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In-vitro investigation of remineralization efficacy of nanotechnological products and herbal-derived agents in primary teeth

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Abstract

Objective: The objective of this study was to evaluate the remineralization efficacy of nanotechnological products including casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), nanohydroxyapatite (nHA), bioactive glass, and herbal agents, such as cocoa, propolis, grape seed extract, rosemary, and ginger, in the initial enamel caries of primary teeth.

Materials and methods: In this study, 80 primary tooth enamel samples were used and categorized into groups 1–10 as follows: intact enamel, caries group, CPP-ACP, nHA, bioactive glass, cocoa, propolis, grape seed extract, rosemary, and ginger. To create initial enamel caries, all groups except group 1 were placed in a demineralization solution for 72 hours and then subjected to pH cycling for 7 days. Except for groups 1 and 2, remineralization materials were applied to the teeth in the other groups for 4 minutes for 7 days. Scanning electron microscopy-energy dispersive X-ray (SEM/EDX) analysis was subsequently performed on all teeth. Statistical analyses were performed using Kruskal–Wallis, H test, Dunn, Tukey's honestly significant difference, and one-way analysis of variance tests.

Results: Examination of the SEM images demonstrated that all remineralization materials formed calcified deposits on the enamel surface. EDX analysis revealed that, when Ca/P ratios were examined, there was a significant difference between the intact enamel group and the CPP-ACP group, and between the caries group and the CPP-ACP, nHA, bioactive glass and rosemary groups ($p<0.05$). No significant difference was noted between the remineralization materials ($p>0.5$).

Conclusions: All nanotechnological products and propolis, grape seed extract, cocoa butter, rosemary oil, and ginger oil showed remineralization activity in the initial caries of deciduous tooth enamel. Of all the remineralization groups, the grape seed extract group had the most homogeneous SEM image. CPP-ACP, nHA, bioactive glass, and rosemary increased the Ca/P ratio in enamel more than other remineralization materials.

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Introduction

Dental caries is most common in childhood and is considered a chronic disease by the World Health Organization (WHO) (1). Enamel structure of primary teeth in children is weaker than that of permanent teeth and caries lesions progress faster in such teeth; this consequently highlights the importance of preventive applications. The presencehealthyalth oral flora affects children's overall health, growth, and development. In addition to providing adequate dietary control and oral hygiene education to prevent caries in children, the application of remineralization agents at home by the patient's parents or professionally by dentists is very important, especially in those with high caries risk (2-4).

Currently, many agents are used for remineralization purposes. Although fluorine is considered the gold standard among these agents, the claims that high doses of fluorine may cause cognitive problems in children are met with concern by parents, although this has not been confirmed in the literature (5). Therefore, studies have examined other remineralization agents as alternatives to fluorine. Nanohydroxyapatite (nHA), bioactive glass (calcium sodium phosphosilicate, NovaMin®), toothpastes containing tricalcium phosphate (TCP), and nanotechnological agents— such as casein phosphopeptide amorphous calcium phosphate (CPP-ACP) and chitosan— are reportedly used for remineralization; in addition, herbal agents such as licorice root, Galla chinensis, theobromine, propolis, grape seed extract, rosemary, and ginger may have applications in remineralization treatments (6-8).

The aim of this study was to investigate the remineralization efficacy of nanotechnological products and plant-derived agents in primary tooth enamel in vitro.

Material and methods

This study was approved by the Ethics Committee of Dicle University Faculty of Dentistry. (29/12/2021-2021/60). Eighty extracted primary teeth that had fallen, had not undergone any dental treatment, and had no defects including caries and erosion, among others, on the enamel surface were used in the study. Soft residues on the tooth surface were removed with periodontal curettage. A fluorine-free fine-grain polishing paste was applied to the enamel surfaces with a polishing brush. The collected teeth were stored in 0.1% thymol at +4°C until the time of the procedure. The teeth were separated from their crowns using a micromotor device (NSK FX23, Japan) with a diamond separator at low speed under water cooling. A 2 × 2-mm tape was placed on the buccal surfaces of the teeth and the surrounding areas were painted with two coats of acid-resistant nail polish. The tooth specimens were randomly selected and divided into 10 groups with 8 specimens in each group as follows (**Table 1**).

The demineralization solution (1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 50 mM Acetic acid) was prepared with a pH of 4.8 and the remineralization solution (1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl, 20 mM Hepes solution) was prepared with a pH of 7.0. After the teeth were soaked in an artificial saliva

Table 1: Study groups

Groups	Experiment
Group 1	:Positive control group (Intact enamel group)
Group 2	:Negative control group (Caries group)
Group 3	:CPP-ACP group (Tooth Mousse, GC, Japan)
Group 4	:Toothpaste group containing nHA (Splat Kids Natural Toothpaste, Splat, UK)
Group 5	:Fluoride toothpaste group with bioactive glass (Sensodyne Repair & Protection, NovaMin Tech. Inc, GSK, UK)
Group 6	:Cocoa butter group (Arifoglu, Turkey)
Group 7	:Propolis group (Arifoglu, Turkey)
Group 8	:Grape seed extract group (Arifoglu, Turkey)
Group 9	:Rosemary oil group (Arifoglu, Turkey)
Group 10	:Ginger oil group (Arifoglu, Turkey)

Table 2: pH cycle and remineralization agents of the study groups

Duration	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9	Group 10
18 Hours	REMINERALIZATION							
5 Seconds	DISTILLED WATER							
4 Minutes	CPP-ACP	nHA	Bioactive glass	Cocoa butter	Propolis	Grape seed extract	Rosemary oil	Ginger oil
5 Seconds	DISTILLED WATER							
4 Minutes	CPP-ACP	nHA	Bioactive glass	Cocoa butter	Propolis	Grape seed extract	Rosemary oil	Ginger oil
6 Hours	DEMINERALIZATION							

solution for 30 minutes, all the teeth were dried with an air spray for approximately 5 seconds. Except for group 1, the teeth in all other groups were placed in the demineralization solution for 72 hours and then subjected to pH cycling in an oven at 37°C for 7 consecutive days. The teeth were incubated in the demineralization solution for 6 hours within a day. The teeth were then washed with distilled water for 5 seconds to prevent mixing of the solutions. The teeth were maintained in the remineralization solution for 18 hours. Group 1 was not subjected to any solution, and group 2 was subjected only to pH cycling without any remineralization material. Remineralization materials were applied to all teeth in all groups except groups 1 and 2. Remineralization materials were applied to the teeth with a bonding brush (Microbrush, medium thickness, USA) 2 times a day for 4 minutes before and after demineralization. After the application of the materials, the specimen surfaces were washed with distilled water for 5 seconds to remove the material and then kept in remineralization solution. These procedures were performed sequentially for 7 consecutive days (**Table 2**). After this cycle, the teeth were placed in artificial saliva with a pH of 7.0 at 37°C for 30 minutes to mimic the oral environment. The teeth were dried in an oven at 37°C for one day and prepared for analysis. The elemental distributions of all samples were visualized by energy dispersive spectroscopy using a Scanning electron microscopy-energy dispersive X-ray (SEM/EDX) instrument (SEM, LEO-EVO40, Cambridge UK and EDX unit, Bruker-125 eV, Berlin Germany). The surfaces on the samples were examined at ×1000 magnification. The elemental distributions of the samples scanned via EDX analysis were determined according to the atomic percentage (at%) and mass percentage (wt%) of the elements. The Ca, P, and O elements were evaluated for analysis. The SEM/EDX analysis spectra for each surface were obtained by scanning five times on each surface. Mean spectra values were used in the statistical analyses of the elements.

Statical analysis

In this study, an effect size of 0.50, 5% margin of error, 95% confidence level, the theoretical power value of the study was taken as 81% for the parameters to be used within the scope of SEM/EDX analysis, and the study was conducted with a total of 80 observations for 10 groups, 8 observations in each group. A licensed GPower 3.1 package program was used for power calculation. The data obtained from this study were analyzed with a licensed SPSS V23 (IBM, Armonk, NY, USA) package program. The compliance with normal distribution was examined via the Shapiro–Wilk test. Kruskal–Wallis test was used for intergroup

Table 3: Comparison of mean ± standard deviation (SD) and median (minimum-maximum) wt% and at% Ca, P and Ca/P values by group

	wt% Ca		at% Ca		at%P		wt% Ca/P		at% Ca/P	
	Mean ± SD	Median (min–max)	Mean ± SD	Median (min.–max)	Mean ± SD	Median (min.–max)	Mean ± SD	Median (min.–max)	Mean ± SD	Median (min.–max)
Group 1	36.85 ± 3.04	35.90 (bc)	21.40 ± 2.42	20.68 (bc)	13.85 ± 0.71	13.72 (bc)	2.00 ± 0.14	1.91 (bc)	1.54 ± 0.11	1.48 (bc)
Group 2	32.17 ± 1.18	32.65 (b)	17.88 ± 0.87	18.26 (b)	13.08 ± 0.58	13.03 (b)	1.77 ± 0.06	1.75 (b)	1.37 ± 0.05	1.36 (b)
Group 3	46.76 ± 3.14	45.70 (a)	29.65 ± 2.72	28.74 (a)	14.94 ± 0.50	15.01 (ac)	2.58 ± 0.30	2.47 (a)	1.99 ± 0.23	1.92 (a)
Group 4	42.50 ± 5.40	40.64 (ac)	26.17 ± 4.78	24.52 (ac)	15.02 ± 0.80	14.93 (ac)	2.25 ± 0.36	2.12 (ac)	1.74 ± 0.28	1.64 (ac)
Group 5	43.15 ± 3.35	42.26 (ac)	26.70 ± 3.02	25.87 (ac)	15.48 ± 0.91	15.42 (a)	2.23 ± 0.18	2.18 (ac)	1.72 ± 0.14	1.68 (ac)
Group 6	40.23 ± 4.38	40.16 (ab)	24.24 ± 3.57	24.11 (ab)	14.84 ± 1.18	15.02 (ac)	2.11 ± 0.25	2.05 (ab)	1.63 ± 0.19	1.58 (ab)
Group 7	40.52 ± 3.05	40.36 (ab)	24.51 ± 2.57	24.42 (ab)	15.35 ± 0.87	15.30 (ac)	2.06 ± 0.13	2.04 (ab)	1.60 ± 0.10	1.58 (ab)
Group 8	39.62 ± 6.83	38.10 (ab)	23.81 ± 5.75	22.49 (ab)	14.18 ± 0.96	14.35 (ab)	2.16 ± 0.42	1.95 (ab)	1.67 ± 0.32	1.51 (ab)
Group 9	45.26 ± 3.60	45.73 (ac)	28.15 ± 3.00	28.69 (ac)	14.09 ± 1.49	14.17 (ab)	2.62 ± 0.43	2.65 (ac)	2.02 ± 0.33	2.05 (ac)
Group 10	38.67 ± 3.18	37.75 (ab)	22.97 ± 2.65	22.21 (ab)	14.84 ± 0.98	15.03 (ac)	2.00 ± 0.12	1.95 (ab)	1.55 ± 0.09	1.51 (ab)
Test ist.	44.659		44.255		5.103		44.702		44.785	
p	<0.001*		<0.001*		<0.001**		<0.001*		<0.001*	

comparisons of non-normally distributed data. One-way analysis of variance was used to compare the normally distributed data according to the groups and multiple comparisons were analyzed with Tukey's honestly significant difference test. The analysis results were analyzed as mean ± standard deviation and median. Statistical significance was indicated by $p<0.05$.

Results

When the SEM images obtained were examined, it was observed that heterogeneous calcified mineral deposits covered the porous structures in all remineralization materials. The grape seed extract group showed the most homogeneous image. Examination of the SEM images of the enamel of primary teeth revealed that the regular keyhole structure of the enamel in the intact enamel group and the regular keyhole structure of the enamel in the caries group were disrupted (**Figure 1A-1B**). In the CPP-ACP group, it was observed that the porous structures in the demineralization group were lost and covered with calcified deposits. Since the observed calcified deposits were irregular, the remineralization formed can be considered heterogeneous. The nHa group showed that the heterogeneity of the calcified deposits with crack lines and irregular calcified deposits on the enamel surface was similar to the CPP-ACP group; however, not as dense as that of the CPP-ACP group, and the porous structures could not be completely covered. The fluoridated group

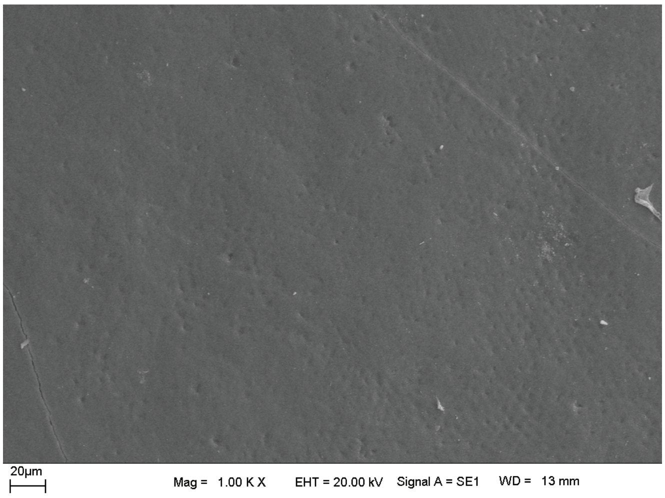

Figure 1A: SEM image of intact enamel surface at

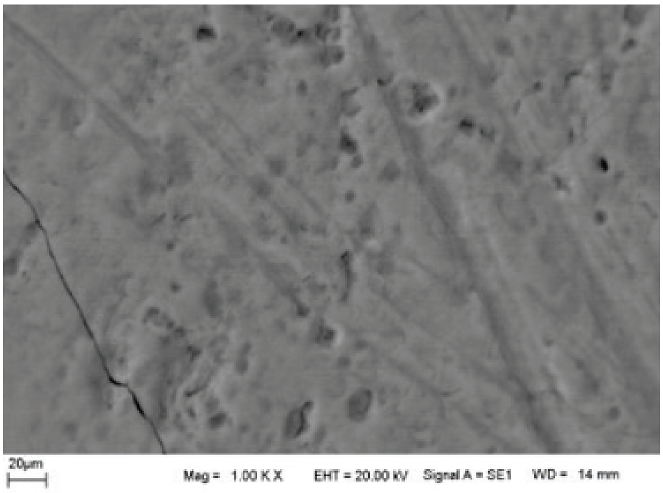

Figure 1B: SEM image of demineralized enamel surface

containing bioactive glass showed that the irregular calcified deposits largely covered the enamel surface, and the heterogeneity of the calcified deposits was similar to that of nHa group. (**Figure 2A**). In the cocoa butter group, crack line and irregular calcified deposits were observed on the enamel surface. Furthermore, the density of calcified deposits was lower than that of CPP-ACP, nHA and bioactive glass groups. In the propolis group, crack lines and irregular calcified deposits were observed on the enamel surface. The density of calcified precipitates was similar to that of nHA group. In the grape seed extract group, a crack line and irregular calcified deposits are observed on the enamel surface. It was observed that the density of calcified deposits was similar to that of nHA and propolis group and the deposits were more homogeneous than the other groups. In the rosemary oil group, irregular calcified deposits were observed on the enamel surface. The density of calcified precipitates was similar to that of nHA, propolis and grape seed groups (**Figure 2B**). In the ginger oil group, crack line and irregular calcified deposits were observed on the enamel surface. Furthermore, in this group, the heterogeneity of calcified deposits was higher than the other groups.

Comparison of mean and median wt% and at% Ca, P and Ca/P values according to the groups are given in **Table 3**. The mean wt% and at% values of Ca, P, and Ca/P ratios increased in all remineralization groups compared to the intact enamel and caries groups. No statistically significant difference was noted between the groups treated with remineralization materials in terms of EDX values ($p>0.001$). A statistically significant difference was found between the CPP-ACP group and the intact enamel group ($p<0.001$). A statistically significant difference was found between the caries group and the CPP-ACP, nHA, bioactive glass, and rosemary groups ($p<0.001$). CPP-ACP, nHA, bioactive glass, and rosemary increased Ca values more than other remineralization materials.

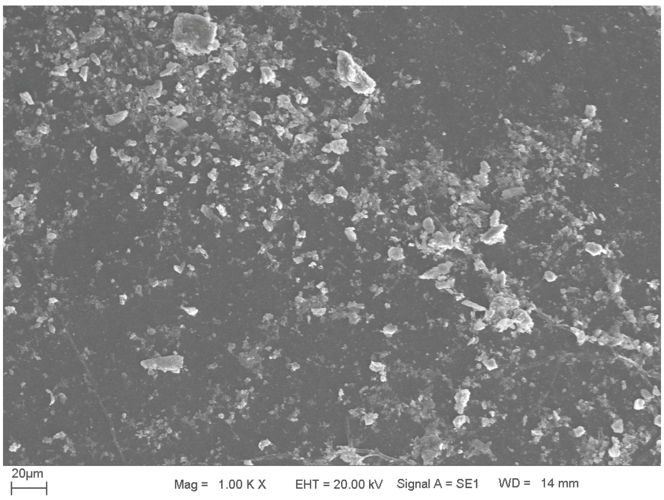

Figure 2A: SEM image of the remineralized enamel surface of the bioactive glass group

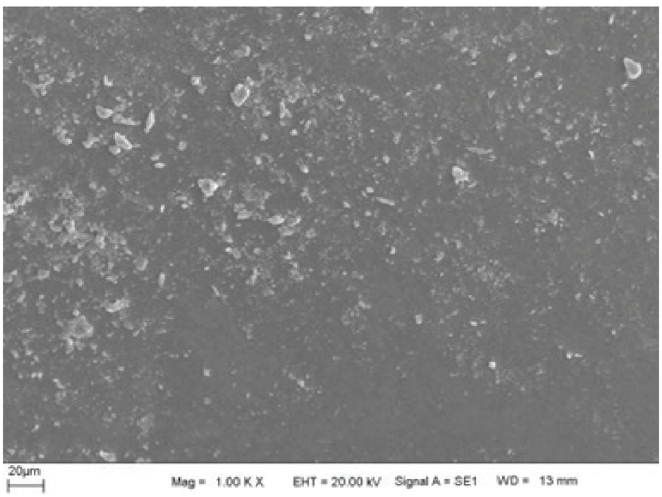

Figure 2B: SEM image of the remineralized enamel surface of the rosemary group

A statistically significant difference was found between the intact enamel group and the bioactive glass group ($p<0.001$). A statistically significant difference was found between the caries group and the CPP-ACP, nHA, bioactive glass, cocoa, propolis, and ginger groups ($p<0.001$). CPP-ACP, nHA, bioactive glass, cocoa, propolis, and ginger increased P values more than other remineralization materials.

A statistically significant difference was found between the intact enamel group and the CPP-ACP group ($p<0.05$). A statistically significant difference was found between the caries group and the CPP-ACP, nHA, bioactive glass, and rosemary groups ($p<0.05$). CPP-ACP, nHA, bioactive glass, and rosemary increased Ca/P value more than other remineralization materials. Although there was no significant difference between the remineralization groups, the highest Ca deposit density was noted in the CPP-ACP and rosemary groups.

Discussion

To the best of our knowledge, no study has quantitatively examined the remineralization activities of nanotechnological products and propolis, grape seed extract, cocoa butter, rosemary oil, and ginger oil in the initial caries of primary tooth enamel.

Studies have reported that dental caries, which is a general public health problem, is preventable (9,10). The progression of initial enamel caries, which occurs with recurrent acid attacks and continuous decrease in pH during the demineralization process, can be reversed by increasing implementation of preventive practices (11). Early childhood caries (ECC), which is defined as caries lesion with or without multiple cavities in children aged 71 months and younger, and tooth loss caused by filled surfaces or caries, is a global public health problem that requires complex treatment procedures and causes high costs when it is not prevented (12,13). Fluorine and fluorine compounds are recognized as the most preferred materials in preventive dentistry applications owing to their high remineralization efficiency, antibacterial properties and easy accessibility. However, owing to the parents' rejection of fluorine and the tendency towards natural remedies, the remineralization effects of herbal agents have started to be investigated (6,14). Therefore, in our study, the efficacy of different remineralization agents against fluorine in primary tooth caries, which threatens the health of the permanent dentition as well as the general health of children, was investigated comparatively.

Kamath et al (15) determined that nHA, TCP, CPP-ACPF, and fluoride had remineralization efficiency in primary teeth and no significant intergroup difference was noted. Manoharan et al (16) found no significant difference between the remineralization efficiency of bioactive glass and CPP-ACPF in permanent teeth. Shawky et al (17) reported that the remineralization efficiency of cocoa and fluoride in permanent teeth was significantly higher than that in the caries group. Taneja et al (18) reported that bioactive glass, NHA, fluoride, and cocoa caused remineralization in permanent teeth and no significant intergroup difference was noted. Amalina et al (19) reported that CPP-ACP, propolis, and the combination of these two had remineralization capacity in permanent teeth; however, no significant intergroup difference was noted. Mirkarimi et al (20) reported that the remineralization efficiency of grape seed extract in primary teeth was high. Hassan et al (21) reported that rosemary and ginger caused more remineralization in permanent teeth, but there was no significant difference between these two herbal products. Similar to the EDX studies in the literature, all agents used in our study were found to be effective in the remineralization of primary teeth and there was no significant difference between the groups.

Huang et al (22) and Möller et al (23) observed in their studies that the structure of intact enamel in permanent teeth showing integrity in the SEM analysis disappeared after the formation of the initial enamel caries and appearance of the micropores. Herein, we noted that this regular structure was lost and micropores increased in initial enamel caries. This observation was consistent with those reported in the literature. Kamath et al (15) reported irregular calcified deposits in the SEM imaging of nHA group in primary teeth, Manoharan et al (16) reported irregular calcified deposits in the SEM analysis of bioactive glass group, and Özalp et al (24) reported irregular calcified deposits in the SEM analysis of propolis. In the study conducted by Nagi et al (25), in which the SEM images of grape seed extract were examined, irregular small calcifications were observed. In our study, in accordance with the literature, SEM analysis of all remineralization materials showed that heterogeneous calcified mineral deposits covered the porous structures.

Conclusions

Remineralization efficacy of fluoride toothpaste containing cocoa butter, propolis, grape seed oil, rosemary oil, ginger oil, CPP-ACP, nHA, bioactive glass was observed in the primary teeth. In addition to fluoride and nanotechnological products, we suggest that plant-derived formulas can be used in routine oral hygiene practices in children.

Conflict of interest: The authors report no conflict of interest.
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Ethical approval: This study was approved by the Ethics Committee of Dicle University Faculty of Dentistry (29/12/2021-2021/60).
Informed consent: Written informed consent was obtained from all individual participants and/or their gaurdians.
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Peer-review: Externally. Evaluated by independent reviewers working in at least two different institutions appointed by the field editor.
Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Contributions
Research concept and design: EBH, EA
Data analysis and interpretation: EBH
Collection and/or assembly of data: EBH, EA
Writing the article: EBH
Critical revision of the article: EBH, EA
Final approval of the article: EBH, EA
All authors read and approved the final version of the manuscript.

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