ABSTRACT

Purpose: This study evaluated the systemic and local effects of doxycycline (DOX) and low-intensity laser (LIL) treatment as adjuvants to scaling and root planing (SRP) in the treatment of experimental periodontitis in rats.

Methods: The sample consisted of 180 male rats (Rattus norvegicus albinus, Wistar), of which 30 did not receive induction of periodontal disease (negative control [NC] group) and 150 received induction of periodontal disease in the lower first molar. After 7 days, the ligature was removed, and the animals were divided into the following groups: NT (no treatment), SRP (SRP), DOX (SRP and DOX irrigation), LIL (SRP and laser irradiation), and DOX+LIL (SRP, DOX, and LIL). The animals were euthanized at 7, 15, and 30 days; thereafter, biochemical, radiographic, histological, and immunohistochemical analyses were performed.

Results: In the intragroup analysis, lower concentrations of \(\alpha\)-1-glycoprotein acid (\(\alpha\)-1-Ga) and complement 3 (C3) were observed in the DOX+LIL group than in all other groups at all time points, as well as lower levels of complement 4 (C4) at 15 and 30 days \((P<0.001)\). Less bone loss was observed in the DOX, LIL, and DOX+LIL groups than in the NC and SRP groups at all time points \((P<0.001)\). There was a smaller number of tartrate-resistant acid phosphatase (TRAP)-positive cells in the DOX+LIL group than in the other groups at all time points \((P<0.001)\). Positive correlations were observed between the systemic levels of \(\alpha\)-1-Ga, C3, and C4 and the number of TRAP-positive cells.

Conclusions: The combination of DOX with LIL as SRP adjuvants was effective both systemically and locally for the treatment of experimental periodontitis in rats.

Keywords: Doxycycline; Lasers; Periodontal disease; Periodontitis; Rats

INTRODUCTION

Periodontal disease involves host immune-inflammatory responses triggered by microbial biofilms that accumulate around the dentition and destroy periodontal tissues. Periodontal disease is a complex, multifactorial condition, the progression of which depends on an imbalanced host response to the attacking agents; if the host response is exacerbated,
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Conflict of interest
No potential conflict of interest relevant to this article was reported.

The organism itself can destroy normal structures of the periodontium [1]. Clinically, the periodontal tissues show an initial manifestation (gingivitis) characterized by inflammatory processes, hyperemia, edema, and gingival bleeding, and gingivitis, which may progress to periodontitis [2]. Collagen fibers are cleaved, triggering apical migration of the junctional epithelium and periodontal pocket formation [2].

The immunoinflammatory response results in the release of cytokines such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α that are involved in bone resorption and destruction of periodontal connective tissue [1]. The endotoxins present in periodontal pockets can initiate a local and systemic inflammatory response [3]. Systemic changes are possible since the cytokines (IL-1, IL-6, TNF-α) released in the immunoinflammatory process can, through the bloodstream, stimulate the production of glycoproteins and components of the complement system such as complement 3 (C3) and complement 4 (C4) [3].

Bone resorption in periodontal disease is controlled by proteins such as receptor activator of nuclear factor κB (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG). RANK is present in osteoclast membranes and its dimerization allows the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells responsible for bone resorption [4]. RANKL, which is produced by osteoblasts, bone stromal cells, and activated T lymphocytes, promotes bone resorption when it forms a complex with RANK. In contrast, OPG inhibits the formation of osteoclasts in the development stage by preventing RANKL from binding to RANK [5].

One of the goals of periodontal treatment is to eliminate adherent bacteria through scaling and root planing (SRP). This procedure attenuates the host’s response to these aggressive agents, thereby protecting the periodontal tissues from destruction [6]. However, SRP treatment alone may be unable to eliminate pathogenic bacteria lodged within soft and hard tissues or areas that cannot be accessed by periodontal instruments, such as the furcation region and root depressions [7,8].

Due to these limitations, adjunctive methods can be combined with conventional periodontal therapy [9,10]. In a systematic review, Chambrone et al. [11] demonstrated that in smokers with chronic periodontitis (CP), the use of local antimicrobials as adjunctive treatment to SRP improved the efficacy of non-surgical periodontal therapy in reducing the probing depth (PD) and improving the clinical attachment level at sites presenting PD ≥5 mm before treatment. Doxycycline (DOX) is one of the most widely used antibiotics in periodontal therapy due to its anti-inflammatory action, which inhibits the release of enzymes that destroy collagen, its ability to prevent bacterial protein synthesis and bone resorption, and the fact that it can interact positively with periodontal tissues [12]. Another option for adjunctive treatment in combination with SRP is photobiomodulation therapy using a low-intensity laser (LIL), which also demonstrates positive effects in periodontal treatment, such as inhibition of the inflammatory process and bone resorption by reducing the production of prostaglandin E2. Furthermore, LIL therapy can modulate the behavior of gingival fibroblasts, inducing the expression of growth factors responsible for better healing [13].

The literature contains studies that investigated DOX or LIL alone for periodontal treatment, but no studies have yet evaluated their effects in combination with other therapies. Thus, the objective of the present study was to assess the systemic and local effects of the combination of DOX and LIL as adjuvants to SRP in the treatment of experimental periodontitis in rats through biochemical, radiographic, histological, and immunohistochemical analyses.
MATERIALS AND METHODS

Animals
The present study was approved (57/2017) by the Animal Ethics Committee (CEUA) of the Federal University of Alfenas (UNIFAL-MG), following the current standards adopted by the Brazilian College of Animal Experimentation (COBEA) and the ARRIVE 2.0 animal research report guidelines. The sample consisted of 180 male rats (Rattus norvegicus albinus, Wistar), of which 30 did not receive induction of periodontal disease (negative control [NC] group) and 150 received induction of PD. These animals were 2 to 3 months old, weighed approximately 200 to 250 g, and were kept under standard conditions with water and food ad libitum, at room temperature and with a consistent light/dark cycle of 12 hours.

Induction of experimental periodontitis
The animals were anesthetized using an intramuscular injection of 75 mg/kg of ketamine hydrochloride (Rhobifarma Pharmaceutical Industry Ltd., Hortolândia, SP, Brazil) combined with 6 mg/kg of xylazine hydrochloride (Rhobifarma Pharmaceutical Industry Ltd.,) to induce periodontal disease. With the aid of a modified forceps (Quinelato, Rio Claro, SP, Brazil), a No. 10 cotton thread (Coats Corrente, São Paulo, SP, Brazil) was adapted around the lower left first molars [14].

Local treatments and group categorization
After 7 days of induction and development of PD, the ligature was removed from the animals, which were randomly divided (by lot) into 5 groups according to the local treatment they received. In the NT group, the animals received no treatment; in the SRP group, the animals received SRP; in the DOX group, the animals received SRP and DOX application; in the LIL group, the animals received SRP and irradiation with LIL; and in the DOX+LIL group, the animals received SRP, irrigation with DOX, and irradiation with LIL.

The SRP procedures were performed using Gracey Mini-Five Curette 5 and 6 (Hu-Friedy MFG, Corporation, Inc., Chicago, IL, USA) with 3 movements in the mesiodistal direction on both the buccal and lingual surfaces [15]. The 10% DOX gel was made with the following components: 10% DOX hydrochloride, 15% ethylcellulosegel, 3% triethanolamine, and distilled water (Fagron Brazil, São Paulo, SP, Brazil). For irrigation with 10% DOX gel, a 1-mL insulin syringe (Becton Dickinson Indústrias Cirurgicas, Curitiba, PR, Brazil) containing 1 mL of the substance was used, and the tip of the needle was directed into the subgingival region. DOX remained in the tissue for 1 minute and was then aspirated.

The DOX gel was administered topically in the present study, because topical administration has advantages over systemic use; for instance, topically administered DOX does not cause side effects, and it can be easily applied to periodontal pockets, where it then solidifies and releases the drug. In addition, the gel form of DOX has slower resorption, enabling a more lasting and prolonged effect than occurs with its liquid form [16].

In the DOX+LIL group, treatment with LIL was applied after the drug had remained in the tissue for 1 minute. Irradiation with LIL was performed using an InGaAlP Twin Flex® diode laser (MM Optics, São Carlos, SP, Brazil) operating at a 660-nm wavelength (visible red light), with the following dosimetric parameters: aperture diameter, 0.23 cm; beam area, 0.04 cm²; tip not initiated. The application was carried out continuously, in a localized (punctual) manner, with the tip parallel to the long axis of the alveolus, in contact with the area of
the periodontal pocket at 3 points equidistant from the buccal surface (mesial, middle, and distal) and 3 points equidistant from the lingual surface (mesial, middle, and distal). Each point received a power of 0.04 W for 4 seconds. Thus, the dose applied per point was calculated by the following equation: dose (energy density) = power (W) × time (seconds) / area (cm²), resulting in: dose = 0.04 W × 4 seconds / 0.04 cm² = 1 J/cm². Therefore, the radiant energy applied per point was 0.16 J. The power density (irradiance) was calculated using the following equation: power (W) / area (cm²), resulting in 0.04 W / 0.04 cm² = 1 W/cm².

**Experimental periods**

Thirty animals from each experimental group were euthanized by anesthetic overdose at 7, 15, and 30 days after local treatment by administration of a lethal dose of thiopental (150 mg/kg) (Cristália Ltd, Itapira, SP, Brazil), so that biochemical, radiographic, histological, and immunohistochemical analyses could be performed.

**Blood collection and sample preparation**

The animals were anesthetized intramuscularly using a combination of 75 mg/kg of ketamine hydrochloride (Rhoibafarma Pharmaceutical Industry Ltd.) with 6 mg/kg of xylazine hydrochloride (Rhoibafarma Pharmaceutical Industry Ltd.). Thereafter, incisions were made in the thoracic cavity to visualize the heart, and approximately 10 mL of blood per animal was collected through cardiac puncture in the left ventricle. The blood was immediately poured into heparinized tubes previously identified according to each experimental group, centrifuged for 15 minutes at 2,000 rpm for blood plasma separation, and then stored at −70°C for biochemical analyses [17]. The animals were then euthanized through anesthetic overdose, the mandibles were removed and sectioned in the middle, and their left sides—where periodontal disease had been induced—underwent radiographic and immunohistochemical analyses.

**Plasma biochemical analyses**

For the biochemical analyses, the plasma concentrations of α-1-glycoprotein acid (α-1-Ga), C3, and C4 were evaluated using spectrophotometry. The α-1-Ga level was determined by an immunoturbidimetry reaction with an α-1-Ga reagent (Biotécnica, Varginha, MG, Brazil). In this immunoturbidimetric method, anti-α-1-Ga antibodies form an insoluble complex with α-1-Ga, resulting in turbidity with an intensity proportional to the concentration of α-1-Ga of the sample. This concentration was determined using a spectrophotometer at 340 nm [18]. The concentration (mg/dL) of α-1-Ga in each sample was calculated using the equation of the line obtained in the calibration step, through the absorbances from each experimental group obtained in the spectrophotometer.

The concentrations of C3 and C4 (mg/dL) were obtained using the same methodology, despite the following modifications in the reaction parameters: 1) for C3 (Biotécnica), 2.5 μL of sample was used in 250 μL of R1, and then incubated at 37°C for 2 minutes; 2) for C4 (Biotécnica), 5 μL of sample was used in 200 μL of R1, with incubation at 37°C for 2 minutes.

**Radiographic imaging and digital analyses**

After euthanasia of the animals, the jaws were removed and fixed in 10% formalin solution for 48 hours. The right and left sides of the pieces were then divided and the left side was subjected to X-ray imaging.

On a table, the left hemimandibles were positioned with the buccal surfaces facing the radiographic film (Kodak Dental Intraoral E-Speed Film, Osasco, SP, Brazil). Radiographs
were standardized using an X-ray machine (Pampas-E, General Electric Company, Milwaukee, WI, USA), with settings of 65 kVp and 10 mA. A central beam of X-rays was focused on the perpendicular surface of the optical plate, with a focal length of 30 cm and an exposure time of 0.8 seconds.

The radiographs were scanned and the images were analyzed with the Image Lab software (Softium Computer Systems, São Paulo, SP, Brazil), using the tool for distance and angle measurement. With this feature, the distance from the cementoenamel junction to the bone crest was measured at the mesial surface; measurements were recorded in millimeters. The cursor was positioned in the region corresponding to the cementoenamel junction. The left mouse button was then pressed and dragged down to the level of the alveolar crest, and the program automatically measured the distance (Figure 1) [19].

**Histologic processing**

The specimens were demineralized in 50% formic acid solution (Multichemie, Cotia, São Paulo, SP, Brazil) and 20% sodium citrate (Multichemie) in equal proportions. After this stage, the specimens were included in paraffin. Semi-serial cuts were performed in the mesio-distal direction in relation to the teeth, with a thickness of 4 µm, and the specimens were dyed with hematoxylin and eosin (HE) (Multichemie). Immunohistochemical reactions were performed using primary antibodies against TRAP (1:100, SC 30833, goat anti-TRAP, Santa Cruz Biotechnology, Dallas, TX, USA) and proteins involved in the process of bone repair in the furcation region of the first left lower molars.

**Histological analysis**

The sections dyed with HE were analyzed under light microscopy at a magnification of ×40 to characterize the periodontal ligament of the furcation region of the first molar [20].

**Immunohistochemical analysis**

The immunohistochemical marker TRAP was assessed by counting TRAP-positive cells located in the boundary of the most coronal portion of the bone tissue of the furcation region (area in millimeters squared) in mandibular first molars without or with periodontal disease, extending between the roots (width), and from the bone margin to the root trunk (height). Mature osteoclasts containing 3 or more nuclei were considered positive TRAP cells. A blinded trained examiner (L.A.F.) selected the sections for the histological and immunohistochemical analyses. Another blinded calibrated examiner conducted the data analysis. The values for each specimen section were measured 3 times by the same examiner on different days to reduce variations in the data [21].

![Figure 1](https://doi.org/10.5051/jpis.2100420021)

Figure 1. (A) Radiographic image illustrating the evaluation of bone loss in the NT group at 7 days (scale bar=1 mm). NT: no treatment.

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https://jpis.org

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Intra-examiner reproducibility
Before the biochemical, radiographic, and immunohistochemical analyses, the examiner was trained by performing 2 measurements of 30 specimens at a 1-week interval. The paired t-test was calculated, and no differences were observed in the mean values that were compared (P>0.05). In addition, a calculation of Pearson correlation coefficients revealed a very high correlation (0.95) between the 2 measurements for both the radiographic and immunohistochemical analyses.

Statistical analyses
The number of animals required was calculated using the formula: n = 1+\left[2\times10.51\times(0.2/0.172)\right]^2, accounting for 30 animals per experimental group, with a study power of 90%. Therefore, the sample was composed of 150 2- to 3-month-old male rats (Rattus norvegicus albinus, Wistar), weighing approximately 200 to 250 g, from the Central Vivarium of the Federal University of Alfenas (UNIFAL-MG). Statistical analyses of the biochemical, radiographic, and immunohistochemical data were performed using the BioEstat 5.0 software (BioEstat Windows 1995 Sonopress, Indústria Brasileira, Manaus, AM, Brazil). The hypothesis that there was no statistically significant difference between the data among the groups and time points was tested in the teeth with induced periodontitis. After the normality of the data distribution was analyzed using the Shapiro-Wilk test, comparative analyses among groups and periods were performed by 2-way analysis of variance, with the Bonferroni correction yielding a statistical significance threshold of P<0.001 (groups) or P<0.003 (time points). In addition, the Pearson correlation test was performed for the biochemical, radiographic, and immunohistochemical data, with P values <0.01 considered to indicate statistical significance.

RESULTS
Plasma biochemical results
The DOX+LIL group showed significantly lower concentrations of α-1-Ga and C3 than all other groups at all experimental time points (P<0.001). The concentration of C4 protein was significantly lower in the DOX+LIL group than in the NT group at all time points and the SRP, DOX, and LIL groups at 15 and 30 days (P<0.001). The concentrations of α-1-Ga, C3, and C4 decreased significantly over time in the DOX+LIL group at 7, 15, and 30 days (P<0.003) (Table 1).

Table 1. Plasma concentrations (in the same animals) of α-1-Ga, C3, and C4 (mg/dL) in each group and time point

| Groups/time points | α-1-Ga concentrations | No. | C3 protein concentrations | No. | C4 protein concentrations | No. |
|--------------------|------------------------|-----|---------------------------|-----|---------------------------|-----|
|                    | 7 days                 | 15 days | 30 days | 7 days | 15 days | 30 days | 7 days | 15 days | 30 days |
| NC                 | 15.0±1.10^s            | 12.29±0.32^s | 7.5±2.39^s | 30    | 4.5±1.43^s            | 20.1±0.44^s | 8.2±1.35^s | 30    | 10.7±4.03^s | 3.0±1.67^s | 0.3±0.14^s | 30    |
| NT                 | 208.29±23.69^aa         | 190.79±51.00^aa | 189.49±51.36^aa | 30    | 377.05±24.46^aa         | 337.87±43.61^aa | 319.50±36.71^aa | 30    | 84.36±0.56^aa         | 76.80±7.91^aa | 77.80±5.85^aa | 30    |
| SRP                | 196.16±3.06^AA          | 163.26±12.36^AA | 144.03±18.76^AA | 30    | 288.52±28.21^AA         | 238.41±38.49^AA | 173.41±40.03^AA | 30    | 35.84±10.16^AA         | 45.68±18.11^AA | 61.14±10.74^AA | 30    |
| DOX                | 146.81±21.53^AA         | 132.82±17.66^AA | 99.03±14.82^AA | 30    | 259.03±22.24^AA         | 227.43±40.01^AA | 153.96±23.93^AA | 30    | 36.80±3.27^AA         | 18.17±6.67^AA | 21.47±0.66^AA | 30    |
| LIL                | 124.95±12.36^BB         | 47.27±20.21^BB | 25.42±11.16^BB | 30    | 61.17±22.77^BB         | 121.20±22.09^BB | 90.15±44.68^BB | 30    | 28.80±3.21^BB         | 12.92±7.66^BB | 15.47±4.33^BB | 30    |
| DOX+LIL            | 43.01±14.89^CC          | 18.94±15.77^CC | 0.83±1.76^CC | 30    | 68.01±26.38^CC          | 31.57±24.04^CC | 0.33±10.44^CC | 30    | 27.44±3.86^CC          | 5.05±41.81^CC | 0.38±1.50^CC | 30    |
| Total              | 180                    | 180     | 180                   | 180   | 180                     | 180    | 180     | 180    | 180                     | 180    | 180     | 180    |

Different capital letters in the columns indicate differences between groups at the same time point (2-way analysis of variance and the Bonferroni correction, with P<0.003).
Different lowercase letters in the rows indicate differences between time points in the same group (2-way analysis of variance and the Bonferroni correction, with P<0.001).

α-1-Ga: α-1-glycoprotein acid, C3: complement 3, C4: complement 4, NC: negative control, NT: no treatment, SRP: scaling and root planing, DOX: doxycycline, LIL: low-intensity laser.
Radiographic results
Significantly less bone loss (BL) was observed in the DOX, LIL, and DOX+LIL groups than the NT and SRP groups at all time points \((P<0.001)\). In the NT group, significantly less BL was found at 30 days than at 7 days \((P<0.003)\) (Table 2).

Histological results
In the NT group, at all time points, the connective tissue was disorganized, with a high number of neutrophils and a discrete number of fibroblasts. The bone tissue showed areas of necrosis and thin bone trabeculae.

In the SRP and DOX groups, at all time points, the connective tissue was more organized, with a predominantly chronic inflammatory process and a moderate number of fibroblasts. The bone tissue did not show areas of necrosis, and the bone trabeculae were thin.

In the NC, LIL, and DOX+LIL groups, at 7 and 15 days, the connective tissue was well developed, with a moderate number of fibroblasts, a distinct chronic inflammatory infiltrate, and moderately developed bone tissue. At 30 days, the periodontal ligament was organized and intact, with collagen fibers parallel to each other. The connective tissue was well developed, intact, and without inflammatory infiltrates (Figures 2-4).

Immunohistochemical results
Significantly fewer TRAP-positive cells were observed in the DOX+LIL group than in the other groups at all experimental time points \((P<0.001)\). In the DOX+LIL group, the number of TRAP-positive cells was significantly lower at 30 days than at 7 days \((P<0.003)\) (Table 3, Figures 2-4).

Correlations between local parameters and systemic biomarkers
A strong positive correlation was observed between the systemic levels of \(\alpha-1\)-Ga, C3, and C4 and the local levels of the number of TRAP-positive cells in the furcation region of the lower left first molars with induced periodontitis. As the levels of those proteins decreased, there was also a decrease in the number of TRAP-positive cells \((P<0.01)\). There was no correlation between BL observed on radiographs and systemic biomarkers (Table 4).

### Table 2. Distances between the cementoenamel junction and the alveolar bone crest (mm) on the mesial surface of the lower left first molars according to each group and time point

| Groups/time points | 7 days       | 15 days      | 30 days      | No. |
|--------------------|--------------|--------------|--------------|-----|
| NC                 | 0.33±1.00\(^{a}\) | 0.35±0.01\(^{a}\) | 0.31±0.09\(^{a}\) | 30  |
| NT                 | 1.89±0.16\(^{a}\) | 1.79±0.10\(^{a}\) | 1.52±0.08\(^{a}\) | 30  |
| SRP                | 1.43±0.26\(^{a}\) | 1.40±0.13\(^{a}\) | 1.37±0.04\(^{a}\) | 30  |
| DOX                | 0.59±0.20\(^{a}\) | 0.56±0.11\(^{a}\) | 0.52±0.10\(^{a}\) | 30  |
| LIL                | 0.53±0.21\(^{a}\) | 0.50±0.04\(^{a}\) | 0.49±0.13\(^{a}\) | 30  |
| DOX+LIL            | 0.44±0.06\(^{a}\) | 0.42±0.14\(^{a}\) | 0.41±0.01\(^{a}\) | 30  |
| Total              |              |              |              | 180 |

Different capital letters in the columns indicate differences between groups at the same time point (2-way analysis of variance and the Bonferroni correction, with \(P<0.001\)).

Different lowercase letters in the rows indicate difference between time points in the same group (2-way analysis of variance and the Bonferroni correction, with \(P<0.003\)).

NC: negative control, NT: no treatment, SRP: scaling and root planing, DOX: doxycycline, LIL: low-intensity laser.
DISCUSSION

The present study was based on the model of induction of periodontal disease proposed by Swerts et al. [14], by placing a cotton thread around the molars of rats. In this model, the ligature promotes bacterial accumulation, leading to the development of periodontal disease. This phenomenon was demonstrated by Theodoro et al. [22], who evaluated the antimicrobial effects of antimicrobial photodynamic therapy in the treatment of induced periodontitis. The authors observed microbiologically the significant accumulation of Actinobacillus actinomycetemcomitans (Aa) and Porphyromonas gingivalis (Pg) in the ligatures used to induce periodontal disease. In the present study, we observed that this model was efficient in the induction of experimental periodontal disease because the ligature induced bacterial plaque formation and a local inflammatory response. Periodontal disease was characterized by clinical signs of gingival inflammation, such as edema, redness, and loss of gingival consistency.

In the present study, the adjuvant usage of DOX systemically demonstrated that there was a lower C4 level in the DOX group than in the SRP group at 15 and 30 days. Corroborating our results, Madi et al. [23] observed that periodontal treatment in humans with DOX gel, as an adjunct to SRP, had an anti-inflammatory effect, improving both clinical
parameters and levels of inflammatory markers. Regarding the local evaluation through radiographic analyses, there was less BL in the DOX group than in the NT and SRP groups at all the experimental time points. Corroborating these results, a recent clinical study [24] demonstrated that periodontal treatment with SRP combined with the application of DOX increased radiographic bone density and significantly improved the periodontal clinical parameters evaluated in treated patients, such as clinical insertion level, bleeding on probing, and the plaque index.

In the systemic evaluation, the concentration of $\alpha$-1-Ga was significantly lower in the LIL group than in the NT, SRP, and DOX groups at 15 and 30 days, and the C3 level was significantly lower in the LIL group than in the NT, SRP, and DOX groups at 7 days. Locally, the LIL group presented well-developed connective and bone tissue, with fewer TRAP-positive cells than in the NT group at all experimental time points. In a study of patients with diabetes who had CP, Dengizet Eltas et al. [25] investigated the effects of a diode laser combined with non-surgical periodontal treatment on periodontal parameters, the systemic inflammatory response, and serum hemoglobin levels. The results showed that clinical parameters such as the gingival index, bleeding on probing, and PD were significantly reduced; however, they did not show beneficial effects on the systemic inflammatory response and glycemic
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Figure 4. (A) Photomicrograph illustrating the histological results in the furcation region of the mandibular left first molar with induced periodontal disease in the LIL group at 7 days; (B) Immunostaining for TRAP (arrow). (C) Photomicrograph illustrating the histological results in the furcation region of the mandibular left first molar with induced periodontal disease in the LIL group at 15 days; (D) Immunostaining for TRAP (arrow). (E) Photomicrograph illustrating the histological results in the furcation region of the mandibular left first molar with induced periodontal disease in the LIL group at 30 days; (F) Immunostaining for TRAP (arrow). (G) Photomicrograph illustrating the histological results in the furcation region of the mandibular left first molar with induced periodontal disease in the DOX+LIL group at 7 days; (H) Immunostaining for TRAP (arrow). (I) Photomicrograph illustrating the histological results in the furcation region of the mandibular left first molar with induced periodontal disease in the DOX+LIL group at 15 days; (J) Immunostaining for TRAP (arrow). (K) Photomicrograph illustrating the histological results in the furcation region of the mandibular left first molar with induced periodontal disease in the DOX+LIL group at 30 days; (L) Immunostaining for TRAP (arrow). Scale bar=mm

LIL: low-intensity laser, TRAP: tartrate-resistant acid phosphatase, DOX: doxycycline.

Table 3. Number of TRAP-positive cells in the furcation region of the lower left first molar according to each group and time point

| Groups/time points     | 7 days       | 15 days      | 30 days      | No. |
|------------------------|--------------|--------------|--------------|-----|
| NC                     | 0.88±1.30a   | 0.80±1.05a   | 0.70±1.15a   | 30  |
| NT                     | 9.10±0.60a   | 8.02±1.16a   | 7.89±0.23a   | 30  |
| SRP                    | 6.23±0.02a   | 6.00±0.34a   | 5.79±0.87a   | 30  |
| DOX                    | 5.71±1.24a   | 5.45±1.55a   | 4.85±0.13a   | 30  |
| LIL                    | 4.31±1.19a   | 3.99±1.26a   | 3.68±0.57a   | 30  |
| DOX+LIL                | 2.05±0.34c   | 1.89±0.70c   | 1.02±0.83c   | 30  |
| Total                  |              |              |              | 180 |

Different capital letters in the columns indicate differences between groups at the same time point (2-way analysis of variance and the Bonferroni correction, with \( P<0.001 \)).

Different lowercase letters in the rows indicate difference between periods in the same group (2-way analysis of variance and the Bonferroni correction, with \( P<0.003 \)).

NC: negative control, NT: no treatment, SRP: scaling and root planing, DOX: doxycycline, LIL: low-intensity laser.

Table 4. Correlation (\( r \)) between systemic (biochemical) and local (radiographic and immunohistochemical) parameters according to each group and time point

| Systemic/local          | Radiographic bone loss | Number of TRAP-positive cells |
|-------------------------|------------------------|------------------------------|
| \( \alpha-1 \)-acid glycoprotein | 0.10                   | 0.74\(^a\)                  |
| C3                      | 0.02                   | 0.81\(^h\)                  |
| C4                      | 0.04                   | 0.70\(^h\)                  |
| TRAP                    |                        |                             |

\(^a\)Strong positive correlation between systemic (biochemical) and local (radiographic and immunohistochemical) data (Pearson correlation coefficients, with \( P<0.01 \)).

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control. Other authors in the literature [14] who studied the use of laser adjuvants to SRP treatment during periodontal treatment showed positive results in the improvement of periodontal parameters, unlike the present study, which demonstrated a significant decrease in TRAP-positive cells only in comparison to the NT group. These conflicting results may be due to methodological differences, mainly in relation to the laser protocols used and the different irradiation parameters used. Regarding the beneficial local effects of lasers, authors have shown that the use of lasers inhibits the production of inflammatory mediators by periodontal ligament cells, benefits cellular chemotaxis [13], and assists in vasodilation and local angiogenesis. These events can increase the diffusion of oxygen in the tissue, thereby benefiting the repair process since the secretion of collagen by fibroblasts in the extracellular area occurs only in the presence of high oxygen pressures [26]. It is important to highlight that this biomodulation takes place because of the increase in ATP production, which is generated by the application of a low-power laser and, consequently, by increasing the speed of mitosis. The anti-inflammatory action takes place because the irradiation causes an increase in the degranulation of mast cells, which consequently causes a rise in histamine levels, provoking local circulatory changes such as vasodilation and increased vascular permeability [27].

The DOX+LIL group systematically demonstrated lower concentrations of α-1-Ga and C3 than the other groups at all time points. For C4, the DOX+LIL group showed lower concentrations than the NT group at all time points and the SRP, DOX, and LIL groups at 15 and 30 days. In addition, the DOX+LIL group presented significantly less BL than the NT and SRP groups at all experimental time points. Supporting the results of BL, the histological evaluation demonstrated an organized and intact periodontal ligament, with well-developed connective tissue and without inflammatory infiltrate. These findings demonstrate that SRP, DOX, and LIL generated positive effects in the treatment of periodontal disease. Another study in the literature also demonstrated the beneficial effects of DOX and laser irradiation on the expression of collagen I and metalloproteinase matrix 8 (MMP-8) in cultured fibroblasts of the human periodontal ligament. The results showed that treatment with DOX significantly increased the secretion of collagen I, and its combination with laser treatment significantly reduced the expression of MMP-8 [28]. DOX is effective against periodontopathogens, such as Aa, Pg and Tannerella forsythia, and it demonstrated clinical efficacy in modulating the clinical signs of periodontitis [29]. These factors can be attributed to the effects of DOX, such as the inhibition of MMPs (collagenase and gelatinase) and positive impact on the bone repair process. In addition, it exerts anti-inflammatory effects by suppressing the number of polymorphonuclear leukocytes and blocking the release of prostaglandin E2 [12,30]. Another important factor is the way DOX is used, since it has been reported that DOX gel can remain in the subgingival site for a minimum period of 10 days, and its mechanism of action reduces the volume of gingival crevicular fluid and transforming growth factor-beta 1, with improved clinical signs of inflammation and decreased PD [31,32]. In addition, DOX, due to its action in the conditioning of the dentin, could allow fibrin binding, resulting in the formation of a new fixation and promoting the insertion of fibroblasts to the root surface, thereby increasing the production of collagen and bone and facilitating periodontal repair [32]. In contrast, LIL modulates biological processes, such as the acceleration of tissue repair, the reduction of pain and inflammation, and the activation of immune system cells [27,33].

The animals in the DOX+LIL group showed lower immunoreactivity to TRAP at 7 days. Wang et al. [34], when applying a DOX hydrogel solution to periodontal pockets of dogs, also observed positive results of this treatment, such as the reduction of pro-inflammatory IL-8 levels and improvement of gingival consistency. A possible explanation for these results is
that DOX works by directly preventing bone resorption, inducing osteoclastic apoptosis, and indirectly inhibiting RANKL in osteoclast genesis. Moreover, it is beneficial for the formation of bone tissue through the activation of osteoblasts [30,31]. Significantly fewer TRAP-positive cells were observed in the DOX+LIL group than in the other groups at all experimental time points. Zhang et al. [35] investigated the effect of 650-nm LIL irradiation as an adjunctive treatment of experimental periodontitis, and also demonstrated that the laser significantly decreased the number of osteoclasts as noted by TRAP staining. The authors concluded that the 650-nm LIL irradiation might be a useful treatment modality for periodontitis. The decreased BL in this group could be explained by the fact that the treatment inhibited the binding between the RANK receptor and its ligand, preventing its dimerization and causing a decreasing osteoclastogenic activity, as well as inhibition of the formation of TRAP-positive multinucleated cells, which are mainly expressed in osteoclasts [4].

The correlation between oral health and general health has been studied in recent years [36]. The challenge is to understand the relevant biological mechanisms and the effects that periodontal treatment can have on the systemic condition of individuals, as well as how to incorporate this knowledge into clinical practice [37]. After periodontal treatment, a strong positive correlation was observed between the systemic levels of α1-Ga, C3, and C4 and the number of TRAP-positive cells in the furcation region of the lower left first molars with periodontitis induction. As the protein level decreased, there was also a decrease in the number of TRAP-positive cells. Supporting these results, Rasperini et al. [38] evaluated the effects of dietary supplementation on periodontal clinical parameters and systemic inflammatory markers in patients with severe CP. Supragingival debridement was performed, and periodontal clinical parameters were monitored and correlated with both serum C-reactive protein and MMP levels. Longitudinal analyses revealed that MMP-8 and MMP-9 levels decreased over time. The correlation between gingival bleeding and MMP-8 levels in both groups was significant. Although authors suggested an association between the periodontal treatment of periodontal disease and systemic pathologies, as reported by Li and Xu [39], other authors, such as Joshi et al. [40], did not find such a correlation. Further studies are needed to clarify and determine the real association between periodontal disease and the impacts of its treatment on systemic changes.

Within the limits of this study, it is possible to conclude that the combination of DOX with LIL as SRP adjuvants was effective both systemically and locally for the treatment of experimental periodontitis in rats.

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