Evaluation of discoloration of sound/demineralized root dentin with silver diamine fluoride: In-vitro study

Mahmoud SAYED1, Naoko MATSUI1, Noriko HIRAISHI1, Go INOUE1, Toru NIKAIDO1, Michael F. BURROW2 and Junji TAGAMI1

1 Cariology and Operative Dentistry, Oral Restitution Department, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
2 Faculty of Dentistry, University of Hong Kong, Hong Kong SAR, China
Corresponding author, Naoko MATSUI; E-mail: Matsui.ope@tmd.ac.jp

The objective was to evaluate the effect of application of silver diamine fluoride (SDF) on discoloration of demineralized dentin over time. Dentin specimens were divided into four groups according to time of dentin demineralization. A 38% SDF solution was then applied to the dentin surfaces. Half of the specimens were placed in light-proof boxes while the remainder were exposed to light. Both groups were maintained at 37°C. Color change was determined using a spectrophotometer at different time intervals. SEM/EDS analysis were also undertaken. The 13 h EDTA demineralized group showed the highest values for color change among different time intervals, with the control being the lowest. The light exposed groups showed more color change compared to the unexposed groups. We concluded that the degree of dentin demineralization leads to a significant increase of the rate of dentin color change after application of SDF.

Keywords: Silver diamine fluoride, Dentin discoloration, Spectrophotometer, Demineralized dentin

INTRODUCTION

Dental caries continues to remain a global health problem, despite various advancements in the dental care. As the philosophy of caries management has changed from a surgical approach to a medical model, and managing caries as a disease1, a variety of chemical agents have been developed with the aim of arresting dentin caries2.

Silver-containing compounds have proved to be successful antimicrobial agents, with one of these compounds being silver diamine fluoride (SDF). SDF was introduced by Yamaga et al., in late 1960’s3. Studies recently reported that topical application of SDF can be considered a cost effective, simple and non-invasive technique for caries management4-6. Laboratory studies reported that SDF exhibited a high anti-bacterial effect on cariogenic biofilm6 and inhibited the effect of matrix metallo-proteinase7. SDF action can increase mineral density of the carious enamel lesions8,9 and the micro-hardness of the carious dentin lesions9,10.

A literature review reported that SDF is considered a safe and effective caries preventive agent that seems to meet the criteria of the WHO millennium goals and the FDA US institute11. Demineralized dentin occurs due to acid produced by cariogenic biofilm. SDF application is recommended as a mean to inhibit further dentin demineralization and collagen degradation in addition to promoting remineralization12,13.

Despite its benefits, SDF application is often clinically limited due to formation of dark stains within tooth structure caused by silver deposition, that can lead to esthetic concerns14. Therefore, the aim of this study was to evaluate the effect of different degrees of dentin demineralization on the amount of dentin color change after application of SDF with and without light exposure at different time intervals. The null hypothesis in this study was that there is no effect of dentin demineralization on color change of dentin after application of SDF.

MATERIALS AND METHODS

This study protocol was approved by the ethics committee of Tokyo Medical and Dental University (Institutional Research Board approval number: 725)

Specimens preparation
Eighty dentin blocks (5×5×2 mm) were prepared from the labial and lingual surfaces of bovine incisor roots being free from caries and cracks. The specimens were cut using a low-speed diamond saw (IsoMet 1000, Buehler, Lake Bluff, IL, USA) under copious water coolant, and embedded in acrylic resin (Unifast III, GC, Tokyo, Japan). The surfaces were ground flat exposing the dentin using 600–2000-grit silicon carbide papers (Fuji Star, Sankyo Rikagaku, Saitama, Japan) under running water, then ultra-sonicated in deionized water (DW) (Milli-Q water, Millipore, Billerica, MA, USA).

The dentin specimens were randomly assigned into the following four groups. The first group received no treatment (control), while the remaining three groups were demineralized with 0.5 M ethylene diamine tetra-acetic acid (EDTA: Decalcifying Soln. B, Wako Pure
Fig. 1 Illustration for specimen preparation.

Chemical Industries, Osaka, Japan, pH 7.5) for either 30 min, 5 or 13 h respectively (EDTA 30 min, EDTA 5 h and EDTA 13 h), and then rinsed with distilled water for 10 s. The specimen preparation sequence is illustrated in Fig. 1.

A swept-source optical coherence tomography (SS-OCT) system (IVS-2000, Santec, Komaki, Japan) was used to measure the depth of dentin demineralization treated with the EDTA solution. This system uses OCT that utilizes the coherence and magnitude of reflected light from the subject into a lesion depth-profile. The system integrates a high-speed frequency and swept external-cavity laser. The wavelength ranges from 1,260 to 1,360 nm centered at 1,310 nm at 20 kHz sweep rate15).

A custom code in the image analysis software (Image J, version 1.48; National Institutes of Health, Bethesda, MD, USA) was used to read the raw data of SS-OCT.

SDF application
Thirty-eight percent SDF (Saforide, Bee Brand Medico Dental, Osaka, Japan) was applied to all specimens according to the manufacturer’s instructions. SDF was applied and agitated with a micro-brush for 1 min, left for 2 min then rinsed for 30 s with copious amounts of distilled water. Each group was divided into two subgroups according to light exposure namely, specimens under dark condition and specimens under light one (n=10). The former was kept in light-proof containers while the latter was kept in the clear containers and exposed all the time to a fluorescent light source (FBL18EXL, 18W, Mitsubishi, Tokyo, Japan) with intensity of approximately 4 mW/cm² and wavelength 410–460 nm. All containers were incubated at 37°C.

Color assessment
Color assessments of the dentin specimens (n=10) were recorded at nine time-interval points: baseline (before SDF application), immediately after SDF application, 3, 24, 48, and 72 h, then 7, 10, 14 days after SDF application. The color of the dentin surface was recorded using a spectrophotometer (JP7200F, Juki, Tokyo, Japan) with wave length range of 400–700 nm. In addition, photographs were taken using digital camera at each time interval. The spectrophotometer was calibrated before each examination time according to the manufacturer’s instructions. Each color was explained using the 3-dimensional CIELAB color space system (frequently denoted as \( L^*a^*b^* \)), where \( L^* \) represents brightness ranging from dark (0) to bright (100), \( a^* \) describes red (+a*) to green (−a*), and the \( b^* \) represents yellow (+b*) to blue (−b*). Color measurements were replicated three times for each specimen at each time period by single operator and the mean values were recorded.

The color difference (\( \Delta E \)) of each specimen between baseline and each time-interval point was calculated using the equation:

\[
\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}
\]

EDS analysis
For elemental analysis of the SDF treated dentin surfaces, five samples were prepared for each group. The dentin was treated in the same manner described for specimen preparation for the color measurement. The specimens were then fixed using 2.5% glutaraldehyde for 2 h at 4°C for primary fixation, followed with 0.1% osmium solution for 2 h at 4°C for secondary fixation, and finally dehydrated in an ascending ethanol series (50, 70, 80, 90 and 95%) for 25 min each and twice in 100% for 25 min each, and sputter-coated with carbon. The surfaces of the prepared specimens were examined under a scanning electron microscope (SEM; H-4500, Hitachi High-Technologies, Tokyo, Japan) with energy dispersive X-ray spectroscopy. The specimens were analyzed with SEM under operating conditions of 15 KV. The surface area analysis was performed for detection of phosphorous (P), calcium (Ca) and silver (Ag) ion levels.
Fig. 2 SS-OCT image of the lesion depth for 13 h EDTA-treated dentin. Arrow refers to the thickness of demineralized area.

Table 1 Mean and SD for lesion depth of the demineralized dentin groups

|            | 30 min EDTA | 5 h EDTA | 13 h EDTA | p-value |
|------------|-------------|----------|-----------|---------|
| Mean       | 120.35      | 195.45   | 581.05    | ≤0.001* |
| SD         | 7.25        | 10.59    | 10.69     |         |

Table 2 Mean and SD for color change (ΔE) for the tested groups at different time intervals within the dentin group under dark condition

|            | control | EDTA 30 min | EDTA 5 h | EDTA 13 h | p-value |
|------------|---------|-------------|----------|-----------|---------|
| Mean       |         | Mean       | Mean     | Mean      |         |
| SD         |         | SD         | SD       | SD        |         |
| Immediate  | 2.05c   | 2.33c       | 5.86b    | 10.93b   | ≤0.001* |
| 3 h        | 5.72d   | 8.81c       | 11.95b   | 17.97b   | ≤0.001* |
| 24 h       | 10.30b  | 10.66b      | 11.55b   | 20.89b   | ≤0.001* |
| 48 h       | 11.64b  | 12.43b      | 13.46b   | 23.45b   | ≤0.001* |
| 72 h       | 13.32c  | 14.36c      | 16.26c   | 25.61c   | ≤0.001* |
| 7 days     | 15.96c  | 16.33c      | 19.21b   | 30.23b   | ≤0.001* |
| 10 days    | 19.32c  | 19.87c      | 22.90b   | 31.48b   | ≤0.001* |
| 14 days    | 22.29c  | 22.92c      | 24.40b   | 33.09b   | ≤0.001* |

Means with same letter within each row are not significant at p>0.05



Statistical analysis
Data were analyzed for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Repeated Measures ANOVA test was used to compare the effect of different degrees of demineralization and time intervals on mean ΔE. The test was followed by pairwise comparison with Bonferroni correction. The significance level was set at p<0.05. Statistical analysis was performed using IBM® SPSS® (SPSS, IBM, Armonk, NY, USA) Statistics Version 23 for Windows.

RESULTS

Lesion depth
SS-OCT images (Fig. 2) showed the lesion as a bright zone with increased signal intensity. The 30 min, 5 and 13 h EDTA treated groups (n=10) showed mean lesion depths of approximately 120, 200 and 580 µm respectively (Table 1).

Spectrophotometric measurement of color change
A repeated measure ANOVA was conducted to compare different tested groups under dark and light conditions for color change (ΔE) of the dentin surface over a 14-day time interval. The data were obtained immediately, 3, 24 and 72 h followed by 7, 10 and 14 days. Mauchly’s test indicated that the assumption of sphericity had been violated; therefore, the Greenhouse-Geisser corrected tests were used. There was a significant interaction between time and dentin groups at F (19.517, 172.8)=31.159, p≤0.001). Post hoc comparisons indicated that there was difference between tested groups at all follow-up periods (p≤0.001).

Specimens under dark conditions showed demineralization time with EDTA had a highly significant effect on dentin surface color change after treatment with SDF. For each dentin surface, ΔE showed a gradual increase over the 14-day observation periods. Sound dentin (control) recorded the least color change.
Table 3  Mean and SD for color change (ΔE) for the tested groups at different time intervals within the dentin group under light condition

| Light condition | control | EDTA 30 min | EDTA 5 h | EDTA 13 h | p-value |
|----------------|---------|-------------|----------|-----------|---------|
| Mean          | SD      | Mean        | SD       | Mean      | SD      |
| Immediate     | 2.47b   | 0.57        | 2.06b    | 1.18      | 2.58b   | 1.27    | 7.11a   | 3.38     | ≤0.001* |
| 3 h           | 6.91c   | 2.57        | 8.43c    | 1.23      | 18.89b  | 3.04    | 25.77a  | 4.23     | ≤0.001* |
| 24 h          | 14.40c  | 1.67        | 13.78c   | 1.41      | 26.32b  | 1.88    | 28.07a  | 2.99     | ≤0.001* |
| 48 h          | 19.16c  | 1.72        | 18.92c   | 1.02      | 30.24b  | 1.83    | 32.91a  | 2.04     | ≤0.001* |
| 72 h          | 21.30c  | 1.21        | 21.72c   | 1.11      | 33.15b  | 2.18    | 36.61a  | 1.46     | ≤0.001* |
| 7 days        | 23.91c  | 1.94        | 23.80c   | 1.30      | 37.58b  | 1.77    | 41.24a  | 1.46     | ≤0.001* |
| 10 days       | 27.94b  | 1.18        | 29.21b   | 0.84      | 41.70a  | 1.25    | 42.60a  | 0.87     | ≤0.001* |
| 14 days       | 27.52c  | 1.15        | 30.57b   | 1.32      | 42.74a  | 1.10    | 44.25a  | 1.08     | ≤0.001* |

Means with same letter within each row are not significant at p>0.05

Fig. 3  Representative images of dentin discoloration after SDF application at different time intervals.

change values for all groups (p≤0.001), while the 13-h EDTA group recorded the highest color change for all time intervals (Table 2).

The same pattern occurred for the light exposed groups; sound dentin (control) recorded the least color change (ΔE) values in all groups, while the 13-h EDTA group recorded the greatest color change (ΔE) values in all groups (Table 3).

Figure 3 shows images of dentin color change after SDF application at different time intervals which also proves that light exposure dramatically influenced the color change (ΔE) compared with samples kept in dark conditions.

SEM/EDS observations

Figure 4 shows SEM observation for normal dentin (control) 24 h after application of SDF. Small and discrete silver crystals were observed on the surface. While, the EDS analysis showed that calcium has the highest atomic ratio % (52.34%) followed by phosphorus (42.03%), silver was lowest (5.63%).

Figure 5 shows SEM observation of the demineralized dentin (13-h EDTA) 24 h after application of SDF. Silver crystals had precipitated and aggregated into larger clusters. In this case, the EDS analysis showed that silver has the highest atomic ratio % (53.54%) followed by phosphorus (30.76%) with calcium recording the lowest (15.70%).

DISCUSSION

Based on the results of the current study, dentin demineralization was demonstrated to have an effect on color change, therefore the null hypothesis was
rejected.

SDF is comprised of large amounts of silver and fluoride in addition to ammonia $[\text{Ag (NH}_3\text{)}_2\text{ F}]$. The ammonia and silver ions produce diamine silver ion complex $[\text{Ag (NH}_3\text{)}_2\text{]}$, which is reversible and more stable than silver fluoride (AgF). Thus, it can be stored for a longer time with stable concentration$^{17}$. Thirty-eight percent SDF is a highly alkaline (pH 12.5)$^{18}$ colorless solution, that contains 24–27% silver, 8.5–10% ammonia and 5–6 % fluoride$^{19}$, however, the downfall of SDF is the strong darkening discoloration of dentin limiting its use, not to mention the strong ammonia-based smell.

Bovine teeth were used in this study, due to the ability to easily handle these teeth due to their size and availability. Studies reported the similarity of bovine and human teeth in many aspects such as radio-density$^{20}$, enamel thickness and dentin surface hardness$^{21}$. Bovine teeth also have greater homogeneity in respect of mineral composition compared with human teeth$^{22}$. This is regarded as an advantage in color evaluation experiments, as it will aid in limiting the variability among teeth.

A spectrophotometer was used in this study as it can measure the full visible spectrum with low light intensity using the 3D color space system LAB system$^{23}$.

In order to simulate the in-door ambient lighting conditions, a conventional “white” 18-W fluorescent lamp and intensity of 4 mW/cm$^2$ and wavelength 410–460 nm was used. This kind of fluorescent lamp has a broad visible spectrum that excites silver ions leading to reduction into metallic silver$^{24}$.

Dentin is composed of approximately 40–45% mineral, 30% organic matrix and 20–25% water by volume$^{25}$. The effect of SDF on dentin is mainly due to silver$^{8}$, which results primarily in formation of metallic silver “silver –protein” and lesser amounts of calcium fluoride (CaF$_2$) and silver phosphate (Ag$_3$PO$_4$)$^{18}$.

EDTA acid is a demineralizing solution that has the ability to demineralize dentin by chelation of calcium ions without altering its collagen fibrillar structure$^{26}$.

Optical coherence tomography (OCT) is considered as a noninvasive method to obtain cross-sectional images of internal biological structures. SS-OCT is proven to have higher sensitivity and specificity than radiographic...
methods, and to have comparable accuracy to microfocus X-ray computed tomography (µCT) and confocal laser microscope (CLSM). It has proven to be a promising method for detecting early demineralized enamel and dentin lesions.

From the OCT measurements of dentin treated with EDTA, the mean demineralized dentin thicknesses with EDTA treatment for 30 min, 5 and 13 h were 120, 200 and 580 µm respectively.

Dentin demineralization had a significant impact on dentin color change after application of SDF, especially for the 5 and 13 h EDTA-treated dentin groups. It was reported that silver has a high affinity for proteins (i.e. collagen) as silver ions are known as good electron acceptors and characterized by a large atomic radius with lower affinity for oxidation. Silver has a high polarizing power as it has a high ratio of ionic charge in relation to the ion radius which in turn facilitates formation of strong bonds with nitrogen and sulfur groups present in cysteine and histidine in proteins. Therefore, the application of SDF on proteins leads to a relatively rapid darkening, suggesting direct reduction of silver ions into metallic silver. Therefore, a greater collagen exposure of demineralized dentin leads to more silver uptake and its reduction into metallic silver, resulting in a darker color change within a short time.

Silver phosphate crystals formed due to the reaction of SDF with dentin are yellow in color but become darker due to the gradual reduction into metallic silver by reducing agents such as light exposure, which contributes in the darker dentin color change.

The light exposed group showed a faster rate of color change than the “dark” group. Light is a physical reducing agent, which can reduce silver ions into metallic silver. Hou et al. reported that silver ions can absorb light and are reduced into silver nano-particles. The change in color occurs due to silver nano-particle size aggregation from less than a 20 nm size to particle sizes of sub-micrometer levels. In addition, the shape tends to change from spherical to triangular or hexagonal forms which eventually fuse together. The increase in silver particle size is associated with darkening in color. This explains the color measurements which were also noted from the SEM observation findings.

SEM/EDS image findings for sound dentin suggest that a portion of the free silver ions were reduced into small separated rounded silver crystals, as an initial stage of silver crystal formation, mainly due to the effect of light and collagen. On the other hand, the findings of the demineralized dentin suggest that a larger portion of the free silver ions were reduced, mainly due to the greater exposure of the collagen fibers, as many aggregated silver crystals fuse together resulting in larger silver crystals with different shapes, some with triangular shape indicating fully grown crystals identified.

Demineralized dentin has the ability to take up larger amounts of silver, which in turn increases the rate of color change. However, absorbing more silver is proven to be more effective in arresting caries, inhibition of further dentin demineralization, prevention of dentin degradation and reduction of bacteria within the dentinal tubules. From the results of this study, discoloration by SDF may be an indicator to detect carious dentin. However, further evaluation of the degree of discoloration and characteristics of natural caries dentin are needed. The benefits of SDF may outweigh the aesthetic concerns.

Further studies are needed to observe the effects of SDF on the morphology and physical form of exposed collagen fibers along with the penetration of silver into the demineralized dentin.

CONCLUSION

The increase in degree of dentin demineralization was associated with an increased rate of dentin color change associated with SDF application. Light exposure significantly increased the rate of color change of dentin treated with SDF.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest in the present study.

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