

COMPARISON OF THE EFFECTIVENESS OF FLUORIDE
VERSUS GRAPE SEED EXTRACT IN BIOMIMETIC
REMINERALIZATION OF DENTIN

Simge Vural[#], Zeynep Ökte*

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Abstract

This study aims to compare the remineralization efficiency of grape seed extract versus fluoride in artificial carious dentin lesions in the biomimetic remineralization procedure. One hundred and twelve extracted impacted human teeth were demineralized for 10 days and divided into 4 groups. Demineralization group (DM), remineralization group (RM group), remineralization fluoride group (RM F), remineralization grape seed extract group (RM GSE). Demineralization (DM) group were stored in physiological saline after demineralization. RM F was kept in 1000 ppm fluorine solution and RM GSE was kept in 6.5% grape seed extract for 10 min. RM, RM F, RM GSE groups were stored in simulated body fluid (SBF) with biomimetic analogues and Portland cement discs for 6 weeks. The specimens were evaluated by microhardness test, elasticity modulus measurement, and scanning electron microscope (SEM) analysis. Kruskal–Wallis H test with Bonferroni correction was used for between-group comparisons. Microhardness and elasticity modulus values differed significantly among the groups, with the highest values in the RM-GSE group, followed by the RM-F group, RM group, and DM group. SEM images showed granular structure formations in the RM-GSE and RM-F groups. It is concluded that although remineralization can occur by mineral precipitation in structures with high organic matrix density such as dentin, the collagen structure must be supported to increase the degree of remineralization.

Key words: dentin remineralization, biomimetic remineralization, grape seed extract, fluoride

[#]Corresponding author.
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Introduction. The early enamel caries can heal or be remineralized through improving the oral hygiene and remineralization treatments. But if the lesion continually develops into the dentin the infected tooth tissue should be removed. However, excess removal of tooth tissues tends to make carious teeth fracture more easily and may cause accidental pulp exposure [1]. Remineralization of demineralized dentin plays an important role in the control of dentinal caries. However, dentin remineralization is more difficult than enamel remineralization due to presence of the organic matrix [2]. Remineralization agents such as fluoride have been shown to act via similar mechanisms in both enamel and dentin tissue. However, as fluoride remineralization requires seed crystals, it is less effective in dentin than enamel due to the scarcity of seed crystals in dentin lesions [3] and because of the collagen matrix components (type 1 collagen and noncollagenous proteins) of dentin. In the dentin remineralization process, it is recognized that remineralization does not occur by spontaneous precipitation or mineral nucleation on the organic matrix, but by the growth of residual crystals in the dentinal lesion [4].

Biomimetic remineralization, also known as functional remineralization, is a promising methodology that could challenge the common belief that demineralized dentin cannot be effectively remineralized. The procedure involves hydroxyapatite (HAP) growth over organic and inorganic substrates and controlled nucleation with substrates in simulated body fluid (SBF), a supersaturated, metastable, calcium phosphate solution containing the same substances as human blood plasma [5]. The biomineralization concept is based on the templating of mineral nucleation through noncollagenous proteins. These polyanionic protein molecules are believed to bind to the collagen substrate at specific sites of the fibre assembly. The bound proteins present anionic charge sites for calcium binding and apatite formation. Immobilization of the proteins at critical sites of the collagen matrix facilitates precipitation at both intrafibrillar and extrafibrillar locations [6]. In biomimetic remineralization, polyanionic molecules (e.g., polyphosphates, polyvinyl phosphonic acid, or polyacrylic acid) are introduced to enable prenucleation clusters of amorphous precursors to infiltrate collagen fibrils and transform into interfibrillar apatite crystals [6,7]. Based on the knowledge that fluoride is not as effective on dentin as enamel [2,3], the search for alternative remineralization agents continues.

Natural, nontoxic therapeutic agents have been used for medical purposes for thousands of years. One of these therapeutic agents is grape seed extract (GSE), which contains polyphenolic compounds. The main components of GSE are anthocyanins, flavonoids, and resveratrol, which have various bioactivities such as antioxidant, cardioprotective, anticancer, anti-inflammation, anti-ageing, and antimicrobial activities [3,8]. Flavonoids strengthen collagen tissue structure by increasing cross-linkage between collagen fibres [9]. They also increase collagen synthesis, accelerate the conversion of soluble collagen into insoluble collagen and increase dentin elasticity [10]. It has been hypothesized that GSE could therefore

stabilize the collagen matrix for dental preservation and thus prevent dental caries.

Our study aimed to compare the remineralization capacity of GSE as a promoter of collagen formation versus fluoride as a promoter of mineral deposition on standardized artificial dentin caries lesions in the biomimetic remineralization model.

Materials and methods. Before the study, power analysis was performed to determine the sample size necessary for microhardness and modulus of elasticity testing and scanning electron microscope (SEM) imaging. With a significance level of 0.05 and sensitivity of 0.35, the minimum sample size was determined to be 112.

One hundred and twelve extracted impacted human third molars with no cracks or deformations, in accordance with standard procedures in the Oral and Maxillofacial Surgery department of Ankara University were collected after obtaining the patients' informed consent. The teeth were brushed and stored in physiological saline containing 0.2% thymol at 4 °C to inhibit bacterial growth before use. All teeth were used within 3 months of extraction.

Specimen preparation. All teeth were embedded in acrylic molds (Meliodent, Heraeus) with the occlusal surfaces facing upwards. A high-speed, water-cooled diamond saw (Meisinger, Germany) was used to slice the specimens horizontally at the dentino-enamel junction and polished on a water-cooled polishing machine (Metkon Gripo 2V, Bursa, Turkey) with abrasive paper (800, 1200, 1500, and 2000 grit) to create a smooth, flat surface.

After this step, the samples were divided into two groups to prepare them for microhardness, SEM, and elasticity modulus tests. For SEM and elasticity modulus testing, specimens were cut with a microcutter (Metkon, Bursa, Turkey) and prepared to a size of $7 \times 3 \times 1$ mm dimension. For microhardness testing, the flat surface was preserved with no other application.

Reagents. The demineralization solution consisted of 1.5 mM CaCl_2 , 0.9 mM KH_2PO_4 , 50 mM acetic acid, and 0.02% NaN_3 adjusted to a pH of 4.5 using NaOH ²⁰.

SBF was prepared by dissolving 136.8 mM NaCl , 4.2 mM NaHCO_3 , 3.0 mM KCl , 1.0 mM $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.5 mM CaCl_2 , and 0.5 mM Na_2SO_4 in deionized water. Sodium azide (3.08 mM) was added to prevent bacterial growth, and 500 $\mu\text{g}/\text{ml}$ polyacrylic acid (M_w : 1800; Sigma Aldrich, St. Louis, MO) and 200 $\mu\text{g}/\text{ml}$ sodium tripolyphosphate (M_w : 367.8; Sigma Aldrich, St. Louis, MO) were added as dual biomimetic analogues [7].

Type-1 Portland cement (Adana Çimento, Adana, Turkey) was used as a source of calcium and hydroxyl ions for biomimetic remineralization. The cement was mixed with deionized water at a ratio of 3:1. The mixture was poured into standard silicone containers to make 1-cm (0.5 g) discs, then stored at 37 °C and 100% humidity for 1 week.

A 6.5% GSE solution was prepared by dissolving 6.5 g of proanthocyanidin-rich ($\geq 95\%$) GSE (MegaNatural-BP capsules; Polyphenolics, Madera, CA) in 100 ml distilled water in a glass container. A homogeneous sodium fluoride solution (1000 ppm) was prepared by diluting 0.1 g of NaF (Merck, Darmstadt, Germany) in 100 ml of distilled water.

Treatment and measurement procedures. All specimens ($n = 112$) were placed in separate containers containing 10 ml of demineralization solution and stored at 37°C for 10 days. The solutions were refreshed and adjusted to pH 4.5 daily. After the demineralization procedure, the specimens were randomly divided into 4 groups ($n = 28$): the demineralization (DM) group, remineralization (RM) group, remineralization fluoride (RM-F) group, and remineralization GSE (RM-GSE) group. Prior to remineralization, samples in the RM-F and RM-GSE groups were immersed for 10 min in GSE or fluoride solution, respectively. Samples from all 3 remineralization groups were then stored in individual containers with a single Portland cement disc and 10 ml of SBF for 6 weeks. The SBF solution was refreshed every other day. From each group, 13 samples were used for microhardness measurement, 13 were used for elasticity measurement, and the remaining 2 were used for SEM analysis (Fig. 1).

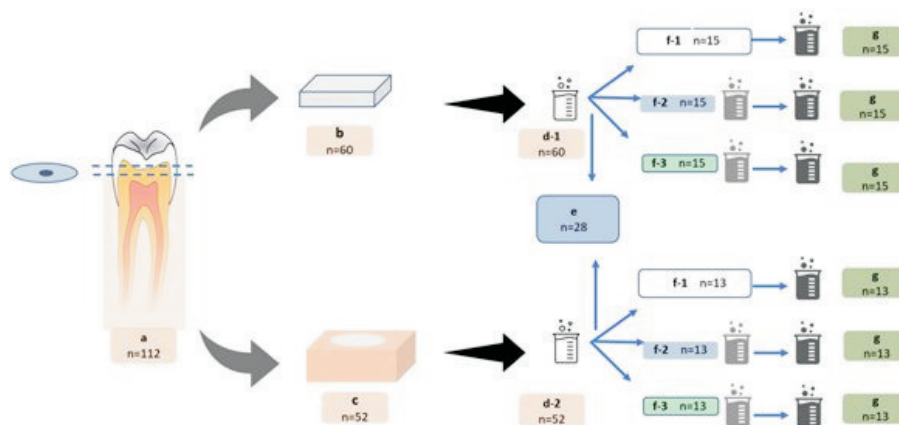


Fig. 1. Diagram of the study groups.

- a. Tooth embedded in acrylic molds and cut at dentino-enamel junction; $n = 112$.
- b. Specimens for elastic modulus and SEM examinations; $n = 60$.
- c. Specimens for microhardness measurements; $n = 52$.
- d. 1. Demineralization of specimens prepared for modulus of elasticity and SEM examination; ($n = 60$).
2. Demineralization of specimens prepared for microhardness measurements; ($n = 52$).
- e. Demineralized specimens for microhardness ($n = 13$), modulus of elasticity ($n = 13$), SEM examination ($n = 2$).
- f. 1. Specimens with no application.
2. Specimens immersed 1000 ppm fluor solution.
3. Specimens immersed %6.5 GSE solution.
- g. Serum body fluid (SBF)

Microhardness and modulus of elasticity measurement. Microhardness was measured at the centre of each specimen with an applied load of 200 g (Zwick/Roell ZHV 10, Germany). The mean of 3 readings was recorded as Vicker's Hardness number (VHN).

Modulus of elasticity of the specimens was assessed by nondestructive three-point bending flexural test at 2% strain with 1 N load cell at 0.5 mm/min crosshead speed in a Universal Test Machine (Lloyd LRX, USA). The results were expressed in megapascals (MPa).

Scanning electron microscopy. After completely drying the specimens, they were plated with 10 nm gold-palladium and examined at 2000×, 10 000×, and 20 000× magnifications using an SEM (FEI Quanta 200F, USA).

Statistical analysis. Microhardness and modulus of elasticity values were statistically analyzed using IBM SPSS Statistics, version 20.0 for Windows (IBM Corp, Armonk, NY, USA). The Kruskal–Wallis H test with Bonferroni correction was used for intergroup comparisons. Results with $p < 0.05$ were considered statistically significant.

Results. Microhardness and elastic modulus results. The lowest microhardness value was observed in the DM group, with a mean VHN of 45.4. Among the biomimetic remineralization groups, the RM-GSE group had the highest mean microhardness value (84.2 VHN), followed by the RM-F group (67.8 VHN) and the RM group (55.3 VHN). There was a statistically significant difference in microhardness values among the groups ($p < 0.05$) (Table 1).

T a b l e 1

Vicker's hardness values of groups [DM; RM; RM F; RM GSE]

Group name	Number	Mean	Std	Median
DM	13	45.4	3.8	47.0
RM	13	55.3	6.7	52.7
RM F	13	67.8	5.5	66.0
RM GSE	13	64.2	4.0	84.0

Similarly, the DM group had the lowest elastic modulus value among the groups, at 61.2 MPa. In the biomimetic remineralization groups, mean values were 95.6 MPa in the RM group, 136.6 MPa in the RM-F group, and 213 MPa in the RM-GSE group. Comparison of mean values among all groups revealed a statistically significant difference ($p < 0.05$). We determined that elastic modulus values were significantly lower in the DM group and higher in the RM-GSE group compared to the other groups ($p < 0.05$) (Table 2).

SEM Findings. Images were obtained at 2000×, 10 000×, and 20 000× magnifications for all groups. In the DM group, all the dentin tubules were exposed and completely empty, with homogeneous demineralized areas observed on the surface (Fig. 2a, b, c).

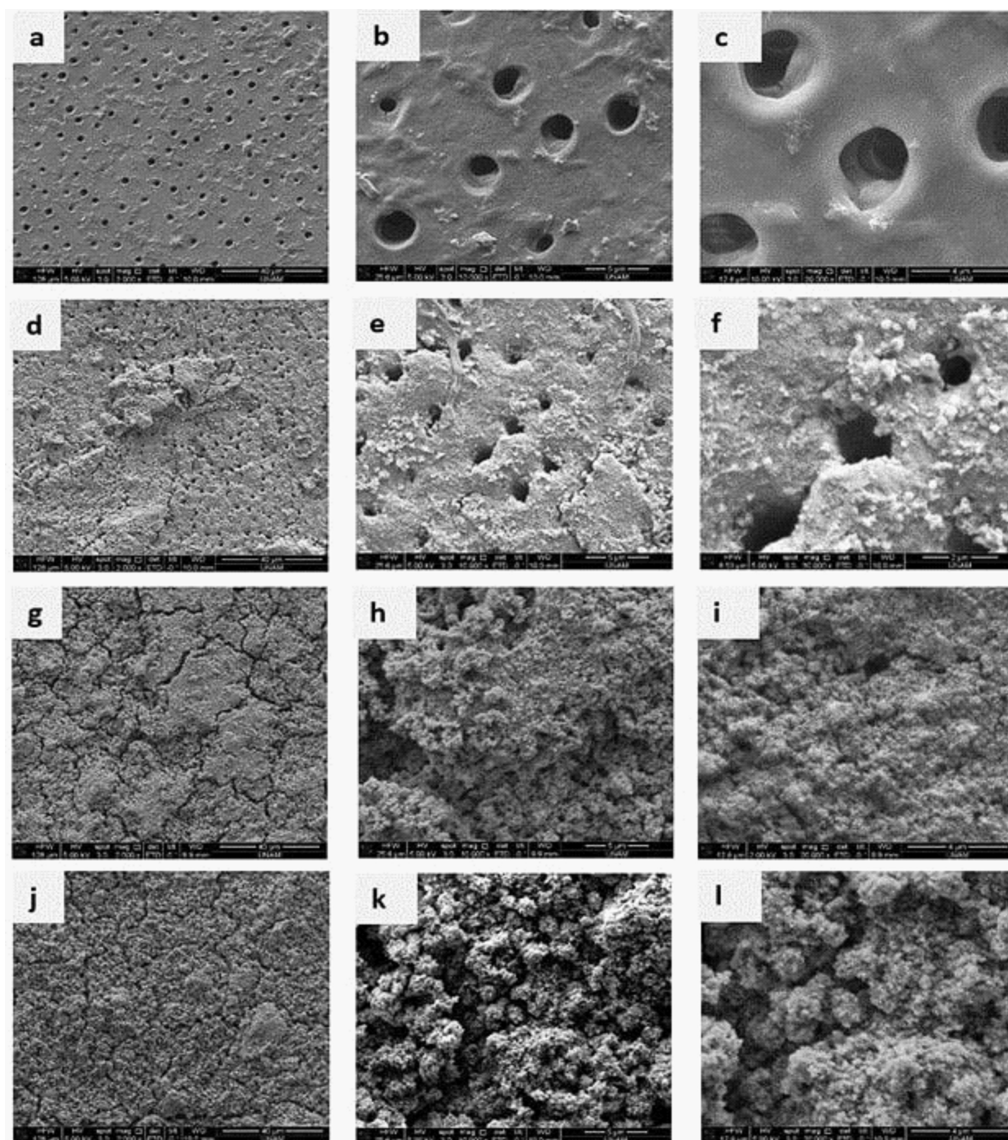


Fig. 2. SEM image of all groups (a: $\times 2000$ DM group, b: $\times 5000$ DM group, c: $\times 20\,000$ DM group; d: $\times 2000$ RM group, e: $\times 5000$ RM group, f: $\times 20\,000$ DM group; g: $\times 2000$ RM F group, h: $\times 5000$ RM F group, i: $\times 20\,000$ RM F group; j: $\times 2000$ RM GSE Group, k: $\times 5000$ RM GSE Group, l: $\times 20\,000$ RM GSE Group) homogenous demineralization of dentin at a, b, c. Partially remineralized areas, blocked dentin tubules and granular structures at d, e, f. Totally remineralized areas at g, h, i. Totally remineralized areas, completely blocked dentin tubules and more dense granular structures at j, k, l

T a b l e 2

Elastic modulus results of the groups [DM; RM; RM F; RM GSE]

Group name	Number	Mean	Std	Median
DM	13	61.2	15.6	59.2
RM	13	95.6	12.8	98.7
RM F	13	136.6	41.3	121.4
RM GSE	13	213.0	19.1	215.2

In the RM group, the dentin tubules were not as open as in the demineralized dentin surface but were not completely blocked (Fig. 2d, e, f). In the RM-F group, the dentin tubules were completely blocked, and granular structures were observed on the surface (Fig. 2g, h, i). In the RM-GSE group, the dentin tubules were completely blocked and had a denser granular structure than in the RM-F group (Fig. 2j, k, l).

Discussion. Fluoride remineralization is dependent upon epitaxial precipitation of mineral deposits over remnant apatite seed crystals [3,11]. While there are studies showing that fluorine provides remineralization in dentin [12,13], it has also been reported that its remineralization capacity is lower compared to enamel [14].

Recently, researchers have also focused on the remineralization potential of medical plants and their extracts. Studies have shown that proanthocyanidins can remineralize dentin by promoting dentin interaction with the collagen structure, thereby increasing dentin mineral density [9,15]. The present study evaluated the use of GSE, a substance rich in proanthocyanidins, as a promoter of collagen formation as compared with fluoride, a substance frequently used as a promoter of mineral deposition.

The remineralization techniques used in clinical dentistry are considered to represent a top-down approach, whereas natural mineralization is considered a bottom-up phenomenon [16]. Elements such as fluoride that are used in top-down mineralization require residual apatite crystals for the storage of newly formed minerals, and remineralization cannot take place in the absence of these precursor elements. In contrast, biomineralization can occur without the presence of apatite seed crystals in the organic collagen scaffold [17], which is the basis for both intra- and interfibrillar remineralization [6,17].

ZHANG et al. [18] noted that biomimetic remineralization could imitate the natural mineralization process. The remineralization solution used in biomimetic remineralization procedures has a composition similar to that of saliva [19]. A number of studies [7,18] have suggested that the use of polyacrylic acid and sodium tripolyphosphate as dentin analogues, with the addition of Portland cement, could be effective in promoting intrafibrillar remineralization in clinical practice.

Experimental studies have employed a variety of demineralization techniques to create artificial dentin caries lesions, including pH cycling, microbial deminer-

alization, and acidic gels and solutions. JOVES et al. [20] developed a demineralization solution containing mineral ingredients similar to those found in the superficial layer of natural caries dentin. Therefore, we used this solution to create a homogeneously demineralized surface that resembles a superficial layer of dentinal caries. However, instead of applying the solution for 7 days, in this study we used a demineralization period of 10 days.

The mechanical properties of dentin are directly related to its mineral content. Therefore, dentin hardness values have been used as an indirect measurement of remineralization. In the present study, VHN values were 84.2, 67.8, and 55.3 for the RM-GSE, RM-F, and RM groups, respectively, whereas the VHN of the DM group (45.2) was significantly lower ($p < 0.05$). The higher hardness value of the RM group compared to the DM group may be attributed to the transfer of Ca, P, and OH ions derived from the SBF and Portland cement. In the RM-F group, fluoride and Ca may have formed CaF_2 and precipitated onto the dentin surface. In the RM-GSE group, proanthocyanidins and type-1 collagen might have facilitated mineral precipitation over dentin tissue to achieve higher levels of remineralization. These findings are consistent with those of previous studies [9,15]. Similarly, we determined that elastic modulus values were lowest in the DM group and increased progressively in the RM, RM-F, and RM-GSE groups. The higher elastic modulus of the RM-GSE group supports a study by CASTELLAN et al. [10] suggesting that strengthening the collagen structure could improve the mechanical behaviour of dentin.

Granular structures observed on micromorphological examination are one of the signs of extrafibrillar mineralization [7]. In this study, granular structures were seen in both the RM-F and RM-GSE groups but were denser in the RM-GSE group. This finding indicates that although both fluoride and GSE enhanced extrafibrillar remineralization, GSE was relatively more effective.

KIM et al. [7] reported that the combination of granular structures observed in micromorphological analysis and increased elastic modulus values are indicators of both extrafibrillar and intrafibrillar remineralization. Based on our findings, we concluded that functional remineralization can occur in the presence of biomimetic analogues in SBF, and that remineralization agents can increase remineralization levels. While fluoride may be a highly popular remineralization agent, GSE is more effective in dentin remineralization.

Conclusion. Our study provides evidence that GSE is a natural promoter of dentin remineralization that was more effective than fluoride application. This finding may be attributed to the capacity of GSE to promote collagen matrix formation, which enables mineral deposition on this newly formed scaffold. The increases in microhardness and modulus of elasticity results observed to varying degrees in all remineralization groups indicate that the dentin analogues and calcium ion sources used in the biomimetic remineralization procedure facilitate biomechanical restoration of dentin. After the positive effects of GSE in rem-

ineralization are understood, in vivo studies will be beneficial for adding it as a component to restorative materials or applying it directly to dentin.

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Department of Pediatric Dentistry
Ankara Medipol University
Hacı Bayram Mahallesi Talatpaşa Bulvarı No:4/1
06050 Altındağ/Ankara
e-mail: vural simge@gmail.com

**Department of Pediatric Dentistry*
Ankara University
Emniyet, Mevlana Blv. No:19/1
06560 Yenimahalle/Ankara
e-mail: zokte62@yahoo.com