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ORIGINAL ARTICLE

Effects of improvement in periodontal inflammation by toothbrushing on serum lipopolysaccharide concentration and liver injury in rats

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Abstract

Objective. Periodontitis increases the serum lipopolysaccharide level, contributing to liver injury. Toothbrushing improves periodontitis and may also affect serum lipopolysaccharide concentration and periodontitis-induced liver injury. The purpose of the present study was to examine whether the improvement in periodontal inflammation by toothbrushing clinically affects the serum lipopolysaccharide level and hepatic pathological changes in rat periodontitis. ***Material and methods.*** Thirty male Wistar rats were divided into 5 groups, 2 groups receiving topical application of pyrogen-free water to the gingival sulcus for 4 or 8 weeks. The next 2 groups received topical application of lipopolysaccharide and proteases for 4 or 8 weeks. The last group received topical application of lipopolysaccharide and proteases for 8 weeks, and the palatal gingiva was brushed with a powered toothbrush once a day for 4 weeks prior to the end of the experimental period. ***Results.*** Topical application of lipopolysaccharide and proteases induced not only periodontal inflammation but also an elevation in the serum lipopolysaccharide concentration, with increasing hepatic inflammation, steatosis and 8-hydroxydeoxyguanosine levels in a time-dependent manner. The rats that received gingival stimulation showed decreased polymorphonuclear leukocyte infiltration and collagen loss levels in the periodontal lesions. Furthermore, this group also showed a decrease in serum lipopolysaccharide concentration and hepatic inflammation, steatosis and 8-hydroxydeoxyguanosine levels, compared with the group receiving no treatment. ***Conclusions.*** Toothbrushing promoted healing of periodontal lesions, decreased serum lipopolysaccharide concentration and suppressed liver injury in a rat periodontitis model.

Key Words: *Lipopolysaccharide, liver injury, toothbrushing, periodontitis, rats*

Introduction

Periodontitis is a chronic inflammatory disease of the tooth-supporting structures [1]. In periodontal lesions, gingival connective tissue exhibits polymorphonuclear leukocyte (PMN) infiltration and collagen loss [2]. These pathological changes result in loose spaces within the periodontal tissue, i.e. conditions that enable easy transfer of bacterial components [e.g. lipopolysaccharide (LPS)] from the periodontal pockets to the blood stream [3]. In fact, LPS applied in the gingival sulcus is transferred to blood vessels 2 h after application [4]. However, it is unclear whether an elevation in serum LPS concentration following periodontitis is detrimental to the general health.

LPS elicits a wide variety of host defense responses to severe tissue injury, including liver injury [5]. Epidemiological studies suggest a relationship between periodontitis and liver diseases [6,7]. In addition, studies on animals have demonstrated that periodontitis may be a risk factor for progression of non-alcoholic fatty liver diseases [8] and alcoholic fatty liver disease [9]. It is therefore conceivable that periodontitis could damage hepatic health due to the increased serum LPS concentration.

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Toothbrushing decreases inflammatory cell infiltration and stimulates collagen synthesis in the periodontal tissue [10], indicating that toothbrushing can reduce tissue destruction in the periodontal lesion. If the level of serum LPS concentration is dependent on the severity of periodontal tissue destruction, the improvement in tissue destruction by toothbrushing may suppress elevated serum LPS concentration induced by periodontitis. Such situations may also have the beneficial effects on suppression of liver injury induced by periodontitis.

In the present work, we hypothesized that the improvement in periodontal inflammation by toothbrushing might suppress liver injury by decreasing serum LPS concentration. Our aim was to investigate the effect of the improvement in periodontal inflammation by toothbrushing on serum LPS concentration and liver injury in a rat periodontitis model. The scores of steatosis, inflammation, and necrosis, and the concentration of 8-hydroxydeoxyguanosine (8-OHdG) (an indicator of oxidative DNA damage), were measured to evaluate the liver injury. In addition, the concentrations of serum tumor necrosis factor (TNF)-α and serum reactive oxygen species (ROS) were quantified as periodontitis-associated circulating risk factors other than LPS [8].

Material and methods

Animals

The 30 male Wistar rats (8 weeks old) used in this study were housed in an air-conditioned room (23–25°C) with a 12 h light-dark cycle. They had free access to water and powdered food (Oriental Yeast Co., Osaka, Japan). All experimental procedures were performed in accordance with the regulations of the Animal Research Control Committee of the Okayama University Dental School.

Experimental design

The rats were randomly divided into 5 groups of 6 rats each. Two groups (control groups) received topical application of pyrogen-free water (Otsuka Medical Co., Tokyo, Japan) to the gingival sulcus for 4 or 8 weeks. The next 2 groups (periodontitis groups) received topical application of LPS and proteases [*Escherichia coli* LPS (Sigma Chemical Co., St. Louis, Mo., USA) and *Streptomyces griseus* proteases (Sigma Chemical Co.)] for 4 or 8 weeks [8]. The last group (the stimulation group) received topical application of LPS and proteases for 8 weeks, and the palatal gingiva was stimulated with a powered toothbrush (TwinPecker, S. E. W. Co., Osaka, Japan) once a day for 4 weeks prior to the end of the experimental period [11]. Twenty-five μg/μl LPS (0.5 μl × 3 times) and 2.25 U/μl proteases (0.5 μl × 3 times) or pyrogen-free water (0.5 μl × 6 times) were introduced by micropipette into the gingival sulcus of both maxillary first molars. Toothbrushing was done at 0.02 N for 10 s after calibration of the brushing force [11]. There was an interval of 10 min between each application of LPS or proteases and between application of LPS/proteases and toothbrushing. No treatment including application of LPS/proteases and toothbrushing was performed on the last day of the experimental period, when the rats were killed and blood samples collected. All treatments were performed under inhalative anesthesia of an O_2-isoflurane mixture.

Measurement of serum LPS, TNF-α, and total ROS

Blood samples were collected directly from the heart of 24-h-fasted animals. Serum was separated by centrifugation at 1500*g* for 15 min. Levels of serum LPS were determined using a kinetic Limulus Amebocyte Lysate test kit (Wako, Osaka, Japan). Serum levels of TNF-α were quantified with a rat TNF-α enzyme-linked immunosorbent assay kit (Biosource International, Camarillo, Calif., USA). The level of serum total ROS was also measured with a spectrophotometer (MDS Inc., Toronto, Canada) with catalytic capability for transition metals [12]. One unit was defined as equivalent to 1 μg H_2O_2/ml.

Histological evaluation

After the experimental period, the animals were killed under general anesthesia. Samples of the maxillary molar regions and the liver were resected from each rat and immediately fixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer (pH 7.4) for 1 day. Periodontal tissues were further subjected to decalcification with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 weeks. The formalin-fixed tissue samples were embedded in paraffin and stained with hematoxylin and eosin. Furthermore, frozen sections obtained from paraformaldehyde-fixed, optimum cutting temperate compound-embedded liver samples were stained with oil red O for detection of the presence of lipids.

A single examiner, blind to the treatment assignment, did the histometric analyses. The number of PMNs in two standard areas (0.05 × 0.05 mm each) of the connective tissue subjacent to the junctional epithelium was determined under a magnification of × 400 [13]. The distances between the cementoenamel junction (CEJ) and the most apical portion of the collagen destruction, and between the CEJ and alveolar bone crest, were also measured with a microgrid at a magnification of × 200 [2].

Liver pathology was scored as described in a previous study [14]: steatosis (the percentage of liver cells containing fat, which was confirmed by staining with oil red O): $<25\% = 1+$; $<50\% = 2+$;

Table I. Histometric analysis of rat periodontal tissue [mean (SD)]

	4 weeks		8 weeks		
	Control ($n=6$)	Periodontitis ($n=6$)	Control ($n=6$)	Periodontitis ($n=6$)	Toothbrushing ($n=6$)
Polymorphonuclear leukocyte density (number/0.05 mm × 0.05 mm)	1.4 (0.5)	2.3 (0.2)[a]	1.1 (0.3)	3.2 (0.7)[b, c]	1.5 (0.4)[d]
Linear distances between the cemento-enamel junction and the most apical portion of collagen destruction (μm)	0 (0)	21 (7)[a]	0 (0)	72 (53)[b, c]	1 (2)[d]
Linear distances between the cemento-enamel junction and the alveolar bone crest (μm)	588 (92)	709 (80)[a]	659 (127)	1,010 (114)[c]	846 (87)

[a] $p<0.05$, compared to the control group (4 weeks), according to the Mann-Whitney *U*-test.
[b] $p<0.05$, compared to the periodontitis group (4 weeks), according to the Mann-Whitney *U*-test.
[c] $p<0.01$, compared to the control group (8 weeks), according to the Kruskal-Wallis test followed by Bonferroni's correction of the Mann-Whitney *U*-test.
[d] $p<0.01$, compared to the periodontitis group (8 weeks), according to the Kruskal-Wallis test followed by Bonferroni's correction of the Mann-Whitney *U*-test.

$<75\% = 3+$; $>75\% = 4+$; inflammation and necrosis: 1 focus per low-power field $= 1+$; 2 or more $= 2+$. In each rat, 10 areas from 3 sections were randomly selected for histological evaluation. In those, a maximum score per rat was used as the results of liver pathology [8].

Assay of 8-OHdG in the liver

The mitochondrial DNA was extracted from liver homogenate using a commercially available kit (Wako). The concentration of 8-OHdG per mitochondrial DNA was determined using an enzyme-linked immunosorbent assay detection kit (Japan Institute for the Control of Aging) [9]. The assays were performed in triplicate.

Statistical analysis

All data were expressed as means and standard deviation. Differences between the groups at the 4-week time-point or between the groups at different time-points were identified by Mann-Whitney *U*-test. Differences at the 8-week time-point among the 3 groups were analyzed using the Kruskal-Wallis test followed by Bonferroni's correction of the Mann-Whitney *U*-test.

Results

There were no significant differences among the groups in terms of food consumption and body weight during the experimental period. In addition, no rats have gingival swelling and redness at baseline.

The density of PMNs, the distance between the CEJ and the most apical portion of the collagen destruction (collagen loss level) and the distance between the CEJ and alveolar bone crest (alveolar bone loss level) were greater in the periodontitis group than in the control group at 4 and 8 weeks (Table I). In the periodontitis group, both the density of PMNs and collagen loss level increased in a time-dependent manner. The density of PMNs and the collagen loss level were lower in the toothbrushing group than in the periodontitis group at 8 weeks. There was no significant difference in the alveolar bone loss level between the periodontitis and toothbrushing groups.

No significant difference in the serum LPS concentration was detected between the control and

Table II. Serum levels of lipopolysaccharide, TNF-α, and total ROS in rats [mean (SD)]

	4 weeks		8 weeks		
	Control ($n=6$)	Periodontitis ($n=6$)	Control ($n=6$)	Periodontitis ($n=6$)	Toothbrushing ($n=6$)
Lipopolysaccharide (pg/ml)	189 (29)	165 (57)	222 (49)	437 (119)[b, c]	153 (106)[d]
TNF-α (pg/ml)	14 (4)	21 (3)[a]	8 (4)	19 (7)[c]	16 (10)[c]
Total ROS (unit)	108 (17)	141 (9)[a]	80 (21)	125 (10)[c]	111 (17)[c]

[a] $p<0.05$, compared to the control group (4 weeks), according to the Mann-Whitney *U*-test.
[b] $p<0.05$, compared to the periodontitis group (4 weeks), according to the Mann-Whitney *U*-test.
[c] $p<0.01$, compared to the control group (8 weeks), according to the Kruskal-Wallis test followed by Bonferroni's correction of the Mann-Whitney *U*-test.
[d] $p<0.01$, compared to the periodontitis group (8 weeks), according to the Kruskal-Wallis test followed by Bonferroni's correction of the Mann-Whitney *U*-test.

periodontitis groups at 4 weeks; however, the level of serum LPS was twice higher in the periodontitis group than in the control group at 8 weeks (Table II). The level of serum LPS was lower in the toothbrushing group than in the periodontitis group at 8 weeks. In addition, the levels of serum TNF-α and serum total ROS were significantly higher in the periodontitis group than in the control group at 4 and 8 weeks.

The liver tissue in the periodontitis group showed mild hepatic steatosis (Figure 1B), while those in the control and toothbrushing groups showed little pathological changes (Figure 1A and C). In the periodontitis group, lipid droplets were also detected by oil red O staining (Figure 1B, inset). Inflammation score in the periodontitis group was higher than in the control group at 4 and 8 weeks (Table III). Furthermore, steatosis score in the periodontitis group was also higher than in the control group at 8 weeks. On the other hand, steatosis and inflammation scores in the toothbrushing group were lower than those in the periodontitis group at 8 weeks. There were no significant differences in the necrosis score among the groups at 4 and 8 weeks.

The level of hepatic 8-OHdG was higher in the periodontitis group than in the control group at 4 and 8 weeks, and the level in the periodontitis group increased in a time-dependent manner (Figure 2). Hepatic 8-OHdG level in the toothbrushing group was lower than that in the periodontitis group at 8 weeks; however, this value was higher compared with the control group.

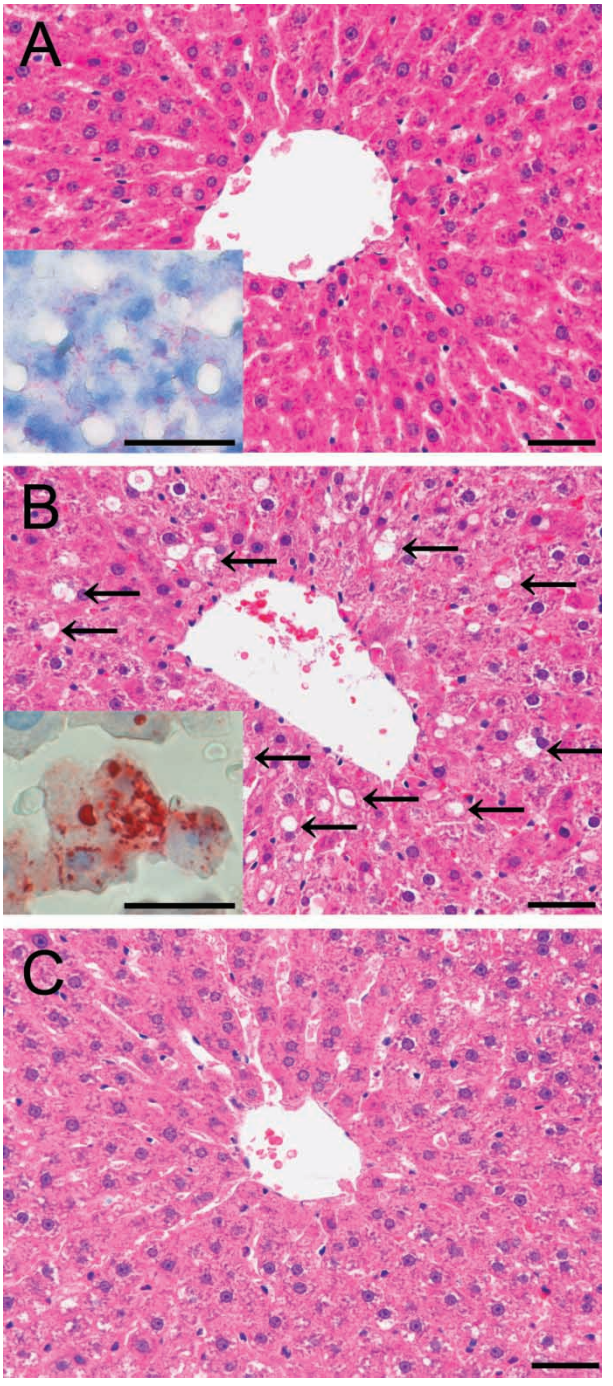

Figure 1. Liver stained with hematoxylin and eosin in the control group (A), periodontitis group (B), and toothbrushing group (C) at 8 weeks. Few pathological changes were found in the control group (A) and toothbrushing group (C). The periodontitis group exhibited mild hepatic steatosis (arrows) (B). Variously sized lipid droplets were stained positively with oil red O in the periodontitis group (inset in B), but not in the control group (inset in A). Bar = 25 μm.

Discussion

In the present study, chronic administration of LPS and proteases to the gingival sulcus increased periodontal inflammation, serum levels for TNF-α and total ROS and hepatic 8-OHdG level at 4 weeks. At 8 weeks, not only serum levels for TNF-α and total ROS but also serum LPS concentration was elevated by induction of periodontitis, with an increase in the hepatic steatosis, inflammation and 8-OHdG level. These findings indicate that in periodontitis-induced rats liver injury was induced through increased serum TNF-α and ROS, and that an increased serum LPS is important in the progression of periodontitis-induced liver injury. In addition, the rats that further received toothbrushing showed a reduction of serum LPS concentration at 8 weeks. It is conceivable that toothbrushing could reduce the load of LPS within the gingival sulcus and therefore decreased the systemic levels. Furthermore, this group also showed suppression in the liver injury. Toothbrushing would inhibit liver injury by decreasing serum LPS in a periodontitis model.

In our findings, the elevation in serum LPS concentration was not found in the periodontitis group at 4 weeks. However, serum LPS concentration was elevated at 8 weeks, with increasing PMN density and collagen loss in the periodontal lesion. These observations suggest that the increased serum LPS concentration in periodontitis depended on the degrees of periodontal PMN density and connective tissue destruction. On the other hand, toothbrushing decreased periodontal PMN density, contributing to

Table III. Scores of steatosis, inflammation, and necrosis of rat liver

	4 weeks		8 weeks		
	Control ($n=6$)	Periodontitis ($n=6$)	Control ($n=6$)	Periodontitis ($n=6$)	Toothbrushing ($n=6$)
Steatosis score					
<25% (1+)	6	3	6	1	3
25% to 50% (2+)	0	3	0	2	3
50% to 75% (3+)	0	0	0	2	0
>75% (4+)	0	0	0	1	0
Inflammation score					
0	6	0	4	0	2
1+	0	6	2	0	3
2+	0	0	0	6	1
Necrosis score					
0	6	4	5	3	3
1+	0	2	1	3	3
2+	0	0	0	0	0

reduction of serum LPS concentration. Toothbrushing may suppress the transfer of LPS from gingival sulcus to blood stream by not only the decrease in LPS within the gingival sulcus but also healing of inflamed gingival tissue and reduction of PMN infiltration [11].

However, the levels of serum TNF-α and serum total ROS did not change following gingival stimulation. Therefore, it may be considered that the improvement in liver injury might be incomplete (Figure 2). One possible explanation is that LPS and proteases can stimulate production of TNF-α and ROS within both the gingival connective tissue subjacent to the junctional epithelium and the periodontal ligament [15], but the histological improvement by toothbrushing may be limited to connective tissue subjacent to the junctional epithelium [16]. In addition to toothbrushing, periodontal treatment that reaches the periodontal ligament (i.e. subgingival scaling and root and planing) would be necessary to decrease the serum levels for TNF-α and total ROS in periodontitis.

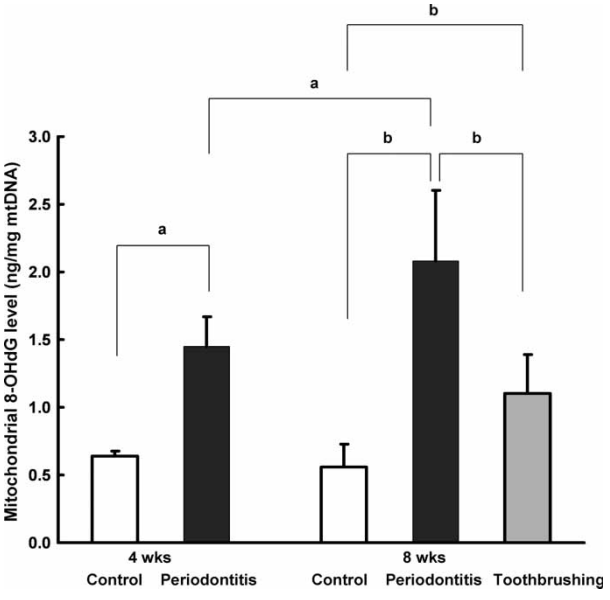

Figure 2. The levels of mitochondrial 8-OHdG in the rat liver. [a]$p<0.05$, using the Mann-Whitney *U*-test. [b]$p<0.01$, using the Kruskal-Wallis test followed by Bonferroni's correction of the Mann-Whitney *U*-test.

In a previous study, we observed that the improvement in periodontitis by toothbrushing decreased plasma 8-OHdG level [11]. In that study, we were unable to explain how reduction of circulating 8-OHdG affected systemic organs. The present study revealed that a decrease in serum LPS by toothbrushing could suppress liver injury in the periodontitis model.

Clinical studies have indicated that periodontal treatment may improve periodontitis-driven impaired serum LDL cholesterol [17], increased C-reactive protein [18], increased interleukin 6 [19], and increased glycated hemoglobin levels [20]. Our present study in animals has revealed that toothbrushing suppresses the increased LPS concentration during periodontitis progression. These observations support the concept that local treatment of periodontitis could be clinically effective not only for periodontal inflammation, but also for prevention of systemic diseases induced by periodontal inflammation.

In the current study, we used a model of topical application of LPS and proteases to the gingival sulcus to induce periodontal inflammation. It was chosen since it mimics several features of human periodontitis, including the inflammatory cellular infiltrate, periodontal pocket formation, and bone loss [2]. However, the LPS and proteases used derived from *E. coli* and *S. griseus*, respectively, which are not periodontal pathogens. The results would have more validity if periodontal pathogens (i.e. *Aggregatibacter actinomycetemcomitans* LPS) had been applied to induce periodontitis.

In conclusion, the improvement in periodontal inflammation by toothbrushing could decrease serum lipopolysaccharide concentration and suppress liver injury in the rat periodontitis model. Periodontal treatment including toothbrushing may

be effective in preventing systemic diseases induced by periodontal inflammation.

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