In vitro effect of hesperidin on root dentin collagen and de/re-mineralization

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The aims of this study were to investigate the effects of hesperidin, a citrus flavonoid, on human root dentin demineralization and collagen preservation, and compare it with chlorhexidine and grape seed extract. Specimens were assigned to different treatment groups: hesperidin, chlorhexidine and grape seed extract. Specimens were subjected to pH cycling by demineralization for 14 h, incubation in testing solutions for 2 h and remineralization in presence of bacterial-derived collagenase for 8 h, for 8 days. Calcium release was measured by means of an atomic absorption spectrophotometer, and degraded collagen matrix was investigated by hydroxyproline assay. Specimens were assessed longitudinally with transverse micro-radiography to investigate lesion depth and mineral loss. In hesperidin and grape seed extract groups, demineralization was reduced when the collagen matrix was preserved. The hesperidin group showed the lowest value in lesion depth and mineral loss, indicating that hesperidin inhibited demineralization and probably enhanced remineralization even under fluoride-free conditions.

Keywords: Hesperidin, Root dentin, Dentin collagen, Dentin demineralization, Dentin remineralization

INTRODUCTION

Root caries is prevalent especially among the elderly population due to gingival recession and exposure of the susceptible root surface11. During root caries development, two stages are distinguished microscopically. In the first stage, dentin minerals are dissolved by acid produced from bacterial bio-films. In the second stage, the demineralized dentin matrix is further degraded and bacteria infiltrate into the inter-tubular area25. It has been suggested that the presence of an organic matrix may reduce the progression of dentin erosion8,9. Dentin is a complex mineralized tissue composed of approximately 70% mineral, 20% organic component and 10% fluid by weight8. Type I collagen fiber accounts for 90% of the organic matrix. The preservation and stability of dentin collagen may be essential during the re-mineralization process since it acts as a scaffold for mineral deposition8. Preservation of collagen matrix would be followed by promotion of remineralization of demineralized dentin, which is one of the important strategies regarding preventive therapies for root caries7,10.

In an attempt to stabilize the collagen matrix, glutaraldehyde and some natural flavonoids such as grape seed extract (GSE) have been used on root caries to investigate their cross-linking effects on demineralized lesion11,12. Another approach which has been considered is to inhibit the proteolytic activity of sound and carious dentin13,14, because the organic matrix of dentin collagen is subjected to degradation by matrix metalloproteinases (MMPs) that present in dentin and saliva15.

Pharmacological studies have reported that MMPs activity was reduced by the application of chlorhexidine (CHX) which in turn leads to the arrest of carious lesion16,17. Natural products have been used as folk medicine for thousands of years and are promising sources for novel therapeutic agents26. They have been the focus of several recent studies potential materials in the prevention of oral diseases, particularly plaque-related diseases, such as dental caries16,17.

A previous study using hesperidin (HPN), a citrus flavonoid, showed that HPN preserved bovine dentin collagen against proteolytic degradation22. It has also been reported that HPN reduced the susceptibility of dentin lesion to acid dependent demineralization with the potential to promote remineralization process22. In this study, we used human root dentin to confirm the effect of HPN to resist collagenous degradation and arrest demineralization. Citrus flavonoids were reported to possess antioxidant20, anti-inflammatory26, anticarcinogenic25, hypoglycemic effects26, and to prevent bone loss27,28. The pH cycling model was employed in the present in vitro study to examine the role of collagenase on remineralization. Demineralized human root dentin was treated using HPN 5,000 ppm, CHX 2,000 ppm or GSE 5,000 ppm for 2 h a day, which were then subjected to pH cycling for 8 days. The purposes of this study were to investigate the effect of HPN on preservation of collagen matrix, and compare it with that of CHX and GSE. The null hypotheses tested were that HPN, CHX and GSE would (i) not prevent collagen degradation and (ii) not influence de/re-mineralization in human root dentin lesion.
MATERIAL AND METHODS

Preparation of human root dentin specimens
Thirty non-caries extracted human third molars were used in this study, according to the protocol approved by the Human Research Ethics Committee, Tokyo Medical and Dental University, Japan. Root slabs were obtained from medial and distal root surfaces of human molar teeth and polished to expose the root dentin and to obtain a smooth surface using a series of silicon carbide papers (280, 400, 600, 800, 1000, 1200, 1500, 2000, 4000) up to 4000-grit. The dentin surfaces were then soaked in 10% H₃PO₄ for 10 s to remove the smear layer. One side of each dentin surface was painted with nail varnish, leaving a base line (1st base line) (Fig. 1).

Lesion formation
After nail varnish application, the specimens were immersed in demineralizing solution (pH=4.5) for 96 h to create pre-lesion. The demineralizing solution contained 50 mL acetic acid, 2.2 mM/L CaCl₂ and 2.2 mM/L KH₂PO₄. After pre-lesion formation, the specimens were painted with another coat of nail varnish adjacent to the 1st coat of the exposed dentin surface to create another baseline (2nd base line) (Fig. 1). The slabs were divided into five groups (Table 1).

Methods of pH cycling
The demineralizing solution contained 50 mmol/L acetic acid, 1.5 mmol/L CaCl₂ and 0.9 mmol/L KH₂PO₄ adjusted to pH 5.0 with KOH. The remineralization solution contained 1.5 mmol/L CaCl₂, 0.9 mmol/L KH₂PO₄, 130 mmol/L KCl and 20 mmol/L HEPES buffer, adjusted to pH 7.0 with KOH. Highly purified collagenase type VII from Clostridium histolyticum (C-0773, Sigma Chemical Co., St. Louis, MO, USA) was further added to obtain a remineralization solution that contains 7.5 U/mL collagenase. The testing solution was prepared by adding HPN (hesperidin, Wako Pure Chemical Industries, Ltd., Tokyo, Japan), CHX (chlorhexidine di-gluconate, Sigma-Aldrich, St. Louis, MO, USA) or GSE (Gravinol® grape seed extract, Kikkoman, Chiba, Japan) to the remineralization solution. The concentrations used for HPN, CHX and GSE were 5,000 ppm, 2,000 ppm and 5,000 ppm, respectively. Each specimen was immersed individually at 37°C in the demineralizing solution for 14 h, in the testing solution with HPN, CHX or GSE for 2 h and in the remineralization solution for 8 h. The specimens were thoroughly rinsed between each immersion with buffer solution containing 130 mmol/L KCl, 20 mmol/L HEPES, adjusted to pH 7.0 with KOH. The pH cycling was performed at 37°C for 8 days.

Chemical analyses
1. Determination of calcium ion release
The demineralizing solution was collected to measure the amount of calcium ion dissolved during the pH cycling. An aliquot of 2.9 mL of 1.67% LaCl₃ 7H₂O in 50 mM HCl was added to each 100 mL of collected solution. The atomic absorption was measured using an atomic absorption spectrophotometer (AA-6300, SHIMADZU. INC, Kyoto, Japan) at 423 nm. The solution after the first demineralization per each sample was used as an individual baseline of calcium release. Then, the total

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Table 1  Study design. Group PC is positive control and group NC is negative control, DS: demineralizing solution; RS: remineralizing solution. \( N=6 \)

<table>
<thead>
<tr>
<th>Group</th>
<th>Demineralization solution for 14 h</th>
<th>Testing solution for 2 h</th>
<th>Remineralization Solution for 8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPN</td>
<td>DS</td>
<td>HPN 0.5%</td>
<td>RS with collagenase</td>
</tr>
<tr>
<td>CHX</td>
<td>DS</td>
<td>CHX 0.2%</td>
<td>RS with collagenase</td>
</tr>
<tr>
<td>GSE</td>
<td>DS</td>
<td>GSE 0.5%</td>
<td>RS with collagenase</td>
</tr>
<tr>
<td>PC</td>
<td>DS</td>
<td>RS</td>
<td>RS without collagenase</td>
</tr>
<tr>
<td>NC</td>
<td>DS</td>
<td>RS</td>
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calcium ion release from the 1st day to the 8th day during the pH cycling was measured and expressed in relative release compared with that of the baseline.

2. Determination of degraded collagen
Degradation of collagen was determined by estimating hydroxyproline, an amino acid characteristic of collagen. The total remineralizing solution was collected after the pH cycling. Sixty micro-liters of the solution (from stock solution collected in each day) were subjected to chemical analysis of degraded collagen using the simplified chloramines-T method. In brief, the solution aliquot was hydrolyzed with 2 N sodium hydroxide by autoclaving at 120°C for 20 min. The chloramines-T was added to the hydrolyzate to allow oxidation, followed by the addition of Ehrlich’s aldehyde reagent for hydroxyproline assay. When chromophore was developed, the absorbance of each specimen was read at 550 nm using a spectrophotometer (BIO-RAD-680 Micro plate reader, BIO-RAD laboratories, Tokyo, Japan) and converted to concentration of hydroxyproline. Amount of hydroxyproline was determined by plotting the value with standard calibration curve.

Transverse micro radiography (TMR) measurements
After the pH cycling for 8 days, the mineral loss (ΔZ, vol% mm) and lesion depth (LD, µm) were examined using TMR. The specimens were sectioned longitudinally through the lesion centre into 220±20 µm thicknesses. The cut sections were put in a solution containing 80% of glycerin and 20% of water to prevent shrinkage. Photo plates (HY2, Konica Minolta Holdings, Inc., Tokyo, Japan) were exposed at 4 mA and 25 kV for 5 min using an X-ray generator (Type SRO-M50, Sofron Company Lit., Tokyo, Japan) together with 15 sheets of an aluminum Al step wedges for calibration. The plates were developed and fixed according to standard techniques. The microradiographs were analyzed under a microscope (BX 41, Olympus Co., Tokyo, Japan) with CCD camera (DP70, Olympus Co., Tokyo, Japan). The LD was defined as the distance from the surface of the second baseline to the lesion where the mineral content was more than 95% of the sound dentin. The ΔZ was determined by plotting the vol% mineral profile towards the LD in each specimen section with the sound dentin set as 48 vol% mineral content.

Statistical analysis
The effect of testing solution on the calcium release, degraded collagen and TMR variables (ΔZ and LD) was tested by one-way ANOVA using statistical software package (SigmaStat Version 16.0, SPSS, Chicago, IL, USA). Where appropriate, post-hoc Tukey multiple comparisons tests were performed on all groups. The level of statistical significance was set at 5%.

RESULTS

The results of chemical analyses are shown in Fig. 2 (a, b). The one-way ANOVA indicated that the effect of testing solutions had a significant difference on the results of chemical analyses (p<0.05 for calcium release; p<0.01 for degraded collagen). A significant difference was revealed in the results between the negative and positive controls (p<0.001 for both calcium release and degraded collagen), indicating that the incubation with the presence of collagenase had an effect of causing mineral dissolution and organic degradation. Regarding calcium release results, the lowest amount was for GSE group, followed HPN and CHX groups, respectively. According to the results of degraded collagen, HPN and GSE groups showed the lowest value with no statistical significant difference when compared with the negative control (p>0.05). The results of TMR measurement are shown in Fig. 3 (a, b). The effect of testing solutions had a significant difference on the TMR variables (p<0.01 for LD and p<0.001 for ΔZ). No significant difference in LD was shown between the positive control and CHX group (p>0.05). The positive control showed a statistical significant ΔZ when compared with HPN and GSE groups. Representative TMR images are shown in Fig. 4 (a–e). The image manifested the effect of HPN on resisting demineralization (Fig. 4a).
DISCUSSION

The chemical analyses demonstrated that HPN, CHX and GSE preserved and stabilized dentin collagen. The calcium release in HPN and GSE groups was significantly different when compared with the positive control, while no significant difference was found between the CHX group and the positive control. Thus, the first null hypothesis was partially rejected. The positive and negative controls were significantly different in TMR measurement. The results of TMR revealed that the LD and ΔZ were lower in groups incubated with HPN and GSE, when compared with the positive control. This result implies that HPN and GSE had the effect to prevent demineralization and/or promote remineralization. Thus, the second null hypothesis was partially accepted.

In a previous study, it was reported that demineralization of the surface was observed on dentin samples when the pH cycling was performed with collagenase even at pH 7.0 in the remineralizing solution. The authors claimed that surface demineralization might occur when mineral contents released as the result of degradation of collagen matrix. Likewise; in our present study, the mineral release might have occurred during remineralizing cycles, which accounted for increased ΔZ for the positive control (using collagenase in the remineralizing solution). Such a mineral release was suppressed for the negative control group (without using collagenase) and those groups using HPN, CHX and GSE where collagen matrix was preserved by the aforementioned agents. Although the amount of mineral release in remineralizing cycles was not chemically analyzed, the results of TMR indicated the effect of stabilized collagen matrix on mineral content in the lesion.

Dentin organic matrix plays an important role in demineralization and remineralization process. The organic layers of dentin are important in hampering the
lesion progression and preventing further demineralization challenges\(^{33}\). In previous studies using collagenase, the proteolytic degradation of demineralized matrix was said to enhance the susceptibility of dentin lesions to acid-dependent demineralization\(^{22,30}\). Recent studies have demonstrated that CHX possesses a potent anti-proteolytic effect due to its ability to inhibit activity of MMPs in carious dentin\(^{10}\). The present study demonstrated a positive effect of CHX in preserving dentin collagen against bacterial proteolysis, which contributed to the low value of organic matrix degradation. However, the TMR measurement showed that the incubation in CHX did not contribute to suppressed \(\Delta Z\). This finding was consistent with that of a previous study involving bovine root dentin\(^{22}\). The preserved organic matrix might hamper further diffusion of calcium and phosphate ions out of the dentinal lesion of caries, resisting further demineralization.

In the HPN and GSE groups, the effect of incubation in HPN and GSE was revealed in terms of preserved collagen and reduced LD and \(\Delta Z\). With the limited findings under TMR analysis, the difference in remineralization process between the flavonoid groups (HPN and GSE) and CHX group could not be thoroughly explained. Further studies should be conducted to investigate the chemical reactions of HPN and GSE with calcium and/or phosphate ion. The suppressed \(\Delta Z\) in the HPN group may be explained by the contribution of HPN on stabilization of the exposed collagen matrix. Xie et al. reported that GSE is a promising agent to be used in non-invasive root caries therapy as remineralization was evident on root dentin most likely through the interaction between GSE and proteins in dentin thus stabilizing of dentin matrix\(^{19}\). Previous studies using glutaraldehyde demonstrated that fixing the collagenous/non-collagenous protein and cross-linking collagen matrix reduced the progression of the root caries development\(^{34,35}\). The stabilized collagen matrix behaves as a mechanical barrier to mineral diffusion\(^{25}\), thereby resisting demineralization and promoting remineralization. The preservation and stability of dentin collagen may be essential during the remineralization process since it acts as a scaffold for mineral deposition\(^{18}\). Furthermore, non-collagenous proteins in dentin that possess the mineral-inductive capacity have a crucial function on the organic matrix for apatite precipitation\(^{50}\). In our speculation, the effect of HPN on remineralization may be related to its interaction with collagen and/or non-collagenous proteins, resulting in stabilizing collagen matrix and induction of remineralization. In fact, the binding capacity of HPN to human serum albumin protein indicated that HPN played a comprehensive role in homeostatic process\(^{36}\).

Within the limits of this in vitro study, the HPN may have the potential to promote remineralization process. The biochemical mechanism of HPN has not been investigated and its application in dentistry has not been developed. Further studies are needed to elucidate the mode of action of HPN on human dental tissue.

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