The effect of silver diammine fluoride application time on dentine remineralisation: An *in vitro* study

BY

MR. SURAPONG SRISOMBOON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER SCIENCE (GERODONTOLOGY) FACULTY OF DENTISTRY THAMMASAT UNIVERSITY

ACADEMIC YEAR 2021

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ENTITLED

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Abstract

The use of silver diammine fluoride (SDF) is a cost-effective strategy to arrest active caries lesions. The current guidelines suggested to apply SDF on the lesions for 1 to 3 min. However, the recommended application time might not be feasible in some clinical situations which may affect remineralising actions of SDF. The aim of this study was therefore to assess the effect of different application times of SDF (30 s, 60 s, and 180 s) on remineralisation in demineralised dentine. Dentine slices were prepared and demineralised in 17% EDTA followed by the immersion in simulated body fluid for 336 h. The formation of apatite precipitated in specimens was assessed using FTIR-ATR (n = 13) followed by SEM-EDX. The ratio of the absorbance at 1023 cm^{-1} versus 1663 cm^{-1} which represent phosphate group and amide I in mineral apatite and collagen in dentine respectively was recorded. The Synchrotron radiation X-ray tomographic microscopy (SRXTM) (n = 6) was employed to quantify the degree of mineral precipitation (vol%) in demineralised dentine at 336 h. Additionally, fluoride concentration in the storage solution after immersion for 336 h was measured using fluoride ion specific electrode (n = 7). The mean absorbance ratio of 30 s, 60 s, and 180 s groups were increased upon immersion time, but the values obtained from these groups were comparable ($p > 0.05$). SEM images showed mineral precipitation occluding patent tubules which are expected to be silver chloride particles. SRXTM results showed the increase of mineral density from 0 to ~ 60 vol% upon applying SDF. However, the degree of mineral precipitation in all groups treated with SDF were comparable ($p > 0.05$). The fluoride release from the specimens treated with SDF for 180 s was significantly higher than other groups ($p < 0.05$). The different application times of SDF for 30 s, 60 s, or 180 s showed similar

remineralising properties in simulated demineralised dentine. However, fluoride released from the dentin was increased upon the increase of application time.

Keywords: silver diamine fluoride, mineral precipitation, application time

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List of Abbreviation

Symbols/Abbreviations	Terms
AAPD	American Academy of Pediatric Dentistry
Ab	Absorbance
BS ISO	British Standard International Organization for Standardization
EDTA	Ethylenediaminetetraacetic acid
FHA	Fluorohydroxyapatite
FTIR-ATR	Fourier Transform Infrared Spectroscopy-Attenuated Total Reflections
FDA	Food and Drug Administration
GV	Grayscale Value
HA	Hydroxyapatite
KI	Potassium Iodide
Micro-CT	Microtomography
MMP	Matrix metalloproteinases
NaF	Sodium fluoride

NH_4OH	Quaternary ammonium compound
NRCC	Non-restorative cavity control
SBF	Simulated body fluid
SDF	Silver diammine fluoride
SEM-EDX	Scanning Electron Microscope - Energy Dispersive Spectroscopy
SRXTM	Synchroton radiation x-ray tomographic microscopy
TISAB III	Total Ionic Strength Adjustment Buffer
XRD	X-ray diffraction
XTM	X-ray tomographic microscopy

Chaper 1 Introduction

Untreated dental caries is a common oral disease affecting people at all age groups. In 2010, it was reported that untreated dental caries in permanent teeth was the most prevalent health burden affecting almost 35% of global population (Peres et al., 2019). Elderly population may become more susceptible to dental caries if the daily physical activities of oral hygiene care were compromised by chronic diseases or medications. The suboptimal oral health care may subsequently lead to the progression of dental caries forming irreversible carious cavitation which may require invasive restorative treatment or extraction. This could later cause the reduction of oral health-related quality of life for patients (Gil-Montoya et al., 2015).

Currently, the primary aim for management of dental caries is to arrest the active carious lesions and to promote the prevention of new lesions. One of the cost-effectiveness strategies is to apply silver diammine fluoride (SDF) to promote remineralisation in cavitated active carious lesions in addition to the motivation of patient to improve oral hygiene. The technique of SDF application is simple. Hence, it is suitable for the management of active caries in patients who are less cooperative or require a special care needs.

Currently, the application time of SDF can be ranged from 1 to 3 min (Horst et al. 2016 and Seifo et al. 2020). However, the application time of SDF may be shorter than the recommendation if the patient cooperation is limited. Although the previous study proposed that different application time was not directly related to treatment success

(Horst et al. 2016), the evidence or mechanisms to support this observation remain limited and uncleared.

The aim of this study was therefore to assess the effect of different SDF application times on apatite formation, degree of mineral precipitation, and fluoride release after SDF application in the *in vitro* demineralised dentine. The null hypothesis was that the use of different application times was not significantly affect the remineralising actions of SDF in demineralised dentine.

Chaper 2 Review of literature

2.1 Dental caries

Dental caries is the multifactorial disease, biofilm-mediated, sugar-taken leading to the imbalance between demineralisation and remineralisation of dental hard tissue (Hendre et al., 2017). It was reported that the untreated caries was the most prevalent chronic disease affecting almost 2.4 billion people worldwide (Kassebaum et al., 2015). The progress of caries lesion can lead to severe pain and tooth loss which could negatively affect the quality of life (Gil-Montoya et al., 2015). Currently, the burdens of untreated caries are shifting from children to adults, with three peaks in the prevalence of age 6, 25, and 70 years. Thailand is now going to become the ageing society. It is additionally expected that the population over 60 years old may reach 20% of the overall population in 2037 (Prasartkul et al., 2019). Furthermore, the most recent 8th Thai National Oral Health survey also reported that the prevalence of untreated caries in the elderly group (60-74 years old) was now reached 53% (Bureau of Dental Health, 2017).

2.2 Non-restorative cavity controls (NRCC)

The aim of the current caries management is to arrest and promote remineralisation of cavitated active carious lesions. Non-restorative cavity controls (NRCC) are considered a minimally invasive treatment aiming to inactivate carious lesions with minimal cavity preparation. The primary goal of NRCC is to prevent the vicious repetitive restoration cycle that may eventually lead to the non-restorable situation. NRCC can be applied for caries management in primary dentition, root caries, and cavitated coronal smooth surface

lesions. In addition, NRCC is considered feasible for using in challenging clinical situation in non-cooperative patients.

The treatment concept of NRCC are comprised of the reduction of caries risk and the promotion of lesion remineralisation, which was in agreement with the concept of medical model in caries management (Yon et al., 2019). NRCC can be divided into 3 stages (van Strijp and van Loveren, 2018). The first stage is to improve patient's oral hygiene. The second is to make the cavity accessible for home toothbrushing by removing overhanging enamel with hand instrument (Figure 2-1). The third is to arrest caries by the application of topical fluoride such as 38% SDF or 5% sodium fluoride (NaF) varnish. SDF seems to be more effective than NaF varnish (Trieu et al., 2019). If the aesthetic is of concern, the placement of tooth-colored restorations such as resin composites or glass ionomer cement can be provided.

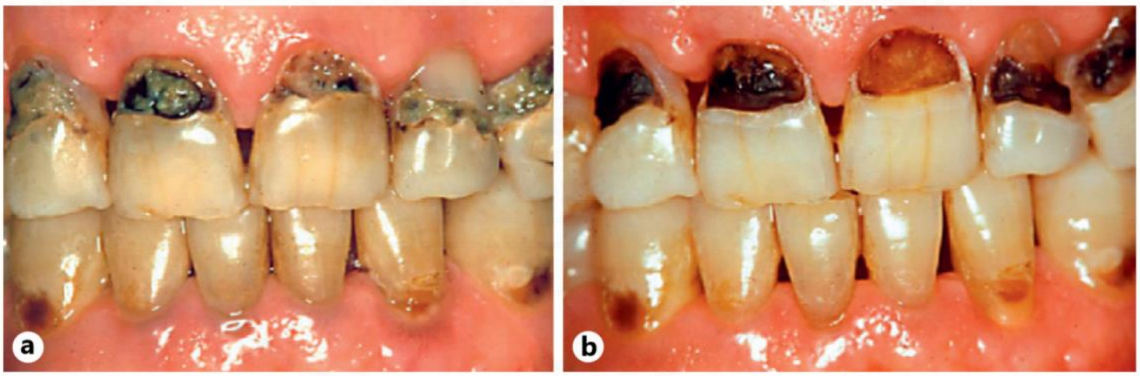

Figure 2-1 a) Multiple active carious lesion covered by plaque in upper anterior teeth b) appearance after 14 days. Overhanging enamel has been removed to make the cavity accessible for cleaning. Reprinted with permission from Monographs in Oral Science: *No removal and Inactivation of Carious Tissue: Non-Restorative Cavity Control,* Guus van strijp and Cor van Loveren Copyright © (2012) Karger Publishers, Basel, Switzerland.

2.3 Silver diammine fluoride (SDF)

Silver di-ammine fluoride or silver diamine fluoride (SDF) has been introduced to promote therapeutic effects to dental caries due to the high fluoride concentration (44,800 ppm) and antibacterial actions of silver ions. SDF is commonly misspelled as silver diamine fluoride, but “diamine” can be used in both scientific and marketing purposes (Figure 2-2). The release of fluoride and silver ions are expected to enable remineralising and antibacterial actions. In the US, FDA approved SDF in 2014 for treating hypersensitivity in adults. Later in October 2016, FDA awarded SDF as the designation of “breakthrough therapy” based on its promising caries arresting effects (Sarvas, 2018).

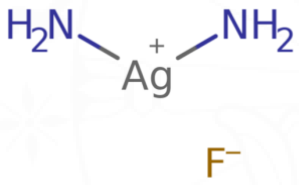

Figure 2-2 Chemical structure of silver diammine fluoride (SDF).

The use of SDF is safe which showed no incident of acute or serious systemic illness (nausea, vomiting, generalized discomfort) (Duangthip et al., 2018). The local adverse effects from SDF were rarely reported in the studies. Additionally, SDF showed minimal histological change and cytotoxic effects on dental pulp cells (Rossi et al., 2017). The major limitation of SDF is the black staining which may affect aesthetic outcome. The use of adjunctive potassium iodide (KI) was suggested to help reverse the black metallic colour (Li et al., 2016; Bimstein and Damm, 2018). In addition, the taste of SDF was bitter/metallic which may reduce patient compliance (Mei et al., 2017). The safety lethal dose 50 by oral administration of SDF is 520 mg/kg. One drop (25 microliters) contains

9.5 mg SDF. It was recommended to use one drop per a 10 kg child per treatment visit (Horst et al., 2016).

2.4 Caries-arresting mechanisms of SDF

The proposed caries arresting mechanism of SDF application included antimicrobial activities, dentin remineralisation, and the reduction of collagen matrix deconstruction (Figure 2-3) (Mei et al., 2013; Zhao et al., 2018).

SDF solution releases both silver ion, fluoride ion, and silver fluoride complexes. Silver ion promoted antibacterial properties by the following mechanisms (Mei et al., 2018). Firstly, silver ion can interact with thiol group and subsequently deactivate the bacterial enzymes (Toh et al., 2014). Secondly, the ion can bind with the anionic portion of the cell membrane causing the leakage of bacterial cell membrane (Marambio-Jones and Hoek, 2010). Thirdly, silver ion may interact with bacterial DNA leading to the death of cell (Dakal et al., 2016). Fourthly, the ion may bind with an amino acid in bacterial cell, resulting in the inactivation of DNA and RNA (Marambio-Jones and Hoek, 2010). Furthermore, fluoride ion from SDF could also interfere with plaque metabolism and help to inhibit acid production (Zhao et al., 2018). Furthermore, SDF enabled the formation of quaternary ammonium compound (NH_4OH), which can inhibit microbial activities and provide buffering effects (Mei et al., 2018).

SDF (38%) contained the high level of fluoride (44,800 ppm) to promote remineralisation of demineralised hydroxyapatite crystals. It was proposed that SDF promoted the formation of silver phosphates ($AgPO_4$) and calcium fluoride (CaF_2) (Zhao et al., 2018). Subsequently, the dissolution of calcium and fluoride enabled the formation of low

soluble fluorohydroxyapatite (FHA) (Mei et al., 2017). The formation of CaF_2 could potentially act as a pH-regulated slow-release reservoir of fluoride ions during caries attack (Zhao et al., 2018).

SDF also helped to preserve dentine collagen which is essential to serve as the template for mineral precipitation. Ag ion inhibited dentine collagenases such as MMP and cathepsin. Additionally, fluoride promotes remineralising effect to promote the repair of apatite crystals that will cover and protect the exposed collagen. Fluoride ions can also directly inhibit collagenases (Mei et al., 2018).

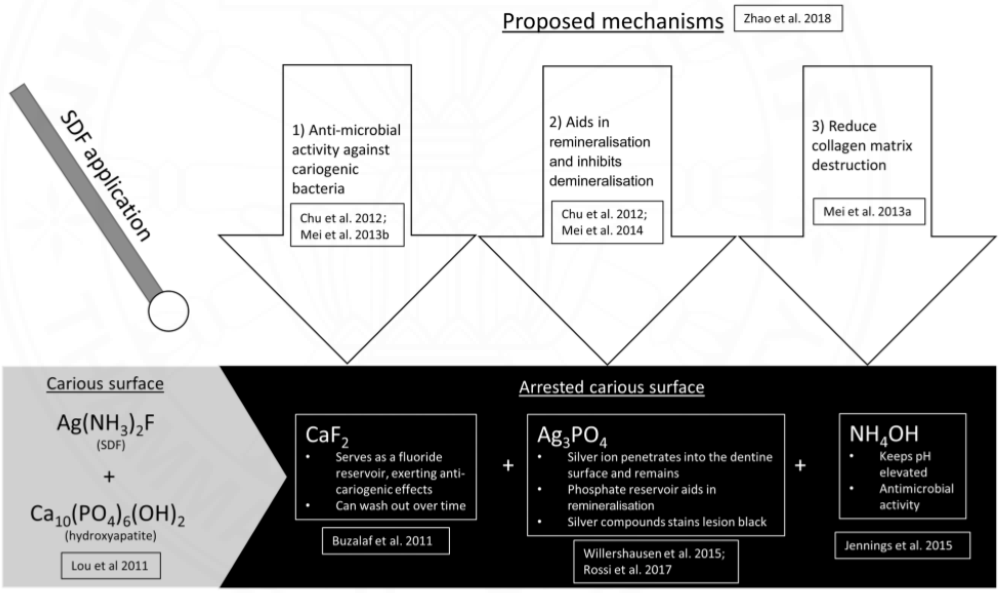

Figure 2-3 Proposed mechanisms of silver diammine fluoride on arresting caries. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, European Archives of Paediatric Dentistry: *A silver renaissance in dentistry*, S. Hu et al., copyright (2018).

The mineral precipitation in dentine after SDF application have been examined by assessing surface morphology, mineral density, or crystal characteristic. Various techniques have been employed to assess the precipitation such as scanning electron microscope-energy dispersive spectroscopy (SEM-EDX), X-ray diffraction (XRD), Fourier transform infrared spectroscopy with attenuated total reflections (FTIR-ATR), and microtomography (micro-CT). Surface morphology after SDF application in *in vitro* demineralised dentine covering mineral has been examined which showed the dense granular structure of spherical grains in inter-tubular area (Zhao et al., 2017)(Figure 2-4).

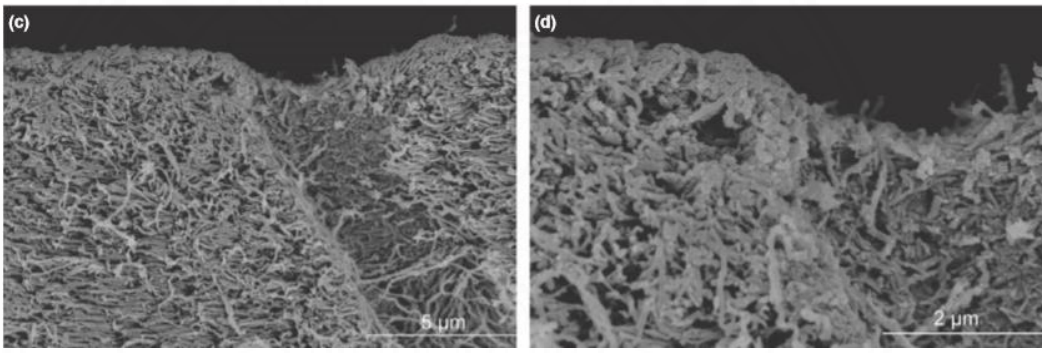

Figure 2-4 Scanning electron microscopy images of the cross-section of artificial dentine caries, (c) 8,000 x (d) 20,000 x magnification views of caries lesions treated with 38% SDF. Reprinted by permission from John Wiley and Sons and Copyright Clearance Centre: Wiley, International Dental Journal: *Arresting simulated dentine caries with adjunctive application of silver nitrate solution and sodium fluoride varnish: an in vitro study*, IS. Zhao et al, copyright (2017).

A study reported that the lesion depth in simulated carious dentine treated with SDF was significantly less than that of the control group (Zhao et al., 2017) (Figure 2-5). The surface of dentine treated with SDF was also assessed using XRD to analyse the crystal characteristic that was precipitated on the surface (Zhao et al., 2017) (Figure 2-6). The result showed that AgCl was the main precipitation on the surface.

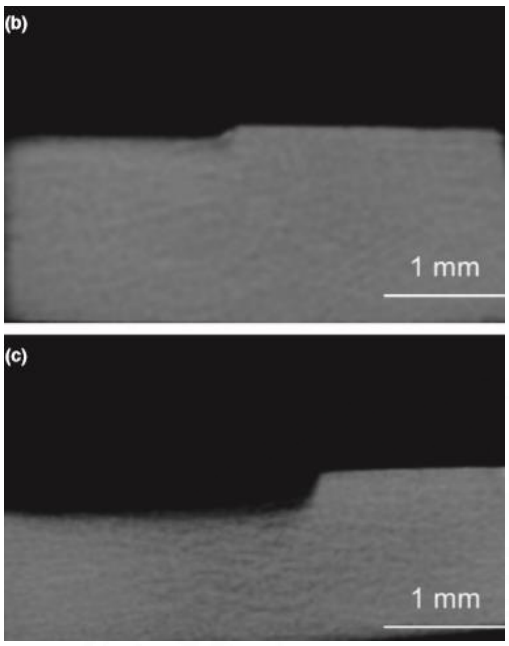

Figure 2-5 Micro-computed tomography images of the cross-section of artificial dentine caries, left side: lesion body; right side: internal control. (b) treatment with 38% SDF application; (c) treatment with deionised water. Reprinted by permission from John Wiley and Sons and Copyright Clearance Centre: Wiley, International Dental Journal: *Arresting simulated dentine caries with adjunctive application of silver nitrate solution and sodium fluoride varnish: an in vitro study*, IS. Zhao et al, copyright (2017).

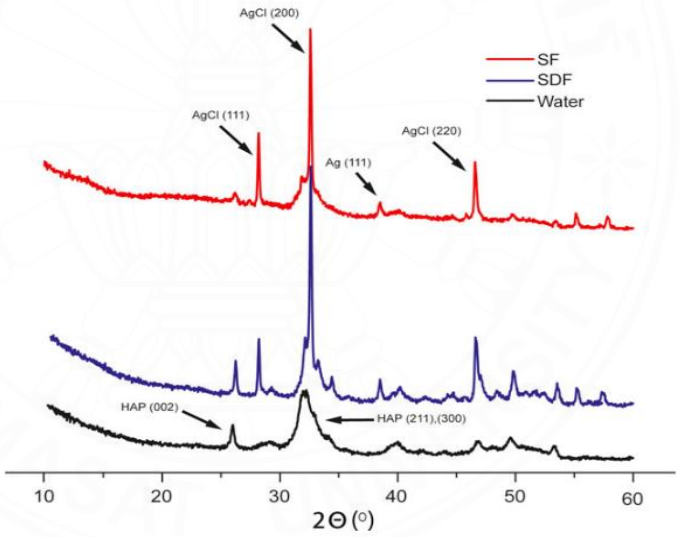

Figure 2-6 Typical X-ray diffraction (XRD) patterns of the experimental groups. SDF, treatment with topical application of 38% silver diamine fluoride solution; SF, treatment with topical application of 25% silver nitrate solution followed by 5% sodium fluoride varnish; W, treatment with deionized water. Reprinted by permission from John Wiley and Sons and Copyright Clearance Centre: Wiley, International Dental Journal: *Arresting simulated dentine caries with adjunctive application of silver nitrate solution and sodium fluoride varnish: an in vitro study*, IS. Zhao et al, copyright (2017).

2.5 Clinical application of SDF

The protocol for applying SDF in is simple. However, the application time suggested by various sources may by slightly varied across the different protocols. For example, the University of California, San Francisco suggested to apply SDF for 1-3 min (Horst et al., 2016) whilst the American Academy of Paediatric Dentistry (AAPD) was recommend to apply SDF for 1 min (Crystal et al., 2017). The another recommended application time of SDF reported in the published literature was 3 min (Seifo et al., 2020). The less application time of SDF may help facilitate clinical application in the areas where the moisture control is not ideal or when the patients are co-operative is limited.

It was proposed that the application of SDF was not related with the success of treatment (Horst et al., 2016). However, the actual mechanisms that explain the observation were not yet reported. The aim of this study was therefor to assess the effect of different application times of SDF on apatite formation, degree of mineral precipitation, and fluoride release in the *in vitro* demineralised dentine.

Chaper 3 Materials and methods

3.1 Specimen preparation

The research ethic for collecting extracted third molar (n=3) with no visible cavitated caries lesions at Oral Health Care department, Thammasat University Hospital, Pathum Thani, Thailand was granted from the Ethics Review Sub-Committee for Research Involving Human Research Subjects of Thammasat University, No. 3 (Faculty of Health Sciences and *Science* and Technology, approval number: 150/2562, date of approval 2nd October 2019). Consents obtained from patients was waived as the tooth identification related with patient was not required. The collected teeth were stored in 1% thymol solution at room temperature for less than 3 months prior the test.

The tooth was cleaned and fixed into plaster block. Crown was cut horizontally using a diamond blade (Accumtom-50, Struers, Ballerup, Denmark) to produce the dentine slice with 2 mm in thickness. The tooth was cut with a speed of 2700 round/min and feed time of 0.2 mm/s. Then, 13 dentine slice was further divided into four pieces (13 x 4 = 52 specimens). The surface of the dentine slice was polished manually by microfine 4,000-grit sandpaper. The dentine slice was cleaned in an ultrasonic cleaner to remove debris from cutting and polishing steps. The polished specimens were examined for the crack and other defects under a stereomicroscope. An acid-resistant nail polish (Revlon, USA) was then applied to cover half of the treatment surface of the specimens.

The specimens were immersed in 17% ethylenediaminetetraacetic acid (EDTA, Faculty of Dentistry, Chulalongkorn university, Bangkok, Thailand) for 72 h to demineralise the

mineral components mimicking carious soft dentine (Chen et al., 2015; Sayed et al., 2019a). The loss of mineral content was then confirmed by using Fourier Transform Infrared Attenuated Total Reflectance (FTIR-ATR, Nicolet iS5, Thermo Fisher Scientific, Massachusetts, USA).

3.2 Treatment of sample

The four specimens from each dentine slices (n=13) were treated with 25 μL of deionised water or 38% SDF (253,900 ppm Ag and 44,800 ppm F, Topamine™, Dentalife, Australia) by agitation with micro brush according to the following 4 protocols.

1) Group 1 (n=13): applied with 25 μL of deionised water (water)
2) Group 2 (n=13): applied with 25 μL SDF, agitated for 10 s, and left for 30 s (30 s)
3) Group 3 (n=13): applied with 25 μL SDF, agitated for 10 s, and left for 60 s (60 s)
4) Group 4 (n=13): applied with 25 μL SDF, agitated for 10 s, and left for 180 s (180 s)

The specimens were then rinsed with deionised water for 10 s. Then, the specimens in each group were immersed in 5 mL of simulated body fluid (SBF) for 14 days. The SBF solution was prepared following BS ISO 23317:2014 (British Standard, 2014). Specimens were kept in SBF in incubator at 37 °C. The method used in this study is presented in Figure 3-1.

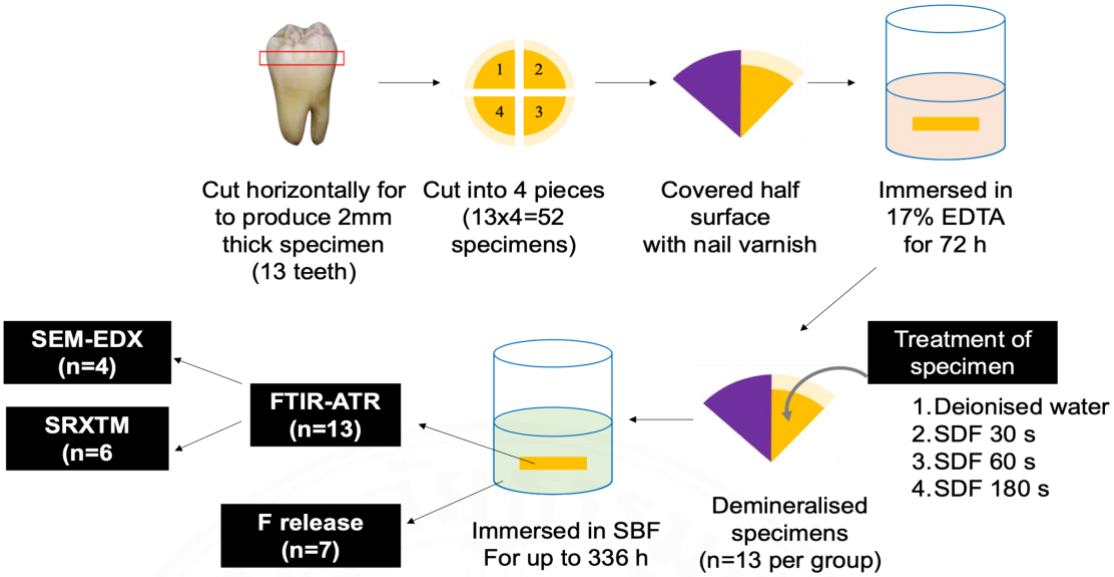

Figure 3-1 Flow chart representing the method used in the current study.

3.3 Assessing apatite formation

An FTIR-ATR (Nicolet iS5, Thermo Fisher Scientific, Massachusetts, USA) was used to assess mineral precipitation in the demineralised dentine after applying SDF following a protocol modified from previous studies (n = 13) (Liu et al., 2014; Lopes et al., 2018; Zhang et al., 2019; Yin et al., 2020). The specimens were placed on the ATR diamond. The FTIR spectra in the region of 700 - 4000 cm^{-1} was then recorded at the resolution of 8 cm^{-1} and 12 repetitions from the bottom surface. The spectra of the specimens before immersion in SBF and after immersion in SBF for 24 h, 168 h, and 336 h were then recorded (n = 13). The current study used the absorbance at 1023 cm^{-1} (Abs_{1023}, phosphate group of apatite) and 1663 cm^{-1} (Abs_{1663}, Amide I in collagen) (Berzina-Cimdina and Borodajenko, 2012) to represent the mineral apatite and collagen contained in the demineralised dentine respectively (Figure 3-2). The ratio of Abs_{1023} versus Abs_{1663} (Abs_{1023}/Abs_{1663}) were calculated to assess the changes of mineral precipitation in the demineralised dentine treated with SDF using different application time.

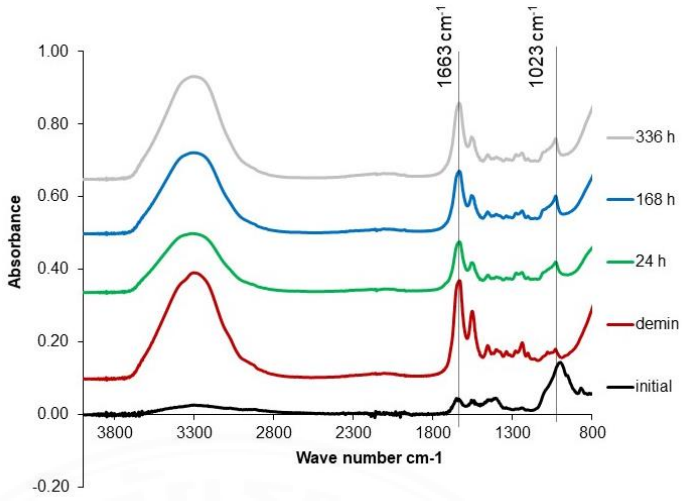

Figure 3-2 An example of FTIR results obtained from specimen treated with SDF for 60 s before demineralization (initial), after demineralization (demin), and after immersion in SBF for 1 day (24 h), 1 week (168 h), and 2 weeks (336 h).

Specimens in each group (n=4) were randomly selected and sputter-coated with gold by a sputter coating machine (Q150R ES, Quorum Technologies Limited Company, West Sussex, UK) using the sputter condition of 23 mA for 45 s. Then, the surface morphologies of the specimens were examined under the scanning electron microscope (SEM, JSM-7800F, JEOL Ltd., Tokyo, Japan). The specimens were then analysed by an energy dispersive X-ray microscope (EDX, X-Max20, Oxford instrument, Oxford, UK) to detect elements such as calcium, phosphorus, fluoride, and silver in the precipitates on the specimens.

3.4 Degree of mineral precipitation

The SRXTM of treated specimens (n = 6) was carried out at the XTM beamline (BL1.2W: X-ray imaging and tomographic microscopy, Synchrotron Light Research Institute Public Organization, Thailand). The synchrotron radiation was generated from 2.2-Tesla multipole wiggler in the 1.2 GeV Siam Photon Source. For the data collection, each

dentine specimen was held in a polyimide tube and mounted on a rotary stage. A total of 900 X-ray projections were obtained for 0° - 180° with an angular increment of 0.2°. The tomography imaging was collected with filtered polychromatic X-ray beam at the mean energy of 14 keV, at the distance of 32 m from the source. The X-ray projections were obtained using detection system equipped with 200-μm-thick YAG:Ce scintillator, the white-beam microscope (Optique Peter, France) and the pco.edge 5.5 sCMOS camera (2560x2160 pixels, 16 bits). All tomographic scans were acquired at a pixel size of 1.44 μm (Figure 3-3).

The X-ray projections were normalized by flat-field correction with open-beam and dark images. The tomograms were reconstructed using Octopus Reconstruction software (Vlassenbroeck et al., 2006). The mineral precipitation of demineralized dentine was determined using Octopus Analysis software and the 3D volume representation was produced using Drishti software (Limaye, 2012) (Figure 3-4).

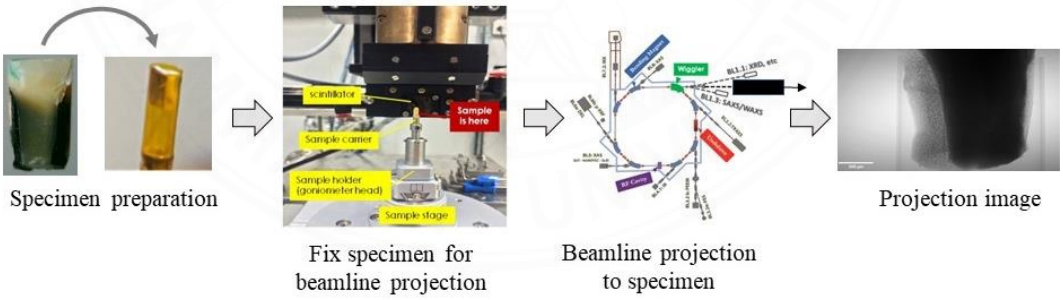

Figure 3-3 Flow chart of making SRXTM analysis.

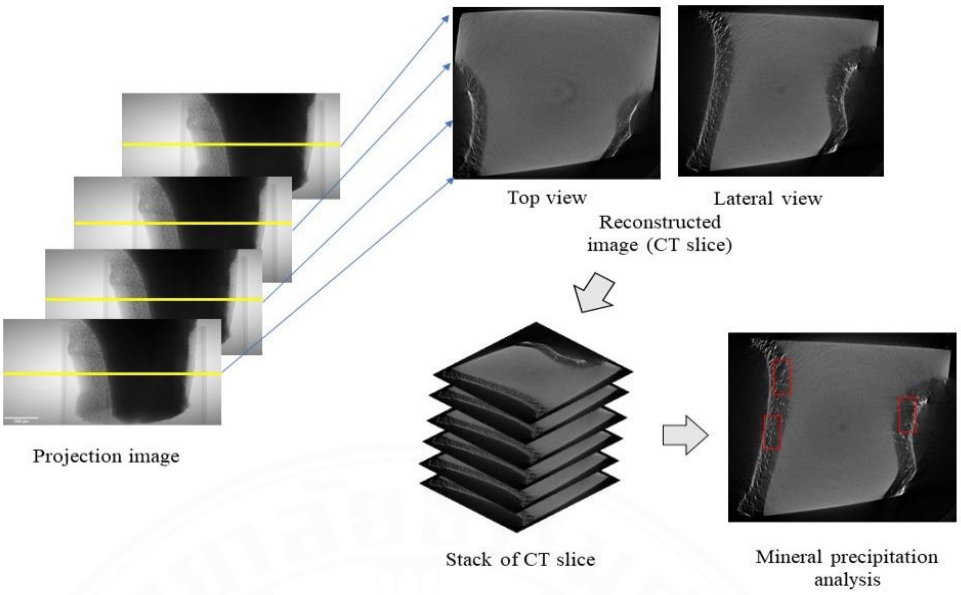

Figure 3-4 Flow chart represent the image analysis technique to quantify the level of mineral precipitation.

3.5 Level of fluoride release

After the specimens were removed from the solution, the storage solution (n = 7) was collected to analyse the fluoride concentration. The collected solution was mixed with TISAB III (Orion ionplus, Thermoscientific, Massachusetts, USA) using 1:10 volume ratio. Fluoride calibration standards (0.1, 1, 10, and 100 ppm) were prepared using the standard fluoride solution (Orion ionplus, Thermoscientific, Massachusetts, USA). Fluoride ion concentration (ppm) was measured using the fluoride specific ion electrode (Orion Versastar Pro, Thermoscientific, Massachusetts, USA)(Panpisut et al., 2020).

3.6 Statistical analysis

Values reported in the present study were mean and SD or median (min,max). Data were analysed using SPSS Statistics version 26 for Window (IBM, Chicago, IL, USA). The normality of the data was assessed using Shapiro-Wilk test. The Abs_{1024}/Abs_{1636} ratio

changes upon immersion time were analysed using the Friedman test followed by Dunn's multiple comparison. One-way ANOVA followed by Tukey multiple comparison was used to compare mean mineral density and fluoride release between groups. The significance level was set at $p = 0.05$. Power analysis was performed using G*Power 3.1 Software (University of Dusseldorf, Germany) confirmed that sample size in each test gave power > 0.95 at $\alpha = 0.05$.

Chaper 4 Results

4.1 Assessing apatite formation

4.1.1 Abs_{1023}/Abs_{1663} ratio

In control group, the median Abs_{1023}/Abs_{1663} ratios after 24 h, 168 h, and 336 h were comparable ($p > 0.05$) (Table 4-1). The median Abs_{1024}/Abs_{1636} ratio of the 30s group was increase from 0.282 to 0.308 after immersion in SBF for 336 h. However, at each time point, the observed the Abs_{1023}/Abs_{1663} ratios in 30 s were comparable ($p > 0.05$). For the 60 s group, the Abs_{1023}/Abs_{1663} ratio was significantly increase after immersion in SBF for 336 h ($p = 0.0143$). For the 180 s group, the Abs_{1023}/Abs_{1663} ratio was significantly increase after immersion in SBF for 168 h ($p = 0.0029$).

At the time point before immersion (0 h), the median Abs_{1023}/Abs_{1663} ratios of all groups were comparable ($p > 0.05$) (Table 4-1). After immersion in SBF for 24 h, 30 s group showed a significant higher Abs_{1023}/Abs_{1663} ratio than the control group ($p = 0.0461$). After immersion in SBF for 168 h, the median Abs_{1024}/Abs_{1636} ratios of 30 and 180 s groups were significantly higher than that of the control groups ($p < 005$). Likewise, after immersion in SBF for 336 h the median Abs_{1023}/Abs_{1663} ratios of both the 30 and 180 s groups were also significantly higher than that of the control group ($p < 0.05$). However, the median Abs_{1023}/Abs_{1663} ratios obtained from 30 s, 60 s, and 180 s groups were comparable at all time points.

Table 4-1 The Abs_{1024}/Abs_{1636} ratios (median, min–max) obtained from specimens in each group for up to 336 h (n = 13). Same lower and uppercase letters within the same row and same column, respectively, indicate significant differences ($p < 0.05$).

Groups/ Time	0 h Median (Min,Max)	24 h Median (Min,Max)	168 h Median (Min,Max)	336 h Median (Min,Max)	Friedman Statistics	*p* Values
Control (Water)	0.134 (0.070,0.453)	0.133 (0.07,0.35) ^{A}	0.123 (0.07,0.38) ^{B,C}	0.117 (0.07,0.38) ^{D,E}	11.34	>0.05
SDF 30 s	0.282 (0.080,0.722)	0.244 (0.093,0.928) ^{A}	0.318 (0.101,0.949) ^{B}	0.308 (0.104,0.935) ^{D}	5.215	>0.05
SDF 60 s	0.116 ^{a} (0.068,0.613)	0.173 (0.040,0.593)	0.148 (0.084,0.804)	0.206 ^{a} (0.088,0.957)	0.48	^{a}0.0143
SDF 180 s	0.180 ^{a,b} (0.048,0.421)	0.202 ^{c,d} (0.061,0.701)	0.316 ^{a,c} (0.093,0.832) ^{C}	0.250 ^{b,d} (0.126,0.895) ^{E}	20.54	^{a}0.0029 ^{b}0.0085 ^{c}0.0085 ^{d}0.0234
Kruskal–Wallis statistics	2.667	8.021	12.80	16.12		
p values	>0.05	^{A}0.0461	^{B}0.168 ^{C}0.0083	^{D}0.0023 ^{E}0.0046		

4.1.2 SEM-EDX

4.1.2.1 Group 1 (water)

In group 1 (water), SEM images showed patent dentinal tubules (Figure 4-1). EDX result showed carbon (C) and Oxygen (O) with no Ca, P was detected (Figure 4-2).

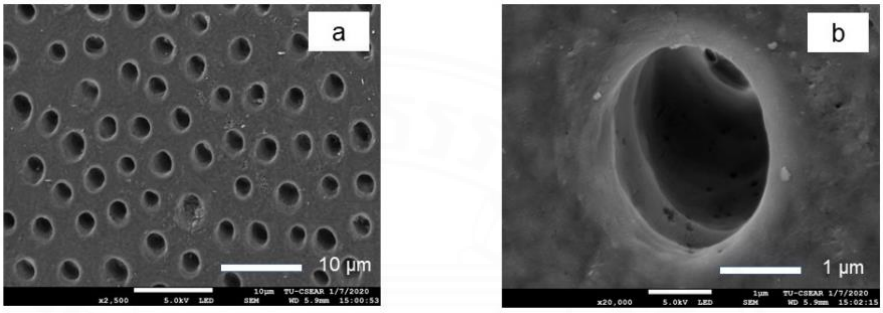

Figure 4-1 SEM images of group 1 (water), surface specimen soaked in SBF for 336 h at low magnification 2500 X (a) and high magnification 20000 X (b).

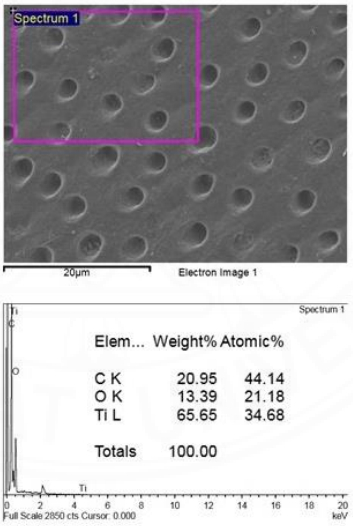

Figure 4-2 EDX images of group 1 (water).

4.1.2.2 Group 2 (SDF 30 s)

In group 2 (SDF 30 s), SEM images showed that the surface of specimen was occluded with precipitated minerals in dentinal tubules. The mineral precipitation was rhomboid and needle-like crystals which can be seen in Figure 4-3. EDX results showed carbon (C), Oxygen (O), Chloride (Cl), Silver (Ag), Calcium (Ca), Phosphate (P) and Nitrogen (N) were contained in the tested areas (Figure 4-4).

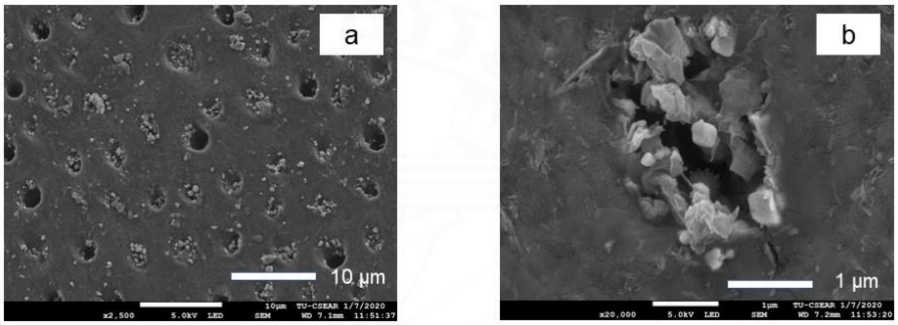

Figure 4-3 SEM images of group 2 (SDF 30 s), surface specimen soaked in SBF for two weeks at low magnification 2500 X (a) and high magnification 20000 X (b).

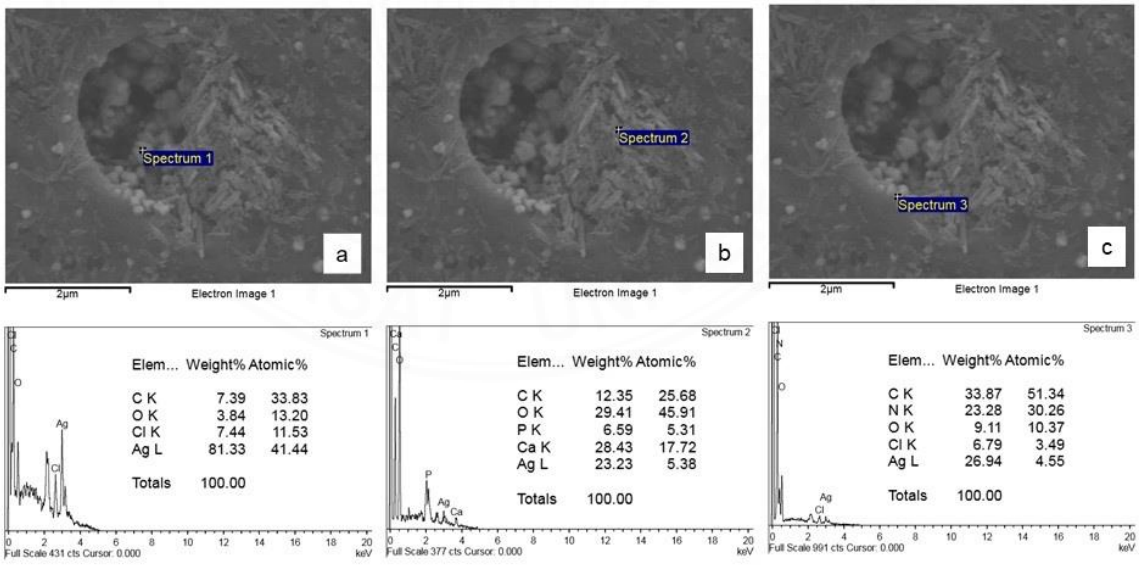

Figure 4-4 EDX images of group 2 (SDF 30 s).

4.1.2.3 Group 3 (SDF 60 s)

In group 3 (SDF 60 s), the rhomboid and needle-like crystals occluding dentinal tubules can be seen in Figure 4-5. Elemental analysis detected by EDX founded carbon (C), Oxygen (O), Chloride (Cl), Silver (Ag), Calcium (Ca) and Phosphate (P) were contained in the crystals (Figure 4-6).

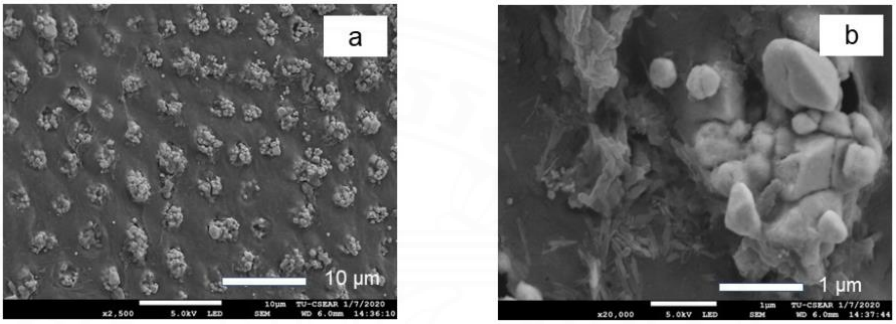

Figure 4-5 SEM images of group 3 (SDF 60 s), surface specimen soaked in SBF for two weeks at low magnification 2500 X (a) and high magnification 20000 X (b).

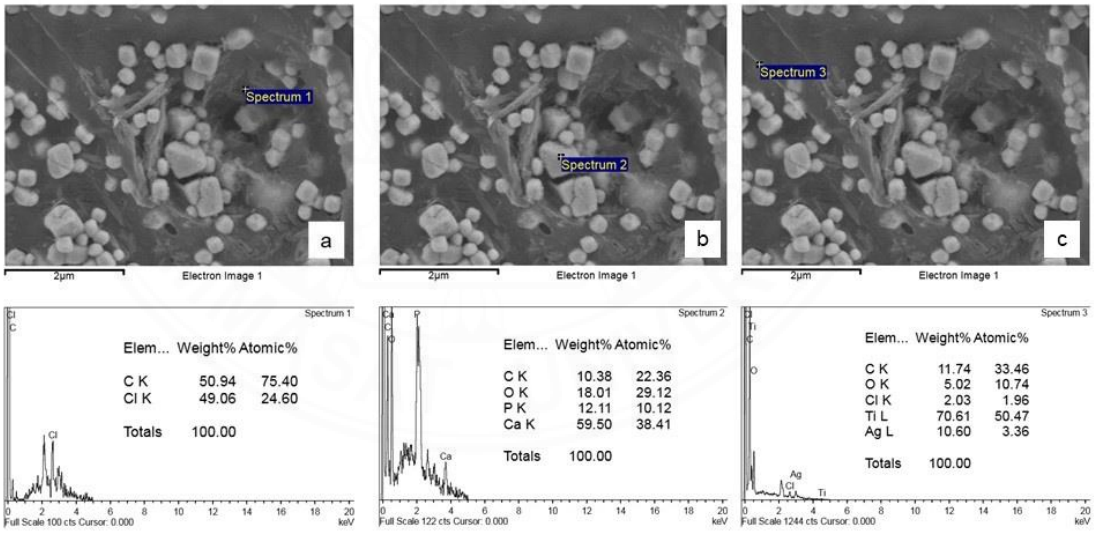

Figure 4-6 EDX images of group 3 (SDF 60 s).

4.1.2.4 Group 4 (SDF 180 s)

In group 4 (SDF 180 s), dental tubules were occluded with similar crystals detected with 30 s and 60 s (Figure 4-7). Elemental analysis detected by EDX founded carbon (C), Oxygen (O), Chloride (Cl), Silver (Ag), Calcium (Ca) and Phosphate (P) (Figure 4-8).

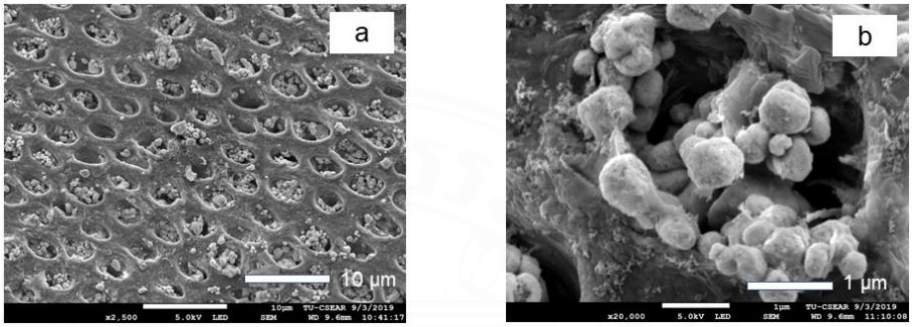

Figure 4-7 SEM images of group 3 (SDF 180 s), surface specimen soaked in SBF for two weeks at low magnification 2500 X (a) and high magnification 20000 X (b).

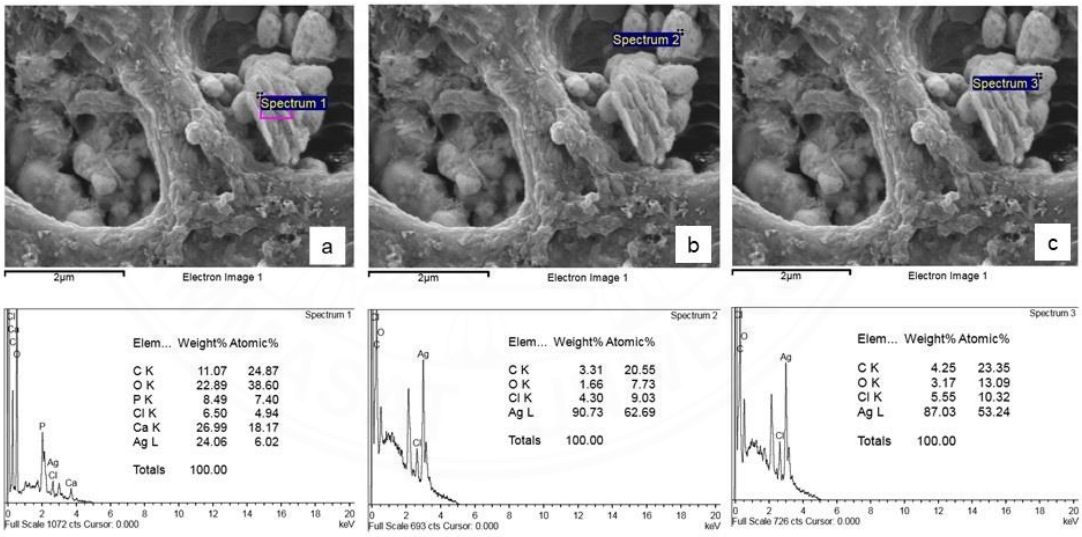

Figure 4-8 EDX images of group 4 (SDF 180 s).

4.2 Degree of mineral precipitation

The reconstructed 3D images of specimens treated with SDF (30 s, 60 s, and 180 s) showed high radiodensity of minerals precipitated in the whole depth of demineralised area (~ 250 μm) (Figure 4-9). From the results, the amount of mineral precipitation (mean ± SD) were 0 ± 0 vol% , 65.57 ± 7.46 vol%, 65.79 ± 1.99 vol%, and 65.19 ±7.97 vol% for water, SDF 30 s, SDF 60 s and SDF 180 s respectively (Figure 4-10). However, there were no significant differences detected among groups treated with SDF ($p > 0.05$).

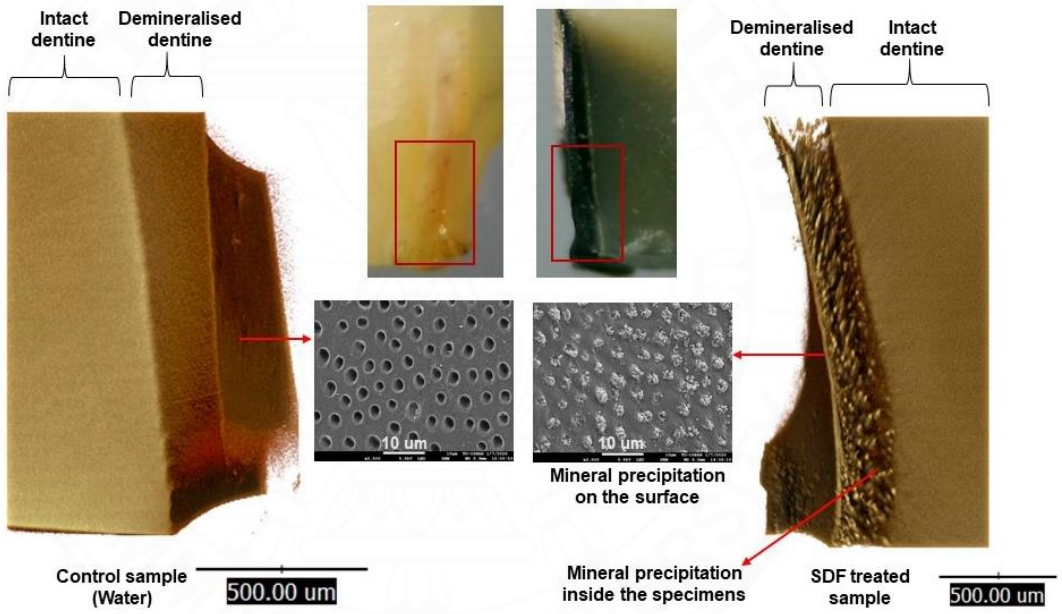

Figure 4-9 Reconstructed 3D images showed mineral precipitation in the demineralised dentine treated with SDF. Reproduced with permission from Srisomboon et al., *Dent J*, 9, 70. published by MDPI, 2021(Srisomboon et al., 2021).

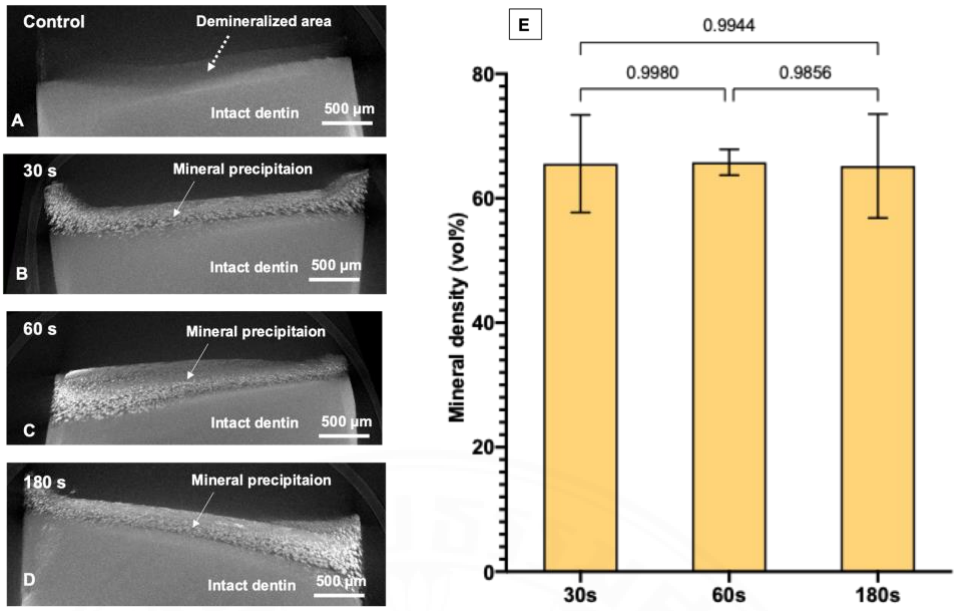

Figure 4-10 A) Reconstructed SRXTM images of a representative specimen from each group after immersion in simulated body fluid for 336 h. (A) The control group showed a demineralized area (~250 µm depth, dashed arrow) without radiodense of minerals. (B,C,D) Reconstructed images from the representative specimen from 30, 60, and 180 s groups, respectively. The images show substantial radiodensse precipitation in the demineralized area (solid arrows). (E) Mean mineral density obtained from 30, 60, and 180 s groups. Error bars are 95% CI (n = 6). Lines indicate $p > 0.05$. Data from the control group were not analyzed because mineral precipitation was not detected. Reproduced with permission under the Creative Commons Attribution (CC BY) license from Srisomboon et al., *Dent J*, 9, 70. published by MDPI, 2021.

4.3 Level of fluoride release

Fluoride release (ppm) (mean ± SD) from each group was 0.02 ± 0.01, 2.82 ± 2.01, 5.15 ± 1.36, and 12.92 ± 2.78 for water, SDF 30 s, SDF 60 s and SDF 180 s respectively (Figure 4-11 A). The fluoride release was linearly increased upon the increase of application time (Figure 4-11 B)(R^2 = 0.99). The *post-hoc* multiple comparisons showed that there were significant statistical differences between group 1 (water) and other groups *(*$p < 0.05$). Fluoride release of group 4 (180 s) was significantly higher than other groups ($p < 0.001$). There were no significant statistical differences found between group 2 (SDF 30 s) and group 3 (SDF 60 s) ($p = 0.112$).

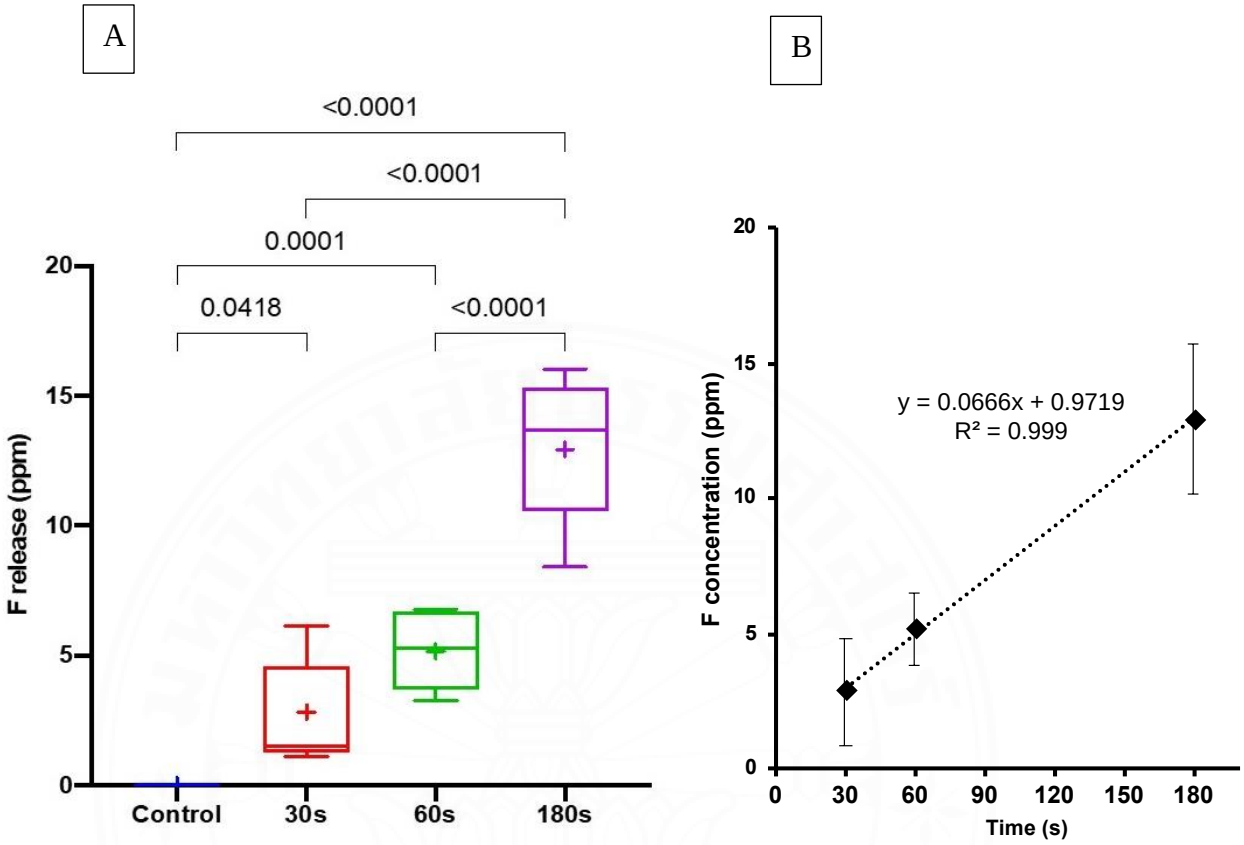

Figure 4-11 A) Boxplot of fluoride release in storage solution. The boxes represent the first quartile (Q1) to the third quartile (Q3), the horizontal lines in the box represent the median, the whiskers represent the maximum and minimum values, and "+" represents the mean value (n =7). Lines and number indicated *P* values between groups. B) the plot of fluoride release versus application time reveals linear trend.

Chaper 5 Discussion

The purpose of this current *in vitro* study was to investigate the effect of different application times of SDF (30 s, 60 s, and 180 s) on apatite formation, degree of mineral precipitation, and fluoride release in demineralised dentine. The results indicated that among the group of specimens treated with SDF in various times (30 s, 60 s, and 180 s), the mineral apatite formation and degree of mineral precipitation were comparable. However, the fluoride released from the specimens obtained from group 3 (SDF 180 s) was significantly higher than other groups. Hence, the null hypothesis was partially rejected.

5.1 Apatite formation

In the current study, dentine surface (Figure 4-10) was completely demineralised for ~ 250 μm by EDTA. This was expected to mimic the aggressive active and soft dentine caries in dentine. The specimen was not subjected to demineralisation-remineralisation cycles because our aim was to produce the completely demineralised layer. This was expected to mimic the severely demineralised dentine in advance caries lesions. This may additionally aid the comparison of remineralising actions between groups. The specimens were immersed in simulated body fluid (SBF), which contained similar ion concentration to blood plasma and body fluid (Kokubo and Yamaguchi, 2019). The use of SBF was expected to mimic the interaction between SDF and dentinal fluid that may occur when the applied SDF penetrate into dentinal tubules.

It is known that biomimetic remineralisation is required to encourage mineral apatite formation in demineralised collagen fibrils (Cao et al., 2015). This may help promote

mechanical recovery for the demineralised dentine. In the current study, the FTIR peak ratio that representing hydroxyapatite versus collagen was increased after SDF application in all groups. This may suggest that the application of SDF for 30 s to 180 s encouraged the suitable condition for apatite precipitation in the *in vitro* demineralised dentine. This current study only employed FTIR to assess mineral precipitation. The further studies should be using XPS or XRD to confirm the type of precipitation.

The SEM images revealed that the main precipitation was metallic crystals which was expected to be silver salts. It is demonstrated that silver phosphate crystals were the main precipitation observed in dentin after applying SDF. The high solubility of silver phosphate (6.4×10^{-3} g/100 mL) may allow the crystals to react with chlorides in remineralising solutions and reprecipitated as the lower soluble silver chloride (8.9×10^{-4} g/100 mL)(Zhao et al., 2018). This might be the explanation that silver chloride is the primary precipitate on the dentin treated with SDF (Mei et al., 2013; Mei et al., 2017), which in an agreement with the result obtained from the current study.

The formation of this low soluble precipitates was expected to act as the protective layer to prevent the loss of calcium or phosphate ions from the inner dentine (Zhao et al., 2018). This silver chloride precipitation from SDF was believed to help increase the hardness of the lesions (Li et al., 2019). Moreover, the silver ion can also played an important role in antibacterial effects and reduce collagen degradation (Zhao et al., 2018), which could arrest a dental caries progression. Furthermore, in the current study this precipitates by SDF was occluded in dentinal tubule. Hence, SDF also reduced sensitivity or protect pulp-dentine complex from various stimuli (Burgess and Vaghela, 2018). This finding may also support the benefit of using SDF as dentine desensitizer (Castillo et al., 2011).

It is expected that fluoride from SDF could potentially substitute hydroxyl groups in hydroxyapatite producing low soluble and acid-resistant fluorohydroxyapatite (Mei et al., 2017). However, the apatite containing fluoride was not easily detected in the specimens even when the application time was 180 s. This may indicate that the increase of application time may not directly enhance in the formation of fluorohydroxyapatite. Additionally, the application of SDF was expected to promote the formation of CaF_2 globules which was believed to act as fluoride reservoir to release fluoride ions during the acid attack (Vogel, 2011; Mei et al., 2017). However, the formation of CaF_2 was not detected in the current study. The possible explanation could be that CaF_2 was not usually form at the detectable amount and it can be washed away easily after rinsing with water (Mei et al., 2017).

5.2 Degree of mineral precipitation

According to Lambert-Beer's law, silver should give greater X-ray absorbance than calcium. The SRXTM images revealed that the precipitates in demineralised layer had higher GV the sound dentine which consisted mainly calcium phosphate apatite (Cai et al., 2019). Hence, it is speculated that silver chloride or silver phosphate particles were the main components in demineralised dentine which was in accordance with the previous study (Li et al., 2019). The current study showed that the degree of mineral precipitation in specimens applied with SDF for 30 s, 60 s, and 180 s was comparable. Furthermore, all specimens treated with SDF exhibited the formation of minerals through the whole depth of demineralised layer (~ 250 μm). The previous study also showed that SDF penetrated enamel rods and dentinal tubules forming silver-enriched protective layer with the thickness of ~ 745 μm)(Li et al., 2019). It should be mentioned that silver give a highly

radiopaque, which could overestimation the mineral density in the demineralized area. Additionally, after SDF application the dentin was shrink, which may have affected the measurements.

The current study was the first study that employed synchrotron-based X-ray microtomography (SRXTM) for analysing dentine remineralisation. The main advantage of SRXTM technique (pixel size ~ 1.44 μm) compared with conventional micro-CT (pixel size ~ 8 μm) is the higher resolution of image. Despite the high resolution, SRXTM technique was still not be able to detect the mineral precipitation which were smaller than the pixel size (< 1.44 μm). It may underestimate the precipitation of CaP apatite which was smaller than 1 μm (Figure 4-4).

5.3 Fluoride release

The release of fluoride from SDF was also expected to help enhance anti-caries actions. The current study showed that the level of fluoride ions released from the specimens in the storage solution was linearly increased upon the increase of SDF application time. It was demonstrated that the level of fluoride deposited in demineralized dentin after SDF application was highest at the surface and decreased toward the inner dentine in the diffusion gradient pattern (Sayed et al., 2019b). Hence, the increase in application may promote the increase the deposition of fluoride into the deeper dentin which could subsequently enable the high level of fluoride release. This could potentially be the possible explanation of high fluoride concentration observed with 180 s group. The limitation of the current study was that the measurement was only performed at final time

point. Hence, future work should assess the releasing versus immersion time to examine the fluoride releasing profiles.

The clinical significance of this study could be the comparable remineralising actions in mineral precipitation of demineralised dentin observed with different application times of SDF different application times. This may partially fulfil the mechanism to support the lack of correlation between SDF application times and clinical success (Horst et al., 2016). This could facilitate the use of SDF in patients with limited cooperation or the use of SDF in suboptimal clinical settings such as domiciliary cares or mobile dental services in remote areas.

However, the current study is an *in vitro* study so the clinically relevant associated with the results shall be carefully interpreted. In future *in vitro* or clinical studies assessing the effects of applying SDF at shorter application time than the current study, such as 5 to 10 s, on remineralisation would be of interested. Moreover, the effect of different application times of SDF may affect to the degree of ion penetration into the pulp-dentin complex which may exhibit different level of cytotoxic effects. Hence, the effect of different application times of SDF on cytoxicity test should be assessed.

Chaper 6 Conclusions

Within the limitations of this study, the following conclusions can be drawn.

1) The different application times of SDF from 30 s, 60 s, and 180 s showed comparable apatite formation and degree of mineral precipitation in demineralised dentin at 336 h (2 weeks).

2) The increase of application times from 30 s, 60 s, and 180 enhanced the level of fluoride release.

Chaper 7 References

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Chaper 8 Appendices

8.1 Abs_{1023}/Abs_{1663} ratio from FTIR studies

8.1.1 Descriptive statistic

The descriptive data of Abs_{1023}/Abs_{1663} ratio from all specimens (water, SDF 30 s, SDF 60 s and SDF 180 s) can be seen in Table 8-1, Table 8-2, Table 8-3 and Table 8-4.

Table 8-1 Abs_{1023}/Abs_{1663} ratio of group 1 (water) (n=13).

time/ specimen	initial	24hr	168hr	336hr
1	0.36	0.13	0.13	0.14
2	0.08	0.07	0.08	0.07
3	0.09	0.07	0.07	0.07
4	0.09	0.16	0.15	0.15
5	0.14	0.09	0.08	0.08
6	0.07	0.07	0.07	0.07
7	0.18	0.11	0.12	0.12
8	0.13	0.13	0.11	0.12
9	0.21	0.16	0.15	0.11
10	0.07	0.17	0.18	0.15
11	0.12	0.09	0.10	0.09
12	0.44	0.35	0.38	0.38
13	0.45	0.28	0.22	0.21
Mean	**0.19**	**0.15**	**0.14**	**0.13**
SD	**0.14**	**0.08**	**0.09**	**0.08**
95% CI	**0.10**	**0.06**	**0.06**	**0.04**

Table 8-2 Abs_{1023}/Abs_{1663} ratio of group 2 (SDF 30 s) (n=13).

time/ specimen	initial	24hr	168hr	336hr
1	0.31	0.27	0.32	0.36
2	0.14	0.93	0.95	0.94
3	0.72	0.41	0.59	0.64
4	0.34	0.12	0.10	0.15
5	0.28	0.24	0.28	0.26
6	0.39	0.18	0.65	0.69
7	0.08	0.17	0.14	0.16
8	0.11	0.40	0.38	0.31
9	0.22	0.24	0.24	0.24
10	0.11	0.09	0.10	0.10
11	0.34	0.37	0.40	0.52
12	0.10	0.13	0.25	0.25
13	0.48	0.64	0.64	0.69
Mean	**0.28**	**0.32**	**0.39**	**0.41**
SD	**0.19**	**0.24**	**0.25**	**0.26**
95% CI	**0.14**	**0.18**	**0.19**	**0.14**

Table 8-3 Abs_{1023}/Abs_{1663} ratio of group 3 (SDF 60 s) (n=13).

time/ specimen	initial	24hr	168hr	336hr
1	0.12	0.10	0.12	0.12
2	0.09	0.17	0.13	0.26
3	0.08	0.15	0.12	0.15
4	0.12	0.11	0.11	0.10
5	0.07	0.14	0.13	0.14
6	0.16	0.17	0.16	0.17
7	0.45	0.40	0.42	0.43
8	0.46	0.49	0.80	0.80
9	0.61	0.59	0.78	0.78
10	0.07	0.32	0.61	0.96
11	0.10	0.14	0.15	0.21
12	0.07	0.04	0.08	0.09
13	0.22	0.22	0.60	0.63
Mean	**0.20**	**0.23**	**0.32**	**0.37**
SD	**0.18**	**0.17**	**0.28**	**0.31**
95% CI	**0.14**	**0.12**	**0.21**	**0.17**

Table 8-4 Abs_{1023}/Abs_{1663} ratio of group 4 (SDF 180 s) (n=13).

time/ specimen	initial	24hr	168hr	336hr
1	0.05	0.06	0.23	0.22
2	0.15	0.50	0.51	0.50
3	0.35	0.20	0.20	0.20
4	0.09	0.08	0.09	0.13
5	0.31	0.26	0.73	0.73
6	0.21	0.18	0.25	0.25
7	0.14	0.14	0.15	0.15
8	0.42	0.70	0.72	0.86
9	0.35	0.25	0.49	0.46
10	0.34	0.68	0.83	0.90
11	0.18	0.28	0.32	0.31
12	0.06	0.12	0.15	0.17
13	0.18	0.20	0.46	0.21
Mean	**0.22**	**0.28**	**0.39**	**0.39**
SD	**0.12**	**0.21**	**0.25**	**0.28**
95% CI	**0.09**	**0.16**	**0.18**	**0.14**

8.1.2 Normality test

The Shapiro-Wilk test (Table 8-5) showed that there was insufficient evidence to accept the hypothesis that the data is distributed (p value < 0.05). Hence, the non-parametric test was therefore selected to analyse the data.

Table 8-5 Shapiro-Wilk test for normality test.

	group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	Df	Sig.	Statistic	df	Sig.
Abs_{1023}/Abs_{1663} ratio	water(initial)	0.248	13	0.028	0.784	13	0.004
	water (24hr)	0.251	13	0.024	0.806	13	0.008
	water (168hr)	0.217	13	0.094	0.788	13	0.005
	water (336hr)	0.256	13	0.020	0.748	13	0.002
	30s(initial)	0.156	13	.200*	0.892	13	0.103
	30s(24hr)	0.199	13	0.167	0.836	13	0.019
	30s(168hr)	0.170	13	.200*	0.911	13	0.188
	30s(336hr)	0.188	13	.200*	0.906	13	0.160
	60s(initial)	0.289	13	0.004	0.736	13	0.001
	60s(24hr)	0.255	13	0.021	0.864	13	0.044
	60s(168hr)	0.337	13	0.000	0.768	13	0.003
	60s(336hr)	0.252	13	0.023	0.820	13	0.012
	180s(initial)	0.161	13	.200*	0.926	13	0.304
	180s(24hr)	0.274	13	0.008	0.824	13	0.013
	180s(168hr)	0.183	13	.200*	0.906	13	0.162
	180s(336hr)	0.234	13	0.050	0.828	13	0.015

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

8.1.3 Kruskal Wallis test

As a result of the data was not normal distribution. A Kruskal Wallis Test was conducted, and the results can be seen in Table 8-6.

Table 8-6 Kruskal Wallis Test.

group		N	Mean Rank	group		N	Mean Rank
Abs_{1023}/Abs_{1663} ratio	water (initial)	13	78.35	Rank of Abs_{1023}/Abs_{1663} ratio	water (initial)	13	78.35
	water (24hr)	13	65.96		water (24hr)	13	65.96
	water (168hr)	13	62.35		water (168hr)	13	62.35
	water (336hr)	13	58.00		water (336hr)	13	58.00
	30s(initial)	13	111.08		30s(initial)	13	111.08
	30s(24hr)	13	123.92		30s(24hr)	13	123.92
	30s(168hr)	13	136.15		30s(168hr)	13	136.15
	30s(336hr)	13	143.00		30s(336hr)	13	143.00
	60s(initial)	13	74.54		60s(initial)	13	74.54
	60s(24hr)	13	99.69		60s(24hr)	13	99.69
	60s(168hr)	13	109.23		60s(168hr)	13	109.23
	60s(336hr)	13	121.46		60s(336hr)	13	121.46
	180s(initial)	13	98.15		180s(initial)	13	98.15
	180s(24hr)	13	110.77		180s(24hr)	13	110.77
	180s(168hr)	13	140.15		180s(168hr)	13	140.15
	180s(336hr)	13	139.19		180s(336hr)	13	139.19
	Total	208			Total	208	

Test Statistics[a,b]

	ratio	Rank of ratio
Kruskal-Wallis H	45.927	45.93
df	15	15
Asymp. Sig.	0.000	0.000

a. Kruskal Wallis Test

b. Grouping Variable: group

The result from Kruskal Wallis Test showed that there was sufficient evidence to reject the null hypothesis that the mean Abs_{1023}/Abs_{1663} ratio in the four groups at any time were the same (p value < 0.05).

8.1.4 Multiple comparisons

A post-hoc multiple comparisons applying Dunn test in Table 8-7 was conducted to test if there are statistically significant differences across Abs_{1023}/Abs_{1663} ratio mean of different application time. The result showed that there were significant differences in the mean Abs_{1023}/Abs_{1663} ratio between the groups.

Table 8-7 Post-hoc comparisons using Dunn Test.

Each node shows the sample average rank of group

sample1-sample2	Test Statistic	SE	Std. Test statistic	Sig.	Adj,Sig.
water(336hr)-water(168hr)	4.346	23.608	0.184	0.854	1.000
water(336hr)-water(24hr)	7.962	23.608	0.337	0.736	1.000
water(336hr)-60 s(initial)	-16.538	23.608	9.701	0.484	1.000
water(336hr)-water(initial)	20.346	23.608	0.862	0.389	1.000
water(336hr)-180 s(initial)	-40.154	23.608	-1.701	0.089	1.000
water(336hr)-60 s(24hr)	-41.692	23.608	-1.766	0.077	1.000
water(336hr)-60 s(168hr)	-51.231	23.608	-2.170	0.030	1.000
water(336hr)-180 s(24hr)	-52.769	23.608	-2.235	0.025	1.000
water(336hr)-30 s(initial)	-53.077	23.608	-2.248	0.025	1.000
water(336hr)-60 s(336hr)	-63.462	23.608	-2.688	0.007	0.862
water(336hr)-30 s(24hr)	-65.923	23.608	-2.792	0.005	0.628
water(336hr)-30 s(168hr)	-78.154	23.608	-3.310	0.001	0.112
water(336hr)-180 s(336hr)	-81.192	23.608	-3.439	0.001	0.070
water(336hr)-180 s(168hr)	-82.154	23.608	-3.48	0.001	0.060
water(336hr)-30 s(336hr)	**-85.000**	**23.608**	**-3.6000**	**0.000**	**0.038**
water(168hr)-water(24hr)	3.615	23.608	0.153	0.878	1.000
water(168hr)-60 s(initial)	-12.192	23.608	9.678	0.498	1.000

sample1-sample2	Test Statistic	SE	Std. Test statistic	Sig.	Adj,Sig.
water(168hr)-water(initial)	16.000	23.608	0.678	0.498	1.000
water(168hr)-180 s(initial)	-35.808	23.608	-1.517	0.129	1.000
water(168hr)-60 s(24hr)	-37.346	23.608	-1.582	0.114	1.000
water(168hr)-60 s(168hr)	-46.885	23.608	-1.986	0.047	1.000
water(168hr)-180 s(24hr)	-48.423	23.608	-2.051	0.040	1.000
water(168hr)-30 s(initial)	-48.731	23.608	-2.064	0.039	1.000
water(168hr)-60 s(336hr)	-59.115	23.608	-2.504	0.012	1.000
water(168hr)-30 s(24hr)	-61.577	23.608	-2.608	0.009	1.000
water(168hr)-30 s(168hr)	-73.808	23.608	-3.126	0.002	0.212
water(168hr)-180 s(336hr)	-76.846	23.608	-3.255	0.001	0.136
water(168hr)-180 s(168hr)	-77.808	23.608	-3.416	0.001	0.118
water(168hr)-30 s(336hr)	-80.654	23.608	-3.416	0.001	0.076
water(24hr)-60 s(initial)	-8.577	23.608	-0.363	0.716	1.000
water(24hr)-water(initial)	12.385	23.608	0.525	0.600	1.000
water(24hr)-180 s(initial)	-32.192	23.608	-1.364	0.173	1.000
water(24hr)-60 s(24hr)	-33.731	23.608	-1.429	0.153	1.000
water(24hr)-60 s(168hr)	-43.269	23.608	-1.833	0.067	1.000
water(24hr)-180 s(24hr)	-44.808	23.608	-1.898	0.058	1.000
water(24hr)-30 s(initial)	-45.115	23.608	-1.911	0.056	1.000
water(24hr)-60 s(336hr)	-55.500	23.608	-2.351	0.019	1.000
water(24hr)-30 s(24hr)	-57.962	23.608	-2.455	0.014	1.000
water(24hr)-30 s(168hr)	-70.192	23.608	-2.973	0.003	0.354
water(24hr)-180 s(336hr)	-73.231	23.608	-3.263	0.001	0.231
water(24hr)-30 s(336hr)	-74.192	23.608	-3.143	0.002	0.201
60 s(initial)-60 s(initial)	-77.038	23.608	-3.263	0.001	0.132
60 s(initial)-180 s(initial)	-23.615	23.608	-1.00	0.317	1.000
60 s(initial)-60 s(24hr)	-25.154	23.608	-1.065	0.287	1.000
60 s(initial)-60 s(168hr)	-34.692	23.608	91.47	0.142	1.000
60 s(initial)-30 s(initial)	-36.231	23.608	-1.535	0.125	1.000
60 s(initial)-30 s(24hr)	-36.538	23.608	1.548	0.122	1.000
60 s(initial)-30 s(24hr)	-46.923	23.608	-1.988	0.047	1.000
60 s(initial)-30 s(336hr)	49.385	23.608	2.092	0.036	1.000
60 s(initial)-30 s(168hr)	61.615	23.608	2.610	0.009	1.000
60 s(initial)-180 s(336hr)	-64.654	23.608	-2.739	0.006	0.740
60 s(initial)-180 s(168hr)	-65.615	23.608	-2.779	0.005	0.654
60 s(initial)-30 s(336hr)	68.462	23.608	2.9000	0.004	0.448
water(initial)-180 s(initial)	-19.808	23.608	-0.839	0.401	1.000

sample1-sample2	Test Statistic	SE	Std. Test statistic	Sig.	Adj,Sig.
water(initial)-60 s(24hr)	-21.346	23.608	-0.904	0.336	1.000
water(initial)-60 s(168hr)	-30.885	23.608	-1.308	0.191	1.000
water(initial)-180 s(24hr)	-32.423	23.608	-1.373	0.170	1.000
water(initial)-30 s(initial)	-32.731	23.608	-1.386	0.166	1.000
water(initial)-60 s(336hr)	-43.115	23.608	-1.826	0.068	1.000
water(initial)-30 s(24hr)	-45.577	23.608	-1.931	0.054	1.000
water(initial)-30 s(168hr)	-57.808	23.608	-2.449	0.014	1.000
water(initial)-180 s(336hr)	-60.846	23.608	-2.577	0.010	1.000
water(initial)-180 s(168hr)	-61.808	23.608	-2.618	0.009	1.000
water(initial)-30 s(336hr)	-64.654	23.608	-2.739	0.006	0.740
180 s(initial)-60 s(24hr)	1.538	23.608	0.065	0.948	1.000
180 s(initial)-60 s(168hr)	11.077	23.608	0.469	0.639	1.000
180 s(initial)-180 s(24hr)	-12.615	23.608	-0.534	0.593	1.000
180 s(initial)-30 s(initial)	12.923	23.608	0.547	0.584	1.000
180 s(initial)-60 s(336hr)	23.308	23.608	0.987	0.324	1.000
180 s(initial)-30 s(24hr)	25.769	23.608	1.092	0.275	1.000
180 s(initial)-30 s(168hr)	38.000	23.608	1.610	0.107	1.000
180 s(initial)-180 s(336hr)	-41.038	23.608	-1.738	0.082	1.000
180 s(initial)-180 s(168hr)	-42.000	23.608	-1.779	0.075	1.000
180 s(initial)-30 s(336hr)	44.846	23.608	1.900	0.057	1.000
60 s(24hr)-60 s(168hr)	-9.538	23.608	-0.404	0.686	1.000
60 s(24hr)-180 s(24hr)	-11.077	23.608	-0.469	0.639	1.000
60 s(24hr)-30 s(initial)	11.385	23.608	0.482	0.630	1.000
60 s(24hr)-60 s(336hr)	-21.769	23.608	-0.922	0.356	1.000
60 s(24hr)-30 s(24hr)	24.231	23.608	1.026	0.305	1.000
60 s(24hr)-30 s(168hr)	36.462	23.608	1.544	0.122	1.000
60 s(24hr)-180 s(336hr)	-39.500	23.608	-1.673	0.094	1.000
60 s(24hr)-180 s(168hr)	-40.462	23.608	-1.714	0.087	1.000
60 s(24hr)-30 s(336hr)	43.308	23.608	1.834	0.067	1.000
60 s(168hr)-30 s(336hr)	-1.538	23.608	-0.065	0.948	1.000
60 s(168hr)-180 s(24hr)	1.846	23.608	0.078	0.938	1.000
60 s(168hr)-30 s(initial)	-12.231	23.608	-0.518	0.604	1.000
60 s(168hr)-60 s(336hr)	14.692	23.608	0.622	0.534	1.000
60 s(168hr)-30 s(24hr)	26.923	23.608	1.140	0.254	1.000
60 s(168hr)-30 s(168hr)	-29.962	23.608	-1.269	0.204	1.000
60 s(168hr)-180 s(336hr)	-30.923	23.608	-1.310	0.190	1.000
60 s(168hr)-30 s(336hr)	33.769	23.608	1.430	0.153	1.000

sample1-sample2	Test Statistic	SE	Std. Test statistic	Sig.	Adj,Sig.
180 s(24hr)-30 s(initial)	0.308	23.608	0.013	0.990	1.000
180 s(24hr)-30 s(336hr)	10.692	23.608	0.453	0.651	1.000
180 s(24hr)-30 s(24hr)	13.154	23.608	0.557	0.577	1.000
180 s(24hr)-30 s(168hr)	25.385	23.608	1.075	0.282	1.000
180 s(24hr)-180 s(336hr)	-38.423	23.608	-1.204	0.229	1.000
180 s(24hr)-180 s(168hr)	-29.385	23.608	-1.245	0.213	1.000
180 s(24hr)-30 s(336hr)	32.231	23.608	1.365	0.172	1.000
30 s(initial)-60 s(336hr)	-10.385	23.608	-0.440	0.660	1.000

Asymptotic significance (2- sided tests) are displayed. The significance level is 0.05

Significance values have been adjusted by the Bonferroni correction for multiple test

8.1.5 Friedman test

The differences between the absorbance ratio of each group in respect of immersion times were tested using Friedman test. The analysis was performed using Prism 9 (GraphPad Software LLC., San Diego, CA, USA).

Table 8-8 Friedman test (control group).

Number of families	1					
Number of comparisons per family	6					
Alpha	0.05					
Dunn's multiple comparisons test	**Rank sum diff.**	**Significant?**	**Summary**	**Adjusted P Value**		
control_0 vs. control_24	11.00	No	ns	0.5683	A-B	
control_0 vs. control_1w	16.00	No	ns	0.0904	A-C	
control_0 vs. control_2w	21.00	Yes	**	0.0085	A-D	
control_24 vs. control_1w	5.000	No	ns	>0.9999	B-C	
control_24 vs. control_2w	10.00	No	ns	0.7724	B-D	
control_1w vs. control_2w	5.000	No	ns	>0.9999	C-D	
Test details	**Rank sum 1**	**Rank sum 2**	**Rank sum dif**	**n1**	**n2**	**Z**
control_0 vs. control_24	44.50	33.50	11.00	13	13	1.671
control_0 vs. control_1w	44.50	28.50	16.00	13	13	2.431
control_0 vs. control_2w	44.50	23.50	21.00	13	13	3.190
control_24 vs. control_1w	33.50	28.50	5.000	13	13	0.7596
control_24 vs. control_2w	33.50	23.50	10.00	13	13	1.519
control_1w vs. control_2w	28.50	23.50	5.000	13	13	0.7596

Table 8-9 Friedman test (30 s)

Number of families	1					
Number of comparisons per family	6					
Alpha	0.05					
Dunn's multiple comparisons test	**Rank sum diff.**	**Significant?**	**Summary**	**Adjusted P Value**		
30s_0 vs. 30s_24	-1.000	No	ns	>0.9999	E-F	
30s_0 vs. 30s_1w	-8.000	No	ns	>0.9999	E-G	
30s_0 vs. 30s_2w	-13.00	No	ns	0.2897	E-H	
30s_24 vs. 30s_1w	-7.000	No	ns	>0.9999	F-G	
30s_24 vs. 30s_2w	-12.00	No	ns	0.4099	F-H	
30s_1w vs. 30s_2w	-5.000	No	ns	>0.9999	G-H	
Test details	**Rank sum 1**	**Rank sum 2**	**Rank sum dif**	**n1**	**n2**	**Z**
30s_0 vs. 30s_24	27.00	28.00	-1.000	13	13	0.1519
30s_0 vs. 30s_1w	27.00	35.00	-8.000	13	13	1.215
30s_0 vs. 30s_2w	27.00	40.00	-13.00	13	13	1.975
30s_24 vs. 30s_1w	28.00	35.00	-7.000	13	13	1.063
30s_24 vs. 30s_2w	28.00	40.00	-12.00	13	13	1.823
30s_1w vs. 30s_2w	35.00	40.00	-5.000	13	13	0.7596

Table 8-10 Friedman test (60 s)

Number of families	1					
Number of comparisons per family	6					
Alpha	0.05					
Dunn's multiple comparisons test	**Rank sum diff.**	**Significant?**	**Summary**	**Adjusted P Value**		
60s_0 vs. 60s_24	-4.000	No	ns	>0.9999	I-J	
60s_0 vs. 60s_1w	-10.00	No	ns	0.7724	I-K	
60s_0 vs. 60s_2w	-20.00	Yes	*	0.0143	I-L	
60s_24 vs. 60s_1w	-6.000	No	ns	>0.9999	J-K	
60s_24 vs. 60s_2w	-16.00	No	ns	0.0904	J-L	
60s_1w vs. 60s_2w	-10.00	No	ns	0.7724	K-L	
Test details	**Rank sum 1**	**Rank sum 2**	**Rank sum dif**	**n1**	**n2**	**Z**
60s_0 vs. 60s_24	24.00	28.00	-4.000	13	13	0.6076
60s_0 vs. 60s_1w	24.00	34.00	-10.00	13	13	1.519
60s_0 vs. 60s_2w	24.00	44.00	-20.00	13	13	3.038
60s_24 vs. 60s_1w	28.00	34.00	-6.000	13	13	0.9115
60s_24 vs. 60s_2w	28.00	44.00	-16.00	13	13	2.431
60s_1w vs. 60s_2w	34.00	44.00	-10.00	13	13	1.519

Table 8-11 Friedman test (180 s)

Number of families	1					
Number of comparisons per family	6					
Alpha	0.05					
Dunn's multiple comparisons test	**Rank sum diff.**	**Significant?**	**Summary**	**Adjusted P Value**		
180s_0 vs. 180s_24	-2.000	No	ns	>0.9999	M-N	
180s_0 vs. 180s_1w	-23.00	Yes	**	0.0029	M-O	
180s_0 vs. 180s_2w	-21.00	Yes	**	0.0085	M-P	
180s_24 vs. 180s_1w	-21.00	Yes	**	0.0085	N-O	
180s_24 vs. 180s_2w	-19.00	Yes	*	0.0234	N-P	
180s_1w vs. 180s_2w	2.000	No	ns	>0.9999	O-P	
Test details	**Rank sum 1**	**Rank sum 2**	**Rank sum dif**	**n1**	**n2**	**Z**
180s_0 vs. 180s_24	21.00	23.00	-2.000	13	13	0.3038
180s_0 vs. 180s_1w	21.00	44.00	-23.00	13	13	3.494
180s_0 vs. 180s_2w	21.00	42.00	-21.00	13	13	3.190
180s_24 vs. 180s_1w	23.00	44.00	-21.00	13	13	3.190
180s_24 vs. 180s_2w	23.00	42.00	-19.00	13	13	2.886
180s_1w vs. 180s_2w	44.00	42.00	2.000	13	13	0.3038

8.2 Degree of mineral precipitation

8.2.1 Descriptive statistic

The descriptive data of amount of mineral precipitation (vol %) in demineralised dentin from all specimens (n=6) of treatment groups (water, SDF 30 s, SDF 60 s and SDF 180 s) can be seen in Table 8-12.

Table 8-12 mineral precipitation (volume %) of all specimen (n=6)

Treatment group/ specimen	water	SDF 30 s	SDF 60 s	SDF 180 s
1	0	64.63	66.34	63.76
2	0	65.73	64.75	65.96
3	0	59.41	67.32	72.24
4	0	72.03	63.39	50.27
5	0	55.85	68.64	67.7
6	0	75.74	64.3	71.21
Mean	**0**	**65.57**	**65.79**	**65.19**
SD	**0**	**7.46**	**1.99**	**7.97**
95% CI	**0**	**5.97**	**1.59**	**6.38**

8.2.2 Normality test

The Shapiro-Wilk test result from Table 8-13 showed that there was sufficient evidence to accept the hypothesis that the data is normally distributed (p value ≥ 0.05). Levene's test was therefore applied to check the variances of the groups.

Table 8-13 Shapiro-Wilk test for normality test

group		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
% mineral precipitation	water		6			6	
	30s	0.158	6	.200*	0.969	6	0.886
	60s	0.199	6	.200*	0.960	6	0.823
	180s	0.262	6	.200*	0.844	6	0.141

8.2.3 Homogeneity of variance test

The Levene's test result from Table 8-14 showed that there was sufficient evidence to reject the hypothesis that the variances between the groups were equal (p value < 0.05).

Table 8-14 Levene's test for equality of variances.

		Levene Statistic	df1	df2	Sig.
% mineral precipitation	Based on Mean	4.031	3	20	0.021
	Based on Median	3.353	3	20	0.039
	Based on Median and with adjusted df	3.353	3	9.414	**0.067**
	Based on trimmed mean	3.750	3	20	0.028

8.2.4 Analysis of variance

As a result of the data was normal distribution and the Levene's test were unequal. A Welch analysis of variance (Welch ANOVA) was conducted, and the results can be seen in Table 8-15.

Table 8-15 Welch analysis of variances (Welch ANOVA).

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19316.071	3	6438.690	209.113	.000
Within Groups	615.808	20	30.790		
Total	19931.879	23			

Robust Tests of Equality of Means[b]

crystal_volume

	Statistic[a]	df1	df2	Sig.
Welch	.	.	.	.

a. Asymptotically F distributed.

b. Robust tests of equality of means cannot be performed for crystal_volume because at least one group has 0 variance.

The result from Welch ANOVA showed that there was sufficient evidence to reject the null hypothesis that the mean amount of mineral precipitation (vol %) in the four groups were the same ($p < 0.05$).

8.2.5 Multiple comparisons

A post-hoc multiple comparisons applying Dunnett T3 (Table 8-16) was conducted to test if there are statistically significant differences across the mean mineral precipitation (vol %) at different application time. The results showed that significant differences were detected in the mean mineral precipitation (vol %) between the groups.

Table 8-16 Post-hoc multiple comparison with Dunnett T3.

Dunnett T3

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
water	30s	-65.56500*	3.04682	.000	-77.5315	-53.5985
	60s	-65.79000*	.81297	.000	-68.9830	-62.5970
	180s	-65.19000*	3.25314	.000	-77.9668	-52.4132
30s	water	65.56500*	3.04682	.000	53.5985	77.5315
	60s	-.22500	3.15342	1.000	-11.9858	11.5358
	180s	.37500	4.45713	1.000	-13.8969	14.6469
60s	water	65.79000*	.81297	.000	62.5970	68.9830
	30s	.22500	3.15342	1.000	-11.5358	11.9858
	180s	.60000	3.35318	1.000	-11.9757	13.1757
180s	water	65.19000*	3.25314	.000	52.4132	77.9668
	30s	-.37500	4.45713	1.000	-14.6469	13.8969
	60s	-.60000	3.35318	1.000	-13.1757	11.9757

*. The mean difference is significant at the 0.05 level.

8.3 Level of fluoride release

8.3.1 Descriptive statistic

The descriptive data of Fluoride release (ppm) from all specimens n= 7 of treatment groups (water, SDF 30 s, SDF 60 s and SDF 180 s) can be seen in Table 8-17.

Table 8-17 Fluoride release (ppm) of all specimen (n=7).

Treatment group/ specimens	water	SDF 30 s	SDF 60 s	SDF 180 s
1	0.02	1.10	5.29	16.03
2	0.03	1.52	3.25	10.57
3	0.03	4.57	3.69	14.63
4	0.03	1.27	6.77	15.33
5	0.01	6.15	6.68	13.70
6	0.02	3.79	4.80	8.40
7	0.02	1.36	5.58	11.80
Mean	**0.02**	**2.82**	**5.15**	**12.92**
SD	**0.01**	**2.01**	**1.36**	**2.78**
95%CI	**0.003**	**1.10**	**0.74**	**1.51**

8.3.2 Normality test

The Shapiro-Wilk test result from Table 8-17 showed that there was sufficient evidence to accept the hypothesis that the data is normal distribution (p value ≥ 0.05). Levene's test was therefore applied to check the variances of the groups.

Table 8-18 Shapiro-Wilk test for normality test.

group		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
F release	water	0.242	7	.200*	0.911	7	0.401
	30s	0.313	7	0.037	0.830	7	0.080
	60s	0.155	7	.200*	0.934	7	0.583
	180s	0.181	7	.200*	0.941	7	0.651

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

8.3.3 Homogeneity of variance test

The Levene's test result from Table 8-19**Error! Reference source not found.** showed that there was sufficient evidence to reject the hypothesis that the variances between the groups were equal (p value < 0.05).

Table 8-19 Levene's test for equality of variances

		Levene Statistic	df1	df2	Sig.
F release	Based on Mean	9.952	3	24	0.000
	Based on Median	3.487	3	24	0.031
	Based on Median and with adjusted df	3.487	3	14.121	0.044
	Based on trimmed mean	9.376	3	24	0.000

8.3.4 Analysis of variance

As a result of the data was normal distribution. A one-way analysis of variance (ANOVA) was conducted and the results can be seen in Table 8-20.

Table 8-20 One way analysis of variances (one-way ANOVA)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	644.751	3	214.917	63.241	0.000
Within Groups	81.561	24	3.398		
Total	726.312	27			

The result from one-way ANOVA showed that there was sufficient evidence to reject the null hypothesis that the mean fluoride release (ppm) in the four groups were the same (p value < 0.05).

8.3.5 Multiple comparisons

A post-hoc multiple comparisons applying Tukey-HSD Table 8-21 was conducted to test if there are statistically significant differences across Fluoride release (ppm)mean of different application time. The result showed that there were significant differences in the mean Fluoride release (ppm) between the groups.

Table 8-21 Post-hoc multiple comparison with Tukey-HSD.

(I) group		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
water	30s	-2.80048*	0.98537	0.042	-5.5187	-0.0822
	60s	-5.12857*	0.98537	0.000	-7.8468	-2.4103
	180s	-12.90096*	0.98537	0.000	-15.6192	-10.1827
30s	water	2.80048*	0.98537	0.042	0.0822	5.5187
	60s	-2.32810	0.98537	0.112	-5.0464	0.3902
	180s	-10.10048*	0.98537	0.000	-12.8187	-7.3822
60s	water	5.12857*	0.98537	0.000	2.4103	7.8468
	30s	2.32810	0.98537	0.112	-0.3902	5.0464
	180s	-7.77238*	0.98537	0.000	-10.4906	-5.0541
180s	water	12.90096*	0.98537	0.000	10.1827	15.6192
	30s	10.10048*	0.98537	0.000	7.3822	12.8187
	60s	7.77238*	0.98537	0.000	5.0541	10.4906

*. The mean difference is significant at the 0.05 level.