REMINERALIZATION EFFECT OF EGG SHELL POWDER AND NOVAMINE ON INITIAL CARIES-LIKE LESIONS IN YOUNG PERMANENT TEETH (IN-VITRO STUDY)

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ABSTRACT

INTRODUCTION: Despite the cariostatic effect of fluoride, the continuous use of fluoridated toothpastes increases the risk of dental fluorosis. Therefore, new safe alternative technologies of teeth remineralization must be introduced to arrest dental caries and remineralize the early enamel carious lesions.

OBJECTIVES: The aim of the study was to evaluate the remineralizing effect of eggshell powder and novamine on initial caries-like lesions in young permanent teeth compared to fluoride.

MATERIALS AND METHODS: A total of 96 permanent teeth were selected. Each tooth was coated with nail varnish, leaving squares of 4x4 mm. Each tooth was sectioned into 2 halves longitudinally in a buccolingual direction through the center of the window to obtain a mesial and a distal half (192 specimen). One half was treated and the other half remained untreated to serve as its negative control. Teeth were assigned into three groups according to treatment agent; group I (eggshell powder solution), group II (novamine toothpaste) and group III (fluoride toothpaste) Specimens were immersed in the demineralizing solution for 72 hours to produce artificial carious lesions on the exposed enamel surface.

RESULTS: There was a statistically significant mean microhardness and lesion depth values between test subgroups IA (eggshell powder), IIA (novamine) and their controls (P<0.001). There was no statistically significant difference in mean microhardness and lesion depth values between the test subgroup IIIA (fluoride) and its control (P>0.001). There was statistically significant difference in mean percent difference values of enamel microhardness among three groups (P<0.001). There was no statistically significant difference in mean percent difference values of lesion depth between the three groups (P>0.001).

CONCLUSION: Both eggshell powder and novamine were effective for remineralization of initial caries-like lesions of young permanent teeth.

KEYWORDS: Remineralization, eggshell powder, novamine, fluoride.

INTRODUCTION

Dental caries is considered one of the most common chronic diseases throughout the whole world (1). The World Health Organization (WHO) in 2003 reported that there is an international widespread distribution of global caries epidemiology with 60-90% caries prevalence among school-age children. It also affirmed that dental caries in 12-years-olds is increasing in developing countries (2). Moreover, Selwitz et al. in 2007 (3) concluded that the prevalence of dental caries is a continuous public health problem in most developed countries.

Rebuilding of carious lesions through tooth preparation and use of restorative material is an irreversible cycle which results in loss of tooth structure over time (4). Preventive treatment and early intervention of initial carious lesions are the key components of the recent dental caries management (5). Remineralizing agents play an important role in improving the remineralization process and prevention of dental caries (6).
multipurpose new regenerative material that can be used as a bone graft substitute because of its biocompatibility, lack of disease transfer risks, ease of use, unlimited availability, cost-effectiveness, efficiency and also it can be prepared in a very economical way. The minerals of ESP when come in contact with enamel caries-like lesions; they diffuse into the superficial layer and obstruct the surface porosities (14). In 2016, Haghgoo et al. (10) reported that ESP solution can be applied in preventive dentistry as a remineralizing agent and it can be used as a supplement or even an alternative to fluoride.

Novamine is a known synthetic component of bio-active glass (15). The bioactivity of the glass is extremely related to the surface growth rate of hydroxyapatite (HA) layer which is considered the main mineral phase of teeth. It consists of silica, calcium, phosphorus and sodium ions. When they come in contact with saliva, they bind to the tooth structure and initiate the remineralization process (16). Taha et al. in 2017 (17) systematically reviewed the effect of bioactive glasses on enamel remineralization and concluded that bioactive glasses had the ability to enhance enamel remineralization more effectively than other topical remineralizing agents regardless of its formulation or mode of application technique.

The aim of this is to evaluate the remineralizing effect of eggshell powder and novamine on initial caries-like lesions in young permanent teeth compared to fluoride. The null hypothesis tested is that there is no difference between the remineralizing effect of eggshell powder and novamine on initial caries-like lesions in young permanent teeth compared to fluoride.

MATERIALS AND METHODS
This study was an experimental in vitro study which was approved by the Scientific Research Ethical Committee, Faculty of Dentistry, Alexandria University, Egypt. It was performed in the Departments of Pediatric Dentistry and Dental Public Health, Oral Biology and Dental Materials in Faculty of Dentistry, Department of Geology in Faculty of Science and Department of Production in Faculty of Engineering, Alexandria University.

Sample size estimation
The minimal sample size was calculated based on a study aimed to evaluate the remineralization potential of enamel surface lesion using chicken egg shell powder (CESP) solution. Mony et al., (18) (2015) reported microhardness values for all the 4 groups of their study. A sample size of 32 teeth per group (number of groups=3) (total sample size=96) or 16 measurements (half-tooth) per subgroup (number of subgroups =6) (total sample size = 192 half-tooth), each subgroup was measured by 2 different devices, was the enough required sample, as statistically significant with 80% power and at a significance level of 95%(accepted alpha of 0.05). Sample size per group did not need to be increased to control for attrition bias. The sample size was calculated using GPower version 3.1.9.2.

Sample
Ninety six human premolar teeth extracted for orthodontic reasons were collected from Public Hospitals, Department of Orthodontics, Faculty of Dentistry, Alexandria University and Private Dental Clinics. The sample was randomly divided according to exposure media into three equal groups (I, II and III). Each group was further subdivided into two subgroups by sectioning the teeth of each group. Subgroups IA, IIA and IIIA were treated by the agents eggshell powder, novmine and fluoride respectively and served as experimental subgroups, while subgroups IB, IIB and IIIB were left untreated and served as negative control.

Criteria of teeth selection
Teeth were selected according to the following criteria:
1. Young permanent teeth free from caries.
2. No enamel cracks.
3. No developmental defects.

Preparation of the teeth specimens
Teeth were thoroughly cleaned using fluoride free pumice and were stored in normal saline solution until required for use. Teeth were dried, and then squares of self-adhesive labels 4x4 mm were stuck at the center of the buccal surface of each tooth. All teeth surfaces were coated with acid-proof nail varnish. Then, the adhesives were removed exposing only a small window of enamel. The crown of each tooth was separated from its root using a diamond disc mounted on a straight hand piece then each crown was sectioned longitudinally in a buccolingual direction through the center of the window into two equal halves; a mesial half and a distal half (192 specimens). Each specimen in the control group was the other half of the same tooth in the test group. Teeth that were assessed by Vickers microhardness tester were mounted in a self-cure acrylic resin inside cylindrical plastic mold. The buccal surface of each tooth specimen was set perpendicular to the long axis of the block.

Remineralizing agent's formulation
The novamine toothpaste (GSK consumer Healthcare, Brentford, U.K) contained: Calcium Sodium Phosphosilicate, Glycerin, polyethylene glycol ester-8, Hydrated Silica, Cocamidopropyl Betadine, Sodium Methyl Cocomyl Taurate, Aroma, Titanium Dioxide, Carbomer, Sodium Saccharin, Ligninene and 1450 ppm Sodium Fluoride. Fluoride tooth paste (B&S Healthcare, Ruislip, UK) contained: 5000 ppm Sodium Fluoride, 70% Sorbitol solution, Silica dental type, Precipitated Silica dental type, Macrogol 600, Tetrapotassium Pyrophosphate, Xanthan gum, Sodium Benzoate, Sodium Laurilsulfate, Spearmint Flavouring, Saccharin Sodium, Brilliant Blue FCF and Purified water. Eggshell powder (cdVet Germany, dispatched from U.K.).

Preparation of egg shell powder solution: 10% solution of egg shell powder solution was prepared by dissolving 10 grams of egg shell powder in 100 ml of distilled water (10).

Demineralization phase: (Caries-like lesion formation)
Each tooth specimen was immersed in a demineralizing solution (10ml) at 37°C for 72 hours without stirring in order to produce artificial carious lesions (white spot lesion without cavitation) (14). After the lesion formation, teeth specimens were rinsed with distilled water and stored in artificial saliva for 7 days (18).

Remineralization phase:
For Subgroups IA: Each specimen was treated with eggshell powder solution for 3 minutes.
For subgroup IIA and IIIA: Tooth pastes were applied with a microbrush according to the manufacturer’s instruction for 3 minutes. Then specimens were rinsed with distilled water and stored in the artificial saliva (19). These procedures were performed once/day for 7 days.

For Subgroups IB, IIB and IIIB: Each specimen was left untreated and stored in artificial saliva. After 7 days, all specimens were rinsed with distilled water and prepared for final evaluation.

Methods of assessment of enamel surface: Half of the specimens were randomly selected for assessment by Vickers microhardness tester and the other half was assessed by Polarized light microscope.

1- Vickers Microhardness Device:
Microhardness testing was done before any cariogenic challenge (initial base line assessment), after initial caries formation (second assessment) and at the end of the study (final assessment). A load of 50 g was applied to the surface of the specimens for 10 seconds. Five indentations were equally placed over a circle of 1 mm diameter at the cervical third of the specimens. The surface microhardness was obtained using the following equation: \( HV = \frac{1.854 \cdot P \cdot d}{d^2} \) where, \( HV \) is Vickers hardness in Kgf/mm\(^2\), \( P \) is the load in Kg and \( d \) is the length of the diagonals in mm (20-22).

2- Polarized light microscope:
At the end of the study, from each subgroup (IA, IIA and IIIA), 16 specimens and their corresponding controls (IIB, IIB and IIIB) were evaluated quantitatively (mean lesion depth) and qualitatively (histologically) using polarized light microscope.

Lesion depth evaluation: (Quantitative evaluation)
The subsurface depth measurements were evaluated using software (Image J 4.6) (23). The readings by the software were represented in pixel unit. To turn the pixel unit into millimeter (mm), a photomicrograph was taken to a reference slide. The reference slide was projected onto the computer image processing software (Image J 4.6) as a scale. The reference slide was the mounting medium used to hold the specimen in place between the slipcover and the glass slide.

Lesion depth evaluation: (Qualitative evaluation)
Qualitative evaluation was used to describe the interpretation of enamel birefringence and the course of the enamel rods. The different sections were examined by the polarized microscope to evaluate the changes in enamel as a result of demineralization and remineralization process. Photomicrographs were taken with a digital camera with magnification of X40 (eyepiece X10 & objective lens X4) to achieve comparison in histological features between the test and control specimens. Furthermore, they were compared with the reference section.

Statistical analysis:
Data were collected and entered to the computer using SPSS program for statistical analysis (version 25.0). Normality was checked using descriptive statistics, plots (histogram and box plot) and Shapiro Wilk test. Differences in lesion depth and enamel microhardness between test and control in each group were analyzed using parametric test; Student’s t test and the non-parametric test; Mann-Whitney U test. Meanwhile, differences between the three remineralizing agents were assessed by using ANOVA for normally distributed data and Kruskal Wallis H test for the not normally distributed. The level of statistical significance was set at 0.05. Percent difference of the lesion depth and enamel microhardness among the three groups relative to their controls was calculated according to the formula [(lesion depth in test-lesion depth in control)/lesion depth in control] x 100 and the same for enamel microhardness. Post hoc tests were needed for comparison between percent differences of enamel microhardness among three groups. Dunn’s pairwise comparisons were carried out for the three pairs of groups (24).

RESULTS
Quantitative results:
There was a statistically significant difference in mean enamel surface microhardness between subgroup IA (egg shell powder) and subgroup IB (its control) (P=0.004). There was a statistically significant difference in mean enamel surface microhardness between subgroup IIA (novamine) and subgroup IIB (its control) (P<0.001). Whereas, there was no statistically significant difference between subgroup IIIA (fluoride) and its control where P=0.54. Using Kruskal Wallis H test there was a statistically significant difference in mean percent difference values of enamel microhardness among three groups (P<0.001), with group II having a higher mean percent difference value of enamel microhardness 12.47±20.99 than group I and III (9.17±12.06, 1.61±0.49), (Table 1).

Table 1: Showing mean enamel surface microhardness values between test and control in each group and mean percent difference values of enamel surface microhardness among three groups relative to their controls.

<table>
<thead>
<tr>
<th>Enamel microhardness</th>
<th>Test</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg shell powder : Mean±SD</td>
<td>270.63±34.62</td>
<td>248.06±19.54</td>
<td>0.004*</td>
</tr>
<tr>
<td>Novamine : Mean±SD</td>
<td>271.46±18.48</td>
<td>243.13±61.43</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Fluoride : Mean±SD</td>
<td>269.03±19.67</td>
<td>246.78±19.87</td>
<td>0.54</td>
</tr>
<tr>
<td>Group I %</td>
<td>Group II %</td>
<td>Group III %</td>
<td></td>
</tr>
</tbody>
</table>
Mean±SD   9.17±12.06   12.47±20.99   1.61±0.49
P value <0.001*

*: Significant difference (P<0.05).

Post-Hoc test for multiple comparisons between mean percent difference of enamel microhardness among three groups using Dunn's pairwise test showed that there was a statistically significant difference between group I and III (P=0.014) and between group II and III (P=0.001). Whereas, there was no statistically significant difference between group I and group II (P=0.022), (Table 2).

Table 2: Showing post hoc test for multiple comparisons between the mean percent differences of enamel microhardness among three groups.

<table>
<thead>
<tr>
<th>Groups Compared to</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>0.022</td>
</tr>
<tr>
<td>Group III</td>
<td>0.014*</td>
</tr>
<tr>
<td>Group II</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*Statistically significant with adjustment for multiple comparison for multiple testing (P<0.016)

There was a statistically significant difference in mean lesion depth between subgroup IA (egg shell powder) and subgroup IB (its control) (P<0.001). There was a statistically significant difference in mean lesion depth between subgroup IIA (novamine) and subgroup IIB (its control) (P<0.001). Whereas, there was no statistically significant difference between subgroup IIIA (fluoride) and its control (P=0.36). Using Kruskal Wallis H test there was no statistically significant difference in mean percent difference values of lesion depth among three groups (P=0.15), (Table 3).

Table 3: Showing mean lesion depth values between test and control in each group and mean percent difference values of lesion depth among three groups relative to their controls.

<table>
<thead>
<tr>
<th>Lesion depth</th>
<th>Test</th>
<th>Control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg shell powder :</td>
<td>95.63±56.09</td>
<td>203.13±61.43</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mean±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Novamine :</td>
<td>79.38±26.70</td>
<td>236.25±61.19</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Mean±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoride :</td>
<td>76.25±34.23</td>
<td>190.00±53.54</td>
<td>0.36</td>
</tr>
<tr>
<td>Mean±SD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Group I %          | 54.21±16.05| -62.96±18.12| -59.15±14.04| 0.15

*: Significant difference (P<0.05).

Qualitative results

Subgroup IA (Eggshell powder solution)
Most of the representative lesions exhibited noticeable reduction in the depth of the lesion seen in subgroup IB. Other specimens showed complete absence of the dark bands. This appeared in the form of negative birefringence emphasizing the effect of this solution for remineralization, (Figure 1A, Figure 1B).

![Figure 1A](image1.png) Polarized light photomicrograph of a longitudinal ground section of the control specimen (subgroup IB) exhibiting a dark and deep demineralized enamel segment, magnification X40.

![Figure 1B](image2.png) Polarized light photomicrograph of a longitudinal ground section of enamel treated with eggshell powder solution (subgroup IA) showing homogenous remineralization of the caries like lesion comparable to the control specimen figure 1B-A, magnification X40.

Subgroup IIA (Novamine toothpaste)
In this subgroup the observed degree of remineralization was comparable to that seen in subgroup IA. Almost all the representative lesions showed absence of the dark zones that were seen prior to remineralization and this emphasized the remineralization effect of this paste, (Figure 2A, Figure 2B).

![Figure 2A](image3.png) Polarized light photomicrograph of a longitudinal ground section of the control specimen...
Mohamed et al. Remineralization effect of eggshell powder and novamine toothpaste (subgroup IIIA) showing a decrease in the extent of the lesion, magnification X40.

**DISCUSSION**

In the present study, the quantitative evaluation of the enamel surface was carried out by measuring the surface microhardness. Microhardness evaluation is a convenient method for a material having non-homogenous, fine microstructure and prone to cracking like enamel. Surface microhardness indentation provides relatively a simple, reliable, non-destructive and rapid method for evaluation of remineralization changes of the tooth surface (18).

The results obtained in the present study revealed that there was a statistically significant difference in the mean enamel surface microhardness between subgroup IA (treated with eggshell powder solution) and subgroup IB (its control). This finding was in agreement with Mony et al. in 2015 (18) who concluded that eggshell powder has the potential to favor remineralization due to its high pH and rich bioavailable calcium content. It was also supported by Haghoo et al. in 2016 (10) who revealed that eggshell solution can be used as a remineralizing agent for incipient enamel carious lesions and it is as effective as nano-hydroxyapatite for enamel remineralization. Moreover, Yaberi and Haghoo in 2018 (25) reported that microhardness of the enamel significantly increased after treatment with eggshell extract solution.

Statistical analysis of the present study indicated that there was a statistically significant difference in the mean enamel surface microhardness between subgroup IIA (treated with novamine toothpaste) and subgroup IIB (its control). This result was in agreement with Golpayegani et al. in 2012 (26) who concluded that novamine dentifrice has a greater effect on remineralization of carious-like lesions. Results of a study conducted by Taha et al. in 2017 (17) was also in agreement with the results of the present study. They reported that bioactive glass has the ability to enhance enamel remineralization regardless of its formulation or mode of application technique. Moreover, Huang et al. in 2018 (27) affirmed that novamine can remineralize the demineralized dentin specimens and occlude the dentinal tubules in the hydroxyapatite-like crystal structure.

Although there was a slight increase in enamel surface microhardness in subgroup IIIA (treated with fluoride toothpaste) than in subgroup IIIB (its control); this difference was not statistically significant. The possible explanation of this result is that, fluoride is not considered as a source of calcium (Ca) as with novamine and eggshell powder. Its action is limited by the availability of phosphate (P) and Ca ions (28). The real remineralizing effect of fluoride is to enhance the redeposition of Ca and P ions that are present in the biofilm of the demineralized enamel when the pH of the oral cavity rises (6). This interpretation was further supported by Reynolds et al. in 2008 (29) who proved that Ca and P ions from saliva are important factors in the remineralization of previously demineralized enamel by fluoride. It was also in agreement with Neel et al. in 2016 (30) who concluded that fluoride acted as a catalyst for

**Subgroup IIIA (Fluoride toothpaste)**

The enamel showed variable degrees of remineralization, but the body of the lesions showed less degree of enamel remineralization than in subgroups IA and IIA, (Figure 3A-Figure 3B).

**Figure 2B:** Polarized light photomicrograph of a longitudinal ground section of enamel treated with novamine toothpaste (subgroup IIA) showing complete homogenous extensive remineralization of the caries like lesion, magnification X40.

**Figure 3A** Polarized light photomicrograph of a longitudinal ground section of a control specimen (subgroup IIIB) showing dark demineralization segment, magnification X40.

**Figure 3B:** Polarized light photomicrograph of a longitudinal ground section of enamel treated with fluoride toothpaste (subgroup IIIA) showing a decrease in the extent of the lesion, magnification X40.
diffusion of Ca and P ions in the tooth which aided in remineralization of crystals in the lesion. In the current study, there was no significant difference in mean percent difference of enamel microhardness between group I (eggshell powder) and group II (novamine). On the other hand, they showed significant higher mean percent difference of enamel microhardness than group III (fluoride). These findings were in agreement with Golpayegani et al. in 2012 (26) who reported that novamin dentifrice appears to have a greater effect on remineralization of carious-like lesions when compared to that of fluoride containing dentifrice. On the other hand, these results were not in line with the findings of Mony et al. in 2015 (18) who, revealed that fluoride showed more remineralization on early enamel carious lesions than eggshell powder. The difference with the current results could be due to the difference in the type of toothpaste used in both studies. This study is a pioneer work to use polarized light microscope (PLM) to evaluate remineralizing effect of eggshell powder and novamine in comparison to fluoride. PLM was used for qualitative and quantitative evaluation of the lesion changes in conjunction with microhardness analysis, because it is extremely sensitive to small changes in the optical properties. It can qualitatively show the areas of mineral loss and mineral gain represented by the visualization of areas with different porosities and birefringence (31).

In the present study, qualitative data obtained from PLM showed that most of the representative lesions treated by eggshell powder solution or novamine toothpaste exhibited a shift from positive to negative birefringence with a striking reduction in the depth of the lesion. Other specimens showed complete absence of the dark bands within the body of the lesion that is present in the control specimens. This was proven quantitatively by the significant reduction in the mean lesion depth of test subgroups IA (egg shell powder) and IIA (novamine) in comparison with their corresponding controls. Qualitative data revealed that the enamel lesions of subgroup IIIA (treated by fluoride toothpaste) showed variable degrees of remineralization, but the body of the lesions showed less degree of enamel remineralization than in subgroups IA and IIA. This was proven quantitatively as there was no significant lesion depth reduction between test subgroup IIIA (fluoride) and its control. This finding is not in a line with the finding of Pulido et al. in 2008 (32) who reported that the highly concentrated sodium fluoride (NaF) 5000 ppm reduced lesion depth and had a protective effect against demineralization. Moreover, Prabhakar et al. in 2013 (33) concluded that NaF 5000ppm showed significant percentage of remineralization, measured as a decrease in lesion depth. The difference with the current results could be due to the use of different methodology. Although there was a significant difference in mean percent values of enamel microhardness among the three groups, there was no statistically significant difference in the mean percent values of the lesion depth among them. The possible explanation of these results is that, histologically, the overall lesion depth usually did not change after remineralization because the reduction of the pore volume inside the body of the lesion is due to the reduction in the overall pore size but the number of pores remained high. In addition to that, due to volume loss from demineralization, the lesion body did not repair by an organized crystal arrangement (34). Moreover, remineralization is a very slow process. In fact, while subsurface lesions remineralize in vitro within weeks, years are required for complete remineralization in vivo (35). Several studies have confirmed that the remineralization process restores minerals to a greater extent near the surface than in the underlying body of the lesion (36-38). This explanation was supported by ten Cate in 2008 (39) who concluded that the effect of the tested agents were found in the outer enamel and did not significantly affect mineral precipitation in the inner enamel and dentin. It was also in agreement with Kim et al. in 2013 (40) who, proved that the histological observations of lesions by PLM revealed higher recovery percentage for shallow lesions than deep lesions. Based on the overall qualitative and quantitative results of the present study, it could be concluded that both egg shell powder and novamine can be used as remineralizing agents. Moreover, they appear to have a greater effect on remineralization of initial caries-like lesions when compared to fluoride in young permanent teeth. The limitations of this study were the difficulty in reproducing the oral environment; cariogenic bacteria and different salivary compounds which might influence the outcome during application of these agents. In addition, the present study was limited to a 7 days period, while the de/remineralization processes are long-term processes. Furthermore, in vivo salivary flow and turnover cannot be sufficiently mimicked in vitro. Within the limitations of the current study, it is indicated that eggshell powder and novamine showed better remineralization capacity than fluoride, this rejects the proposed null hypothesis in the present study.

CONCLUSION
Based on the results of the present study and its limitations, it could be concluded that:

1. Both egg shell powder and novamine can be used as remineralizing agents.
2. Both egg shell powder and novamine appear to have a greater effect on remineralization of initial caries-like lesions when compared to fluoride in young permanent teeth.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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12.

10.


