academicJournals

Vol. 5(7), pp. 68-76, July 2013
DOI 10.5897/JDOH2013.0089
ISSN 2006-9871 © 2013 Academic Journals
http://www.academicjournals.org/JDOH

Journal of Dentistry and Oral Hygiene

Full Length Research Paper

In vitro assessments of white-spot lesions treated with NaF plus tricalcium phosphate (TCP) toothpastes using microtomography (micro-CT)

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Accepted 23 May, 2013

X-ray microtomography (micro-CT) was used to assess the densities of white-spot lesions (WSL) treated with either a fluoride-free paste, or 0.21 or 1.1% NaF toothpastes containing functionalized tricalcium phosphate (TCP). Bovine enamel specimens were ground, polished and demineralized to form WSL. Specimens (N=10) were treated with one of the following NaF silica-based toothpastes in a 10-day pH cycling model: (1) Tom's of Maine (0% NaF), (2) Clinpro® Tooth Crème (0.21% NaF plus TCP), and (3) Clinpro® 5000 (1.1% NaF plus TCP). Each day consisted of four 2 min treatments, one 4 h acid challenge (pH=5.0), and immersion in artificial saliva (pH=7.0) between these events. After cycling, specimens were analyzed using micro-CT. Statistical analysis (Student's t-test, Welch t-test or Tukey HSD test) was performed at the 95% confidence level. Compared to sound enamel, significantly ($p<0.05$) lower densities in WSL were only found at 24 µm from Clinpro Tooth Crème, and at 36 µm from Clinpro 5000. In contrast, the densities measured at 12, 24, 36, and 48 µm from the fluoride-free toothpaste were lower ($p<0.05$) than sound enamel. Micro-CT analyses revealed NaF toothpastes containing TCP led to increased WSL densities relative to the fluoride-free toothpaste.

Key words: Toothpaste, microtomography (micro-CT), density, remineralization.

INTRODUCTION

Caries decay in tooth enamel remains a worldwide problem, and international global summits are organized to bring research together from locations around the world to examine the evidence and plan strategic efforts to deal with changes in dental caries prevalence (Bagramian et al., 2009). Orthodontists have to select patients very carefully and provide detailed oral hygiene instructions. When combined with dietary advice by an auxillary at regular intervals throughout treatment, this remains the most cost-effective approach (Mitchell, 1992). However, these approaches may not be sufficient, so other strategies are often employed, such as at-home topical fluoride formulations. In the United States, dental practitioners may recommend professional-strength 1.1% sodium fluoride tooth pastes for high-risk patients instead of the typical range in fluoride toothpaste (e.g. 0.20 to 0.32% NaF) readily available over-the-counter (Nordström and Birkhed, 2009). For instance, it has been estimated that using professional-strength and over-the-counter fluoride toothpastes leads to an average clinical reduction in caries by approximately 70 and 22%, respectively (Tavss et al., 2003). While such therapies might be recommended for

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various at-risk dental populations, from an orthodontic perspective, the inherent cleaning difficulties that are the direct result of the installation of dental appliances could lead to the recommendation of a high-fluoride treatment and/or multiple-step treatment regime to help fight against tooth decay. A relatively new and promising approach is the incorporation of a fluoride-compatible functionalized tricalcium phosphate (TCP) ingredient, which is a hybrid material that comprised of β–tricalcium phosphate and sodium lauryl sulfate, to NaF formulations (Karlinsey and Pfarrer, 2012). In particular, 3 M ESPE's Clinpro® Tooth Crème (0.21% NaF) and Clinpro® 5000 (1.1% NaF), which contain the TCP ingredient, are two of the newest professional-grade toothpastes commercially available. Inclusion of the func-tionalized TCP ingredient in NaF formulations has been shown to produce stronger, more acid-resistant mineral relative to fluoride alone in laboratory and clinical evalua-tions (Karlinsey et al., 2010a, b, c, 2009a, b, 2011a; Amaechi et al., 2012).

While transverse microradiography, cross-polarization microscopy and microhardness measurements are valuable in the assessment of remineralization/demineralization, another method of assessing efficacy may be through the use of X-ray microtomography (micro-CT). Micro-CT has been utilized in the observation of time-course changes in remineralization (Nakata et al., 2012) and demineralization (Watanabe et al., 2012); however, the use of micro-CT to assess changes in density of white-spot lesions (WSL) treated with NaF plus TCP systems has not yet been reported. As such, the purpose of this *in vitro* study was to use micro-CT to assess the densities of WSL treated with one of the following dentifrices: a fluoride-free, TCP-free control, or a 0.21 or 1.1% sodium fluoride toothpaste containing TCP. These densities were then compared to those measured for baseline sound and WSL enamel.

MATERIALS AND METHODS

Specimen preparation and WSL formation

Three millimeter enamel cores were drilled from the lingual and labial surfaces of bovine incisors using a bench top drill press (Power Glide, China) affixed with a hollow core drill bit. The cores were embedded into hollowed out acrylic rods using DuraBase resin (Reliance Dental Mfg. Co., Worth, IL, USA). Each specimen was ground by hand with 600 grit SiC sandpaper under water cooling for 30 s using a Leco Spectrum System 1000 Grinder/Polisher (St. Joseph, MI, USA) set to 300 rpm. Then, with the unit set to 200 rpm, each specimen was polished by hand for 1 min using 3 µm diamond compound in conjunction with microid extender solution (Leco). After rinsing with distilled (DI) water, Vickers surface micro hardness was performed with a 200 gf load and 15 s dwell time (Leco LM247AT micro hardness tester, St. Joseph, MI, USA) to confirm the presence of sound enamel in the polished specimen as indicated by a Vickers hardness number (VHN) of 300 VHN or greater. Acceptable sound specimens were then immersed in vials containing a 0.2 wt% 450 kDa polyacrylic acid (Sigma-Aldrich, St. Louis, MO, USA) and 0.1 M lactic acid (Sigma-Aldrich) solution saturated 50% with hydroxyapatite (BioRad, Hercules, CA, USA) and adjusted to a pH of 5.0 (Karlinsey and Pfarrer, 2012). These vials were subsequently loaded into an incubator for 40 h at 37°C to establish 'white-spot' (non-cavitated) lesions, the characteristics and histology of which have been discussed in detail previously by White (1987). Briefly, the ensuing caries-like lesions produced by this solution have a dense surface mineral zone approximately 15 µm thickness, while the lesion depth extends to about 70 µm (White, 1987). Upon white-spot formation, acceptable baseline surface micro hardness ranging from 25 to 50 VHN (200 gf for 15 s made using a LecoLM247AT indenter) were included in the study.

Treatment groups and study protocol

Bovine enamel specimens (N=10 per group) were then grouped for treatment with one of the following silica-containing dentifrices: toothpaste A, Tom's of Maine fluoride-free toothpaste; toothpaste B, Clinpro® Tooth Crème (0.21% NaF plus TCP); toothpaste C, Clinpro® 5000 (1.1% NaF plus TCP); sound (N=10) and WSL (N=10) specimens were also produced for control purposes.

As outlined in Table 1, the three groups of enamel specimens were then cycled in a remin/demin pH cycling model lasting 10 days. This daily cycling model comprised immersion of inverted specimens in two 2 min treatment events performed an hour apart in the morning, followed by one four-hour polyacrylic acid-lactic acid challenge (15 mL, pH=5.0), and finally two more two-minute treatment events in the afternoon. Specimens were inverted and immersed in artificial saliva in between the daily treatments and acid challenge, as well as overnight (Cate, 1988). The treatments were diluted three-fold with DI water (5 g dentifrice: 10 ml DI water) to create the dentifrice slurry. The treatments and saliva events were magnetically agitated at 300 rpm, while the acid challenge was static. After each treatment and acid challenge, the specimens were rinsed with DI water prior to placement into artificial saliva. Fresh treatment slurries and acid solution were used daily, with the artificial saliva solution changed once daily during the acid challenge.

Micro-CT analysis

A commercial micro-CT (Toscaner-30000 µhd®, Toshiba, Japan) was used for density measurement at Advanced Composite Technology Center, Japan Aerospace Exploration Agency (JAXA) in Tokyo, Japan. The parameters used to collect micro-CT data, which were optimized based on the best image collected, are shown in Table 2. The 'Laks' (standard parameters) filter function was used to probe densities of specimens. An example view of the micro-CT apparatus and the mounted specimen fixed to the turn table is as shown in Figure 1. From this setup, an example 'field of view' (FOV) image was taken, as shown in Figure 2. The raw data from the 50 bovine enamel specimens (N=10 for each treatment groups, plus 10 baseline sound and 10 baseline WSL) were collected, the window level and width were adjusted, and then they were converted to bitmap files using Toscaner-30000V.17.46 operating software. CTAn® software version 1.12.0 (SkyScan, U.S.A.) was used to determine density based on the 256 gray scale collected from each enamel specimen. Data reconstruction did not include ring free correction (RFC). Five regions of interest (ROI) were randomly selected from one slice picture indicated at the position 50.0 ± 1.0 grayscale value (Figure 3). The grayscale baseline was set at 50.0 points ± 1.0 in ROI based on the brightness generated from the surface in one circular ROI from the top-view perspective as shown in Figure 3. Since the bovine enamel surfaces were not perfectly flat, a small ROI helped to reduce inclinations at the top surface. In doing so the possibilities of involving ring and/or beam-

Table 1. Outline of daily events and duration employed in the remin/demin pH cycling model.

Event	Duration
Treatment 1*	2 min
Saliva, pH=7.0	1 h
Treatment 2	2min
Saliva, pH=7.0	1 h
Acid challenge, pH=5.0	4 h
Saliva, pH=7.0	1 h
Treatment 3	2 min
Saliva, pH=7.0	1 h
Treatment 4	2 min
Saliva, pH=7.0	Overnight

On day one, specimens were pre-conditioned for 1 h in artificial saliva prior to the first treatment. Artificial saliva refreshed daily after the acid challenge. The treatment will consist of a three-fold dilution of test product to water (that is, 5 g paste, 10 ml distilled water). The carbopol-lactic acid system will be used to prepare the artificial lesions and will also be used throughout the 10-day cycling study.

Table 2. Micro-CT exposure parameters.

Parameter	Value
Scan. time (s)	355.8
Recon. time (s)	5.7
Tube voltage (kV)	90.000
Tube current (mA)	385.0000
number of views	800
Integration number	3
FPD gain (pF)	0.25
FPD integration time (ms)	124
FOV (mm)	6.987782
Slice thickness (mm)	0.0120
System name	TOSCANER-30000
Matrix size	1024
Scan mode	Half
Filter function	Laks
RFC	none
Image bias	0
Image slope	100
Data mode	Cone beam
Image direction	From the top
FDD	800.000
FCD	16.500
Window level	767
Window width	1230
Pixel size (mm)	0.0068240059
Magnification ratio	40.92858
Scale (mm)	0.6987782

hardening artifacts in the ROIs also were reduced (Figure 4), and careful consideration was used in selecting ROIs lacking these types of artifacts. Densities were then determined at each slice depth at baseline, z1=12 µm, z2=24 µm, z3=36 µm, z4=48 µm, z5=60 µm, z6=72 µm, and z7=84 µm. Only toothpaste B group (that is, Clinpro Tooth Crème) had specimens that produced X-rays out of range of the FOV: in this instance, only eight of the ten specimens provided data and were measured at 40 places with 8 slice depths per place for a total of 320 ROIs. Otherwise, all ten specimens from each of the other two toothpaste groups, along with the baseline sound and baseline WSL, were measured at 50 places with 8 slice depths per place for a total of 400 ROIs. To obtain absolute densities, the baseline density was then subtracted from the z1 through z7 densities for each group.

Statistics

All statistics were determined using the statistical package SAS-JMP (SAS Institute, USA). The mean densities at each depth (z1 through z7) for each of the dentifrice groups (A, B and C) along with the two control groups (sound and baseline WSL) were defined as independent variables. Comparisons between groups were made by comparing either the baseline sound enamel or the baseline WSL as the control group to the WSL treated with each of the three toothpastes as the experimental group. Each measurement was considered of equivalent variance so parametric testing (Student's t-test) of the mean densities between the control groups and experimental groups were performed, with $p<0.05$ considered significant. For multiple comparison testing, one-way analysis of variance (ANOVA) was used followed by Tukey highest significant difference (HSD) test to determine where the differences existed ($p<0.05$).

RESULTS

Density versus slice depth comparison between sound enamel and toothpaste-treated groups

Mean density values at each slice depth were compared between baseline sound enamel and lesions treated with either Clinpro Tooth Crème (Figure 5), Clinpro 5000 (Figure 6) or Tom's of Maine fluoride-free toothpaste (Figure 7). Significant differences were found between sound enamel and lesions treated with Clinpro Tooth Crème at z2 and z7 (Figure 5). At these slice depths, the mean density of lesions treated with Clinpro Tooth Crème was significantly lower ($p<0.05$) at z2 and significantly higher ($p<0.05$) at z7 compared to baseline sound enamel. With respect to comparisons between sound enamel and lesions treated with Clinpro 5000 (Figure 6), the mean density of lesions treated with Clinpro 5000 was significantly lower ($p<0.05$) than the baseline sound enamel at the z3 slice depth. Comparisons of density values versus slice depth between sound enamel and lesions treated with Tom's of Maine (Figure 7) revealed the density values were significantly lower ($p<0.05$) at z1, z2, z3 and z4 for lesions treated with Tom's of Maine.

Density versus slice depth comparison between WSL and toothpaste-treated groups

Mean density values at each slice depth were compared between baseline WSL and lesions treated with either Clinpro Tooth Crème (Figure 5), Clinpro 5000 (Figure 6) or Tom's of Maine fluoride-free toothpaste (Figure 7). Significant differences were found between baseline WSL

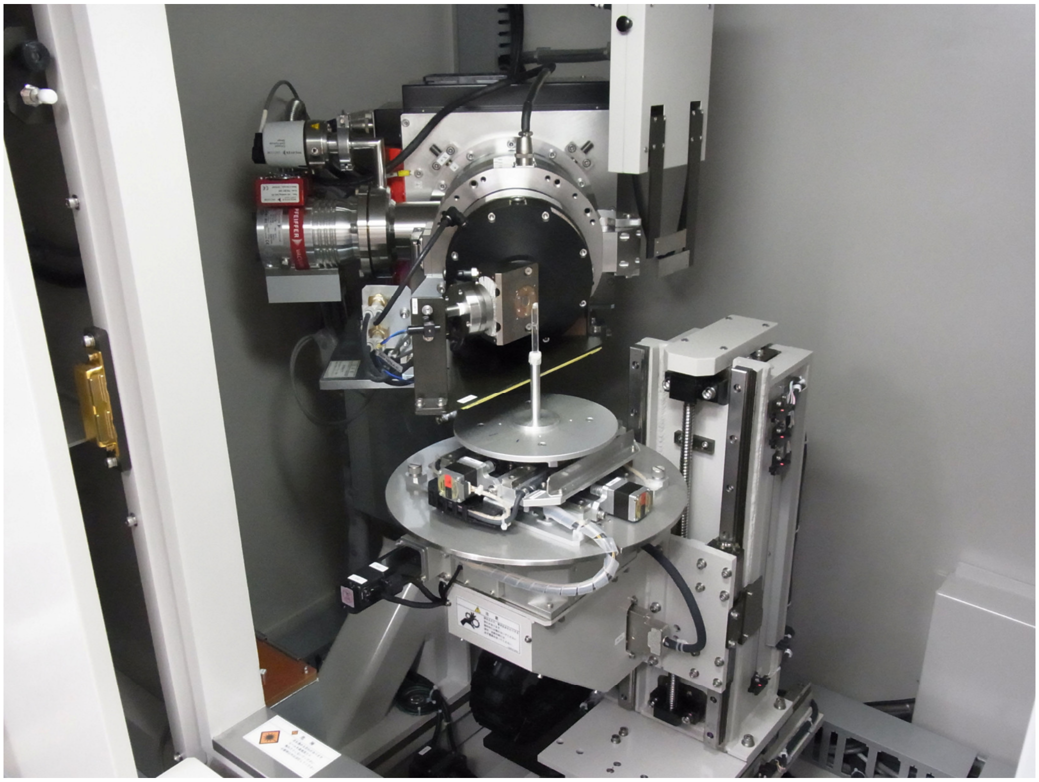

Figure 1. Representative view of an enamel specimen positioned on the turntable in the micro-CT apparatus.

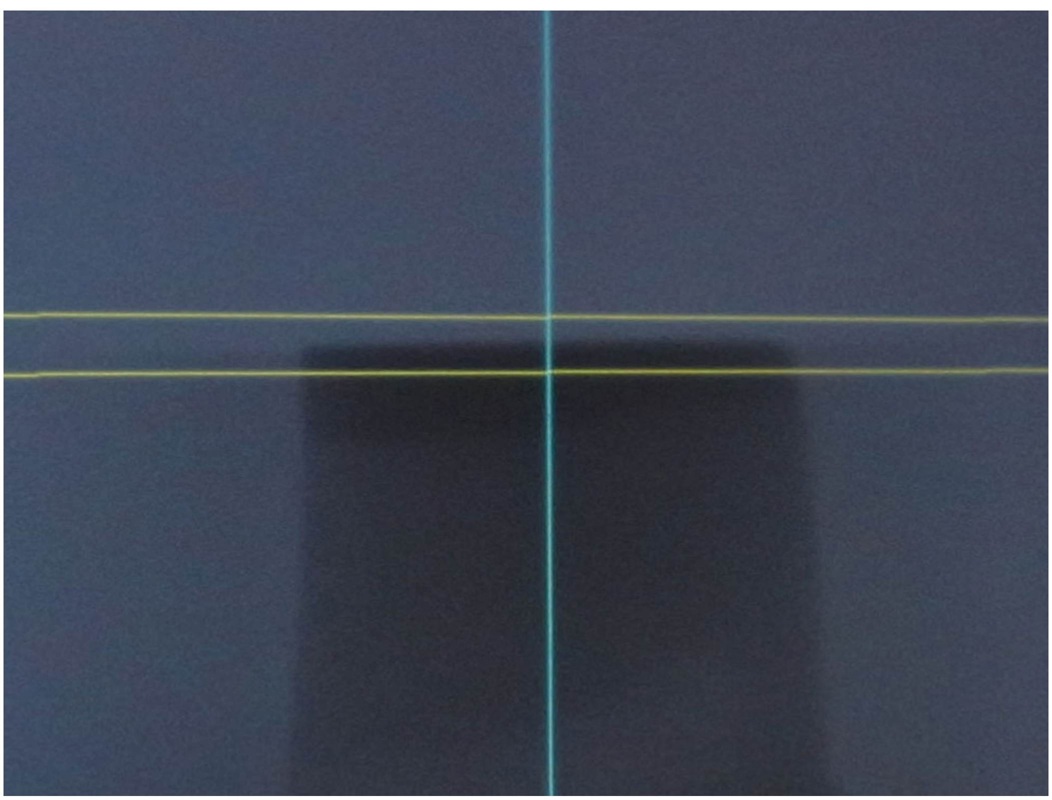

Figure 2. Two lines showing the X-ray FOV in the vertical position.

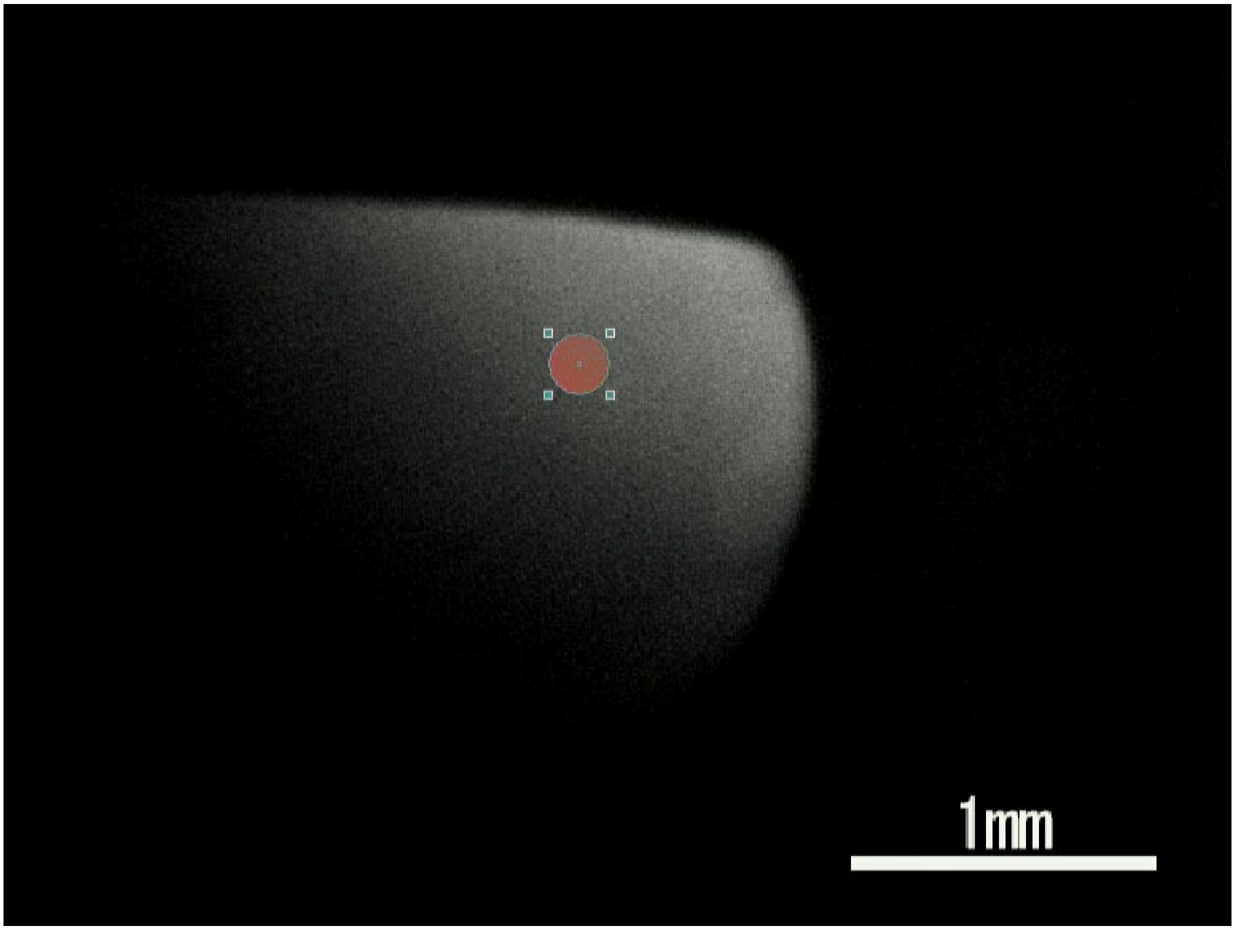

Figure 3. ROI is composed of 664 pixels in the horizontal position.

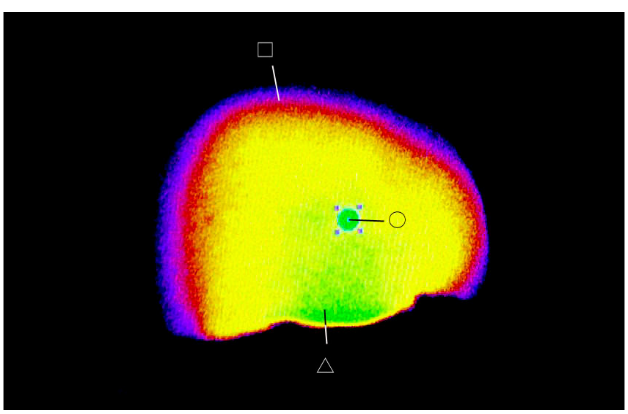

Figure 4. Within the yellow-shaded area, a randomly selected ROI (small green circle) is shown. Also shown are beam-hardening (purple outline) and ring (green in the center) artifacts.

and lesions treated with Clinpro Tooth Crème at z1 through z6 (Figure 5). At these slice depths, the mean density values of lesions treated with Clinpro Tooth Crème were significantly higher ($p<0.05$) as compared to the baseline WSL. With respect to comparisons between baseline WSL and lesions treated with Clinpro 5000 (Figure 6), the mean density of lesions treated with Clinpro 5000 was significantly higher ($p<0.05$) than the baseline WSL at z1, z2, z3 and z4 slice depths. Comparisons of mean density values versus slice depth between baseline WSL and lesions treated with Tom's of Maine (Figure 7) revealed the density values were significantly higher ($p<0.05$) at z1 through z5 for lesions treated with Tom's of Maine.

Density versus slice depth comparison among WSL treated with each of the toothpastes

Comparison of the mean densities at each slice depth for WSL treated with each of the three toothpastes is as shown in Figure 8. At z1, Clinpro Tooth Crème and Clinpro 5000 were not significantly different ($p=0.91$), but were both superior to the fluoride-free Tom's of Maine ($p=0.04$ and 0.01, respectively). Similarly, at z2, Clinpro Tooth Crème and Clinpro 5000 were not significantly different ($p=0.99$) but again were both superior to the

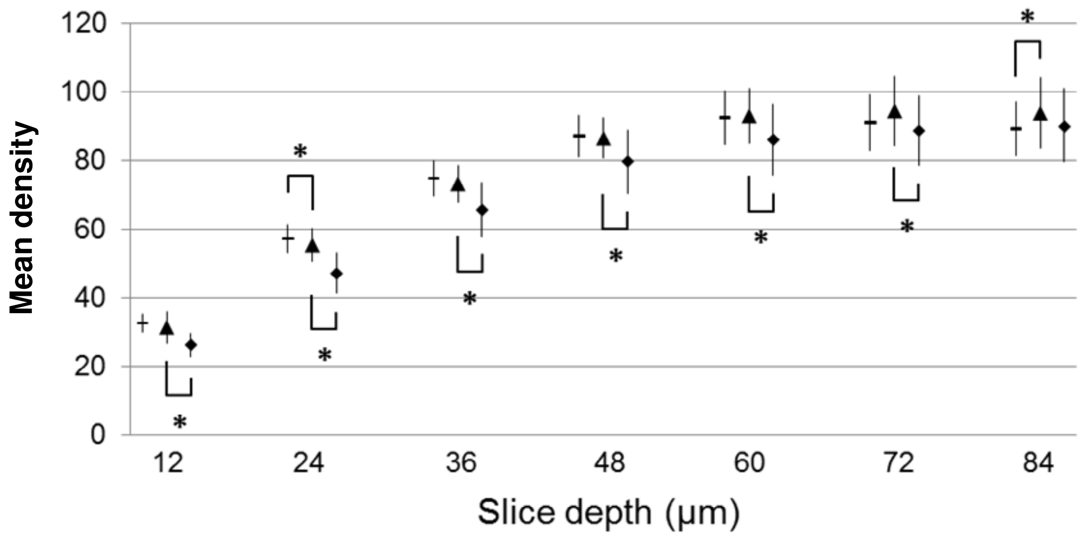

Figure 5. Mean density (standard deviation) versus slice depth for white-spot lesions treated with Clinpro Tooth Crème (B, ▲) and baseline sound enamel (-) and baseline white-spot lesions (♦). Asterisks (*) mark significant differences.

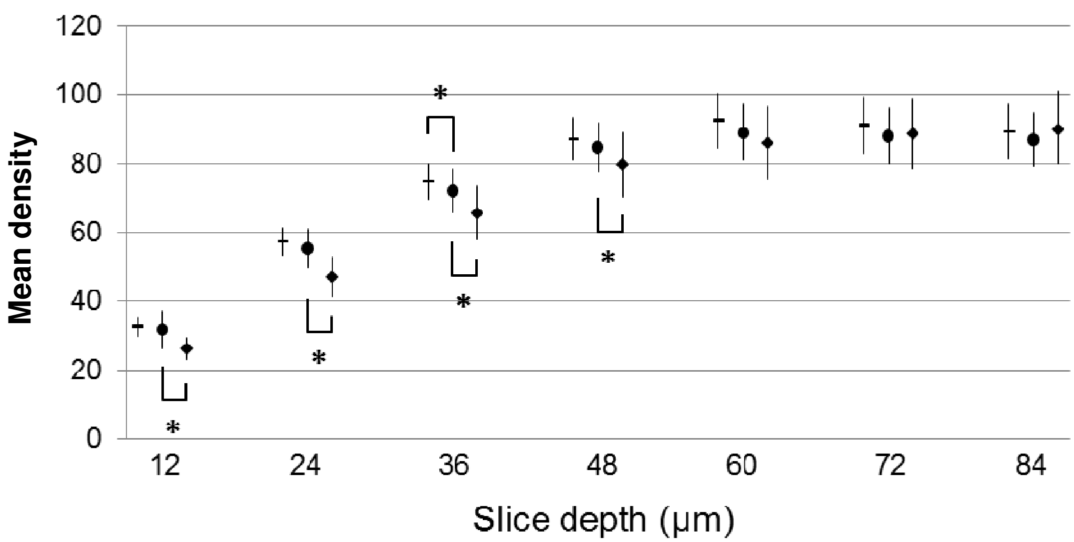

Figure 6. Mean density (standard deviation) versus slice depth for white-spot lesions treated with Clinpro 5000 (C, ●) and baseline sound enamel (-) and baseline white-spot lesions (♦). Asterisks (*) mark significant differences.

fluoride-free Tom's of Maine (p=0.0009 and 0.0004, respectively). At z3, Clinpro Tooth Crème was found to be statistically superior to Tom's of Maine (p=0.01), but statistically similar to Clinpro 5000 (p=0.63), which was not significantly different compared to Tom's of Maine (p=0.08). No significant differences were observed among the three toothpastes at z4 or z5. At z6, Clinpro Tooth Crème was found to be significantly different compared to Clinpro 5000 (p=0.002), but Clinpro Tooth Crème and Clinpro 5000 were not significantly different compared to Tom's of Maine (p=0.28 and 0.11, respectively). At z7, Clinpro Tooth Crème and Tom's of Maine were not significantly different from one another (p=0.68) but were each significantly different than Clinpro 5000 (p=0.001 and 0.01, respectively).

DISCUSSION

This study utilized micro-CT as a tool to characterize the

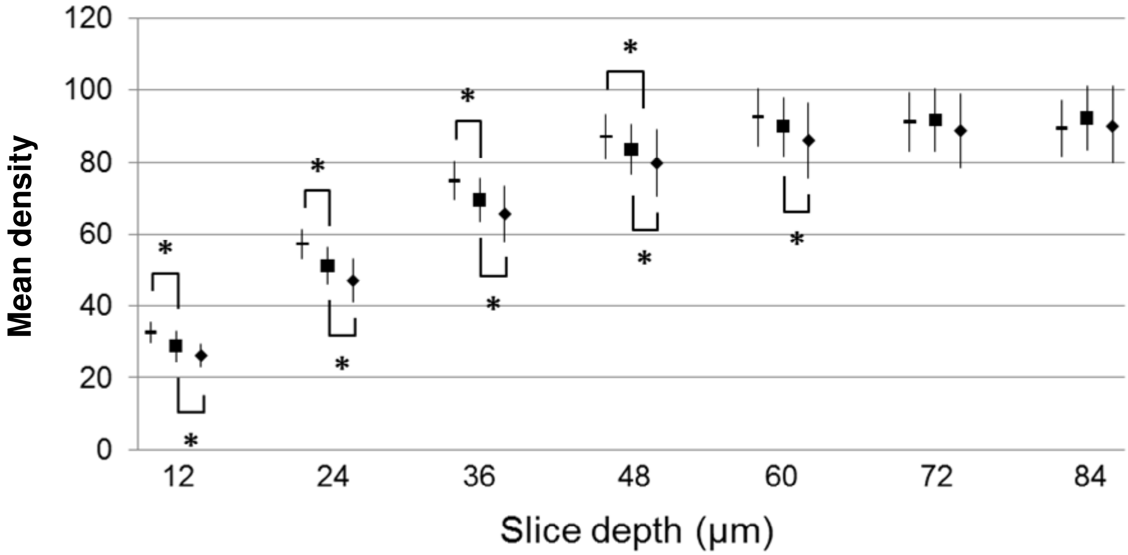

Figure 7. Mean density (standard deviation) versus slice depth for white-spot lesions treated with Tom's of Maine baseline (A, ■) and baseline sound enamel (-) and baseline white-spot lesions (♦). Asterisks (*) mark significant differences.

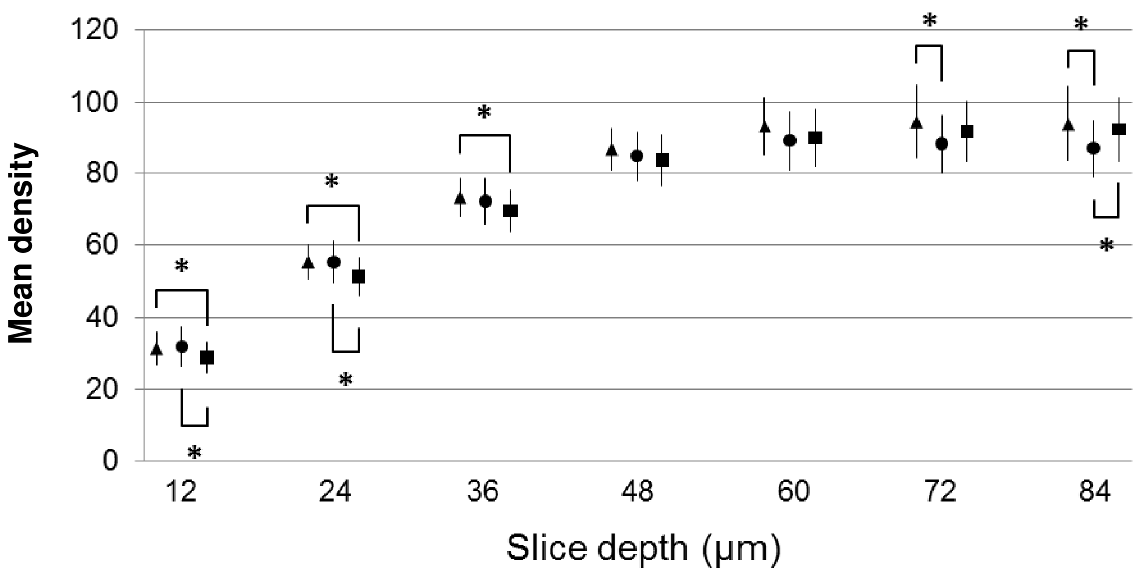

Figure 8. Mean density (standard deviation) versus slice depth for white-spot lesions treated with Tom's of Maine (A, ■) and Clinpro Tooth Crème (B, ▲) and Clinpro5000 (C, ●). Asterisks (*) mark significant differences.

response of incipient lesions subjected to a 10-day pH cycling model manifesting remineralization and demineralization periods and treated with one of three toothpastes. Among the elemental constituents of enamel, calcium is present in the largest concentration (Cate et al., 1988) and is most sensitive to X-ray absorption (Wildenschild et al., 2002). The loss of calcium during initial demineralization of enamel to create the 70 µm-deep lesion accompanies the loss of other enamel constituents as well, including phosphate, carbonate and hydroxyl groups. Micro-CT has previously been used to probe such compositional changes (Watanabe et al., 2012; Wong et al., 2004). Additionally, the exposure to acid challenges, different toothpaste treatments and remineralization periods in simulated saliva solutions in a pH cycling model leads to chemical and physical changes of the lesion framework (Watanabe et al., 2012).

An important result of this study was that micro-CT resolved significant differences between either baseline WSL or sound enamel and lesions treated without or with fluoride from three different toothpastes. As shown in Figures 5 to 7, the densities of WSL exposed to the different treatments in the pH cycling model manifest positions between sound and softened (that is, WSL) enamel. With respect to the densities of baseline WSL, each of the toothpastes produced increased densities when evaluated in the 10-day remineralization model. This result demonstrates that the artificial saliva solution encourages remineralization by virtue of its ionic constituents and is consistent with the important role that natural saliva has on the remineralization and/or protection against demineralization of the dentition (Stookey, 2008). But in WSL treated with each of three dentifrices, significant differences were observed as shown in Figure 8. In particular, within the first 25 µm (that is, z1 and z2) of the approximately 70 µm-deep lesion, both fluoride toothpastes produced greater densities relative to the fluoride-free Tom's of Maine toothpaste. Because integration of fluoride with the tooth structure improves resistance to demineralization (Karlinsey et al., 2010a it is possible that the lesions treated with the fluoride-free paste succumbed more readily to the daily acid challenges as compared to the two fluoride pastes. Alternately, the lesions treated with fluoride may have increased in density due to the formation of fluoridated mineral such as fluorapatite or calcium fluoride (Cate and Rempt, 1986). Due to the pharmacokinetics of fluoride, its penetration into enamel is limited and decreases exponentially with enamel depth (Stearns, 1970). It may be possible that the functionalized TCP present in the Clinpro dentifrices helps extend the depth of fluoride penetration, and therefore lesion remineralization (Karlinsey et al., 2010d; Mensinkai et al., 2012), as evidenced by Clinpro Tooth Crème having a statistically significant density increase over baseline WSL from 12 to 72 µm. However, high fluoride levels can still restrict penetration depth even with TCP included, considering the Clinpro 5000 group only had statistically significant density increases over baseline WSL from 12 to 48 µm. The data in Figure 8 demonstrate that no significant differences in density among the three toothpastes were observed around 48 µm. Although some differences were observed beyond the bottom of the 70 µm lesion and into sound enamel, these densities are on par with those for sound enamel and it may be possible that the observed differences might be attributed to possible leaching or redistribution of mineral constituents due to penetration of acid during the acid challenge.

Compared with sound enamel at z2 (that is, ~ 24 µm) no significant differences in WSL density were observed with Clinpro 5000, while the density for Clinpro Tooth Crème was significantly lower. This observation suggests Clinpro 5000, which contains 1.1% NaF, delivers remineralization benefits at the surface of the WSL better than the 0.21% NaF Clinpro Tooth Crème. Since the densities of lesions treated with Clinpro 5000 were similar to those of sound enamel down to 24 µm, this result suggests the rapid reaction kinetics and concentration of fluoride from the high-fluoride dentifrice manifests limitations to the extent of subsurface remineralization relative to a lower fluoride level (that is, Clinpro Tooth Crème). At z3 (that is, ~ 36 µm) the density of Clinpro 5000 was significantly lower, and trended lower at subsequent depths. This response suggests that high-fluoride Clinpro 5000 may have limited penetration into WSL, a position that is consistent with characteristics of high-fluoride treatments (Tavss et al., 2003), and is also consistent with a previous report on WSL treated with Clinpro 5000 and analyzed using transverse micro-radiography (TMR) (Karlinsey et al., 2011a). And in the absence of fluoride, there exists an increased suscepti-bility for enamel dissolution, which appears to contribute to the markedly lower densities at z1 through z4 for lesions treated with fluoride-free Tom's of Maine relative to sound enamel.

The comparison between baseline sound enamel and the WSL treated with each of the pastes was performed to assess the degree to which each toothpaste remineralized incipient enamel lesions. These comparisons suggest fluoride assists in mineral integration, and subsequently affects the bonding environments of enamel constituents, especially with respect to calcium. Based on previous research of surface and subsurface enamel strengthening (Karlinsey and Pfarrer, 2012; Karlinsey et al., 2010b), it is possible that the functionalized TCP ingredient in the Clinpro Tooth Crème and Clinpro 5000 contributes to the observed density measurements; however, additional studies, including those involving fluoride dentifrice without functionalized TCP, would be needed to further understand the nature of these effects. In any case, these observations suggest micro-CT might be useful in resolving effects due to different treatments. As such, studies incorporating different laboratory models that include treatments from dental preparations with various fluoride concentrations and/or other agents could be investigated. Additionally, studies involving lesions of various depths and porosities could be performed to further assess the sensitivity of micro-CT.

Conclusion

Micro-CT was used to assess the remineralization of combination dentifrices having fluoride and functionalized TCP, with both dentifrice systems leading to WSL densities approaching those of sound enamel. Additionally, observations regarding the extent of remineralization with respect to fluoride concentrations were also made. Although the pH cycling model employed in this study has clinical relevance (Karlinsey et al., 2010d; O'Reilly and Featherstone, 1987), a pilot clinical study should be performed to strengthen support for clinical recommendations.

ACKNOWLEDGEMENTS

The authors specially thank Sunao Sugimoto, Chief Manager of Advanced Composite Technology Center (JAXA) and Junko Aoki, technical support worker of IHI Jet Service Co., Ltd. for performing micro-CT. Toscaner – 30000 µhd® was used in the Facilities Utilization Program (JAXA). Dr. Karlinsey is the CEO and Mr. Mackey is the quality director of Indiana Nanotech, which has a commercial relationship with 3M ESPE and whose products were investigated in this manuscript. Drs. Asaizumi, Kato and Kuga report no commercial, proprietary, or financial interest in the products or companies described in this article.

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