Inhibitory effect of zinc-containing desensitizer on bacterial biofilm formation and root dentin demineralization

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This study compared the effect of a novel zinc containing, Caredyne Shield (CS), and a fluoroaluminocalciumsilicate-based, Nanoseal (NS) desensitizers on dentin tubule occlusion, inhibition of Streptococcus mutans (S. mutans) biofilm formation, and resistance to bacterial demineralization. Desensitizers were applied to simulated hypersensitive bovine dentin, with distilled water used as a control. S. mutans biofilms were grown on the surface of each specimen in an oral biofilm simulator. CS showed the least bacterial count and water insoluble glucan amount followed by NS. Transverse micro radiography revealed that both CS and NS showed significant reduction in mineral loss and lesion depth of the associated lesion. Scanning electron micrographs showed that the two desensitizers formed obvious depositions on the dentin surfaces, occlusion of tubules and mineral tag formation.

Keywords: Desensitizer, Demineralization, Root dentin, Antibacterial, Streptococcus mutans

INTRODUCTION

Gingival recession is a common clinical condition which is often induced by periodontal diseases, aging, or traumatic tooth brushing habits which results in the exposure of the root surface. Since root surfaces and cementum are not able to resist the effect of actions such as intense tooth brushing or root planing1, the dentin of the root can often be exposed, and will therefore become an area for bacterial biofilm formation, plaque buildup, and site for possible root caries2. Although dental caries involves various types of acidogenic and aciduric bacteria, S. mutans is regarded as the primary bacterial species associated with the initiation and progress of caries lesion formation3. The bacteria produce water-insoluble glucans (WIGs) which form a barrier that entrap the organic acids formed by the bacteria and in turn come in contact with the tooth surfaces, leading to a cariogenic biofilm4. Therefore, a root coating material that possesses inhibitory effects against cariogenic bacteria, including S. mutans, may show significant protection against the initiation of root caries lesions.

In addition, exposed root dentin is also prone to dentinal hypersensitivity. The removal of the protective cementum and dentin smear layer can expose patent dentinal tubules. It is generally accepted that the short, sharp pain in case of dentin hypersensitivity is due to the stimulation of pulpal nerve fibers by fluid movement within the patent dentinal tubule. The pain is triggered by thermal, evaporative, osmotic, chemical or tactile stimuli, and has been known as the hydrodynamic theory5.

A common treatment approach for dentin hypersensitivity is sealing or occluding the open dentinal tubules, hence, reducing the intratubular fluid movement. This can be achieved by resins and adhesives, or desensitizing materials which contain ingredients that form insoluble mineral precipitates resulting in dentinal tubule occlusion, for example calcium-fluoroaluminosilicate is one such desensitizing agent.

The calcium-fluoroaluminosilicate based desensitizer, Nanoseal (NS), is a two-component system. Its mechanism of action is based on a chemical reaction resulting in nano-sized insoluble mineral deposits that aggregate on the tooth surface to block exposed patent dentinal tubules6,7.

NS has shown desensitizing activity by dentinal tubule occlusion (and mineral tag formation), along with root surface protection from demineralization8,9 and an anti-erosive effect10, which is suggested to be a result of the fluoride in this material. NS may show an inhibitory effect against Streptococcus mutans (S. mutans) biofilm formation and demineralization of dentin induced by the bacterial biofilm, however, it has yet to be assessed.

A recent tooth surface coating material, Caredyne Shield (CS), has been developed. It possesses the ability to act as a dentin desensitizer including antibacterial, and anti-demineralization effects. Its ionic content is mainly fluoride, calcium, and zinc, which has shown to aid in the prevention of demineralization of dentin10,11, inhibit matrix metalloproteinase (MMP) activity12,13, and moreover, has antibacterial activity14,15. Due to its antibacterial and anti-MMP activity, zinc has been incorporated into experimental resin composites14, glass ionomer cements16, adhesive resins17,18, and
Table 1 Desensitizers used, manufacturer, composition and method of application

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<th>Material</th>
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<th>Method of application</th>
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| Nanoseal (NS)  | Nippon Shika Yakuhin, Yamaguchi, Japan | Solution A: fluoroaluminocalciumsilicate glass  
Solution B: 10% phosphoric acid | Mixing of two equal proportions of liquid thoroughly using micro-brush and application to the dentin surface for 20 s, then washed with water for 5 s |
| Caredyne Shield (CS) | GC, Tokyo, Japan               | Solution A: BioUnion nanofiller, median size 400 nm  
Solution B: 10–15% phosphoric acid | Same as NS but applied with gentle rubbing                  |

Materials
The materials used in this study were two desensitizing agents namely, Nanoseal (calcium-fluoroaluminosilicate based desensitizer) and Caredyne Shield (BioUnion nanofiller based desensitizer). Their composition, manufacturer information and method of application are listed in Table 1.

Specimen preparation
This study was approved by the human research ethics committee of Tokyo Medical and Dental University (no. 725). Thirty extracted bovine teeth were used in this study, the roots were separated from the crown 1 mm below the cemento-enamel junction using a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under water coolant. Dentin blocks from the buccal cervical area of the root were obtained (5×5×3 mm). The buccal surface was ground flat with 1200-grit silicon carbide papers under running water. To mimic a surface of hypersensitive dentin; the dentin blocks were placed in a 1.0% citric acid solution for 20 s\textsuperscript{21,22}, then washed thoroughly with water.

Among the prepared dentin specimens, a total of 42 specimens were randomly selected and prepared for the biofilm formation in two separate experiments to ensure reproducibility. Acid resistant nail varnish (Revlon, Paris, France) was used to coat all the specimen surfaces except for the buccal area of the tooth, additionally, only 1.5 mm of the exposed buccal surface was covered with the nail varnish to serve as the sound portion for the Transverse microradiography (TMR) analysis. Following this, specimens for this test were disinfected with 99% ethanol to minimize, and kept in artificial saliva (1.5 mM CaCl\textsubscript{2}, 0.9 mM KH\textsubscript{2}PO\textsubscript{4}, 130 mM KCl and 20 mM Hepes, pH 6.5\textsuperscript{9}) which was filtered with a sterile MF-millipore filter unit (Millex-GS, Millipore, Burlington, MA, USA) for 24 h.

Material application
The specimens were randomly divided into three groups, and each of the two desensitizers was applied to the buccal exposed dentin surface according to the manufacturers’ instructions. Briefly, the two solutions of either NS or CS were mixed and then applied to the test surface with a micro-brush-CS was applied with a gentle rubbing motion- then left for 20 s, and rinsed gently with water. For the negative control group, distilled water was applied for 20 s instead of the desensitizer. Each specimen was stored separately at 37°C in 20 mL of filtered artificial saliva for 24 h. Preparation of specimens and material application are shown in Fig. 1.

Biofilm formation on the specimens and quantity measurement assay
To evaluate the behavior of the desensitizers relative to biofilm formation, a procedure similar to that previously reported\textsuperscript{23,24} was followed. Briefly, a suspension of S. mutans.
*S. mutans* MT8148 in phosphate buffered saline (PBS) at $\text{OD}_{490}=2$ (approximately $2 \times 10^8$ colony forming units/mL) was prepared from a 16-h fresh culture in Brain Heart Infusion (BHI; Becton Dickinson, Sparks, MD, USA) broth. For the growth of the biofilms, a solution of Heart Infusion (HI; Becton Dickinson) broth with sucrose (1% final concentration) was used.

Specimens were placed on a Teflon holder of an OBR (Fig. 2) and fixed by using red utility wax (GC, Tokyo, Japan). Only the experimental surface of each specimen was exposed for the biofilm formation. *S. mutans* biofilms were then grown on the specimens inside two identical chambers of the OBR encircled with a water jacket. The Teflon holder carrying the specimens was placed on silicone plug at the bottom of the chamber around a flat bulb of a pH electrode. The chamber was sealed from above with another silicone plug fitted with five stainless steel tubes (21 gauge) so that the chamber itself served as an incubator at a temperature of 37°C. The other ends of the five stainless steel tubes were connected to silicone tubes passing through peristaltic pumps regulated by a computer operated controller (EYELA EPC-2000, Tokyo Rika, Tokyo, Japan). One tube was used to collect the *S. mutans* suspension, two more to collect BHI and the other two tubes to collect PBS from the prepared stock as described above. The pumps continuously fed all of these liquids onto the center of the specimen holder at a rate of 6 mL per hour per tube. Both of the chambers were simultaneously operated and the pH on the flat bulb electrode was continuously recorded.

After 20 h, the retained biofilm on each specimen was measured by separating the bacterial cells and WIG according to a method previously reported:

Each retained biofilm was transferred carefully from the PBS to 1 mL of 0.5 mol/L sodium hydroxide solution, incubated for 15 min vortexed, and centrifuged at 5,000 rpm for 10 min to separate the WIG and bacterial cells embedded in the biofilms. Each bacterial pellet was resuspended in 1 mL of PBS and 100 μL of each bacterial cell suspension, and then transferred to separate wells of a 96-well flat-bottom microplate. Turbidimetric analysis (OD490 nm) was performed with a spectrophotometer (Model 680 Microplate reader, Bio-Rad, Hercules, CA, USA) to quantify the bacteria. The amount of dissolved WIG was measured by the phenol-H$_2$SO$_4$ method, and absorbance at 490 nm was determined using a spectrophotometer. To calculate the WIG amount (μg/mL), 250 μL of WIG solution from each sample was dissolved in phenol-H$_2$SO$_4$ and 200 μL of each of the resulting solutions was subjected to analysis. The dentin specimens were kept for lesion analysis using TMR.

TMR analysis

After demineralization by artificial biofilm formation, the specimens were cut into approximately 220 μm thick sections using a low-speed diamond saw and then immediately immersed in a 70% aqueous glycerin solution to prevent lesion shrinkage. To perform TMR imaging, the excess glycerin was removed from the sections, then placed on an X-ray glass plate (High Precision Photo Plate, Konica Minolta Photo, Tokyo, Japan), with a 15-step aluminum step wedge. A soft X-ray generator (SOFTEX CMR-2, Softex, Kanagawa, Japan) was used at a voltage of 20 kV, current of 2.5 mA, and exposure time of 9 min. The obtained TMR images were digitally photographed using an optical microscope (SMZ1000, Nikon, Tokyo, Japan) and CCD camera (DS-Fi1, Nikon), then the digitized images were analyzed using image analysis software (Image J, version 1.42q, Wayne Rasband, NIH, Bethesda, MD, USA) and customized image processing software. The output parameters obtained were the mineral content profile of the lesion, lesion depth (LD) and integrated mineral loss ($\Delta Z$). LD is defined as the depth from the base of the sound dentin surface (the dentin surface covered with the nail varnish used as a reference) to the lesion base where the mineral content was 95% of the sound dentin mineral content, while ($\Delta Z$) was defined by the integrated mineral loss from the surface of the lesion to the LD. In this analysis, the mineral content of sound dentin was assumed to be 48 vol%.

Scanning electron microscopy (SEM) analysis

Specimens were prepared for SEM analysis by mounting on aluminum stubs, while specimens for cross section observation were fractured with a chisel. All specimens were desiccated for 24 h, then gold sputter-coated for SEM observation (JSM-IT 100, Jeol, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS V.23, SPSS, Chicago, IL, USA). Data showed parametric distribution using
RESULTS

Quantity of attached biofilm
The amounts of bacteria and WIG of the biofilm on the specimen surfaces are shown in Fig. 3. Both results showed that NS and CS groups had low susceptibility for biofilm attachment, with significantly lower values than the control group in bacterial amount and WIG tests ($p<0.001$), however, CS group showed the lowest values of bacterial amount and WIG.

TMR analysis of mineral profiles, LD and mineral loss
Figure 4 shows mineral profiles of representative lesion specimens from each group, customized analysis software was used to calculate LD and $\Delta Z$ of each specimen.

The measured values of the LD and mineral loss ($\Delta Z$) of the control, NS and CS groups are shown in Fig. 5. Both NS and CS showed significantly lower LD and $\Delta Z$ than the control group, without a significant difference between them ($p<0.001$).

Scanning electron microscope observation
Figure 6 shows SEM images of dentin surfaces and longitudinally sectioned dentin specimens respectively for the three groups.

The distilled water (control) group showed open dentinal tubules without smear layer. The scratches formed by the SiC paper were still visible.

NS application showed heavy aggregate formation, covering the dentin surface and sealing most of the dentinal tubules, however, with NS, the mineral deposit particles had different sizes and shapes compared with CS, and more precipitated nanoparticles were integrated within the openings and internal walls of the dentinal tubules.

The application of CS showed abundant mineral
Fig. 5 Graphs comparing: A: measured values of the mineral loss (ΔZ), and B: LD in the control, NS and CS groups. Different letters indicate statistical significance ($p<0.05$).

Fig. 6 Scanning electron microscope micrographs of the dentin surfaces (×1,000) and cross sections (×5,000) of the distilled water (Control), NS and CS groups. Arrows indicate mineral tags blocking the dentinal tubules.

Deposit formation on the dentin surface, resulting in covering most of the dentinal tubules, in addition, partial and complete obliteration of dentinal tubule orifices. The dentin scratches formed by the SiC papers were almost covered by CS deposition.

Examination of the fractured specimens in both CS and NS groups detected a marked layer of deposits on dentin surface, total or partial sealing of the orifices and formation of mineral tags within the dentinal tubules.

**DISCUSSION**

NS is a two-component tooth surface coating material. The first component is calcium-fluoroaluminosilicate, similar to silicate cements, with the second component being phosphoric acid. Upon mixing the two components and applying to the dentin, an acid-base reaction proceeds, the phosphoric acid in the mixture etches the dentin surface, releasing calcium and phosphate to the mixture, at the same time, it dissolves the calcium-fluoroaluminosilicate. Finally, the pH rises gradually and precipitations of acid resistant deposits of undissolved powder particles, phosphate salts, silicates and fluorides are created.

On the other hand, CS works with a similar mechanism. However, it has different components, as it is formed mainly of a BioUnion nanofiller, containing zinc, calcium and fluoride. Similarly, upon mixing the two components and application on the dentin surface, an acid-base reaction occurs, forming a mixture containing Zn$_3$(PO$_4$)$_2$ and BioUnion nanofiller. Calcium and phosphate are taken from the teeth into the mixture, forming a nanoparticle-sized layer on the dentin surface. It is speculated that this layer contains the BioUnion nanofiller, Zn$_3$(PO$_4$)$_2$, CaF$_2$ and Ca$_3$(PO$_4$)$_2$.

In this experiment, the SEM observations showed that both desensitizers showed occlusion of dentinal tubules by formation of inorganic particles/crystal salts on the surface and mineral tags inside the dentinal tubules. These were integrated with the internal walls of the dentinal tubules, sealing them, hence reducing the movement of intertubular fluid and greatly reducing the dentin permeability. However, CS showed denser material deposits, while NS showed a greater number of tubules that were partially or totally sealed internally. It should be mentioned that storage of the specimens for 24 h in artificial saliva containing calcium and phosphate salts before SEM examination was considered as it promotes the crystal formation of the deposits and sealing of the dentinal tubules, which simulates the oral condition.

Regarding the antibacterial test; after 20 h of
incubation in the OBR, thick biofilms were formed on all specimen surfaces. After collecting these biofilms and measuring the quantity of retained bacteria and WIG, both desensitizers showed a significant reduction in both the quantity of bacteria and WIG, indicating an inhibitory effect on biofilm formation with CS's effect being superior.

However, TMR analysis of the lesion formed by the *S. mutans* biofilm showed similarity in LD and mineral loss for both desensitizers, which was significantly less than the control group.

The two desensitizers contain fluoride, which is known to have activity against acid production, acid tolerance and extra polysaccharide (WIG) formation of *S. mutans*\(^{29}\), its mechanism of action could be due to the inhibition potentials on enzymatic activities and proton-transporting ATPase, which play important role in the process of glucose uptake and metabolism by the bacteria. hence, affecting its metabolic activity, growth, and multiplication. The disruption of these enzymatic functions reduces the production of intracellular polysaccharide and WIG. This happens to be the consequence of the suppression of glycogen synthesis, which is the polysaccharide store that enables bacteria to produce acids even after the sugars are cleared from the mouth. As a result, lactic acid secretion from glucose metabolism is hindered and consequently causes slowing down the progression of caries. Therefore, these influences of fluoride on bacteria can be advantageous out of the desensitizers tested in this study\(^{29,30}\).

CS showed better inhibitory effects on biofilm formation compared to NS due to its zinc content in addition to fluoride. Zinc oxide and zinc nanoparticles have comparable antibacterial properties with silver, but —unlike silver— does not have any potential dental discoloration effects\(^{31}\). Zinc alone is known to have bacteriostatic rather than bactericidal effects\(^{15,32}\), its mechanism of action has been studied previously, but briefly, it showed inhibitory potency on bacterial glycolysis, which works better at neutral pH —while effects of fluoride works much greater at acidic pH values. Besides this, zinc ions inhibit acid tolerance and transmembrane proton translocation in the bacterial cells\(^{15,33}\), overall, zinc does not only inhibit the growth of the caries related bacteria, but also inhibits the essential cariogenic virulence factor of *S. mutans*, which is glucan production. Zinc inhibits the extracellular polysaccharide-producing enzymes of plaque-forming organisms, the reduction in formation of extrapoly saccharides, especially WIGs, as shown by this experiment, will reduce the accumulation of more cariogenic streptococci on a tooth surface, and will reduce the bulk and cariogenicity of the dental plaque.

Koo et al.\(^{34}\), showed that fluoride and zinc, when used together, have an additive inhibitory effect on *S. mutans* glucosyl transferase production and polysaccharide synthesis in biofilms, meaning that their combination showed enhanced results than using each agent alone, and this could be the rationale behind the results of CS on the bacterial count, WIG production and dentin LD.

The chemical composition of CS and NS may have inhibited the demineralization process directly in this study. NS was reported to have a protective effect against demineralization by acidic attack and pH cycling\(^{7-9}\), those studies demonstrated that fluoride, calcium and silica from NS were incorporated into the tooth surface. These elements have cariostatic, and protective effects. Accordingly, they increased dentin resistance to bacterial acids, led to reduction in LD and mineral loss. Zinc, fluoride and calcium in the Biounion Nanofiller of CS were able to demonstrate inhibitory effects on dental hard tissue demineralization\(^{10,25,35,36}\) and enhance remineralization\(^{37-39}\), in addition to the ability of fluoride to form fluorapatite, which decreases crystal solubility and makes it more resistant to acid attack\(^{40}\). Future studies including SEM/EPMA analysis of both materials are essential to understand more about the exact constituents of the formed precipitates.

**CONCLUSIONS**

1. The NS and CS desensitizers showed inhibitory effects on biofilm formation on root dentin and also root dentin demineralization.
2. Application of the zinc-containing CS desensitizer may show good potential as a new therapeutic treatment to prevent root caries formation.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**REFERENCES**


