 12 (IL-12), and interleukin 17 (IL-17) [17].

Many studies have identified associations between RA and periodontitis, and evaluated the consequences of both diseases [18–20]. Patients who suffered from RA and simultaneously periodontitis showed an increase in joint damage [21]. A study showed that pre-existing periodontitis influenced the severity of RA [22].

The literature reports several animal studies where the two diseases were induced in different ways [22–25]. However, no studies have used, as an experimental model, the association of ligature-induced periodontitis and the model proposed by Gomes et al. [26] for the induction of RA. Since these induction methods are widely used in isolation [25,27,28], added to the evidence that the impairment is accentuated when both diseases are present, the investigation of new forms of experimental models in this research becomes plausible.

Thus, the main hypothesis of the present study was that this experimental model potentiates deleterious effects on functional capacity, leukocyte migration, increased pro-inflammatory cytokine IL-17, morphological impairment of the SM, and alveolar bone resorption in male *Wistar* rats. Furthermore, in the RA induction model established by Gomes et al. [26] and used in this study, two injections of FCA were required for RA development. With the aim of investigating this new experimental model, the second hypothesis was that, in the presence of periodontitis, only one injection of FCA will be sufficient to cause the same effects as the administration of two injections of FCA.

The main objective of this investigation was to evaluate whether the novel experimental model potentiates deleterious effects on functional capacity, periodontal and synovial tissues, leukocyte migration, and IL-17 levels and to evaluate whether the repercussions caused by a single FCA injection in the presence of periodontitis would be similar to the repercussions caused by administration of two FCA injections.

Materials and methods

Animals and experimental groups

This experimental and randomised study involved 51 3-month-old male *Wistar* rats, weighing 325 ± 39 g, who were kept in polypropylene boxes, with access to water and food *ad libitum*, under controlled conditions of temperature (21°C ± 1 °C) and a light/dark photoperiod of 12 h. The sample size was calculated, totalling 42 animals, by including a 28.56% increment in the sample size, equivalent to 54 animals, considering the risk of possible loss. Animals were randomised into six groups: control (CG, $n = 8$), RA (RAG, $n = 9$), periodontitis (PG, $n = 9$), periodontitis and RA (PRAG, $n = 9$), periodontitis and intra-articular (PIAG, $n = 9$), and periodontitis and intradermal (PIDG, $n = 7$). The difference in the number of samples was due to the exclusion of two animals, one animal from the PIDG due to ligature drop and one outlier sample excluded from the CG. The study was approved by the Ethics Committee for Animal Use (ECAU) of the University of Western Paraná (Protocol no. 19-19) 25 July 2019.

Experimental model of rheumatoid arthritis induced by Freund's Complete Adjuvant

To induce the arthritis model [26], two injections of 50 µl of FCA (Difco®, 0.5 mg/mL, *Mycobacterium butyricum*) were administered, the first as pre-sensitization through an intradermal injection at the base of the tail on day 1, and on day 8, in the tibiofemoral joint (Figure 1). The animals in the RAG and PRAG were subjected to these two injections with FCA for the induction of RA, while the animals in the CG and PG received these injections only with sodium chloride saline solution (0.9%, Aster®). However, the animals in the PIDG received only the first injection on day 1 intradermally, while animals in the PIAG received only the second injection on day 8 intraarticularly, and both were subjected to ligature placement. Before the injection, the animals were gently immobilised with a flannel by an experienced researcher only for the time necessary to perform the injection. The application sites were trichotomized and asepsis was performed with iodised alcohol (1%), and a 1 ml syringe and 13 × 4.5 mm needle was used for the injection performed at the base of the tail, inserted approximately 1 cm into the subcutaneous region and/or into the right tibiofemoral joint of the animals.

Induction of periodontitis by ligature

To induce periodontitis [29] on day 5 (Figure 1), the animals had their food restricted for 8 h, and after this period, they were anaesthetised (xylazine 0.04 mL/100 g and ketamine 0.08 mL/100 g) and placed on an appropriate operating table, which allowed the rats' mouths to remain open, facilitating access to the teeth in the posterior region of the mandible. Using modified forceps and an exploratory probe, a 4.0 cotton ligature was placed around the right and left lower first molars. This ligature acts as a gingival irritant, favouring plaque accumulation and the development of periodontitis.

Functional evaluations

To evaluate the animals for motor disability, nociceptive threshold, paw edema, and muscle strength, they were trained and adapted to the functional evaluation equipment the week prior to the evaluations by previously calibrated researchers. In total, nine evaluations were performed on all animals during the 30 days of the experiment: on day 1 (Basal 0 – B0) prior to the intradermal injection; on day 8 (Basal 1 – B1) prior to the intra-articular injection; on day 9, 24 h after the intra-articular injection (Evaluation 1 – EV1); on day 11 (Evaluation 2 – EV2) after 3 days; on day 13 (Evaluation 3 – EV3) after 5 days; on day 15 (Evaluation 4 – EV4) after 7 days; on day 20 (Evaluation 5 – EV5) after 12 days; on day 25 (Evaluation 6 – EV6) after 17 days; and on day 29 (Evaluation 7 – EV7), 21 days after the intra-articular injection (Figure 1).

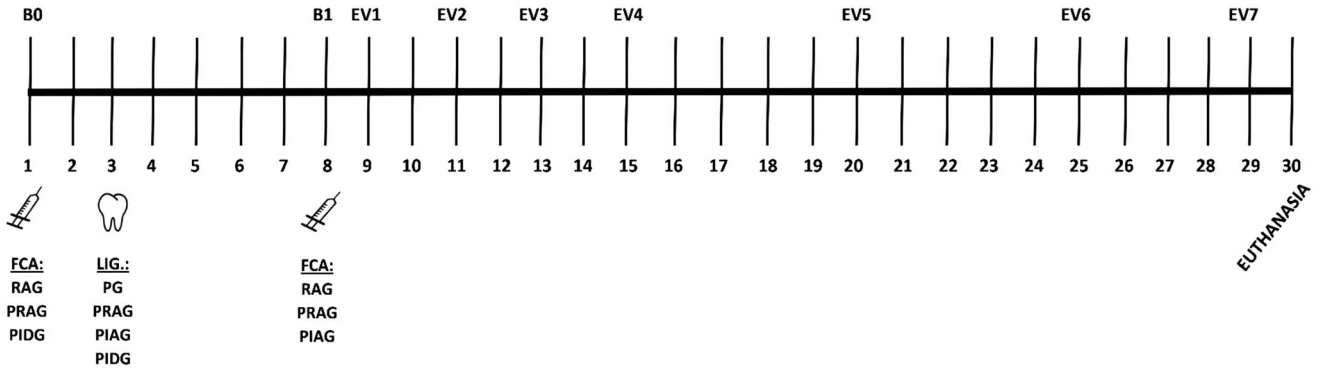

Figure 1. Schematisation of pathology inductions and functional evaluations. B0: Basal 0; B1: Basal 1; EV1: Evaluation 1; EV2: Evaluation 2; EV3: Evaluation 3; EV4: Evaluation 4; EV5: Evaluation 5; EV6: Evaluation 6; EV7: Evaluation 7. RAG: Rheumatoid Arthritis group; PRAG: Periodontitis and rheumatoid arthritis group; PIDG: Periodontitis and intradermal group; PG: Periodontitis group; PIAG: Periodontitis and intra-articular group.

Joint disability test

Joint disability was measured using paw elevation time (s) (PET). For this, the animals were subjected to walking on a metal cylinder (30 cm high and 30 cm in diameter) covered with stainless steel braided mesh (2 mm) connected to a motor that generated three rotations per minute. The animals were fitted with metallic shoes connected to both plantar regions, and the right shoe was connected through a wire to a computer that contained the Rise-Step program. Each animal then walked for one minute on the cylinder, and the values during which the sensitised paw remained without touching the surface were obtained through the program [30]. The PET of animals without any intervention is approximately 10 s, and an increase in PET after intra-articular injection with phlogistic agents indicates the development of joint disability [31].

Nociception evaluation

To evaluate the nociceptive threshold during the experiment, the animals were placed in wooden boxes with individual stalls whose floor was elevated and composed of a non-malleable wire mesh, where they remained for a few minutes without any intervention for acclimatisation. Subsequently, a digital Von Frey type analgesimeter (Insight®) was used, consisting of a pressure transducer connected to a digital force metre expressed in grams, and the contact of the pressure transducer with the right pelvic limb (RPL) was made through a disposable polypropylene tip, 0.5 mm in diameter. The experimenter then applied increasing pressure to the centre of the plantar surface of the RPL through the meshes of the net until the animal withdrew the limb. Three values were obtained, and the mean was used for further analysis [32].

Evaluation of joint edema

To quantify edema, the animal was gently immobilised with flannel and, with the help of a non-digital calliper positioned in the region of the right knee joint interline, three measurements were recorded mid-laterally, and the mean of the values was used for analysis [33].

Muscle strength evaluation

Muscle strength of the right pelvic limb was evaluated using grip strength equipment (Insight®). For this, the animal was immobilised by the researcher so that only the evaluated limb remained free and grasped the grid, after which the animal was pulled with increasing force until it lost its grip. Three measurements were performed, and the mean value was used for analysis [34].

Synovial fluid leukogram

After the experimental period, the animals were subjected to the euthanasia procedure (30th day) by overdosing with an anaesthetic (ketamine, 240 mg/kg) and alpha 2 adrenoreceptor agonist (xylazine, 45 mg/kg) administered intraperitoneally. After checking the animal's state of consciousness (absence of motor response to tail clenching and interdigital folds), synovial fluid was collected. For this, the joint capsule of the right knee was exposed, and a joint wash was performed with 100 µL of 0.9% saline solution with 4 µL of 5% ethylenediamine tetra-acetic acid, and 20 µL of the fluid was collected and diluted in Turk's fluid (2 mL glacial acetic acid, 1% methylene blue, and 98 mL distilled water) with a dilution factor ranging from 80 to 380 µL according to the concentration of cells in the fluid. The total cell count was performed in a Neubauer glass chamber (cells/mm^3) with a light microscope at 40× objective, and this count was determined in four quadrants and multiplied by the dilution factor used [28].

Interleukin-17 analysis (ELISA)

A portion of the gingival tissue around the teeth of the right hemimandibles, with or without ligature placement, was removed and used for IL-17 cytokine dosage by the enzyme-linked immunosorbent assay (ELISA) method. The RAT IL17A ELISA Kit (Biosource, INVITROGEN®, CA, USA) was used according to the manufacturer's instructions. Briefly, 96-well plates previously sensitised with anti-IL-17 monoclonal antibodies were incubated with the gingival tissue supernatants. The detection antibody, specific for the cytokine, conjugated to peroxidase was added to the wells, and after the specific incubation time, the wells were washed, and the reaction was

revealed by adding the developing solution, following the manufacturer's guidelines. The reaction was blocked with a stop solution, and the absorbance was read at 450 nm in a microplate reader. The cytokine concentration was calculated using a standard curve with known IL-17 concentrations and then plotted through a linear regression curve to obtain a straight-line equation to calculate the IL-17 concentration in the sample. The results are expressed in pg/ml.

Synovial membrane morphology and histometry of the jaw

The right knee joints and the hemimandibles of the left side were collected and dissected, fixed in Metacarn (70% methanol, 20% chloroform, 10% glacial acetic acid) for 48 h, and then placed in 70% alcohol (Neon®) for 15 days. Then, the material was washed in running water for 24 h, and the tissues were immersed in 5% trichloroacetic acid (TCA) (Neon®) for 7 days, following the routine histological process for paraffin embedding (Alphatec®). For slide preparation, cuts were made in the sagittal plane on an Olympus CUT 4055 microtome, 7 μm thick, and mounted on glass slides. The haematoxylin and eosin (Synth®) protocol was used for staining. Microscopic analyses were performed using a light microscope, and the fields of interest were photomicrographed using an Olympus DP71 photomicroscope (USA). In the morphological analyses, normal aspects and changes in the SM were observed, and in the photomicrographs of the hemimandibles, the shortest distance between the alveolar bone crest apex and the cemento-enamel junction was measured using the image analyser program Image Tools 3.0 (University of Texas Health Science Centre, San Antonio, TX). The measurements were repeated once a day on three different days, and the mean values were used for analysis [35,36].

Radiographic analyses of the mandible

The right hemimandibles were collected, dissected, and fixed in Metacarn (70% methanol, 20% chloroform, 10% glacial acetic acid) for 48 h, after which Metacarn was replaced with 70% alcohol. For the radiographs, the hemimandibles were positioned with the lingual surface on a digital radiographic sensor (Kodak RVG 6100) with image resolution 20 pl/mm, theoretical sensor resolution 27.03 pl/mm, optical fibre 1, active surface dimensions 22 × 30 mm, and matrix dimensions (pixels) 1200 × 1600 (1.92 million), such that the buccal and lingual cusps of the first molars were in the same vertical plane. A GE-1000 X-ray machine was used, set at 15 mA, 65 Vp, 18 pulses, and focus/film distance of 50 cm, with X-ray incidence perpendicular to the samples. Image Tools software (version 3.0; University of Texas Health Science Centre, San Antonio, TX, USA) was used to analyse the scanned images, and linear measurements were taken from the cemento-enamel junction to the alveolar bone crest on the mesial side of the right mandibular first molar [37].

Data analyses

The SPSS 20.0® program was used for the statistical analysis of functional assessments. Comparisons were made using generalised linear models with Fisher's post hoc test. The significance value was set at $p < .05$, and the results were expressed as the mean and standard deviation. For statistical analysis of the radiographic and histometric evaluations of the mandible, the Bioestat@ Program - version 5.3 (Mamirauá Sustainable Development Institute, Brazil, AM) was used. Normal distribution and homogeneous variance were tested. As the distribution was considered normal and the variance was homogeneous, the parametric one-way ANOVA test was used, followed by Tukey's multiple comparison test. Differences were considered significant when $p < .05$ (5%).

Results

In the clinical analyses, the animals did not show any signs of systemic disease except RA during the entire study period. Body weight was also monitored during the experiment, and no statistically significant difference was found between the groups. With respect to weight evaluation on day 3, there was a statistically significant difference between weight 1 and weight 3 ($p < .001$), indicating weight gain compared to the beginning of the experiment (Additional File: Table 1; Figure 1). No deaths occurred.

In the evaluation of PET, there was a significant difference in the comparison between groups ($p < .001$), between evaluations ($p < .001$), and in the interaction between groups and evaluations ($p < .001$). The RAG, PRAG, and PIAG showed a statistically significant difference from the CG starting at EV1 ($p < .05$). The RAG had a higher PET than the PRAG and PIAG, and between these groups, the PIAG had a higher impairment than the PRAG. The PG and PIDG were similar to the CG in all or most evaluations ($p > .05$). In the intragroup evaluations, in the RAG and PIAG there was a statistically significant difference from EV1 until EV7 ($p < .05$). In the PRAG there was an increase in PET at EV2, but this parameter was re-established based on the statistical similarity between baseline and EV7 ($p > .05$). In the PG and PIDG there was a similarity observed between the evaluations ($p > .05$) (Figure 2).

Table 1. Radiographic and histometric evaluation of the hemimandibles.

	Radiographic evaluation	Histometric evaluation
CG	57.8 ± 2.3^{A}	222.83 ± 48.76^{A}
RAG	58.3 ± 3.2^{A}	232.49 ± 36.96^{A}
PG	70.4 ± 3.3^{B}	354.59 ± 96.52^{B}
PRAG	77.3 ± 1.7^{C}	513.17 ± 86.35^{C}
PIAG	70.4 ± 5.2^{B}	370.15 ± 33.41^{B}
PIDG	70.9 ± 4.8^{B}	372.50 ± 50.73^{B}

Data expressed as mean and standard deviation. Similar capital letters show similarity between groups. Control group (CG); Rheumatoid arthritis group (RAG), Periodontitis group (PG), Periodontitis and rheumatoid arthritis group (PRAG), Periodontitis and intra-articular group (PIAG) and Periodontitis and intradermal group (PIDG).
Similar capital letter show similarity between groups.
[A]RAG were similar to CG
[B]PG, PIAG and PIDG were similar among themselves, but different of CG, RAG and PRAG
[C]The PRAG showed difference of all others groups

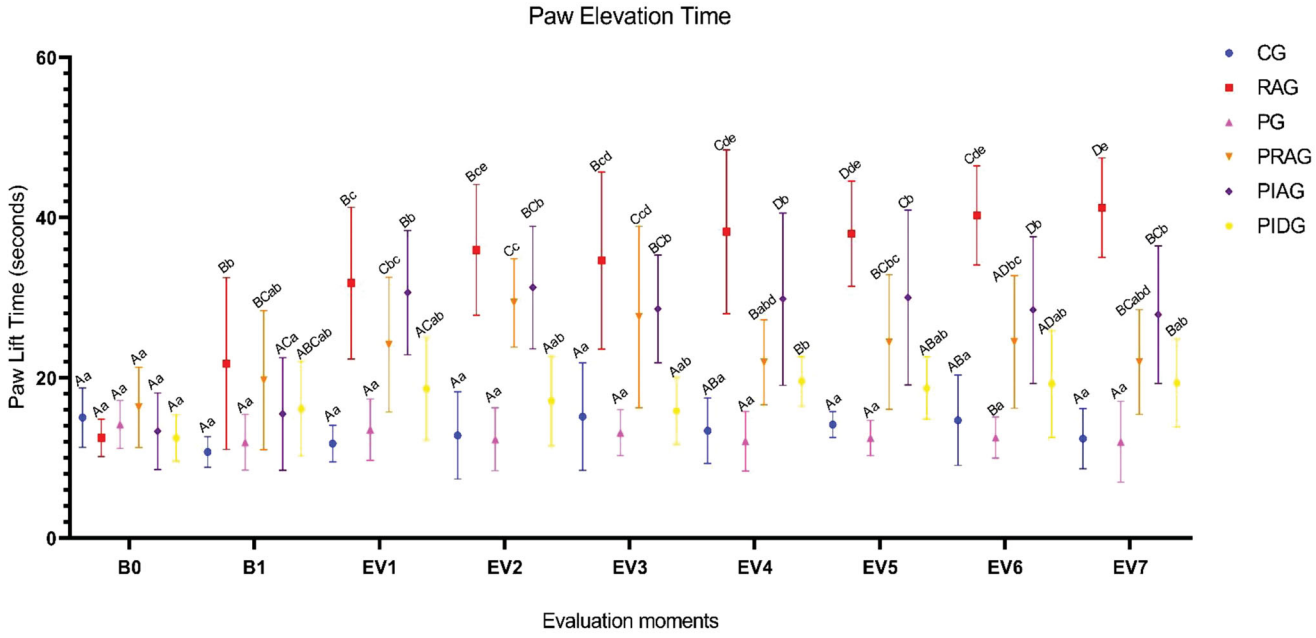

Figure 2. PET evaluation data. Data expressed as mean and standard deviation. Similar capital letters show similarity between groups. Similar lowercase letters show similarity within the same group. CG: Control group; RAG: Rheumatoid Arthritis group; PG: Periodontitis group; PRAG: Periodontitis and rheumatoid arthritis group; PIAG: Periodontitis and intra-articular group; PIDG: Periodontitis and intradermal group.

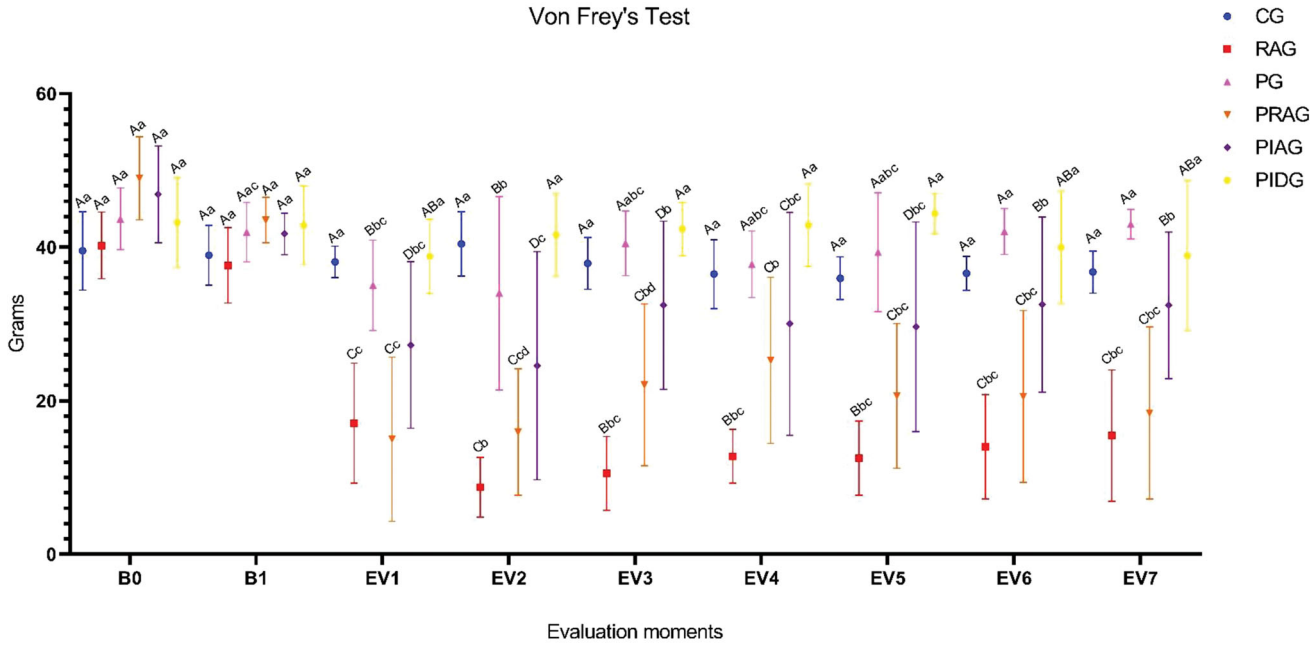

Figure 3. Nociception evaluation data. Data expressed as mean and standard deviation. Similar capital letters show similarity between groups. Similar lowercase letters show similarity within the same group. CG: Control group; RAG: Rheumatoid Arthritis group; PG: Periodontitis group; PRAG: Periodontitis and rheumatoid arthritis group; PIAG: Periodontitis and intra-articular group; PIDG: Periodontitis and intradermal group.

In the evaluation of the nociceptive threshold, there was a significant difference in the comparison between groups ($p < .001$), between evaluations ($p < .001$), and in the interaction between groups and evaluations ($p < .001$). The RAG, PRAG, and PIAG showed a statistically significant difference from the CG starting at EV1 ($p < .05$). The RAG had a lower nociceptive threshold than the PRAG and PIAG, among which the PRAG had greater impairment than the PIAG. The PG and PIDG were similar to the CG in all or most evaluations ($p > .05$). In the intragroup evaluations in the RAG, PRAG, and PIAG there was a statistical difference from EV1 until EV7 ($p < .05$). In the PG, EV1 and EV2 denoted hyperalgesia, but this parameter was re-established over the evaluations, as noted by the statistical similarity between basal, EV6, and EV7 ($p > .05$). In the PIDG there was a similarity observed between evaluations ($p > .05$) (Figure 3).

In the evaluation of edema, there was a significant difference in the comparison between groups ($p < .001$), between evaluations ($p < .001$), and in the interaction between groups and evaluations ($p < .001$). The RAG, PRAG, and PIAG showed a statistically significant difference from the CG starting at EV1 ($p < .05$), the RAG showed higher mean values in the joint

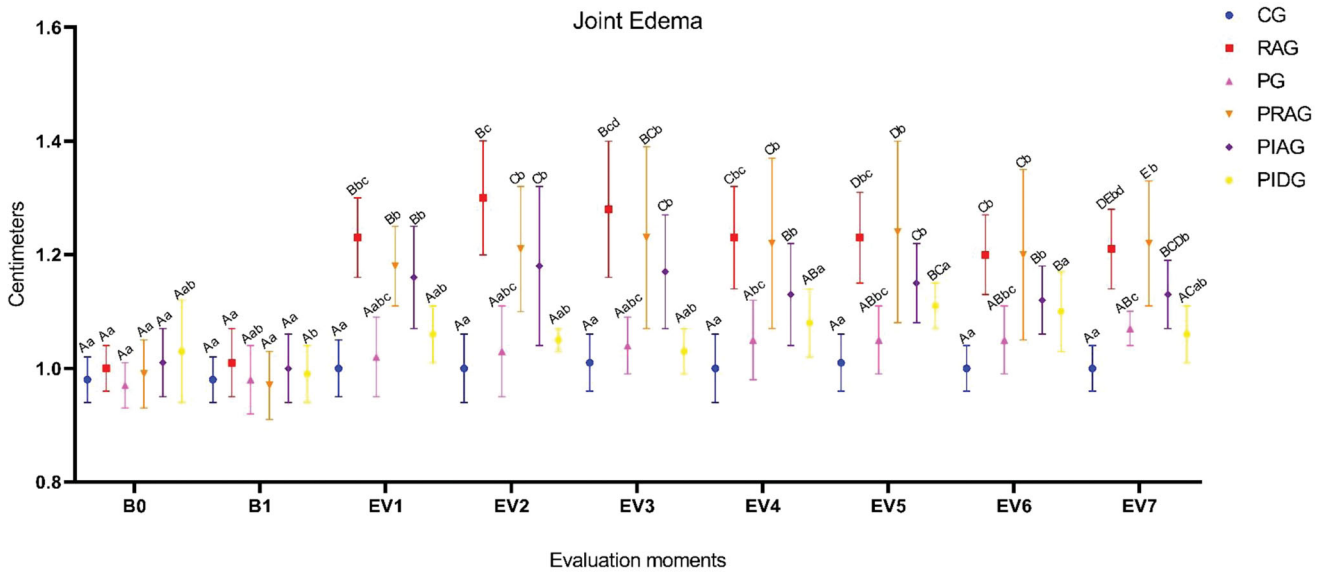

Figure 4. Edema joint evaluation data. Data expressed as mean and standard deviation. Similar capital letters show similarity between groups. Similar lowercase letters show similarity within the same group. CG: Control group; RAG: Rheumatoid Arthritis group; PG: Periodontitis group; PRAG: Periodontitis and rheumatoid arthritis group; PIAG: Periodontitis and intra-articular group; PIDG: Periodontitis and intradermal group.

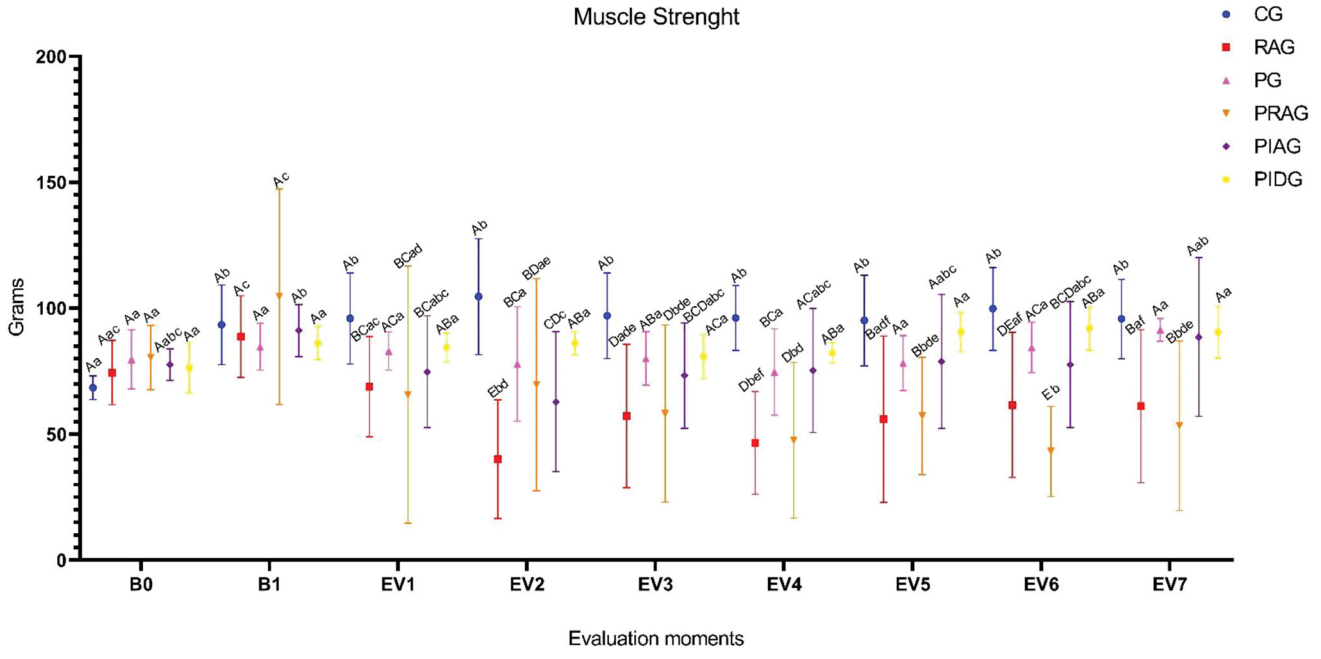

Figure 5. Muscle strenght evaluation data. Data expressed as mean and standard deviation. Similar capital letters show similarity between groups. Similar lowercase letters show similarity within the same group. CG: Control group; RAG: Rheumatoid Arthritis group; PG: Periodontitis group; PRAG: Periodontitis and rheumatoid arthritis group; PIAG: Periodontitis and intra-articular group; PIDG: Periodontitis and intradermal group.

diameter than PRAG and PIAG. The PG and PIDG were similar to the CG in all or most evaluations ($p > .05$). In intragroup evaluations in the RAG, PRAG, and PIAG there was a statistical difference from EV1 until EV7 ($p < .05$). In terms of mean values, the RAG showed a reduction in values in the final evaluations, which was different from PRAG and PIAG, which did not show a reduction in values. In the PIDG and PG, there was a similarity observed between the evaluations ($p > .05$) (Figure 4).

In the evaluation of muscle strength, there was a significant difference in the comparison between groups ($p < .001$), between evaluations ($p < .001$), and in the interaction between groups and evaluations ($p < .001$). The RAG and PRAG showed a statistically significant difference compared to the CG from EV1 onwards ($p < .05$). In the PIAG, there was a change in the strength parameter in some evaluations, but this was not maintained, based on the statistical similarity noted with the CG in some evaluations ($p > .05$). The PG and PIDG were similar to the CG in all or most evaluations ($p > .05$). In the intragroup evaluations, the CG initially obtained a low value, possibly due to the adaptation of the animal to the equipment, but in the rest of the evaluations, there was an increase and maintenance of strength. The mean values in the RAG showed a reduction in strength in EV2, but these values increased in the final evaluation. The PRAG maintained a low value showing muscle weakness with no increase in mean values. The PIAG showed a

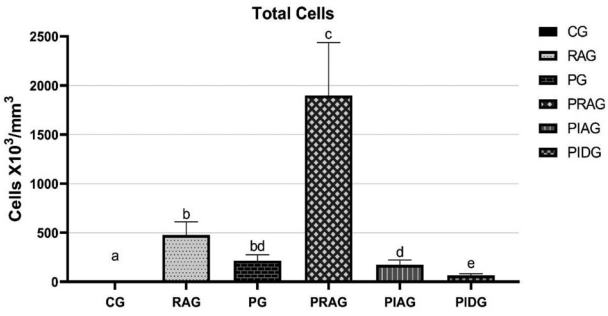

Figure 6. Total leukocyte count. Data expressed as mean and standard deviation. Similar letters show similarity between groups. CG: Control group; RAG: Rheumatoid Arthritis group; PG: Periodontitis group; PRAG: Periodontitis and rheumatoid arthritis group; PIAG: Periodontitis and intra-articular group; PIDG: Periodontitis and intradermal group.

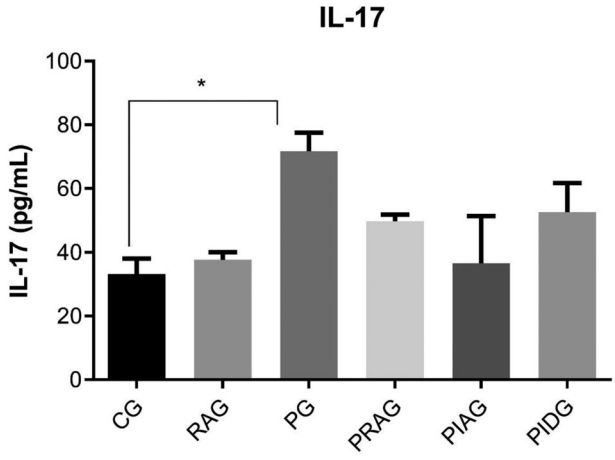

Figure 7. Interleukin-17 analysis. Data expressed as mean and standard deviation. CG: Control group; RAG: Rheumatoid Arthritis group; PG: Periodontitis group; PRAG: Periodontitis and rheumatoid arthritis group; PIAG: Periodontitis and intra-articular group; PIDG: Periodontitis and intradermal group. *Statistically different values between groups ($p < .05$).

reduction in this parameter only in one evaluation. In the PIDG and PG there was a similarity noted between the evaluations ($p > .05$) (Figure 5).

In leukocyte migration, the RAG showed an increase in the number of leukocytes but remained similar to PG ($p > .05$), while PG resembled PIAG ($p > .05$). In the PRAG there was a statistically significant difference in leukocyte migration compared to that in the other groups ($p < .05$). The CG and PIDG did not show an increase in the number of leukocytes ($p > .05$) (Figure 6).

Regarding the analysis of IL-17, Figure 7 shows a statistically significant difference between the CG and PG ($p < .05$), where it was significantly higher in the PG. In the remaining groups, there was a statistical similarity ($p > .05$) (Figure 7).

In the morphological analyses of the SM, the CG (Figure 8(A)) showed normal characteristics, with the SM arranged in two layers of cells (synoviocytes) in the synovial intima. In the subintima layer, there was a predominance of fat cells, distributed in an organised manner, with unaltered blood vessels and connective tissue. The animals in the PG (Figure 8(C)) and PIDG (Figure 8(F)) had morphological characteristics similar to those in the CG. However, the RAG and PIAG (Figure 8(B,E)) demonstrated the presence of inflammatory infiltrate, with thickening of the synovial intima with fibrosis and disorganisation of synoviocytes, and altered adipocyte arrangement in the subintima layer, with loss of organisational compliance. Moreover, the PRAG (Figure 8(D)) showed an intense inflammatory process, with thickening and disorganisation of the synovial intima and subintima in the absence of adipocytes.

Hemimandibular histometric and radiographic analyses showed that the RAG was similar to the CG ($p > .05$). In contrast, the PG, PIAG, and PIDG had significantly higher values than the CG ($p < .05$) and a statistical similarity between them ($p > .05$). In PRAG, there was a significant difference ($p < .05$) compared to all other groups, indicating that periodontitis associated with rheumatoid arthritis caused the greatest alveolar bone loss (Table 1).

Discussion

The induction of periodontitis by ligature and the induction of RA with two injections of FCA containing *Mycobacterium butyricum* are standard models well established in the literature. The primary hypothesis of this study was that the association between these two diseases as a novel experimental model would potentiate the deleterious effects caused by these diseases in male *Wistar* rats. The group that contained these two pathologies showed persistent edema and muscle weakness, with no prospect of improvement, including leukocyte migration, morphological alteration of the SM, and greater alveolar bone resorption. The group that presented the greatest impairment was also the group that contained the two pathologies, proving the hypothesis of the study and the efficacy of the experimental model used.

In further investigation of this experimental model, the second hypothesis formed was that in the presence of periodontitis, one injection of FCA would be enough to cause the same effects as when two injections were administered. However, it was found that the group that had periodontitis and was subjected to an intra-articular injection (PIAG) showed fluctuating results; for example, in PET, there was greater impairment than in PRAG but not more than in RAG. In the remaining variables analysed, PIAG remained similar to the other groups or showed no significant differences, indicating that the association between periodontitis and intra-articular injection of FCA was not more effective than periodontitis associated with the two FCA injections. In the PIDG, which was subjected to periodontitis and intradermal injection, the results obtained were even less significant, and most evaluations resembled the CG, indicating the non-effectiveness of the experimental model.

Therefore, the second hypothesis was not confirmed, since the group that underwent periodontitis associated with only intradermal injection and the group that underwent periodontitis associated with only intra-articular injection did not show similar or more significant changes than the group that underwent periodontitis and both injections of FCA. Again, the effective model was the association of the two models already existing and established in the literature: ligature-induced periodontitis and RA induced by two injections of FCA.

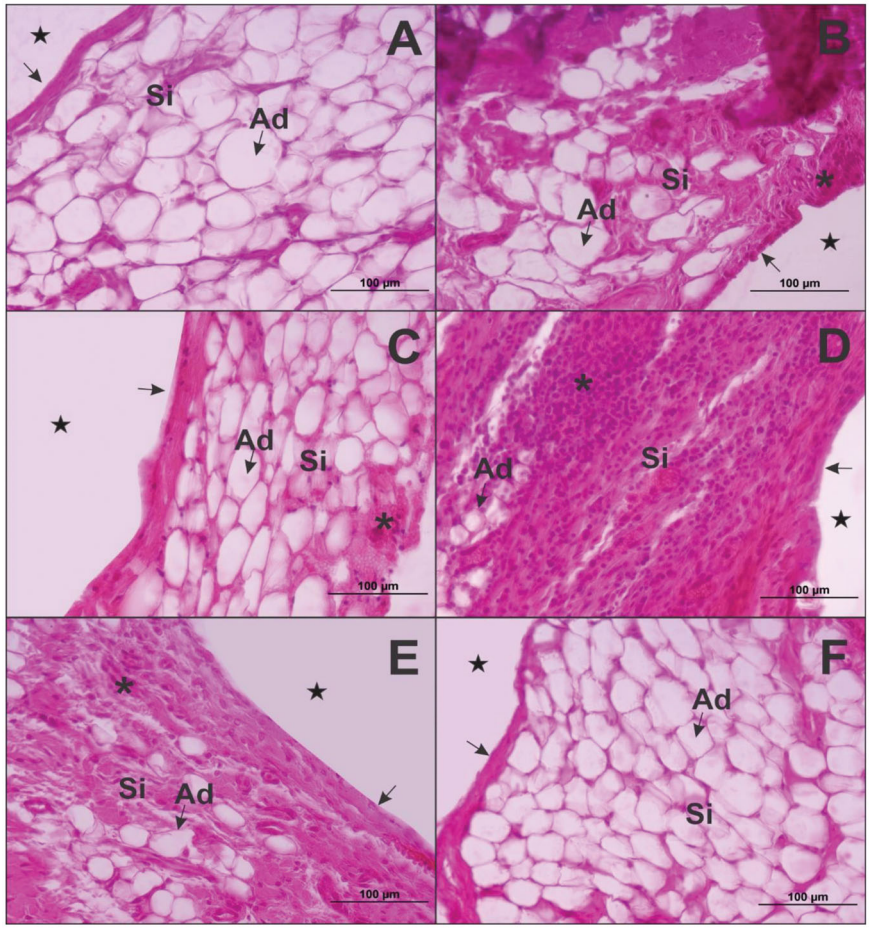

Figure 8. Photomicrographs of the synovial membrane of the knee joint of *Wistar* rats; sagittal section, haematoxylin and eosin staining. In A: Control Group (CG), membrane with thin synovial intima (black arrow) and subintima (Si) with adipose cells (Ad); In B: Rheumatoid Arthritis Group (RAG), synovial membrane thickening with fibrosis (asterisk) and subintima (Si) with change in adipocytic characteristic; In C: Periodontitis Group (PG) membrane with thin synovial intima (black arrow) and subintima (Si) with adipose cells (Ad); In D: Periodontitis and Rheumatoid Arthritis Group (PRAG), thickening of synovial membrane with fibrosis (asterisk) and subintima (Si) with loss of adipocytic characteristic (Ad); E: Periodontitis and Intra-articular Group (PIAG), synovial membrane thickening with fibrosis (asterisk) and subintima (Si) with change in adipocytic characteristic; F: Periodontitis and Intradermal Group (PIDG), membrane with thin synovial intima (black arrow) and subintima (Si) with adipose cells (Ad). Joint cavity (star). 40× magnification.

In the present study, it was possible to verify the efficacy of ligature induction of periodontitis in the groups subjected to the procedure, confirmed by histometry and radiography of the gingival tissue. It was also possible to verify the efficacy of the induction of RA by the two injections in the groups that underwent the procedure by increasing the articular diameter of the knees.

In PET, the association of pathologies did not result in increased significant changes compared to the group containing only RA, since this evaluation was directly linked to the evaluation of joint function, and the presence of periodontitis did not cause significant changes in motor ability. In the nociceptive threshold, the RAG reached a lower threshold, but during the evaluations, these values progressively increased. In the PRAG, there was an increase in values, but it was not permanent; therefore, the presence of both pathologies favoured the maintenance of the alteration in the nociceptive threshold.

In relation to edema and muscle strength, RAG reached very accentuated values of impairment, but in the final evaluations, these values were re-established, which did not occur in the PRAG; in other words, in the group with both pathologies, the impairment demonstrated maintained values with no high accentuation, and without the perspective of obtaining better values in the final evaluations. Similarly, in a study by Corrêa et al. [38], which evaluated the impact of experimental RA and periodontitis in rats, in paw swelling analysis using a plethysmometer, the RA showed a reduction in edema, while the RA and periodontitis showed a maintained volume.

In the investigation by Gomes et al. [26], animals that received only the intra-articular injection showed an increase in edema and a subsequent decrease. In the present study, the group with periodontitis and intra-articular injection did not show this reduction in edema but rather maintained this parameter in an altered form.

In view of this, immuno-inflammatory mechanisms analogous to inflammatory dysregulation increase the susceptibility of patients with RA and periodontitis to develop aggressive, advanced, and severe forms of both pathologies [39]. In addition, muscle weakness is a common complaint in RA because it alters the patient's ability to contract the muscles [40].

The PIAG presented an alteration in joint diameter, but this alteration was not greater than that in the groups that received the two injections. There was muscle strength impairment in this group; however, in the final evaluations, it was possible to observe that this parameter was re-established. In relation to

the group that received the intradermal injection associated with periodontitis, there were no significant changes because of the similarity with the control group in most of the variables analysed, indicating that periodontitis associated with the injection at the base of the tail did not cause changes in functional parameters. Our findings corroborate the study of Gomes et al. [26], which showed that the preferred route of immunisation was at the base of the tail, and this did not show significant signs of pain or edema.

Patients with periodontitis tend to have higher arthritis activity scores than those without periodontitis, and there is a proportional relationship between periodontitis severity and RA activity [39]. Thus, it was plausible that the highest leukocyte migration with a statistically significant increase, represented by the total cell count, was in the group with associated pathologies.

Interestingly, the level of IL-17 was higher in the PG, indicating that periodontitis alone seems to increase the local IL-17 production. The fact that IL-17 levels did not increase or decrease in the presence of both pathologies may be related to other inflammatory mechanisms or may depend on different experimental models of arthritis and periodontitis. In a study [38] using two immunizations of type II collagen and a third immunisation with FCA associated with periodontitis by ligature, the IL-17 level was higher in the group that had both diseases.

Synovitis affects the SM, which is the site of primary inflammation, and is therefore accompanied by hyperplasia and cellular inflammation [12]. This occurred in the animals in the RAG, which showed inflammatory infiltrate, thickening of the synovial intima, and changes in the adipocytic characteristics of the SM. However, these changes were more pronounced in the group with both pathologies. Similar to our results, in a study [21] with patients to determine the association of periodontitis, *Porphyromonas gingivalis*, and RA, the patients with both diseases showed increased joint damage, seen through higher radiographic scores and significantly higher swollen joints leading to further involvement of the synovial joint.

In radiographic and histometric analyses, the distance from the cementoenamel junction to the alveolar bone crest to assess alveolar bone resorption was evaluated, and the group that showed greater deficiency was the group with both pathologies. Both diseases share common molecular mechanisms, in which low OPG levels cause reduced vascular protection and high levels of the active receptor ligand RANKL and TNF-related apoptosis-inducing ligand (TRAIL). With the association of inflamed tissue, vascular damage and osteoclast activation occur, contributing to bone resorption [41].

A study [42] conducted to characterise periodontal disease associated with experimental RA in rats identified greater significant alveolar bone loss in the group with both pathologies. Similar destructive mechanisms, such as pro-inflammatory cytokines and inflammatory cells, lead to chronic gum destruction in periodontitis, and bone erosion in RA. Recent findings support the idea that periodontitis may be a factor in initiating and maintaining the autoimmune inflammatory responses that occur in RA [43].

To the best of our knowledge, this is the first study to use the association of two pre-established experimental models used in the literature: periodontitis through ligature and RA through the two injections of FCA containing *Mycobacterium butyricum*. This experimental model accentuated the deleterious effects on synovial and periodontal tissues, functional capacity, and leukocyte migration, confirming the hypothesis of this study. Furthermore, as a secondary investigation, the repercussions of the application of a single injection associated with periodontitis were observed. However, these repercussions were no more significant at the functional and histomorphological levels than when the two injections were administered, indicating the non-effectiveness of this experimental model.

Thus, this study contributes to and expands the possibilities of developing new research that mirrors the experimental model used, such as the proposal of new forms of treatment in the presence of the two diseases, as well as the application of new evaluations that contemplate inflammatory or histomorphological parameters that can be discussed with those used in this research.

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