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***In Vitro* Remineralization Effects of Grape Seed Extract on Artificial Root Caries**

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Abstract

Grape seed extract (GSE) contains Proanthocyanidin (PA), which has been reported to strengthen collagen-based tissues by increasing collagen cross-links. We used an *in vitro* pH-cycling model to evaluate the effect of GSE on the remineralization of artificial root caries. Sound human teeth fragments obtained from the cervical portion of the root were stored in a demineralization solution for 96 hr at 37°C to induce artificial root caries lesions. The fragments were then divided into three treatment groups including: 6.5% GSE, 1,000 ppm fluoride (NaF), and a control (no treatment). The demineralized samples were pH-cycled through treatment solutions, acidic buffer and neutral buffer for 8 days at 6 cycles per day. The samples were subsequently evaluated using a microhardness tester; polarized light microscopy (PLM) and confocal laser scanning microscopy (CLSM). Data were analyzed using ANOVA and Fisher's tests ($p < 0.05$). GSE and fluoride significantly increased the microhardness of the lesions ($p < 0.05$) when compared to a control group. PLM data revealed a significantly thicker mineral precipitation band on the surface layer of the GSE treated lesions when compared to the other groups ($p > 0.05$), which was confirmed by CLSM. We concluded that grape seed extract positively affects the demineralization and/or remineralization processes of artificial root caries lesions, most likely through a different mechanism than that of Fluoride. Grape seed extract may be a promising natural agent for non-invasive root caries therapy.

Introduction

Approximately 8% of the population are expected to acquire one or more new root caries lesions yearly in North America (1). Root caries is especially prevalent among the elderly population due to gingival recession and exposure of the susceptible root surface (2). During root caries development, two stages are distinguished microscopically. In the first stage, dentin mineral is dissolved by acid produced from the bacterial biofilm. In the second stage, the demineralized dentin matrix is further degraded and bacteria infiltrate the intertubular area (3). It has been suggested that the presence of an organic matrix may reduce the progression of erosion in dentin (4,5). Dentin is a complex mineralized tissue composed (by weight) of approximately 70% mineral, 20% organic component, and 10% fluid (6). Fibrillar type I collagen accounts for 90% of the organic matrix, while the remaining 10% consists of noncollagenous proteins such as

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phosphoproteins and proteoglycans (6,7). The preservation and stability of dentin collagen may be essential during the re-mineralization process since it acts as a scaffold for mineral deposition.

One of the important strategies regarding preventive therapies for root caries is to promote remineralization of demineralized dentin (8–11). Natural products have been used as folk-medicines for thousands of years, and are promising sources for novel therapeutic agents (12). They have been the focus of much recent research as potential agents in the prevention of oral diseases, particularly plaque-related diseases, such as dental caries (13–15). A majority of the studies of natural products in the field of oral health have focused on their antibacterial activities (16,17). Very few reported on the effects of natural products or phytochemicals on the demineralization and remineralization processes of dental hard tissues (18).

Proanthocyanidin (PA) is a naturally occurring plant metabolite widely available in fruits, vegetables, nuts, seeds, flowers and barks (19). As a bioflavonoid, it contains a benzene-pyran-phenolic acid molecular nucleus (referred to as flavin) as part of its much larger molecular structure. PA are a mixture of monomers, oligomers, and polymers of flavan-3-ols (known as catechins), which are ubiquitous in plants. Widely used as natural antioxidants and free-radical scavengers, PAs have been proven to be safe in various clinical applications and as dietary supplements (20,21). PA from grape seed extract (GSE) have been thought to prevent ischemia/reperfusion damage caused by reactive oxygen species such as superoxides and peroxynitrites (22). PA from cranberry inhibited the surface-adsorbed glucosyltransferases and F-ATP activities, and the acid production by *Streptococcus mutans* (23). Studies have also shown that PAs increased collagen synthesis and accelerated the conversion of soluble collagen to insoluble collagen during development (24,25). PA-treated collagen matrices were demonstrated to be nontoxic and resisted enzyme digestion *in vitro* and *in vivo* (26). At present, there have been few studies investigating PA's effect on the remineralization and demineralization of the collagen rich root tissue of human teeth.

The current study tests the hypothesis that grape seed extract, consisting mainly of PA, may positively affect the remineralization of artificial root caries, which offers a potential novel therapy for root caries.

MATERIALS AND METHODS

Materials

The grape seed extract (GSE) used in the present study was purchased from MegaNatural, Polyphenolics, (Madera, CA). It consisted of 97.8% proanthocyanidin (PA) according to data provided by the manufacturer. The PA in the GSE is composed mainly of monomers (Catechins). A 6.5% (w/v) solution in phosphate buffer (0.025 M KH_2PO_4 , 0.025 M K_2HPO_4 , pH 7.4) was used in this study. All GSE solution was prepared from the same lot of extract. The fluoride concentration in the GSE was less than 0.01 ppm as measured by a fluoride electrode (Thermo Scientific Orion Ion-selective Solid State Combination Electrodes, 960900).

Specimen Preparation

Twenty-five extracted sound human third molars were obtained from the College of Dentistry, University of Illinois at Chicago (IRB research protocol # 2006-0229). They were cleaned and the organic contaminants were removed with a scalpel blade. Forty five root fragments (5×5×5 mm) obtained from the cervical portion of the 25 teeth were used in the study. The root fragments were sealed with acid-resistant nail polish (Revlon Corp., NY, USA) except for a 3×4 mm window.

Lesion Formation

The root fragments were placed in a demineralization solution (2.2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.2 mM KH_2PO_4 , 50 mM acetate, pH 4.6) for 96 hr at 37 °C to create lesions of 70 to 100 μm -deep (confirmed by pilot study, data not shown). Following lesion development, the fragments were rinsed thoroughly with deionized water, and half of the specimens' windows were covered with an acid-resistant nail polish (Revlon Corp., NY) to maintain the baseline lesion.

Remineralization Regimen

The demineralized root fragments were randomly divided into 3 groups ($n=15$) based on treatments: 6.5% w/v GSE; 1000 ppm fluoride solution as NaF (Fisher Chemical, US); control (deionized water). All samples were pH-cycled through treatment solutions (10 min), acidic buffer (50 mM acetate; 2.25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 1.35 mM KH_2PO_4 ; 130 mM KCl; pH 5.0; 30 min) and neutral buffer (20 mM HEPES; 2.25 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 1.35 mM KH_2PO_4 ; 130 mM KCl, pH 7.0; 10 min) for 8 days. For *in vitro* pH cycling experiments, 250 ml polystyrene jar were used. All solutions were made freshly daily prior to use. Six cycles per day were performed. Root fragments were kept in a neutral buffer over night. The study design is presented in Fig. 1

Post-treatment analyses

Following pH-cycling, root fragments were rinsed with deionized water for 2 min and sectioned into two halves. One half was embedded perpendicular to the demineralized surface in epoxy resin for microhardness evaluation. The other half was further sectioned and used for polarized light microscopy (PLM) and confocal laser scanning microscopy (CLSM) analysis.

Microhardness test—The embedded samples in epoxy resin were polished on a water-cooled polishing unit (EcoMet 3000, Buehler, Lake Bluff, IL, USA) with abrasive paper (400-, 600- and 1200-grit) followed with 0.9, 0.6, 0.3 and 0.1 μm diamond mask alumina suspensions (Metaldi Supreme, Buehler, Lake Bluff, IL). The polished samples were cleaned ultrasonically in deionized water for 15 min to remove the residues from the polishing procedure. Cross-sectional microhardness measurements perpendicular to the demineralized surface (Leica, Tukon 200, Germany) were performed below the surface at (20 μm , 50 μm , 80 μm , 110 μm , 140 μm , 170 μm and 200 μm) using Knoop hardness indentation (KHN) at a 25 g load force for 15 sec. The measurements were performed at 3 different locations at each depth for all samples.

Polarized Light Microscopy (PLM) and image analysis—Samples were sectioned longitudinally through the lesions with a low speed water-cooled diamond saw (Isomet 1000, Buehler, Lake Bluff, IL, USA) to obtain 300 μm thick sections that were further polished to approximately 100 μm thickness. Each specimen was sectioned perpendicularly to the varnished area so that each section included the varnish-covered, baseline lesion area and the uncovered post-treatment lesion area. Lesion depths and the depth of the remineralized band were quantified using polarized light microscopy (Leica Microsystems GmbH, 020-525.025, Germany) and an image analysis system (Image Pro-Plus version 5.1, Media Cybernetics, Inc., Silver Spring, MD, USA).

Confocal Laser Scanning Microscopy (CLSM)—Following polarized microscopy analysis, the same sample was stained with a freshly prepared 0.1% Rhodamine B solution (Aldrich Chem. Co., Milwaukee, WI, USA) for 1 hr, and rinsed for 3 times with deionized water. Samples were analyzed with a CLSM (Zeiss LSM 510, Carl Zeiss, Inc. Germany), using an argon laser with a 529 nm excitation wavelength. Areas were scanned between 10 to 50 μm below the cut surface to reduce the influence of the smear layer created during the cutting

and polishing procedure. The images of stained post-treatment lesions were quantitatively analyzed for the optical intensity with a image-analysis system (Image Pro-Plus, 5.1). The optical density is directly related to the porosity of the demineralized dentin, where increased porosity coordinates with decreased optical density. If remineralization occurs, the optical intensity will increase accordingly (27). In order to avoid interference of the differences among individual samples and fluorescent elimination, the relative optical density (ROD) was calculated as $OD_R = OD_l / OD_s \times 100\%$, where OD_l was the OD of the lesion, OD_s was the OD of sound root tissue of the same sample at the same level (depth from the surface). OD_R was quantitatively calculated at the same depth where the microhardness was evaluated.

Statistical Analysis

For each sample group, the mean and standard deviation were calculated for all measured parameters. The microhardness data was transformed (log10) to satisfy hypothesis of homogeneity on intra-samples variances and analyzed using 2-Way ANOVA test and Pos-hoc multiple comparison Games-Howell test. The data collected from PLM and CLMS were analyzed using one-way ANOVA and Scheffe post hoc comparison test (SPSS for windows Version 11, SPSS Inc., Chicago, IL, USA). A level of significance of $\alpha=0.05$ was explored in all statistical tests.

Results

The cross-sectional KHN values of the artificial root caries lesions of different treatment groups at different lesion depths from the surface are presented in Table 1. There was no significantly interaction between treatment and depth ($p=0.160$). Samples treated with GSE and fluoride were significantly higher in microhardness values when compared to the control group ($p=0.005$ and $p=0.012$, respectively). There was no statistically significant difference between the fluoride and GSE treatment group as well ($p=0.838$). The hardness value increased according to the increased depth below the surface, regardless of the group evaluated.

Representative PLM micrographs of root fragment sections from each group as viewed in water are presented in Fig. 2. The profile obtained from the polarized light microscopy showed the presence of a baseline lesion in all samples after lesion formation (Fig. 2a). Upon pH cycling, a mineral precipitation band (Fig. 2, P) was evident on the superficial layers in the control and treated groups lesions. An increase in the advanced demineralization band (Fig. 2, AD) was also noted in every sample due to the acid challenge during pH cycling procedure. As shown in Fig. 3, the lesion depths data obtained from PLM of fluoride treated samples were significantly smaller than the control and the GSE (Fig. 3) ($p<0.001$). A significantly wider ($p<0.001$) mineral precipitation band was observed in the GSE treated group when compared to those of fluoride and control groups (Fig. 2c, Fig. 3)

When the samples were stained with rhodamine B and examined under the CLSM, a red fluorescent band was observed (Fig. 4, L). The lesions further demineralization after pH-cycling could be evidenced by the increase in fluorescence (or less optical intensity). This observation was consistent with the findings by PLM. A condensed band with almost no fluorescence (Fig. 4, P) was again noted at the surface of the lesions. The relative optical density (ROD) values of different treatment groups at different sites of the lesion are presented in Table 2. At the superficial layer of the lesions (50 μm and 80 μm), the ROD values of GSE group ($73.23\% \pm 36.46\%$, $41.55\% \pm 24.21\%$) were significantly higher when compared to the control group ($38.60\% \pm 18.26\%$, $22.48\% \pm 9.57\%$; $P=0.006$, $P=0.033$ respectively), indicating less tissue porosity. A schematic average ROD distribution evaluated by CLSM of each group is presented in Fig. 5. It was evident that the pattern of the fluoride and GSE were different (Fig. 5).

Discussion

The *in vitro* pH cycling models are designed to simulate the dynamic variations in mineral saturation and pH associated with the natural caries process (28). In order to find the treatment's effect on existing root lesions, artificial caries lesions were produced according to ten Cate and Duijsters (29). The pH cycling procedure employed in this study exposed artificial caries lesions to repeated acid challenge followed by remineralization.

It is well documented that fluoride's anti-cariogenic effects take place via two principal mechanisms, inhibiting demineralization when fluoride is present at the crystal surfaces during an acid challenge, and enhancing remineralization by forming a low solubility veneer similar to the acid-resistant mineral fluorapatite (FAP) on the remineralized crystals (30). Fluoride in this study was employed as a positive control, which was found to be effective in the process of demineralization and remineralization of the artificial root caries. Fluoride treatment inhibited further demineralization of existed artificial root lesions (Fig. 3) and increased the microhardness value of lesions (Table 1).

The GSE used in the present study consisted mainly of 97.8% proanthocyanidin (PA). PAs are potent antioxidants known to possess vasodilation, anticarcinogenic, anti-inflammatory, antibacterial and immunostimulating effects (31). High molecular mass PA (condensed tannin) from cranberry affected acid production and biofilm development by *S. mutans* (23,32,33). However, the effect of PA, on the remineralization and demineralization of dental hard tissues is less well understood.

The rationale for the CLSM analysis of demineralization and remineralization relies on the hypothesis that imbibition of a fluorescent dye into porosities of demineralized enamel decreases following remineralization and enables quantitative analysis of thick samples using confocal microscopy (27). A precipitation band was formed at the superficial layer of the lesion after the pH cycling which was observed in both CLSM (Fig. 4, P) and PLM (Fig. 2, P). The precipitation band was wider in GSE treated group when compared to the fluoride treated and the control groups (Fig. 3). The ROD values of the GSE group were also higher than that of any other group (table 2). Combined with the increase in the microhardness values, GSE promoted mineral deposition on the superficial layer of the lesion.

Based on data obtained from this study, GSE may positively affect the remineralization process through two distinct mechanisms. First, GSE may contribute to mineral deposition on the superficial layer of the lesion. Although not presented, GSE formed insoluble complexes when mixed with the remineralizing solution at pH 7.4. These complexes remained visually insoluble at pH range of 2–7 (data not show). This is consistent with reports made previously by other researchers (34,35). Thus, it is likely that after treatment with GSE, the latter present in the lesion may combine with Ca^{2+} from the remineralizing solution, thereby enhancing remineralization. Secondly, GSE may interact with the organic portion of the root dentin through PA-collagen interaction, thereby stabilizing the exposed collagen matrix. Interactions between PA and proteins have been suggested to involve covalent (36), ionic (37), hydrogen bonding or hydrophobic interactions (26). Bedran-Russo *et al.* (38) reported that GSE treatment significantly increased the ultimate tensile strength of demineralized dentin, indicating the potential of GSE to induce cross-links in the dentin collagen. In the present study, GSE treated dentin demonstrated increased microhardness value and wider precipitation band. This effect was not contributed by fluoride since the fluoride concentration in 6.5% GSE solution was less than 0.01 ppm. Thus the potential remineralizing effect of GSE may also be attributed to the changes in the organic matrix, specifically by the presence of newly induced collagen cross-links. The presence of exogenous collagen cross-links have been reported to increase collagen stability by reducing enzymatic degradation (39,40). Further studies involving

microradiography and electron microscopy are warranted to confirm mineral deposition at the superficial layer of the lesion.

Based on data obtained in this *in vitro* study, we believe that GSE inhibits the demineralization and/or promote the remineralization of artificial root carious lesions under dynamic pH-cycling conditions. The remineralization effect of GSE appears to be distinct from that of fluoride treatment. GSE may serve to be a promising adjunct or alternative to fluoride in the treatment of root caries during minimally invasive therapy.

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References

1. Leake JL. Clinical decision-making for caries management in root surfaces. *Journal of Dental Education* 2001 Oct;65(10):1147–53. [PubMed: 11699992]
2. Banting DW. Epidemiology of root caries. *Gerodontology* 1986 Spring;5(1):5–11. [PubMed: 3470253]
3. ten Cate, AR. Oral Histology: development, structure, and function. 5. 1998. p. 150
4. Vanuspong W, Eisenburger M, Addy M. Cervical tooth wear and sensitivity: erosion, softening and rehardening of dentine; effects of pH, time and ultrasonication. *Journal of Clinical Periodontology* 2002 Apr;29(4):351–7. [PubMed: 11966933]
5. Hara AT, Queiroz CS, Paes Leme AF, Serra MC, Cury JA. Caries progression and inhibition in human and bovine root dentine in situ. *Caries Research* 2003 Sep–Oct;37(5):339–44. [PubMed: 12925824]
6. Linde A. The extracellular matrix of the dental pulp and dentin. *Journal of Dental Research* 1985 Apr;64 Spec No 523–9. [PubMed: 3857252]
7. Birkedal-Hansen H, Butler WT, Taylor RE. Proteins of the periodontium. Characterization of the insoluble collagens of bovine dental cementum. *Calcified tissue research* 1977 May 31;23(1):39–44. [PubMed: 890542]
8. Mellberg JR, Sanchez M. Remineralization by a monofluorophosphate dentifrice in vitro of root dentin softened by artificial caries. *Journal of Dental Research* 1986 Jul;65(7):959–62. [PubMed: 3458756]
9. Clarkson BH, Rafter ME. Emerging methods used in the prevention and repair of carious tissues. *Journal of Dental Education* 2001 Oct;65(10):1114–20. [PubMed: 11699987]
10. Lynch E, Baysan A. Reversal of primary root caries using a dentifrice with a high fluoride content. *Caries Research* 2001;35 (Suppl 1):60–4. [PubMed: 11359061]
11. ten Cate JM. Remineralization of caries lesions extending into dentin. *Journal of Dental Research* 2001 May;80(5):1407–11. [PubMed: 11437209]
12. Cragg GM, Newman DJ, Snader KM. Natural products in drug discovery and development. *Journal of Natural Products* 1997 Jan;60(1):52–60. [PubMed: 9014353]
13. Duarte S, Koo H, Bowen WH, Hayacibara MF, Cury JA, Ikegaki M, et al. Effect of a novel type of propolis and its chemical fractions on glucosyltransferases and on growth and adherence of mutans streptococci. *Biological & pharmaceutical bulletin* 2003 Apr;26(4):527–31. [PubMed: 12673037]
14. Ooshima T, Osaka Y, Sasaki H, Osawa K, Yasuda H, Matsumura M, et al. Caries inhibitory activity of cacao bean husk extract in in-vitro and animal experiments. *Archives of Oral Biology* 2000 Aug;45(8):639–45. [PubMed: 10869475]
15. Nostro A, Cannatelli MA, Crisafi G, Musolino AD, Procopio F, Alonzo V. Modifications of hydrophobicity, in vitro adherence and cellular aggregation of *Streptococcus mutans* by *Helichrysum italicum* extract. *Letters in applied microbiology* 2004;38(5):423–7. [PubMed: 15059215]
16. Cai L, Wu CD. Compounds from *Syzygium aromaticum* possessing growth inhibitory activity against oral pathogens. *Journal of Natural Products* 1996 Oct;59(10):987–90. [PubMed: 8904847]
17. Li XC, Cai L, Wu CD. Antimicrobial compounds from *Ceanothus americanus* against oral pathogens. *Phytochemistry* 1997 Sep;46(1):97–102. [PubMed: 9276981]

18. Chu JP, Li JY, Hao YQ, Zhou XD. Effect of compounds of *Galla chinensis* on remineralisation of initial enamel carious lesions in vitro. *Journal of Dentistry* 2007 May;35(5):383–7. [PubMed: 17196320]
19. Sarni-Manchado P, Cheynier V, Moutounet M. Interactions of grape seed tannins with salivary proteins. *Journal of Agriculture and Food Chemistry* 1999 Jan;47(1):42–7.
20. Fujii H, Sun B, Nishioka H, Hirose A, Aruoma OI. Evaluation of the safety and toxicity of the oligomerized polyphenol Oligonol. *Food and Chemical Toxicology* 2007 Mar;45(3):378–87. [PubMed: 17081671]
21. Yamakoshi J, Saito M, Kataoka S, Kikuchi M. Safety evaluation of proanthocyanidin-rich extract from grape seeds. *Food and Chemical Toxicology* 2002 May;40(5):599–607. [PubMed: 11955665]
22. Aldini G, Carini M, Piccoli A, Rossoni G, Facino RM. Procyanidins from grape seeds protect endothelial cells from peroxynitrite damage and enhance endothelium-dependent relaxation in human artery: new evidences for cardio-protection. *Life Sciences* 2003 Oct 17;73(22):2883–98. [PubMed: 14511773]
23. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. *FEMS Microbiology Letters* 2006 Apr;257(1):50–6. [PubMed: 16553831]
24. Rao CN, Rao VH, Steinmann B. Bioflavonoid-mediated stabilization of collagen in adjuvant-induced arthritis. *Scandinavian Journal of Rheumatology* 1983;12(1):39–42. [PubMed: 6836239]
25. Cetta G, Pallavicini G, Tenni R, Bisi C. Influence of flavonoid-copper complexes on cross linking in elastin. *Italian Journal of Biochemistry* 1977 Jul-Aug;26(4):317–27. [PubMed: 562863]
26. Han B, Jaurequi J, Tang BW, Nimni ME. Proanthocyanidin: a natural crosslinking reagent for stabilizing collagen matrices. *Journal of Biomedical Materials Research A* 2003 Apr 1;65(1):118–24.
27. Gonzalez-Cabezas C, Fontana M, Dunipace AJ, Li Y, Fischer GM, Proskin HM, et al. Measurement of enamel remineralization using microradiography and confocal microscopy. A correlational study *Caries Research* 1998;32(5):385–92.
28. White DJ. The application of in vitro models to research on demineralization and remineralization of the teeth. *Advances in Dental Research* 1995 Nov;9(3):175–93. 94–7. [PubMed: 8615942]
29. ten Cate JM, Duijsters PP. Alternating demineralization and remineralization of artificial enamel lesions. *Caries Research* 1982;16(3):201–10. [PubMed: 6953998]
30. ten Cate JM, Duijsters PP. Influence of fluoride in solution on tooth demineralization. I. Chemical data. *Caries Research* 1983;17(3):193–9. [PubMed: 6573960]
31. Rice-Evans CA, Miller NJ. Antioxidant activities of flavonoids as bioactive components of food. *Biochemical Society Transactions* 1996 Aug;24(3):790–5. [PubMed: 8878849]
32. Steinberg D, Feldman M, Ofek I, Weiss EI. Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. *Journal of Antimicrobial Chemotherapy* 2004 Jul;54(1):86–9. [PubMed: 15163648]
33. Gupta K, Chou MY, Howell A, Wobbe C, Grady R, Stapleton AE. Cranberry products inhibit adherence of p-fimbriated *Escherichia coli* to primary cultured bladder and vaginal epithelial cells. *The Journal of Urology* 2007 Jun;177(6):2357–60. [PubMed: 17509358]
34. Kosasi S, Hart LA, van Dijk H, Labadie RP. Inhibitory activity of *Jatropha multifida* latex on classical complement pathway activity in human serum mediated by a calcium-binding proanthocyanidin. *Journal of Ethnopharmacology* 1989 Nov;27(1–2):81–9. [PubMed: 2615430]
35. Kim S, Don S, Mainwaring DE. Effect of ion-binding on the formation of temporary viscoelastic networks of proanthocyanidin biopolymers. *Journal of Applied Polymer Science* 1997;65(9):1795–805.
36. Pierpoint WS. o-Quinones formed in plant extracts. Their reactions with amino acids and peptides. *Biochemical Journal* 1969 May;112(5):609–16. [PubMed: 4980678]
37. Loomis WD. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. *Methods in Enzymology* 1974;31(Pt A):528–44. [PubMed: 4418408]
38. Bedran-Russo AK, Pereira PN, Duarte WR, Drummond JL, Yamauchi M. Application of crosslinkers to dentin collagen enhances the ultimate tensile strength. *Journal of Biomedical Materials Research B Applied Biomaterial* 2007 Jan;80(1):268–72.

39. Miguez PA, Pereira PN, Atsawasuwan P, Yamauchi M. Collagen cross-linking and ultimate tensile strength in dentin. *Journal of Dental Research* 2004 Oct;83(10):807–10. [PubMed: 15381724]
40. Kleter GA, Damen JJ, Buijs MJ, Ten Cate JM. The Maillard reaction in demineralized dentin in vitro. *European Journal of Oral Science* 1997 Jun;105(3):278–84.

Fig. 1.
In vitro artificial lesion formation and pH-cycling procedure.

Fig. 2. Polarized light microscopy photomicrographs of sections of artificial root lesions: a. baseline lesion; b. control; c. Fluoride; d. Grape seed extract (GSE); (L, lesion; D, sound dentin; P, mineral precipitation band; AD: Advanced demineralization band).

Fig. 3. Effect of Fluoride (F), grape seed extract (GSE) on artificial root lesion depth as determined by polarized light microscopy. D: post-treatment lesion depth (μm), P: precipitation band depth (μm). * Statistically significant difference detected between the treatment group and control group ($p < 0.05$).

Fig. 4. Confocal Laser Scanning Microscopy representative image of artificial root lesions treated by Fluoride, grape seed extract. (L, lesion; D, sound dentin; P, precipitation band). A. Baseline lesion; B. Control group; C. Fluoride group; D. grape seed extract group.

Fig. 5. CLSM profile of each group and the schematic relative optical density versus depth (μm) for the four experimental groups. Baseline: represents the samples after demineralization in acid buffer for 96 hr. Control: no treatment; GSE: Grape seed extract; F: Fluoride.

Table 1

Cross-sectional microhardness values of the artificial root caries lesions after pH cycling.

Depth from surface	Knoop Hardness Numbers (SD)			
	Control	Fluoride (1000ppm)	GSE (6.5%)	
20 μ m	3.78 (6.02)	6.52 (3.76)	8.62 (7.62)	f
50 μ m	6.73 (9.20)	10.38 (4.17)	11.81 (6.83)	ef
80 μ m	14.10 (15.57)	20.57 (8.40)	20.60 (9.54)	e
110 μ m	30.91 (17.83)	41.74 (14.12)	33.83 (11.26)	cd
140 μ m	43.58 (18.24)	57.00 (13.72)	47.95 (10.43)	bc
170 μ m	51.84 (15.13)	63.55 (11.74)	58.13 (9.18)	ab
200 μ m	61.30 (12.17)	67.81 (7.68)	63.78 (10.19)	a
	B	A	A	

Upper cases indicate statistically significant differences between treatments ($p < 0.05$). Lower cases indicate statistically significant differences between depths ($p < 0.05$) GSE: grape seed extract.

Table 2

Relative optical density (ROD) of artificial root lesions treated by Fluoride, grape seed extract (GSE) at different depths.

Depth from surface	Control Mean (SD)(%)	Fluoride (1000ppm) Mean (SD) (%)	GSE (6.5%) Mean (SD) (%)
20 μ m	86.35 (26.76)	58.33 (17.57) a	105.83 (45.90) b
50 μ m	38.60 (18.26) b	32.70 (14.58) b	73.23 (36.46) a
80 μ m	22.48 (9.57) b	21.27 (8.65) b	41.55 (24.21) a
110 μ m	19.11 (9.47)	25.37 (6.96)	21.50 (11.91)
140 μ m	17.97 (9.18)	25.96 (8.37)	18.46 (13.75)
170 μ m	33.36 (18.12)	29.06 (9.86)	45.01 (36.40)
200 μ m	55.69 (34.15)	40.16 (16.87)	66.69 (41.11)
230 μ m	68.35 (33.17)	67.46 (14.58)	81.26 (32.66)
250 μ m	81.86 (28.84)	84.62 (18.41)	91.67 (13.93)

ROD was calculated as $OD_R = OD_I / OD_S \times 100\%$; OD_I was the OD of the lesion, OD_S was the OD of sound root tissue of the same sample at the same level. Different letters (a,b) indicate statistically significant differences in each row.