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Effects of electrical stimulation on periodontal tissue remodeling in rats

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Background and Objective: Electric current is used to promote wound healing. However, it is unclear whether electrical stimulation contributes to gingival tissue remodeling. This study examined the effects of electrical stimulation on gingival tissue remodeling in a rat periodontitis model.

Material and Methods: Male Wistar rats (n = 28, 8 wks of age) were divided into four groups of seven rats each. The control group did not receive any treatment for 6 wks. In the other groups, periodontitis was ligature-induced for 4 wks. After 4 wks, the rats with periodontitis were given daily electrical stimulation of 0, 50 or 100 μA for 2 wks.

Results: The periodontitis group stimulated with 0 μA showed a higher density of polymorphonuclear leukocytes and a lower density of collagen in gingival tissue compared with the control group ($p < 0.05$). The two remaining groups treated with 50 or 100 μA of electrical stimulation exhibited a lower density of polymorphonuclear leukocytes ($p < 0.05$) and a higher density of collagen than the group stimulated with 0 μA ($p < 0.05$). They also showed higher expression of fibroblast growth factor-2 than the group treated with 0 μA of electrical stimulation ($p < 0.05$).

Conclusion: Electric stimulation may offer a novel approach to promote gingival tissue remodeling in periodontal lesions.

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Periodontitis is an inflammatory lesion of the supporting tissues of teeth, characterized by soft-tissue destruction and alveolar bone loss (1). One of the objectives of periodontal treatment is to regenerate the structure lost as a result of periodontitis. The removal of bacterial pathogens is essential for the treatment of periodontitis. In addition, an approach to reduce tissue inflammation would also help to accelerate periodontal healing (2–4). Recently, tissue-engineering procedures using growth factors have been developed to accelerate periodontal healing and regeneration (2). Topical application of basic fibroblast growth factor (bFGF) was shown to be a powerful pharmacological tool in periodontal healing (3,4).

The therapeutic effects of electricity, in terms of promoting tissue remodeling, have been investigated (5,6). Studies have demonstrated that externally applied electrical stimulation could stimulate bone remodeling (7,8) and impose a bacteriostatic effect on bacterial biofilms (9,10). One of the mechanisms by which electrical stimulation induces tissue remodeling is through the regulation of growth factors. A recent study revealed that electrical stimulation directly induces expression of the *bFGF* gene in cultured retinal Mueller cells (11). Therefore, electrical stimulation may accelerate tissue remodeling through increased production of bFGF. However, it is still unclear how electrical stimulation affects inflamed gingival tissue.

In the present work, we hypothesized that electrical stimulation could induce tissue remodeling by increasing bFGF production in the inflamed gingiva. The purpose of the present study was to investigate the effects of electrical stimulation on gingival tis-

sue remodeling and bFGF production in a rat periodontitis model. MMPs are membrane-bound, zinc-dependent endopeptidases, and MMP-3, MMP-8 and MMP-9 are involved in the pathogenesis of periodontitis (12,13). Studies have also demonstrated that regulating the balance between MMPs and TIMPs plays an important role in tissue remodeling (14,15). Therefore, for better insight into the mechanism of action, we also evaluated the expression of *Mmp* and *Timp* genes. Furthermore, the linear distance between the cemento–enamel junction and the alveolar bone crest was measured to evaluate the degree of alveolar bone loss (16).

Material and methods

Experimental design

Male Wistar rats (n = 28, 8 wks of age), which had an intact dentition with a healthy periodontium, were used in this 6-wk study. The procedures and experimental protocol complied with guidelines approved by the Animal Research Control Committee of Okayama University. The rats were housed, two per cage, in standard conditions (12-h light–dark cycles and at 23–25°C), with free access to food and water.

The rats were randomly allocated into four groups of seven rats each. One group (the control group) received no treatment during the 6-wk study period. In the other three groups, a 3/0 cotton ligature (Alfresa Pharma Co., Osaka, Japan) was placed in a submarginal position on the maxillary second molars for 4 wks to induce periodontitis (16). The animals with periodontitis were then given electrical stimulation, daily, for 2 wks, after removing the ligature. For electrical stimulation, a unit consisting of a signal generator (Wave Factory Co., Ltd., Tokyo, Japan), a power supply (Kikusui Electronics Co., Ltd., Tokyo, Japan), a circuit board, a metal clip and a one-tuft conductive silicon bristle (Panasonic Corporation, Osaka, Japan) was assembled (Fig. 1). The silicon bristle was applied on the palatal side gingiva of the maxillary second molars after connecting the metal clip to the paw of the rat, and an alternating current of 0 μA (periodontitis group), 50 μA (periodontitis + 50 μA group) or 100 μA (periodontitis + 100 μA group), at 9 kHz, was delivered. A single application of electrical stimulus was given for 10 s (5 s on the right palatal side and 5 s on the left palatal side), once a day, under general anesthesia (administered by inhalation of 2–4% isoflurane delivered in O_2 gas through a face mask). The electric current was optimized visually using an oscilloscope.

Histological analysis

After the experimental period, the rats were killed under general anesthesia. The samples in the left maxillary molar region were decalcified with 10% tetrasodium-ethylenediaminetetraacetic acid (EDTA) aqueous solution (pH 7.4) (Nacalai Tesque. Inc., Kyoto, Japan) for 2 wks at 4°C, dehydrated and embedded in paraffin. Bucco-lingual sections (4 μm thick) were then prepared and stained with hematoxylin and eosin, or with other stains, as described below.

For immunostaining of bFGF, the tissue section was first deparaffinized and then rehydrated. After washing with phosphate buffer and quenching of endogenous peroxidase (with 3% hydrogen peroxide) (Nacalai Tesque. Inc.), the gingival and periodontal tissue slices were incubated with polyclonal antibody against bFGF (diluted 1 : 200 in phosphate-buffered saline; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C (15). After washing with phosphate buffer, the slices were incubated with secondary antibody (Nichirei Co., Tokyo, Japan). The color was developed with 3-3-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

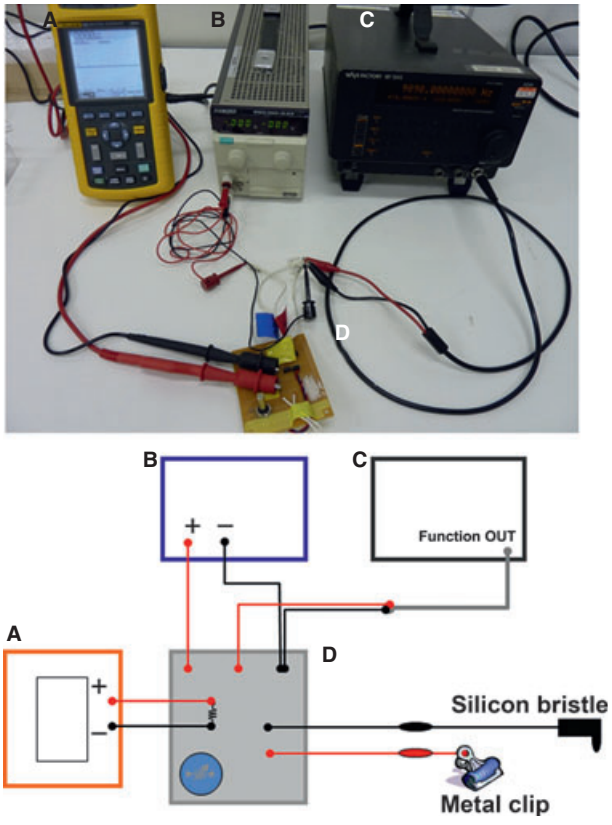

Fig. 1. The electric signal producer and the appliance. The unit includes an oscilloscope (A), a power supply (B), an electric signal producer (C), a current circuit board (D) and a metal clip and one tuft conductive silicone bristle.

Histometric analysis was performed using a microscope by a single examiner (T.T.), who was blinded to the treatment allocation. The linear distance between the cemento–enamel junction and the alveolar bone crest (an indicator of alveolar bone loss) was measured using a microgrid at a magnification of × 200 (16). The numbers of polymorphonuclear leukocytes (an indicator of inflammation), gingival fibroblasts and bFGF-positive fibroblasts per unit area (0.1 mm^2) of the connective tissue subjacent to the junctional epithelium were determined under a magnification of × 400 (15).

Histological sections stained with Mallory's aniline blue were used to determine the collagen density (17). Digital images of the sections were transferred to a microcomputer and analyzed using mathematical morphology software (WinROOF; Mitani Co., Fukui, Japan) (18). Measurements were performed in areas (0.05 mm^2 each) subjacent to the junctional epithelium.

Quantitative RT-PCR analysis

The mRNA levels of *Mmp3*, *Mmp8*, *Mmp9*, *Timp1*, *Timp2* and *Timp3* in the gingival biopsy samples were analyzed by RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The purity of mRNA was determined by the 260/280 nm absorbance ratio, and only samples with a ratio of > 1.8 were used. Samples (2 μg) of total RNA from each group, which was reverse transcribed by AMV Reverse Transcriptase (TAKARA, Kyoto, Japan) at 42°C for 30 min, were used to perform synthesis of first-strand complementary DNA using a commercial kit (Roche, Tokyo, Japan). The complementary DNA thus prepared was diluted 10-fold with yeast RNA (10 μg/mL). The primer sequences of the gene-encoding rat *Mmp*s, *Timp*s and β-actin are shown in Table 1 (19). The cycling conditions using TOYOBO SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) in a LightCycler™ (Roche Applied Science, Mannheim, Germany) system were as follows: 95°C for 30 s, 59°C for 30 s and 72°C for 30 s, for 45 cycles. The mRNA levels were expressed in terms of the relative copy number ratio of *Mmp3*, *Mmp8*, *Mmp9*, *Timp1*, *Timp2* or *Timp3* to β-actin for each sample (20). Gene expression in the periodontitis, periodontitis + 50 μA and periodontitis + 100 μA groups was calculated in terms of the relative copy number ratio of each mRNA to the control group for each sample.

Table 1. Primer sequences for *Mmp*s and *Timp*s

Gene	Sense (5′–3′)	Antisense (5′–3′)	Length (bp)	Accession no.
Mmp3	TGGGAAGCCAG TGGAAATG	CCATGCAATGGG TAGGATGAG	81	NM_133523
Mmp8	ACCTACGAAAAT TCTACCACTTACCAA	CCTTAAGCTTCTC GGCAATCA	81	AJ007288
Mmp9	TGCTCCTGGCTC TAGGCTAC	TTGGAGGTTTT CAGGTCTCG	88	NM_031055
Timp1	CTGAGAAGGGCT ACCAGAGC	GTCATCGAGAC CCCAAGGTA	88	NM_053819
Timp2	AGGACCTGACAA GGACATCG	TTCTTTCCTCCA ACGTCCAG	84	NM_021989
Timp3	TACACAGGGCTG TGCAACTT	CCAGGTGGTAG CGGTAATTG	85	U27201
β-actin	TGTTGCCCTAGAC TTCGAGCA	GGACCCAGGAA GGAAGGCT	115	NM007393

Statistical analysis

Parameters were analyzed statistically using one-way analysis of variance, followed by Tukey's method, using a statistical software package (SPSS 17.0J for Windows; SPSS Japan, Tokyo, Japan). The results were considered significant at $p < 0.05$.

Results

The periodontitis group showed a greater distance between the cemento–enamel junction and the alveolar bone crest ($p < 0.05$), a higher density of polymorphonuclear leukocytes ($p < 0.05$) and a lower density of collagen ($p < 0.05$) compared with the control group at 6 wks (Table 2, Fig. 2). Although the periodontitis + 50 μA group also showed a greater distance between the cemento–enamel junction and the alveolar bone crest ($p < 0.05$), a higher density of polymorphonuclear leukocytes ($p < 0.05$) and lower density of collagen ($p < 0.05$) than the control group, they exhibited a lower density of polymorphonuclear leukocytes ($p < 0.05$) and a higher density of

Table 2. Histopathological evaluation in periodontal tissues at 6 wk

	Control	Periodontitis + 0 μA	Periodontitis + 50 μA	Periodontitis + 100 μA
Distance between the cemento–enamel junction and the alveolar bone crest (μm)	380 ± 60	686 ± 47[a]	609 ± 22[a]	537 ± 89[a, b]
Polymorphonuclear leukocytes (numbers/ 0.05 × 0.1 mm)	1.1 ± 0.6	5.1 ± 0.8[a]	2.2 ± 0.9[a, b]	1.9 ± 0.5[b]
Collagen density (%)	71.0 ± 6.2	52.9 ± 6.5[a]	62.2 ± 1.9[a, b]	65.1 ± 4.4[b]

Values are given as mean ± standard deviation ($n = 7$).
Significantly different from [a]the control group and [b]the periodontitis group ($p < 0.05$; Tukey's method.

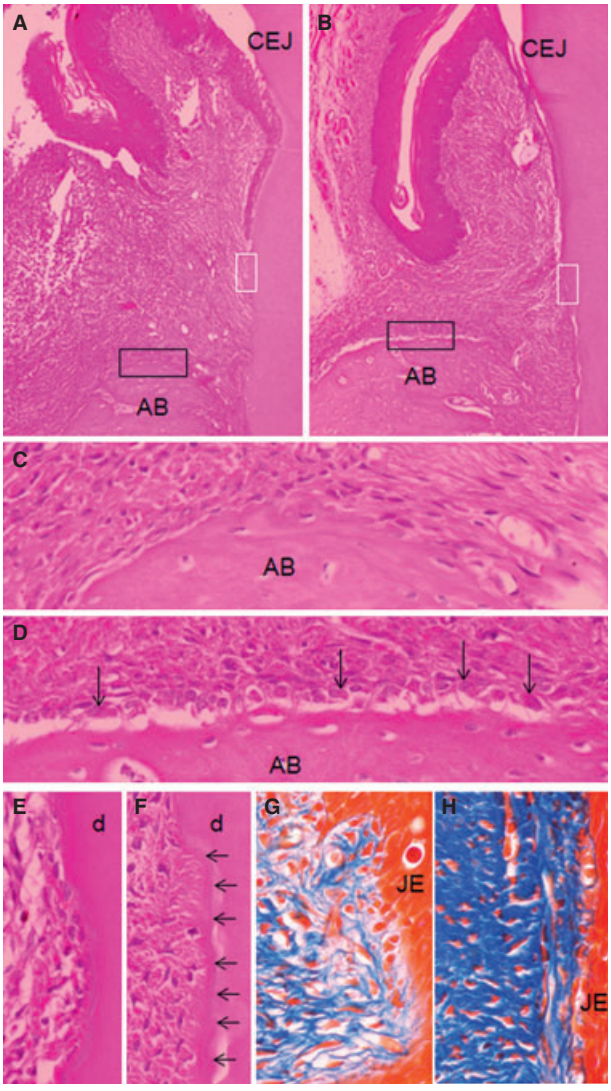

Fig. 2. Photomicrographs of rat periodontal lesions. The linear distance between the cemento–enamel junction (CEJ) and the alveolar bone (AB) crest was greater in the periodontitis group (0 μA electric stimulation) (A) than in the periodontitis + 100 μA electric stimulation group (B). On the surface of alveolar bone (C and D; black boxes in A and B, respectively), there were elliptic osteoblast-like cells (arrows) in the periodontitis + 100 μA electric stimulation group (D) but not in the periodontitis group (C). On the dentin (d) (E and F; white boxes in A and B, respectively), root resorption and odontoclast-like cells were observed in the periodontitis group (E), but these histological findings were not evident in the periodontitis + 100 μA group (F). Furthermore, thin cementum (arrows), in which collagen fibers were inserted vertically, was observed in the periodontitis + 100 μA group (F). In addition, gingival connective tissue subjacent to the junctional epithelium (JE) receiving 100 μA electric stimulation (H) showed greater collagen density than tissue without electric stimulation (G), respectively. (A–F) Original magnification × 4 and × 20; hematoxylin and eosin staining. (G, H) Original magnification × 20; Mallory's aniline blue staining.

collagen ($p < 0.05$) than the periodontitis group. On the other hand, the periodontitis + 100 μA group showed a smaller distance between the cemento–enamel junction and alveolar bone crest ($p < 0.05$), a lower density of polymorphonuclear leukocytes ($p < 0.05$) and a higher density of collagen ($p < 0.05$) than those of the periodontitis group. In addition, in the periodontitis + 100 μA group (Fig. 2D) but not in the periodontitis group (Fig. 2C), elliptic osteoblast-like cells were evident on the surface of alveolar bone. However, the distance between the cemento–enamel junction and th alveolar bone crest was greater in the periodontitis + 100 μA group than in the control group ($p < 0.05$). Furthermore, evident root resorption and odontoclast-like cells were found in the periodontitis group (Fig. 2E), but these histological findings were not evident in the periodontitis + 100 μA group (Fig. 2F).

In the rat periodontium, bFGF staining was evident in the cytoplasm of gingival cells, including fibroblasts (Fig. 3). The ratio of bFGF-positive fibroblasts to total fibroblasts was higher in the periodontitis, periodontitis + 50 μA and periodontitis + 100 μA groups than that in the control group at 6 wks ($p < 0.05$)(Fig. 4). Furthermore, the periodontitis + 50 μA and periodontitis + 100 μA groups showed a higher ratio of bFGF-positive fibroblasts to total fibroblasts compared with the periodontitis group ($p < 0.05$).

The expression of *Mmp3*, *Mmp8* and *Mmp9* genes in the periodontitis group was seven times higher than the expression of those genes in the control group (Table 3). The corresponding values in the periodontitis + 50 μA and periodontitis + 100 μA groups were less than half of those in the periodontitis group. On the other hand, in the periodontitis group the expression of *Timp2* and *Timp3* genes was almost double that of the control group. There were no marked differences in expression of the *Timp1* gene among the four groups.

Discussion

In the present investigation, the periodontitis group showed higher levels of polymorphonuclear leukocyte infiltration, collagen loss and alveolar bone loss than did the control group at 6 wks. On the other hand, the density of polymorphonuclear leukocytes within the connective tissue subjacent to the junctional epithelium was lower in the periodontitis + 50 μA and periodontitis + 100 μA groups

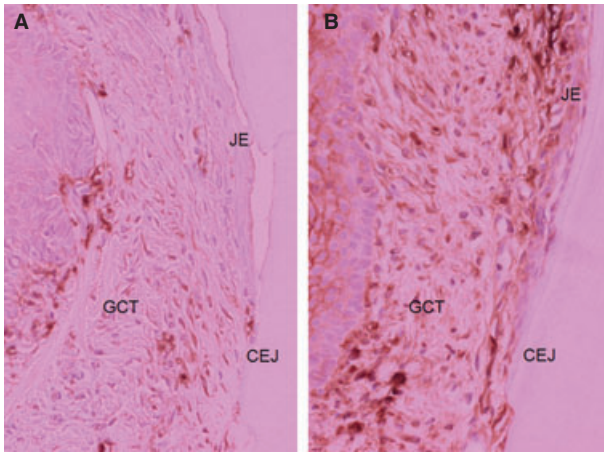

Fig. 3. Photomicrographs [original magnification × 20; basic fibroblast growth factor (bFGF) immunostaining] of rat periodontal lesions. Gingivae receiving 100 μA of electric stimulation (B) showed more bFGF-positive cells than did those without electric stimulation (0 μA electric stimulation). (A) CEJ, cemento–enamel junction; GCT, gingival connective tissue; JE, junctional epithelium.

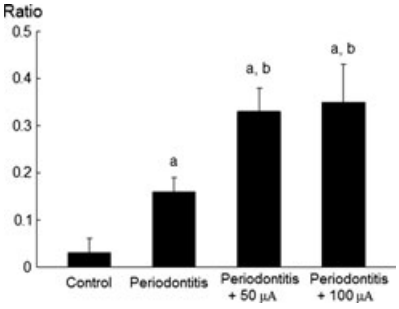

Fig. 4. Changes in the ratio of basic fibroblast growth factor (bFGF)-positive fibroblasts to total fibroblasts following electric stimulation. Values are presented as means ± standard deviation of seven rats. Differences compared with [a]the control group and [b]the periodontitis group were evaluated using the Tukey test ($p < 0.05$).

than in the periodontitis group. In addition, the collagen density in the gingival connective tissue was higher in the periodontitis + 50 lA and periodontitis + 100 lA groups than in the unstimulated periodontitis group. Furthermore, thin cementum was observed in the periodontitis + 100 μA group. It is feasible that electrical stimulation with 50 or 100 μA could promote periodontal tissue remodeling in periodontal lesions.

Recent *in-vitro* studies have shown that electrical stimulation can induce the expression of growth factor genes (11,21). In the present study, the ratio of bFGF-positive fibroblasts to total fibroblasts in the periodontitis + 50 μA and periodontitis + 100 μA groups was higher than that in the periodontitis group. Studies have demonstrated that bFGF is a potent indicator of periodontal regeneration (3,4). It is possible that electrical stimulation with 50 or 100 μA contributes to gingival tissue remodeling by inducing bFGF production.

The results also show that expression of *Mmp3*, *Mmp8* and *Mmp9* genes in the periodontal lesion was suppressed by electrical stimulation with 50 or 100 μA, indicating that electrical stimulation suppressed the expression of MMPs in gingival cells. In addition, expression of *Timp2* and *Timp3* genes was down-regulated by electrical stimulation. This may represent negative feedback for inhibition of collagen destruction.

The distance between the cemento –enamel junction and the alveolar bone crest is an indicator of alveolar bone loss (16,22). In the present study, the distance between the cemento–enamel junction and the alveolar bone crest was lower in the periodontitis + 100 μA group than in the periodontitis group. In addition, elliptic osteoblast-like cells were found in the periodontitis + 100 μA group but not in the periodontitis group. It is conceivable that electrical stimulation with 100 μA may induce alveolar bone regeneration. This is consistent with a previous study, which reported that the application of 60-kHz electrical fields enhanced alveolar bone regeneration in experimental bony defects in dogs (23). However, a greater distance was observed between the cemento–enamel junction and the alveolar bone crest in the periodontitis + 100 μA groups than in the control group, suggesting that alveolar bone regeneration following electrical stimulation was incomplete. In this study, we applied electrical stimulation for a period of 2 wks. Longer application of electrical stimulation may be necessary to induce completer alveolar bone regeneration.

A previous study demonstrated that ligature placement enhances dental-plaque formation in rats (24). In the present study, we applied electrical stimulation after removing the ligature. This indicates that the healing of inflamed gingival tissue by electrical stimulation occurred when the source of plaque formation was removed. In other words, elec-

Table 3. Ratios of gene expression, following stimulation with different intensities of electrical current, for *Mmp*s and *Timp*s relative to the control group at 6 wks

Gene	Periodontitis + 0 μA	Periodontitis + 50 μA	Periodontitis + 100 μA
Mmp3	8.0	0.4	1.0
Mmp8	7.7	2.3	2.3
Mmp9	7.0	2.2	3.3
Timp1	1.9	1.3	1.5
Timp2	4.3	0.8	1.4
Timp3	2.4	0.4	1.1

trical stimulation following reduction of the bacterial load helped to promote tissue remodeling in the periodontal lesion.

The therapeutic effects of electricity have been investigated largely in terms of promotion of tissue repair. For instance, it was reported that micro-electrical fields of $\geq$ 100 mV/mm appeared to have a crucial role in the control of cultured nasal fibroblast activity in the process of wound healing (25). It is also known that high-voltage pulsed current can stimulate healing of pressure ulcers of people with spinal cord injury (26). Furthermore, the present results reveal that electrical stimulation with 50 or 100 μA promoted gingival remodeling in the rat periodontitis model. These observations are in agreement with the concept that electrical stimulation has a strong influence on wound healing.

A previous *in-vitro* study (27) demonstrated that fibroblast ingrowth and collagen fiber alignment were increased in collagen sponges stimulated with currents of 20–100 μA. An animal study also revealed that the histologic fusion rate of titanium interbody fusion cages in the spine increased as the current increased from 0 to 100 μA (28). Therefore, we selected electrical stimulation currents of 50 and 100 μA in the present study. However, we did not collect data with regard to the optimal voltage, Hz and daily frequency of stimulation required to induce gingival tissue remodeling. Further studies are needed to clarify these points.

This study has other limitations. First, because the bacterial flora in the sulcus of ligature-placed rat is different from that in human, we did not investigate the effect of electrical stimulation on pathogenic bacteria in the present model. However, it is possible that electrical stimulation has direct effects on bacteria or bacterial products (9,10), and we need to perform more studies to clarify this point. Second, we only counted the number of polymorphonuclear leukocytes in the ligature-induced periodontal lesions. The measurement of T- and B-lymphocytes by immunostaining would increase the reliability of our data on periodontal inflammation.

In conclusion, electrical stimulation with 50 or 100 μA may offer a novel approach to promote gingival tissue remodeling in periodontal lesions.

Acknowledgement

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