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Supplementation of green tea catechins in dentifrices suppresses gingival oxidative stress and periodontal inflammation

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ABSTRACT

Objective: This study examined the effects of a dentifrice containing green tea catechins on gingival oxidative stress and periodontal inflammation using a rat model.

Design: Twenty-four male Wister rats were randomly divided into four groups. The first group (Control group) received no treatment for 8 weeks. Periodontal inflammation was induced in the second group for 8 weeks. Periodontal inflammation was induced in the last two groups for 8 weeks and dentifrices with or without green tea catechins were topically applied to the gingival sulcus daily for 4 weeks prior to the end of the experimental period.

Results: Rats that had experimental periodontal inflammation showed apical migration of the junctional epithelium, alveolar bone loss and inflammatory cell infiltration in the connective tissue subjacent to the junctional epithelium at 8 weeks, whilst the control group showed no pathologic changes. Topical application of a green tea catechin-containing dentifrice reduced inflammatory cell infiltration in the periodontal lesions to a greater degree than the control dentifrice at 8 weeks. The gingiva in which green tea catechin-containing dentifrice was applied also showed a lower level of expression of hexanoyl-lysine (a marker of lipid peroxidation), nitrotyrosine (a marker of oxidative protein damage), and tumour necrosis factor-α (an indicator of pro-inflammatory cytokines) at 8 weeks compared to gingiva in which the control dentifrice was applied.

Conclusions: Adding green tea catechins to a dentifrice may contribute to prevention of periodontal inflammation by decreasing gingival oxidative stress and expression of pro-inflammatory cytokines.

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Abbreviations: ABC, alveolar bone crest; CEJ, cemento-enamel junction; HEL, hexanoyl-lysine; JE, junctional epithelium; LPS, lipopolysaccharide; PMNs, polymorphonuclear leucocytes; NF-κB, nuclear factor-κB; ROS, produce reactive oxygen species; TNF-α, tumour necrosis factor-α.

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1. Introduction

Periodontitis is a chronic inflammatory disease caused by oral bacterial infection.[1] Imbalance between the levels of bacterial pathogens (e.g., lipopolysaccharide (LPS) and proteases) and the host immune response to infection contributes to the initiation or progression of periodontitis. In a periodontal lesion, polymorphonuclear leucocytes (PMNs) produce reactive oxygen species (ROS) as the initial host defence against bacterial pathogens.[2] However, excessive production of ROS has a detrimental effect on the host defence system,[3] and induces the oxidation of lipids and protein, contributing to tissue damage (oxidative stress).[4] Investigations have demonstrated that oxidative stress plays a key role in the progression of periodontal inflammation.[5–8] Therefore, antioxidant therapy targeting periodontal lesions may offer clinical benefits in the management of periodontal inflammation.

Green tea is a non-oxidized and non-fermented product that contains several polyphenolic components, also called catechins, including epigallocatechin-3 gallate, epicatechin-3 gallate, epicatechin, and epigallocatechin.[9] Green tea catechins have been shown to possess potent antioxidant activity several times higher than that of vitamin C and vitamin E.[10] It has been reported that a dentifrice containing vitamin C was effective in inhibiting gingival oxidative stress in periodontal lesions.[11] Topical green tea catechins may also be an effective therapeutic agent, acting to suppress periodontal inflammation with decreasing gingival oxidative stress.

An epidemiological study indicated an inverse association between green tea intake and clinical periodontal parameters (probing depth, clinical attachment level, and bleeding on probing).[12] A clinical study also reported that the slow-release local delivery of green tea catechins strengthened the effects of traditional periodontal treatment on improvement of periodontitis.[13] Furthermore, an animal study demonstrated that injection of green tea catechins into LPS-induced inflamed gingivae suppressed alveolar bone loss by inhibiting pro-inflammatory cytokine production.[14] However, it is still unclear how topical application of a dentifrice containing green tea catechins to periodontal lesions affects gingival oxidative stress and periodontal inflammation.

In the present work, we hypothesized that topical application of a dentifrice containing green tea catechins to periodontal lesions might suppress periodontal inflammation with decreasing gingival oxidative stress. The present study examined the preventive effects of supplementation of green tea catechins to the dentifrice on gingival oxidative stress and periodontal inflammation using a rat model. Immunohistochemical analyses of hexanoyl-lysine (HEL) (a marker of lipid peroxidation),[15] nitrotyrosine (a marker of oxidative protein damage),[16] and nuclear factor-κB (NF-κB) (an oxidative stress-sensitive nuclear transcription factor)[17] were performed to evaluate gingival oxidative stress. In addition, expression of tumour necrosis factor-α (TNF-α) was analysed as a parameter related to periodontal inflammation.[18]

2. Materials and methods

2.1. Animals

All experimental procedures were performed in accordance with the institutional guidelines of the Animal Research Control Committee of Okayama University Dental School. The 24 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 standard food (Oriental Yeast Co., Tokyo, Japan) during the experimental period.

2.2. Experimental design

The rats were randomly divided into four groups of six rats each. The first group (Control group) received no treatment for 8 weeks. In the next group (Periodontal inflammation group), periodontal inflammation was induced for 8 weeks. The last two groups (Periodontal inflammation + Control dentifrice [Periodontal inflammation + CD] group and Periodontal inflammation + green tea catechin-containing dentifrice [Periodontal inflammation + GTCD] group) had experimental periodontal inflammation for 8 weeks and received topical application of a green tea catechin-containing dentifrice or a control dentifrice not containing tea catechins to the gingival sulcus daily for 4 weeks. Treatment with a dentifrice was started 4 weeks after periodontal inflammation induction, and has further been continued until 8 weeks were completed.

The green tea catechin-containing dentifrice included 1.0% green tea catechins (Sunphenon 100S; Taiyo Kagaku, Mie, Japan),[14] whilst the control dentifrice did not contain any catechin (Table 1). To induce experimental periodontitis, 25 μg/μL lipopolysaccharide (LPS) from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO, USA) and 2.25 U/μL proteases from *Streptomyces griseus* (Sigma Chemical Co.) were applied to the palatal gingival sulcus of both maxillary first molars.[19] In the Periodontal inflammation + CD and Periodontal inflammation + GTCD groups, after application of LPS and protease, the dentifrices were further applied to the same area of the maxilla.[11] LPS (0.5 μL × 2 times), proteases (0.5 μL × 3 times), or dentifrices (0.5 μL × 1 time) were introduced daily with a micropipette under inhalative anaesthesia with an O_2–isoflurane mixture. There was an interval of 10 min between application of LPS/proteases and dentifrices. The applied dentifrice was wiped off using a cotton ball after 10 min.

2.3. Histological and immunohistochemical analysis

After the experimental period, rats were sacrificed under deep anaesthesia with diethyl ether. 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 weeks at 4 °C. Paraffin-embedded buccolingual sections (4 μm thick) were stained with haematoxylin and eosin or other stains, as described below.

Commercial kits (Histofine Simple Stain MAX PO; Nichirei Co., Tokyo, Japan)[20] were used to determine the level of expression of TNF-α (R&D Systems, Minneapolis, MN, USA),[18] NF-κB (Abcam, Tokyo, Japan),[15] HEL (Japan Institute for the

Table 1 – Composition of control and experimental dentifrice (%).

Component	Control dentifrice	Green tea catechin-containing dentifrice
Glycerine	6	6
Carageenan	1	1
Sodium carboxymethyl cellulose	1	1
Ethanol	4	4
Sucrose esters of fatty acids	0.2	0.2
Flavour	0.1	0.1
Tea extract[a]	0	1.4
Distilled water	Balance	Balance

[a] Tea extract was composed of 18.0% (−)-epigallocatechin-3-O-gallate, 11.6% (−)-gallocatechin-3-O-gallate, 4.6% (−)-epicatechin-3-O-gallate, 15.0% (−)-epigallocatechin, 14.8% (+)-gallocatechin, 7.0% (−)-epicatechin, and 3.5% (+)-catechin. Final concentration of tea catechin in dentifrice was 1%.

Control of Aging, Shizuoka, Japan), and nitrotyrosine (Upstate Biotechnology Inc., Lake Placid, NY, USA) with antibodies against each substance diluted to 1:200, 1:100, 1:50, and 1:50 in phosphate-buffered saline, respectively. The colour was developed with 3-3′-diamino bentizine tetrahydrochloride, and sections were counterstained with Mayer's haematoxylin.

A single examiner (T.T.), blinded to the treatment assignment, performed the following histometric analyses using a microscope (Olympus Co., Tokyo, Japan). The distances between the cemento-enamel junction and the alveolar bone crest (CEJ–ABC) and between the cemento-enamel junction and the most apical portion of the junctional epithelium (CEJ–JE) were measured with a microgrid at a magnification of 200×.[21] The PMNs per unit area (0.05 mm × 0.05 mm) of the connective tissue subjacent to the junctional epithelium were counted at a magnification of 400×.[21] The numbers of TNF-α, NF-κB, HEL, and nitrotyrosine-positive cells and total cells per unit area (0.01 mm × 0.01 mm) were determined in the connective tissue subjacent to the junctional epithelium at a magnification of 400×. The ratios of each substance-positive cell to total cells were calculated. We evaluated the intra-examiner reproducibility by double-scoring 15 randomly selected sections at 2-week intervals. Agreement with one cell of PMNs and TNF-α, NF-κB, HEL, and nitrotyrosine-positive cells was more than 90%, respectively.

2.4. *Statistical analysis*

All data were expressed as means and standard deviation. Differences at the 8-week time-point amongst the four groups were analysed using the Kruskal–Wallis test followed by Bonferroni's correction of the Mann–Whitney *U*-test. A $p < 0.01$ was considered to be statistically significant.

3. Results

There were no significant differences in body weight and food consumption amongst the four groups during the experimental period.

In the periodontal tissue, the Periodontal inflammation + CD group showed apical migration of junctional epithelium and inflammatory cell infiltration in the connective tissue at 8 weeks, whilst the Control group showed no pathologic changes (Fig. 1). The CEJ–JE, CEJ–ABC, and the number of PMNs were significantly greater in the Periodontitis group than in the Control group at 8 weeks (Table 2). These parameters were also greater in the Periodontal inflammation + CD group than in the Control group at 8 weeks, and there was no significant difference between the Periodontal inflammation and Periodontal inflammation + CD groups. On the

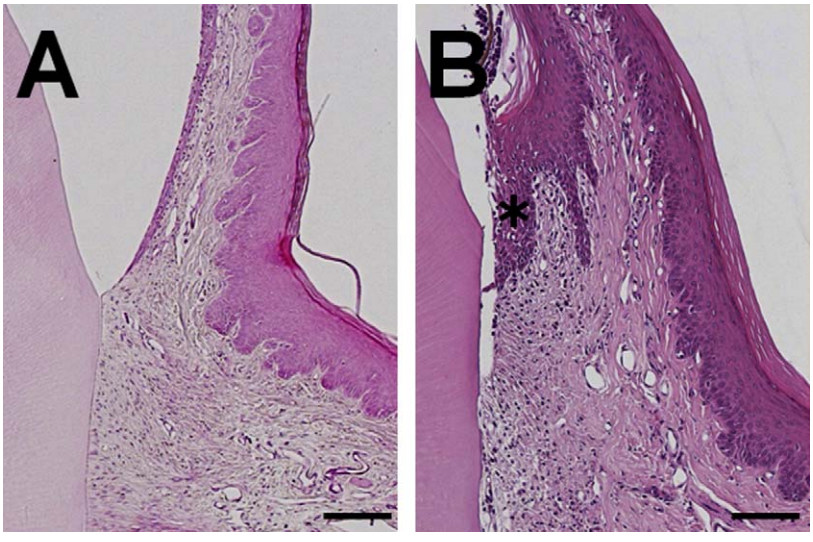

Fig. 1 – Histological analysis of rat periodontal tissue at 8 weeks. No pathological changes were observed in the periodontium in any of the samples in the control group (A). The Periodontal inflammation + Control dentifrice group (B) showed apical migration of the junctional epithelium (asterisk) and polymorphonuclear leucocytes infiltration within the connective tissue subjacent to the junctional epithelium. Scale bar = 100 μm.

Table 2 – Histometric analysis of rat periodontal tissue at 8 weeks (mean ± SD).

	Control ($n = 6$)	Periodontal inflammation ($n = 6$)	Periodontal inflammation + Control dentifrice ($n = 6$)	Periodontal inflammation + green tea catechin-containing dentifrice ($n = 6$)
Linear distances between the cemento-enamel junction and the most apical portion of the junctional epithelium (μm)	0 ± 0	133 ± 102[a]	143 ± 56[a]	29 ± 30[a,b]
Linear distances between the cemento-enamel junction and the alveolar bone crest (μm)	466 ± 40	691 ± 87[a]	697 ± 67[a]	603 ± 131[a]
Polymorphonuclear leucocytes density (number/0.05 mm × 0.05 mm)	1.7 ± 0.2	2.8 ± 0.4[a]	2.5 ± 0.6[a]	1.3 ± 0.3[b]

[a] $p < 0.01$, compared with the Control group, according to the Kruskal–Wallis test followed by the Bonferroni's correction of the Mann–Whitney *U*-test.
[b] $p < 0.01$, compared with the Periodontal inflammation group, according to the Kruskal–Wallis test followed by the Bonferroni's correction of the Mann–Whitney *U*-test.

other hand, the CEJ–JE and the number of PMNs were significantly lower in the Periodontal inflammation + GTCD group than in the Periodontal inflammation + CD group at 8 weeks. The CEJ–ABC was lower in the Periodontal inflammation + GTCD group than in the Periodontal inflammation + CD group at 8 weeks; however, the difference was not significant.

TNF-α, NF-κB, HEL and nitrotyrosine were strongly detected in the cytoplasm of inflammatory cells, endothelial cells, epithelial cells and fibroblasts in the Periodontal inflammation + CD group compared to the Periodontal inflammation + GTCD group (Fig. 2). The ratios of these substance-positive cells to total cells were significantly lower in the Periodontal inflammation + GTCD group than the Periodontal inflammation + CD group by 71, 63, 48 and 75%, respectively (Table 3). No significant difference was detected in the total number of gingival cells between the Periodontal inflammation + CD group and the Periodontal inflammation + GTCD group.

4. Discussion

In the present study, topical application of LPS and proteases to the gingival sulcus induced periodontal inflammation, including apical migration of the junctional epithelium, alveolar bone resorption, and polymorphonuclear leucocyte infiltration at 8 weeks. The degree of apical migration of the junctional epithelium and the number of PMNs within the

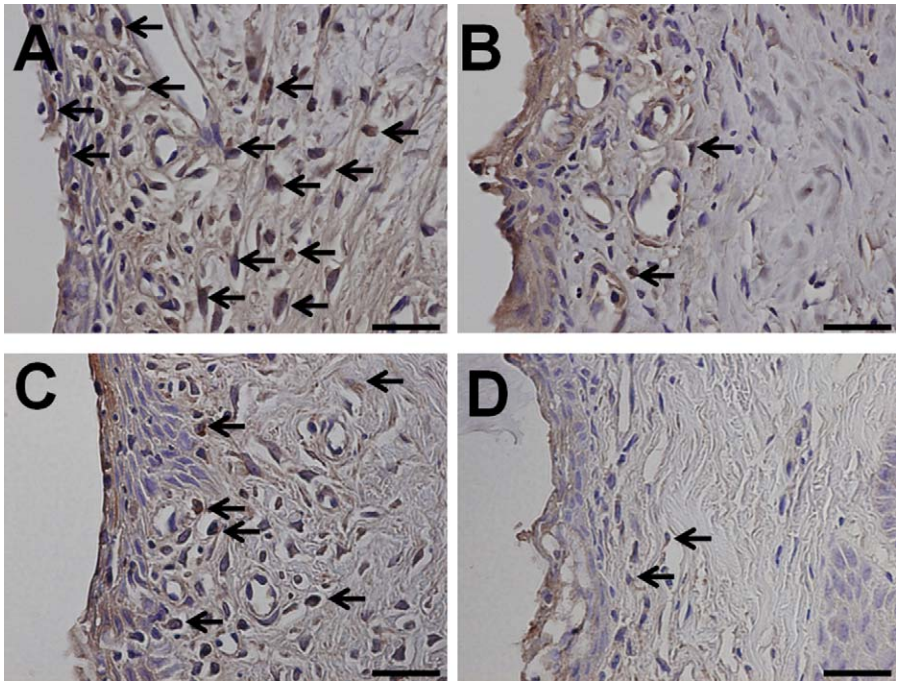

Fig. 2 – HEL-positive cells (A and B) and nitrotyrosine-positive cells (C and D) in rat gingivae. Gingivae received green tea catechin-containing dentifrice (B and D) exhibited less HEL-positive cells (arrow, brown-stained cytoplasm) and nitrotyrosine-positive cells (arrow, brown-stained cytoplasm) than those received control dentifrice (A and C). Bar = 50 μm.

Table 3 – Differences in TNF-α, NF-κB, HEL and nitrotyrosine expression in rat gingiva at 8 weeks (mean ± SD).

Parameters	Periodontal inflammation + Control dentifrice ($n = 6$)	Periodontal inflammation + green tea catechin-containing dentifrice ($n = 6$)
Total number of gingival cells (cell number/0.1 mm × 0.1 mm)	15.7 ± 1.8	14.8 ± 1.4
Ratio of TNF-α positive cells	0.24 ± 0.05	0.07 ± 0.05[a]
Ratio of NF-κB positive cells	0.30 ± 0.06	0.11 ± 0.05[a]
Ratio of HEL-positive cells	0.33 ± 0.09	0.17 ± 0.06[a]
Ratio of nitrotyrosine-positive cells	0.20 ± 0.08	0.05 ± 0.03[a]

[a] $p < 0.01$, compared with the Periodontal inflammation + Control dentifrice group, according to the Mann–Whitney *U*-test.

connective tissue subjacent to the junctional epithelium were lower in the Periodontal inflammation + GTCD group than in the Periodontal inflammation + CD groups. In our model, green tea catechin-containing dentifrice was applied under the condition that the induction of periodontal inflammation was continued. Therefore, these results reveal a preventive aspect of green tea catechin on periodontal inflammation. Furthermore, the periodontal tissue in the Periodontal inflammation + GTCD group showed decreased ratios of gingival cells positive for HEL and nitrotyrosine compared to those in the Periodontal inflammation + CD group. HEL and nitrotyrosine are markers of lipid peroxidation and oxidative protein damage, respectively.[15,16] Oxidative stress plays an important role in the initiation and progression of periodontal inflammation.[5–8] It is feasible that suppression of periodontal inflammation associated with green tea catechin supplementation was in part the result of reduction in oxidative damage.

Recently, considerable interest has arisen concerning the health-promoting potential of green tea, particularly as an anti-oxidant. It has been reported that oral administration of green tea catechins to diabetic rats reduced the level of lipid peroxidation in serum, heart and aorta.[22] It is also known that intraperitoneal administration of green tea catechins (100 mg/kg) reduced the mean hepatic level of lipid peroxidation in rats fed on an atherogenic diet compared to the values in rats fed an atherogenic diet and treated with saline.[23] These observations were consistent with the current findings showing that topical application of a dentifrice containing green tea catechins decreased lipid peroxidation and protein oxidative damage.

Periodontal inflammation induces gingival oxidative stress by potentiating inflammatory responses.[5] In the present study, expression of TNF-α in the periodontal lesions were more than 70% suppressed by green tea catechin supplementation. Green tea catechins may alleviate gingival oxidative stress by decreasing production of pro-inflammatory cytokines. This concept is supported by a previous study reporting that green tea catechins decreased interleukin-1β-positive cells in gingival tissue in response to LPS administration.[14] It is conceivable that green tea catechins could decrease gingival oxidative stress via their immunomodulatory action.

In this study, treatment of the marginal gingivae with a dentifrice containing green tea catechins decreased the number of NF-κB-positive gingival cells. The transcriptional factor NF-κB is involved in regulating the expression of cytokines and other mediators that participate in inflammatory responses, many of which are associated with increased generation of ROS.[24] NF-κB blockade with the green tea catechin-containing dentifrice would also mitigate gingival oxidative stress. This is in agreement with the results of a previous *in vitro* study, which revealed that green tea catechins inhibited NF-κB activation by suppressing I kappa B kinase activity in an intestinal epithelial cell line.[25]

Green tea catechins have been found to counteract inflammation-induced bone loss in animal studies.[26,27] However, in this study, the degree of alveolar bone loss did not change following topical migration of dentifrices containing green tea catechins. One explanation is that the period of application of green tea catechins was too short to influence the mechanisms of alveolar bone loss. Also, because the dentifrice containing green tea catechins was applied to the gingival margin and was influenced by the gingival crevicular fluid, the volume of green tea catechins absorbed through the epithelium, which actually affected the connective tissue, may not have been enough to improve alveolar bone loss.

The use of therapeutic agents in dentifrices is a well-established approach for improving periodontal health.[28] A previous study reported that supplementation of vitamin C in dentifrices is effective to improve gingival oxidative stress and periodontal inflammation.[11] The present study revealed that green tea catechins are also an effective agent that prevents gingival oxidative damage and periodontal inflammation. Supplementation of antioxidants in dentifrices would offer a beneficial option for improvement and prevention of periodontal inflammation.

In this study, the treatment with a green tea catechin was performed 10 min after application of LPS and proteases, since an interval of 10 min between each application of LPS or proteases was judged enough to ensure that the solutions were penetrated into the gingival sulcus in our previous study.[19] In other words, we had an interval of 10 min between application of LPS/proteases and dentifrices in order to avoid mixing LPS/proteases and dentifrices in the sulcus. However, it is possible that green tea catechins had the direct effects on applied LPS and proteases within the periodontal tissue.

The present study used topical application of LPS and proteases to the gingival sulcus to induce periodontal inflammation. The LPS and proteases used in this study were derived from *E. coli* and *S. griseus*, respectively, which are not periodontal pathogens. Our results would have been more valid if products from periodontal pathogens (i.e., *Aggregatibacter actinomycetemcomitans* LPS) were applied to induce periodontal inflammation. In addition, the LPS/proteases-induced periodontal inflammation differs from human peri-

odontitis. In the future, it might be necessary to examine how green tea catechin affects human periodontitis.

This study has other limitations. For instance, we did not examine the effect of green tea catechins on bacterial pathogens in the periodontium. Further studies are needed to investigate the changes in periodontal microorganisms (e.g., *Porphyromonas gingivalis*) following topical application of green tea catechins. In addition, further detailed investigations such as semi-quantitative western blot analysis for assessing HEL and nitrotyrosine might be helpful to improve the reliability of our data about gingival oxidative stress.

In conclusion, adding green tea catechins to a dentifrice could suppress periodontal inflammation but not bone loss by decreasing gingival oxidative stress in a rat model.

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Competing interests: The authors have no conflicts of interest to declare.

Ethical approval: The experimental protocol was approved by the Animal Research Control Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (OKU-2009373).

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