A Modified Experimental Model of Periodontitis in Rats: Morphological and Cytokine Profile

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Abstract

Periodontitis is a chronic disease with inflammatory damage to the tissues surrounding the tooth and progressive alveolar bone loss, and it remains one of the most common dental diseases. Despite advances in the experimental studies of periodontitis, existing experimental rodent models of this disease remain technically challenging. In this regard, the objective of this study was to develop a modified, simplified model of ligation-induced periodontitis in rats, providing morphological characteristics and a tissue mRNA expression profile of target cytokines.

Based on the identified gross and histopathological findings and changes in the level of relative mRNA expression of genes (TNF-α, IL-1β, IL-10, RANK, and OPG), it was concluded that the proposed modified model of periodontitis in rats with a placement of ligature around the frontal groups of teeth with additional fixation of the ligature by suturing the interdental papilla is relevant and can act as an accessible and less labor-intensive alternative to the existing methods of experimental modeling of periodontitis in rodents.

The observed histopathological changes and increased levels of relative mRNA expression of the TNF-α, IL-1β, IL-10, and RANK genes allow us to conclude that key pathogenetic mechanisms of the development of the inflammatory process in periodontal tissues in the experimental model are preserved.

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Introduction

Periodontitis is a chronic disease with inflammatory damage to the tissues surrounding the tooth and progressive alveolar bone loss.[1] The evolution of modern ideas about the etiopathogenesis of periodontitis owes much to the results of preclinical experimental studies, which demonstrated the role of local dysbiosis-induced immune inflammation.[2] At the same time, despite progress in understanding the key mechanisms and patterns of damage to periodontal tissues, periodontitis still remains an extremely common and socially significant disease with a significant burden on the dental care system, continuing to be one of the leading causes of non-traumatic tooth loss throughout the world.[3] Currently available approaches and means of therapy often do not allow achieving the desired results, which makes the search for new methods of adjuvant prevention and therapy of periodontitis, including nutraceuticals, still relevant.[4] An equally urgent task remains to elucidate the key mechanisms of intercellular interaction in the focus of periodontal damage in conditions of dysregulation of the immune response.[5]

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Preclinical animal studies have been and remain the main approaches aimed at solving these scientific problems, provided that appropriate experimental models are available that meet the following criteria, among others: resemblance between animal species and humans in terms of physiological and/or pathophysiological aspects; availability of evaluation during the experimental model; capacity to reproduce the disease or pathology at

the same level as that of humans; reproducibility; simplicity and cost.[6] Several models were proposed as experimental models of periodontitis, including the bacteria oral gavage or inoculation models,[7,8] the bacterial lipopolysaccharide inoculation model,[9-11] as well as ligature-based models with or without bacteria.[12-14] The latter experimental model of periodontitis has recently become widespread due to the fact that disease can be predictably initiated at a known time and significant host inflammatory responses and alveolar bone loss occur in a defined location within a short period of time.[12] The experimental animals used to model periodontitis are non-human primates[15,16], dogs[17], rats[18], and mice[19], as well as other animals, such as rabbits, ferrets, hamsters, etc.[20] However, the use of rodents is preferable, which is associated with the relative simplicity of their breeding and maintenance in standard vivarium conditions, the possibility of using genetically modified lines, as well as the wide availability of various reagents for immunohistochemical, immunofluorescent, and molecular genetic studies. Along with this, when using small laboratory animals (rats and mice), researchers often encounter technical difficulties in inducing experimental periodontitis, related to the tiny size of the oral cavity and teeth in these animals. In this regard, several different modifications of the standard ligation-induced periodontitis model have recently been proposed.[21-24] Despite this, such an experimental model with a ligature placed around one of the molars on the upper or lower jaw still remains technically complex and difficult to implement without specialized equipment such as a stereoscopic microscope.

Considering the above, the objective of this research was to develop a modified and simplified model of ligation-induced periodontitis in rats, providing morphological characteristics and tissue mRNA expression profile of targeted cytokines.

Materials and methods

Animals

Twelve male Wistar rats (Rattus norvegicus) weighing 180–200 g were used, with ad libitum access to food and water. The rats were randomly housed in standard polycarbonate cages in a temperature-controlled environment, maintained at 21–23ºC and 55–60% humidity, with 12 h of light and 12 h of darkness cycle. The rats were randomly (using a randomization table) allocated into two experimental groups: the control group (CG) (n = 6) and the periodontitis group (PG) (n = 6).

Periodontitis induction

After acclimatization of animals, experimental animals from the CG and PG groups were anesthetized with a xylazine (1 mg/mL) solution. In the periodontitis group, the modified protocol for ligature-induced periodontitis was used. Before the procedure, ligatures were soaked in P. gingivalis suspension (10^9 CFU/mL) at controlled room temperature (25 ± 2°C).[25] To perform the ligature-induced damage, using a needle holder and an atraumatic needle, the interdental space in the area of the lower incisors was sutured from the vestibular surface using a 5–0 silk suture material. After this, a suture thread was tied around the lower incisors in an “8” shape, followed by fixation of the thread using surgical knots. The ligature was applied as close as possible to the interdental papilla from the lingual surface. In the control group, animals were subjected to sham surgery with similar suturing of the interdental space using a needle holder and an atraumatic needle but without suture filament. Animals were monitored for the position of the applied ligature and the accumulation of dental plaque every other day. 30 days after the ligation or sham surgery, experimental animals were sacrificed by cervical dislocation under anesthesia, followed by decapitation. Samples of gingival tissue and jaw segments were collected.

Real-Time qPCR

Gingival tissues were homogenized for RNA extraction. Total RNA was extracted using the RNA-Extran Kit (Syntol) following the manufacturer’s instructions. The quantity and purity of the RNA were determined from the absorbance at 260/280 nm using a Nano-500 spectrophotometer (Helicon). 20 ng of total RNA was reverse transcribed into cDNA using the MMLV RT kit (Syntol) in accordance with the manufacturer’s protocol. The CFX 96 TOUCH Real-Time PCR system (Bio-Rad) and real-time PCR kit were used based on the manufacturer’s instructions. Polymerase chain reaction (PCR) thermal cycling included three steps: step 1 at 50°C lasting 2 minutes, step 2 at 95°C lasting 10 minutes, and a final step 3 with 40 cycles at 95°C lasting 15 seconds and at 60°C lasting 1 minute.

The relative mRNA expression levels of IL-1β (interleukin-1 beta), IL-10 (interleukin-10), TNF-α (tumor necrosis factor alpha), RANK (receptor activator of nuclear factor kappa beta), and OPG (osteoprotegerin) (Table 1) were assessed. Fold changes in the gene expression were calculated by the ΔΔCt method, where ΔCT is the difference between the CT value of a target gene and the CT value of the internal control gene. ΔΔCT is the difference between the ΔCT value of a target sample and the ΔCT value of a control sample.[26] The $2^{-\Delta\Delta CT}$ is the fold change of the target gene expression in a target sample relative to a control sample. An analysis of the results of tissue mRNA expression was made by a blinded observer who had no knowledge of the identity of the experimental groups.

mRNA	Forward (F) and Reverse (R) Primer Sequences
β-actin	F: AAGTACCCCATTGAACACGG
	R: ATCACAATGCCAGTGGTACG
IL-1β	F: TTGAGTCTGCACAGTTCCCC
	R: GTCCTGGGGAAGGCATTAGG
IL-10	F: ATCCGGGGTAATAACTGC
	R: TGTCAGCAGTATGTTGTECAGC
TNF-α	F: CCAGGTTCTCTTCAAGGGACAA
	R: CTCCTGGTATGAAATGGCAAATC
RANK	F: GTACCATGATCGAGGCTGGG
	R: GATAGTCCGCAGGTACGCTC
OPG	F: ACACACCAACTGCAGCTCAC
	R: TGTCCACCAGAACACTCAGC

Table 1. Primers used for RT-qPCR.

Histological analysis

Samples of the jaw segment were fixed in neutral buffered 10% formalin for ~24 hours, followed by decalcification in 10% EDTA (changed every 3 to 4 days) for 2 weeks, with subsequent histological processing using the Logos Hybrid Histological Processor (Milestone Medical, Italy) and the Leica EG1150 Modular Tissue Embedding Center (Leica Biosystems, Germany). Histologic sections (~ 4 μm) were obtained from 10% formalin-fixed paraffin-embedded blocks using an automatic rotary microtome Leica RM2255 (Leica Biosystems, Germany) and stained with hematoxylin and eosin (H&E) for microscopic evaluation. Histological slides were scanned using an Aperio CS2 Digital Pathology Slide Scanner (Leica Biosystems, Germany), followed by digital image analysis using the Aperio ImageScope and ImageJ software.[27] Histopathological evaluation was performed by a blinded observer who had no knowledge of the identity of the experimental groups.

Statistical analysis

Statistical analyses of RT-PCR results were performed for each target mRNA, and the data were expressed as the geometric mean ± SEM. Significant differences were determined using the Mann-Whitney U test. Values of $P < 0.05$ were considered to be significant. Variables were analyzed by statistics software (Statistica, StatSoft, v.10.0).

Ethics statements

All procedures were performed under the recommendations of the Guide for the Care and Use of Laboratory Animals[28] and were approved by the local Institutional Ethics Committee (A16-116072810135-5). The experimental part of the study was carried out in specialized premises of the vivarium of the Medical Institute named after S.I. Georgievsky, which are intended for keeping laboratory animals in standard and appropriate conditions to meet the physiological and environmental needs of experimental rats. The morphological part of the study and real-time qPCR analysis were carried out in the Central Research Laboratory of the Medical Institute named after S.I. Georgievsky using certified equipment.

Results

During the experiment, when assessing the condition of the oral cavity and jaw segment of the experimental rats, a 30-day period was chosen as optimal for evaluation of the developed changes. 30 days after the modified procedure of placing a ligature around the lower incisors with suturing of the interdental papilla, pronounced changes in the periodontal tissues were observed in the PG group, in the absence of such in the CG (sham-operated animals). The most significant changes included extensive dental plaque accumulation and atrophic changes in the segment of the alveolar process of the lower jaw (Figure 1).

Histopathological findings

The descriptive histological analysis was performed to confirm the development of periodontitis in a modified ligature-induced periodontitis model. Analysis of the segment of the jaw related to the lower incisors region in CG rats did not reveal any significant changes, with

the presence of normal gingiva, periodontal ligament, tooth structures, and alveolar bone.

The major histopathological changes in PG animals included pronounced inflammatory changes in the gingival lamina propria and periodontal ligament. As observed in Figure 2, the mixed inflammatory infiltrate was represented by a moderate focal or diffuse accumulation of lymphocytes and macrophages with an admixture of neutrophils and plasma cells, along with moderate hemodynamic disturbances with dilation and congestion of the microvasculature.

The periodontal membrane was wider, with signs of active resorption of the underlying alveolar bone. Most of the surface of the alveolar bone tissue was represented by lacunae, with typical multinucleated cells corresponding to osteoclasts.

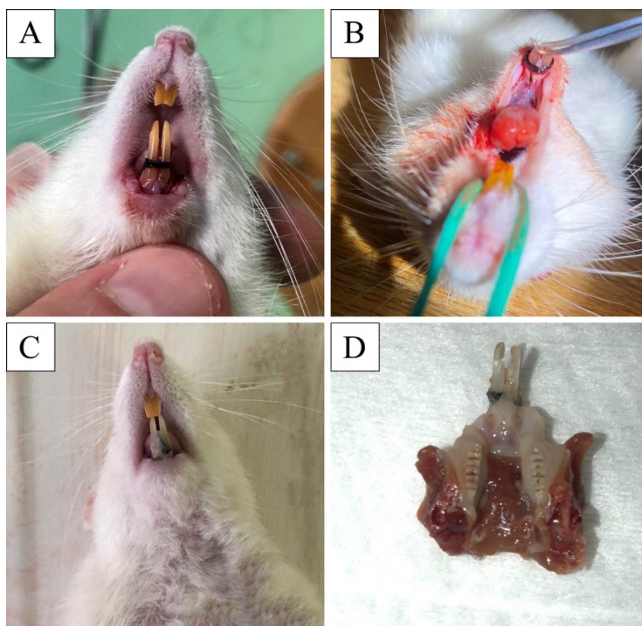

Figure 1. A ligature was placed around the lower incisors with suturing of the interdental papilla (A, B: at the beginning of the experiment, day 0; C, D: at the end of the experiment, day 30). By the 30th day, a significant accumulation of dental plaque was noted in the area of the retention ligature.

Cytokine mRNA tissue expression

After 30 days of the ligature placement, to confirm the inflammatory damage to periodontal tissues, an analysis of the relative mRNA expression of a number of genes in the gingival tissue was carried out. The results obtained regarding the mRNA expression of the TNF-α, IL-1β, IL-10, RANK, and OPG genes were generally consistent with the data obtained from the descriptive histopathological analysis. The levels of relative mRNA expression of the proinflammatory cytokines TNF-α and IL-1β were increased by 3.2 ($p = 0.0008$) and 3.9 ($p < 0.0001$) times, respectively, compared to the control group (Figure 3). The relative mRNA expression of the RANK gene, which regulates the differentiation and activation of osteoclasts with subsequent resorption and remodeling of bone tissue, was also statistically significantly increased by 3.1 times ($p < 0.0001$). Changes in the levels of relative mRNA expression of the gene for the anti-inflammatory cytokine IL-10 and another marker of bone tissue, OPG, tended to increase but did not reach the level of statistical significance ($p = 0.15$ and $p = 0.12$, respectively).

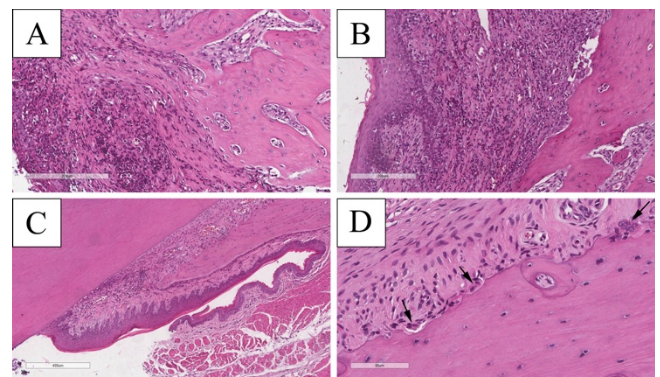

Figure 2. Histopathological periodontal changes 30 days after ligature placement. A, B: Moderate to severe focal and diffuse mixed lymphohistiocytic infiltration in the gingival connective tissue plate (H&E, x400); C: Moderate inflammatory infiltration and expansion of the periodontal ligament zone (H&E, x200); D: extensive alveolar bone lacunar resorption by osteoclasts (arrows) (H&E, x600).

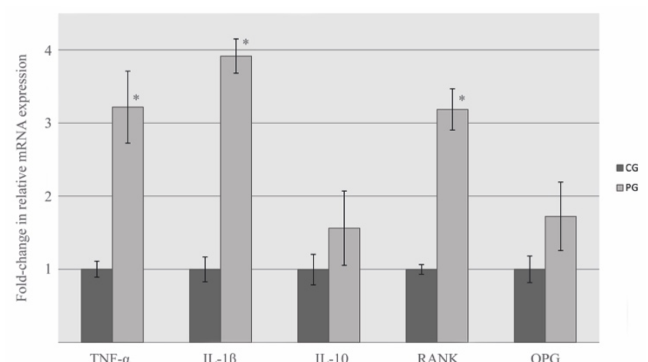

Figure 3. The bars represent geometric mean fold changes in relative mRNA expression of TNF-α, IL-1β, IL-10, RANK, and OPG, and the

vertical lines represent the standard errors of the mean fold change in each experimental group. mRNA expression was normalized to the expression of the housekeeping β-actin gene. Note: * Statistically significant differences ($p < 0.05$) compared to the control group.

Discussion

The identified gross and histopathological findings and changes in the mRNA expression levels of target cytokines, including bone-specific cytokines (RANK and OPG), suggest that the presented modified procedure with ligation around the lower incisors with suturing of the interdental papilla can serve as a simplified, reproducible, and relevant model of periodontitis in rodents.

Previously, attempts have been made to simplify the modeling of periodontitis by placing a ligature around the frontal teeth in rodents.[29-32] However, the obtained results remained contradictory: some studies demonstrated the development of inflammation in periodontal tissues, while other studies showed that this approach is not very effective in terms of inducing inflammatory damage and resorption of the alveolar bone.[33] The main reason for the ineffectiveness of the traditional incisor ligature approach is that these teeth in rodents continue to grow throughout life, gradually wearing down[34], which makes it impossible to securely fix the suture in close proximity to the gingiva. Another factor is the anatomical features of the incisors, which do not have tubercles. This predisposes the ligature to slip. As a result, the lack of reliable fixation of the ligature near the interdental papilla does not allow for the accumulation of bacteria and plaque formation in direct contact with periodontal tissues.

In the presented modified model, after tying the lower incisors with a ligature, the suture thread was fixed by suturing the interdental papilla, which avoids its slipping or displacement during chewing movements or physiological tooth growth. Periodontitis was confirmed by the identified characteristic histopathological changes, including the presence of a mixed focal or diffuse inflammatory infiltrate in the gingival connective tissue, as well as typical signs of destruction of the alveolar bone adjacent to the periodontal ligament with active lacunar resorption due to osteoclasts. The observed changes are consistent with those demonstrated in other studies using traditional approaches to modeling periodontitis, both using bacterial inoculation[9] and ligation around maxillary or mandibular molars.[14] Similarly, the level of tissue mRNA expression of cytokine genes that are actively involved in the inflammatory response (IL-1β, IL-10, and TNF-α), as well as bone-specific cytokines (RANK and OPG), also indicates the relevance of the developed modified method. Currently, the TNF-α/NF-κB-mediated signaling mechanism is considered one of the main ones in the development and further progression of periodontitis.[35,36] This mechanism triggers a deregulatory immune response with a characteristic predominance of pro-inflammatory cytokines (including IL-1β and IL-6)[37], as well as enhanced differentiation and activation of osteoclasts with progressive alveolar bone loss through increased expression of the RANK/RANKL signaling system.[38,39] Similar changes in the expression of these cytokines were demonstrated in other experimental periodontal studies.[5,9,14,22,33] Thus, the presented modified model of periodontitis reflects the key pathogenetic mechanisms of the development of the inflammatory process in periodontal tissues and can be used to study the cellular and molecular genetic features of the disease, including from the standpoint of searching for novel treatment approaches.

Despite its simplicity, relatively low labor intensity, and no need to use specialized equipment, this modified method of ligature-induced periodontitis in rodents has a number of limitations. In particular, it should be noted that the experiment takes a longer period of time compared to traditional models of ligature-induced periodontitis (30 days compared to 7–14 days). Taking into account the anatomical features of the structure of the teeth of the frontal group, investigators may face a problem with technical difficulties in determining the level of alveolar bone loss measured as a distance between the cemento-enamel junction and the alveolar bone crest. However, assessment of the level of bone resorption can still be carried out using X-ray methods, histomorphometric analysis on histological preparations, analysis of the distribution of cell subpopulations, including osteoclasts, as well as molecular genetic methods.

Conclusions

Based on the identified gross and histopathological findings and changes in the level of relative mRNA expression of genes (TNF-α, IL-1β, IL-10, RANK, and OPG), it was concluded that the proposed modified model of periodontitis in rats with a placement of ligature around the frontal groups of teeth with additional fixation of the ligature by suturing the interdental papilla is relevant and can act as an accessible and less labor-intensive alternative to the existing methods of experimental modeling of periodontitis in rodents. The observed histopathological changes and increased levels of relative mRNA expression of the TNF-α, IL-1β, IL-10, and RANK genes allow us to conclude that key pathogenetic mechanisms of the development of the inflammatory process in periodontal tissues in the experimental model are preserved.

Declaration of Interest

The authors have no relevant interests to declare.

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