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Cellular responses of rat gingival connective tissue to mechanical stress produced by a brush

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Abstract
The objective of the present study was to compare and analyze the effects of toothbrushes with different types of bristles on the activation of gingival connective tissue. In this study, 2 types of toothbrushes were used: those with fewer bristles than normal (the experimental group) and those with a normal number of bristles (the normal group). The upper and lower gingival tissues of the incisor regions of rats were brushed by applying 30 g of pressure with both types of toothbrushes. In terms of the brushing stroke used, the neck of each toothbrush was held as if gripping a pen, and teeth were brushed 30 times in the horizontal direction against the gingiva. Tooth brushing was done twice a day for one week. As a control, tooth brushing was not performed in some rats (the control group). After the brushing period, the rats were sacrificed and expression levels of mRNAs encoding VEGF, osteopontin, type I collagen and HSP70 were measured by RT-PCR. Some rats from each group were used for morphological analyses and immunohistochemical analyses of PCNA. VEGF mRNA expression in the experimental group was significantly higher compared to the control and the normal groups. HSP70 mRNA expression in the normal group was significantly higher when compared to the control and the experimental groups. Moreover, no significant difference existed in type-1 collagen mRNA expression among the 3 groups. While no statistically significant difference was found in osteopontin mRNA expression among the 3 groups, it tended to be higher in the experimental group. Histological analyses showed that collagen formation in the experimental group was greater compared to the normal and the control groups. Furthermore, dilation of capillaries and edema were seen in the normal group. In conclusion, when compared to the normal and the control groups, the activation of gingival connective tissue was marked in the experimental group, and for the normal group, distinct reactive inflammatory changes were observed.

Key words: toothbrush, gingival, HSP 70, VEGF, osteopontin, type I collagen, mRNA

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Introduction
Tooth brushing exerts a favorable effect on the gingiva not only through plaque removal \cite{34} but also through mechanical stimulation of the tissues \cite{13,14}. In monkeys and dogs, tooth brushing stimulation can help resolve gingival inflammation, as measured through the use of the visible gingival index \cite{20} and histometric analysis of inflammatory cell infiltrates. Horiuchi et al. \cite{7}, Tomofuji et al. \cite{8} and Kusano et al. \cite{9} reported that tooth brushing promotes gingival cell proliferation, which may occur as the result of the physical stimulation of the gingiva.

The mechanical effects of tooth brushing are known to be affected by the brushing method, brushing time, mechanical pressure, usage conditions, and bristle thickness, count, length, shape and alignment. Previous studies were conducted to eliminate dental plaque attached to tooth surfaces. In addition, various mechanical stresses are known to increase tissue activities. Mechanical stresses vary in various forms, including continuous pressure, traction force, centrifugal force and weightlessness, but the mechanical stress generated by brushing is thought to be a form of intermittent pressure.

The level of vascular endothelial growth factor (VEGF) increases with various stimuli, including mechanical stress, and VEGF is known to facilitate vascular growth during wound healing \cite{13,14}. Type I collagen is the major component of bone and fibrous substrates, and it is secreted by activated fibroblasts \cite{15}. Moreover, osteopontin is expressed during bone formation prior to calcification, and its expression is high in activated fibroblasts \cite{15,16}. Heat-shock proteins are known to respond to various stimuli and maintain homeostasis \cite{12,17}.

The objective of the present study was to use molecular biological techniques to compare the effects of two different types of toothbrushes, i.e., conventional toothbrushes and toothbrushes with fewer bristles, on the activation of gingival tissue.

Materials and Methods
1. Brushes and Animals
In this study, in the experimental group we used microtufts, which have 12 bristles fixed in a small oval hole (25% of normal), and in the normal group, we used normal tufts, which have 48 bristles fixed in a normal round hole (as used in a commercial toothbrush ‘CLINICA’ (Lion Corporation).

Thirty male Sprague-Dawley rats, each weighing approximately 150 g, were used in this study. Under general ether anesthesia, the gingival region of the right maxillary incisor of each rat was brushed using a toothbrush on which strain gauges (Kyowa, KFG-1-120-C1-11L3M2R) were mounted. Each brush was controlled by a computer (Kyowa, PCD30A) with 20 g weight measured by strain gauges. Brushing was done 30 times twice a day, morning and evening, in a direction parallel to the gum horizontally. The left maxillary gingival of incisor where no brushing occurred was used as a control.

2. Morphological observation
Rats were sacrificed with an overdose of sodium pentobarbital 7 days after the start of the experiment. The jaw of each rat was removed and fixed in 4% paraformaldehyde for 2 days and then de-mineralized with 10% EDTA for 14 days at 4degree C before embedding in paraffin. Sections were deparaffinized with xylene, re-hydrated in 100% alcohol, and washed in distilled water. Serial sections were cut and stained with hematoxylin-eosin using routine procedures.

3. Immunohistochemical study
Endogenous peroxidase activity was blocked by incubating each section with 3% H2O2 in methanol for 30 min. To prevent nonspecific binding, sections were incubated in 10% serum solution for 30 min in a 100% humidity chamber. Sections were stained immunohistochemically with an anti-PCNA antibody (1:100, PC-10, DAKO). Slides were washed in PBS and were then incubated with the secondary antibody for 30 min in a humidity chamber. After washing in PBS, sections were stained with 3,3’-diaminobenzidine for 5 min, washed in distilled water, counterstained in hematoxylin and cover-slipped.

4. Finite Element Method
Mechanical stresses generated by each toothbrush were analyzed on the artificial gum by finite element methods.

5. Quantitative RT-PCR using the LightCyclerTM
To evaluate osteocalcin and VEGF mRNA levels, rats were sacrificed on day 7 with an overdose of sodium pentobarbital. The jaw of each rat was removed and put into an Eppendorf tube. For the quantitative analysis of osteopontin, Type 1 collagen, HSP70 and VEGF mRNA levels, total RNA was extracted using the Isogen reagent (Nippon Gene, Japan) according to the manufacturer’s instructions. Briefly, cells were homogenized and solubilized in Isogen/chloroform solution at 4 degree C. Supernatants were obtained following centrifugation at 12,000 x g for 20 min.

| Table 1 Primers used in this study |
|-------------------------------|-----|----------------|
| primer | bp | sequence |
| Collagen type I | 252 | F: 5’ CAA GAC AGT CTA GCA ATA CA 3’ |
| | | R: 5’ TGT ATT CGA TGA TCT TG C’ |
| VEGF | 201 | F: 5’ TAC CAG GCC ACC TAT TGC CGT 3’ |
| | | R: 5’ TTT GGT GAG GTC TTA CCA GCA TG 3’ |
| HSP70 | 180 | F: 5’ GTG TGC AAC CGC ATC ATC AG 3’ |
| | | R: 5’ CAC CAG CAC CCA TCA AGA GT 3’ |
| Osteopontin | 207 | F: 5’ CTC GGA GGA GAA GCC GCA TTA 3’ |
| | | R: 5’ CCA TCG TCA TCG TCG TCA 3’ |
| β -actin | 260 | F: 5’ CTT GTA TGC CTC TGG TGG TA 3’ |
| | | R: 5’ CCA TCT CTT GCT CGA AGT CT 3’ |
Fig. 1 The stress received by the experimental group was approximately 5.8 g/mm² and was 7.4 g/mm² in the normal group. The stress was less both in the superficial layer and in deep areas of experimental group compared to the normal group.

Fig. 2 In the experimental group (a), the keratinized layer was thinner than in the control group(b), however, connective tissues were no significant differences [HE staining] PCNA-positive cells were observed in the basal cell layer and sometimes peri-vascular cells were also seen in both the experimental (c) and the control groups(d). However, there were no significant differences between two groups. (PCNA Immunohistochemical staining)

at 4 degree C. The precipitates were recovered following decantation and were washed with 75% ethanol. The RNA pellets were then dissolved in RNase-free water, and were preserved at -20 degree C until used. Using the extracted RNA as a template, reverse transcription reactions were conducted with an RT-PCR kit RNA-PCR kit ver 2.1, (Takara Biomedicals, Japan) to synthesize cDNA. Quantitative PCR was then conducted using primers for VEGF, osteocalcin and HSP70 with a LightCyclerTM (Roche Diagnostics, Germany) using the double-stranded DNA dye SYBR Green I (Roche Diagnostics). The primer sequences used are shown in Table 1. Quantification was performed by comparing the levels obtained with standard samples. In the present study, the concentrations of cDNA in the unstimulated samples were 0.2, 0.5, 1.0 and 2.0 µl. The PCR conditions used in the LightCyclerTM were 40 cycles, while PCR conditions were 95 degree C for 10 sec, 60 degree C for 5 sec and 72 degree C for 12 sec. After the PCR amplification, melting curve analyses were performed to confirm the absence of primer dimers in the PCR products. The ratios of osteopontin mRNA expression were adjusted by

Fig. 3 The expression of VEGF mRNA was significantly higher in the experimental group than in the control group (P<0.05).

Fig. 4 The expression of HSP70 mRNA was significantly higher in the control group than in the experimental group (P<0.05).

Fig. 5 The expression of Type 1 collagen mRNA was not significantly different among the 3 groups, although a higher tendency was observed in the experimental group.

Fig. 6 The expression of osteopontin mRNA was not significantly different among the 3 groups, although a higher tendency was observed in the experimental group.
the value of the housekeeping gene beta-actin. Four independent experiments were performed for all assays of cell behavior, and 2 specimens of each sample type were used in each experiment.

6. Statistical analysis
Results were analyzed using Fisher-LSD and Tukey-Kramer tests.

Results
1. Finite Element Method
The stress received by the experimental group was approximately 5.8 g/mm² and was 7.4 g/mm² in the normal group. The stress was less both in the superficial layer and in deep areas of experimental group compared to the normal group (Fig. 1)

2. Histopathology (Fig.2)
In the control group, the surface of the stratified squamous epithelium was covered by a keratinized layer with hyperkeratosis and the thickness was just one-half the size between the experimental and the normal groups.

In the experimental group, the keratinized layer was thinner than in the control group and fibrous connective tissue was mainly composed of thick collagen bundles and capillaries. However, there were no significant differences between the control and the experimental groups. There were no inflammatory cell infiltrations in the connective tissue.

In the normal group, there was a tendency to keratinize compared to the control group, however, there was no significant difference compared to the experimental group. Furthermore, elongation of rete ridges tended to be prolonged and edema and dilation of capillaries was sometimes observed. Many capillaries and lymphocyte infiltrates were frequently observed in the connective tissue.

PCNA-positive cells were observed in the basal cell layer and sometimes peri-vascular cells were also seen. However, there were no significant differences between the control and the experimental groups.

3. Expression of mRNAs
The expression of VEGF mRNA (Fig.3) was significantly higher in the experimental group than in the control group (P<0.05). On the other hand, the expression of HSP70 mRNA (Fig.4) was significantly higher in the control group than in the experimental group (P<0.05). The expression of Type I collagen (Fig.5) and osteopontin mRNAs (Fig.6) were not significantly different among the 3 groups, although a higher tendency was observed in the experimental group.

Discussion
Tomofuji et al. evaluated the effects of temperature and silicone rubber bristles of a sonic toothbrush on gingival cell proliferation in dogs using PCNA as a primary antibody. They concluded that the sonic toothbrush with silicone rubber bristles induced gingival fibroblast proliferation to a greater degree than did a manual toothbrush and they concluded that warming the silicone rubber bristles increased their stimulatory effects on the proliferative activity of gingival cells.

Recently, Kusano et al. 9) evaluated the effects of ultrasonic and/or vibrating toothbrushes on cell proliferation and collagen synthesis and concluded that vibratory toothbrushes, but not ultrasonic toothbrushes, induced an increase in collagen density without gingival over-growth.

The level of VEGF increases with stimuli such as hypoxia and mechanical stress, to facilitate vascular growth during wound healing. In the present study, VEGF mRNA expression in the experimental group was significantly higher when compared to the other groups, and this suggests that brushing stress in the experimental group worked favorably to facilitate wound healing. Moreover, while there was no statistically significant difference, osteopontin mRNA expression tended to be higher in the experimental group. During bone formation, osteopontin is expressed before calcification, and its expression is high in activated fibroblasts. Furthermore, finite element method analysis showed that the force reached deep tissue in the control group. In the present study, for the experimental group, brushing applied a greater force to the superficial layer when compared to the control group, thus possibly increasing connective tissue activation and periosteal reactions.

Heat shock proteins are known to respond to various stimuli and maintain homeostasis. For example, Amemiya et al. 10) proved that, in a hypoxic environment, pulp cells express HSP70 to resist ischemia. In the present study, HSP70 expression in the normal bristle group was significantly higher than in the other groups. Furthermore, morphological analyses demonstrated inflammatory reactions, which suggests that normal toothbrushes excessively stimulate gingival connective tissue.

No significant differences existed in type-1 collagen mRNA expression among the 3 groups, and the reason for this is that the study was conducted for only 1 week. However, morphological analyses confirmed numerous collagen fibers, and this suggests that significant differences would be seen in long-term studies. Elevated VEGF stimulates glucose metabolism to induce cell growth and to elevate collagen production.

The above findings suggest that when compared to the normal bristle and control groups, the experimental group was more effective in activating gingival connective tissue.

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References


