Original

Effect of a test dentifrice containing nano-sized calcium carbonate on remineralization of enamel lesions *in vitro*

Syozi Nakashima[1], Makoto Yoshie[2], Hiroshi Sano[2] and Armasastra Bahar[3]

[1]Oral-care Research Laboratories, Lion Corporation, Odawara, Japan
[2]Product Development Department, International Division, Lion Corporation, Tokyo, Japan
[3]Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia

Abstract: The efficacy of a test dentifrice containing nano-sized (several tens to hundreds of nm) calcium carbonate (hereafter NC) on enamel lesion remineralization was studied in an *in vitro* system that employed collagen-coated wells for cell culture, as a model of oral surfaces for NC retention. The well surfaces were treated with the test dentifrice and briefly rinsed with distilled water. Thin sections of enamel with artificial subsurface demineralization were remineralized in the plate wells containing remineralizing solution. The dentifrice treatment was repeated twice a day (in the morning and evening) for 20 days. After remineralization, microradiographic analysis was performed to evaluate the rate of lesion remineralization on the sections. The test dentifrice showed a statistically significant mineral gain (48.8% decrease in ΔZ % $\times$ μm from the baseline value), indicating lesion remineralization, whereas the placebo dentifrice without NC did not. An elevated Ca concentration in the remineralizing solution was also observed after a single treatment with the test dentifrice. We conclude that the test dentifrice has potential to remineralize incipient enamel lesions due to the unique properties of NC, which is retained on oral surfaces, thereafter releasing Ca ions into oral fluids (saliva, plaque). (J Oral Sci 51, 69-77, 2009)

Correspondence to Dr. Syozi Nakashima, Oral-care Research Laboratories, Lion Corporation, 100 Tajima, Odawara, Kanagawa 256-0811 Japan
Tel: +81-465-49-4471
Fax: +81-465-48-4079
E-mail: naka-s@lion.co.jp

Introduction

The fundamental process of enamel de- and remineralization is governed by the degree of saturation (hereafter DS) of oral fluids (saliva, plaque) with respect to apatitic minerals, a model of stoichiometric hydroxyapatite (1). To enhance lesion remineralization as well as inhibit demineralization, elevation of the concentrations of calcium, phosphate, and hydroxide, as well as fluoride ions, in oral fluid seems reasonable from a theoretical viewpoint.

For this purpose, fluoride has been used widely in various products, and its caries-preventive mechanism can be explained by increased driving force for fluoridated apatite precipitation (2,3). On the other hand, calcium compounds such as calcium glycerophosphate (4) and calcium carbonate (usually as an abrasive agent) have been employed, especially in dentifrice formulations (5,6), and their caries-preventive action can be explained by increased driving force for hydroxyapatite precipitation (7).

Furthermore, the use of amorphous calcium phosphate containing casein phosphopeptide (ACP-CPP) (8-10) and phosphorylated oligosaccharide containing calcium (11) in chewing gum has been reported for enhancement of lesion remineralization. The use of a dentifrice formulation comprising a dual phase, one phase containing calcium compounds and the other containing phosphate compounds, has been proposed for stable delivery of Ca and phosphate ions (12).

This study was carried out to investigate the in vitro

remineralization efficacy of a test dentifrice containing nano-sized calcium carbonate (hereafter NC). Previously we had performed a comparative test of the topical effect (two applications per day) of water slurries of calcium carbonate abrasive as a dentifrice and NC for caries prevention in a hamster model. It was demonstrated that calcium carbonate abrasive was not effective, whereas NC reduced the caries score by 31.1% compared with placebo (distilled water) treatment (13). Moreover, a preliminary experiment in humans showed that a higher Ca concentration delivered by rinsing with the slurry at night before sleep was detectable in saliva collected from the tongue surface just after getting up the following morning. The Ca concentration in saliva collected in the morning was found to be elevated about 2.6-fold in comparison with that collected before sleep.

Therefore we expected that the NC-containing dentifrice would show promising remineralization efficacy on enamel lesions in view of its good retention properties on oral proteinaceous surfaces (mucous membrane, tongue, pellicle) and effective Ca ion delivery together with a pH elevation effect when dissolved in oral fluids.

The purpose of this study was to examine the efficacy of a NC-containing test dentifrice on remineralization of artificially produced subsurface enamel lesions using collagen-coated plate wells as a model of oral proteinaceous surfaces.

Materials and Methods

Preparation of enamel thin sections

Twelve human extracted sound molar teeth stored in 70% ethanol solution in a refrigerator were selected. The smooth enamel surface was cleaned with sandpaper (#240 Sharp-Mini, Ohki Chemical Co., Hiroshima, Japan) fixed to the handle of a micro-motor (Tas-35L, Shofu, Kyoto, Japan), and cut parallel to the tooth axis with a thickness of approximately 500 μm. From one tooth enamel surface, 3 - 5 sections were obtained, and 48 sections were prepared for this study. On the cleaned enamel surface, a "window" (500 μm × 2.0 mm) for de- and remineralization treatments was prepared by varnishing the cut surface with waterproof nail varnish except for the "window".

Demineralization of the thin sections

Organic acid buffer solution containing Ca and phosphate ions ($CaCl_2$: 12 mM, KH_2PO_4: 10 mM, NaCl: 100 mM, acetic acid: 100 mM, lactic acid: 50 mM, pH 4.2 adjusted using NaOH solution) was prepared by modifying the ion concentrations of the original that had been previously demonstrated to produce subsurface lesions (14). The varnished sections were demineralized in the organic buffer solution (5 ml/section) at 37°C for 2 weeks. The extent of demineralization was estimated by observing the whiteness of the "window". When the whiteness was not obviously notable, the period of demineralization was extended by 1 to 2 weeks.

Remineralization treatment of thin sections by test and placebo dentifrices

The 48 sections were randomly allocated to 3 groups (16 sections/group): the first for the baseline group (demineralization only), the second for the test dentifrice group (sodium monofluorophosphate: 0.76%, silica abrasive: 23.0%, sodium laurylsulfate: 2.3% sorbitol: 21.0%, xanthan gum: 0.8%, NC: 1.0%, flavor: 1.2%, water: 33.7%, others) and the third for the placebo dentifrice group without NC. The NC (product name KOROKARUSO; primary particle size determined by SEM several tens to hundreds of nm; application food additive) was supplied by Shiraishi Calcium Kaisha, Ltd. (Osaka, Japan).

The dentifrice slurries were prepared by dispersing 8.0 g of the dentifrice in a 50-ml centrifugal plastic tube and adding 16.0 ml of distilled water and 4 g of glass balls followed by a thorough mixing. The slurries were then centrifuged at 9,000 rpm (7,245 ×g) for 10 mins. The supernatant of the slurry was added to 6 wells (1.0 ml /well) on the plate (Sumilon Cell Tight^{TNC} - 1 Plate 6F, Sumitomo Bakelite, Co., Ltd., Tokyo, Japan) for treatment of the well surface (well diameter 3.4 mm, area 9.2 cm^2, bottom surface coated with type 1 collagen). The slurry was stirred gently for 3 min, to allow a proportion of the NC to adhere to the collagen-coated surface of the well. The supernatant was then discarded and the bottom surface of the well was rinsed twice with 0.5 ml distilled water to remove any excess supernatant, and 0.5 ml of artificial saliva (i.e., remineralizing solution: $CaCl_2$ 1.0 mM, KH_2PO_4 3.0 mM, NaCl 100 mM, pH 6.30 adjusted with NaOH solution, DS = 1.5×10^6 with respect to hydroxyapatite) was added to the treated well. The demineralized thin sections were then placed at the bottom of the wells (4 sections/well) for the two dentifrice groups. The treatment was performed 2 times per day (in the morning and evening). During the rest of the day, the plates were kept in an incubator at 37°C for remineralization with tight sealing to prevent evaporation of the remineralizing solution. The remineralization treatment was continued for 4 weeks (5 days/week). During each of the weekends, the sections were immersed in 100 mM saline solution in refrigerator without the dentifrice treatment.

Preparation of thin sections for TMR analysis

After completion of the remineralization treatment, the thin sections were immersed in ethanol to remove the nail varnish, and the sections were dehydrated by sequential 60-min immersions in ethanol solutions (70%, 80%, 90%, and 100%). Finally, the sections were immersed in acetone overnight. The dehydrated sections were embedded in resin (Styrene Monomer, Rigolac^{TM} 70F/2004, Okenshoji, Tokyo, Japan) for polymerization, then ground to a thickness of 100 μm for TMR (transverse microradiography) analysis.

Monitoring the change in Ca concentration and pH in the remineralizing solution

The collagen-coated wells of the plates were treated with the NC supernatant in the same way as described above (see section 3). The remineralizing solution (0.5 ml/well) was added to 6 wells of the plate and kept in an incubator for 1, 3 and 16 h (overnight) at 37°C. For each incubation time, 3 plates were used for repetition. After incubation, the remineralizing solutions in 6 wells of the plate for each incubation time were put together in a vial (total volume about 3 ml) and centrifuged at 10,000 rpm (8,944 $\times g$) for 20 min to separate the supernatants containing Ca ions released from the retained NC and the unsolved NC. The supernatants were diluted with distilled water, and 1.0 N HCl and 10% LaCl$_3$ as La solutions were added to the diluted supernatants. The Ca concentrations were determined by Ca atomic absorption spectroscopy (Z-5310, Air-acetylene flame, Hitachi, Tokyo, Japan).

In parallel with measurement of Ca concentrations, the pH of the remineralizing solutions after incubation was measured using a pH electrode with a flat rod end (type GST-5723S, DKK-TOA Corp., Tokyo, Japan).

TMR analysis of mineral gain resulting from the remineralization treatment

The TMR analytical method employed in this study was described in detail in a previous paper (15), so a brief description is given below. Microradiographs of the thin sections were taken on High Precision Photo Plates (Konica Minolta Opto, Tokyo, Japan) at 3 mA and 18 kV for 10 min using an X-ray generator (SOFTEX CMR 2, Tokyo, Japan) together with an Al step-wedge (15 sheets of Al foil, 15 μm in thickness) for calibration. After X-ray irradiation, the photo plates were developed and fixed in accordance with the manufacturer's instructions.

The fixed plates were observed using a light microscope (BH2, Olympus, Tokyo, Japan) equipped with a color CCD video camera (IK-T40, Toshiba, Tokyo, Japan). The CCD camera cable was connected to a video image analyzer (PIAS-V, PIAS, Osaka, Japan). The analyzer was also connected to a monitor with a video-out board for image visualization, and was interfaced to the board (VME, PIAS) of a personal computer (PC-9821Xa, NEC Corp., Tokyo, Japan) equipped with image analysis software (P-Analyzer, PIAS). The mean gray value of a lesion area was converted to the number of Al steps, and mean mineral profiles of the lesion were acquired on the basis of the Al step number (on the y axis) versus the lesion depth (on the x axis). Finally, the mineral profiles were converted to mineral % (on the y axis) as the mineral content, taking the sound enamel portion as 100%.

Values of lesion parameters (mineral loss ΔZ [% × μm], lesion depth LD [μm], maximum mineral density at the lesion surface layer Max [%], and minimum mineral density at the lesion body Min [%]) were calculated from the mean mineral profiles (Fig. 1).

SEM observation of NC particles

A small amount of NC powder was placed and spread on a microscope cover glass under dry conditions and sputter-coated with osmium and platinum. The powders were observed using a Field Emission Scanning Electron Microscope (FE SEM: JEOL JSM6300F, Tokyo, Japan). For comparison, calcium carbonate powders for dentifrice abrasive (Lot # 9020, BIHOKU FUNKA KOGYO, Okayama, Japan) were observed in the same way.

Statistical analyses of lesion parameters

The Tukey-Kramer test was applied to analyze differences in the mean values of the lesion parameters (ΔZ, LD, Max and Min) among the baseline, test and placebo dentifrice groups.
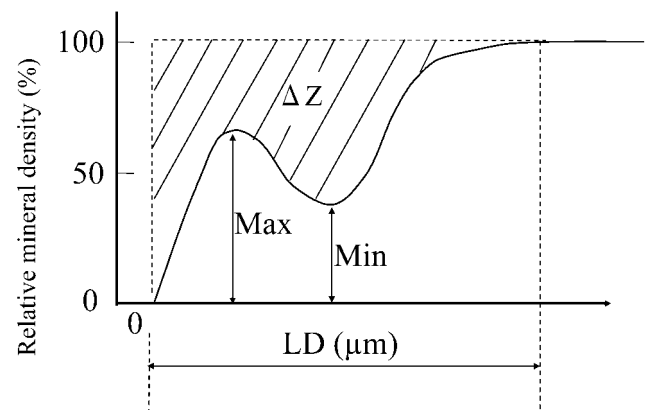

Fig. 1　Determination of lesion parameters of ΔZ, LD, Max and Min.

Results

Before quantitative TMR analysis, we carefully observed the TMR images of the sections and noted severe destruction of the subsurface layer in some cases. The destruction probably occurred during preparation of the 100-μm sections for TMR analysis. Therefore, we discarded these sections for TMR analysis (3, 1 and 5 sections for the baseline, test and placebo dentifrice groups). The final number of sections used for TMR analysis was 13, 15 and 11 for the baseline, test and placebo dentifrice groups, respectively.

Figure 2 shows ΔZ values (% × μm, mean ± SD) at the baseline (no remineralization treatment; 8,029 ± 2,176) and for the test (4,108 ± 1,181) and placebo dentifrice groups (6,515 ± 2,062) after the remineralization treatment. There

was a significant difference between the baseline and test dentifrice groups, indicating progression of lesion remineralization with a 48.8% recovery rate. However, no significant difference was observed between the baseline and placebo dentifrice groups, although there was quite a large difference (1,514) in the mean values between them (numerical recovery rate, 18.9%). Moreover there was a significant difference between the test and placebo dentifrice groups.

Figure 3 shows LD values (μm, mean ± SD) at the baseline (184 ± 41), and for the test (148 ± 39) and placebo dentifrice groups (156 ± 40) after remineralization. No significant difference was observed between the baseline and test/placebo dentifrice groups.

Figures 4 and 5 show the Max and Min values (%,

Fig. 2 ΔZ values at the baseline and after remineralization in the test and placebo groups.
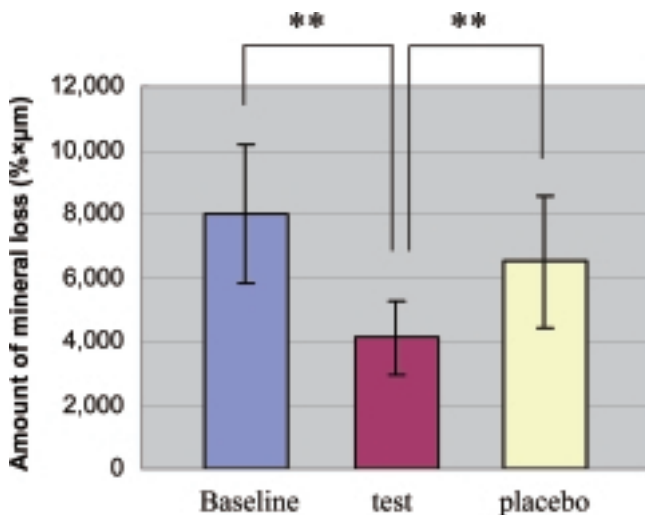

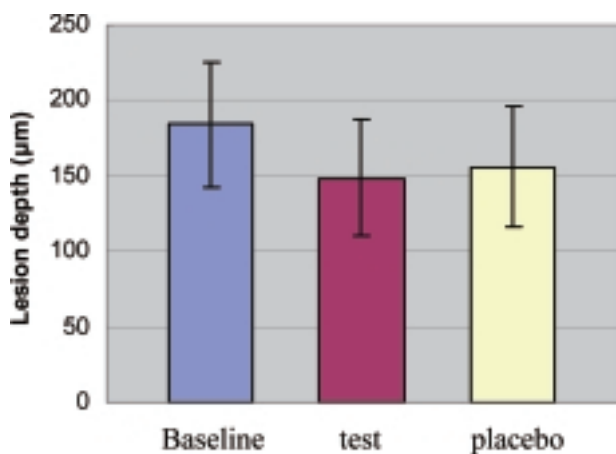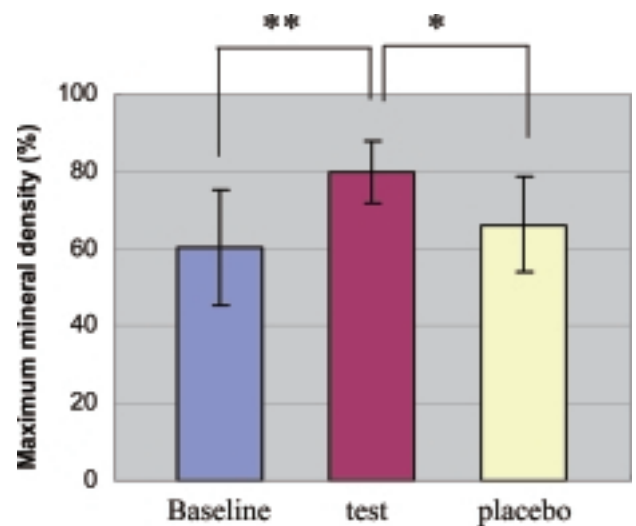Fig. 4 Maximum values at the baseline and after remineralization in the test and placebo groups.

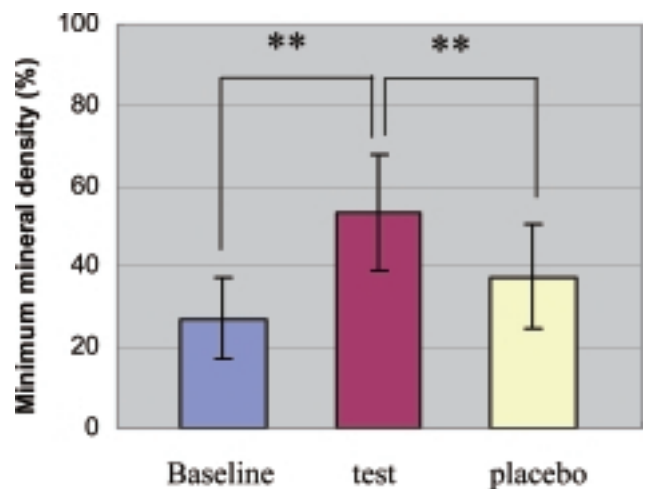Fig. 3 LD values of the baseline and those after the remineralization in the test and placebo groups.

Fig. 5 Minimum values at the baseline and after remineralization in the test and placebo groups.

mean ± SD) at the baseline (Max: 60.2 ± 14.9, Min: 27.2 ± 10.1), and for the test (Max: 79.6 ± 8.1, Min: 53.5 ± 14.4) and placebo dentifrice groups (Max: 66.3 ± 12.3, Min: 37.6 ± 13.1), respectively. There were significant differences between the baseline and test dentifrice groups, but no significant differences between the baseline and placebo dentifrice groups. Also there were significant differences between the test and placebo dentifrice groups.

Typical TMR images at the baseline, and those after remineralization treatment with the test and placebo dentifrices are shown in Fig. 6. Higher mineral density was noted at the surface layer and the lesion body in the test dentifrice group than in the baseline and placebo dentifrice groups. This finding can be confirmed by reference to the corresponding mineral profiles attached to Fig. 6.

Table 1 shows the longitudinal changes in Ca concentration (mM) and pH in the remineralizing solutions (originally Ca: 1.0 mM, pH 6.30, respectively). An increased Ca^{2+} concentration (7.11 mM) was observed just after 1 h of incubation, and slight increases were seen after 3 and 16 h (7.39 mM). Similar behavior in terms of a pH increase was noted after 1 h (6.79) and 16 h (6.93) of incubation, whereas for the placebo dentifrice, a slight increase in pH (6.42, data not shown) was observed after 16 h of incubation.

Figures 7A and 7B show FE SEM images of calcium carbonate powders for the dentifrice abrasive (× 7,000) and that of NC particles (× 60,000), respectively. The former image indicates an aggregate composed of very large (10 ~ 12 μm) and smaller (~ 1 μm or less) particles compared with the NC particles (several tens to hundreds of nm).

Discussion

The present study employed conventional culture plates with wells whose bottom surface had been collagen-coated. Such plates have been used to facilitate cell culture and adhesion to the collagen-coated surface (16). The collagen-coated surface has been also used for adhesion studies of oral bacteria such as *Streptococcus mutans* and *Lactobacillus*, assuming that these bacteria recognize dentinal collagen molecules for their adhesion (17,18). We considered the feasibility of using collagen-coated wells as a model of proteinaceous oral surfaces (mucous membrane, tongue, pellicle-coated tooth surfaces). Generally oral surfaces are covered by saliva, and more characteristically by various salivary proteins, and these

Table 1 Released Ca^{2+} concentrations (mM) and pH after the NC incubation

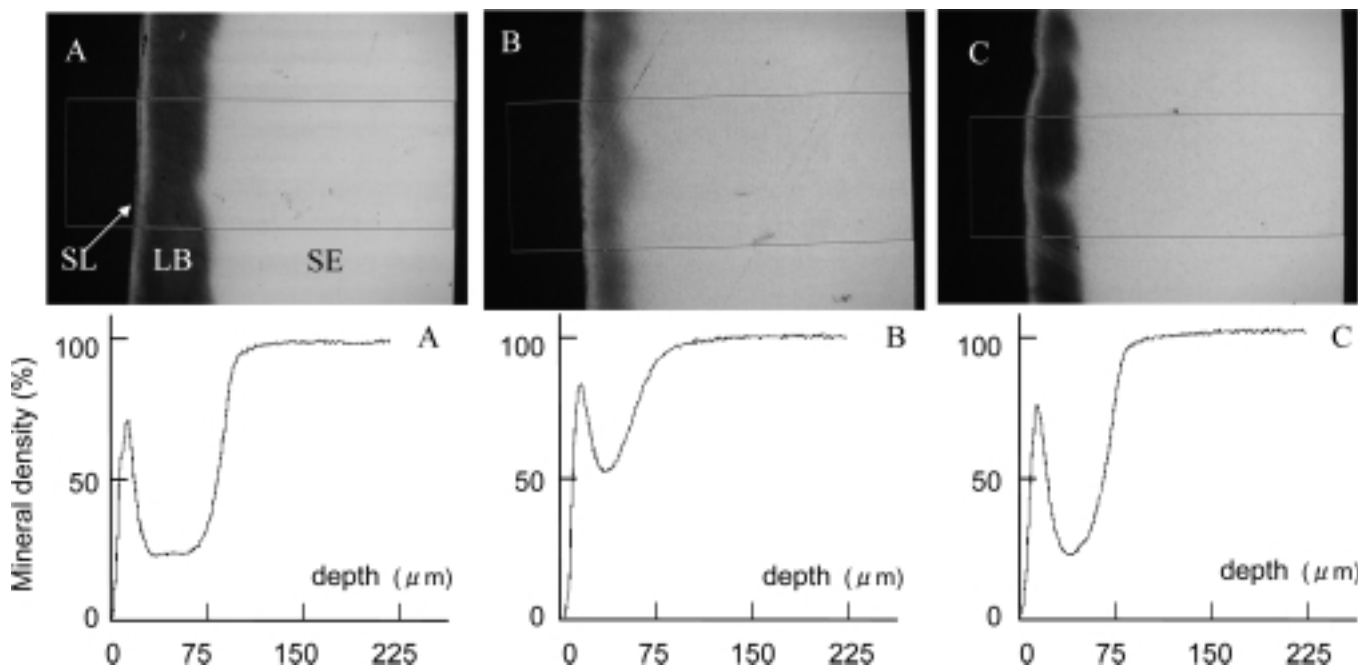|	Time after incubation (hr)		
	1	3	16
Ca^{2+} : mean ± SD	7.11 ± 0.15	7.27 ± 0.40	7.39 ± 0.19
pH : mean ± SD	6.79 ± 0.04	6.86 ± 0.03	6.93 ± 0.01

Fig. 6 Typical TMR images at the baseline (A), and in the test (B) and placebo (C) groups after remineralization.
SL: surface layer, LB: lesion body, SE: sound (no demineralized) enamel.

proteinaceous films probably cover both soft and hard tissue surfaces. The salivary thin film formed on tooth surfaces is called a pellicle, and various kinds of proteins have been identified in it (19). In fact, we observed by SEM that the NC particles (secondary aggregates) were retained on the collagen-coated surface as well as saliva-coated enamel surface after topical treatment with the slurry followed by several rinses with distilled water, suggesting similarity in terms of NC retention behavior between collagen-coated and saliva-coated surfaces. Moreover, the use of 6-well plates is advantageous because such plates are commercially available and thus have substantially the same quality of the coated surfaces. These advantages would make it possible to establish an in vitro standardized and controlled comparison study protocol.

It is important to point out that this study used a small amount of remineralizing solution (0.5 ml/well). It is known that saliva flows as a kind of thin film covering the oral surfaces, and that the volume of resting saliva is small (0.4 - 1.4 ml) (20), with a low flow rate (0.3 - 0.4 ml/min) (21). As the thickness of the salivary film is estimated to be 10 - 70 µm (22), we presumed that a small amount of remineralizing solution would provide reasonable simulation of the salivary film. The volume of the present remineralizing solution was chosen as a minimal one for certainty of quantitative and reproducible handling. Mathematical calculation indicated that the thickness of the remineralizing solution in each well (9.2 cm^2) would be 540 µm if spread evenly over the coated surface of the well. This thickness was sufficient for 500 µm of 4 thin sections per well to be immersed in 0.5 ml of the remineralizing solution.

There was a statistically significant difference in ΔZ value between the baseline and test dentifrice groups (Fig. 2), indicating lesion remineralization. This efficacy would be attributable mostly to the marked elevation of Ca^{2+} concentration (Table 1), and also the pH increase in the remineralizing solution would exert a favorable effect on lesion remineralization.

Generally, calcium carbonate is sparingly soluble in distilled water (1.4 - 1.5 mg/100 ml or 0.14 - 0.15 mM). Chemically when it is placed in aqueous solution, it is equilibrated with Ca and carbonate ions (CO$_3^{2-}$, HCO$_3^-$) in solution, and the concentrations of carbonate ions are equilibrated with the partial pressure of CO$_2$ in air. Therefore calcium carbonate placed in contact with pure water loses CO$_2$ into the atmosphere and yields Ca and OH$^-$ ions in the solution (23). Experimentally, the water slurry of the present NC gave a pH value of 9.1.

We think that the specific surface area (SSA) of the NC would be extremely large because of its nano-sized particles (several tens to hundreds of nm) compared with calcium carbonate powders for dentifrice abrasive (Fig. 7). Therefore, the rate of dissolution (release of Ca^{2+} ions) of NC would be much faster than that of other calcium carbonate products. The elevation of Ca concentration and pH would result in an increase in the DS value with respect to hydroxyapatite, favoring apatitic mineral deposition in the lesions (1,7). The observed Ca concentration (approximately 7 mM), which was higher than that of inherent calcium carbonate in distilled water, was ascribed to buffering (in this case, dissolving) action by the phosphate in the remineralizing solution with a lower pH of 6.30.
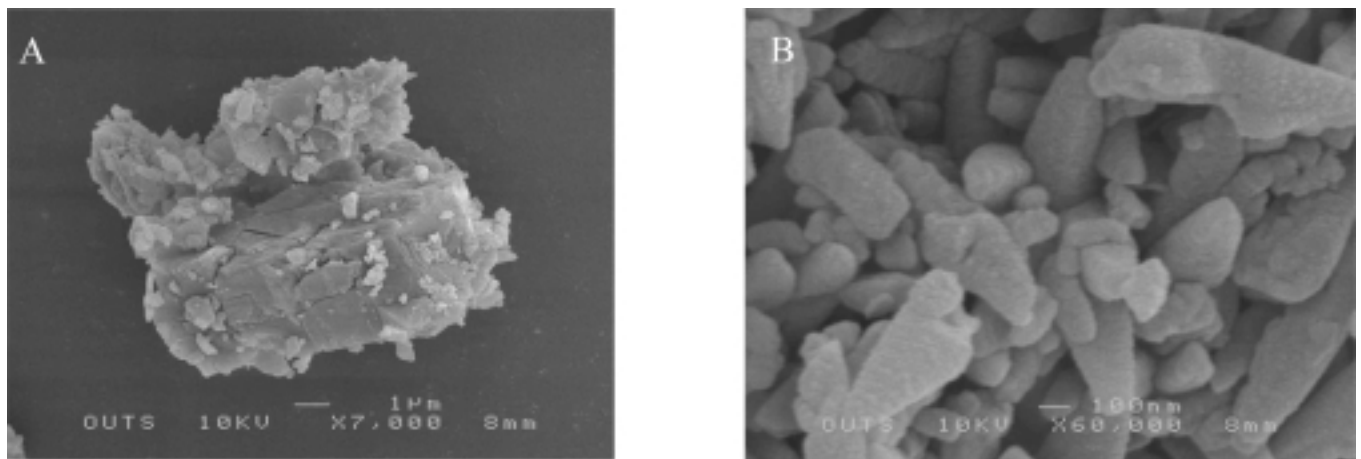

Fig. 7   FE SEM images; A: calcium carbonate powders for dentifrice abrasive (× 7,000), B: Magnified image of NC particles (× 60,000).

No significant difference in ΔZ value was observed between the baseline and placebo dentifrice groups, although there was quite a large difference in the mean values between them. This might have been due mostly to a relatively low remineralizing potential of the solution, similar to that of resting saliva, and also to a larger deviation (SD) in the sample specimens. Nakashima et al., reported an approximately 4-fold difference in acid susceptibility among 43 extracted human teeth (24). Many more sections would be required to conclude whether the difference was significant.

Furthermore, it is possible to speculate about possible influences of lesion depth and volume of the remineralizing solution on the overall rate of lesion remineralization, as follows. If the lesion depth had been less, and the volume of remineralizing solution much greater than those used in the present study, a greater rate of remineralization might have been detected. This possibility needs to be discussed on the basis of rate-determining factors. Obviously two factors need to be considered. The first is the rate of penetration of Ca/phosphate/OH ions into the lesion. The penetration rate is dependent on at least three sub-factors: 1) the volume of remineralizing solution, 2) the lesion depth that is related to the capacity of the maximal remineralizable volume in the lesion body of the specimen, and 3) the degree of porosity in the lesion surface layer. The second factor is the rate of apatitic mineral formation in the lesion. If the rate of apatitic mineral formation is sufficiently slow compared with the rate of Ca/phosphate/OH ion penetration, the volume of remineralizing solution would not be the rate-determining factor in the overall remineralization process. It would be quite difficult to determine the two rates. We believe that the volume of the remineralizing solution would not be the rate-determining factor in this experimental condition if the Ca^{2+} concentration in the remineralizing solution is kept high and steady during 16 h of incubation (Table 1).

On the other hand, no significant difference was found in lesion depth (LD) between the baseline and test/placebo dentifrice groups, although the mean LD value of the test dentifrice group was the lowest among the three groups. This might be due to the steep lesion profile, indicating that the Min values were rather lower before the remineralization treatment (Fig. 5; mean value at the baseline: 27.2 % and Fig. 6A). The mineral deposition around at the lesion front after remineralization might be insufficient to reduce the lesion depth due to the steep lesion with a lower mineral density at the lesion front (25).

As demonstrated in terms of ΔZ, there were statistically significant differences in Max and Min values between the baseline and test dentifrice groups (Figs. 4 and 5),

supporting the remineralization efficacy of the test dentifrice. However, no significant difference was observed between the baseline and placebo dentifrice groups.

It is believed that monofluorophosphate would exert its caries-preventive efficacy after the splitting of the molecule to free fluoride (F^-) and phosphate ions by phosphatase enzymes in the oral cavity (26,27). In this study, no such enzyme was used because of several difficulties in use of enzymes, i.e., no commercially available and validated enzyme identical to phosphatase enzymes in the oral cavity, no information on how to use them in active conditions using a standardized procedure without interfering with dentifrice ingredients such as sodium laurylsulfate, and others (28). Although we did not determine the concentration of F^- originating from monofluorophosphate, we thought that the F^- concentration would be negligibly low because of the stability (no splitting) of monofluorophosphate in calcium carbonate-based dentifrice formulations without forming sparingly soluble CaF_2 (6). Thus the present efficacy could be ascribed solely to the presence of NC, which released Ca ions (Table 1) and probably OH^- ions (pH increase). Of course one could anticipate the combination effects of NC and monofluorophosphate based on theoretical consideration in terms of elevation of driving force for fluoridated apatite precipitation in the lesion. Since the driving force would increase in proportion to the concentrations of mineral ions (Ca^{2+}, phosphates, F^-, OH^-) in oral fluids, the precipitation of fluoridated apatite would be promoted to a far greater extent in the presence of increased mineral ion concentrations derived from NC. In other words, even a lower F^- concentration would exert a remineralizing effect in the presence of NC. However, as mentioned above, it would be very difficult to evaluate it in an *in vitro* system. A future intra-oral study would be needed to confirm this in humans.

It should be pointed out that NC has an advantage over water-soluble Ca salts. As demonstrated in this study, NC possessed a good retention property on the surfaces of the oral cavity due to its colloidal particle size, followed by Ca ion delivery. In order for Ca ions to exert a caries prevention effect, continuous Ca delivery with a higher Ca concentration would be required. Water-soluble Ca salts are thought to be readily washed away from the oral cavity. On the other hand, NC adsorbed to oral surfaces as solid particles would be capable of continuously supplying Ca ions to oral fluids due to the slow release of Ca ion. Moreover, NC has the potential to increase the fluid pH surrounding the lesion enamel. These favorable effects would lead to promotion of fluoridated apatite in the lesion in conjunction with fluoride in the oral fluids, as mentioned

above. Also, owing to the favorable properties of NC, an inhibitory effect of NC on acid demineralization would be anticipated by neutralizing acids in plaque and subsequent supply of Ca^{2+} ions. Future study is planned to examine these possibilities.

On the other hand, it should be emphasized that a non-fluoridated dentifrice containing NC would be acceptable for children or their parents who are concerned about the dental fluorosis.

NC, however, has some disadvantages for preparation of dentifrice formulations. For instance, inclusion of NaF in the formulation is not allowed due to the formation of sparingly soluble CaF_2 that is assumed to be a non-cariostatic (inert) fluoride agent in dentifrices. Also acidic and Ca chelating ingredients are not employed in formulations, as they would destroy the NC colloidal property.

In conclusion, this study has demonstrated that a test dentifrice containing NC has promise for remineralization of incipient enamel lesions.

Acknowledgments

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