Original Article

# Effect of Grape Seed Extract and CPP-ACP on Microhardness of Artificial Root Dentin Caries

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## Abstract

This study was an *in vitro* pH-cycling model that aimed to study and compare the effect of grape seed extract (GSE) and CPP-ACP on the microhardness of artificial root caries. Sixty sound human premolars extracted for orthodontics treatment were sectioned at the cervical portion of the root and embedded in resin. Nail varnish was coated on the root dentin surfaces except on a window of 4 mm. x 4 mm. Knoop hardness indentations were tested for baseline ($KHN_b$) and sixty specimens with KHNb between 60-70 were included. All specimens were stored in a demineralization solution for 96 hours to induce artificial root caries lesions and measured initial Knoop microhardness ($KHN_i$). Sixty specimens were randomly divided into four groups randomly (n=15 per group) including: the deionized water group (control group), the GSE group, the GSE + CPP-ACP group and the CPP-ACP group. The demineralized specimens were pH-cycled, six cycles per day for eight days, then a final microhardness test ($KHN_f$) was performed. The means of the four indentations of $KHN_b$, $KHN_i$ and $KHN_f$ from each specimen were analyzed. The means of differences in microhardness values between $KHN_f$ and $KHN_i$ (ΔKHN) among groups were analyzed using one-way ANOVA, followed by the Games-Howell post-hoc test at a significance level of 0.05. The results of this study indicated that all test groups had a significantly higher ΔKHN than the control group at ($p$<0.01). Both the GSE group and the GSE + CPP-ACP group showed a significantly higher ΔKHN compared to the CPP-ACP group at ($p$<0.01). The GSE group and the GSE + CPP-ACP group showed no statistically significant difference of ΔKHN ($p$<0.01). It was concluded that the GSE and the GSE+CPP-ACP increased microhardness values more than the CPP-ACP. All three interventions increased microhardness compared to the control group.

**Keywords :** CPP-ACP, Grape seed extract, Knoop microhardness

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## Introduction

Dental caries is a major cause of oral pain, and infection. It can be found in patients of various ages especially in older patients due to the decrease of the salivary flow rate which is the result of increased underlying diseases.[1-3] In addition, attachment loss and gingival recession occur frequently in older people, which increases the risk of bacterial adherence to root surface leading to incidences of root caries.[4] The success in treatment of root caries is challenging, because of the difficulty in moisture control on subgingival root surfaces. Furthermore, the bonding effectiveness between restorative materials to root dentin is less predictable.[5] According to minimal intervention concepts, if root caries have occurred, treatment options depend on the progression of the lesion. The most effective chemical agent for a non-surgical treatment of root caries lesions is the daily use of dentifrice containing 5,000 ppm fluoride.[6] However, fluoride concentrations of regular dentifrices of only around 1000 to 1500 ppm are widely used.[7] The prevalence of root caries has increased; therefore, additional chemical agents have been suggested. Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) can inhibit demineralization, and enhance remineralization.[8]

CPP-ACP is composed of caseinphosphopeptide (CPP) and amorphous calcium phosphate (ACP). Under acidic conditions, the application of CPP-ACP on the tooth surface increases the level of free calcium and phosphate ions, which substantially maintains a supersaturated state, increases buffer capacity, inhibits demineralization and enhances remineralization. It is a remineralizing agent that can promote dentin remineralization as well as enamel remineralization.[9-11] Furthermore, CPP-ACP has beneficial microbial ecological effects which can reduce microbial numbers, inhibit lactic acid production from polymicrobial biofilms and reduce adherence of bacteria to the tooth surface.[12]

Proanthocyanidins (PA) is a phenolic compound composed of a hydroxyl group that induces a chemical bond with collagen fibers. PA acts as a naturally-derived cross-linker which increases strength and stability of a collagen matrix.[5,13] PA has positive effects on oral health such as antimicrobial potential against cariogenic bacteria, inhibition of demineralization and remineralization enhancement in initial caries.[14,15] This phenolic compound can be found in natural extracts. Grape seed extract (GSE) is an over the counter nutritional supplement that is rich in PA. GSE from MegaNatural, Polyphenolics, (Madera, CA) was used in this study. It consisted of 97.8% PA according to data provided by the manufacturer. The concentration of GSE that can inhibit demineralization, and enhance remineralization is 6.5%.[16] At this concentration, it is harmless to humans.[17]

However, the inhibitory effect of GSE on demineralization and the synergistic effect of GSE and CPP-ACP on remineralization are still controversial. Hence, the objective of this study was to compare the effect of GSE and CPP-ACP on the microhardness of artificial root caries. The null hypothesis was the effects of GSE and CPP-ACP on microhardness of artificial root dentin caries were not different.

## Materials and methods

### Root dentin specimen preparations

The sample size of this study was calculated using the G Power program version 3.1. The reference data was referred from a previous study with an alpha of 0.05 and an 0.8 power of test. The effect size from the calculation was 0.72 based on the F test.[15] Sixty human premolars with completed root formation extracted for orthodontic treatment were used. The teeth were checked under a stereomicroscope with 10x magnification (StereomicroscopeSZ61, Olympus, Japan) in order to exclude teeth with defects at the crown and root of the teeth such as craze lines, crack lines, dental caries and restorations. Teeth were cleaned with ultrasonic scaler and non-fluoridated pumice and stored in 0.1% thymol solution no longer than one month before being used. The protocol was approved by The Research Ethics Review Committee for Research Involving Human Research Participants, Faculty of Dentistry, Chulalongkorn University.

Root dentin blocks, 6 mm. long, were prepared using a slow speed cutting machine (Isomet™ 1000, Buehler, IL, USA) which horizontally sectioned at the cementoenamel junction (CEJ) and 6 mm. apical to CEJ under water irrigation. The blocks were embedded in resin, and the buccal root surfaces emerged 1 mm. from the resin and were polished using 1000 grit and 2000 grit silicon carbide papers and aluminum oxide 0.05 micron, respectively. The specimens were cleaned with deionized water in an ultrasonic cleanser (Ultrasonic cleanser 5210, HEIDOLPH, Germany) for 15 min. Nail varnish (Revlon, USA) was coated on the root dentin surface except for a window of 4 mm. x 4 mm., measured by a digital caliper. The microhardness of root dentin blocks were tested (FM 700e type D, FUTURE-TECH, Japan) using the Knoop hardness indentation at a 25 g. load force for 15 s.[18] For a baseline ($KHN_b$), four indentations were performed below the top margin of nail varnish (started from CEJ of root) at 0.5 mm., 1.5 mm., 2.5 mm., 3.5 mm., respectively. Every indentation performed 1 mm. away from the left margin of the nail varnish (Fig. 1). Sixty specimens with $KHN_b$ between 60-70 were included in this study.

**Artificial root dentin caries lesions formation**

Sixty specimens were placed in a demineralization solution (2.2 mM $CaCl_2 2H_2O$, 2.2 mM $KH_2PO_2$, 50 mM acetate and KOH; pH 4.6) for 96 h. at 37 °C to create a 70–100 µm. depth of artificial root dentin caries lesions.[19] Specimens were cleaned with deionized water in ultrasonic cleanser for two mins. All specimens were measured for initial Knoop microhardness ($KHN_i$) using the Knoop hardness indentation at a 25 g. load force for 15 s.[18] Four indentations were performed below the top margin of the nail varnish (started from the CEJ of root) at 0.5 mm., 1.5 mm., 2.5 mm., 3.5 mm., respectively. Every indentation performed 2 mm. away from the left margin of the nail varnish (Fig. 1).

**Preparation of testing solutions**

**Group 1** deionized water (control group), **Group 2** GSE (LOT 1715601) (MegaNatural ™; Polyphenolics, Madera, CA, USA) ; The solution of 6.5% (w/w) GSE was prepared by dissolving 6.5 g. of GSE in 93.5 g. of deionized water.[20],**Group 3** 6.5% (w/w) GSE (LOT 1715601) + CPP-ACP (LOT 180220B) (GC Tooth Mousse ™; GC, Tokyo, Japan) ; 6.5 g. of GSE was added to CPP-ACP 93.5 g. and stirred well by a magnetic stirrer.[15], **Group 4** CPP-ACP (LOT 180220B) ; The commercially available form of CPP-ACP was used.

**Method of pH cycling**

The prepared specimens were randomly divided into four groups (n=15 per group) based on the remineralization treatment. All specimens were immersed in an acidic buffer (50 mM acetate, 2.25 mM $CaCl_2 2H_2O$, 1.35 mM $KH_2PO_4$, 130 mM KCl; pH 5.0) and stored in an incubator at 100% relative humidity 37 °C for 30 min. Then cleaned with deionized water in an ultrasonic cleanser for two min. All specimens were immersed in a neutral buffer (20 mM HEPES; 2.25 mM $CaCl_2 2H_2O$; 1.35 mM $KH_2PO_4$; 130 mM KCl; pH 7.0) and stored in an incubator at 100% relative humidity at 37 °C for ten min. Then, they were cleaned with deionized water in an ultrasonic cleanser for two min. Twenty mg. of treatment solution was measured by using a digital balance (HL-400, AND, Japan). Then 20 mg. of treatment solution was applied to the prepared surface using a paintbrush according to the allocated groups. The specimens were stored in an incubator at 100 % relative humidity 37 °C for ten min before cleaning with deionized water in an ultrasonic cleanser for two min. The pH cycles were repeated six times a day for eight days (Fig. 1). All solutions were freshly prepared prior to use and the specimens were kept in a neutral buffer overnight.[18]

The final microhardness test ($KHN_f$) was performed below the top margin of the nail varnish (starting from CEJ of root) at 0.5 mm., 1.5 mm., 2.5 mm., 3.5 mm., respectively. Every indentation was performed 3 mm. away from the left margin of the nail varnish.

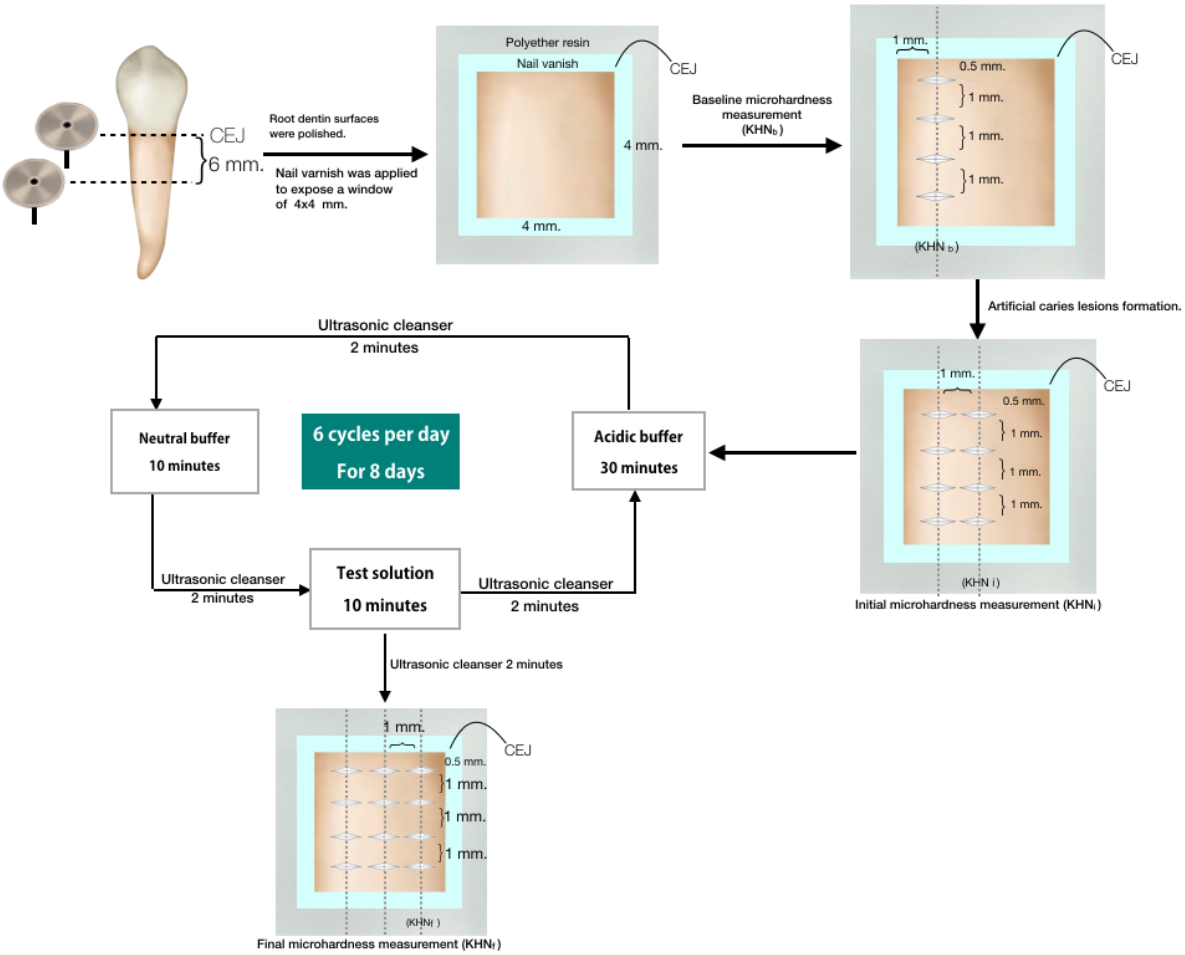


***Figure 1*** *Specimen preparation, pH cycling model and microhardness testing locations*

## Statistical Analysis

The means of four indentations of $KHN_b$, $KHN_i$ and $KHN_f$ from each specimen were analyzed. The differences of means of $KHN_b$ and $KHN_i$ among groups were compared using Kruskal-Wallis analysis followed by Dunn test. The differences of means of $KHN_f$ among groups were analyzed by one-way ANCOVA with a Bonferroni post-hoc test. For each group, means and standard deviation of the differences in microhardness values between $KHN_f$ and $KHN_i$ ($\Delta KHN$) were calculated. The differences in means of $\Delta KHN$ among groups and the differences in means of $KHN_b$, $KHN_i$ and $KHN_f$ from each group were compared using one-way ANOVA, followed by a Games-Howell post-hoc test. Normal distribution was analyzed by the Kolmogorov-Smirnov test. Homogeneity of variance was tested by the Levene's test. A significance level of $\alpha = 0.05$ was set for all statistical tests. The statistical analysis was performed using the SPSS program version 21.0.

## Results

The results presented in Table 1 revealed that there were no statistically significant differences between the means of $KHN_b$ and $KHN_i$ among four groups. The mean of $KHN_f$ of group 1 was significantly less than the three remaining groups. There were no statistically significant differences between the means of $KHN_f$ of group 2 and group 3. The mean $KHN_f$ value of group 4 was significantly more than the three remaining groups. The results of one-way ANOVA were presented in Table 1 indicating that the means of the $\Delta KHN$ of all test groups (group 2-4) were significantly higher than group 1 ($p<0.01$). Both groups

2 and 3 showed significantly higher means of the ΔKHN compared to group 4 ($p$<0.01). There was no statistically significant difference in the mean of the ΔKHN between group 2 and group 3 ($p$<0.01). The means of $KHN_f$ of groups 2, 3 and 4 were significantly more than the means of $KHN_i$. The means of $KHN_i$ and $KHN_f$ were significantly less than the means of $KHN_b$ in every group ($p$<0.01).

*Table 1* *Means and standard deviation of $KHN_b$, $KHN_i$, $KHN_f$ and the ΔKHN of the root dentin from each treatment group (n=15 per group)*

| Groups | Mean $KHN_b$ (SD) | Mean $KHN_i$ (SD) | Mean $KHN_f$ (SD) | Mean ΔKHN (SD) |
|---|---|---|---|---|
| Group 1 (DI) | 63.13 (1.86)$^{Aa}$ | 11.69 (2.93)$^{Ab}$ | 12.07 (2.28)$^{Cb}$ | 0.37 (1.01)$^{C}$ |
| Group 2 (GSE) | 62.86 (1.79)$^{Aa}$ | 13.42 (6.24)$^{Ac}$ | 25.39 (5.0)$^{Ab}$ | 11.97 (3.80)$^{A}$ |
| Group 3 (GSE+CPP-ACP) | 62.45 (1.37)$^{Aa}$ | 10.10 (2.4)$^{Ac}$ | 22.98 (3.41)$^{Ab}$ | 12.88 (2.62)$^{A}$ |
| Group 4 (CPP-ACP) | 64.10 (2.83)$^{Aa}$ | 10.36 (3.90)$^{Ac}$ | 16.23 (3.01)$^{Bb}$ | 5.88 (2.71)$^{B}$ |

*Different capital letters represent statistically significant differences in each column. Different small letters represent statistically significant differences in each row; SD : Standard deviation; DI : Deionized water; GSE : Grape Seed Extract*

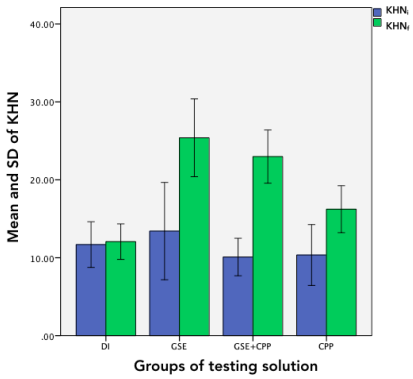


*Figure 2* *Bar chart represents means and standard deviation of $KHN_i$ and $KHN_f$ of group 1 DI, group 2 GSE, group 3 GSE+CPP-ACP, group 4 CPP-ACP (n=15 per group)*

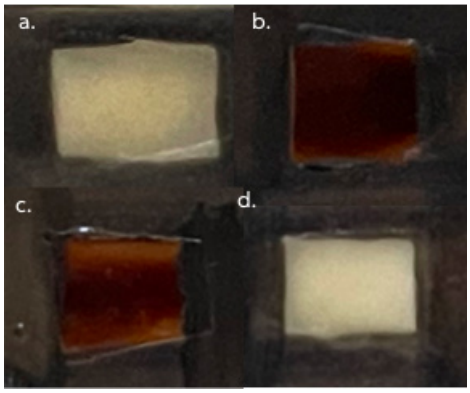


*Figure 3* *Specimens after being treated with testing solutions a. DI, b. GSE, c. GSE+CPP-ACP and d. CPP-ACP*

## Discussion

Based on the results of this study, the null hypothesis, that the effects of GSE and CPP-ACP on microhardness of artificial root dentin caries were not different, was rejected. The results of this study showed that GSE increased surface microhardness of artificial root dentin caries. The GSE used in this study was mainly composed of PA. PA is a natural collagen cross-linker which enhances the mechanical properties of both sound and caries-affected dentin.[13] PA forms a hydrogen bond with amine group of collagen fibers, which can replace water molecules and increase the modulus of elasticity resulting in strengthening dentin collagen matrix and improving the mechanical properties of teeth.[16, 21] These findings were confirmed by the study of Xie *et al.*[18] which used the same testing solutions, artificial root dentin caries formation method and pH cycling method.[18]

The mean of ΔKHN of the CPP-ACP group was significantly higher than the control group. The highest mean of ΔKHN was found in the GSE+CPP-ACP group. Although there were differences of artificial caries formation, testing solutions preparation and pH cycling method, these

results were in accordance with the study performed by Khamverdi *et al.*[20] The explanation is that CPP-ACP is a supplemental source of calcium and phosphate, thus they can keep oral cavity environment supersaturated with calcium and phosphate.[22] CPP contains casein peptides and a cluster of phosphoseryl residues. Casein peptides form a complex with calcium and phosphate and a cluster of phosphoseryl residues can stabilize nanocluster of ACP in supersaturated solution.[23] Combining CPP-ACP and GSE releases more amorphous calcium phosphate to remineralize the lesions due to the calcium-binding effect of PA and slightly acidity of PA, which decrease the pH of CPP-ACP.[24] Although this study showed that both of the GSE+CPP-ACP group and the GSE group gained surface microhardness values, there was no significant differences between the two groups. These results may infer that the increase of surface microhardness values were mainly because of GSE. Increasing the concentration of GSE and the contact time to the demineralized root dentin surface may gain more microhardness values.

In this study, demineralization solution (pH 4.6) was used to simulate the caries process to generate artificial dentin caries lesions. The pH-cycling model, employed in this study, alternated between remineralization solution (pH 7) and demineralization solution (pH 5). It intended to mimic the *in vivo* periodic alteration of pH. A neutral environment is periodically interrupted by an acid challenge when sugar is metabolized. An ultrasonic cleanser with deionized water was used to clean all specimens before changing the solution to maintain the real pH of the solutions. Although this was an in vitro study, pH-cycling models are widely used for dentin caries study because of reliability, efficiency and stability of the experiment. It can simulate the dynamic change of pH and variations in mineral saturation in an oral cavity that resemble the natural caries process.[25, 26]

Demineralized root dentin specimens were dried before $KHN_i$ and $KHN_f$ measurements. The demineralized dentin matrices might have collapsed and hydrogen bonds between collagen peptides may occur. Although hydrogen bonds between collagen peptides can be broken down by immersing the collapsed demineralized dentin in water for at least 20 min., the collapsed matrices could not be fully re-expanded.[27] The collapse of demineralized dentin collagen can reduce the remineralization efficacy of testing solutions. So the proper storage of specimens during the study is a concern to maintain proper demineralized dentin matrices.

The results of this study were represented in microhardness values. The microhardness test is a well-known method and commonly used for measuring demineralization and remineralization of dentinal caries.[28] Knoop microhardness value has a linear relationship with the depth of artificial caries. Furthermore, the results from the Knoop microhardness test were related to the results from microradiograph.[29,30] However, the microradiography can be helpful for further study to confirm the results of the microhardness test and to study the characteristics of demineralized and remineralized dentin. This study measured the surface microhardness values with different methods from the previous studies,[15,18] which measured the cross-sectional microhardness values. The results of this study were in agreement with Xie *et al.,*[18] who reported that the GSE group was significantly higher in microhardness values when compared to the control group. On the contrary, Epasinghe *et al.*[15] revealed no significant difference among the CPP-ACP, the PA and the control group. There was no significant interaction between depth and treatments in both studies.

According to the manufacturer's recommendation, CPP-ACP should be left undisrupted on the teeth for a minimum of three minutes. The longer CPP-ACP remains in the mouth, the more effective the results are. In this study, CPP-ACP was applied to the specimens for ten min. It may have gained more microhardness values than the three-min. application.

The preparation method of the GSE solution in this study was not complicated so patients can routinely prepare it themselves. In this study, GSE solution and GSE+CPP-ACP were applied on the root dentin for ten

minutes. In the oral cavity, there are clearance effects from oral salivary flow and movement of oral structures such as the tongue, the buccal mucosa and the lips. Because the GSE solution was in a liquid form that can be easily washed-out. In clinical situations, tray application may be helpful to retain the GSE solution longer in the oral cavity. The use of GSE and GSE+CPP-ACP can stain the teeth (Fig. 3), because the main composition of GSE is PA that contains red, purple and green pigments.[31] Stained teeth may have a negative impact on appearance, so the use of GSE in young patients and esthetic areas should be a concern. The stained teeth that are caused by GSE can be misunderstood as caries, so it is important to inform patients about this side effect and when doing an oral examination of these patients, it should be noted.

According to the results of this study, although all test groups gained significantly higher microhardness values than the control group, they were not able to gain the microhardness values to the same level as the normal root dentin. In Previous studies of GSE efficiency[15,18,20] and the present study were *in vitro* studies. So they could not simulate the real situation of the oral environment such as complex structures of the oral cavity and the hydrodynamics of saliva. The artificial dentin caries formation hardly imitated the complex microbiological aspect of natural caries formation. Further randomized control trials in situ studies or *in vivo* studies are needed in order to confirm the results of this *in vitro* study.

## Conclusions

Within the limitations of this study, it can be concluded that the GSE and GSE+CPP-ACP increased the microhardness values of artificial root dentin caries than CPP-ACP did. However, all three interventions increased more microhardness values of artificial root dentin caries compared to the control group.

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