

GRAPE SEED EXTRACT AND DENTIN REMINERALIZATION

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ABSTRACT

Objective: The motivation of this study was to investigate the effect of grape seed extract on dentin remineralization.

Methodology: Sound dentin disks were prepared from the buccal surfaces of human impacted third molars. The specimens were stored in demineralizing solution for 72 hours at room temperature to create artificial carious lesions. They were divided into four groups according to the treatment used; 15% GSE, 1000 ppm sodium fluoride, artificial saliva meanwhile teeth in the fourth group were kept without treatment as a negative control group. An in vitro pH cycling model for eight successive days was done. Subsequently micro-hardness of the specimens and their micro-morphological appearance were evaluated using micro-indenter and Environmental scanning electron microscope (ESEM) respectively. Data were statically analyzed.

Results: The results indicated that there was a significant increase in dentin micro-hardness values of GSE and NaF groups in comparison to the demineralized and artificial saliva groups. The ESEM micrographs confirmed the micro-hardness results.

Conclusions: GSE is a promising natural remineralizing agent for treatment of the demineralized dentin.

KEY WORDS: Dentine, Demineralization, Remineralization, Grape seed

INTRODUCTION

Dentin constitutes the main bulk of the human tooth⁽¹⁾. It carries the vitality and hydration to the whole tooth. Albeit, it is typically covered by overlying enamel and cementum and it can be exposed following their loss or gingival recession⁽²⁾. Once uncovered, dentin is negatively affected by the fluctuation in the oral environmental conditions

through the demineralization and remineralization processes⁽³⁾. Its demineralization occurs much faster than the enamel as it is less mineralized and contains higher organic portion. Dentin Remineralization is the process in which regaining of the lost minerals and repairing of the damaged collagenous and non-collagenous proteins are included⁽⁴⁾. Various reviews bolster the idea that dentin

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remineralization happens through the development of leftover crystals inside the demineralized structures⁽⁴⁻⁸⁾. Others enrolled the role of the non-collagenous proteins which stick to the collagen matrix and play their roles through both inhibitory and accelerating capacities^(9,10). Improving dentin remineralization solves the problems of dentinal hypersensitivity, cervical wear and root caries⁽⁴⁾. The most commonly used remineralizing agent is the fluoride. However, there are reported constraints regarding its use as a traditional remineralizing agent. This is because of the risk of overexposure and rise of fluoride-resistant *Streptococcus Mutans* strains. These side effects have limited its utilization as a perfect therapeutic agent. Therefore, an extraordinary measure of dentin demineralization management has centered on looking for replacing treatment options which are, natural, effective and non-fluoride based^(11,12). Back to nature and searching for a natural biocompatible remineralizing agent, directed us towards the use of grape seed extract. It has an active ingredient which is the Proanthocyanidins (PA). This agent has antioxidant and anti-inflammatory properties. It has been accounted to fortify collagen-based tissues by increasing collagen cross-links. There is a confirmation that PA increases collagen synthesis during development and quickens the transformation of soluble collagen to insoluble collagen⁽¹³⁾. The target of this study was to investigate the remineralizing impact of a PA-rich GSE on the demineralized dentine utilizing an in vitro pH cycling model. The null hypothesis established for this study was that there was no difference between the GSE, sodium fluoride and artificial saliva on the remineralization of the demineralized dentin.

MATERIALS AND METHODS

Specimen preparation

Ten human impacted third molars freshly extracted from patients in the age range 20-30 years were gathered, cleaned and put away in

distilled water containing 0.2% thymol antiseptic solution for one month at 4°C till testing⁽¹⁴⁾. Only intact teeth without having any enamel defects were incorporated. The utilization of extracted human teeth was confirmed by the Research Ethics Committee of the Faculty of dentistry, Suez Canal University, Egypt. Enamel was thoroughly removed from the coronal portion. Two guiding grooves were prepared on the mesial and distal surfaces utilizing cylindrical flat ended diamond stone ((ISO #111/012), Mani inc. Japan) mounted on high hand-piece with overflowing air-water spray then the two surfaces were ground flat. During enamel, removal color was used as a criterion to differentiate enamel from dentin. With the utilization of lead black pencil, a line was drawn on the proximal surfaces parallel to the DEJ yet underneath it by 0.5 mm. The lines on the proximal surfaces were then connected painstakingly through the buccal surface at the DEJ level. The enamel tooth structure above the line was removed, and the buccal surface was ground flat to uncover dentin level utilizing a micro saw under running water. Forty dentin specimens with measurements of 3.0 mm × 3.0 mm × 2.0 mm were set up from the buccal and lingual surfaces of the selected teeth (4 from each molar) with a water-cooled slow speed Isomet diamond saw. Dentin specimens from each tooth were ultra-sonicated in a deionized water bath (Unique, Ultrasonic Cleaner, Indaiatuba, SP, Brazil) to expel any debris. All dentin specimens were investigated under a reflected light microscope and discovered free of any imperfections, like cracks or pores. They were inserted in self-cured acrylic resin and permitted to set to make dentin blocks and then specimens were labeled for easy identification of each tooth under different treatment. Specimens were identified by letters (A-J) for knowing the tooth and digit (1-4) to the group. So different treatments were conducted on the same tooth. The dentin specimens were then stored in distilled water for 24 hours.

Baseline Micro-hardness tests

The baseline microhardness estimations were taken at three different points on the dentin specimens. The indentations were made around 0.5 mm from the interface and 1mm separated from each other ⁽¹⁵⁾. Every estimation was completed utilizing a 100g load for 15s, applied perpendicularly to the dentin surface. The diagonal lengths of indentations were measured by built-in scaled micrometer and estimations were changed over into Vicker's numbers. The values were averaged to produce one hardness value for each specimen. The microhardness estimations were performed utilizing a Vickers Microhardness Tester (Wilson miniaturized scale hardness analyzer, display Tukon 1102, Germany) with a Vickers diamond indenter and a 20X lens. Micro-hardness was acquired utilizing the accompanying equation: $HV=1.854 P/d^2$ Where, HV is Vickers hardness in Kgf/mm², P is the load in Kgf and d is the average length of the diagonals in mm

Preparation of grape seed extract

The grape seed solution was prepared by measuring 15 grams of grape seed (GSE) powder (MegaNatural, Polyphenolics, Madera, California, USA) with sensitive balance. Then they were added to 100ml of ethyl alcohol anhydrous $\geq 99.5\%$ solvent and dissolved carefully in water bath shaker at 1200 rpm for 15 min to make a concentration of 15% GSE solution. The pH of the solution was adjusted by pH meter to make 7.2 utilizing few drops of NaOH. The solution was filtered through filter paper no 6 ⁽¹⁶⁾

Preparation of artificial carious lesion

Lesions were produced by immersing the specimens into glass tubes containing 20ml of demineralizing solution (50 mM acetic acid derivation, 2.25 mM CaCl₂ 2H₂O, 1.35 mM KH₂PO₄; 130 mM KCl for pH=5.0), for 72 hours, at room temperature ⁽¹⁷⁾.

Remineralization protocols

The demineralized dentin specimens were distributed among the four groups (n = 10):

- Group 1: Demineralized specimens were left as a negative control group.
- Group 2: Demineralized specimens were immersed in 15% (w/v) GSE.
- Group 3: Demineralized specimens were immersed in 1000 ppm aqueous solution of NaF.
- Group 4: Demineralized specimens were immersed in artificial saliva (Na₃PO₄ (3.90mM), NaCl (4.29mM), KCl (17.98mM), CaCl₂ (1.10mM), MgCl₂ (0.08mM), H₂SO₄ (0.50mM), NaHCO₃ (3.27mM) and distilled water the pH adjusted to 7.2 ⁽¹⁶⁾.

PH cycling

The specimens were subjected to pH-cycling (demineralization and remineralization) to simulate the cariogenic challenge ⁽¹⁸⁾ in conjunction with the treatments according to the abovementioned groups except for the 1st group. At first, the specimens were put in 50 ml of treatment solution according to each group for 10 minutes, then in 50 ml of demineralizing solution for 30 minutes lastly in 50 ml of artificial saliva for 10 minutes. All the solutions were in constant agitation. These pH cycles/treatments were performed six times a day for eight days. Between the treatment and pH cycling, the specimens were washed thoroughly with distilled water. Between the daily cycling, the specimens were stored in the artificial saliva at 37°C ⁽¹⁹⁾.

Final surface Micro-hardness test

Following the pH-cycling/treatment process, the specimens were put in the ultrasonic cleaner for cleaning any outstanding debris on their surfaces. At that point, the micro- hardness estimation of

the surface was taken and it was made between the initial hardness indentations. In this way, the final indentations were not in the same place as the initial ones and the markings were moved 100 μm far from the past indentations through a digital microscope. The loads utilized were the same as the initial ones (300 grams for 10 seconds).

Micromorphological examination

Four specimens from the same tooth were chosen representing the different groups to be examined under the ESEM (Philips 505, Eindhoven, Netherlands). The four recovered specimens were put in the ultrasonic cleaner for cleaning any outstanding deposits on their surfaces. Images were taken at 25 kV and a magnification of 2000X.

Statistical Analysis

Data analysis was performed by Kruskal-Wallis test followed by Dan Bonferroni Post hoc test to detect significance between groups. Statistical analysis was performed using SPSS version 22 software for Windows. P values < 0.05 were considered to be statistically significant.

RESULTS

Micro-hardness results

Table 1 and figure 1 demonstrate the correlation between micro-hardness results (Mean \pm SD and level of significance) for all groups. They demonstrate that the GSE recorded the highest mean value followed by NaF then the artificial saliva. Meanwhile, the demineralized group recorded the lowest mean value. The difference between GSE and NaF groups was statistically non-significant. Also there was an insignificant difference between the group of artificial saliva and demineralized one. Meanwhile, there was a significant difference between both of them and the artificial saliva and the demineralized group respectively as indicated by Duncan's Post hoc tests ($p < 0.05$).

TABLE (1) Comparison between micro-hardness results for all groups

Group	Mean baseline assessment	Mean Assessment after treatment	P value
Demineralized	68.3 \pm 4.7	20.2 \pm 2.7 ^b	0.005*
GSE	67.0 \pm 4.7	49.3 \pm 3.8 ^a	0.005*
NaF	66.9 \pm 4.1	49.2 \pm 5.0 ^a	0.005*
Artificial saliva	67.5 \pm 3.6	21.1 \pm 1.7 ^b	0.005*
P value	0.834	<0.001*	

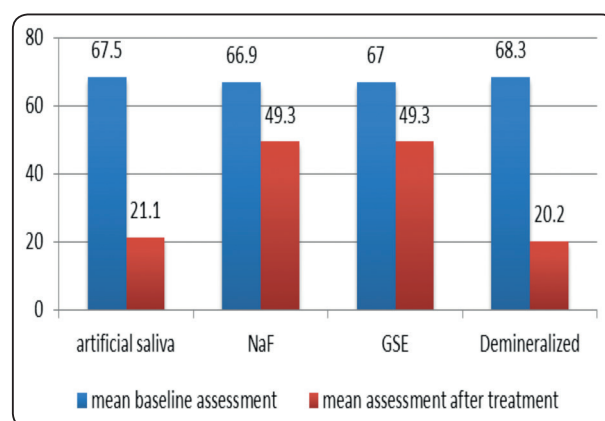


Fig. (1) Bar chart of micro-hardness means values for all groups

Environmental Scanning electron microscope (ESEM) results

The ESEM micrograph of the demineralized representative specimen, revealed widely opened dentinal tubules creating spongy like appearance. Some areas were completely demineralized and others were with partially demineralized peritubular dentin fig. (2). However, in the artificial saliva specimen, the micrograph showed opened dentinal tubules with different shapes, some had circular shape and others were with oval shape with small aggregates within the dentinal tubules fig. (3). The micrograph of the representative NaF and GSE specimens respectively fig. (4 & 5), revealed almost complete obliteration of dentinal tubules and peritubular dentin leaving a smoother surface.

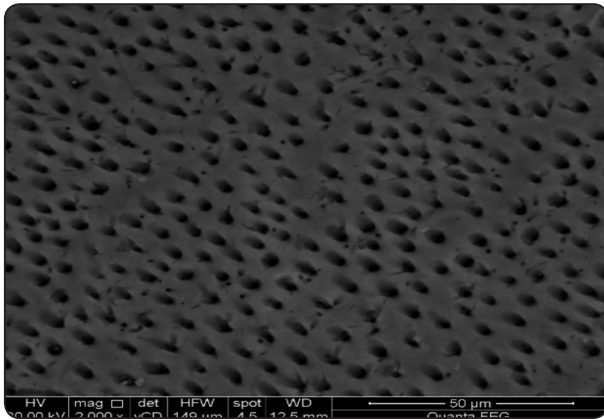


Fig. (2) ESEM showing demineralized dentin surface having opened dentinal tubules with different shapes, (circular or oval).

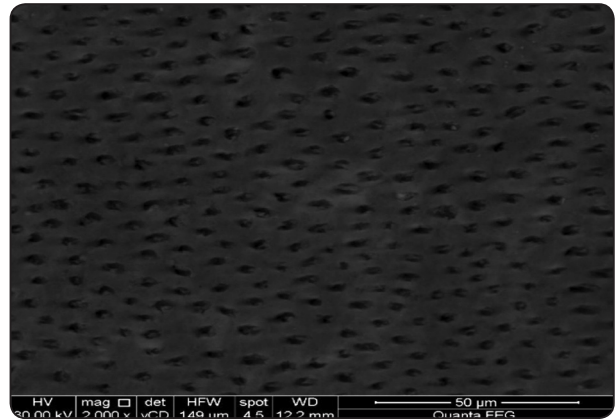


Fig. (3) ESEM showing dentin surface after storage in artificial saliva with small aggregates within the dentinal tubules.

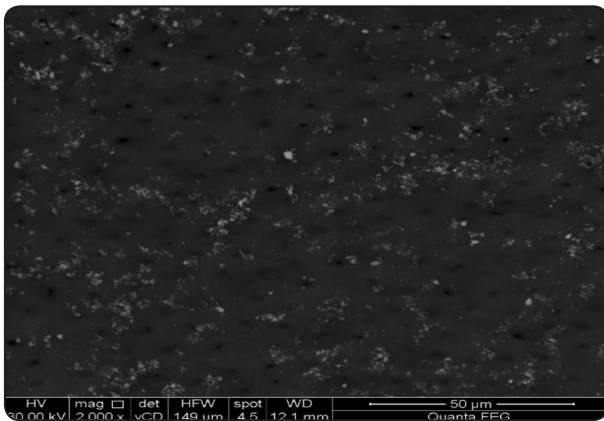


Fig. (4) ESEM showing dentin surface treated with NaF with almost complete obliteration of dentinal tubules and peritubular dentin.

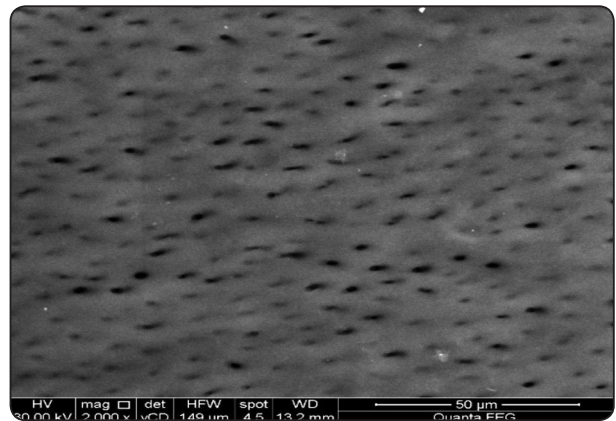


Fig. (5) ESEM showing dentin treated with GSE with almost complete obliteration of dentinal tubules and peritubular dentin.

DISCUSSION

By regulating the mineral balance favorably towards the remineralization, the caries lesion can be arrested or repaired through the regaining of some dissolved crystals, growth of surviving crystals and arrangement of new crystals⁽²⁰⁾. Because of the littler extent of dentinal crystallites and presence of tubules filled with fluids, the dentin demonstrates great affinity towards the demineralization. It occurs at a critical pH higher than that for enamel (critical $\text{pH} \approx 6.7$)^(21,22).

In addition, after the mineral disintegration, exposed dentine organic matrices are additionally broken down by proteolytic enzymes, for example, bacterial derived collagenases and host derived matrix metalloproteinases. All these constraints drive the dentin deterioration to be an irreversible process and less well-suited to remineralization^(23, 24). Therefore, the management of dentine caries is much more challenging, highlighting an urgent need to seek novel and alternative strategies. Many agents were used for inducing remineralization for such depleted weak dentin. The fluoride anti-cariogenic

effect can be credited to different reasons including enhanced calcium phosphate precipitation and fluoroapatite formation. Although caries preventive role of fluoride has been proved beyond any doubt, controlling this procedure of dentin deterioration utilizing fluoride alone seems questionable.

Collagen bio-alteration by exogenous collagen crosslinks, has been proposed to maintain, reestablish and enhance tissue biochemical and biomechanical properties⁽²⁵⁾. In particular, these properties are desirable for prevention and restoration of dentin caries. Also they enhanced collagen stability against proteolytic degradation. The stabilized collagen can additionally inhibit demineralization and improve remineralization. Albeit different crosslinking agents, for example, glutaraldehyde, formaldehyde, carbodimide and epoxy compounds, have been utilized to initiate exogenous crosslinks, their *in vivo* application has been generally constrained because of the cytotoxicity and instability after some time⁽²⁶⁾, that is why other natural alternatives are recommended.

The current study utilized an artificial caries model, in which a demineralization time of three days was chosen as a longer period may deplete the collagen matrix of mineral and consequently hinder the remineralization^(27, 28). Following such protocol, a lesion depth of around 150µm with a micro hardness being similar to a natural carious lesion at the same depth was obtained⁽²⁹⁾. Micro-hardness was utilized as an indirect method for detecting changes in mineral content that may reflect a decrease in mineral content⁽³⁰⁻³²⁾. Standardization of the obtained results were done by obtaining four specimens representing the different groups from the same tooth and so following the change in micro-hardness after the different treatments was definite. A natural remineralizing agent was represented by the artificial saliva and PA-rich GSE in contrast with fluoride were also utilized in the current study to stimulate dentin remineralization.

It had been proved that GSE successfully enhances the mechanical properties^(33, 34) and decreases the degradation rates of sound and caries affected dentine.⁽³⁵⁾ It has the capacity to interact with and modify the dentine collagen. GSE may also play a role in diminishing collagen degradation by its inhibitory impact on proteases, for example intrinsic metalloproteinases⁽³⁶⁾. Moreover, the protection of the collagen network may create a mechanical barrier to acid diffusion and mineral release and furthermore encourage mineral precipitation during the remineralization procedure. The GSE-treated group (fig. 5) delineated the formation of a well-defined outer layer when contrasted with the demineralized group (fig. 2). This layer might be the result of insoluble complex depositions formed by GSE when blended with the artificial saliva. In addition the GSE particles would link to the collagen, reinforcing the intertubular dentin to permit a more prominent remineralization process, as previously suggested for remineralization of artificial root caries⁽³⁷⁾. Similar findings were found in the fluoride treated specimen as nearly obliterated dentinal tubules were found (fig. 4) which indicated evidence for dentin remineralization. This finding was supported by micro hardness values.

Straightforward precipitation of minerals into the demineralized dentin matrix in the artificial saliva group (fig.3) might provide an increased mineral content however it may not really give an optimal interaction with the organic components of the dentin matrix⁽³⁸⁾. This could be the explanation behind the diminished micro-hardness values after submersion of specimens in the artificial saliva despite the fact that there were little deposits inside the dentinal tubules. The present study highlights the critical positive remineralizing impact of GSE. However, no one from the used remineralizing agents regained the micro-hardness of the intact enamel. The null hypothesis was partially rejected as both fluoride and GSE produced the same remineralizing agent but the artificial saliva was

inferior to them. Additional studies should be conducted to distinguish the active constituents of GSE and expand its impact on the tooth substrate and to find the optimal biocompatible remineralizing agent.

CONCLUSION

Within the conditions of the present study, grape seed extract is a promising nature driven remineralizing agent that can be compared with the gold standard fluoride. Artificial saliva alone cannot face the dentin demineralization.

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