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EFFECT OF WHEY EXTRACT AND NANO-HYDROXY APATITE POWDER MIXED WITH OLIVE OIL ON MICROHARDNESS AND MICROMORPHOLOGY OF WHITE SPOT LESION

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ABSTRACT

Background: This study was performed to examine the effect of Whey extract and nano-hydroxyapatite powder mixed with olive in different times of application on the microhardness and micromorphology of white spot lesion.

Materials and Methods: A total of 16 premolars were selected. The samples were sectioned mesiodistally into two halves to obtain 32 specimens, then divided into two groups according to materials used; A1: Whey extract, A2: Nano-hydroxyapatite paste. The two groups were subdivided into two subgroups according to the time of assessment; B1: after 10 minutes and B2: after 8 days. All the samples were treated by demineralizing solution for 72 hours, and then the tested materials were applied. Microhardness test was performed three times for each sample, at baseline, after demineralization, and after treatment for each period of time (after 10 minutes and after 8 days). Two representative samples from each group (one representing 10 minutes and the other representing 8 days) and two samples of sound and one for demineralized enamel were selected for SEM.

Results: Microhardness results revealed that the two materials show a significant increase in microhardness with no significant difference between them. SEM demonstrated improvement in demineralized enamel surface defects in the two test groups.

Conclusion: Whey extract and nano-hydroxyapatite powder mixed with olive oil are considered an effective therapy to re-mineralize the white spot lesion of the enamel surface regardless of the time of application (10 minutes or 8 days).

KEYWORDS: Nano-hydroxy apatite paste, Whey extract, White spot lesion.

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INTRODUCTION

Dental caries is the most common dental disease and a significant public health issue [1]. Early detection and treatment are possible, but the condition is irreversible once the incipient lesion turns into cavitation. Because of this, it's crucial to prevent caries from forming in its early stages rather than creating treatment options for its advanced stages [2].

A white patch of demineralized enamel known as a white spot lesion (WSL) can be seen on the surface of the teeth. It is the first stage of a carious lesion, and the activity of bacterial plaque determines its cause. Due to the positioning of the bracket, which encourages plaque buildup and the development of white spots, this lesion is a frequent adverse reaction for patients wearing fixed orthodontic appliances [3].

Nowadays, remineralization of non cavitated carious lesions is prioritized as a non-invasive treatment option in order to slow the advancement of the disease, and improve the appearance, strength, and functionality of teeth [4].

Dairy products such as milk, cheese, and yogurt containing Casein Phosphopeptide Amorphous Calcium Phosphate (CPP- ACP) are effective in preventing dental caries. During the process of cheese manufacturing Whey is left over when milk is coagulated and has everything soluble from milk after PH dropping to 4.6 during the coagulation procedure. It is 5% of lactose solution in water with lactalbumin and few liquid contents [5].

Nano-hydroxyapatite (Nano-HA) was considered promising in tooth re-mineralization due to its resemblance to the bone and mineral composition of teeth, biocompatibility, and bioactivity. Nano-HA particles resemble dental apatite in terms of crystal structure and morphology [6]. Due to its tiny size, Nano-HA has a significant potential to penetrate through the enamel rods and has a great ability to repair lesions [7].

Therefore, this in vitro study was conducted to examine the effect of Whey extract and nano-hydroxyapatite powder mixed with olive oil at different times of application on the microhardness and micromorphology of white spot lesions. The null hypothesis for the present study was that the Whey extract and nano-hydroxyapatite paste can produce re-mineralization to white spot lesions.

MATERIALS AND METHODS

This research was performed after the approval of the research ethical committee of the Faculty of Dental Medicine, Al-Azhar University for Girls, in accordance with international guiding principles, Code: REC-OP-23-02.

Sample size determination

Based on Rezvani et al (2015)[8] ,and Haghgoo et al (2011)[9]; and Using G power statistical power Analysis program (version 3.1.9.4) for sample size determination[10] , a total sample size (n=32 subdivided to16 in each group) will be sufficient to detect a large effect size (f)=0.71, with an actual power (1-β error) of 0.8 (80%) and a significance level (α error) 0.05 (5%) for two-sided hypothesis test.

Preparation of Whey extract solution

It was prepared after the centrifugation of yogurt at 4000 g at 25°C for 10 minutes by using an electric lab centrifuge (80-2). After three rounds of centrifugation, the yogurt was separated into 2 fractions; Yogurt precipitate is the insoluble fraction at the bottom, while Whey (supernatant), the soluble fraction containing CPPs, is still present in suspension [11]. In this study, the soluble fraction was used.

Preparation of nano-hydroxyapatite paste

The nHA powder was obtained from Nano-Tech Egypt for Photo-Electronics Company, and then the powder and liquid were measured on a paper pad by using a sensitive balance in a 1:1 weight ratio of olive oil to nHAP powder to form a paste which can be applied on the tooth surface [12].

Teeth selection and preparation

The study involved a total of 16 sound human premolars. From the ages of the patient (18 to 25) years, all collected teeth were extracted for orthodontic reasons. To rule out fractures, cavities, enamel abnormalities, or other flaws, the chosen teeth were visually inspected. In order to get rid of any plaque, blood, or remaining periodontal ligaments, they were scaled by hand scaler [13]. The teeth were prepared by sectioning the crown horizontally to separate the coronal portion from the radicular portion of each tooth under water coolant using low speed double faced diamond disc (Besqualdia –disc NY 11373, USA size 22mm), mounted in a grinding machine (Demco alloy grinder, dental maintenance co., INC..BONSALL, CALIF., U.S.A), after which the crowns were cut in half in a mesiodistal orientation to produce 32 samples [5]. On the buccal and lingual surfaces of the sectioned samples, a window measuring 4x4 mm was made in the middle; the remaining surface was then painted with acid-resistant nail polish and left to dry [11]. The samples were mounted with their enamel surface facing upward in a mold filled with self-curing acrylic resin (figure 1). The samples were taken out of the mold when the acrylic resin had dried.

Preparation of white spot lesion

All the samples were immersed for 72 hours at room temperature in 20 ml of the demineralizing solution. It consisted of 2.2 mM calcium chloride dihydrate, 2.2 mM sodium hydrogen phosphate dihydrate, and 0.05 M lactic acid solution ($2H_2O$, $CaCl_2$ = 2.2 mM; $2H_2O$, NaH_2PO_4 = 2.2 mM; lactic acid = 0.05 M) at pH 4.5[12]. The solution and the paste were exchanged every 24 hours to keep the pH constant. All the samples were rinsed with distilled water for 10 minutes while being stirred after artificial carious lesions were created, and they were then allowed to air dry.

Remineralization procedure

After demineralization, depending on the re-mineralizing material, the samples were split into two groups of 16 each. Group (A1): Whey extract solution; the demineralized samples were immersed into 20 ml of Whey extract solution, group (A2): NHA paste group; the demineralized samples were painted with a paste of nano-hydroxyapatite powder mixed with olive oil using micro brush(figure 2). Each group was subdivided into 2 subgroups (8 each) according to the time of application (B1) for 10 minutes and (B2) for 8 days. In 8-day subgroups, the solutions and the paste were daily exchanged. This procedure was carried out at room temperature.

Microhardness assessment

The microhardness measurement of all the samples was performed three times. At baseline, following demineralization and following treatment application (after ten minutes and after eight days) (figure 3). The samples' microhardness was measured using the digital display Vickers Micro-hardness tester (Model HVS-50, LaizhouHuayin Testing Instrument Co., Ltd., China) with a Vickers diamond indenter and a 20X objective lens. The specimen's surfaces were subjected to a 100g load for 15 seconds. Each specimen's surface was indented three times, each one evenly spaced around a circle and not more than 0.5 mm apart from one another. Vickers values were transformed into micro-hardness values by measuring the indentations' diagonal length with the built-in scaled microscope.

Scanning electron microscope (SEM)

Two samples from each group were examined (one sample representing 10 minutes and the other representing 8 days). Also, two samples of sound enamel and one of demineralized were examined to assess the change in surface morphology after treatment. Images were captured using an X1000 magnification lens at a distance of 11.8 mm from the sample and an excitation voltage of 20 kV.

Fig. (1): Sample imbedded in acrylic

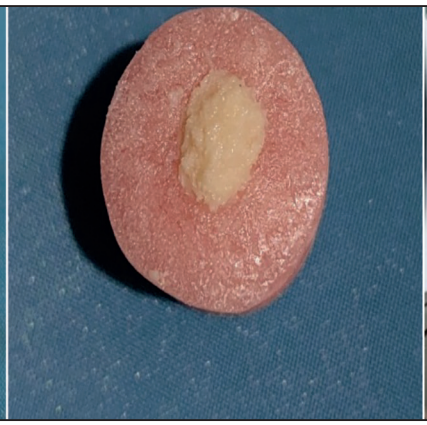

Fig.(2): nHAP paste on tooth sample resin

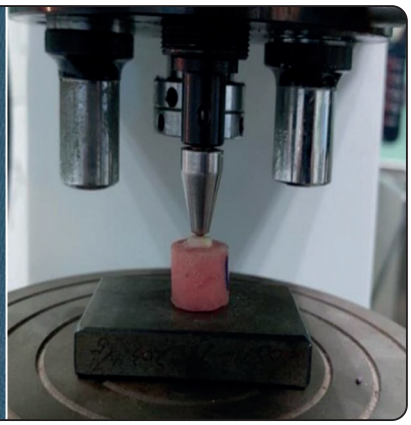

Fig. (3): Sample on Vickers microhardness machine

Statistical Analysis

All the information was gathered, collated, and analyzed. In each test, the mean and standard deviation values were computed for each group. Using the Shapiro-Wilk and Kolmogorov-Smirnov tests to determine whether the data were normal, a parametric (normal) distribution of the data was found. To contrast the two groups in unrelated samples, the t-test for independent samples was employed. $P \leq 0.05$was used as the criterion of significance. The statistical analysis was carried out using IBM® SPSS® Statistics Version 20 for Windows.

RESULTS

Microhardness results:

Regarding the effect of the re-mineralizing agents, the comparisons between the microhardness values of each re-mineralizing agents' group within each time of application (10 minutes and 8 days) are shown in Table (1) &Figure (4). Results showed that, in the Whey extract group, there was a statistically significant difference between baseline, after demineralization, and after re-mineralization, where (p<0.001) at 10 minutes and (p=0.003) at 8 days. The highest mean values were found in baseline, while the least mean value was found after de-mineralization and there was a significant increase in the microhardness values after re-mineralization compared with the values after de-mineralization, as the microhardness values of Whey extract group after demineralization and re-mineralization at 10 minutes of application were 254.60±3.17 and 257.57 ±4.65 respectively and at 8 days of application were 257.15±3.75 and 260.53±3.71 respectively. Also, in the nano-hydroxyapatite group, a statistically significant difference was found between baseline, after de-mineralization, and after re-mineralization, where (p<0.001) at 10 minutes and (p=0.025) at 8 days. The highest mean values were found in baseline, while the least mean value was found after de-mineralization and there was a significant increase in the microhardness values after re-mineralization compared with the values after de-mineralization, as the microhardness values of nano-hydroxyapatite group after de-mineralization and re-mineralization at 10 minutes of application were 253.89±2.67 and 257.17±3.72 respectively and at 8 days of application were 256.72±3.15 and 259.54±5.60 respectively.

Comparisons between the microhardness values of each re-mineralizing agents' group regardless of the time of application (overall of 10 minutes and 8 days) are shown in Table (2) & Figure (5). In all re-mineralizing agents' groups, Whey and nano-hydroxyapatite, a statistically significant difference

(p<0.001) existed between the baseline, after demineralization, and after re-mineralization. The considerably least mean value was discovered after demineralization, whereas the significantly greatest mean values were discovered in baseline, followed by after re-mineralization.

TABLE (1) Descriptive statistics and comparison between microhardness values of each of the two re-mineralizing agents' groups at baseline, after demineralization, after remineralization within each time of application (10 minutes and 8 days)

Re-mineralizing agents		Baseline		After demineralization		After remineralization		p-value
		Mean	SD	Mean	SD	Mean	SD	
Whey	10 minutes	268.67 [a]	5.59	254.60 [c]	3.17	257.57 [b]	4.65	<0.001*
	8 days	266.47 [a]	8.20	257.15 [c]	3.75	260.53 [b]	3.71	0.003*
Nanohydroxyapatite	10 minutes	268.93 [a]	4.63	253.89 [c]	2.67	257.17 [b]	3.72	<0.001*
	8 days	264.75 [a]	9.79	256.72 [b]	3.15	259.54 [a]	5.60	0.025*

Means with different letters in the same row indicate significant difference. ****: significant (p<0.05)***

TABLE (2) Descriptive statistics and comparison between microhardness values of each of the two re-mineralizing agents' groups at baseline, after demineralization, and after remineralization regardless of the time of application (overall of 10 minutes and 8 days)

Re-mineralizing agents	Baseline		After demineralization		After remineralization		p-value
	Mean	SD	Mean	SD	Mean	SD	
Whey	267.57 [a]	6.99	255.88 [c]	3.65	259.05 [b]	4.40	<0.001*
Nanohydroxyapatite	266.84 [a]	7.82	255.31 [c]	3.21	258.35 [b]	4.83	<0.001*

Means with different letters in the same row indicate significant difference. ****: significant (p<0.05)***

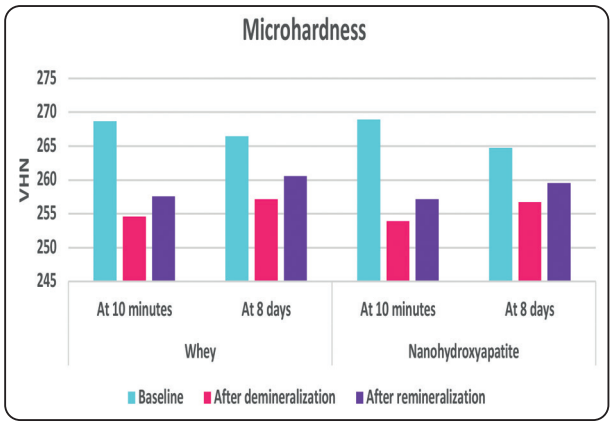

Fig. (4) Bar chart illustrating mean microhardness values at baseline, after demineralization, and after remineralization of Whey and Nanohydroxyapatite re-mineralizing agents' groups at 10 minutes and 8 days times of application.

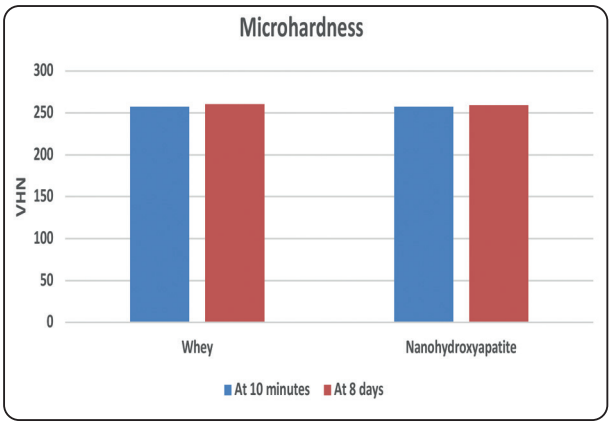

Fig. (5) Bar chart illustrating mean microhardness values at baseline, after demineralization, and after remineralization of Whey and Nanohydroxyapatite re-mineralizing agents' groups regardless of the time of application.

Regardless of the time of application, the two tested re-mineralizing agents showed statistically similar re-mineralizing effects, as the microhardness values did not differ statistically significantly between nano-hydroxyapatite and Whey (p=0.854). Whey recorded the highest mean value, while nano-hydroxyapatite recorded the lowest mean value, which recorded 259.05+4.40 and 258.35+4.83 respectively as shown in Table (3) & Figure (6).

TABLE (3) Descriptive statistics and comparison between microhardness values of the two re-mineralizing agents after remineralization regardless of the time of application (overall of 10 minutes and 8 days)

Re-mineralizing agents	After remineralization	
	Mean	SD
Whey	259.05	4.40
Nanohydroxyapatite	258.35	4.83
p-value	0.854ns	

ns: non-significant (p>0.05)

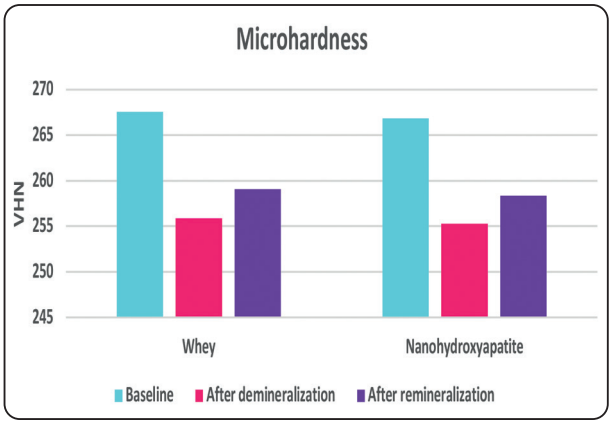

Fig. (6) Bar chart illustrating mean microhardness values, representing the effect of re-mineralizing agents regardless of the time of application.

Regarding the effect of the time of application, the comparisons between the microhardness values of the two times of application (10 minutes and 8 days) within each re-mineralizing agent are shown in Table (4) & Figure (7). For Whey and nano-hydroxyapatite re-mineralizing agents, no statistically significant difference existed between the application times of 10 minutes and 8 days (*p*=0.064), and (p=0.183), respectively. The 8-day period had the highest mean value, while the 10-minute period had the lowest.

TABLE (4) Descriptive statistics and comparison between microhardness values after re-mineralization of the two times of application (10 minutes and 8 days) within each re-mineralizing agent

Time of application	Whey		Nano-hydroxyapatite	
	Mean	SD	Mean	SD
10 minutes	257.57	4.65	257.17	3.72
8 days	260.53	3.71	259.54	5.60
p-value	0.064ns		0.183ns	

ns: non-significant (p>0.05)

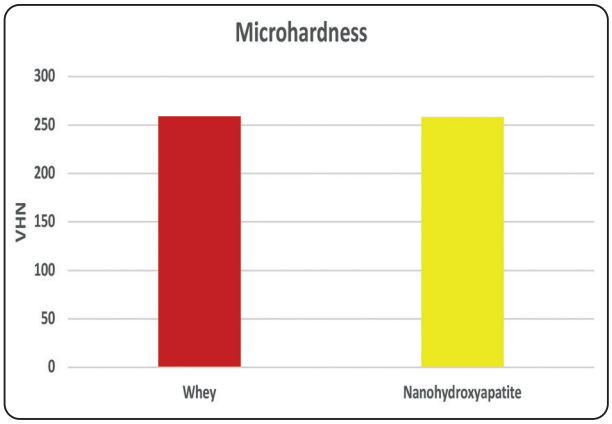

Fig. (7) Bar chart illustrating mean microhardness values, representing the effect of time of application at each re-mineralizing agent.

SEM results:

The SEM pictures are shown in (Figure 8). Scanning electron microscope of sound enamel showed a smooth homogenous surface with some

pores that could be easily seen and there were some areas that had scratches; no enamel prisms were observed (Figure 8a). While demineralized enamel showed loss of typical enamel architecture, as well as micro-porosities and surface irregularities in some areas (Figure 8b). In the Whey extract group after 10 minutes, there was an area of partial and complete occlusion of the rod (Figure 8c), while after 8 days there was a large surface area of the rod showing complete occlusion, the surface was irregular and not returned to sound enamel (Figure 8d). In the nano-hydroxyapatite group after 10 minutes, there was a more homogenous surface with obliteration of some rod spaces (Figure 8e), while after 8 days there was deposition of minerals on the enamel surface with obliteration of rods in some areas (Figure 8f).

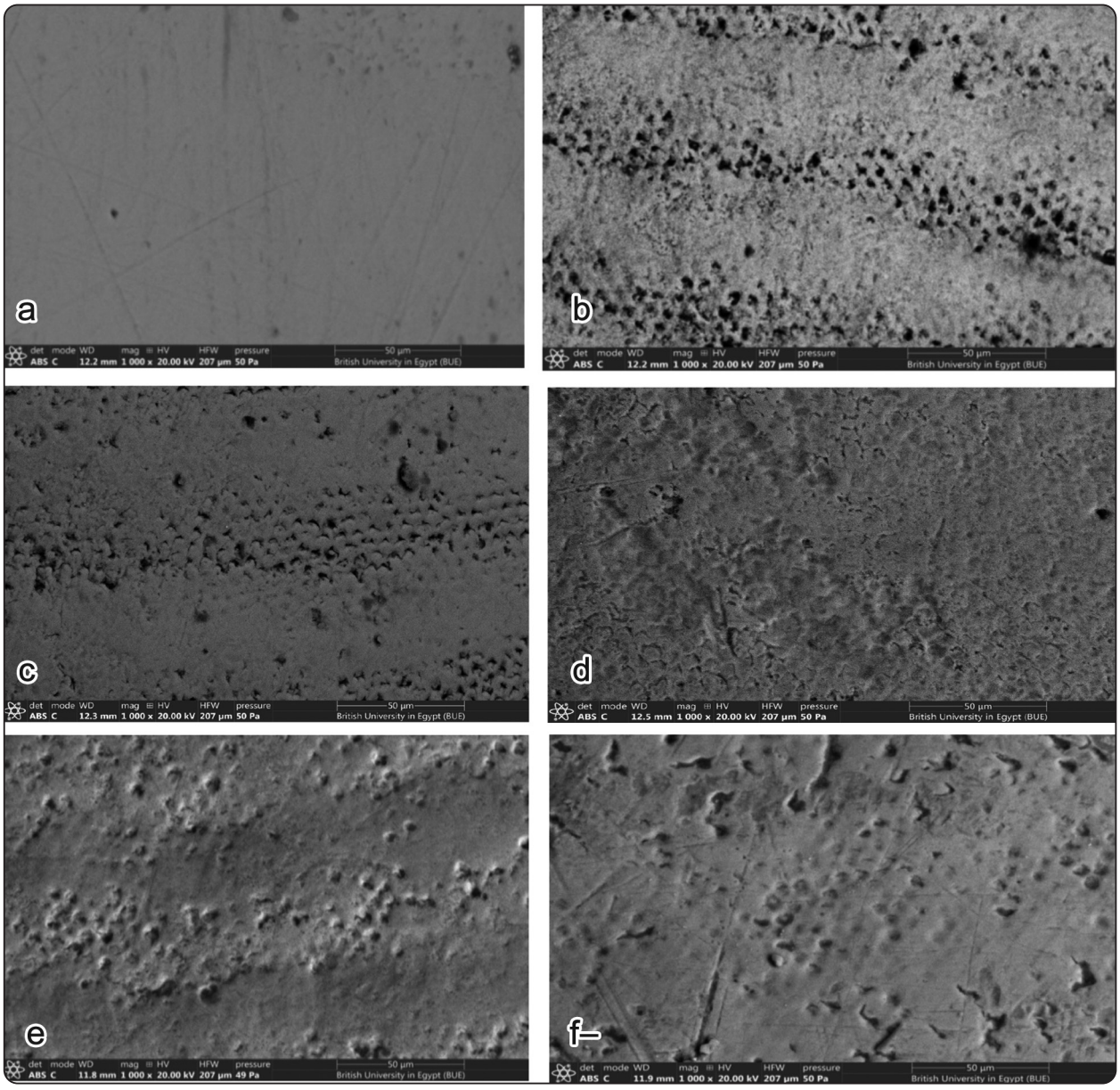

Fig. (8): Scanning electron microscope of different samples (a): Sound enamel.(b): Demineralized enamel (c): Whey extract after 10 min. (d): Whey extract after 8 days. (e): Nano-hydroxyapatite after 10 min (f): Nano-hydroxyapatite after 8 days

DISCUSSION

White spot lesions are likely the early indicators of dental caries disease. These demineralized patches of enamel, which often form because of persistent plaque formation, seem opaque due to substantial underlying porosity brought on by demineralization. Demineralization may advance to non-cavitated lesions, then cavitated lesions, if the process is not prevented and reversed [14].

The primary technique for avoiding caries and remineralizing early lesions has been fluoride treatment, but caries still develop in high-risk patients regardless of the fluoride amount given [15]. Additionally, dental fluorosis has been criticized as an esthetic problem and is seen to be an unfavorable side effect of fluoride's preventive routine. Because of this, there is an increasing need to use other safer compounds that have re-mineralizing abilities at least as effective as fluoride [16].

Therefore, this study was performed to examine the re-mineralizing effect of Whey extract and nano-hydroxyapatite powder mixed with olive oil at different times of application on white spot lesions.

Whey extract is a dairy product containing high amounts of Casein phosphopeptide amorphous calcium phosphate (CPP-ACP) which is one of the most important factors in dairy products that can affect the caries process by inhibiting demineralization alone or combined with the enhancement of remineralization [17].

One of the crystalline calcium phosphate re-mineralizing agents is nano-hydroxyapatite (Nano-HAP). It is among the materials that are highly bioactive and biocompatible. In the crystalline form of hydroxyapatite (HA), it contains calcium (Ca) and nanophosphate [14]. Due to its tiny size, Nano-HA has a significant potential to penetrate through enamel rods and has a strong ability to repair lesions [7].

Olive oil is a kind of vegetable oil; it shows a high content of monounsaturated fatty acids up to 85% of its composition, due to its high content in oleic acid which might range between 70–85%[18]. It is used in this study instead of water for the preparation of NHA paste. Hydroxyapatite powder is soluble in olive oil and the food-grade olive oil is harmless to human health. Olive oil has been used in the production of hydroxyapatite paste and as an additive agent to hydroxyapatite [12].

In this study microhardness of the samples was assessed three times, at baseline, following demineralization, and following treatment application (after ten minutes and after eight days). As substrate-like enamel has a fragile surface that is prone to cracking and a small microstructure, microhardness measurement was used for this study because it is a suitable technique for such materials and is also simple, quick, and non-destructive [19].

Due to its ability to produce highly detailed images of hard objects, the scanning electron microscope (SEM) is adapted for the investigation of the composition of tooth enamel [20]. One of the greatest ways to investigate the enamel surface is by using this technique since it allows you to view structures at extremely high magnification without changing the gross specimen [21].

The results of this study revealed that Whey extract showed an increase in the percentage of microhardness ($p<0.001$), this may be due to Whey extract containing calcium and phosphate ions and has a buffer ability that plays an important role in the process of tooth remineralization. The process of remineralization begins with the attachment of Whey proteins to the surface of demineralized enamel. The calcium and phosphate ions attach to dental plaque, resulting in a supersaturation state of calcium and phosphate minerals. The calcium and phosphate ions undergo a chemical reaction, thus forming hydroxyapatite crystals. The formed hydroxyapatite fills the interprismatic gap of the enamel, thus increasing the microhardness and decreasing the surface roughness of the enamel [22].

The results of this study are in agreement with another study that demonstrated the protective effect of yogurt extract on dental enamel demineralization and reported that Whey extract has an inhibitory effect on demineralization and promotes the remineralization of dental enamel [23]. The finding of this study is also in agreement with another study which showed that the effect of Whey extract was higher than that of casein phosphopeptide-amorphous Calcium phosphate in decreasing the surface roughness of enamel after tooth bleaching [22]. Also, the results of this study are in agreement with another study which showed that whey extract can be used as a natural remineralizing agent as a substitute to fluoride and has long-lasting remineralization potential compared to MI varnish and Xylitol mouthwash [11].

In this study, the microhardness of the enamel surface increased significantly ($p<0.001$) after treatment with nano-hydroxyapatite paste, demonstrating that after treatment, nHAP causes an even remineralization of the enamel by creating a uniform coating apatite on the demineralized surfaces of enamel, which is explained by the hydrophilic and wetting properties of nHA enable it to form a thin but securely bounded coating on the tooth surface, leading to higher surface hardness and re-mineralization. Due to the tiny size of the particles that make up nano-hydroxyapatite, it also functions as filler and can be used to restore the enamel surface microscopic depressions and holes [24].

Additionally, olive oil has been used in the preparation of nano-hydroxyapatite paste instead of water. The water provides a suitable environment for bacteria growth and proliferation [12], so the replacement of the water by the oil helps in decreasing bacteria accumulation and hence gives a better chance for re-mineralization.

This result coincides with a previous study that assessed how toothpaste containing nanohydroxyapatite affected white spot lesions in individuals with orthodontics and showed that nano-hydroxyapatite plays a function in remineralization and reducing the severity of the lesion [7]. Also, it is in the same line with the study that evaluated the effect of nano-hydroxyapatite and olive oil paste on the remineralization of early caries lesions and exhibited a positive effect of the paste on early carious lesions [12]. Additionally, it agrees with a study that evaluated the effectiveness of remineralizing tri-calcium phosphate, nano-hydroxyapatite, and resin infiltration system on early caries lesions and determined that the resin infiltration method and the remineralizing agent based on nano-hydroxyapatite are the most efficient treatments for carious enamel[14].

However, the result of this study is at odds with another study that demonstrated the nHA alone, without association, would not have been able to reach the deeper regions of the lesion this may be due to the presence of a dense layer in the surface layer would be an impediment to remineralization in the innermost regions of the WSL[25].

Results for the time effect showed a considerable rise in microhardness in the two groups after 10 minutes and after 8 days, and the greatest proportion of microhardness was recorded after 8 days, although this increase was statistically insignificant. This may be due to in the Whey extract group the calcium and phosphate ions can readily diffuse into the porous lesion and penetrate deep into the demineralized lesion. As a result of long-term treatment and adequate exposure time, apatite crystals will form again even in deeper parts [26], and in nHA group the long duration increases the capacity of nHA to better integrate into the prismatic and interprismatic enamel structure, producing a more homogeneous surface. [24].

SEM investigations indicated that the demineralized enamel's surface morphology after being treated with Whey extract and nano-hydroxyapatite showed partially obliterated enamel rods and crystals deposition on the enamel surface, supporting the microhardness finding.

CONCLUSION

Whey extract and nano-hydroxyapatite powder mixed with olive oil are considered an effective therapy to re-mineralize the white spot lesion of the enamel surface regardless of the time of application (10 minutes or 8 days).

Recommendations

Additional research is required to determine the effect of various other natural and synthetic materials on the re-mineralization of white spot lesions; also, further in vivo researches regarding the application of natural re-mineralizing materials is needed.

Declaration of funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that there is no conflict of interest.

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