A New Approach for Determining the Minimum Concentration of Proanthocyanidin for Preservation of Collagen in H Dentin

Keywords

Collagen
Dentin
Matrix Metalloproteinase
Proanthocyanidin
Maldi-Tof Mass Spectrometry

Authors

Dr. Azadeh Balalaie*
(DDS, MScD)

Dr. Mohammad Bagher Rezvani§
(DDS, MScD)

Dr. Mahshid Mohammadi Basir§
(DDS, MScD)

Dr. Hasan Rezadoost^
(PhD)

Prof. Saeed Balalaie†
(PhD)

Address for Correspondence

Dr. Azadeh Balalaie

Email: azadehbalalaie1@gmail.com

* *Assistant Professor, Department of Restorative Dentistry, Dental Faculty, Shahed University*

§ *Associate Professor, Department of Restorative Dentistry, Dental Faculty, Shahed University*

^ *Assistant Professor, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University*

† *Professor, Chemistry Department, K.N.Toosi University of Technology*

Received: 15.01.2019
Accepted: 10.07.2019

doi: 10.1922/EJPRD_01904Balalaie10

ABSTRACT

Purpose: Introducing a minimum concentration and clinically relevant application time for grape seed extract (GSE) proanthocyanidin as a dentin preserver. Materials and Methods: Dentin beams were demineralized in 10% phosphoric acid for 24h. Then, the following groups were prepared: G1: no treatment, G2: 2% CHX + 1min, G3: 1% GSE + 1min, G4: 1% GSE +5min, G5: 2% GSE + 1min, G6: 2% GSE + 5min, G7: 5% GSE + 1min and G8: 5% GSE + 5min. After 30 days, MALDI-TOF mass spectrometry was used to confirm the availability of digested peptide fragments and monitor the pattern of collagen digestion. Gravimetric assessment and HPLC-UV were utilized for quantitative measurement of dentin destruction. Glycine quantities were considered as measures of collagen digestion. Results: 7% to 25% loss of dry mass was measured in experimental groups. Regarding the liberated Glycine, GSE dose- and time-responses were observed, so that, 5% GSE showed considerable protecting effect on collagen compared to 1 and 2% GSE (P<0.001) and 5min GSE application could establish superior dentin preservation compared to 1min application (P<0.001). Conclusion: 5-minute treatment of dentin at 2% GSE and above is essential for protecting the demineralized dentin collagen against biodegradation.

INTRODUCTION

Since their introduction, the bond strength of dentin adhesives are known to deteriorate over time. In fact, apart from collagen and resin as desirable constituents of the hybrid layer, remnants of hydroxyapatite crystals, solvents and water are also present.[1] Besides, regardless of the used adhesive system, the dentin surface is not often completely hybridized and in consequence, some available collagen fibrils available in the hybrid layer will remain devoid of resin encapsulation.[2] Unfortunately, this resultant suboptimal resin-dentin inter-diffusion zone is susceptible to deterioration over time, as evidenced by TEM analysis that showed 70% of available collagen within the adhesive interface will degrade after 44 months of water storage.[3] Both resin elution via nano-sized channels in the hybrid layer and enzymatic attack of the denuded collagen fibrils are known to be responsible for this phenomenon.[4,5] MMPs (matrix metalloproteinases) are zinc and calcium dependent endopeptidases trapped

within mineralized dentin during tooth development and have specific roles in development and remodeling of dentin.[6] Most MMPs are synthetized and released from odontoblasts as pro-enzymes which must be activated for their function.[7] Studies have shown that MMPs can attenuate or remove exposed collagen fibers in an aged hybrid layer and therefore, result in a significant loss of bond strength ranging between 36-70% in 12 and 14 months, respectively.[8,9] Chlorhexidine (CHX) was introduced as an inhibitor of MMP-2, -8 and -9[10] which is able to inhibit MMPs by binding calcium and zinc ions that are imperative for the activity of these enzymes.[11] However, owing to the electrostatic bonding nature of CHX to different substrates, it may be prone to desorption from demineralized collagen. Furthermore, large and water-soluble CHX molecules may leach out of the hybrid layer.[12] Also, low concentrations of CHX have been shown to have a mild cytotoxic transdentinal effect on odontoblast-like cells.[13] Up to now, despite all of these aforementioned shortcomings, CHX has been recommended for use as an effective guard for hybrid layer degradation. On the other hand, utilizing some exogenous natural cross-linkers can also enhance the mechanical properties of collagen matrix and simultaneously it can diminish the enzymatic degradation due to MMPs without any cytotoxic effect.[14] As stated in our recent review article, Proanthocyanidin (PA) has a dual function as both a natural biocompatible cross-linker and a non-specific MMP-inhibitor. Moreover, preconditioning of dentin with PA-incorporated primers does not interfere with resin polymerization and creates more stable bonding properties over time. In this regard, GSE (grape seed extract) PA has demonstrated the most satisfying results.[15] However, the majority of studies have shown that PA-incorporated preconditioners may only exert their beneficial effects following relatively long application times which may not be clinically feasible.[16,17]

This study has followed three main objectives. First, utilizing MALDI-TOF MS (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) to confirm the applied method of measuring collagen degradation and to characterize the degradation pattern of human dentin collagen. Second, make use of both the gravimetric method and HPLC (High Performance Liquid Chromatography) for quantitative measurement of degraded collagen. Finally, introducing an optimal concentration and a clinically relevant application time for utilization of GSE PA as a dentin preconditioner. The null hypothesis tested was that the minimum GSE PA concentration and application time, i.e. 1min application of 1% GSE PA, cannot protect the dentin collagen from biodegradation.

MATERIALS AND METHODS

PREPARATION OF DEMINERALIZED DENTIN

Eighty extracted sound human third molars were obtained from 18- to 25-year-old patients under a protocol approved by the ethics committee of Shahed University (REC.1395.55) and written informed consent was granted from all subjects. The teeth were stored at 4ºC in 9.6% (w/v) NaCl containing 0.02% sodium azide (NaN_3) to inhibit bacterial growth. The enamel and superficial dentin of each tooth were removed by an ISOMET saw under water cooling. Dentin beams with dimensions of 6mm× 2mm× 1mm were sectioned from mid-coronal dentin (80 beams). The beams were demineralized in 10% H_3PO_4 (pH =1) for 24 hours at 25°C. Then, beams were washed thoroughly and digital radiography was used to approve complete mineral loss. Demineralized beams were dehydrated in the vicinity of anhydrous calcium chloride salt for 24 hours in a sealed container. The initial dry mass was measured by a digital analytical balance (XP6 Microbalance). The beams were rehydrated in deionized water for 1 hour and according to the planned dentin treatments, randomly assigned to one of the 8 groups (N = 10) as follows: G1: no treatment (control), G2: 2% CHX + 1 min, G3: 1% GSE + 1 min, G4: 1% GSE + 5 min, G5: 2% GSE + 1 min, G6: 2% GSE + 5 min, G7: 5% GSE + 1 min and G8: 5% GSE + 5 min. In the CHX group, dentin beams were treated with 2% chlorhexidine gluconate (Consepsis), and blot dried without any additional washing step. The experimental groups, 1, 2 and 5% of GSE PA were solved in ethanol and demineralized dentin samples from each experimental group were dipped into GSE solution according to designated concentration and time period and then washed thoroughly. As GSE PA has a dark brown color, the washing step is essential for removal of the color. In the next step, each beam was dropped into 1 mL of a simulated body fluid (SBF) containing zinc and calcium in a labeled polypropylene tube. SBF was composed of 5 mM HEPES buffer, 2.5 mM $CaCl_2H_2O$, 0.05 mM $ZnCl_2$, 0.3 mM NaN_3 and 4.8 mM $NaHCo_3$. Finally, all the tubes were sealed and incubated in Heidolph incubator shaker machine with a 60 cycles/min shaking movement at 37°C for 30 days.

GRAVIMETRIC ASSESSMENT OF DENTIN COLLAGEN OVER TIME

After the 30-day incubation period, dentin beams were dehydrated again and final dry mass was measured. Lost dry mass percentage was calculated as an indicator of total protease activity.

MALDI-TOF BASED CHARACTERIZATION OF DIGESTED COLLAGEN

After 30-day incubation, MALDI-TOF MS was used to analyze SBF samples. Before MALDI-TOF analysis, SBF solution were desalted by passing through C18 Zip-Tip reverse phase chromatography pipette tip (Millipore, Bedford, USA) according to the manufacturer's instructions. In order to eliminate the interference related to the signals of matrix molecules, a spot containing only α-cyano-4-hydroxycinnamic acid was analyzed simultaneously, so that samples were spotted on MALDI plate mixed with an equal volume of matrix solution of α-cyano-4-hydroxycinnamic acid in 50% Acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). After air drying, the samples were analyzed with MALDI-TOF MS operated in reflector positive mode. Thereafter, the data were collected in an automated fashion using random sampling over the sample spot to minimize the effects of operator bias and then interpreted and processed using Data Explorer software version 4.0.

QUANTITATIVE DETERMINATION OF DIGESTED COLLAGEN

After 30-day storage and detection of the presence of peptide fragments in the SBF via MALDI-TOF MS, liquid phase hydrolysis was performed to break the peptide bonds of peptide fragments and convert them to their structural amino acids detectable by HPLC. For the hydrolysis process, 1 ml of available SBF in each polypropylene tube was poured into a hydrolysis vial, and freeze dried. 10 mg phenol was added to the resultant powder and the vial was capped under vacuum condition. Then, 1 mL of 6M Hydrochloric acid was injected through the septum. Eventually, the samples were hydrolyzed at 110°C for 60 hours. For HPLC-UV analysis, 1 ml water was added to the vial containing the hydrolyzed sample. 0.4 ml of the sample diluent (triethylamine) was poured into the shaking vial and allowed to dissolve for 15 minutes. Afterward, 30 µl water, 30 µl triethylamine, 200 µl ethanol and 30µl phenyl isothiocyanate were added and shaken for 15 minutes. The sample was filtrated through a 0.22 µm nylon filter and injected to the HPLC system equipped with KNAUER UV detector.

Separation was performed on a C18 column (5 µm, 250×4.6 mm, Agilent TC-C18). The injection volume was 20 µL and UV detection was performed at 254 nm. The column was eluted with a linear gradient at a flow rate of 1 mL/min using 3 mobile phases of A, B and C. Solvent A was made by dissolving 5.6 ml triethylamine (TEA) in 0.5-liter water and pH was adjusted to 6.8 with orthophosphoric acid. Solvent B is a mixture of 70 volumes of HPLC grade acetonitrile and 30 volumes of Mobile Phase A. Solvent C was HPLC grade acetonitrile. Identifications were carried out using comparison of retention times of the samples with standard compounds. The retention time of Glycine was approximately 17 min. According to the external Glycine calibration curve (0.5-3 mg/L), Glycine quantities available in experimental samples were calculated via the equation of the resultant line (y = 153,095.2016x + 285.3191, R^2=0.9977).

For better illustration of the experimental design, a flow chart depicting the methodological steps is shown in Figure 1.

STATISTICAL ANALYSES

Sample size calculation was performed for 8 experimental groups considering first type error (Alpha=0.05) and the corresponding statistical power (1-Betta=0.80) via NCSS-PASS 2015 software.

The percentage of lost dry mass and liberated Glycine from all groups was tested for normality (Kolmogorov-Smirnov test) and homogeneity of variance (Leven's test). For lost dry mass, the normality and equality variance assumption of the data were valid and it was analyzed using One-way ANOVA. Post hoc multiple-comparisons were performed with the Tukey's test at α=0.05. However, for liberated Glycine, the homogeneity assumptions of the variance were violated, so Glycine quantities were analyzed with Kruskal-Wallis one-way ANOVA and Dunn's multiple-comparison tests at α=0.05 using IBM SPSS v.24. Besides, Two-way ANOVA was performed just for GSE groups, to analyze the effect of two parameters (PA concentration and time) on lost dry mass and liberated Glycine. The correlation between lost dry mass and liberated Glycine were tested by Pearson correlation coefficient and Bland-Altman plot. The level of significance was set at 5%.

RESULTS

LOST DRY MASS

Over the 30-day incubation period, GSE treated dentin samples showed 7% to 25% decrease in mean dry mass, which was significant compared to the 36% decrease in the control group ($p<0.05$), although GSE 1%-1min, did not show any significant difference compared with controls ($p=0.06$). CHX group had a weight loss of 13% which was significantly lower compared with that of the PA1%-1min and control group ($p=0.03$, 0.00). Among GSE groups, 5% GSE exhibited significantly higher dry mass compared with 1 and 2% GSE ($p<0.001$), whilst no significant difference regarding lost dry mass percentage was observed between 1 and 2% GSE ($p=0.76$) *(Figure 2, Table 1)*.

MALDI-TOF MASS SPECTROMETRY ANALYSIS

SBF samples were analyzed by MALDI-TOF MS. To determine peptide fragments that resulted from dentin degradation, Figure 3 illustrates the MALDI-TOF spectrum pertaining to an analyzed SBF sample. According to the spectrum, it was assumed that there were several signals (ranging from 146 to 586 m/z) representing both di-and tri-peptide chains. Quasi-molecular ions corresponding to adduct with both proton and sodium were predominant. Signals were recorded at 211.07, 261.05, 345.14 and 336.05 m/z for $[Gly\text{-}Hyp+Na]^+$, $[Gly\text{-}Tyr+Na]^+$, $[Gly\text{-}Arg\text{-}Hyp+H]^+$ and $[Gly\text{-}Pro\text{-}Tyr+Na]^+$, respectively *(Figure 3)*.

LIBERATED GLYCINE QUANTITIES

For each sample, the solubilized collagen was expressed as µg of Glycine/mg of the dry mass of demineralized dentin before incubation. GSE dose- and time-dependent responses were observed with the solubilized peptides, so that the mean liberated Glycine in all three GSE concentrations, was considerably higher (with 1min application) than that compared with 5min application time ($p<0.001$). 5% GSE significantly liberated lower Glycine compared with 1 and 2% GSE ($p<0.001$), and also, 1% GSE released significantly greater Glycine quantities in comparison with 2% GSE in both 1 and 5-minute application time ($p=0.008$).

Mean (SD) value of µg Glycine/mg dentin liberated from control dentin beams was 28.15 (0.80), whereas samples pretreated with various concentrations and times of GSE, liberated between 27.4 (0.30) and 13.38 (3.68) µg Glycine/mg dentin. CHX pretreated dentin liberated 13.47 (0.17) µg Glycine/

ejprd.org - Published by Dennis Barber Journals.

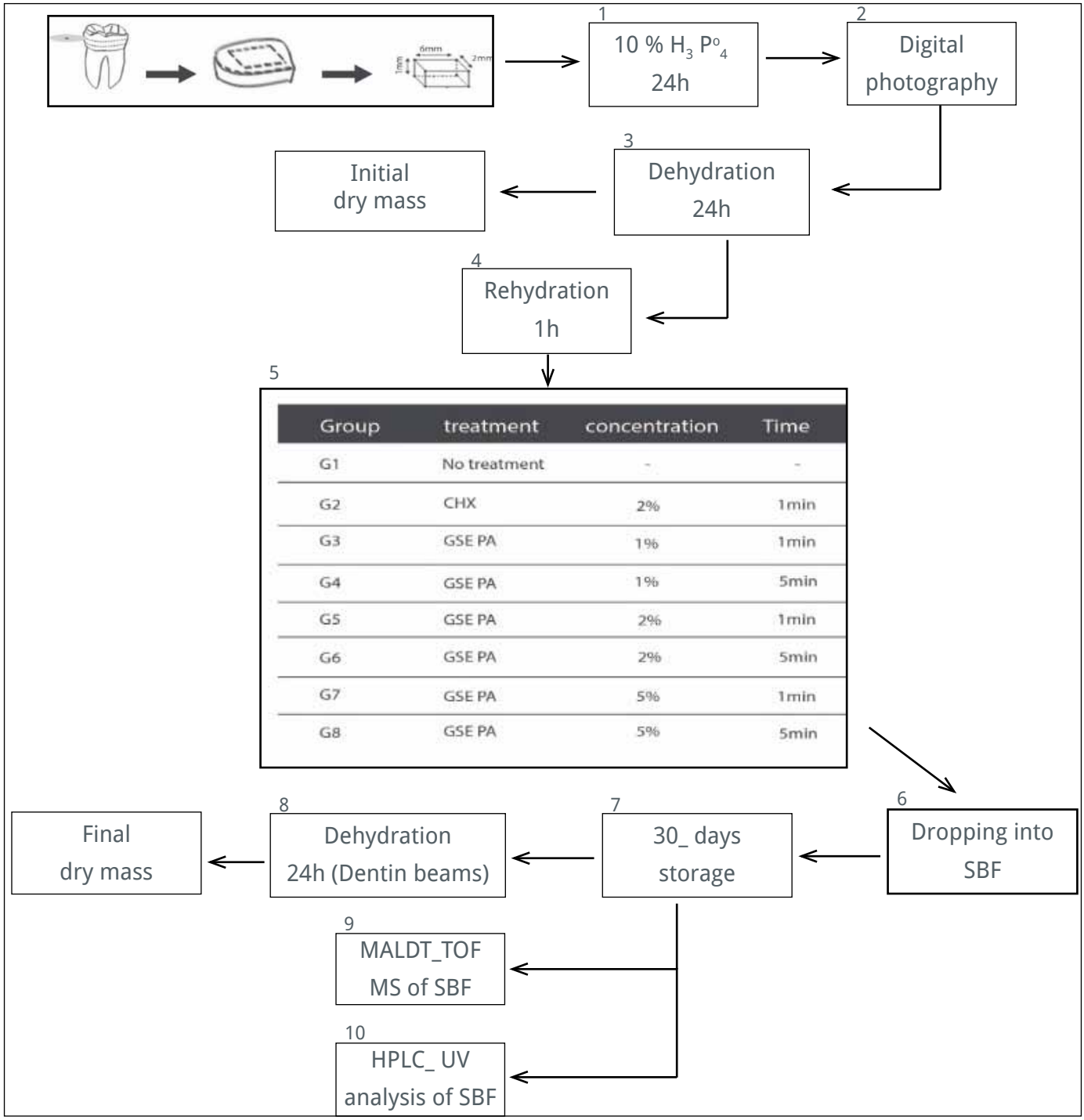

Figure 1: A flow chart illustrating the methodological steps in the current investigation

mg dentin which was significant compared with the control and GSE 1%-1min (p=0.001, 0.005). All GSE groups (1, 2, 5%) with 1min application time could not protect dentinal samples from biodegradation, as no significant difference was observed compared with the control group (p>0.05) *(Figure 4, Table 1)*.

CORRELATION BETWEEN LOST DRY MASS AND LIBERATED GLYCINE

Bland-Altman plot and Pearson correlation coefficient showed a significant correlation (p=0.000) between lost dry mass percentage of dentin beams and the liberated glycine quantities measured in SBF medium (r^2=0.656), as shown in Figure 5.

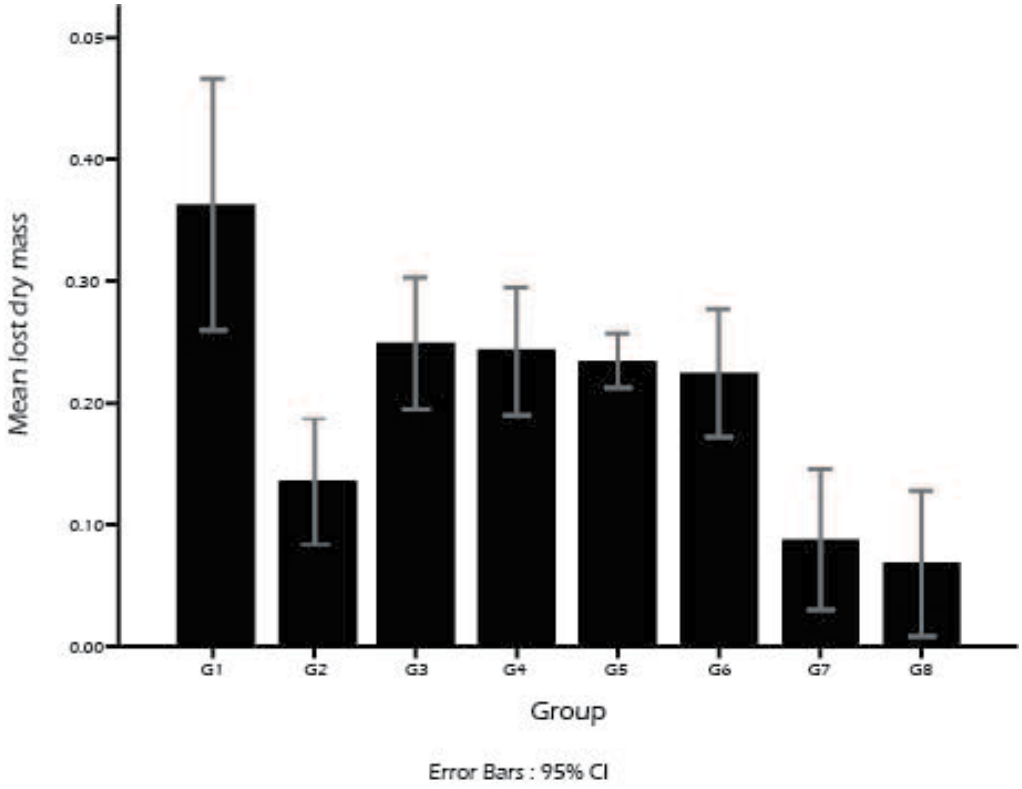

Figure 2: Percent total dry mass loss of completely demineralized human dentin beams after 30 days of incubation. G1 : no treatment (control), G2 : 2% CHX + 1min, G3 : 1% GSE + 1min, G4 : 1% GSE + 5min, G5 : 2% GSE + 1min, G6 : 2% GSE + 5min, G7 : 5% GSE + 1min and G8 : 5% GSE + 5min (n=10)

Table 1. The effect of different treatments on lost dry mass% and µg/mg of liberated Glycine (mean ± SD, n=10)

Group	Lost dry mass%	P–Value*	µg/mg of liberated Glycine	P–Value**
CHX	0.1352 ± 0.07204	0.000	13.4760 ± 0.17573	0.001
1%PA-1	0.2485 ± 0.07524	0.060	27.4040 ± 0.30819	1.000
1%PA-5	0.2421 ± 0.07262	0.038	18.8750 ± 1.06250	0.549
2%PA-1	0.2346 ± 0.03070	0.022	22.6467 ± 2.71063	1.000
2%PA-5	0.2245 ± 0.07123	0.010	16.0060 ± 0.07197	0.036
5%PA-1	0.0870 ± 0.08083	0.000	17.3080 ± 2.86178	0.190
5% PA- 5	0.0679 ± 0.08423	0.000	13.3860 ± 3.68643	0.001
control	0.3619 ± 0.12396	-	28.1520 ± 0.80707	-

*significance of comparison of lost dry mass between treatment and control group based on Bonferroni post-hoc test

** significance of comparison of µg/mg of liberated Glycine difference between treatment and control group based on Dunn post-hoc test

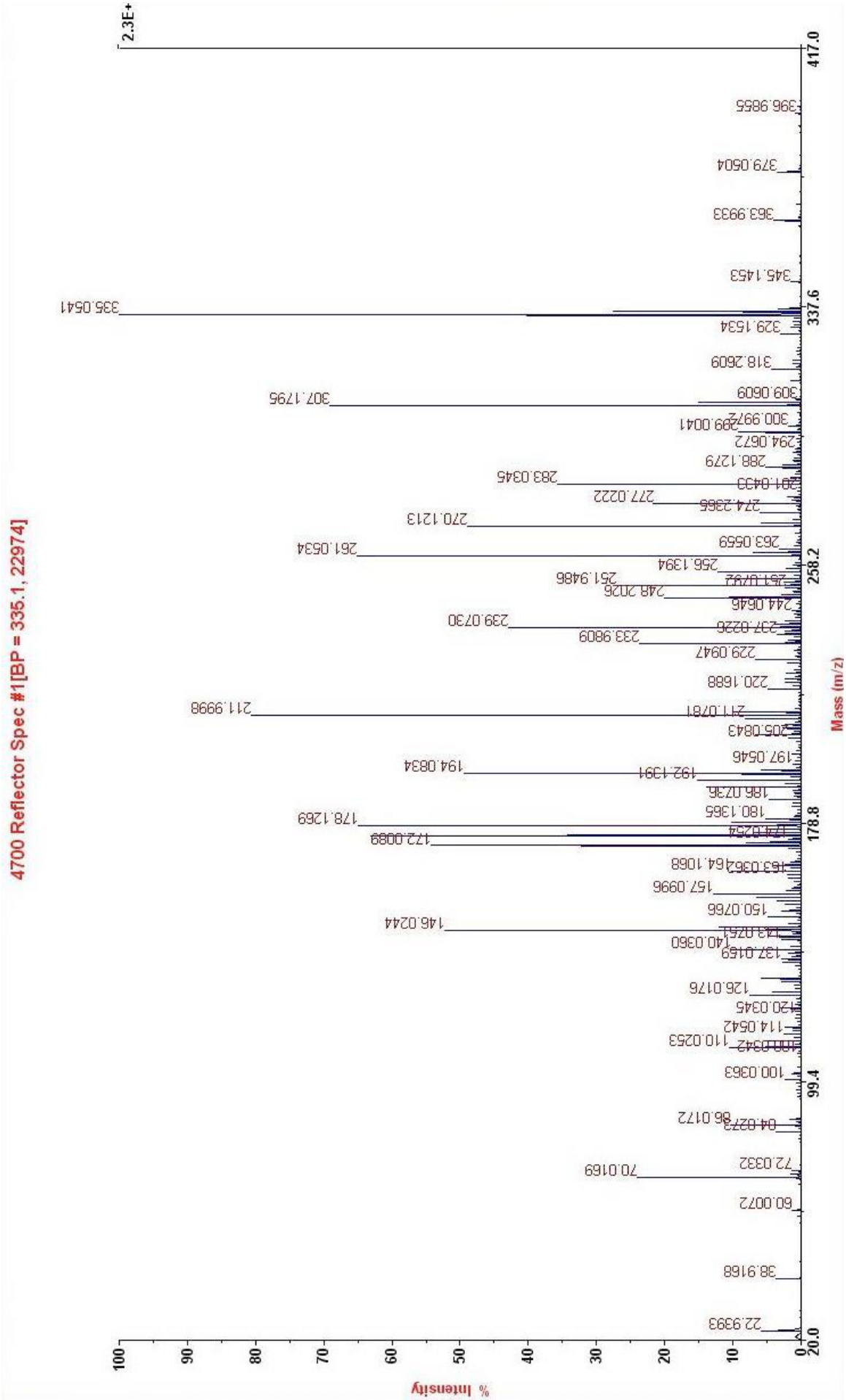

Figure 3: Positive ion MALDI-TOF mass spectra resulted from SBF solution pertaining to control group. Signals recorded at 211.07, 261.05, 345.14 and 336.05 m/z can introduce [Gly-Hyp+Na]$^+$, [Gly-Tyr+Na]$^+$, [Gly-Arg-Hyp+H]$^+$ and [Gly-Pro-Tyr+Na]$^+$ respectively.

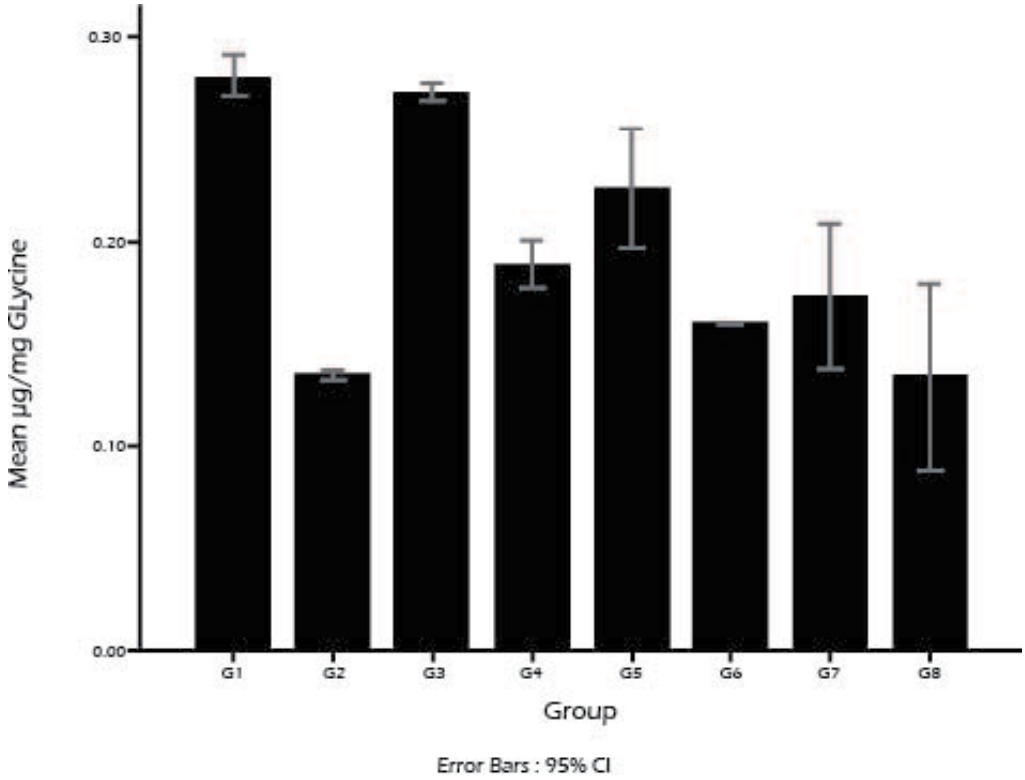

Figure 4: Glycine release from dentin collagen matrices after 30 days of incubation. G1 : no treatment (control), G2 : 2% CHX + 1min, G3 : 1% GSE + 1min, G4 : 1% GSE + 5min, G5 : 2% GSE + 1min, G6 : 2% GSE + 5min, G7 : 5% GSE + 1min and G8 : 5% GSE + 5min (n=10).

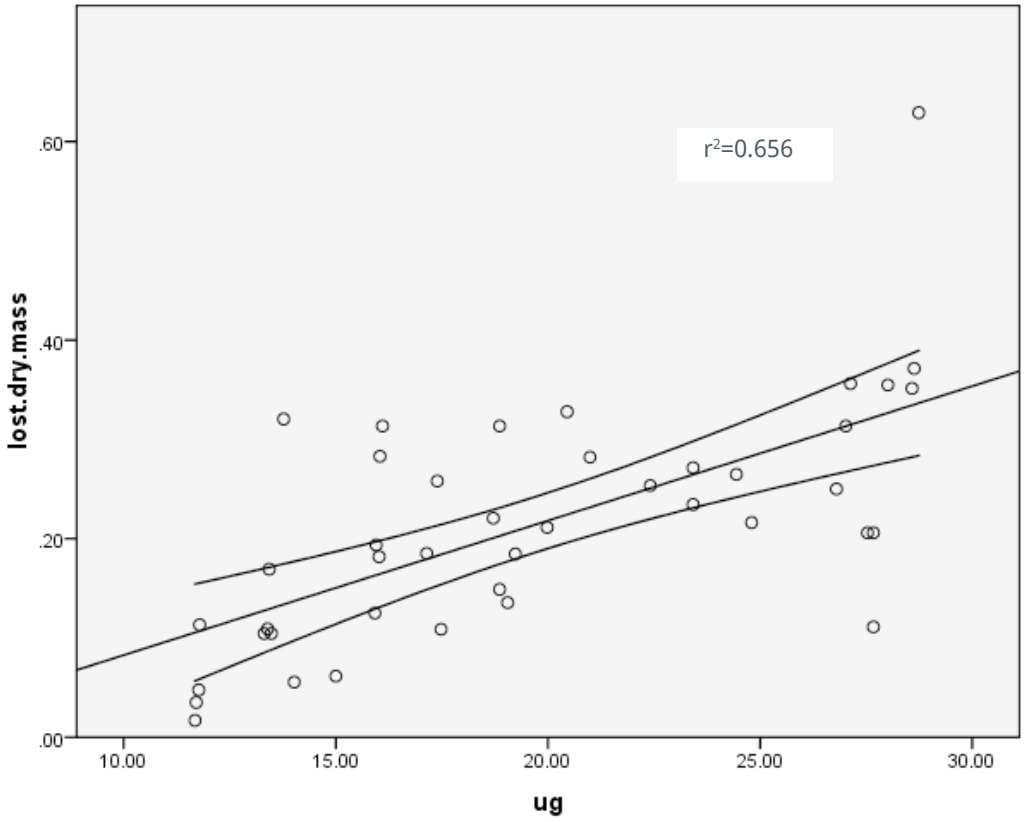

Figure 5: Correlation between lost dry mass and Glycine quantities (µg/mg). scatterplot of lost dry mass against µg.

DISCUSSION

To date, a myriad of studies have revealed the detrimental effect of dentin endopeptidases on the dentin collagen matrix. Type I collagen as a heterotrimeric molecule is composed of one α2 chain and two α1 chains. Both inter- and intra-molecular covalent pyridinium crosslinks are the key factors responsible for stability, strength and viscoelasticity of the dentin collagen matrix.[18] The quantity and type of cross-linking affect collagen thermal stability and resistance to degradation. If the cross-links available in the length of collagen fibrils are cleaved by endogenous proteases, depending on the involved enzyme, dentin collagen will break into two 1/4- and 3/4-length fragments by MMP-8[19] or several smaller peptide pieces by Cathepsin-K.[20] So, inserting additional cross-links between collagen microfibrils via exogenous cross-linkers, not only can improve the mechanical properties of collagen matrix,[21] but it may also ensure long-term stability of the hybrid layer via increasing its collagen matrix resistance against biodegradation.

Chlorhexidine shows desirable MMP-inhibitory properties, however, some of its disadvantages include a reversible electrostatic bond to demineralized dentin, leaching out of large and water soluble molecules from the hybrid layer[12] and trans-dentinal cytotoxic effects on odontoblast-like cells in the areas with minimum dentin thickness,[13, 22] which jeopardize the long-term effect of CHX, and hence, a suitable replacement is sought. Proanthocyanidin, a natural phytochemical agent, has shown positive effects as both a cross-linker and non-specific MMP inhibitor in the biomodification of dentin.[15] In the present study, the impact of various concentrations and treatment times of proanthocyanidin on dentin collagen was assessed and compared with CHX. Based on the proanthocyanidin origin, concentration and treatment time, several studies have been previously conducted in laboratory conditions. The majority of previous studies have utilized grape seed extract proanthocyanidin, with its main units as flavan-3-ol units, i.e. Catechin (C), Epicatechin (EC), Catechingallate (CG) and Epicatechingallate (ECG) which show extraordinary physical properties, reactivity and interactivity with collagen.[15] Regarding treatment time, longer application times (1-72 hours) definitely showed the beneficial impact of PA on preservation of dentin collagen against enzymatic digestion,[16,17] unfortunately, these time periods are not practicable in clinic.

Higher PA concentrations, on one hand, have a greater percentage of flavan-3-ol units responsible for protecting the collagen fibrils, but on the other hand have an intensified dark color which are not desirable beneath a tooth-colored restoration. In this study, both low concentration and low application time were combined to test their probable outcome in dentin protection.

It has been illustrated that 24h storage of dentin in 10% phosphoric acid can cause maximum activation of dentin MMPs,[23] hence, this approach was utilized to reconstruct the degeneration conditions mediated by native dentin proteases. Eventually, the applied digestion assay was confirmed, so that the collagen derived peptides were diagnosed in the incubation media via MALDI/TOF MS. A simulated body fluid containing zinc and calcium was utilized in this study, as these ions are crucial for optimal activity of MMPs and common use of water as the aging medium may underestimate the hydrolytic activity of dentin endopeptidases.[24]

As hydrogen bonding between phenolic hydroxyl and protein amide carbonyl groups is the main supposed force for PA-collagen interactions, the solvent which is used for preparing a proanthocyanidin solution can affect these interactions. Hansen solubility parameter (δH), which indicates the quantity of hydrogen bonding, is lower in ethanol compared with distilled water, so if ethanol is used as the PA solvent, more hydrogen bonding sites will remain on the proanthocyanidin molecule for interaction with collagen.[25] Also, according to the Hagerman and Klucher report, ethanol can stimulate PA-collagen interactions and protect its stability via diminishing the dielectric constant of the media.[26] Consequently, ethanol was chosen as the solvent of choice in this study to prepare proanthocyanidin–containing preconditioner.

MALDI/TOF MS confirmed the presence of peptide fragments resulting from collagen. Type I collagen is the major protein of intertubular dentin (90%) and Glycine is found at the highest levels in this type of collagen, so that every third amino acid is Glycine[27] Also, looking at the results of MALDI/TOF MS, it seemed that Glycine was extremely abundant in the determined amino acid sequences, so the Glycine quantity was measured and reported as the degradation product of dentin collagen.

For quantitative evaluation of this degradation, UV-vis-spectrophotometry is a common technique utilized in literature for measuring Hydroxyproline amino acids liberated from dentin[28] This method monitors the absorbance of the red chromophore at ~550 nm resulting from peptide chains composed of hydroxyproline. However, this method could have some shortcomings in this study, which was mainly due to its high detection limits. Moreover, the red color exerted by GSE-treated dentin beams in the incubation media could interfere with the absorbance of the red chromophore and the resultant spectral interference would skew the test results. So, to overcome these shortcomings, HPLC-UV was utilized as the separation technique for Glycine. HPLC has some merits versus UV-vis-spectrophotometry, including higher sensitivity and higher validity for detection of quantitative measurements. Also HPLC-UV is devoid of any spectral interferences[29]

According to the lost dry mass results, only a dose-related response was evident; however, results relating to liberated Glycine demonstrated both dose- and time-related responses. Moreover, the patterns of dose-dependency were different in the results of these two methods. Interpreting this inconsistency, several reasons can be considered. First, the inherent faults could be related to dry mass preparation and measurements,

for example, an optimal dehydration of dentin may need a strong vacuum pump and it is likely that calcium chloride salt could not extract all the water molecules through the collagen fibers during two different dehydration processes or one of them (before and after incubation). Second, the lower detection limit relating to the HPLC method, i.e. HPLC can perform a very meticulous estimation of the quantities available in the incubation media. As the last reason, it should be mentioned that after collagen cleavage, the resultant fragments may be cross-linked to adjacent peptides and are not free to solubilize. Also, collagen peptides cleaved in the center of collagen fibrils may be too large to diffuse into the incubation medium[30,31] Thus, solely monitoring loss of dry mass, may underestimate the total degradation. In addition, and according to Bland-Altman plot and Pearson correlation coefficient (r=0.656), a positive correlation between dry mass loss and liberated Glycine was observed.[32] Therefore, the measurement of liberated Glycine quantities seemed more meticulous and rational.

According to the HPLC results for Glycine quantities, 5% GSE PA could exert a considerably higher protecting effect on dentin collagen compared with 1 and 2% GSE PA (p=0.005). Also, compared to 1min application, 5min application can establish superior dentin preservation (p<0.001). 2% GSE with 5 min application time showed a considerable dentin protection in comparison with the control group (p=0.036). However, comparing to CHX results, GSE PA groups showed no significant differences with CHX, except GSE 1%-1min which had a significant difference comparing with CHX (p=0.005). None of the GSE groups (1, 2, 5%) with 1min application time could protect dentinal samples from biodegradation, as no significant difference was observed compared with the control group (p>0.05).

The results of the present work suggest that a saturated incorporation of PA into demineralized dentin collagen beams can be achieved in 5min, on the condition that the treatment solution is at a concentration of 2% or above. However, Liu, *et al*. found that application of 2% or more concentrated PA for 30s was enough to protect the demineralized dentin collagen from degradation[33] Such a quick saturation (30 s) was never reported before and it seems that utilizing ultra-thin demineralized dentin collagen films (6 μm thick) has increased the diffusion rate of PA into samples and is the main reason for this difference.

Boteon, *et al*. utilized profilometry method and showed that 1 min application of 1% GSE PA gel can subside collagen degradation.[34] The result of this study stands in contradiction to the results of the present study. Some probable causes can justify this apposition, including the applied method for quantification of dentin loss, i.e. profilometry. In another study by Boteon, *et al*. they merged profilometry with hydroxyproline assay for quantitative evaluation of dentin loss and the results explained that 1min application of 10% GSE PA could significantly diminish dentin wear.[35] Higher PA concentration and a more meticulous approach have been utilized in this study and it seemed that higher PA concentration (10%) may compensate the effect of lower application time (1 min). In other words, higher PA concentration might accelerate the diffusion rate.

This study confirmed that small amounts of collagen derived di- and tripeptides can be determined by MALDI-TOF MS analysis of dentin degradation. Moreover, this study quantified proanthocyanidin efficacy in protecting collagen against enzymatic degradation and it was shown that the inhibitory effect of GSE PA on dentin endopeptidases is both time- and dose-dependent. It was established that 5-minute application of 2 % GSE PA and above could be indispensable to render the demineralized dentin collagen protection from degradation.

ACKNOWLEDGMENTS

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article.

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article.

MANUFACTURER'S DETAILS

- ISOMET saw (Buehler Ltd., Lake Bluff, IL, USA)
- XP6 Microbalance (Mettler Toledo, Hightstown, USA).
- Consepsis (Ultradent Products, Inc., South Jordan, Utah, USA)
- GSE PA (Santa Cruz biotechnology., Dallas, TX, USA)
- Except the CHX and GSE PA, all other chemicals were purchased from SIGMA-ALDRICH Company (SIGMA-ALDRICH Co, St. Louis, Missouri, United States).
- Heidolph incubator shaker machine (Heidolph Instruments, GmbH & Co .KG, Schwa Bach, Germany)
- MALDI-TOF MS (Applied Biosystems 4800 MALDI TOF/TOF, Framingham, MA, USA)
- KNAUER UV detector (KNAUER Wissenschaftliche Geräte GmbH., Berlin, Germany).

REFERENCES

1. Nakabayashi N, Ashizawa M, Nakamura M. Identification of a resin-dentin hybrid layer in vital human dentin created in vivo: durable bonding to vital dentin. *Quintessence Int (Berl)* 1992;**23**:135–141.
2. Yiu CK, Pashley EL, Hiraishi N. Solvent and water retention in dental adhesive blends after evaporation. *Biomaterials* 2005;**26**:6863–6872.
3. Armstrong SR, Vargas MA, Chung I, *et al*. Resin-dentin interfacial ultrastructure and microtensile dentin bond strength after five-year water storage. *Oper Dent* 2004;**29**:705–712.
4. Sano H. Microtensile testing, nanoleakage, and biodegradation of resin-dentin bonds. *J. Dent. Res* 2006;**85**:11–14.

5. Spencer P, Ye Q, Park J, *et al.* Adhesive/Dentin Interface:the Weak Link in the Composite Restorations. *Ann. Biomed. Eng* 2010;**36**:1989–2003.
6. Visse R1, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;**92**:827–839.
7. Zhang SC, Kern M. The role of host-derived dentinal matrix metalloproteinases in reducing dentin bonding of resin adhesives. *Int J Oral Sci* 2009;**1**:163–176.
8. Carrilho MR, Geraldeli S, Tay F, *et al.* In vivo preservation of the hybrid layer by chlorhexidine. *J Dent Res* 2007;**86**:529–533.
9. Koshiro K, Inoue S, Tanaka T, *et al.* In vivo degradation of resin-dentin bonds produced by a self-etch vs. a total-etch adhesive system. *Eur. J. Oral Sci* 2004;**112**:368–375.
10. Gendron R, Grenier D, Sorsa T, *et al.* Inhibition of the activities of matrix metalloproteinases 2, 8, and 9 by chlorhexidine. *Clin Diagn Lab Immunol* 1999;**6**:437–439.
11. Boushel LW, Swift EJ Jr. Critical Appraisal.Dentin Bonding :Matrix Matalloproteinases and Chlorhexidin. *J Esthet Restor Dent* 2011;**23**:347–352.
12. Kim J, Uchiyama T, Carrilho M, *et al.* Chlorhexidine binding to mineralized versus demineralized dentin powder. *Dent Mater* 2010;**26**:771–778.
13. de Souza LB, de Aquino SG, de Souza PP, *et al.* Cytotoxic effects of different concentrations of chlorhexidine. *Am J Dent* 2007;**20**:400–404.
14. Epasinghe DJ, Yiu CK, Burrow MF, *et al.* The inhibitory effect of proanthocyanidin on soluble and collagen-bound proteases. *J Dent Res* 2013;**41**:832–839.
15. Balalaie A, Mohammad Bagher Rezvani MB, Mohammadi Basir M. Dual function of proanthocyanidins as both MMP inhibitor and crosslinker in dentin biomodifcation: A literature review. *Dent Mater J* 2018;**37**:173-182.
16. Macedo GV, Yamauchi M, Bedran-Russo AK. Effects of chemical cross-linkers on caries-affected dentin bonding. *J Dent Res* 2009;**88**:1096–1100.
17. Walter R, Miguez P.A, Arnold RR, *et al.* Effects of natural cross-linkers on the stability of dentin collagen and the inhibition of root caries *in vitro*. *Caries Res* 2008;**42**:263–268.
18. Kuboki Y, Mechanic GL. Comparative molecular distribution of cross-link in bone and dentin collagen. Structure-function relationships. *Calcif Tissue Int* 1982;**34**:306–308.
19. Sulkala M, Tervahartiala T, Sorsa T, *et al.* Matrix metalloproteinase-8 (MMP-8) is the major collagenase in human dentin. *Arch Oral Biol* 2007;**52**:121–127.
20. Li Z, Hou WS, Escalante-Torres CR, *et al.* Collagenase activity of cathepsin K depends on complex formation with chondroitin sulfate., *J Biol Chem.* 227 (2002) 28669–28676.
21. Castellan CS, Pereira PN, Viana G, *et al.* Solubility study of phytochemical cross-linking agents on dentin stiffness. *J Dent* 2011;**38**: 431–436.
22. Lessa FC, Nogueria I, Huck C, *et al.* Transdentinal cytotoxic effects of different concentrations of chlorhexidine gel applied on acid-conditioned dentin substrate., *J. Biomed. Mater. Res.* Part B Appl. *Biomater* 2010;**92**:40–47.
23. Tezvergil-Mutluay A, Seseogullari-Dirihan R, Mutluay M, *et al.* The effect of demineralization duration and concentration on dentin protease activity, in: AADR Annu. Meet. Exhib 2014.
24. Tezvergil-Mutluay A, Agee KA, Hoshika T, *et al.* The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices. *Dent Mater* 2010;**26**:1059–1067.
25. Nalla RK, Balooch M, Ager JW, *et al.* Effects of polar solvents on the fracture resistance of dentin: role of water hydration. *Acta Biomater* 2005;**1**:31–43.
26. Hagerman AE, Klucher KM. Tannin-protein interactions. P*rog Clin Biol Res* 1986;**213**:67–76.
27. Weiner S, Veis A, Beniash E, *et al.* Peritubular dentin formation: crystal organization and the macromolecular constituents in human teeth. *J Struct Biol* 1999;**126**:27–41.
28. Jamall IS, Finelli VN, Que Hee SS. A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal Biochem.* 1981;**112**:70–75.
29. Kabiri M, Rezadoost H, Ghassempour A. A comparative quality study of saffron constituents through HPLC and HPTLC methods followed by isolation of crocins and picrocrocin. *LWT - Food Science and Technolog* 2017;**84**:1-9.
30. Takahashi M, Nakajima M, Tagami J, *et al.* The importance of size-exclusion characteristics of type I collagen in bonding to dentin matrices. *Acta Biomater* 2013;**9**:9522–9528.
31. Toroian D, Lim JE, Price PA. The size exclusion characteristics of type I collagen: implications for the role of noncollagenous bone constituents in mineralization. *J Biol Chem* 2007;**282**:22437–22447.
32. Gail M. Sullivan, Feinn R. Using Effect Size—or Why the P Value Is Not Enough. *J Grad Med Educ* 2012;**4**: 279–282.
33. Yi Liu and Yong Wang. Proanthocyanidin's efficacy in stabilizing dentin collagen against enzymatic degradation: MALDI-TOF and FTIR analyses. *J Dent* 2013; **41**:535-452.
34. Boteon AP, Prakki A, Rabelo Buzalaf MA, *et al.* Effect of different concentrations and application times of proanthocyanidin gels on dentin erosin. *Am J Dent* 2017;**30**:96-100.
35. Boteon AP, Kato MT, Buzalaf MAR, *et al.* Effect of proanthocyanidin-enriched extracts on the inhibition of wear and degradation of dentin demineralized organic matrix. *Arch Oral Biol* 2017;**84**:118-124.