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Effects of mechanical stimulation by a powered toothbrush on the healing of periodontal tissue in a rat model of periodontal disease

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Background and Objective: Elimination of pathogens is the main aim of periodontal treatment; however, modulation of the host immune response should also be considered. This study aimed to evaluate the effects of mechanical stimulation on periodontal healing in rats.

Material and Methods: Before starting the experiment, lipopolysaccharide and proteases were applied once a day, for 4 wk, to both maxillary first molars of 30 rats to induce periodontal disease, and the application was stopped at the end of the 4-wk period. The experiment started immediately following this pretreatment. In the experiment, the left palatal gingiva was stimulated once daily using a powered toothbrush and the right gingiva served as a control (no mechanical stimulation). Pathological changes, and proliferation and cell death in periodontal tissues, were evaluated histometrically and immunohistochemically at baseline (0 wk), and at 1 and 3 wk of stimulation.

Results: The control showed a reduction of polymorphonuclear leukocyte infiltration in connective tissue and an increase in the numbers of gingival and periodontal ligament fibroblasts. Mechanical stimulation reduced polymorphonuclear leukocyte infiltration and the area of destroyed collagen in connective tissue, and increased the number of gingival fibroblasts; however, it had no effect on alveolar bone and root resorption or on the number of periodontal ligament fibroblasts.

Conclusion: Mechanical stimulation accelerated the healing of gingival inflammation by reducing the infiltration of polymorphonuclear leukocytes and enhancing fibroblast proliferation and collagen synthesis.

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Periodontitis is an infectious disease caused by bacteria and their products (1). The elimination of periodontal pathogens is the main aim of periodontal treatment. To remove bacteria or to reduce the bacterial challenge, scaling, root planing and/or application of antimicrobial agents are carried out. Reduction of the bacterial load is

required to allow the spontaneous healing of damaged tissues. It is also recognized that modulation of the host response can be a component of periodontal therapy, and this can be achieved by microbial chemotherapeutic intervention, the use of nonsteroidal anti-inflammatory drugs (2) and the mechanical stimulation of gingiva (3).

It has been reported that the proliferation of epithelial basal cells and fibroblasts is accelerated by mechanical stimulation of the gingiva with a toothbrush (3). A high turnover rate of the junctional epithelium results in desquamation and prevents the invasion of bacteria into periodontal tissue (4). Proliferation of gingival fibroblasts is followed by collagen production by the cells (3), which helps to replace the destroyed connective tissue. The biological effects that occur following mechanical stimulation of the gingiva include enhancement of the gingival capillary circulation (5) and the formation of connective tissue (6). Stimulation of the gingival cells may help to repair the gingival tissues in periodontitis.

Both removal of bacterial pathogens and mechanical stimulation by toothbrushing act synergistically on the healing of gingivitis. However, little information is available regarding the additional effects of mechanical stimulation on the periodontal healing process. The biological effects of mechanical stimulation on gingiva are limited to the area beyond the alveolar bone ridge (7). Therefore, the individual effects of elimination of periodontal pathogens and mechanical stimulation on the periodontal healing process need to be identified. Understanding the distinct effects of elimination of bacterial pathogens and the mechanical stimulation of gingiva might help in planning treatment for periodontal diseases.

In the present study, the effects of mechanical stimulation on the healing of periodontal disease were evaluated using a rat model in which collagen destruction of the subepithelial connective tissue of the junctional epithelium, rete ridge elongation of the junctional epithelium and alveolar bone resorption were induced by topical application of lipopolysaccharide (LPS) and bacterial proteases.

Material and methods

Experimental design

Thirty male rats (6 wk of age) of the Wistar strain were used in this study. The rats received topical application of 25 μg/μL of *Escherichia coli* LPS (Sigma Chemical Co., St Louis, MO, USA) suspended in pyrogen-free water (Otsuka Medical Co., Tokyo, Japan) and 2.25 U/μL of proteases (Sigma Chemical Co.) from *Streptomyces griseus* during a 4-wk pre-experimental period (8). Three successive applications of 0.5 μL of LPS and 0.5 μL of proteases were performed once daily into the palatal gingival sulcus of both maxillary first molars.

After the pre-experimental period, the application of LPS and proteases was discontinued. The palatal gingiva of the left maxillary first molar was then mechanically stimulated once daily using a powered toothbrush (TwinPecker®; S. E. W. Co., Osaka, Japan) with a pulsating movement (9). The toothbrush has 100 filaments (diameter, 0.1 mm; length, 65 mm; nylon #5/1000) and produces a pecking motion with an amplitude of 5 mm, 3000 times/min. The toothbrush was placed on the rat gingiva with a force of 0.02 N. The mechanical stimulation was performed for 10 s, once daily, for 0, 1 or 3 wk (n = 10/group). The right maxillary first molar served as a control (no treatment). These procedures were carried out under general anesthesia with sodium pentobarbital (0.5 mL/kg body weight).

All rats were fed a powdered diet (MF; Oriental Yeast Co. Ltd, Osaka, Japan) during the entire experimental period. The animal experiments complied with guidelines approved by the Animal Research Control Committee of Okayama University Dental School.

Histological and immunohistochemical analyses

After 0, 1 or 3 wk of treatment, the rats were killed by intracardiac perfusion of 4% paraformaldehyde in 0.1 mol/L of phosphate buffer (pH 7.4), under general anesthesia. Following initial fixation, the maxillary molar regions were resected en bloc from each rat. Tissues were decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 wk at 4°C. Paraffin-embedded bucco-lingual sections (4 μm thickness) were stained with hematoxylin and eosin (H&E), or other stains, as described below.

The method involving the avidin–biotin complex (10) was used to determine the level of expression of proliferating cell nuclear antigen (PCNA). The monoclonal antibody against PCNA (PC-10; Novocastra Laboratories, Ltd., Newcastle, UK) was diluted 1:200 in phosphate-buffered saline (3). The color was developed with 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer's hematoxylin.

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling (TUNEL) method (11) was used for the *in situ* detection of nuclear DNA fragmentation. Briefly, the deparaffinized sections were incubated with 2% H_2O_2 for 30 min and then treated with 0.02 mg/mL of proteinase K (Sigma Chemical Co.) for 10 min at 25°C. After rinsing, the sections were incubated with a mixture of terminal deoxynucleotidyl transferase enzyme and fluorescein–deoxyuridine triphosphate for 60 min at 37°C.

A single, blinded examiner performed the following histometric analyses using a microscope at 200× (H&E staining) or 400× (TUNEL staining, and staining for PCNA) magnification, after the preparations were assigned random numbers. The tissue sections stained with H&E were used to evaluate histological changes. The level of destruction of collagen fibers (the distance from the cemento–enamel junction to the most apical portion of the destroyed collagen fibers in the connective tissue), the level of root resorption (from the cemento–enamel junction to the most apical portion of the root resorption), the level of alveolar bone crest (from the cemento–enamel junction to the alveolar bone

crest level), the apical migration of junctional epithelium (from the cemento–enamel junction to the most coronal portion of the connective tissue attachment) and the distance between the most apical portion of the junctional epithelium and the most apical portion of the destroyed collagen fibers were measured on a microgrid under 200× magnification (Fig. 1) (9,12).

The polymorphonuclear leukocytes per unit area (0.05 × 0.05 mm) of connective tissue subjacent to the junctional epithelium and within the junctional epithelium were counted under 400× magnification (Fig. 1). The gross area of the junctional epithelium was measured using image-analysis software (WINROOFl; Mitani Co., Fukui, Japan) and the density of polymorphonuclear leukocytes was calculated (Fig. 1).

The numbers of PCNA-positive, TUNEL-positive and total gingival fibroblasts were determined in two gingival connective tissue zones (0.1 × 0.1 mm each) adjacent to the cementum from the most apical portion of the junctional epithelium (Fig. 1). In the periodontal ligament, the number of PCNA-positive fibroblasts, TUNEL-positive fibroblasts and total fibroblasts were counted in two standard areas (0.1 × 0.1 mm each) (Fig. 1) (12). The numbers of PCNA-positive and total basal cells in the junctional epithelium were also determined and the densities were calculated per 0.1 mm length of basement membrane (Fig. 1) (13).

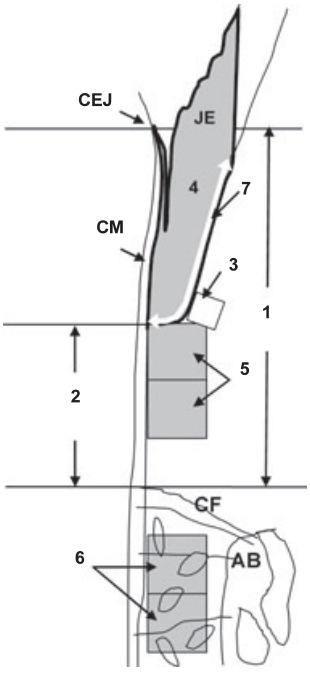

Fig. 1. Schema of rat periodontal tissue for histological analysis: 1, level of the apical portion of the destroyed collagen fibers; 2, distance between the apical portion of the junctional epithelium and the apical portion of the destroyed collagen fibers; 3, number of polymorphonuclear leukocytes; 4, gross area of the junctional epithelium; 5, number of gingival fibroblasts; 6, number of periodontal ligament fibroblasts; 7, basal cells of the junctional epithelium. AB, alveolar bone crest; CEJ, cemento–enamel junction; CF, collagen fibers; CM, cementum; JE, junctional epithelium.

Statistical analysis

The mean of three tissue sections from each gingiva was used for statistical analysis. Analyses using the Mann–Whitney *U*-test and the Wilcoxon signed-rank test were carried out using a statistical software package (SPSS version 10.0 J; SPSS, Tokyo, Japan).

Results

Histological changes in the connective tissue

The 4-wk pretreatment with LPS and proteases induced slight destruction of collagen fibers, vasodilatation and infiltration of polymorphonuclear leukocytes in the subepithelial connective tissue of the junctional epithelium (Fig. 2A). Evident root resorption and odontoclast-like cells were observed in the control group at the 3-wk treatment time-point (Fig. 2B), but these histological changes were not evident at 0 wk (Fig. 2A).

In the control group, the level of destroyed collagen fibers decreased over time; however, no changes were observed in the distance between the apical portion of the junctional epithelium and the apical portion of the destroyed collagen fibers (Table 1). The levels of the apical portion of the root resorption and the alveolar bone crest increased from 0 to 3 wk of treatment, even

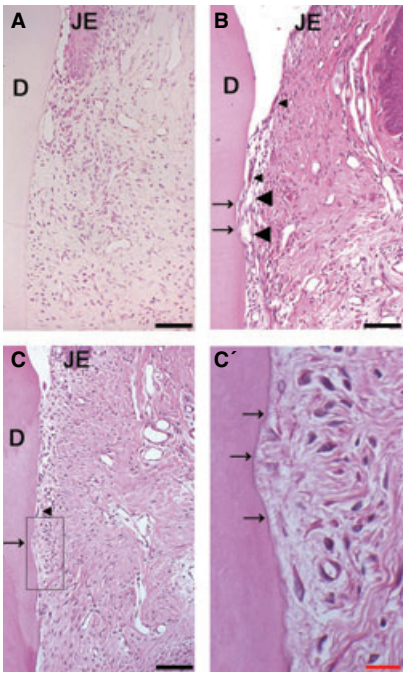

Fig. 2. Hematoxylin and eosin staining of subepithelial connective tissue at 0 (A) and 3 wk, without (B) and with (C and C' which is a close-up of the black box in C) mechanical stimulation. Root resorption (arrows) and odontoclast-like cells (small arrowheads) were observed at 3 wk in both control (B) and stimulation (C) groups. In the destroyed connective tissue of the control group at 3 wk, edema was observed (large arrowheads) (B). Collagen destruction was observed in the connective tissue along the root surface, and the degree of collagen destruction in the stimulation group (C) was lower than that in the control group (B). New connective tissue attachment (i.e. thin cementum), in which collagen fibers were inserted vertically, was observed in the stimulation group (C'). D, dentin; JE, junctional epithelium; black scale bar, 100 μm; red scale bar, 20 μm.

though the application of LPS and proteases had been discontinued. In the periodontal ligament, the number of the fibroblasts decreased and the number of TUNEL-positive fibroblasts showed an increase at the 3-wk treatment time-point, but the number of PCNA-positive fibroblasts did not change throughout the experimental period.

The stimulation group showed a lower degree of collagen destruction and a lower level of edema in the connective tissue along the root surface

Table 1. Histological changes in connective tissue at 0, 1 and 3 wk with or without (Control) mechanical stimulation

	Week	Control	Stimulation
Level of the apical portion of the destroyed collagen fibers (μm)	0	106.6 ± 15.5	–
	1	79.3 ± 10.7	103.2 ± 19.4
	3	47.0 ± 9.2**	57.7 ± 9.4*
Distance between the apical portion of the junctional epithelium and the apical portion of the destroyed collagen fibers (μm)	0	34 ± 8.1	–
	1	36 ± 5.5	32 ± 5.0
	3	35 ± 6.1	13 ± 3.2*,†
Level of the apical portion of root resorption (μm)	0	76 ± 49	–
	1	336 ± 48**	299 ± 55**
	3	315 ± 62**	310 ± 47**
Level of the alveolar bone crest (μm)	0	612 ± 18	–
	1	639 ± 16	617 ± 24
	3	672 ± 15*	647 ± 21
Polymorphonuclear leukocytes (number/0.05 × 0.05 mm)	0	2.5 ± 0.1	–
	1	1.9 ± 0.1**	1.4 ± 0.1***,†
	3	2.1 ± 0.2	1.5 ± 0.1***,†
Total gingival fibroblasts (number/0.1 × 0.1 mm)	0	22 ± 0.5	–
	1	23 ± 0.5*	24 ± 0.9**
	3	23 ± 0.4*	25 ± 0.2**,†
PCNA-positive gingival fibroblasts (number/0.1 × 0.1 mm)	0	2.4 ± 0.2	–
	1	2.7 ± 0.2	3.9 ± 0.4**,†
	3	2.8 ± 0.3	2.9 ± 0.2
TUNEL-positive gingival fibroblasts (number/0.1 × 0.1 mm)	0	2.3 ± 0.7	–
	1	2.4 ± 0.3	2.7 ± 0.3
	3	2.7 ± 0.2	2.3 ± 0.3
Total periodontal ligament fibroblasts (number/0.1 × 0.1 mm)	0	26.2 ± 0.3	–
	1	24.6 ± 0.8	23.2 ± 1.1
	3	23.9 ± 2.8*	28.8 ± 1.0*
PCNA-positive periodontal ligament fibroblasts (number/0.1 × 0.1 mm)	0	4.0 ± 0.3	–
	1	3.7 ± 0.4	3.8 ± 0.4
	3	3.1 ± 0.4	3.0 ± 0.2
TUNEL-positive periodontal ligament fibroblasts (number/0.1 × 0.1 mm)	0	7.8 ± 0.7	–
	1	8.4 ± 1.3	8.5 ± 1.0
	3	9.9 ± 0.6*	10.3 ± 0.9*

Values are presented as mean ± standard error ($n = 10$).
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; significantly different compared to 0 wk of the control group (Mann–Whitney *U*-test).
†$p < 0.05$; significantly different compared to the control group at the corresponding week (Wilcoxon signed-rank test).
PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labeling.

than the control group at 3 wk of treatment (Fig. 2C). Collagen fibers in the stimulation group were arranged more regularly and parallel to the root surface at 1 and 3 wk of treatment than those in the control group. Moreover, seven out of 10 rats in the stimulation group showed new connective tissue attachment at 3 wk of treatment, in contrast to the control group in which new connective tissue attachment was not observed in any rats. In the new connective tissue attachment, thin cementum had formed and collagen fibers were inserted vertically into the cementum (Fig. 2C').

The distance between the apical portion of the junctional epithelium and the apical portion of the destroyed collagen fibers in the stimulation group was significantly shorter than that in the control group at 3 wk of treatment (Table 1). The stimulation group had fewer polymorphonuclear leukocytes and more PCNA-positive gingival fibroblasts and total gingival fibroblasts than the control group (Table 1, Fig. 3).

Histological changes in the junctional epithelium

After the application of LPS and proteases was stopped, the degree of apical migration decreased over time in the control group (Table 2). However, the number of polymorphonuclear leukocytes, PCNA-positive basal cells and total basal cells did not change throughout the 3-wk experimental period.

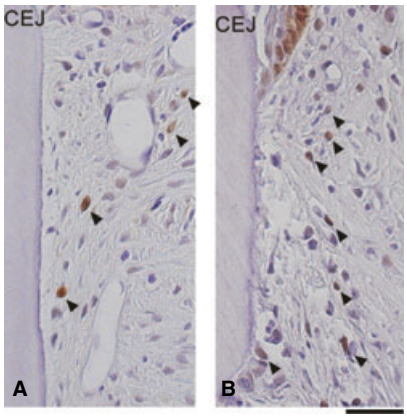

Fig. 3. Proliferating cell nuclear antigen (PCNA)-positive cells in connective tissue adjacent to the root surface at 1 wk without (A) or with (B) mechanical stimulation. The stimulation group (B) exhibited more PCNA-positive fibroblasts (arrowheads) in the connective tissue adjacent to the root surface than the control group (A). CEJ, cemento–enamel junction; scale bar, 100 μm.

In the stimulation group, the degree of apical migration was significantly greater than that of the control group at 3 wk (Table 2). At 3 wk, fewer polymorphonuclear leukocytes were observed in the junctional epithelium of the stimulation group than in the junctional epithelium of the control group (Table 2). An increase in the number of PCNA-positive basal cells of junctional epithelium was observed at 1 and 3 wk of treatment as a result of the application of mechanical stimulation (Table 2, Fig. 4).

Discussion

The application of LPS and proteases was discontinued to simulate the removal of subgingival plaque and/or the use of antimicrobial agents in the treatment of periodontitis. A decreased number of polymorphonuclear leukocytes, an increased number of gingival fibroblasts and restored collagen fibers in the connective tissues, and reversed apical migration of the junctional epithelium, were observed in the control group. However, the root surface

Table 2. Histological changes in junctional epithelium at 0, 1 and 3 wk with or without (Control) mechanical stimulation

	Week	Control	Stimulation
Apical migration (μm)	0	72.3 ± 11.5	–
	1	43.2 ± 9.8	72.7 ± 15.7
	3	12.3 ± 6.3**	44.7 ± 7.0††
Polymorphonuclear leukocytes (number/0.05 × 0.05 mm)	0	6.6 ± 0.4	–
	1	6.5 ± 0.5	5.7 ± 0.6
	3	6.2 ± 0.3	5.1 ± 0.3†
PCNA-positive basal cells (number/0.1 mm)	0	4.6 ± 0.2	–
	1	4.9 ± 0.3	6.3 ± 0.3***,††
	3	4.8 ± 0.3	5.8 ± 0.3**,†
Total basal cells (number/0.1 mm)	0	12 ± 0.4	–
	1	12 ± 0.2	12 ± 0.3
	3	12 ± 0.3	12 ± 0.3

Values are presented as mean ± standard error (n = 10).
**p < 0.01; ***p < 0.001: significantly different compared to 0 wk of the control group (Mann–Whitney U-test).
$^{\dagger}p$ < 0.05; $^{\dagger\dagger}p$ < 0.01: significantly different compared to the control group at the corresponding week (Wilcoxon signed-rank test).
PCNA, proliferating cell nuclear antigen.

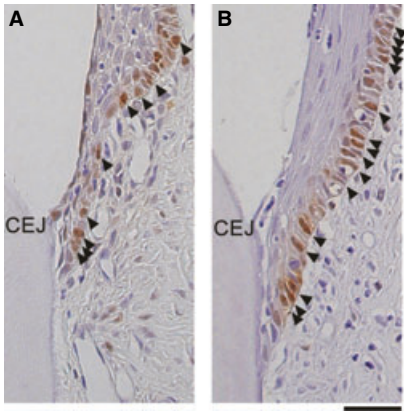

Fig. 4. Proliferating cell nuclear antigen (PCNA)-positive cells in the junctional epithelium at 1 wk without (A) or with (B) mechanical stimulation. More PCNA-positive basal cells (arrowheads) of the junctional epithelium were found throughout the basal layer of the junctional epithelium in the stimulation group (B) than in the control group (A). CEJ, cemento–enamel junction; scale bar, 100 μm.

continued to resorb in spite of discontinuation of the application of LPS and proteases. These results suggest that discontinuation of the application of LPS and proteases may relieve inflammation in the junctional epithelium and subepithelial connective tissue area, but inflammation may progress in the deeper areas of the periodontium, including the root surface and alveolar bone.

The results of the present study, in which alveolar bone resorption was observed 1 and 3 wk after discontinuing the application of LPS and proteases, were in agreement with those of another study using a rat model of periodontitis (14). In that study, alveolar bone loss and increased osteoclastic activity were observed 7 and 10 d, but not 3 d, after a single injection of LPS in saline into the mandibular gingiva. There may be a time lag of about 1 wk between application of a bacterial pathogen to gingival sulcus and alveolar bone resorption.

In addition to alveolar bone resorption, root resorption was observed in both the stimulation and control groups. A similar level of root resorption was reported in human periodontitis (15) and in experimental periodontitis using dogs (16). Similar mechanisms might be involved in the resorption of alveolar bone and cementum.

The effects of toothbrushing include the removal of dental plaque and the mechanical stimulation of gingiva (3). The differences in histological changes between the stimulation group and the control group can be attributed mainly to mechanical stimulation by toothbrushing. In our previous study, little inflammation was observed in the gingiva of rats fed the same diet (8). These results suggest that the indigenous bacteria of rats have little influence on gingival inflammation and that the effects of disruption of plaque biofilm resulting from toothbrushing are negligible.

Mechanical stimulation might be a possible method of accelerating healing of periodontitis when combined with the conventional approach (i.e. the elimination of periodontal pathogens). A decrease in the numbers of polymorphonuclear leukocytes in both connective tissue and junctional epithelium, and an increase in the numbers of total and PCNA-positive gingival fibroblasts, were observed in the stimulation group compared with the control group. In addition, histological findings showed that the destroyed collagen fibers were repaired and new connective tissue attachment had occurred as a result of mechanical stimulation. These histological changes are similar to those observed in dog gingiva that was mechanically stimulated with a toothbrush (3) and indicate that mechanical stimulation accelerates wound healing in gingiva.

The effects of mechanical stimulation on healing as well as on epithelial and fibroblast turnover have been reported in other research fields. Frictional massage facilitated healing of tendonitis by recruitment and activation of fibroblasts in rats (17). Low-intensity ultrasound was reported to have beneficial effects on the treatment of chronic venous leg ulcers (18) and recurrent aphthous stomatitis (19). These findings support the results of the present study.

It has been reported that mechanical strain delivers anti-apoptotic stimuli (20) and promotes the proliferation of fibroblasts (20, 21). In the present study, it was observed that the numbers of TUNEL-positive, PCNA-positive and total periodontal ligament fibroblasts did not change following mechanical stimulation. As the cells respond directly to stimulation (22), the interference of mechanical stimulation by alveolar bone may be responsible for the unchanged numbers of TUNEL-positive, PCNA-positive and total fibroblasts in periodontal

ligament. The findings of the present study are in agreement with those reported previously, where mechanical stimulation by toothbrushing had no effects on the proliferation of periodontal ligament fibroblasts in dogs (7).

During periodontal pocket formation, the junctional epithelium migrates apically along the root surface. Apical migration, which is a typical feature of periodontitis, is followed by accelerated proliferation of basal cells in the junctional epithelium (12). As mechanical stimulation enhances the proliferation of basal cells in junctional epithelium (3), it was speculated that mechanical stimulation induces apical migration of junctional epithelium in gingiva affected with periodontitis. However, the results of the present study showed that mechanical stimulation did not accelerate the apical migration of junctional epithelium, though it arrested the apical migration. These results suggest that promotion of the proliferative activity alone might not induce further apical migration of junctional epithelium.

In addition to excess proliferation of junctional epithelium, the connective tissue substratum that has been modified by the inflammatory process may be involved in the formation of periodontal pockets (23). Degradation of the collagenous structure of periodontal tissues is a critical outcome of periodontal diseases, and MMPs may play a significant role in this process (24). Gingival epithelial cells express several MMPs in inflamed periodontal tissues, including MMP-2, MMP-3, MMP-8 and MMP-13 (25–27). MMP-2 and MMP-9 can degrade basement membrane collagen (28) and other matrix components, such as elastin (29) and collagen types V, VII and X (30–32). Polymophonuclear neutrophils (PMNs) have been recognized as an important source of MMP-9 in human periodontitis (33), and periodontal tissue destruction has been associated with high levels of active MMP-9 in gingival crevicular fluid (34). Further studies are required to clarify the mechanism of periodontal pocket formation from the viewpoint of degradation of collagen structure.

Another issue that was not explored in the present study was whether the change in blood flow caused by mechanical stimulation altered the nature of the host immune response. As the innate immune response might be involved in the destruction of periodontal tissue in this rat model (35), pro-inflammatory cytokines, such as interleukin-1 and tumor necrosis factor, should be evaluated in the future. Moreover, the transition from the tissue destruction phase to the repair phase needs to be assessed by measuring mediators such as transforming growth factor-β (36) and TIMPs (37).

The expression of PCNA and the presence of nuclear DNA fragmentation (determined using the TUNEL assay) were used to evaluate the proliferation and apoptosis of fibroblasts, respectively. It might be useful to investigate the role of other molecules, such as vimentin, to obtain more detailed information on fibroblast proliferation. Vimentin is the major intermediate filament protein of mesenchymal cells, and recent studies have revealed several key functions of vimentin, such as DNA recombination and repair (38).

In conclusion, mechanical stimulation accelerated the proliferation of gingival fibroblasts, the repair of collagen fibers and the reduction of polymorphonuclear leukocyte infiltration induced by the removal of periodontal pathogens in a rat model of periodontal disease.

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