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Effectiveness of local Tocilizumab administration in mitigating alveolar bone loss and inflammation in experimental periodontitis

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To examine the impact of locally applied Tocilizumab (TCZ), an anti-interleukin-6 receptor antibody, on experimental periodontitis. 50 Wistar male rats were divided into three groups: (1) Healthy ($n = 10$), (2) Experimental Periodontitis ($n = 20$), and (3) Experimental Periodontitis + TCZ ($n = 20$). The animals received an intrapapillary injection of TCZ (2 mg/kg). After three days, periodontitis was induced by placing a ligature around the upper left 2nd molar for ten days. In euthanasia, blood, maxillae, and gingival samples were removed for biochemical, microtomography, histologic, immunohistochemistry, cytokine assay and RT-qPCR analyses. TCZ was able to attenuate linear alveolar bone loss ($p < 0.05$) and the loss of the number of bone trabeculae ($p < 0.05$), and to reduce the severity of inflammation ($p < 0.05$). Its local administration decreased gingival tissue levels of IL-6 and TNF-α ($p < 0.05$), the intensity of RANKL immunostaining ($p < 0.0001$) and mRNA expression of SOD ($p < 0.001$) and GPx ($p < 0.01$). These findings suggest the anti-inflammatory efficacy of Tocilizumab administered in periodontitis. Cytokines play a major role in destroying periodontal support tissue during periodontitis. Inhibition of IL-6 receptor demonstrated to reduce periodontal inflammation and bone loss, and may be considered as an adjunctive approach in refractory or severe periodontitis cases that do not adequately respond to conventional therapy.

Keywords Tocilizumab, Periodontitis, Interleukin-6, Alveolar bone loss, Inflammation

Periodontitis is a chronic, multifactorial inflammatory disease with a high global prevalence and is considered the leading cause of tooth loss among adults[1]. While biofilm accumulation is considered its primary etiological factor, the dysregulated host's inflammatory response, usually exacerbated, significantly contributes to the disease's progression[2].

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The clinical manifestations of periodontitis, including bleeding on probing and attachment loss, correlate with molecular events due to the host's immune response. This includes the release of prostaglandins, matrix metalloproteinases, and cytokines[3]. Interleukin-6 (IL-6), a critical inflammatory mediator in response to tissue damage and infection, plays a pivotal role in this process[4]. This cytokine is instrumental in differentiating B cells and promoting cellular proliferation[5]. Furthermore, it potently regulates osteoclastogenesis and bone resorption[6]. Elevated levels of IL-6 have been linked to periodontitis[7].

Scaling and root planing, accompanied by personalized oral hygiene instruction, is considered the gold standard in treating this periodontitis[8]. Additionally, various adjunctive therapies have been explored to enhance the outcomes of non-surgical periodontal treatment. These supplementary treatments include physical agents (such as high- and low-level lasers, as well as antimicrobial photodynamic therapy), chemical agents (e.g., chlorhexidine), antibiotic drugs, and adjunctive host-modulating agents[9]. More specifically, regarding the host-modulating agents, those used to regulate the immune-inflammatory response include local administration of statins and sub-antimicrobial doses of doxycycline[10]. Other approaches that have also been explored comprise probiotics, bisphosphonates, non-steroidal anti-inflammatory drugs, metformin, and omega-3 polyunsaturated fatty acids[10].

Tocilizumab (TCZ), a drug that can modulate the host's inflammatory response, has recently emerged as a noteworthy treatment, particularly for rheumatoid arthritis (RA). It is a recombinant humanized IgG1 monoclonal antibody designed to block both the soluble and membrane-bound forms of the IL-6 receptor (IL-6R), preventing IL-6 from binding to the IL-6R/gp130 complex. This inhibits the classical and trans-signaling pathways mediated by the JAK-STAT pathway. Consequently, TCZ lowers neutrophil counts and infiltration in inflamed tissues, reduces monocytes and macrophage migration inhibitory factor levels, while decreasing Th17 cells and increasing regulatory T cells[11]. Beyond its systemic anti-inflammatory effects, TCZ also influences bone remodeling. By interfering with IL-6–driven osteoclastogenesis, it has been shown to reduce osteoclast numbers and RANKL production[12]. Kanbe et al. (2012) further proposed that TCZ may upregulate OPG expression, thereby inhibiting RANKL/RANK signaling and protecting against inflammation-induced bone resorption in RA[13].

The impact of TCZ on the periodontal tissues of patients suffering from rheumatoid arthritis and periodontitis has already been studied. Systemic administration of TCZ has been linked to improvements in gingival index scores, reductions in bleeding on probing and periodontal pocket depth, and gains in clinical periodontal attachment[14,15].

Despite the promising results observed with TCZ, its impact on periodontal disease not linked to any systemic condition is still poorly understood[16]. Moreover, clarifying the direct effects and mechanisms of IL-6 receptor inhibition on periodontal inflammation is still necessary. The present study aimed to evaluate the effect of locally administered TCZ in a preventive experimental model of periodontitis in rats, designed to explore its early modulatory effects on periodontal inflammation. This approach helps to expand our understanding of the mechanisms underlying periodontitis pathophysiology and the potential role of IL-6 receptor inhibition in its modulation.

Materials and methods

Ethics statement

The research was submitted and approved by the Ethics Committee on Teaching and Research in Animals (CEUA) of the Federal University of Rio Grande do Norte (UFRN) (certificate nº 238.004/2021). The authors followed the ARRIVE (Animal Research: Reporting of in vivo Experiments) guidelines, and all methods were performed in accordance with the relevant guidelines and regulations.

Animals

Fifty 60-days-old male Wistar rats (*Rattus norvergicus*), weighing 180–200 g, obtained from the animal facilities of the Biosciences Center (UFRN) were kept at the bioterium of the Department of Biophysics and Pharmacology in environmental conditions of humidity (45–55%) and temperature (22 °C ± 2 °C) controlled with a standard light/dark cycle of 12/12 hours, water and feed for laboratory animals being provided *ad libitum*. The number of animals per group was determined according to the sample size formula n = DF/k + 1, which can be used for three common ANOVA designs applicable to animal studies, where k = number of groups, n = number of subjects per group, and DF = degrees of freedom[17]. Considering ethical implications that recommend the sample size refinement, we chose to use the minimum number of animals (5 animals for each analysis) to carry out experiments.

Periodontitis induction protocol

The animals were anesthetized through intraperitoneal (i.p) injection using 10% Ketamine Hydrochloride (Vetnil, São Paulo, Brazil) (80 mg/kg) and 2% Xylazine Hydrochloride (Calmium, São Paulo, Brazil) (10 mg/kg). Following a previously described method, periodontitis was induced by placing a sterile nylon 3.0 suture thread (Polysuture, NP45330, São Paulo, Brazil) in the left maxillary second molar's cervical region[18]. The same operator consistently performed this procedure for all animals involved in the study. The animals were euthanized with sodium thiopental (Thiopentax®, Cristália, São Paulo, Brazil) (150 mg/kg) on the 11th day following the induction of experimental periodontitis. All animals undergoing periodontitis induction retained their ligatures, ensuring no exclusion from the study.

Experimental groups

The animals were allocated randomly into three experimental groups, including two control groups, using a simple randomization sequence: (1) A control group not subjected to experimental periodontitis, which

received a saline solution (Healthy; $n = 10$); (2) A control group subjected to experimental periodontitis, which also received a saline solution (EP; $n = 20$); (3) A group subjected to experimental periodontitis and treated with Tocilizumab (Acterma®, Roche) at a dosage of 2 mg/kg (EP + TCZ; $n = 20$). The drug was administered through a 20 µL injection using a Hamilton Microliter™ syringe into each lingual papilla surrounding the left maxillary second molar, 3 days before periodontitis induction.

Local administration was employed to better characterize the effects of TCZ on the periodontium and to model potential local delivery in humans. The 2 mg/kg dosage was selected based on the lowest systemic dose reported to be effective in inhibiting alveolar bone loss in rats with periodontitis[16], and a single dose was administered, considering TCZ's prolonged half-life of up to 13 days (which is sufficient to maintain pharmacological activity throughout the critical period of disease initiation)[19]. This overall preventive experimental model was specifically designed to explore its early modulatory effects on periodontal inflammation and to expand our understanding of the mechanisms underlying periodontitis pathophysiology and the potential role of IL-6 receptor inhibition in its modulation.

The saline solution was administered similarly for the healthy and EP groups. The same operator performed both the periodontitis induction and the injections for all animals. A researcher not involved in the experimental procedures was informed about the group allocations at various experiment stages. Throughout the study, no animals were lost.

Biochemical analysis of blood serum levels for AST (aspartate aminotransferase), urea, and creatinine

On the 11th day after the induction of experimental periodontitis, 5 mL blood samples were collected through cardiac puncture in all animals anesthetized with an intraperitoneal (i.p.) injection of 10% Ketamine Hydrochloride and 2% Xylazine Hydrochloride. The blood was centrifuged at 2500 rpm for 15 min to separate the serum. Aspartate aminotransferase (AST) serum concentration was evaluated as liver function, while urea and creatinine were used as renal function markers. Specific kits were used (LABTEST®, Vista Alegre, Lagoa Santa, MG, Brazil), and the methodology followed the manufacturer's instructions. The results were expressed as mg/dL.

Alveolar bone loss

Immediately following euthanasia, the maxillae were harvested for the assessment of alveolar bone loss using Radiographic Micro-computed Tomography (µCT). Non-demineralized specimens ($n = 5$/group) were digitized by a micro-computed tomography (µCT, micro-CT) system (Model 1172; SkyScan, Bruker, Kontich, Belgium) with a resolution of 20 micrometers. The X-ray generator was operated with an acceleration potential of 80 kV, a current of 124µA, and an exposure time of 883 ms per projection. Images were produced with a voxel size of 6 × 6 × 6 µm. Micro-CT files were converted into DICOM files (Digital Imaging and Communications in Medicine) and imported into Dolphin software (Dolphin software - Imaging & Management Solutions) for linear bone level analysis and CT-Analyser software (V.1.16 Bruker, Billerica, MA) for volumetric analysis. These analyses were performed as described by Silva et al.[20].

For linear analysis, the middle of the crown was identified in the axial plane. Linear measurements, in millimeters, from the cementoenamel junction (CEJ) to the alveolar crest (AC) were recorded in the sagittal plane. Five measurements were taken at the mesial, distal, buccal, and palatal surfaces, as well as in the furcation region, where the distance (mm) was measured from the furcation roof to AC. The five linear measurements obtained in each animal were summed to express the value of the alveolar bone level, and a single-blinded and calibrated examiner guided the images and performed the analyses[20].

Volumetric measurements were performed by manually delineating the region of interest (ROI), encompassing the entire internal and interradicular circumscribed bone area at the furcation region of the four roots, excluding the periodontal ligament space[20]. Then, the software CT-Analyser (Bruker, Kontich, Belgium) was used to determine the following parameters: percentage of bone volume divided by the tissue volume (BV/TV%), and number and separation of trabeculae.

Histopathological analysis

Histopathological analysis was performed according to Martins et al.[21]. First, the maxillae were excised, fixed in 10% neutral-buffered formalin, and demineralized in EDTA 10% for three months. The specimens ($n = 5$/group) were then dehydrated, embedded in paraffin, sectioned in the sagittal plane along the molars, and subsequently stained with hematoxylin and eosin. Sections of 4 µm, corresponding to the area between the first and second molars were evaluated under conventional light and florescence microscopy using a 0 to 3 score grade[20,22], as follows: 0- absence or discrete cellular infiltration (inflammatory cell infiltration is space and restricted to the region of the marginal gingival) and preserved alveolar process and cementum; 1—moderate cellular infiltration (inflammatory cellular infiltration present all over the insert gingival), some but discrete alveolar process resorption and intact cementum; 2—Accentuated cellular infiltration (inflammatory cellular infiltration present in gingival and periodontal ligament), moderate degradation of the alveolar process and partial destruction of cementum; 3—Accentuated cellular infiltrate, complete resorption of the alveolar process and severe destruction of cementum.

Immunohistochemical analysis of RANKL and OPG

Immunohistochemical analysis for RANKL and OPG was performed on 4 µm-thick formalin-fixed, paraffin-embedded tissue sections (derived from the same blocks used for the previous histopathological analysis), mounted on poly-L-lysine-coated microscope slides ($n = 3$). The sections were deparaffinized and rehydrated through xylene and graded alcohols, followed by antigen retrieval using Proteinase K (Thermo Fisher®, Waltham,

MA, USA). Endogenous peroxidase was then blocked (30 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4 °C) with primary polyclonal rabbit anti-RANKL antibody (1:400) or primary polyclonal rabbit anti-OPG (1:400). Negative control sections were processed simultaneously as described above but with the first antibody replaced by 5% PBS–BSA. For visualization, the HiDef Detection™ HRP Polymer System (Cell Marque, Rocklin, CA, USA) was used, and slides were then counterstained with hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped[21].

Semiquantitative RANKL and OPG expression analyses carried out on five standardized fields per slide in areas surrounding the left second molar at 400x magnification. The analyses were categorized into scores based on immunostaining intensity, with 1 indicating absence, 2 representing weak, 3 denoting moderate, and 4 signifying intense staining. Histological sections underwent evaluation by two pathologists who were blinded and calibrated to ensure objectivity and consistency in the assessment.

IL-1β, IL-6 and TNF-α gingival levels

Gingival tissue samples (n = 5/group) restricted to the maxillary molar region were collected on the 11th day and immediately frozen at -80 °C for preservation. The samples' IL-1β, IL-6, and TNF-α concentrations were quantified using an enzyme-linked immunosorbent assay (ELISA), as previously described[23]. IL-1β, IL-6, and TNF-α levels in the gingival tissue were determined using standard curves. The results are expressed as dg/ml of IL-1β, IL-6, and TNF-α.

Superoxide dismutase 2 and GPX gene expression

Expression of mRNA for Superoxide dismutase 2 (SOD) and GPX was determined by quantitative real-time polymerase chain reaction (qPCR). RNA was extracted from gingival tissue (n = 5/group) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the RNA was analyzed by 260/280 ratio, and quantified by UV absorption using NanoDrop (Thermo scientific, Wilmington, DE, USA). One microgram of total RNA from the gingival samples was transcribed into cDNA in a reaction mixture (High-Capacity cDNA Reverse Transcription Kit, Foster City, CA, USA). The final solution was incubated at 25 °C for 10 min, 37ªC for 120 min, 85 °C for 5 min, and 4 °C ∞. The cDNA was stored at − 80 °C until further use. qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems™), as described in the manufacturer's instructions. The primers were designed for the genes Superoxide dismutase 2- SOD (forward 5'- TGGACAAACCTGAGCCCTAAG-3' and reverse 5'-CCCAAAGTCACGCTTGATAGC-3) and glutathione peroxidase 1- GPx-1 (forward 5'-CCACCGTGTATGCCTTCTCC-3' and reverse 5'- AGAGAGAC GCGACATTCTCAAT-3'). The relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method[19] with β-actin as the housekeeping gene.

Statistical analysis

The animals were considered the study unit for statistical analysis. The results were submitted to descriptive and inferential statistics in the software Prism 8.1 program (GraphPad, La Jolla, CA, USA). Data were analyzed for normality distribution (Shapiro-Wilk test), and ANOVA followed by Bonferroni's test or Kruskal–Wallis followed by Dunn's post-test was applied accordingly. Such statistical tests were performed using a margin of error of 5.0%.

Results

Systemic effects of tocilizumab

Biochemical analysis of the animals' blood indicated that local administration of TCZ did not result in changes in serum levels of AST, urea, and creatinine that exceeded the reference values' lower and upper limits (Table 1).

Linear and volumetric assessment of alveolar bone

Micro-CT analysis revealed pronounced alveolar bone resorption on both the buccal and lingual surfaces in the EP group, compared to the animals not exposed to periodontitis (Healthy), where no significant bone loss was observed. Specifically, root furcation exposure was evident in the EP group (Fig. 1). The distance from the alveolar bone crest to the cementoenamel junction significantly increased in this group of animals compared to the healthy group (Fig. 2a). These observations were further corroborated by two-dimensional X-ray (Fig. 1). Additionally, the EP group experienced a significant bone volume to total volume ratio (BV/TV) reduction, as shown in Fig. 2b. Periodontitis led to a marked decrease in trabecular number (Fig. 2c) and a substantial increase in trabecular separation (Fig. 2d) in comparison to the corresponding measurements from animals not exposed to periodontitis (Healthy group). These findings confirmed the successful establishment of experimental periodontitis in the present study.

Group	AST	Urea	Creatinine
Healthy	87,67 ± 7,84	51,67 ± 5,502	1,067 ± 0,08165
EP	102,8 ± 20,23	54,17 ± 6,940	0,9333 ± 0,08165
EP + TCZ	80,75 ± 6,882	37,50 ± 8,751	0,4025 ± 0,04166

Table 1. Serum dosage of TGO/AST, urea and creatinine expressed as mean ± SD. Standard values: AST 61–210 U/L; Urea 26–58 mg/dL; Creatinine 0,2–1,2 mg/dL.

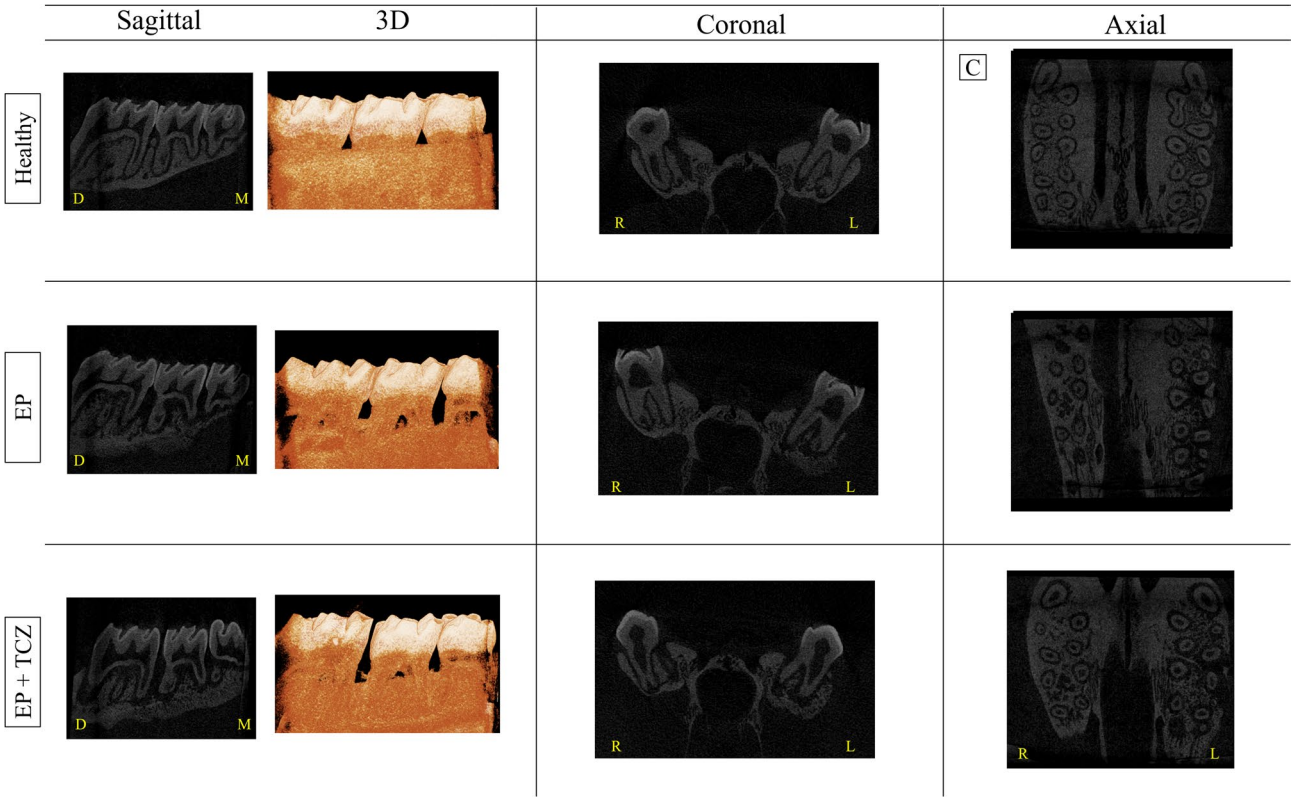

Fig. 1. Representative 2D and 3D reconstructions of the microtomographic sections, and representative microtomographic axial sections showing bone differences in the furcation area at the left maxillary second molars (2 M) for all groups. Healthy, healthy group; EP, experimental periodontitis; EP + TCZ, experimental periodontitis + Tocilizumab.

Local administration of TCZ markedly reduced alveolar bone loss, as illustrated in Figs. 1 and 2, compared to the EP group. The administration of TCZ attenuated the linear bone loss associated with periodontitis ($p < 0.05$) (Fig. 2a). Moreover, a significant increase in trabecular number was observed in the TCZ group compared to the EP group (Fig. 2c). However, no significant differences were noted between these groups in terms of BV/TV (Fig. 2b) or trabecular separation (Fig. 2d).

Histopathological analysis

Histopathological analysis showed significant alveolar bone and cementum resorption, accompanied by considerable inflammatory cell infiltration ($p < 0.001$) in the EP group, receiving a median score and range of 2 (2–3) (Fig. 3c, d), in contrast to the periodontium of the healthy group, which maintained intact structures such as the periodontal ligament, cement, and alveolar bone, receiving a median score and range of 0 (0–1) (Fig. 3a, b). TCZ administration reduced the infiltration of inflammatory cells and partially preserved the cementum and the alveolar process (Fig. 3e, f), receiving a median score and range of 1 (0–2) ($p < 0.05$).

Gingival tissue cytokine levels

A significant increase in IL-1β (Fig. 4a), TNF-α (Fig. 4b), and IL-6 (Fig. 4c) concentrations was observed in the gingival tissue of animals subjected to experimental periodontitis and treated with saline solution (EP group), compared to the healthy group. TCZ significantly reduced the levels of TNF-α (Fig. 4b) and IL-6 (Fig. 4c), but not the concentration of IL-1β, when compared to the EP group.

RT-qPCR analysis of SOD-2 and Gpx-1

The EP group exhibited a significant increase in GPX and SOD gene expression (Fig. 5a and b, respectively) compared to the healthy control group. TCZ treatment significantly reduced GPX and SOD gene expression compared to the EP group (Fig. 5).

RANK, RANKL and OPG immunohistochemistry

The immunostaining of RANKL decreased in the EP + TCZ group compared to healthy and EP groups ($p < 0.001$; Fig. 6a–d). No differences among groups were found for OPG expression (Fig. 6e-h).

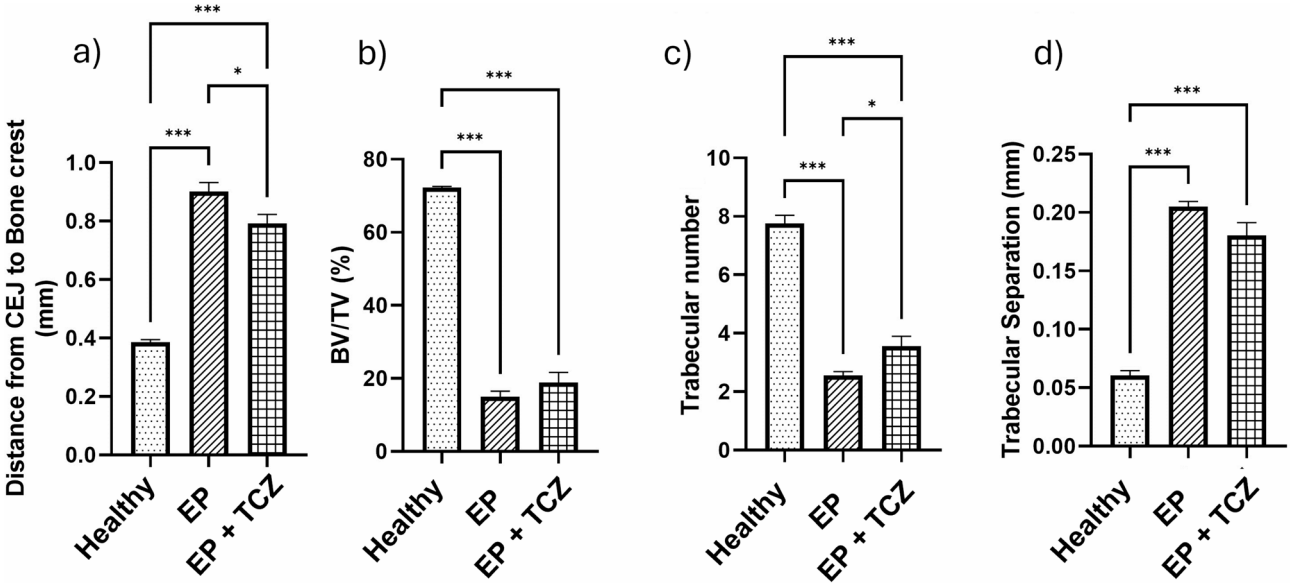

Fig. 2. (**a**) Graph represents the means of alveolar bone level (mm) equivalent to the sum of the distances between cementoenamel junction (CEJ) to the alveolar crest (AC) at the mesial, distal, buccal and palatal surfaces and the distances between the furcation roof and AC of the maxillary left second molars in each group. (**b**) Graph represents the means of BV/TV percentage at the area under the furcation in the maxillary left second molars. (**c**) Graph represents the means of trabecular number under the furcation in the maxillary left second molars. (**d**) Graph represents the means of trabecular separation in the area under the furcation in the maxillary left second molars. $^{*}p < 0.05$ $^{***}p < 0.001$ (ANOVA followed by the Bonferroni's test). Healthy, healthy group; EP, experimental periodontitis; EP + TCZ, experimental periodontitis + Tocilizumab.

Discussion

This study represents a novel investigation into the effects of locally administered Tocilizumab (TCZ), a recombinant humanized monoclonal IgG1 antibody targeting the interleukin-6 receptor, on the periodontal tissues of animals with induced periodontitis. Treatment with TCZ resulted in a reduction of alveolar bone loss typically associated with periodontitis. This positive effect correlates with decreased levels of TNF-α and IL-6 in the gingiva and a significant increase in the expression of GPX and SOD genes within the gingival tissues.

Tocilizumab, a drug widely used in treating rheumatoid arthritis and, more recently, identified as a promising treatment for COVID-19[24], functions through a unique mechanism. It selectively and competitively binds to both soluble (sIL-6R) and membrane-bound (mIL-6) IL-6 receptors, thereby preventing the dimerization of glycoprotein 130 molecules on the cell membrane. This effectively blocks the transmission of IL-6 signals to cells[25]. This inhibition of IL-6 signaling offers potential therapeutic benefits in treating periodontitis. In this condition, the integrity of the epithelial barrier is compromised due to bacterial toxins, leading to the recruitment of neutrophils and lymphocytes to the infection site. This response triggers the production of immunoregulatory and pro-inflammatory mediators, such as TNF-α, IL-1β, and IL-6[7,26]. IL-6 is critical in developing periodontitis, primarily by facilitating osteoclast differentiation and promoting bone resorption[27].

Recent literature has highlighted the beneficial effects of TCZ therapy on periodontal inflammation in patients with rheumatoid arthritis[14,15]. It has been demonstrated that short-term administration of TCZ significantly reduces gingival and periodontal inflammation. This improvement is evidenced by lower gingival index scores, reduced bleeding upon probing, and decreased probing pocket depths, reflecting concurrent improvements in joint health[14]. These results support an earlier clinical study that has similarly identified the efficacy of TCZ therapy in mitigating periodontal inflammation in patients diagnosed with rheumatoid arthritis and periodontitis[15]. Additionally, recent genetic research has revealed that the downregulation of IL-6 signaling correlates with a lower incidence of periodontitis, offering a potential molecular mechanism for the observed clinical benefits[28].

The beneficial effect of systemically administered TCZ on experimental periodontitis in rats, specifically in the absence of systemic disorders, has been documented[16]. TCZ was found to prevent alveolar bone resorption and loss of attachment, underscoring the promise of modulatory therapy as an effective approach for managing periodontal disease. Our work significantly contributes to this field by investigating, for the first time, the local administration of TCZ on experimental periodontitis. Additionally, our findings indicate that TCZ does not affect hepatic and renal function markers, including AST, urea, and creatinine. In contrast to systemic administration[29,30], the local application maintains average plasma concentrations of these markers, indicating a lack of adverse effects on the liver and kidneys. The local administration potentially enhances the direct delivery of active metabolites to periodontal tissues. Therefore, the lack of observed systemic side effects over the 14-day experimental period suggests the potential safety of using TCZ locally in an in vivo model.

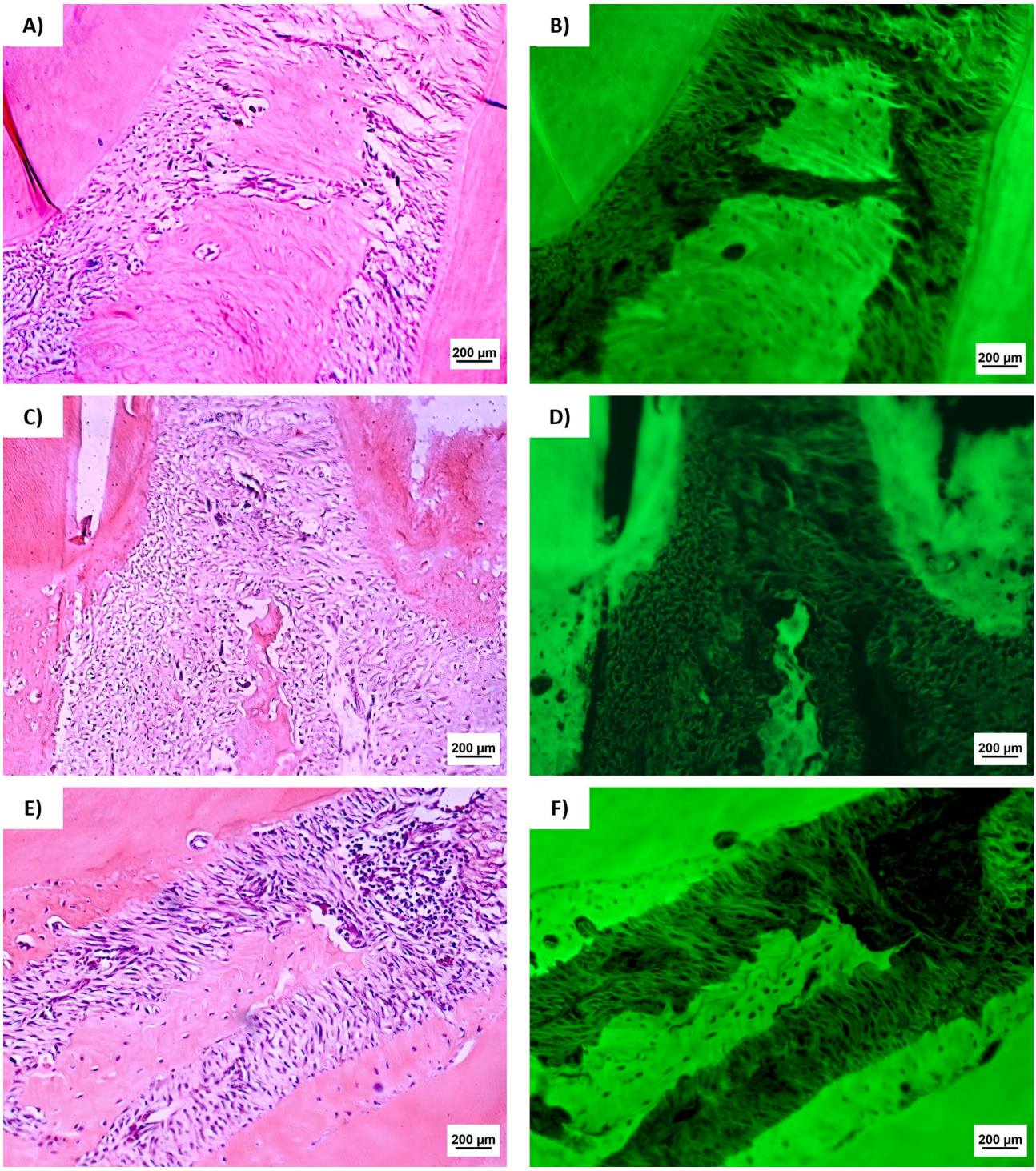

Fig. 3. Histological analysis (H&E). Photomicrographs under conventional light and fluorescence microscopy showing histological sections at the periodontal tissues in the interproximal regions of the maxillary second molars for (**a**) and (**b**) healthy group; (**c**) and (**d**) experimental periodontitis group; (**e**) and (**f**) experimental periodontitis + Tocilizumab group. Staining: Hematoxylin and Eosin. Magnification: 400X.

Among the clinical manifestations of periodontitis, alveolar bone loss is a significant concern and constitutes one of the main challenges in this pathological condition. Our results indicate that TCZ can attenuate linear bone loss and significantly decrease the number of bone trabeculae in animals submitted to experimental periodontitis. The prevailing theory regarding how inflammation leads to bone damage centers on disrupting bone remodeling balance[6]. IL-6 and its soluble receptor negatively impact osteoblastic differentiation[31]. Furthermore, IL-6 seems to stimulate RANKL secretion by osteoblasts, indirectly facilitating osteoclastogenesis[32]. Supporting these studies,

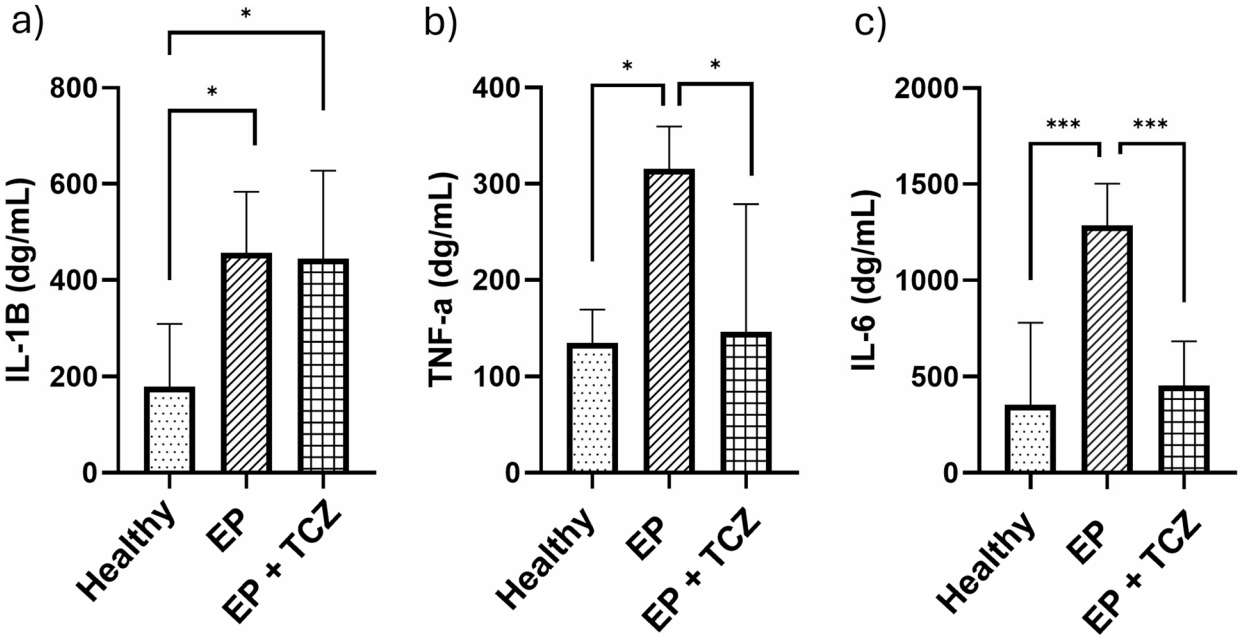

Fig. 4. Graph represents the means of interleukins (**a**) IL-1β; (**b**) TNF-α and (**c**) IL-6 levels measured in gingival tissue samples. $^{*}p < 0.05$; $^{***}p < 0.001$. (ANOVA followed by the Bonferroni's test). Healthy, healthy group; EP, experimental periodontitis; EP + TCZ, experimental periodontitis + Tocilizumab.

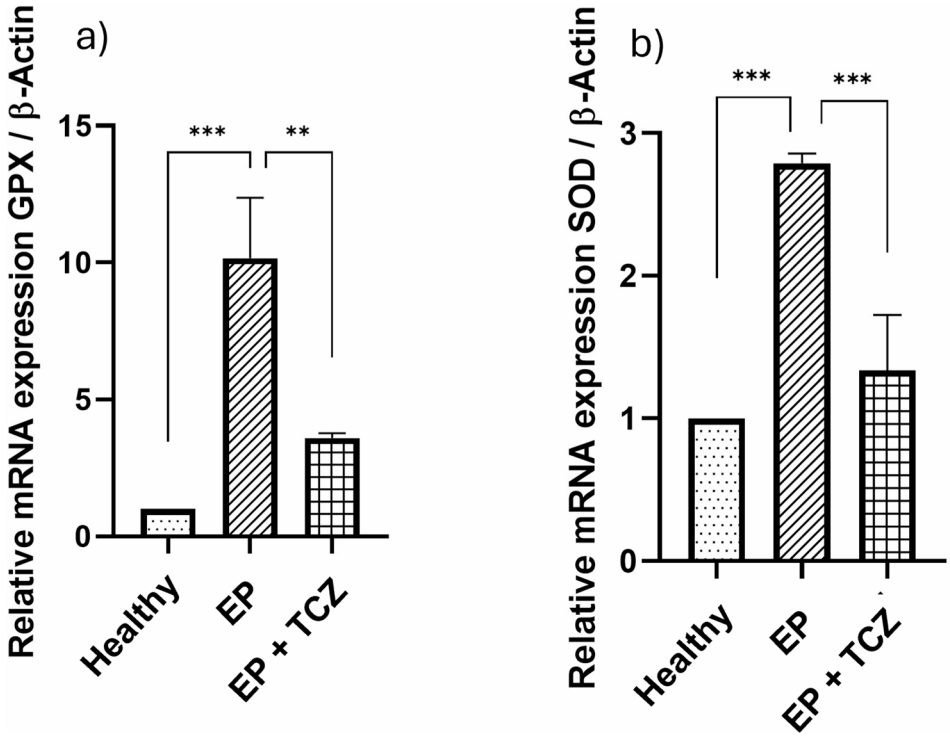

Fig. 5. Graph represents the medians of mRNA expression of (**a**) GPx-1; (**b**) SOD-2. $^{***}p < 0.001$. (Kruskall-Wallis test followed by Dunn's test). Healthy, healthy group; EP, experimental periodontitis; EP + TCZ, experimental periodontitis + Tocilizumab.

our immunohistochemistry analyses have revealed a reduction in RANKL expression in animals subjected to periodontitis and treated with TCZ compared to those in the untreated group. This underscores the impact of IL-6 on bone metabolism, through which TCZ may exert its advantageous effects in treating periodontitis.

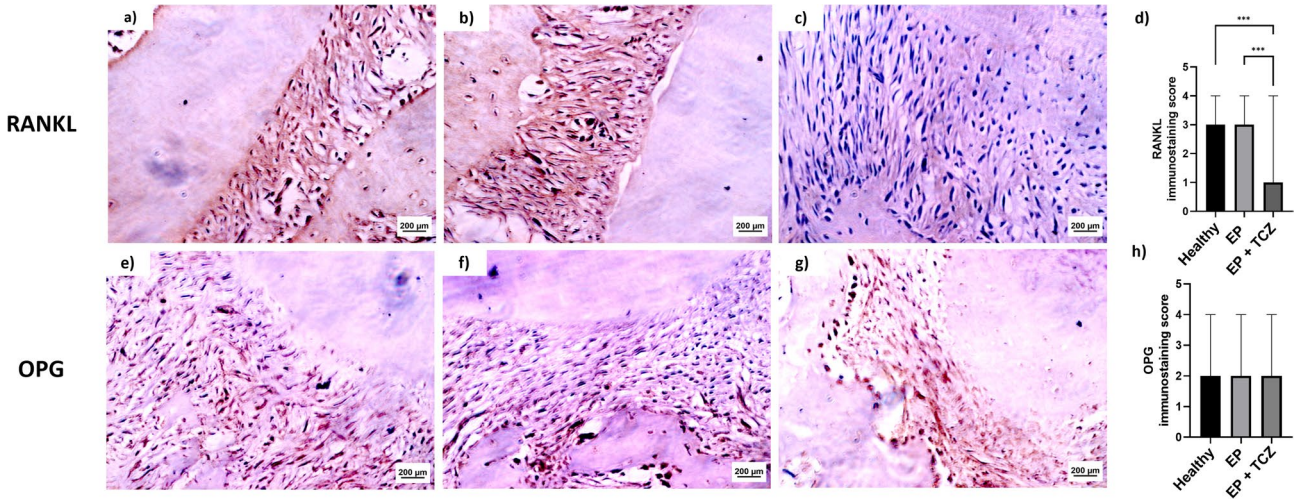

Fig. 6. Immunohistochemical analysis. Photomicrographs of periodontal tissue in the interproximal regions of the maxillary second molars showing immunoreactivity to RANKL (**a**) healthy group; (**b**) experimental periodontitis group; (**c**) experimental periodontitis + Tocilizumab group; and OPG (**e**) healthy group; (**f**) experimental periodontitis group; (**g**) experimental periodontitis + Tocilizumab group. Graphs represent the medians of immunohistochemical scores for (**d**) RANKL and (**h**) OPG. ***$p < 0.001$. (Kruskall-Wallis test followed by Dunn's test). Healthy, healthy group; EP, experimental periodontitis; EP + TCZ, experimental periodontitis + Tocilizumab. Magnification: 630X. Bar = 200 µm.

On the other hand, micro-CT analysis revealed no significant differences in bone volume fraction (BV/TV) or trabecular separation between the periodontitis groups treated or not with TCZ. This discrepancy can be explained biologically, as ligature-induced periodontitis causes localized bone loss at the furcation and alveolar crest, which is more accurately captured by linear measurements. At the same time, volumetric parameters reflect the overall cancellous structure and may be less sensitive to changes. Methodological factors, including the ROI in the maxillary second molar region, irregular trabecular patterns, segmentation thresholds, and partial volume effects, may further limit the detection of subtle changes. Overall, TCZ preserved alveolar crest integrity and local trabecular number without significantly affecting overall bone volume fraction or trabecular structure within the ROI.

Our research further revealed that animals with periodontitis treated with TCZ showed decreased levels of IL-6 and TNF in their gingival tissues compared to those in the untreated group. Interestingly, this decrease was not observed in the levels of IL-1. Our investigation targets gingival tissues, considering that periodontitis originates from a long-term inflammatory response in the gingiva that, without intervention, can lead to the degradation of the periodontal, culminating in periodontitis.

IL-6, TNF-α, and IL-1β are recognized as key pro-inflammatory cytokines intricately linked to the inflammatory response observed in patients with periodontal disease[33,34] as well as in rodents subjected to experimental periodontitis[35]. Aligned with our findings, a clinical trial focusing on TCZ treatment for rheumatoid arthritis patients also observed decreased serum TNF levels[15]. In an in vitro model of antibody-dependent cellular cytotoxicity, TCZ reduced TNF-α production in monocytes, suggesting that the IL-6/IL-6R pathway may play an essential role in antibody-mediated activation of monocytes[36].

The reduction in IL-6 levels observed in our study may seem unexpected, given that TCZ works by competitively binding to the IL-6 receptor. Normally, this mechanism would be expected to decrease IL-6 clearance, which could increase its concentration within the system. This is because serum IL-6 levels are determined by the balance between the production of IL-6 and its clearance[15]. However, we interpret this decrease as a sign of reduced inflammation in the TCZ-treated group. In the context of periodontitis, the generation of inflammatory cytokines is crucial to the disease's pathophysiology. IL-6, in particular, is a prominent player, acting as a significant mediator that influences the disease's course by modulating the activity of various immune cells in the periodontal tissue. Neutralizing IL-6 by inhibiting its binding to its receptor may result in a reduced release of inflammatory cytokines, including IL-6, by the various cells involved in the pathogenesis of periodontitis. Our results align with a previous study examining the impact of systemic TCZ administration in animals with periodontitis. That study reported reduced IL-6 relative expression in gingival samples from animals treated with the exact TCZ dosage we used in the present study[16].

Despite the anti-inflammatory benefits of TCZ treatment identified in our study, IL-1β levels remained elevated in the gingival tissue of treated animals. This persistence of IL-1β levels may be explained by the fact that its synthesis is primarily regulated through inflammasome activation and Toll-like receptor signaling, rather than IL-6–dependent pathways[37]. Thus, IL-6 receptor blockade by TCZ would not directly suppress IL-1β production, allowing its levels to remain elevated despite the overall anti-inflammatory effect. Consistent with our findings, clinical studies involving patients with rheumatoid arthritis[38] or Still's disease[39] also reported no significant alterations in IL-1β levels following TCZ treatment. Moreover, although IL-1 β can facilitate

osteoclastogenesis and increase the expression of matrix metalloproteinases contributing to the degradation of connective tissue[40], the elevated IL-1 levels in TCZ-treated animals might be neutralized by decreased levels of IL-6 and TNF. This observation is supported by our finding of a more preserved alveolar bone in the TCZ-treated group compared to the untreated group.

The efficacy of TCZ in controlling inflammation associated with experimental periodontitis is highlighted by the observed decrease in the Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD) gene expression in the gingival tissue of the TCZ-treated group compared to the untreated group. GPx and SOD are key enzymes in defending against oxidative damage. Their decreased expression indicates a potential reduction in the body's need to counteract oxidative stress. Indeed, oxidative stress, marked by an imbalance between the production of reactive oxygen species (ROS) and a deficit of antioxidant enzymes, is identified as a key pathophysiological mechanism behind tissue damage in periodontal disease[33]. This is due to the ability of ROS, in high concentrations and prolonged exposure, to damage a wide range of biological molecules, such as lipids, proteins, enzymes, and DNA[34,36]. Gaber et al. (2016), in their in vitro study, explored the impact of TCZ on human neutrophils and found that it leads to a dose-dependent decrease in ROS levels within these defense cells. This effect is more pronounced under hypoxic than normoxic conditions[41]. This finding supports our results and contributes to understanding TCZ's role in modulating oxidative stress in periodontal disease.

While the results we presented are promising, it is essential to acknowledge our work's limitations. This study employed a preventive experimental model, in which TCZ was administered locally before periodontitis induction. Although this approach allows for the evaluation of early modulatory effects on periodontal inflammation, it may not fully represent clinical scenarios where patients already present with established disease. However, this methodological choice is justified by the clinical relevance of TCZ, as systemic administration of this medication, clinically utilized to treat rheumatoid arthritis (RA), has previously been associated with improvements in periodontal inflammation in patients with RA and periodontitis[14,15]. Investigating its potential role in preventing or mitigating the early stages of destruction is thus a necessary step in translational research.

In view of this, the timing was specifically selected to ensure the presence of TCZ in the periodontal tissue at the onset of inflammation. This strategy enabled us to precisely define how blocking the IL-6 receptor modulates the initial mechanisms leading to tissue destruction. The overarching goal was to expand our understanding of the mechanisms underlying the pathophysiology of periodontitis. It becomes crucial, however, to conduct further preclinical and clinical studies to evaluate the impact of TCZ therapy initiated after the onset of periodontitis.

Furthermore, focusing on the translational implications of this study, it is worth noting that the high cost of the drug and its potential adverse effects may limit its clinical applicability in periodontics. Nevertheless, TCZ is considered a safe medication[25], and such effects could be minimized through local administration (e.g., intrapapillary injection) or pharmaceutical technologies that concentrate and enhance its local action, such as controlled drug delivery systems, while reducing systemic exposure. It should also be emphasized that, when compared with other host-modulating agents explored for periodontal therapy – such as doxycycline and statins – TCZ presents a distinct mechanism by specifically blocking the IL-6 receptor pathway, which may allow for more targeted inflammatory control. Further investigations are needed to optimize delivery strategies and to assess their efficacy compared to other modulatory approaches. Such research could deepen our understanding of TCZ's benefits for individuals with periodontal disease, enhance the predictability of its effects, and facilitate its targeted clinical use.

In conclusion, the selective interleukin-6 receptor inhibitor administered locally in the oral cavity demonstrated a protective effect in experimental periodontitis. These findings suggest potential clinical implications, particularly for the management of severe or refractory cases of periodontitis. Future research may focus on its therapeutic use or explore its combination with other treatment modalities to enhance clinical outcomes.

Data availability

The experimental data reported in this work is available upon reasonable request by e-mail to the corresponding author.

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Author contributions

All authors have made substantial contributions to conception and design of the study. A.A. M., M.L.S. L., H.E. S.C., S.B.R., D.N.A.S., R.S., V.B.G., R.F.A.J., G.H.A.V. and A.A.A. have been involved in data collection and data analysis. A.A. M., R.F. C.L., C.A.C.X.M., F.Q.P., G.A.C.B., R.D.A.U.L. and A.A.A. have been involved in data in-

terpretation. A.A. M., M.L.S. L., H.E. S.C., S.B.R., D.N.A.S., R.S., V.B.G. have been involved in drafting the work, and R.F.C.L., C.A.C.X.M., F.Q.P., G.A.C.B., R.F.A.J., R.D.A.U.L., G.H.A.V. and A.A.A. have revised the work critically. All authors have given final approval of the version to be published.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
The project of this study was submitted and approved by the Ethics Committee on Teaching and Research in Animals (CEUA) of the Federal University of Rio Grande do Norte (protocol 004/2021; certificate nº. 238.004/2021).

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