Original

The Effects of Calcified Seaweed on Remineralization of Artificially Demineralized Dentin

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Abstract: The study investigated the remineralization effect of calcified seaweed with large amounts of calcium (Ca), magnesium (Mg), sodium (Na), fluorine (F) and phosphorus (P). Human third molars were used in the experiment with the approval of the Ethics Committee of Tokyo Dental College. Cementum was removed from the cervical area around the teeth exposing the dentin. Small dentin blocks were prepared and immersed in acetic acid-sodium acetate buffer for 24 hours. Samples were immersed in remineralized liquid or saliva solution with or without calcified seaweed for 2 weeks. Structural changes in remineralization, depth of demineralization and loss in mineral volume were evaluated by contact microradiogram (CMR). Qualitative analysis was carried out with electron probe microanalyzer (EPMA). Morphology of crystal was observed using high-resolution transmission electron microscopy (HR-TEM). CMR image analysis revealed that part of the samples immersed in the solutions underwent remineralization. However, there was no significant difference in the degree of remineralization. EPMA observation of dentin partly detected Ca and P but not Mg or F. HR-TEM analysis revealed changes in crystal structure caused by Mg and F, which influenced remineralization in a small amount.

Key words: Calcified seaweed, Remineralization, Dentin, Crystal

Introduction

The rise in the percentage of elderly dentate improved the awareness of oral hygiene. However, due to gingival recession and periodontal disease brought about aging, many cases of hyperesthesia and root caries are observed on exposed root after gingival recession¹⁾.

Tooth surface undergo repeated demineralization and remineralization and the balance is maintained or affected by saliva²⁾. Eating causes the saliva to become acidic but saliva provides the buffering action which returns the oral environment immediately to neutral^{3,4)} and has bactericidal activity against cariogenic bacteria^{5,6)}. Supersaturation by minerals like calcium (Ca) and phosphate (P) is essential in suppressing the onset of demineralization and inducing remineralization. In addition, a small amount of fluorine (F) is also needed^{7,8)}. Therefore, saliva plays an important role in the remineralization of tooth. Fluoride and xylitol are used for remineralization of demineralized dental hard tissues⁹⁻¹³⁾, especially F which is highly effective in the prevention and recovery of initial carious lesions⁹⁾. However, fluoride is not a food additive so it cannot be directly added to it. There are only few foods that contain

large amounts of F¹⁴⁾ but in our preliminary study, calcified seaweed contains large amounts of Ca, P and F, which may be effective in remineralization process.

Therefore, we thought to clarify the efficacy of calcified seaweed in remineralizing dentin.

Materials and Methods

1. Preparation of demineralized dentin

A total of 20 human third molars extracted due to pericoronitis from patients 20-40 years old were used in the study and the samples were preserved in 10% formalin solution. The Ethics Committee of Tokyo Dental College, Ethical Clearance Number 189, approved the experiment. The cervical cementum was removed using wet abrasive paper (#4000) and 20 dentin blocks were prepared. Each block was covered with sticky dental wax, leaving a 3 x 4 mm uncovered surface. The blocks suspended by threads were immersed in 0.01 M acetate buffer solution (pH 5.0) at 50°C for 24 hours to form demineralized layers. Then after, the blocks were washed with distilled water and allowed to dry. Controls were prepared by covering half of the demineralized surface with sticky wax. For test samples, 5 blocks were immersed in remineralized liquid alone (Ca²⁺ 1 mM, PO₄³⁻ 0.6 mM, pH7.3) and 5 were immersed in remineralized liquid plus 0.1% calcified seaweed. The other 5

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blocks were immersed in parotid saliva alone and the other 5 blocks were immersed in parotid saliva plus 0.1% calcified seaweed. The parotid saliva was obtained by salivary stimulation, collected using Kirby cup and immediately sterilized. Samples were maintained at 37°C for 2 weeks. The solution (50 ml) for each group was replaced every 3 days. After immersing for 2 weeks, the blocks were washed with distilled water, dried and immersed in xylene for dewaxing.

Calcified seaweed (Aquamineral T®: Biocon Japan Ltd.) was obtained from dead calcareous deposits, which accumulated at the bottom of the sea. The calcified seaweed contains 34.0% Ca, 3.14% Mg, 0.56% P, 0.24% Na, 0.11% F and others. It was dissolved in acid due to its poor solubility. The solution was neutralized and freeze-dried to obtain a powder form.

2. Contact microradiograms (CMR) examination

Samples were dehydrated in graded ethanol and embedded in polyester resin (Rigolac; Nissin EM Co. Ltd, Tokyo, Japan). Ground sections (100 µm thick) were prepared from dentin blocks to include intact demineralized and remineralized areas.

CMRs were taken using soft X-ray generator (Softex CMR-3; Softex Co., Ltd, Tokyo, Japan) with 20 µm Ni filter operated at an accelerating voltage of 10 kV and a specimen current of 3 mA for 7 minutes with Cu-K α . An aluminum step wedge was placed on the X-ray glass film plate (HRP-SN-2; Konica Minolta, Tokyo, Japan) with the sample for the image analysis. The glass film plate was developed (D-19; Kodak, USA) for 5 minutes at 20°C, rinsed with tap water and then fixed (Fujifix; Fuji Film, Tokyo, Japan) for 5 minutes at 20°C.

3. Image analysis

CMR images were taken using Image Analyze System (HC-2500/OL; Olympus, Tokyo, Japan; HDS-N1; Hiroya, Tokyo, Japan). Each digital image was made in 256 level grey scale with a width of 50 µm and depth of 300 µm (from resin base to sound dentin). The demineralization rate (mineral loss value; ΔZ) for each definite area was calculated in terms of aluminum equivalence by setting the photographic density of film (not involving the sample) to 0% and that of intact dentin to 100%. Lesion depth (Ld: demineralization and remineralization area) was defined as the distance from the outer surface to the location of the lesion where the mineral content of sound dentin was 95% larger than that of the lesion. Three areas on each sample were measured and the mean was obtained.

4. Electron probe microanalyzer (EPMA) examination

After coating the ground sections with a thin layer of carbon, qualitative analysis of Ca, P, Mg and F ions was carried out using EPMA (JXA-8200; JEOL, Tokyo, Japan).

5. High resolution-transmission electron microscope (HR-

TEM) examination

Ground sections were re-embedded in epoxy resin (EPON 812; TAAB, England) and ultrathin sections were cut with an ultramicrotome (LKB Type IV, LKB, Sweden) equipped with a diamond knife. Morphology and size of crystals in remineralized area were observed using HR-TEM (Topcon 002-B; Topcon, Tokyo, Japan) at 200kV without staining.

Results

1. CMR images

Dentin was demineralized to an almost uniform depth of about 80µm from the flat surface to the bottom of subsurface lesion. The outer surface of demineralized dentin underwent shrinkage and broke down moderately compared to undemineralized part. The demineralized layer showed extremely weak radiopacity. Most of the surface layers were unmineralized but the degree of remineralization increased from the middle layer towards the deep layer (Fig.1).

Figure 1. Demineralized sample. Undemineralized area is seen in the right edge. Bar=100µm

Figure 2. Sample immersed in remineralized liquid. Undemineralized area is seen in the left edge, scale bar=100µm

The degree of remineralization in samples immersed in remineralized liquid alone slightly increased from the bottom to the middle layer (Fig. 2). The addition of calcified seaweed did not

increase the degree of remineralization of the outermost layer though remineralization of the middle layer was much higher. However, radiopacities in the bottom layer did not increase (Fig. 3).

On the other hand, the outermost surface layer of the samples immersed in saliva alone did not undergo remineralization. In the middle layer between demineralized layer, remineralization occurred just below the outermost surface (Fig. 4). Remineralization of the samples immersed in saliva with calcified seaweed was not observed in the surface layer but remineralization of the middle and deep layers was clear (Fig. 5).

Figure 3. Sample immersed in remineralized liquid plus calcified seaweed, scale bar=100 μ m

2. Image analysis

The lesion depth (Ld: μ m) on each CMR image and mineral loss value (ΣZ : vol% $\cdot \mu$ m) was measured. The surface was destroyed by demineralization experiment therefore the surface before the demineralization experiment was a virtual surface. Ld and ΣZ were measured from this virtual surface to the bottom of demineralization. The table below show measurement results of Ld and ΣZ where D is demineralized sample, R is remineralized liquid, RM is remineralized liquid plus calcified seaweed, S is saliva and SM is saliva plus calcified seaweed.

For Ld, a significant difference was only obtained between R and others ($p < 0.05$). For ΣZ , a significant difference was only obtained between D and R, RM, S ($p < 0.01$) and others were not

Table 1. Results of Ld and ΣZ

	Ld (μ m)	ΣZ (vol% $\cdot \mu$ m)
D	84.35 $\pm$ 14.62	6712 $\pm$ 1116
R	62.98 $\pm$ 17.62	4546 $\pm$ 884
RM	81.86 $\pm$ 9.36	5535 $\pm$ 791
S	88.37 $\pm$ 20.15	4787 $\pm$ 600
SM	85.86 $\pm$ 18.11	5695 $\pm$ 1566

The table shows Ld (lesion depth) and ΣZ (mineral loss value). D: demineralized sample, R: remineralized solution, RM: remineralized liquid plus calcified seaweed, S: saliva, SM: saliva plus calcified seaweed. Data represent the mean $\pm$ S.D. (n=5)

Figure 4. Sample immersed in saliva, scale bar=100 μ m

Figure 5. Sample immersed in saliva plus calcified seaweed, scale bar=100 μ m

significant.

3. EPMA findings

In samples immersed in remineralized liquid or saliva, qualitative analysis of the remineralized area detected Ca and P. In samples immersed in liquid with calcified seaweed Ca and P were detected but the other minerals were not.

4. HR-TEM findings

In cross-section of undemineralized dentin crystal, c -axis showed an irregular oblong shape and the lattice striations crossed each other at 60° angle (Fig. 6). Compared with undemineralized dentin crystal, the crystal immersed in remineralized liquid was large and the observed crystal growth resulted to thickening of the crystal. These crystals are long and hexagonal with clear angle (Fig. 7). The addition of calcified seaweed further increased the size of the crystal creating a large crystal with obscure angles and an irregular crystal growth of a -axis. Furthermore, a large number of lattice defects were observed inside the crystal (Fig. 8). On the other hand, crystal deposition in saliva showed a degree of remineralization of the lower part with a fine needle shape but a high degree of calcification increased the width (Fig. 9). The addition of calcified seaweed caused an increased in the number of crystals, thickness

and crystallinity (Fig. 10). These crystals immersed in saliva were similar to original dentin crystal and had a shape different from the crystal immersed in remineralized liquid.

Figure 6. Undemineralized dentin crystals, scale bar=10nm

Figure 7. Crystals formed in remineralized liquid, scale bar=10nm

Figure 8. Crystals formed in remineralized liquid with calcified seaweed, scale bar=10nm

Discussion

1. Effect of remineralized liquid

CMR shows that demineralization surface is lower than the position of the undemineralized surface. The loss of demineralized dentin seemed to undergo shrinkage due to residual organic matter.

Remineralization of the bottom layer was observed in samples

Figure 9. Crystals formed in saliva, scale bar=10nm

Figure 10. Crystals formed in saliva with calcified seaweed, scale bar =10nm

immersed in remineralized liquid. Residual mineral content in the deep layer is higher than the outer layer as indicated by CMR. Residual crystal growth or new crystal formation, which occurred in response to Ca and P in remineralized liquid, seems to increase the degree of remineralization in the vicinity of deep layer. Further in HR-TEM, good crystallinity and thickness were observed and the crystal growth seemed to occur by itself regardless of the surrounding collagen fibers.

When samples were immersed in remineralized liquid solution with calcified seaweed, a remineralized layer was formed near the advancing middle layer. This is perhaps due to the increased concentration of Ca and P in the liquid solution because the calcified seaweed contains large amounts of Ca and P, which were added into the solution. Moreover, calcified seaweed contains more F. F is known to function on crystal nucleation and crystal growth in small quantities and these ions act synergistically causing an increased in crystallinity or crystal growth from the middle layer. Dentin crystals are known to contain carbonate¹⁵⁾. Carbonate crystals are highly soluble in acid¹⁵⁻¹⁷⁾ and since the elaboration response of hydroxyapatite with hydrolysis creates an acidic solution, there is a possibility of inhibiting remineralization of the deep layer. In other words, apatite crystal formation and growth, which occurred in the middle layer, consumed the Ca and P ions in remineralized liquid. Formation of hydrogen ions caused by

hydrolysis itself will slightly shift to acidic solution inhibiting remineralization. In addition, the action of fluoride during remineralization was inhibited to infiltration of the inner layer that prevented the diffusion of hydrogen ions to diffuse outside not considering the progress of deep remineralization.

On the other hand, calcified seaweed contains lots of Mg. Mg has been reported to increase its solubility to get into apatite crystal^{19, 20)}. In HR-TEM, the crystal grows up in *a*-axis direction irregularly and crystals have many defects. Mg in calcified seaweed was considered to strongly influence the crystal growth.

2. Effect saliva

Immersion in saliva showed a high degree of calcified outer layer but there was no increase in the degree of calcification in the lower layer.

Saliva has always been maintained supersaturated with Ca and P for hydroxyapatite. Saliva contains many free ions in addition to CaHCO_3^+ , CaHPO_4^+ , $\text{CaH}_2\text{PO}_4^+$ and Ca-protein complexes^{21, 22)}. Stimulated parotid saliva contains 1.6 ± 0.8 mM Ca, 3.7 ± 1.0 mM P and $1.0 \pm 0.3 \mu\text{M F}^{23)}$. Proline-rich protein (PRP) or statherin of sialoprotein are associated in the maintenance of supersaturation. These sialoproteins inhibit calcium phosphate deposition and crystal growth of hydroxyapatite²²⁾. These sialoproteins also have the ability to bind with Ca ions and hydroxyapatite at the same time to be selectively absorbed²⁴⁻²⁶⁾.

Thus when demineralized dentin is immersed in saliva, these proteins were absorbed to the surface of collagen fibrils considered as highly calcified layer on the surface because of the deposition of large amounts of Ca and P ions in saliva. In the lower layer, HR-TEM showed thin crystals often worse than that of the crystalline when calcified seaweed was added to the remineralized liquid. There is a possibility that there was inhibition of remineralization from the middle to bottom layer.

On the other hand, remineralization is lower and was not seemingly evident with the addition of calcified seaweed in saliva. HR-TEM revealed better crystal quality formed without the addition of calcified seaweed and that the dentin formed was close to the original. Remineralization was inhibited because the surface is unknown and sialoproteins in the saliva with calcified seaweed especially Mg are likely involved in the inhibition of remineralization. Suppression of surface remineralization may also be caused by the inability of the ions to penetrate the deep surface. Conversely if the experiment were carried out for a long time, remineralization would have reached the entire depth.

3. Results of EPMA image analysis

In this experiment, Ca and P were detected in all samples but P, Mg and F were not detected. Dentin mineral is made of Ca and P, which also contain a small amount of Mg²⁰⁾. In addition, calcified seaweed contains large amounts of Mg and F. In remineralization

as shown in HR-TEM, extremely different form of crystals were observed either with calcified seaweed or not. Mg and F were not detected in calcified seaweed in EPMA suggesting that its effect was very much lower than the detection limit. However, in this experiment, the effect of calcified seaweed did not show any statistical difference. It was thought that a more effective method should be developed as its administration was considered to be effective in remineralization process.

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References

1. Imazato S, Ikebe K, Nokubi T, Ebisu S and Walls AW. Prevalence of root caries in a selected population of older adults in Japan. *J Oral Rehabil* 33: 137-143, 2006
2. Yanagisawa T, Miake Y, Saeki Y and Takahashi M. Remineralization in enamel caries and restoration of carious lesions by enhanced remineralization induced by saliva and xylitol. *Dentistry in Japan* 39: 208-215, 2003
3. Carey CM, Gregory TM, Tatevossian A and Vogel GM. The buffer capacity of single-site, resting, human dental-plaque fluid. *Arch Oral Biol* 33: 487-492, 1988
4. Dreizen S, Mann AW, JK Cline and Tom DS. The buffer capacity of saliva as a measure of dental caries. *J Dent Res* 25: 213-222, 1946
5. Hamon CB and Klebanoff SJ. A peroxidase-mediated, streptococcus mitis-dependent antimicrobial system in saliva. *J Exp Med* 137: 438-450, 1973
6. Bowen WH. Defense mechanisms in the mouth and their possible role in the prevention of dental caries. *J Oral Pathol* 3: 266-278, 1974
7. Shaw L, Murray JJ, Burchell CK and Best JS. Calcium and phosphorus content of plaque and saliva in relation to dental caries. *Caries Res* 17: 543-548, 1983
8. Shannon IL, Suddick RP, Dowd FJ Jr. Saliva: composition and secretion. *Monogr Oral Sci*. 2: 1-103, 1974
9. Zimmer S, Jahn KR and Barthel CR. Recommendations for the use of fluoride in caries prevention. *Oral Health Prev Dent* 1: 45-51, 2003
10. Meyer-Lueckel H and Tschoppe P. Effect of fluoride gels and mouthrinses in combination with saliva substitutes on demineralised bovine enamel in vitro. *J Dent* 38: 641-647, 2010
11. Cochrane NJ, Cai F, Huq NL, Burrow MF and Reynolds EC. New approaches to enhanced remineralization of tooth enamel. *J Dent Res* 89: 1187-1197, 2010
12. Miake Y, Saeki Y, Takahashi M and Yanagisawa T. Remineralization effect of xylitol on demineralized enamel.

- J Electron Microsc 52: 471-476, 2003
13. Thaweboon S, Nakornchai S, Miake Y, Yanagisawa T, Thaweboon B, Soo-ampon S and Lexomboon D. Remineralization of enamel subsurface lesions by xylitol chewing gum containing funoran and calcium hydrogenphosphate. Southeast Asian J Trop Med Public Health 40: 345-353, 2009
14. McClure FJ. Fluorine in foods; survey of recent data. Public Health Rep 64: 1061-1074, 1949
15. Okazaki M, Moriwaki Y, Aoba T, Doi Y and Takahashi J. Solubility behavior of CO₃ apatites in relation to crystallinity. Caries Res 15: 477-483, 1981
16. Brudevold, F. Dental tissue factors in dental caries. Int Dent J 12: 496-507, 1962
17. Brudevold, F, Gardner DE and Smith FA. The distribution of fluoride in human enamel. J Dent Res 35: 420-429, 1956
18. Okazaki M, Aoba T, Doi Y, Takahashi J and Moriwaki Y. Solubility and crystallinity in relation to fluoride content of fluoridated hydroxyapatites. J Dent Res 60: 845-849, 1981
19. Boulet M, Marier JR and Rose D. Effect of magnesium on formation of calcium phosphate precipitates. Arch Biochem Biophys 96: 629-636, 1962
20. Okazaki M, Takahashi J, Kimura H. Unstable behavior of magnesium-containing hydroxyapatites. Caries Res. 20(4):324-331, 1986.
21. Hay DI, Moreno EC. Statherin and the acidic proline-rich proteins. In: Human Saliva: Clinical Chemistry and Microbiology, ed by Tenovuo JO, CRC Press, Florida, 1989, pp 131-150.
22. Hay D I, Schluckbier SK and Moreno EC. Equilibrium dialysis and ultrafiltration studies of calcium and phosphate binding by human salivary proteins. Implications for salivary supersaturation with respect to calcium phosphate salts. Calcif Tissue Int 34: 531-538, 1982
23. Ferguson DB. Salivary Electrolytes. In: Human Saliva: Clinical Chemistry and Microbiology, ed by Tenovuo JO, CRC Press, Florida, 1989, pp 75-99.
24. Hay DI. The interaction of human parotid salivary proteins with hydroxyapatite. Archs Oral Biol 18: 1517-1529, 1973
25. Hay DI. The adsorption of salivary proteins by hydroxyapatite and enamel. Archs Oral Biol 12: 937-946, 1967
26. Koulourides T, Feagin F and Pigman, W. Effect of pH, ionic strength, and cupric ions on the rehardening rate of buffer-softened human enamel. Archs Oral Biol 13: 335-341, 1968