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Long-term effect of ammonia- and water-based silver fluoride on dentin collagen matrix

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ABSTRACT

Objective: To evaluate the long-term effect of ammonia- and water-based silver fluoride treatments on the degradation of the dentin collagen matrix.
Methods: Dentin beams (0.3x3x7mm) were demineralized (10 % H_3PO_4), rinsed and randomly distributed into six groups. Groups (n = 10 beams/group) were treated with (1) ammonia-based silver fluoride = SDF; (2) SDF + potassium iodide = KI (3) water-based silver fluoride = SF (4) SF + KI (5) KI (6) untreated demineralized dentin beams served as control. Following treatments, dry mass, modulus of elasticity and enzymatic activity were assessed. Dentin beams were incubated in calcium- and zinc-containing artificial saliva up to 6 months. After different incubation periods (1 week, 1 month, 3 months or 6 months), dry mass, modulus of elasticity and enzymatic activity were reevaluated. The aliquots of incubation media were analyzed to determine the solubilized telopeptides of collagen (ICTP and CTX immunoassays), hydroxyproline release and total extractable protein (Bradford assay). Scanning electron microscopy imaging and *in situ* zymography analyses were conducted. Data were statistically analyzed with ANOVA followed by Tukey test (α=0.05).
Results: Silver fluoride treatments reduced the total enzymatic activity, but increased the solubilized telopeptides of collagen throughout incubation periods ($p < 0.05$). The addition of KI exacerbated the loss of dry mass, modulus of elasticity, hydroxyproline release and total protein loss ($p < 0.05$).
Significance: Ammonia- and water-based silver fluoride treatments may reduce long-term degradation of dentin collagen. However, potassium iodide can further increase endogenous protease activity and compromise the structural integrity of dentin's organic matrix.

1. Introduction

Dentin caries is a multifactorial, dynamic and the most prevalent oral disease that still constitutes a significant public health challenge and persists as a global health burden [1,2]. The progression of dentin caries involves the demineralization of hydroxyapatite and degradation of dentin collagen matrix. These processes are driven by bacterial acid production and enzymatic biodegradation which together lead to the demineralization of dentin and the weaken of the collagen matrix, respectively [3,4]. Dentin matrices contain proteolytic enzymes such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs). These enzymes are trapped in mineralized dentin. Exposure of dentin to low pH during cariogenic activity or the acid etching step of adhesive procedures [5], can activate endogenous enzymes, leading to degradation of dentin collagen matrix and decreased restoration durability [3,6,7]. Therefore, inhibition of endogenous activity is critical for slowing caries progression and decreasing the bond degradation, in other words, reducing collagen degradation at resin-dentin bonding interfaces over time [4,6,8]. Strategies including the use of collagen crosslinkers, enzyme inhibitors, remineralization agents, selective or non-selective inhibitors have been studied to prevent degradation of dentin collagen matrix by enzymes.

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The contemporary philosophy of caries management has shifted from the traditional drill and fill approach to a seal and heal model, emphasizing preventive strategies such as fluoride therapy [9]. A variety of fluoride-containing agents have been used in dentistry to control the progression of dentin caries and promote remineralization. Combining fluoride with silver, an antimicrobial and anticariogenic agent, silver fluoride-based treatments are formed. Silver fluoride-based treatments have two different chemical formulas depending on the third ion included. When silver and fluoride ions form a complex with ammonia, ammonia-based silver fluoride, known as silver diamine fluoride (SDF), is formed [10]. Recently, a water-based silver fluoride, in other words, an aqueous silver fluoride (SF), has been introduced as the first water-based silver fluoride solution. In this formulation, ammonia ion is replaced by water and provided patient benefits such as improved smell, taste and reduced soft tissue irritation. However, both formulations raise esthetic concerns due to silver staining of tooth and surrounding tissues, caused by oxidation of free silver ions [11], which can limit their acceptance in clinical practice. To address this issue, the application of potassium iodide (KI) immediately after treatment with either ammonia- or water-based silver fluoride leads to the formation of a white silver iodide compound that ameliorates black dentin stains [12]. Therefore, silver fluoride treatments can be achieved either ammonia- and water-based silver fluoride alone (SDF & SF) or in combination with potassium iodide (KI) application.

The use of ammonia-based SDF has gained popularity due to possible inhibition of endogenous proteases [13,14]. It has also emerged as a possible off-label approach to extend the longevity of resin-dentin interfaces [8]. Furthermore, according to the World Health Organization (WHO) Global Strategy Action Plan [15], by 2030, 50 % of countries should include SDF in their list of essential medicines. This highlights the growing potential and increased use of the SDF in preventive and restorative dentistry. Although antimicrobial benefits of SDF are well known on caries arrest, the effect of SF and the combinations with KI treatment require further investigation. Limited number of studies have evaluated the short-term effects of ammonia-based silver fluoride (SDF) [13,14] and water-based silver fluoride (SF) [16] on dentin endogenous activity. To the best of our knowledge, the long-term influence of ammonia- and water-based silver fluoride treatments on dentin endogenous enzymatic activity has yet to be investigated. To date, it remains unclear whether ammonia- and water-based silver fluoride treatments can reduce or inactivate endogenous dentin enzymatic activity over extended periods.

The aim of this study was to evaluate the long-term effect of ammonia- and water-based silver fluoride treatments on their ability to inactivate dentin proteases and prevent dentin matrix degradation. The inactivation of dentin MMPs and CCs were assessed by means of quantitation of ICTP (crosslinked carboxyterminal telopeptide of type I collagen) and CTX (C-terminal crosslinked telopeptide of type I collagen) release, respectively. Furthermore, dentin matrix degradation were evaluated by measuring loss of dry mass, changes in the modulus of elasticity, total enzymatic activity, hydroxyproline release and total extractable protein. To support the findings, imaging techniques, scanning electron microscopy (SEM) and *in situ* zymography were used. The null hypotheses tested were ammonia- and water-based silver fluoride treatments applied on demineralized dentin matrix do not affect the degradation of collagen matrix or dentin protease activity.

2. Materials and methods

Sixty intact third molars extracted during routine dental treatments from anonymous donors were used in the study and exempt from ethical notification in accordance with local regulations (Tissue act, section 20). Teeth were stored at 4 °C in 0.9 % NaCl supplemented with 0.02 % NaN_3 to prevent bacterial growth and used within three months of extraction.

The sample size was determined using G*Power (version 3.1.9.7, Kiel University, Kiel, Germany) based on the results of a prior pilot study [17]. The effect size (f) was 0.40, the type I error (α) was 0.05 and the statistical power (1-β) was 0.95. The total sample size required for this study was 48, with a minimum sample size of 8 specimens per group. In the present study 10 specimens per group were used.

2.1. Preparation of dentin beam specimens

During the preparation of test specimens, a low-speed water-cooled saw (Isomet 1000 Precision Saw, Buehler Ltd., USA) were used. One dentin disc from the mid-coronal dentin region of each tooth (n = 10/group) with the thickness of 0.3 (±0.02) mm was obtained by sectioning perpendicularly to the tooth's long axis. The dentin discs were then sectioned mesio-distally to produce rectangular dentin beams with the dimensions of 0.3 mm thickness x 3 mm width x 7 mm length. To enable repeated measurements to be performed on the same surface, a dimple was made at the corner of each dentin beam on the occlusal surface. Prepared dentin beams were stirred in aqueous 10 wt% H_3PO_4 (Phosphoric acid, Merck Sigma-Aldrich, Germany) for 40 min at 25 °C for complete demineralization and then rinsed in distilled water at 4 °C for 1 h under constant stirring at 60 rpm (Loopster digital, IKA, Germany). Digital radiography was used to confirm the absence of residual minerals [18]. After demineralization process, digital images were obtained on a stereo microscope (Leica M60, Leica Microsystems, Germany) and thickness and width dimension of the demineralized beams were precisely measured using an open-source image software (ImageJ, National Institute of Health, USA). Then, dentin beams were placed in individually labelled 96-well plates. Initial modulus of elasticity of each beam was measured with three point bending test using universal testing machine (Autograph AGS-X Series, Shimadzu, Japan), then beams were dried in vacuum desiccator containing dry silica beads for 72 h and initial dry mass of each beam was weighted. After initial baseline measurements of modulus of elasticity and dry mass, demineralized dentin beams (n = 10/group) were assigned into 6 balanced groups, so that their mean modulus of elasticity and dry mass values were statistically similar. Commercially available ammonia- and water-based silver fluoride treatments (Riva Star and Riva Star Aqua, SDI, Australia) were selected for this study and the pH values of each solution was measured with pH meter (PHM210, Radiometer Analytical, France) as shown in Table 1.

Groups were prepared according to the silver fluoride treatments received (Table 1) and demineralized dentin beams without any silver fluoride treatment were served as control. Group 1: Silver Diamine Fluoride = SDF (Riva Star Bottle 1, SDI, Australia); Group 2: SDF + Potassium Iodide = KI (Riva Star Bottle 1 and 2, SDI, Australia); Group 3: Aqueous silver fluoride = SF (Riva Star Aqua Bottle 1, SDI, Australia); Group 4: SF + KI (Riva Star Aqua Bottle 1 and 2, SDI, Australia); Group 5: KI (Riva Star Aqua Bottle 2, SDI, Australia); Group 6: demineralized dentin beams without any silver fluoride treatment as control group. Demineralized dentin beams in test groups 1–5 were treated with corresponding silver fluoride treatments for 1 min according to the manufacturer`s instructions (Table 1). After silver fluoride treatments, beams were washed with distilled water inside 96-well plate at a microplate shaker at 300 rpm, then beams were blot dried with absorbent paper. Total enzymatic activity (generic MMP assay), modulus of elasticity and dry mass of all dentin beams were measured. Then, each dentin beam was placed into individually labeled O-ring polypropylene tube containing 500 μl artificial saliva (pH 7.4) containing 5 mM HEPES, 2.5 mM $CaCl_2H_2O$, 0.05 mM $ZnCl_2$, and 0.3 mM NaN_3 [19] and incubated in a shaking-water-bath (60 cycles/min) at 37 °C, to facilitate artificial saliva diffusion within collagen fibrils, for designated incubation periods of 1 week, 1 month, 3 months and 6 months. After each incubation period, the incubation medium was stored frozen (-80 °C) until the end of the experiment. Those media were analyzed for solubilized telopeptides of collagen (ICTP & CTX), hydroxyproline (HYP) release and total extractable protein (Bradford assay).

Table 1
Materials, compositions, pH values and application procedures of the ammonia- and water-based silver fluoride treatments.

	Abbreviation	Material	Composition	pH*	Application	Lot number
Ammonia-based silver fluoride		Riva Star (SDI, Australia)				1211902
	SDF	Bottle 1: Silver Diamine Fluoride	silver (32.6 % w/v), fluoride (5.7 % w/v), ammonia (15–20 % w/v), water (40–60 % w/v)	12.05	Active application of 2 µl on each dentin beam surface for 60 s.	1210634
	KI	Bottle 2: Potassium Iodide	potassium iodide (58 % w/w)	8.63	Active application of 4 µl on each dentin beam surface for 60 s until the initially creamy white appearance turns clear.	1211767
Water-based silver fluoride		Riva Star Aqua (SDI, Australia)				1204201
	SF	Bottle 1: Aqueous Silver Fluoride	silver (32.6 % w/v), fluoride (5.7 % w/v), water (50–70 % w/v)	7.73	Active application of 2 µl on each dentin beam surface for 60 s.	221050
	KI	Bottle 2: Potassium Iodide	potassium iodide (58 % w/w)	7.94	Active application of 4 µl on each dentin beam surface for 60 s until the initially creamy white appearance turns clear.	221048

* The pH values of tested materials were determined at Adhesive Dentistry Research Laboratory, Institute of Dentistry, University of Turku, Turku, Finland.

2.2. Loss of dry mass

The loss of demineralized dry dentin mass was used as an indirect measurement of the enzymatic degradation of dentin matrix as a result of total endogenous protease activity as previously described [20,21]. Briefly, dentin beams measuring 0.3 × 3×7 mm (n = 10/group) were demineralized in aqueous 10 wt% H_3PO_4 for 40 min, rinsed in distilled water for 1 h, transferred into individually labelled 96-well plates and placed in a vacuum desiccator for 72 h [18]. Specimens were then weighed individually with an analytical microbalance (XP6 Microbalance, Mettler Toledo, USA). After the initial dry mass measurements, dentin beams were re-hydrated in distilled water at 4 °C for 1 h. According to previous findings, such rehydration period is sufficient for complete re-expansion of the dried dentin beams [22]. After hydration, silver fluoride treatments were applied (Table 1), demineralized dentin beams without any silver fluoride treatment served as control. The measurement of dry mass loss was reassessed immediately after silver fluoride treatments following the same protocol. After each incubation period, dentin beams were rinsed at 4 °C for 24 h in distilled water to remove media salts before dry mass measurements. Calculation of the dry mass was repeated under the same conditions after dehydration in desiccator. The loss of dry mass was calculated as the percentage change of dry mass loss referring to the dry mass recorded for each beam after corresponding silver fluoride treatments. Demineralized dentin beams without any treatment served as control group.

2.3. Modulus of elasticity

The 3-point bending test was selected to investigate the modulus of elasticity since it allows repeated measurements to be performed nondestructively [23]. Dentin beams were placed on a 3-point bending jig (Instron, Instron Inc., USA) with a 5 mm span length between supports, fully immersed in distilled water and tested under flexure to 3 % strain at a displacement rate of 0.5 mm min^{-1} using a 5 N load cell (SMT1–5N, Interface, USA) on a universal testing machine (Autograph AGS-X, Shimadzu, Japan). The load was immediately returned to 0 % stress within 15 s after maximum displacement to prevent creep of the demineralized collagen matrix [20,21]. Load-displacement curves were converted to stress-strain curves and the apparent modulus of elasticity (E) were then calculated using the following equation and expressed in MPa: $E = mL^3/4bh^3$, where m is the slope of the linear portion of the load-displacement curve (N/mm), L is the span length (5 mm), b is the width and h is the thickness of demineralized dentin beam in mm. Each demineralized dentin beam was tested in duplicates and the average was used as the E value of each demineralized dentin beam. E were evaluated initially on demineralized dentin beams which served as baseline measurements, after the application of silver fluoride treatments (Table 1), and after each designated incubation period (1 week, 1 month, 3 months or 6 months). Demineralized dentin beams without any treatment served as control.

2.4. Total enzymatic activity

A generic colorimetric MMP assay (Sensolyte Generic MMP assay, Anaspec, USA) was used to determine whether the ammonia- and water-based silver fluoride treatments could inactivate the dentin-derived endogenous total MMP activity [24]. Demineralized dentin beams were used as the MMP source [21]. After silver fluoride treatments (Table 1), dentin beams were rinsed, blot-dried with absorbent paper and were incubated in 200 µl of the substrate and assay buffer in the 96-well plate for 2 h at 25 ºC to assess the MMP activity. Demineralized dentin beams without any treatment served as control. Every 30 min, dentin beams were removed from the wells and the MMP activity [25] was measured using a spectrometer (Synergy HT, BioTek Inst, USA) at 412 nm wavelength. Data were recorded after each measurement until dentin beam activity reached the peak value. Then, beams were rinsed free of MMP substrate in distilled water and incubated at designated incubation periods (1 week, 1 month, 3 months or 6 months). After each incubation period, beams were rinsed and the total enzymatic activity was reassessed following the same protocol. The percentage inhibition of MMPs were calculated by subtracting the mean values of the control group (demineralized dentin beams without any silver fluoride treatment) from ammonia- and water-based silver fluoride treatment groups, then dividing by the mean value of the control group.

2.5. Solubilized telopeptides of collagen

Release of different C-telopeptide fragments were analyzed from the incubation media after each designated incubation period (1 week, 1 month, 3 months or, 6 months) to evaluate the specific role of MMPs and CCs in type I collagen degradation. Matrix degradation by MMPs were determined by measuring the amount of solubilized type I collagen C-terminal cross-linked telopeptides [26], cathepsin K-induced degradation of type I collagen were measured by the amount of solubilized

C-terminal peptide, using the commercially available kits ICTP (UniQ ICTP RIA, Aidian Diagnostics, Finland) and CTX (Serum Crosslaps ELISA, IDS, Denmark), respectively. The only known source of CTX is cathepsin K [27]. Following each incubation period (1 week, 1 month, 3 months or 6 months), 20–25 µl aliquots of incubation media from each tube were used to quantitate solubilized ICTP and CTX fragments following the kits protocol. The measurements was performed using a gamma counter (Wallac Wizard, Turku, Finland) at 1000 counts for ICTP and a spectrophotometer (Synergy HT, Bio Tek Inst., USA) at 450 nm absorbance, respectively. The amount of collagen degradation as ICTP and CTX telopeptide fragment release was calculated with a standard curve using standards with known concentrations provided by the manufacturer.

2.6. Hydroxyproline quantification

The amount of collagen solubilization was assessed by hydroxyproline (HYP) quantification from the incubation media after each designated incubation period (1 week, 1 month, 3 months or 6 months). Solubilized collagen peptide fragments were assessed following a previously described HYP quantification protocol [28]. Briefly, aliquots of HYP standards (2–20 µg) prepared from stock solutions and 25 µl of incubation media were mixed with 25 µl of 4 N NaOH (2 N final concentration) in a total volume of 50 µl in 2 mL Nalgene O-ring tubes. Specimens were autoclaved at 120 °C for 20 min. 450 µl of chloramine-T was added to hydrolyzed tubes and mixed gently to allow oxidation for 20 min at room temperature. 500 µL Ehrlich's aldehyde reagent was added to each specimen for chromophore formation by incubating the specimens at 65 °C for 20 min. Absorbance values were obtained using a spectrophotometer (Model UV-A180, Shimadzu, Japan) at 550 nm and plotted against the standard HYP curves to determine the HYP release.

2.7. Total extractable protein

Extracted dentin proteins obtained from demineralized dentin were used for the quantification of extractable protein level from the incubation media after each designated incubation period (1 week, 1 month, 3 months or 6 months). Extracted proteins from each dentin beam into the incubation media were tested according to the manufacturer's instructions using a commercial kit based on the Bradford assay (Bio-Rad, Hercules, USA) [29]. Briefly, 1–50 µg/mL series of bovine serum albumin (BSA) standards (50 µl), and aliquots of 50 µl of incubation media were analyzed. After 5 min of incubation to allow for color development, the absorbance was measured using a spectrophotometer (Synergy HT, BioTek Inst, USA) at 595 nm. A linear standard curve was prepared by plotting the average reading for each BSA standard vs. its concentration in µg/mL and the standard curve was used to determine the total extractable protein concentration.

2.8. Scanning electron microscopy (SEM)

Additional 20 dentin beams were prepared, demineralized and treated with ammonia- and water-based silver fluoride treatments (Group 1–4) following the according to the manufacturer instructions (Table 1), demineralized dentin beams without any silver fluoride treatment served as control group (n = 4/group). Group 5, where only KI was applied on dentin beams was not prepared for SEM analysis, because KI application alone is not used in clinical practice. Half of the specimens were evaluated for the surface view of the dentine after silver fluoride treatments, the other half were fractured using liquid nitrogen to obtain a cross-sectional view. Specimens were dehydrated in ascending series of ethanol (25, 50, 75, 95 and 100 %), fixed in HMDS (Sigma Aldrich, USA) [30], mounted on aluminum stubs, sputter coated with gold-palladium and analyzed by SEM (Phenom ProX, Phenom-World, USA) on backscattering mode at 10 kV up to 2500 × magnifications.

2.9. Evaluation of gelatinase activity by in situ zymography

Ten teeth were coronally sectioned under water-cooling to expose flat midcoronal dentin surfaces using low speed diamond saw (Isomet 1000 Precision Saw, Buehler Ltd, USA). Absence of remaining enamel was verified with a stereo microscope (Leica M60, Leica Microsystems, Germany) at 40 × magnification. Smear layer standardization was performed by wet-polishing exposed dentin surfaces with 320-grit silicon carbide (SiC) paper (CarbiMet, Buehler Ltd, USA) for 60 s at 350 rpm (MetaServ 250 Grinder-Polish, Buehler Ltd, USA). Bonding was followed the etch-and-rinse protocol, where dentin surfaces were etched with 37 % phosphoric acid gel (Scotchbond Universal Etchant, Solventum, USA) for 5 s, rinsed with water for 15 s and blot dried with absorbent paper. Ammonia- and water-based silver fluoride treatments (Group 1–4) were applied as in the SEM analysis according to the manufacturer instructions (Table 1) (n = 2/group) after etching step, before adhesive application. The etched dentin without any silver fluoride treatment served as control group. Group 5, where only KI was applied on dentin beams was not prepared for *in situ* zymography evaluation as KI application alone is not used in clinical practice. Active adhesive application (Scotchbond Universal Plus, Solventum, USA) for 20 s, gentle solvent evaporation for 10 s and light curing with a LED light curing unit (Valo Corded, Ultradent, USA) at 1800 mW/cm^2 for 10 s were achieved. Composite blocks were built with a nanofilled composite resin (Filtek Ultimate Universal Restorative, Solventum, USA) in one 2-mm increment and was light cured for 20 s. All bonding procedures were carried out by a single operator.

Bonded specimens were then cut vertically into 0.3 mm thick slabs to expose adhesive/dentin interfaces, wet-polished with 600, 1200 and 2000 SiC paper and ultrasonically cleaned for 5 min after the polishing steps. The four middle specimens were selected for evaluation. 0.3 mm-thick sections were demineralized with 10 % phosphoric acid for 10 s and rinsed with distilled water for 20 s following blot-drying with absorbent paper. A self-quenched fluorescein-conjugated gelatin (E-12055, Molecular Probes, Eugene, USA) from a stock solution of DQ-gelatin (DQ-gelatin, E12055; Molecular Probes, Eugene, USA) were diluted 1:8 in the dilution buffer (NaCl 150 mM, CaCl2 5 mM, Tris-HCl 50 mM, pH 8.0) as described previously [31]. Specimens were wetted with 30 µl of gelatin solution with and without fluorescent gel, to prevent false readings, and then covered with a coverslip. The slides were light protected and incubated in humified chamber at 37 °C for 24 h, interfacial endogenous gelatinase activity were observed using a confocal laser scanning microscope (Zeiss LSM 880, Carl Zeiss, Germany) at 488 nm for excitation wavelength. The entire resin-dentin interface was examined and sequential images (z-stack) of the bonded interface were recorded from each dentin slice. Then, images were quantitatively assessed using an open-source image software (ImageJ, National Institute of Health, USA) by a single-blinded examiner. Readings obtained with the gelatin solution, without fluorescent gel, were used as background readings. The gelatinolytic activity was expressed as the percentage of the fluorescence area.

2.10. Statistical analysis

The percent loss of dry mass, modulus of elasticity, total enzymatic activity, total endogenous activity, hydroxyproline quantification, total extractable protein and *in situ* zymography data were compared for normality (Shaphiro-Wilk test) and homoscedasticity (Levene test). The normality and equality variance assumptions of the data were valid. *In situ* zymography data were analyzed using one-way ANOVA. Remaining data were analyzed using two-way repeated measures of ANOVA. Post-hoc multiple comparisons were performed with the Tukey test. Significance level (α) of 0.05 was used for all statistical tests. Statistical analyses were performed using IBM SPSS Statistics for Windows, version 28 (IBM Corp, USA).

3. Results

3.1. Loss of dry mass

Fig. 1 shows the cumulative loss of dry mass for demineralized dentin beams among all groups and incubation periods. Untreated demineralized dentin beams served as control group, which showed a significant increase in dry mass loss after 1 months ($p < 0.05$). Treatment with SDF alone exhibited significantly lower dry mass loss at all incubation periods compared to the control ($p < 0.05$), without any significant differences within the group ($p > 0.05$). SF did not significantly differ from the control group at 1-week incubation ($p > 0.05$), however, SF treatments resulted in reduced dry mass loss compared to the control beyond 1 week incubation ($p < 0.05$). There were no significant differences within the SF group at different incubation periods ($p > 0.05$). The additional treatment of KI after either SDF or SF significantly increased the dry mass loss compared to their respective single treatments and the control ($p < 0.05$). The SDF + KI treatment (23.8 ± 4.9 %) showed a higher dry mass loss compared to the SF + KI group (11.1 ± 2.2 %) ($p < 0.05$). Both groups displayed significant differences in dry mass loss from 1 week to 6 months incubation ($p < 0.05$). SF + KI treatment revealed similar results with the control group at 3 and 6 months incubation ($p > 0.05$). Treatment with KI alone resulted in the highest dry mass loss among all groups ($p < 0.05$) yet exhibited no significant change over different incubation periods ($p > 0.05$).

3.2. Modulus of elasticity

The average elastic modulus of demineralized dentin collagen for all specimens at baseline (n = 60) was 7.28 ± 1.23 MPa. Changes in elastic modulus across all groups and incubation periods are shown in Fig. 2. Untreated demineralized dentin beams served as control group and did not exhibit significant changes in elastic modulus throughout the incubation periods ($p > 0.05$). Following silver fluoride treatments, at post treatment time point, all treatment groups, except for the KI treatment group, increased the elastic modulus of demineralized dentin beams compared to their baseline measurements and the control group ($p < 0.05$). The elastic modulus for SDF and SF groups remained significantly higher than the control group across all incubation periods ($p < 0.05$). No significant differences in elastic modulus occurred between the incubation periods within the SDF and SF group ($p > 0.05$). The addition of KI significantly reduced the elastic modulus after 1-week incubation. SDF group produced a 3.2-fold increase in elastic modulus compared to SDF + KI group, and SF group exhibited 2.7-fold increase in elastic modulus compared to SF + KI group. SF + KI group showed comparable elastic modulus with the control group up to 3 months incubation ($p > 0.05$), however, at 6 months incubation, this group revealed significantly lower elastic modulus than the control group ($p < 0.05$). Both SDF + KI and KI groups had lower elastic modulus results than the control starting from 1-week incubation ($p < 0.05$).

3.3. Total enzymatic activity

The results of the generic total MMP activity screening assay are shown in Fig. 3 as the percentage inactivation of total enzymatic activity. Untreated demineralized dentin beams served as control group and revealed zero percent inactivation. All treatment groups demonstrated significant reductions in total MMP activity compared to untreated control across all incubation periods ($p < 0.05$). Following silver fluoride treatments, the ammonia-based silver fluoride treatment groups (SDF and SDF + KI) showed higher MMP inactivation than the water-based silver fluoride treatment groups (SF and SF + KI) at post treatment and 1 week incubation ($p < 0.05$). KI treatment alone revealed the lowest MMP inactivation at all incubation periods ($p < 0.05$). At 1 month incubation, SF treatment showed lower MMP inactivation compared to SF + KI, SDF, SDF + KI groups ($p < 0.05$) and no statistically significant differences were found among the three groups ($p > 0.05$). After 3 months incubation, all treatment groups, except for the KI treatment group, showed significant inactivation of around 95 ± 5 % ($p < 0.05$).

3.4. Solubilized of collagen telopeptides (CTX and ICTP)

Fig. 4 illustrated the release of the telopeptide CTX, produced by cathepsin-K activity in demineralized dentin beams among all groups and incubation periods. Untreated demineralized dentin beams served as control group. All treatment groups revealed higher CTX release compared to the control at all incubation periods ($p < 0.05$). Despite a decrease in CTX release over time for all treatment groups, the untreated control group still showed a cumulative CTX release 2.7-fold lower than SDF group, which exhibited the second lowest cumulative release ($p < 0.05$). After 1-week incubation, CC-mediated CTX release was significantly higher for the SDF + KI, SF + KI and KI groups ($p < 0.05$). After 1 month incubation, the CC-mediated CTX release remained higher

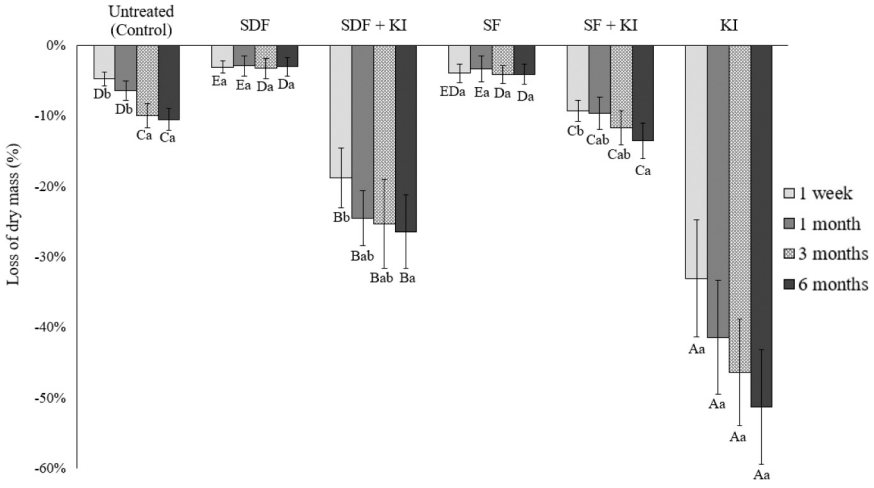

Fig. 1. Cumulative loss of dry mass of demineralized dentin beams submitted to different silver fluoride treatments up to 6 months incubation. Untreated demineralized dentin beams served as control. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

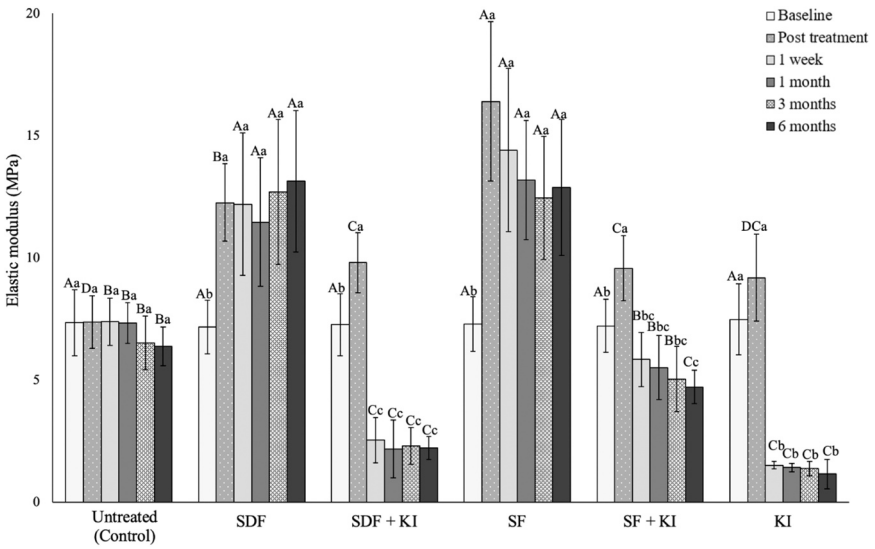

Fig. 2. Elastic modulus means (MPa) and standard deviations of demineralized dentin beams submitted to different silver fluoride treatments up to 6 months incubation. Untreated demineralized dentin beams served as control. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments, within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

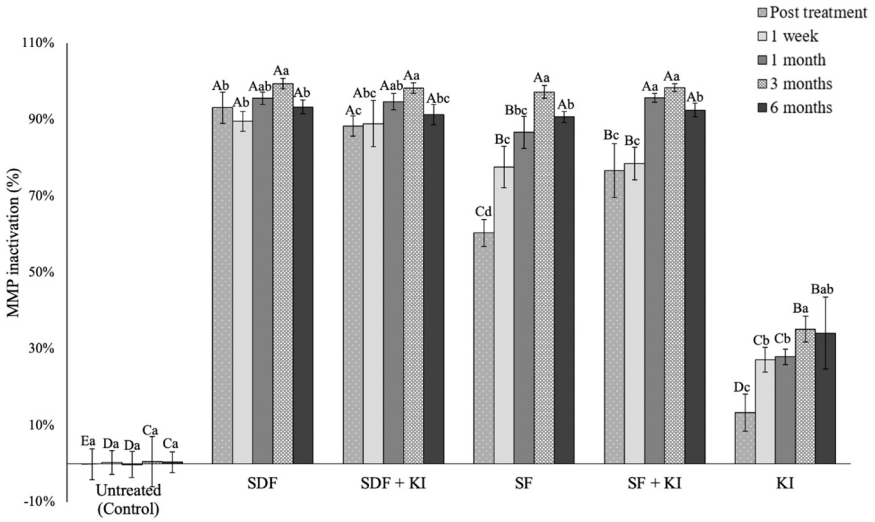

Fig. 3. Total enzymatic activity of dentin MMPs shown as the mean values of percentage inactivation of MMPs of all treatment groups up to 6 months of incubation. Untreated demineralized dentin beams served as control, 0 % MMP inactivation. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

for SDF + KI and KI groups ($p < 0.05$), however, SF + KI group revealed lower CTX release comparable with SF group ($p > 0.05$).

Release of the telopeptide ICTP by the endogenous MMPs for demineralized dentin beams are summarized in Fig. 5. Untreated demineralized dentin beams served as control group and exhibited the lowest MMP-mediated ICTP release, with no significant differences noted compared to KI treatment group ($p > 0.05$). ICTP release decreased in the SDF and SF groups over time ($p < 0.05$); whereas MMP-mediated release increased progressively in SDF + KI and SF+KI treatment groups ($p < 0.05$). MMP-mediated ICTP release from SDF and SF groups up to 1-month incubation showed higher MMP-mediated degradation ($p < 0.05$). Groups combined with KI treatment revealed significantly higher MMP-mediated degradation at 3 months and 6 months incubation periods ($p < 0.05$).

3.5. Hydroxyproline quantification

Hydroxyproline (HYP) release for demineralized dentin beams among all groups and incubation periods is shown in Fig. 6. Untreated demineralized dentin beams served as control group. Analysis of HYP as an indicator of total collagen degradation revealed that SDF + KI, SF + KI and KI groups and SDF and SF groups resulted in 10–20 fold and 2-fold increase in collagen degradation compared to the untreated control at 1 week incubation ($p < 0.05$), respectively. After 1 month of incubation, SDF, SF and KI groups did not significantly affect collagen degradation, yielding results comparable to control group ($p > 0.05$). SDF + KI and SF + KI groups showed higher collagen degradation than SDF, SF and untreated control groups at all incubation periods ($p < 0.05$). At 1-month and 3-month incubations, SF + KI resulted in

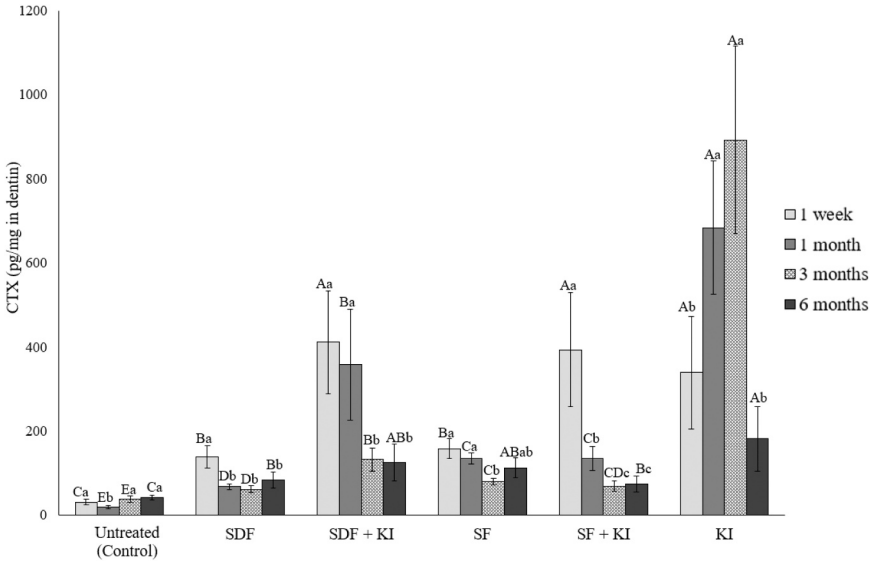

Fig. 4. The rate of release of CTX fragment from demineralized dentin beams submitted to different silver fluoride treatments. Untreated demineralized dentin beams served as control. Aliquots of calcium- and zinc- containing media were analyzed after each incubation period. Values are pg telopeptide/mg dry dentin per unit time. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments, within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

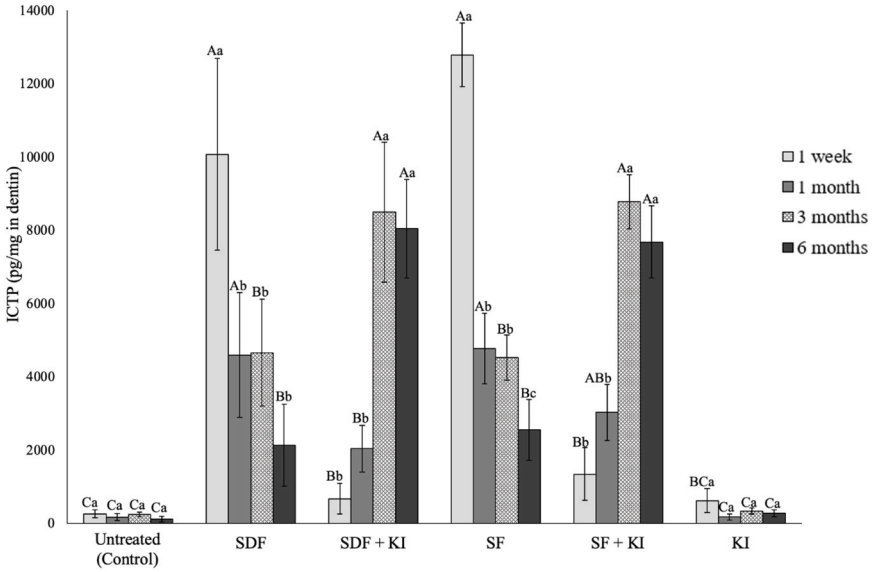

Fig. 5. The rate of release of ICTP fragment from demineralized dentin beams submitted to different silver fluoride treatments. Untreated demineralized dentin beams served as control. Aliquots of calcium- and zinc- containing media were analyzed after each incubation period. Values are pg telopeptide/mg dry dentin per unit time. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments, within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

significantly higher total collagen degradation than SDF + KI group ($p < 0.05$).

3.6. Total extractable protein

Total extractable protein levels for demineralized dentin beams among all groups and incubation periods are shown in Fig. 7. Untreated demineralized dentin beams served as the control group. At 1-week incubation, groups including the KI treatment revealed significantly higher protein levels than SDF, SF and untreated control group ($p < 0.05$). There were no significant differences in extractable protein levels between SDF group and untreated control group ($p > 0.05$), nor between SF group and untreated control group, both of which exhibited protein levels ranging from 2400 to 3000 ng protein/mg in dry dentin ($p > 0.05$). However, there was a significant difference between the SDF and SF groups that SF resulted in higher protein levels compared to SDF ($p < 0.05$). Protein loss decreased in all groups from 1 week to 2 months but then increased from 2 months to 3 months ($p < 0.05$). After 1 week

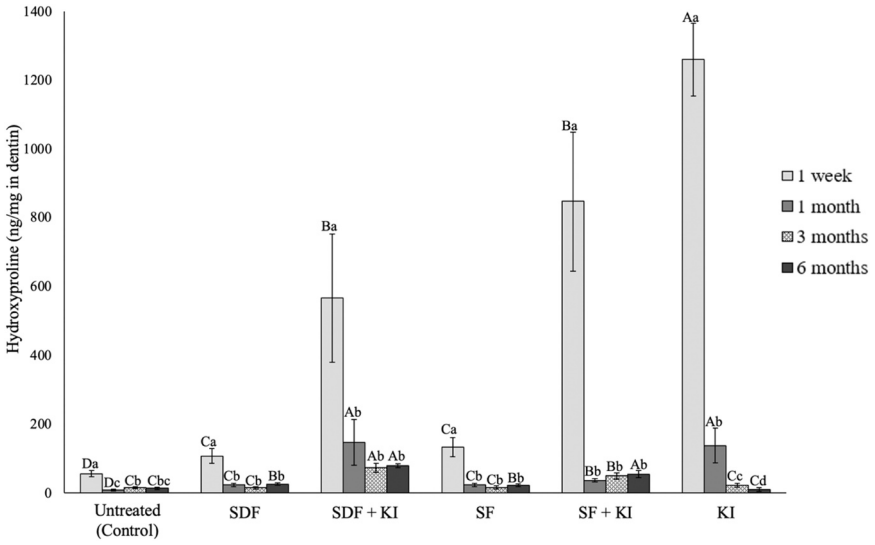

Fig. 6. Hydroxyproline (HYP) levels of demineralized dentin beams submitted to different silver fluoride treatments. Untreated demineralized dentin beams served as control. Aliquots of calcium- and zinc- containing media were analyzed after each incubation period. Values are ng HYP/mg dry dentin per unit time. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments, within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

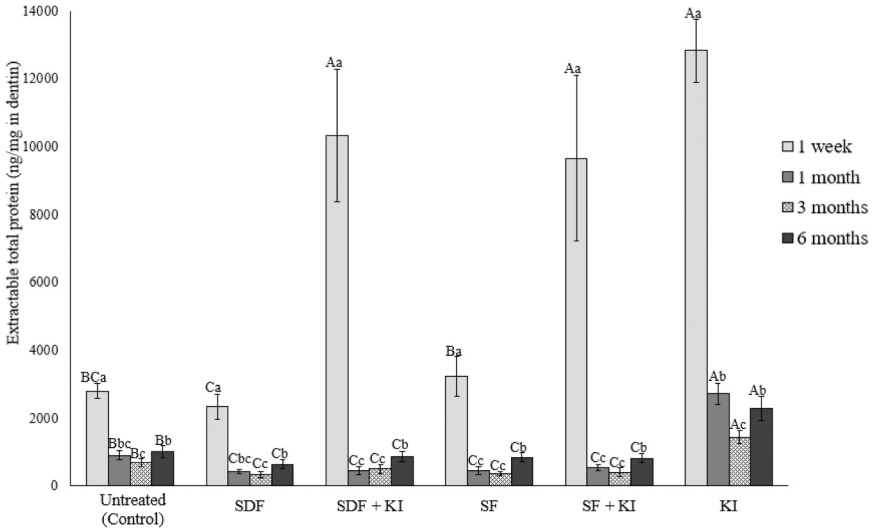

Fig. 7. Extractable protein levels from demineralized dentin beams submitted to different silver fluoride treatments. Untreated demineralized dentin beams served as control. Aliquots of calcium- and zinc- containing media were analyzed after each incubation period. Values are ng total protein/mg dry dentin per unit time. The chart columns represent the mean values and the error bars represent standard deviations (n = 10/group). Different capital letters indicate significant differences between treatments, within incubation periods, according to Tukey test ($p < 0.05$). Different lowercase letters indicate significant differences between incubation times within treatment groups, according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

incubation, ammonia- and water-based silver fluoride treatments revealed lower protein loss compared to control group ($p < 0.05$). KI group revealed higher protein loss than corresponding control groups at all incubation periods ($p < 0.05$).

3.7. SEM analysis

Representative SEM micrographs for demineralized dentin surfaces following ammonia- and water-based silver fluoride treatments to evaluate the surface and the cross sectional view are shown in Fig. 8. H_3PO_4 etching revealed a thick layer of demineralized collagen for both untreated (control) and treated (SDF, SDF + KI, SF, SF + KI) groups (Fig. 8). Untreated demineralized dentin beams presented totally dissolved smear layer, producing clear tubule disobliteration without any precipitations (Fig. 8A, B). Silver precipitates were observed covering most of the surface and within the dentinal tubules after ammonia-based (SDF and SDF + KI) and water-based (SF and SF + KI) silver fluoride treatments (Fig. 8 C, D, E, F, G, H, I, J). Treatments including KI produced precipitates that were more densely packed and extended deeper in the dentinal tubules (Figure E, F, I, J).

3.8. Evaluation of gelatinase activity by in situ zymography

Representative confocal laser scanning microscopy images and the mean results of gelatinolytic activity expressed as the intensity of green fluorescence within hybrid layers (HL) are summarized in Fig. 9. The

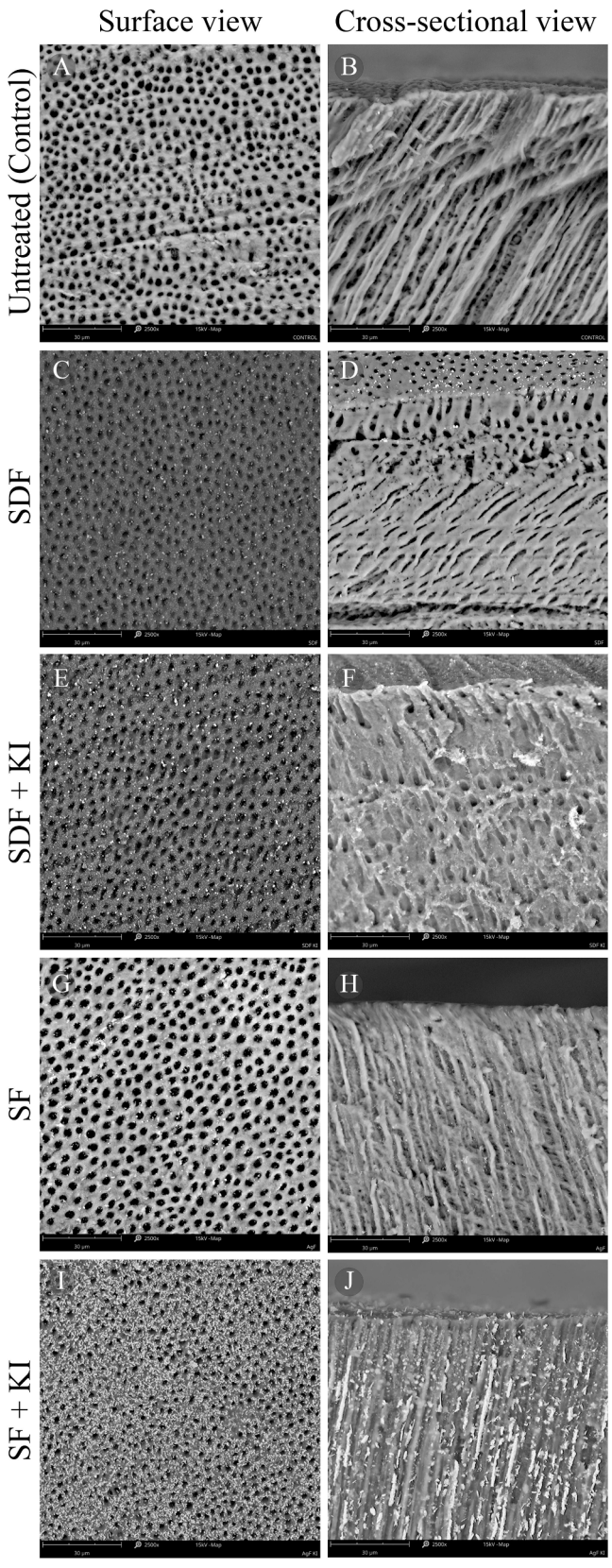

Fig. 8. Representative SEM micrographs showing the surface and cross-sectional views of ammonia- (SDF and SDF + KI) and water- (SF and SF + KI) based silver fluoride treatments. *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride.

untreated 5 s H_3PO_4 .etched group served as control. In all groups, green fluorescence within the HL as well as underlying dentinal tubules were detected. *In situ* zymography images using confocal microscopy revealed a decrease in gelatinase activity at ammonia- and water-based silver fluoride treated groups compared to the untreated control ($p < 0.05$). KI treatment increased the gelatinolytic activity compared to SDF and SF ($p < 0.05$). SF + KI treatment revealed the highest gelatinolytic activity among treatment groups ($p < 0.05$).

4. Discussion

This study focused on the efficacy of ammonia- and water-based silver fluoride treatments in preventing dentin matrix degradation. The null hypothesis was rejected, since ammonia- and water-based silver fluoride treatments affected the activity of dentinal MMPs and CCs, the loss of dry mass, elastic modulus, the degradation of protein and collagen structure in demineralized dentin matrix.

The measurement of dry dentin mass loss and the modulus of elasticity using three-point bending test was selected to evaluate the degradation of demineralized dentin matrices and the mechanical stability of collagen [32], respectively. A decrease in dry dentin mass and modulus of elasticity indicates compromised mechanical properties, primarily due to changes in the collagen structure. These methods allow repeated measurements on the same specimen over time, providing insight into the long-term effects of different treatments [20]. In the current study, dry mass loss and elastic modulus revealed similar trends, silver fluoride formulations and the time elapsed after treatments were critical factors that influence the structural integrity of demineralized dentin. Immediate after treatments, an increase in the elastic modulus of all treated groups were observed, correlating with the deposition of silver particles within dentin tubules (Fig. 8). Among the tested silver fluoride treatments, ammonia-based SDF and water-based SF demonstrated stable dry mass loss and elastic modulus values over the studied period. However, the addition of KI treatment as the second step exacerbated dry mass loss and elastic modulus of the dentin organic matrix. This effect could be explained with denser material deposition on the dentin surface, which extended inside the dentinal tubules (Fig. 8 F; J) and possibly interfere with the collagen matrix. Based on previous studies, there is no consensus regarding ammonia- and water-based silver fluoride treatments depth of penetration into dentin tubules [33]. On the groups included the KI application, visual observations during the experiment revealed increased transparency and signs of structural degradation, such as cracks along the dentin surface, after 1-month incubation period. SF + KI treatment showed better results than the SDF + KI treatment, this could be explained by the differences in water and ammonia content between the solutions, respectively. Clinically, water-based silver fluoride treatments offer benefits such as reduced odor, less irritation and neutral pH, which can enhance both clinician and patient comfort.

A generic MMP Assay kit was used for the detection of total matrix bound MMP activity enables rapid screening of enzymatic activity using a colorimetric assay and allows large number of specimens to be evaluated at the same time. This technique detects the total activity of a variety of MMPs and provides direct information about the efficiency of MMP activation or inactivation [34]. The assay can detect total MMP activity; however, it does not differentiate the specific MMP isoforms. In the current experiment, demineralized dentin beams were used as the MMP source instead of recombinant human MMPs [13], which enhances the clinical relevance of the study. All silver fluoride treatments were effective in inactivating MMP activity, which aligns with the short-term findings of other studies [14]. Consistent with the findings reported by Mei et al. [13] MMP inhibition was observed with 38 % (w/v) silver fluoride concentration [13].

To date, no study has evaluated MMP activity using an MMP assay kit for water-based silver fluoride. In short term, we found that ammonia-based silver fluoride was more effective, which is in agreement with

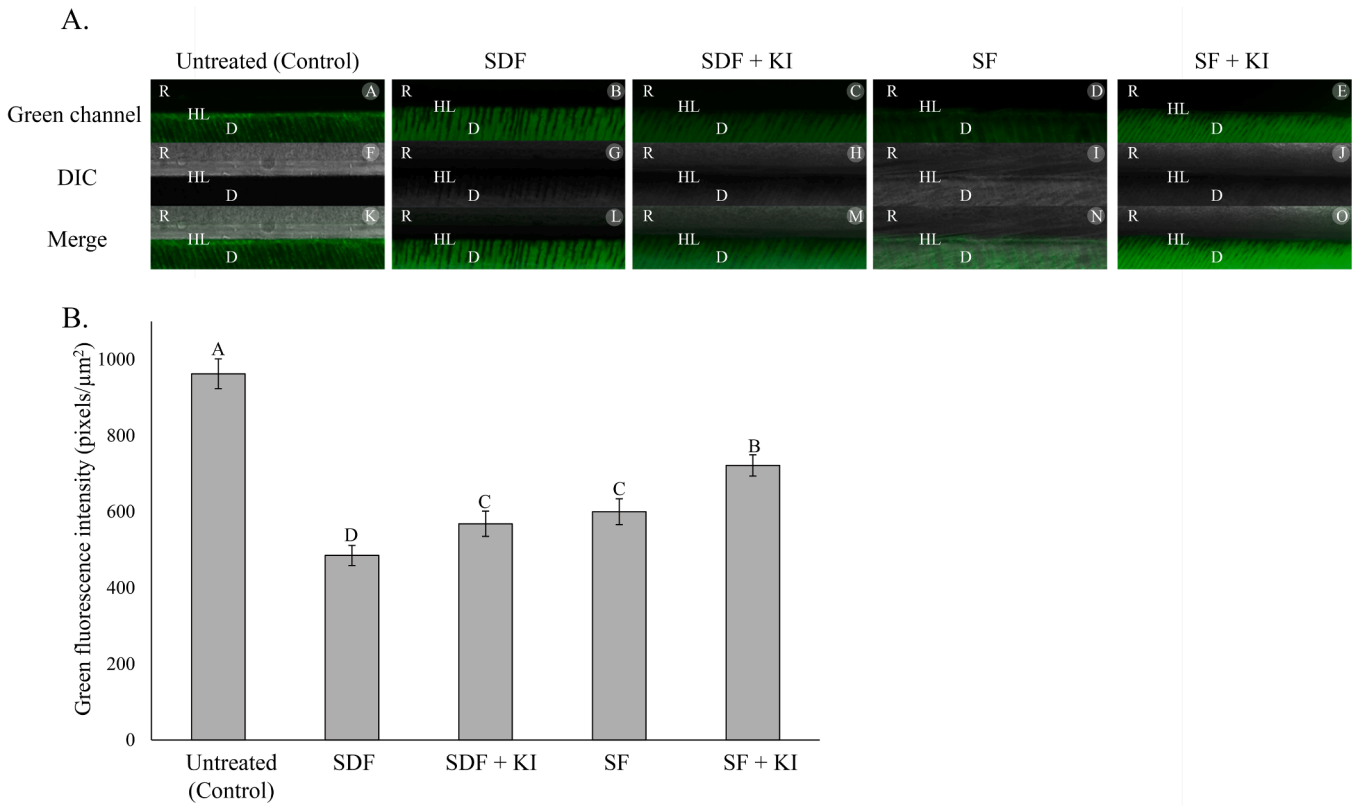

Fig. 9. A. Representative *in situ* zymography images acquired at green channel (A;B;C;D;E), differential interference contrast (DIC) (F;G;H;I;J) and merged channels (K;L;M;N;O) of ammonia- (SDF and SDF + KI) and water- (SF and SF + KI) based silver fluoride treatments. B. Gelatinolytic activity expressed as intensity of green fluorescence (pixels/μm^2) within the hybrid layer. Chart columns represent the mean values and the error bars represent standard deviations of gelatinolytic activity. The untreated group served as control. Different capital letters indicate significant differences between treatments according to Tukey test ($p < 0.05$). *Abbreviations*: SDF = Silver Diamine Fluoride; KI = Potassium Iodide; SF = Aqueous Silver Fluoride; R = Resin composite; HL = Hybrid layer; D = Dentin.

the *in situ* zymography results (Fig. 9) and is consistent with findings from another study [16]. The lower effectiveness of water-based silver fluoride (SF and SF + KI) in inactivating MMP activity compared to ammonia-based silver fluoride (SDF and SDF + KI) could be explained by the higher water content of the water-based formulation [31]. In long-term incubation, our findings confirm that both ammonia- and water-based silver fluoride treatments decreased the total enzymatic activity. Unlike other crosslinking methods which require relatively long application times and therefore clinically impractical [35], silver fluoride treatments are viable alternatives as they can be applied to dentin within minutes while remaining effective in inactivating total enzymatic activity [36,37].

To evaluate collagen degradation, a radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISAs) were used to quantify enzyme-specific degradation products of collagen, specifically C-terminal telopeptide fragments in the incubation media. Both cysteine cathepsins (CCs) and matrix metalloproteinases (MMPs) can cleave type I collagen at distinct sites. MMPs generate ICTP (C-terminal cross-linked telopeptide of type I collagen), whereas cathepsin K produces CTX (C-terminal telopeptide of type I collagen) [3,26,32]. These markers are widely utilized to identify the enzymatic pathways involved in collagen degradation. Notably, KI-treated demineralized dentin beams exhibited an even greater increase in cathepsin K-mediated CTX release during short-term incubation. The KI-treated demineralized dentin beams exhibited lower levels of MMP-mediated ICTP release. An inverse relationship between ICTP and CTX release trends was observed. However, the exact mechanism behind this fluctuation remains unclear, as dentin protease activity is influenced not only by MMPs and CCs [32]. Acid-etching procedures and acidic monomers in dental adhesive techniques expose endogenous dentin proteases, facilitating their activation upon contact with silver fluoride. The commercially available ammonia-based silver fluoride is an alkaline solution (Table 1), which could influence the MMP activity [13]. Since MMPs function optimally at neutral pH, the alkaline pH of ammonia-based silver fluoride may alter their activity. MMPs, a family of zinc- and calcium-dependent endopeptidases present in dentin and saliva, are capable of degrading both native and denatured collagen [19,20], therefore, zinc- and calcium-containing artificial saliva were used as the incubation medium in the current study. Designated incubation periods of 1 week, 1 month, 3 months and 6 months were selected to represent early, intermediate and long-term effect of dentin matrix degradation after silver-fluoride based treatments [18,21,38].

Analysis of hydroxyproline (HYP) as an indicator of total collagen degradation revealed that the amount of released collagen was higher in all groups compared to demineralized dentin during the short-term incubation period. Notably, KI-treated groups exhibited higher collagen release, indicating that KI has a definite effect on collagen degradation. The hydroxyproline assay further revealed that groups treated with ammonia- and water-based silver fluoride combined with KI consistently showed greater total collagen degradation than groups that did not contain KI, a chemical reaction between KI and silver fluoride treatments may be responsible for this effect. Furthermore, the SF + KI group exhibited lower collagen degradation than the SDF + KI group. This trend suggests that the interaction between ammonia and water-based silver fluoride formulations influence collagen degradation.

The Bradford protein assay is one of the most used methods for measuring protein content, first introduced by Bradford in 1976 and has been widely applied to quantify protein concentrations [29]. Bradford assay results showed similar trends observed in elastic modulus and dry mass measurements. KI exhibited excessive protein release, suggesting

that KI contributes to short-term protein degradation before stabilizing over time.

The use of KI as the second step after ammonia- and water-based silver fluoride were found to negatively impact dentin integrity. Specifically, the addition of KI was associated with increased dry mass loss, decreased apparent modulus of elasticity and elevated CC-mediated CTX and MMP-mediated ICTP degradation. Moreover, hydroxyproline and total protein levels indicated that KI treatment accelerated collagen matrix degradation. It has been reported that KI reduces the effectiveness of SDF in arresting or preventing caries [39]. Therefore, the clinical use of KI should be carefully reconsidered, particularly in cases where priority is not esthetics [40]. Moreover, silver fluoride is commonly used in high-caries-risk patients and has demonstrated efficacy in preventing and arresting both early childhood and root caries in the elderly [41,42]. In such cases, esthetic concerns are often secondary [40]. Ammonia-based silver diamine fluoride (SDF) did not cause an immediate color change when applied to demineralized dentin, whereas water-based silver fluoride (SF) resulted in a noticeable yellowish discoloration upon contact with collagen. This discoloration is likely attributed to a chemical interaction between silver ions and exposed collagen, potentially affecting the bonding interface and long-term stability of the material [14,16].

Although KI treatment alone has no direct clinical application, it was included as a separate group to assess its effect on demineralized dentin and to determine whether KI alone contributes to collagen matrix degradation. Our findings revealed that KI treatment alone had a measurable impact on dentin integrity. Specifically, KI significantly increased dry mass loss and total extractable protein levels, suggesting that KI may contribute to dentin dissolution and interfere with protein release. The observed weight loss reflects alterations in both organic and inorganic components of dentin. As an ionic agent, KI interacts with collagen and KI role in modifying dentin composition requires further investigation. Regarding elastic modulus, total enzymatic activity and MMP-mediated ICTP release, KI treatment alone did not appear to have a direct effect. However, KI influenced CTX degradation for up to 6 months and significantly increased CC-mediated CTX release and hydroxyproline release after 1 week of incubation. To our knowledge, no study has evaluated the effect of KI treatment alone on dentin collagen matrix. KI, commonly applied to address tooth's discoloration issues, was not evaluated alone on dentin using scanning electron microscopy or *in situ* zymography. Therefore, future studies should investigate KI treatment independently regarding collagen integrity. Moreover, novel silver-based materials should be developed to address discoloration while preserving the therapeutic benefits of silver fluoride treatments [43,44].

Silver fluoride treatments were initially introduced to the market for the management of dentin hypersensitivity, that explain the precipitation of silver on the dentin surface and within dentinal tubules (Fig. 8). Notably, the SEM specimens were rinsed following silver fluoride treatments, mimicking the clinical scenario where no restorative material is subsequently placed. Furthermore, the high density of silver particles observed within the dentinal tubules in the cross-sectional view can be attributed to the use of fully demineralized dentin beams.

Gelatinolytic activity in hybrid layers using *in situ* zymography was first described by Mazzoni et al. [31]. *In situ* zymography images using confocal microscopy revealed a decrease in gelatinase activity in silver fluoride treatment groups compared to the untreated control group. Moreover, enzymatic activity was higher in water-based silver fluoride treatment. These findings are in agreement with total endogenous enzymatic activity. The alkaline pH of ammonia-based and the neutral pH of water-based silver fluoride could interfere with the collagen matrix (Table 1). Hence, it is reasonable to assume that lower pH may modify silver-crystals formation on demineralized dentin when compared to higher pH [14]. It can be speculated that the high alkalinity of SDF contributes to the neutralization of the acidity of demineralized dentin, which may reduce endogenous proteinase-related collagen degradation [4,14]. In the present study, only immediate collagenolytic activity was evaluated. However, assessing the enzymatic activity using *in situ* zymography over a longer period would be beneficial, as Alessandro et al. [16] conducted a one-year study and reported comparable findings. Ideally, using the same tooth for all groups would improve consistency. To minimize this limitation, non-fluorescent gels were analyzed to subtract each tooth's intrinsic gelatinolytic activity, thereby enabling more accurate comparison across different teeth.

A material's ability to reduce the degradation of dentin's organic matrix can be indirectly associated with the longevity of composite fillings [45]. Currently, there is no universally accepted clinical protocol for the application of ammonia- and water-based silver fluoride treatments. Depending on the clinical scenario, ammonia- and water-based silver fluoride treatments may either be left without subsequent restorative procedures or be followed by restoration with glass ionomer cement or composite fillings [46]. Considering the compelling evidence that silver fluoride treatments may offer a protective effect on collagen structure, future *in vitro* and *in vivo* studies should focus on evaluating long-term resin bonding outcomes to allow clinicians to balance the potential benefits silver fluoride treatments on dentin integrity and bonding performance. Understanding the mechanisms of silver fluoride-dentin interactions regarding different concentrations, formulations and application protocols remain necessary before future clinical applications.

5. Conclusion

The inhibitory efficacy of ammonia- and water-based silver fluoride treatments on dentin protease activity should not be overestimated. Both ammonia-based and water-based silver fluoride treatments, particularly when combined with potassium iodide, revealed complex effects on dentin protease activity and collagen matrix integrity. Silver fluoride treatments do not necessarily fully inhibit endogenous protease degradation of demineralized collagen; however, their use can provide long-term protection regarding collagen's mechanical properties and overall structural integrity. The use of silver-based cariostatic agents associated to potassium iodine in efforts to prevent collagen degradation should be avoided.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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