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Original Article

Inhibitory effect of Salvadora persica extract (Miswak) on collagen degradation in demineralized dentin: In vitro study

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Demineralized dentin;
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Abstract *Background/purpose:* Root dentin is vulnerable to acid attack, suggesting a higher risk of demineralization than coronal enamel. This study aimed to evaluate the inhibitory effect of Miswak extract on collagen degradation of demineralized dentin lesion.
Materials and methods: Demineralized bovine root dentin specimens were treated for 1 h by 20% Miswak extract and 0.12% Chlorehexidine (CHX) as a positive control group, and then subjected to collagenolytic attack (clostridium histolyticum 0.5 CDU/mL, 16 h). These cyclic treatments were repeated for 3 days. After the cyclic treatment, the images of the specimens were captured with a light microscope and the lesion depth of degraded collagen layer of all specimens was measured. The mean lesion depth was calculated and compared between the groups using descriptive and One-way ANOVA followed by Post hoc Tukey's tests. Significant level was set at $p < 0.05$.
Results: The mean lesion depth of CHX (28.6 ± 3.37 μm) had the least value, followed by Miswak (37.5 ± 4.01 μm) then the control (78.4 ± 18.43 μm) group. There was a significant difference in the mean lesion depth among the three groups ($p = 0.000$).

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Conclusion: Miswak aqueous extract from *S. persica* was found to preserve the dentin collagen matrix from collagenase enzyme. This could be due to the organic compounds like flavonoids, saponins, alkaloids, tannins, and others which have been reported in literature. Present finding suggests that Miswak might play a positive effect in dentin caries prevention.

Introduction

Due to the advancement of public health awareness and modern medicine, the life expectancy has increased around the world. Along with the increased expectancy, the number of retained teeth in elderly population is now increasing, resulting in increased risk of root caries occurrence.[1]

Root caries is one of the major reasons of tooth loss in the elderly population.[1] Unlike coronal dentin, root dentin is vulnerable to acid attack, suggesting a higher risk of demineralization than coronal enamel. Therefore, preventing root caries has been recognized as one of the major targets in preventive dentistry in the world.[2]

Several fluoride products such as regular dentifrices and fluoride pastes containing higher concentrations of fluoride were proven to be successful in preventing dentin caries or reverse the incipient dentin lesions.[3,4] Several *in-vitro* studies also reported that inhibition of collagen degradation in demineralized dentin would result in inhibition of mineral loss caused by acid demineralization.[4] The preserved collagen layer act as a diffusion barrier both for inward of proton (H^+) toward the inner dentin tissue and outward diffusion of demineralized mineral ions such as ionized Ca^{2+} and phosphate ions toward the outer demineralization fluids.[5] Thus, inhibition of collagen degradation would be an additive effect on dentin caries development.[5–9]

Dentin collagen degradation is due to the action of collagenolytic enzymes. Several approaches have been studied in an attempt to preserve the dentinal collagen by searching for active materials which might inhibit the degradation processes.[6,7,9–13] Matrix metalloproteinases (MMPs) which are found in dentin tissue and in saliva, are one of the major collagenolytic enzymes responsible for collagen.[11–14] The use of MMPs inhibitors has been suggested as a mean to prevent collagen degradation.[11,12] Inhibition of bacterial growth also prevent collagen degradation by inhibiting the bacteria derived collagenolytic enzyme.[15,16] A further hypothesized theories was that once collagen is denatured to gelatinous substance by chemical, biological and thermal influences, the denatured collagen would be ready to be degraded by other enzymes such as gelatinase and proteinases.[10]

The effect of some chemical agents such as chlorhexidine (CHX) and zinc compounds were assessed for their inhibitory effects on the collagenolytic enzymes. Results of those studies showed positive effects of those products on arresting carious lesion activity.[12,15,17,18] Natural plant derived MMPs inhibitors including green tea polyphenols, particularly epigallocatechin-3-gallate were assessed for the effectiveness in collagen degradation inhibition with positive reported results.[19] In addition, the use of naturally occurring antioxidants or cross-linkers, such as grape seed extract derived proanthocyanidins, fruit extract derived genipin, and citrus fruit derived hesperidin were proved to be effective in preserving the dentin collagen and enhancing the remineralization of dentin caries *in-vitro*.[6,8]

Miswak is an arabic term meaning tooth-cleaning stick, scientifically known as *Salvadora persica* (herein after *S. Persica*) and is the most commonly chewed stick out of 182 plant species.[20,21] Nowadays, Miswak as plant-derivative agent is commonly used in several countries around the world especially in the middle-east. World Health Organization, since 1987, has recommended and encouraged the use of chewing sticks as an active tool for maintaining oral hygiene in areas where such use is habitual.[22] In addition to the mechanical removal of dental bacterial plaque and stimulation of the gingival tissues, there were early reports indicating that Miswak extract has several biological properties including significant anti-bacterial effect,[16,21] antifungal effect,[23] antioxidant effect and anti-cariogenic effect mainly through elevating plaque pH after a sucrose challenge.[24]

Up to our knowledge, there is no study assessed the effect of Miswak extract on collagen preservation. The aim of this study is to evaluate the efficacy of Miswak aqueous extracts on preserving demineralized dentin collagen matrix against bacteria derived collagenase enzyme degradation. The null hypothesis tested was that Miswak extract would not prevent collagen degradation.

Materials and methods

Specimen preparation

Fifty bovine root dentin blocks, approximately 2 mm × 3 mm × 3 mm (width × length × depth) in dimensions, were prepared from freshly extracted sound bovine incisors using a low-speed diamond saw (Isomet, Buehler, IL, USA) under water cooling. They were embedded in self-curing acrylic resin (Shade A2, UNIFAST II; GC, Tokyo, Japan) and the dentin surfaces were polished using 800, 1200 and 2000 grit silicon carbide (SiC) papers (Sankyo, Saitama, Japan). The polished tooth surfaces were

covered by acid-resistant nail varnish (Shiseido, Tokyo, Japan) leaving a window approximately (1.5 mm × 2.5 mm) for treatment. The nail varnished areas served as the reference surface line for measuring the degraded depth of collagen layer.[7] The specimens were demineralized in acetate buffer (0.1 mol/L, pH 4.3) for 3 days at 37 °C (100 mL/10 specimens) to create an incipient lesion.[7] The schematic diagram for specimen preparation and treatment with different test solutions are exhibited in Fig. 1.

Preparation of 20% Miswak aqueous extract and 0.12% chlorhexidine di-gluconate (CHX)

A sample of Miswak was purchased from Saudi Arabia. The Miswak aqueous extract was prepared by following the previous protocol published by Sofrata et al.[20,25] Miswak sticks were dried at room temperature for a few days, then were cut into small pieces and ground to powder in a ball mill or house grinding machine. A concentration of 20% Miswak aqueous extract was prepared by adding 20 g of the powdered Miswak into 100 ml of sterilized and deionized water (SDW) and left for 48 hs at 4 °C.[26] The mixture was then centrifuged at 2200 rpm for 10 min. The supernatant was passed through Millipore filters (0.45 μm pore size; Sigma—Aldrich Chemie GmbH, Germany) to remove possible bacteria contamination. The extract was stored in a sterilized bottle at 4 °C and used within one week.[20,25]

0.12% CHX solution was prepared from 20% CHX (Sigma—Aldrich, St. Louis, MO, USA). It was diluted by SDW and then stocked in refrigerator until use.[18]

Collagenase enzyme preparation

0.5 CDU/mL collagenases enzyme solution was prepared by mixing type IA collagenase (type IA, *Clostridium histolyticum* C-9891, Sigma—Aldrich, Saint Louis, MO, USA) and appropriate amount of SDW buffer solution (50 mmol/L PIPES, 0.15 mol/L NaCl, 5 mmol/L $CaCl_2$, pH 6.5). The collagenase enzyme hereinafter will be Ch-collagenase.[7]

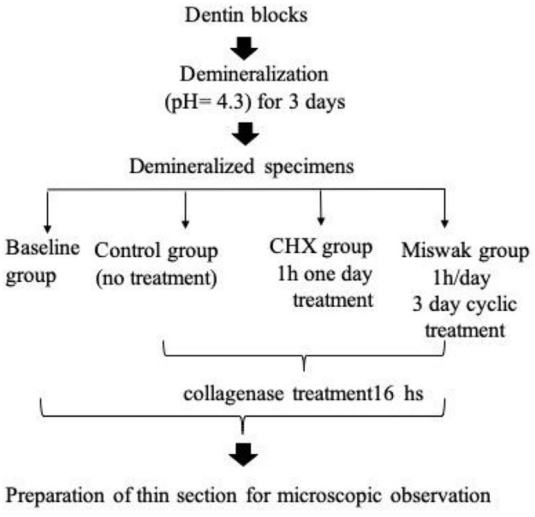


Figure 1 Schematic drawing of experimental steps.

Treatment with test solutions and collagenase challenge

The demineralized specimens were divided into 4 groups, each group having 10 specimens. 1) Miswak group: the specimens were treated for 1 h a day then briefly rinsed by SDW and this treatment was repeated 3 times a day for 3 days. 2) CHX group: the specimens were treated once a day for 1 h and repeated for 3 days and rinsed by SDW. 3) control group: no treatment but only collagenase attack was done. 4) baseline group: no treatment and no collagenase attack were done. All groups were kept in distilled water until collagenase attack was done.

After the treatment, each specimen in the 3 groups without the baseline group was separately incubated in 33 mL of the buffer containing 0.5 CDU/mL Ch-collagenase for 16 hs at 37 °C (Fig. 1).

Light microscope image analysis

The dentin blocks were cut into thin sections (approx. 220 μm) longitudinally with a low-speed diamond saw (Isomet, Buehler, IL, USA) and placed on a glass slide then sandwiched with a cover slide. The images of the specimens were captured with a light microscope (SMZ 1000, Nikon Corp.,Tokyo, Japan) and CCD camera (DS-Fi1, Nikon Corp., Tokyo, Japan). An imaginary line connecting the edges of the lesion window was drawn. The area of degradation and the width of the lesion were calculated from this reference line. The mean depth of degraded collagen layer was obtained by using the following formula;

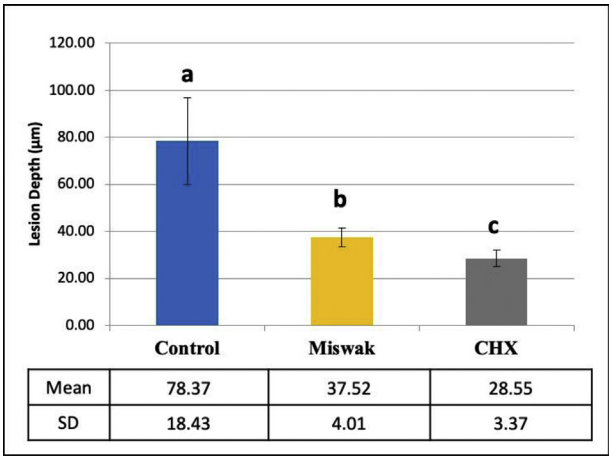
Mean depth of degraded collagen layer (μm) = [area of degraded collagen (μm^2)]/[width of degraded collagen (μm)].[7,27] Image analysis was performed using ImageJ (ImageJ, version1.42q, Wayne Rasband, NIH, USA) by using a customized image processing software.

Statistical analysis

The anti-collagen degradation effects of Miswak and CHX compared to the control group were evaluated using statistical software package (Sigma Stat Version 16.0, SPSS, Chicago, IL, USA). The comparison among the 3 groups (control, Miswak and CHX) were performed by one-way ANOVA test followed by Post hoc Tukey's test when indicated. The level of statistical significance was set at 0.05. Apparent demineralized lesion depth in the baseline group observed by the microscopy was around 170 μm.

Results

Fig. 2 shows comparative data of the mean depth of the degraded collagen layer among the control (78.4 ± 18.43 μm), Miswak (37.5 ± 4.01 μm) and CHX (28.6 ± 3.37 μm) groups, respectively. One-way ANOVA test revealed that there is a significant difference in the mean lesion among the groups ($p = 0.000$). Post Hoc Tukey's test revealed that the mean lesion depth of CHX have the least value, followed by Miswak then the control group.



	Control	Miswak	CHX
Mean	78.37	37.52	28.55
SD	18.43	4.01	3.37

Figure 2 Mean and standard deviations of the lesion depth for the Control, Miswak and CHX group are shown above each bar. Bar plot of means for each level of the independent variable of a one-way analysis of variance (ANOVA). Bars sharing the same letter are not significantly different according to Tukey's HSD test. Chart shows that there is a significant difference in the lesion depth among the three groups ($p = 0.000$).

Fig. 3 shows representative polarized photo images of the dentin sections in the 4 groups: a) baseline, b) control c) Miswak and d) CHX groups, respectively. In Fig. 3a, demineralized layer without loss of collagen matrix layer was observed which was subjected only to demineralization (baseline). In Fig. 3b, cyclic Ch-collagenase treatment in the control group resulted in a remarkable loss of demineralized collagen layer. However, in Fig. 3c and d, Miswak and CHX application treatment resisted the collagen degradation at the demineralized collagen surface,[28] which appeared to be smaller than in the control group.

Discussion

Traditionally, assay of hydroxyproline released from collagen by its degradation has been used as a validated (standard) measure to evaluate the degree of the degradation.[27] Islam et al., in 2016, employed a new method for the assay of collagen degradation, ie, microscopic observation. The latter study demonstrated an excellent correlation between the released amount of hydroxyproline and the depth of the degraded collagen layer with Pearson's coefficient 0.942 and the linearity 0.888. They reported that the new method with microscopic observation was thought to be reliable for collagen degradation assessment. One of the merits using the microscopy is to easily realize the extent of the degraded collagen layer in the demineralized lesion depth just by looking at the visual images of the lesion compared to the method of hydroxyproline assay which provides relative values (µg) among the groups.[27]

The current study compared the efficacy of Miswak extract to CHX as a positive control, because many studies have showed that CHX has effective anti-proteolytic effect by inhibiting the activity of MMPs in carious dentin,[6] as well as inhibiting the effect of Ch-collagenase enzyme.[8] Light microscopy assay of the demineralized dentin specimens in the control group confirmed the loss of collagen matrix due to degradation by the enzyme Ch-collagenase actively (Fig. 3b). Results, under light microscopy, also showed that the lesion depth of the Miswak extract (Fig. 3c) and CHX (Fig. 3d) groups are smaller than the control group. Thus, the present results suggest that Miswak extract has a

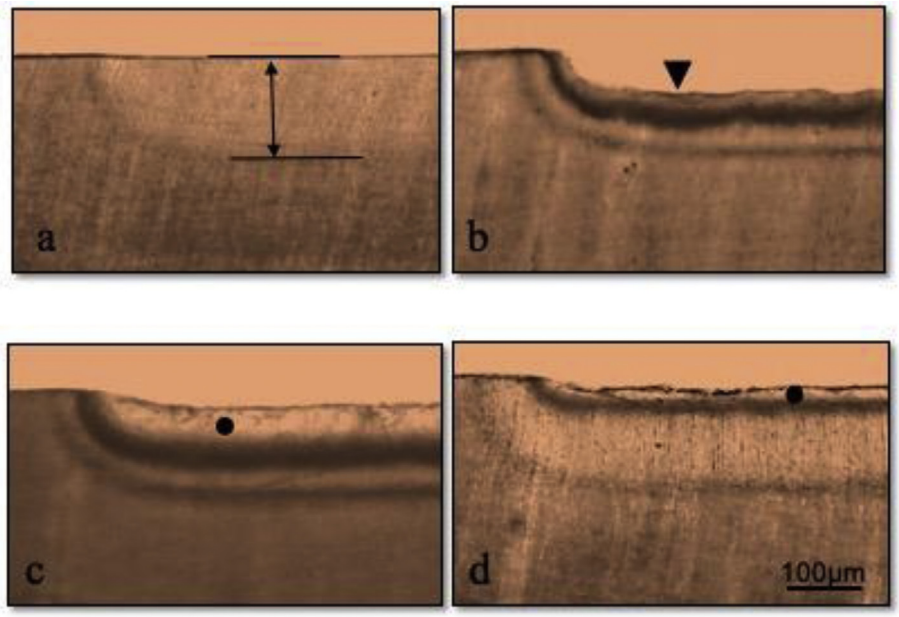


Figure 3 Representative light microscopy images; a) Incipient lesion in baseline. b) Control. c) Miswak treatment. d) CHX treatment. ↕ Indicates the depth of demineralized dentin. The black arrows head (▼) indicates collagen degradation, (●) represents preserved collagen matrix.

potential to inhibit collagen degradation like CHX. Therefore, the null hypothesis of this study was rejected.

The treatment condition in terms of frequency between Miswak and CHX groups was not comparative in the current study, ie, in the former it was 1 h treatment a day and repeated 3 times for 3 days and in the latter it was a single 1 h treatment a day. Although this protocol does not allow a simple comparison, we think this comparison would be justified as mentioned below. 0.12% CHX is known one of the strongest agents to inhibit bacterial growth, thus its use is restrictedly controlled for patients with heavy periodontal diseases,[29] implying less frequency of use of CHX to avoid potential side-effect are recommended. On the other hand, we proposed that Miswak extract could be used daily by taking into consideration the mild efficacy and long history of daily use without notable side-effects.[22] However, this study employed 1 h treatment, which was unlikely in clinical situation. Current treatment protocol was employed just for testing the potential effect of Miswak. Further study is planned for aiming clinical use.

It was reported that Miswak extract (*S. Persica*) contained various organic compounds like flavonoids, salvadorine, cyanogenic glycosides, lignans, saponins, alkaloids, tannins, linoleic acid, stearic acid, salvadourea and others.[24,30] Some molecules of those compounds possess active sites such as phenolic R—OH, carboxylic acid (R—COOH) and N containing sites (amino acid, amido and basic nature of NH). Those sites can form hydrogen bonds with similar sites present in proteinous collagen molecules. Those hydrogen bonds may protect the cleavage sites in collagen molecules from the Ch-collagenase attack. Besides that, similar hydrogen bonds are expected with proteinous Ch-collagenase, which may deform its molecular configuration, resulting in deactivation of the enzyme.[24,30]

In addition, it was reported that flavonoids in *S. Persica* can form complexes with metal ions, implying that flavonoids have potential to bind with metallo enzymes, which can alter or inhibit the degradation activity. As both, Ch-collagenase and MMPs are zinc-containing metalloproteinases, these flavonoid compounds in of *S. Persica* may bind to Zn^{2+} ion within these enzymes, thus affecting the degradation activity.[24]

As mentioned early, *S. Persica* contains chemically active compounds. Beside the potential of the anti-collagen degradation efficacy of Miswak extract, anti-bacterial efficacy would be expected which might contribute to the inhibition of plaque growth and acid production.[31] Miswak has also been found to enhance the growth of fibroblasts.[32]

To our knowledge, this is the first study investigating the action of aqueous Miswak extract on inhibiting dentin collagen degradation; therefore, the study on Miswak efficacy on root caries prevention was warranted for the coming future.

Current study revealed that the use of Miswak aqueous extract from *S. persica* is effective to preserve the dentin collagen matrix from collagenase enzyme, suggesting that it may play a positive effect in dentin caries prevention. Futher studies are needed to explain the mechanism of Miswak preservation actions against collagenase enzymes light microscopy or SEM. Further studies are also needed to confirm *in-vivo* the clinical efficacy on root caries prevention.

Declaration of Competing Interest

We declared that there is no conflict of interest exists with other people or organizations that inappropriately influence (bias) our experiment.

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