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Abstract.

Background: Remineralizing agents such as fluoride and hydroxyapatite (HA) (Ca$_5$(po$_4$)OH) are well-known treatment choices for incipient enamel lesions. Hydroxyapatite has been newly presented to return the color of such enamel lesions.

Objective: The purpose of this prospective in vitro study was to compare commercial sodium fluoride paste to hydroxyapatite paste (HA) made from chicken eggshells powder (CESP) in terms of how it affected the microhardness and color of the enamel surface of artificially demineralized permanent teeth.

Materials and Methods: Fifty healthy maxillary premolars were gathered, decoronated, and the crowns were placed in acrylic moulds with the buccal enamel surfaces exposed. Baseline microhardness evaluation was done for the baseline group, while the color assessment was done at baseline to three treatment groups. Then specimens were randomly divided into the following five groups (n = 10) based on the treatment of enamel surface: Group 1: Baseline group; Group 2: Acid group demineralized only; Group 3: demineralized followed by the application of hydroxyapatite paste (HA); Group 4: demineralized followed by the application of sodium fluoride toothpaste (NaF); and Group 5: demineralized followed by application of combination treatments (HA paste at morning and NaF paste at evening). The specimens were stored in deionized water at room temperature during treatments, after one week they were subjected to a Vickers microhardness test, and colour assessment to three treatment groups after treatment. One-way ANOVA and Tukey’s post hoc multiple comparison tests were used for statistical analysis (P < 0.05).

Results: After artificial demineralization, the enamel's surface microhardness was greatly reduced, and after management, it dramatically increased. The combination group had the greatest mean microhardness value when compared to the HA paste group and the NaF paste group. Statistically, there was no significant variance in microhardness values among the NaF paste and HA paste groups.

Conclusion: Hydroxyapatite sourced from chicken eggshell was as effective as NaF paste in remineralizing and restoring the lost microhardness of artificially demineralized enamel, hydroxyapatite paste changed tooth color, while sodium fluoride paste, a combination group neither changed nor masked color of early caries lesion.

Key words. Chicken eggshells, microhardness, hydroxyapatite paste. Remineralization, colour.

Introduction.

Dental caries is a contagious illness predominantly brought on by the fermentation of carbohydrates by acidogenic bacteria in the mouth, which results in the production of organic acid as a byproduct. As a consequence, the acids penetrate the dental plaque biofilm’s hard tissues and break into hydrogen ions. These hydrogen ions have the power to liquefy the tooth's mineral components, particularly calcium and phosphate. Known as demineralization, this procedure. And if the right treatment is provided, this process can be prevented; otherwise, the teeth would progressively deteriorate [1]. Dental caries is preventable, so many approaches focused on many procedures and measures to conserve the development of caries and treat it at its initial stages trying to preserve more tooth structure whenever possible, Remineralizing agents are created in a variety of products, including dentifrices, fissure sealants, chewing gum, and mouthwash [2,3]. Therefore, modern dentistry emphasizes prevention and minimal intervention, and one of the principles of minimal intervention dentistry is the replacement of missing tooth substances with biomimetic material (MID) [4,5].

In dentistry, the development of biomimetic materials has led to the development of materials that can entirely replace missing tooth structures and possess qualities resembling those of natural tooth structures. HA is one of these materials and is thought to be the most bioactive and compatible. Both natural and artificial sources can be used to prepare HA. The synthetic version of HA has so far been used to demonstrate the positive benefits of the compound. A natural and inexpensive method of manufacturing HA might use chicken eggshells as a raw material [6]. The null hypothesis of the current study: there is no improvement in the microhardness and color of the artificially demineralized enamel teeth when using Hydroxyapatite (HA) paste synthesis from CESP.

Materials and Methods.

Preparation of Hydroxyapatite powder (HAP): Hydroxyapatite powder (HAP) as a white powder prepared manually from chicken eggshell powder (CESP) at Department of Dental Basic Science/College of Dentistry/University of Mosul, (patent 6987, A61C13/08, A61L27/12).

Preparation of Demineralization solution: Consists of CaCl$_2$ (2.2mM), KH$_2$PO$_4$ (2.2 mM) and acetic acid (0.05 M), pH was adjusted with (1M) KOH to (4.4).

Preparation of the specimen: After receiving the ethical approval from the research ethics committee in the University of Mosul/College of Dentistry (UoM. Dent/ HDM.78/22). Fifty extracted non-curious maxillary first premolars were collected and examined under stereomicroscope (OPTIKA /ITALY) to ensure that they were cavity-free, hypo calcifications, and crack then stored in deionized water containing (0.1% PH.7) Thymol (Flukachemie, Switzerland) in a closed container at room temperature and used within 3 months after extraction [7] to prevent dehydration and microbial growth [8]. The teeth were decoronated at the cervical line with abundant water irrigation using straight diamond bur of a high-speed handpiece (continuous water cooling) to evade damaging the enamel. Then, each tooth

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crown was positioned such that the buccal surface faced upward and was parallel to the ring's floor inside a self-curing acrylic resin (VERACRIL/Colombia). Filled cylindrical plastic ring (14mm in diameter, 16mm in depth). A circular 6mm piece of adhesive tape was placed in the middle of the buccal surface of each sample. The tape was removed to reveal an enamel window after applying acid-resistant nail polish to the remaining surface [9]. After that, using fine-grit silicon carbide sheets (1200 grit), the exposed enamel surfaces were ground and wet polished one at a time to provide flat, homogeneous surfaces for the surface microhardness test [10], Then polishing the exposed window with the universal polishing machine (Surf-Corder, Japan). Later, all samples were kept in deionized water until they were soaked in a demineralized solution (Figure 1A).

Establishment of the preliminary Carious Lesion: Every tooth in the research, except the baseline group, was submerged in a demineralizing solution for 48 hours (2 days) at 37°C to cause subsurface enamel caries (white spot lesions without cavities) [11], Specimens were washed with deionized water and stored in deionized water.

Synthesis of hydroxyapatite paste from chicken eggshell powder (CESP): The hydroxyapatite paste (HA paste) was prepared according to Natsir et al [12], which is formulated from Sodium Carboxyl Methyl Cellulose (Na-CMC)(Avonchen, UK.), Distilled water, Glycerol, Methylparaben (NipaginR, Avonchen, UK.), Hydroxyapatite powder extract from chicken eggshells by chemical precipitation method. The most preferable concentration of hydroxyapatite in paste formulation to achieve better remineralization of the enamel tooth surface is (10%) WV according to [13]. Therefore, we prepared 5 ml of 10% Hydroxyapatite toothpaste daily for 7 days.

Infrared Spectroscopy Estimation of Important Bands: The prepared HA powder from CESP, and the prepared HA paste components were characterized by using the FTIR-Alfa-Bruker spectrophotometer (Germany) in the region (400-4000 cm⁻¹). This measurement was carried out at the University of Mosul, College of chemistry sciences, Iraq (Figure 1B).

Grouping and surface treatment: In the current research, there were a total of (50) samples of teeth, which were allocated into (5) groups at random with (n=10) samples in each group.

**Group A (10 teeth):** baseline group without demineralization, (subjected to microhardness test only).

**Group B (10 teeth):** without treatment, demineralized teeth immersed in deionized water only, (subjected to microhardness test only).

**Group C (10 teeth):** after demineralization, HA paste was applied by a micro brush as in figure (1C) and then brushed by a soft brush, which was applied twice daily on the exposed enamel surface to simulate routine oral hygiene in vivo, (Subjected to microhardness test & colour test).

**Group D (10 teeth):** The exposed enamel window of each tooth was coated with a thin layer of fluoridated toothpaste that contains (2500 ppm) fluoride ion (Flurokin, Spain) using a fine brush for 2-3 minutes. This procedure was performed twice daily to simulate routine oral hygiene instructions then washed with deionized water and stored in deionized water change after each application until complete one week, (Subjected to microhardness test & colour test) (Figure 1D)

**Group E (10 teeth):** Combination group (HA toothpaste +fluoridated toothpaste) separately (HA paste) applied once in the morning and (Naf) paste once in the evening until complete one week, (Subjected to microhardness test & colour test).

Surface Microhardness Test (SMH): The Surface Microhardness Test (SMH), assesses a material's resistance to plastic deformation under a standard load source [14]. The microhardness of the samples was determined using a Vickers Microhardness tester (Wilson Wolpert Instruments, Aachen, Germany) outfitted with a 500-g load. It took 15 seconds for the exposed enamel surface to sink and settle before the Vickers hardness number (VHN) could be determined. On each specimen, three indentations were made, and the measurements were averaged. This was used as the sample's first Microhardness value (MHV) for the baseline group, control negative (Acid group) after demineralization, and three treatment groups after remineralization then compare between surface Microhardness of all groups and according to [15].

Colour change evaluation: Color evaluation was assessed at two different stages; at baseline before enamel lesion formation, as well as after therapy application consuming a colour colormeter (NR60CP Precision /3nh/China) as in figure (2). The CIE-lab coordinates were obtained using a colourimeter. L* stands for the light parameter, a* for the green-red chromaticity parameter, and b* for the blue-yellow chromaticity parameter. Each specimen had three readings taken, and the average of those values was recorded. The mean values of colourimeter readings at different stages were compared to evaluate the amount of colour change (ΔE) as follows ΔE = [(ΔL*)² + (Δa*)² + (Δb*)²]¹/².

The colour measurement was performed in a dark room, which could eliminate associated errors [16,17], and the teeth samples
were placed over a white paper during measurements to provide white background [18].

Statistical analysis: Microhardness values of the baseline group, Microhardness values of control negative (acid group) and Microhardness values of three treatments groups and data of the color test were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed parametric (normal) distribution. One-way ANOVA followed by Tukey’s post-hoc test was used to match between different clusters of each test. The significance level was set at P ≤ 0.05. Statistical analysis was performed with SPSS Version 20 for Windows.

Results.

The microhardness test for all groups, including the mean, number, and standard deviation displayed in Table 1. Based on the measurement for tested groups, the Baseline group had the highest surface microhardness mean value and the Combination group had the highest mean between treatment groups, then sodium fluoride group, followed by the HA paste group, and the control negative group.

The contrast of mean values of enamel surface microhardness tested and the results revealed that there were highly statistically significant differences within and in between baseline, Control negative, HA paste, Naf paste, combination groups at p ≤ 0.01 (Table 2).

The mean microhardness for Baseline, control negative, and treatment groups are highly significantly different from each other at p≤ 0.01. The baseline group had surface mean microhardness significantly higher (281.3300) than other groups, the Combination group had a mean microhardness (264.5400), while control negative group had a lesser mean (171.2600) on surface microhardness (Table 3).

We noticed that from mean values of surface microhardness of the enamel teeth samples. The surface microhardness decreased after immersion in demineralizing solution, in comparison to the microhardness of the baseline group.

Color change estimation: To observe the degree of colour change, the mean values of colourimeter readings at two separate phases were calculated, as shown in Table (4).

The hydroxyapatite paste group exhibited an increase in ΔL value more than the other two types of treatment groups associated with a decrease in Δa and Δb, and this reflected that increase in light reflection and decrease in light transmission, through the tooth caused by the HA particles adhering to the enamel (Figure 3).

Discussion.

The efficacy of hydroxyapatite micro-clusters or nanoparticles in toothpaste and mouthwash in promoting caries remineralization and stopping tooth tissue demineralization has been demonstrated via clinical as well as in situ and in

![Figure 2. Display colour measurement of the teeth samples by using a Colorimeter.](image)

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Mean</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline group</td>
<td>10</td>
<td>281.3300</td>
<td>11.48797</td>
</tr>
<tr>
<td>Control negative G</td>
<td>10</td>
<td>171.2600</td>
<td>16.15798</td>
</tr>
<tr>
<td>Hydroxyapatite paste G</td>
<td>10</td>
<td>234.7200</td>
<td>12.57120</td>
</tr>
<tr>
<td>Sodium fluoride paste G</td>
<td>10</td>
<td>237.4000</td>
<td>10.39177</td>
</tr>
<tr>
<td>Combination G</td>
<td>10</td>
<td>264.5400</td>
<td>2.57086</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>237.8500</td>
<td>39.50255</td>
</tr>
</tbody>
</table>

Table 1. Descriptive statistics of surface microhardness measurements among tested groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>70470.940</td>
<td>4</td>
<td>17617.735</td>
<td>132.327</td>
<td>0.000</td>
</tr>
<tr>
<td>Within Groups</td>
<td>5991.185</td>
<td>45</td>
<td>133.137</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>76462.125</td>
<td>49</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**p ≤ 0.01, highly statistically significant using one-way analysis of variance (ANOVA) test**

Table 2. Enamel surface microhardness of the studied groups.

<table>
<thead>
<tr>
<th>Microhardness</th>
<th>ΔL *</th>
<th>Δa *</th>
<th>Δb *</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA paste G</td>
<td>4.782</td>
<td>-0.328</td>
<td>-4.46(2.722)</td>
</tr>
<tr>
<td>Naf paste G</td>
<td>1.154</td>
<td>-0.274</td>
<td>-6.08(2.225)</td>
</tr>
<tr>
<td>Combination G</td>
<td>3.340</td>
<td>-1.353</td>
<td>-6.20(2.996)</td>
</tr>
</tbody>
</table>

Table 4. Mean ΔL*, Δa*, and Δb* values after 1 week of treatment.

Table 3. Microhardness value at baseline G, Control negative, Hydroxyapatite toothpaste, Sodium fluoride paste and combination groups.
effect due to the synergistic effect of two remineralizing agents. Fluoride ions increased hydroxyapatite crystallization utilizing copious amounts of calcium and phosphate ions from the surrounding remineralization material, which promotes crystal deposition and growth [19]. The microhardness of the enamel surface is a good indicator of the mineral makeup of the enamel, and measuring microhardness is sensitive enough to ascertain how the enamel is resistant to demineralization. Additionally, a strong connection was discovered between mineral loss in carious lesions and the enamel’s surface microhardness [20]. According to the findings of the current study, the null hypothesis was not accepted. The enamel bio-mineralization of hydroxyapatite in combination with fluoride following treatment on artificially demineralized enamel of them exhibited promising remineralizing capability in the results. Therefore, based on the results, we observed that enamel microhardness decreased after being exposed to the demineralizing solution, which pointed to the mineral loss triggered by the acid, and the difference in mean surface microhardness values (SMH) between the groups following treatment, which reflected the various remineralization potentials for each treatment procedure, was highly statistically significant. The remineralization system needs to supply constant, bioavailable calcium, phosphate, and fluoride ions since these minerals are lost during demineralization [21]. In the current study and depending on the means values, the remineralization effect of the combination group (HA paste + Naf paste) was better than hydroxyapatite paste alone, and sodium fluoride paste alone but both of them were valuable to protect the microhardness of permanent teeth enamel when compared to the control negative group where the deionized water was used for storing teeth after demineralization. The combination group exhibited the highest mean surface enamel microhardness than the Naf paste alone or HA paste alone, and this agrees with Ebadifar et al. [21-23]. The obtained results of the SMH values found in the combined group denote that the presence of copious amounts of calcium, phosphate from hydroxyapatite paste and fluoride ions increased hydroxyapatite crystalization utilizing the high level of ions available so enhancement remineralization effect due to the synergistic effect of two remineralizing agents. Additionally, fluoride toothpaste is well known for enhancing the remineralization of human enamel following acid assault and is well-regarded by the general population [23]. The results of our experiment on the remineralization of sodium fluoride paste agreed well with those of Oliveira et al [24]. Whose noted that after a week of treatment, toothpaste containing just fluoride significantly increased the microhardness of demineralized specimens. While HA pastes alone group exhibited a minimum mean value than the combination paste group, and Naf paste alone group, this might be due to the paste used in this study being locally synthesized not commercially supplied, the short duration of treatment, and the size of hydroxyapatite used in the toothpaste preparation is micro-sized instead of nanosized, resulting in less entering the lesion and caused HA paste group exhibiting least mean for surface microhardness [10,25]. However statistically speaking, there was no visible difference in the mean SMH value between the HA paste group and the Naf paste group, and this was in good agreement with the study conducted by [26,27]. While regarding color change results, The colourimetric analysis in this study was carried out to describe the ability of hydroxyapatite paste, sodium fluoride toothpaste and a combination of both of them together to induce a color change (ΔE, ΔL, Δa, Δb) in the artificially induced white spot lesion or capability of these remineralizing agents to cover the visible white color of early enamel caries. The attachment of HA particles to the tooth surface may be used to explain why the HA-oral care product can whiten teeth. Fabritius-Vilpoux et al. (2019) concluded that HA particles from a mouthwash adhere to enamel surfaces as in vitro research; this is supported by the scan electron microscope (SEM) examination carried out in the study performed by Sarembe et al. (2020). So, the result of the colour analysis of hydroxyapatite paste of the current study was in good agreement with [28,29]. However, studies on teeth whitening products have shown that remineralization causes a considerable improvement in the yellowness (Δb) and lightness (ΔL) of the teeth. Additionally, the human eye is more sensitive to changes in brightness (ΔL) than the other colour characteristics (Δa, Δb) [30]. According to studies [30,31], the ΔL colour parameter is the most important factor in tooth colour assessments. In the current study, we found that HA paste significantly increased brightness (L) compared to other treatment groups while significantly reducing (b, a), making teeth appear whiter, less red, and less yellow. These optical changes can be attributed to the HA particles sticking to the enamel, which increases light reflection and decreases light transmission through the tooth [16,17,32]. While sodium fluoride paste cannot conceal the whiteness of early caries lesions, this is because a high fluoride concentration can accelerate mineral precipitation on the enamel surface and obturate the enamel surface pores. This procedure may further restrict the remineralization of the subsurface demineralized enamel [33]. The esthetic and structural qualities of the deeper lesions are not significantly enhanced by this surface-only remineralization [21]. Although the high mean ΔE value of the combination group point to the worst result and this can be explained by that, remineralization by using the synergistic effect of HA toothpaste and Naf toothpaste leads to the form of a new product on the tooth surface caused to change in the colour negatively through irregular remineralization making like a bridge on the enamel surface, so further studies we needed.
to observe the outer layer of the demineralized enamel tooth surface treated by this type of synergistic treatment such as evaluation by using scanning electron microscope. However mean ΔE value of HA paste is the lowest mean between the three remineralizing agents and this indicated the best result for colour restoration, and according to the study performed by Abdel-Hakim et al. (2016) [15], who said that the lowest mean of ΔE indicated the best result for changing the colour of the white spot lesion (WSL). Attempt should be made to provide protection of the whole teeth keeping it safe from porosity, enamel loss, and opalescent tooth colour [34].

Conclusion.

The microhardness of artificially demineralized enamel surface is improved by hydroxyapatite paste made from chicken eggshells and sodium fluoride toothpaste (Flurokin), which together have remineralizing properties. Statistically speaking, the ability of sodium fluoride paste, and hydroxyapatite paste to remineralize and restore the enamel's lost microhardness was comparable; however, the combination paste group (HA+ Naf) was thought to be the most successful in remineralizing and regaining the enamel lost microhardness, followed by Naf paste and finally HA paste. Toothpaste containing sodium fluoride had no impact in restoring the colour of lesions with white spots. While hydroxyapatite paste can improve but not mask the white colour of white spot lesions, when it is combined with sodium fluoride as in the combination paste group it is neither improved nor masks the colour of white spot lesions.

REFERENCES