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Remineralization Effect of Eggshell versus Nano-hydroxyapatite on Caries-like Lesions in Permanent Teeth (In Vitro)

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Abstract:

Background: Eggshell (ES) is a rich source of minerals since it contains calcium, phosphorous, magnesium, strontium, and fluoride. This study sought to compare the efficacy of nano-hydroxyapatite (NHA) and ES for remineralization of enamel caries-like lesions by pH cycling.

Materials and Methods: In this *in vitro* experimental study, artificial enamel carious lesions were induced in 47 human teeth using an acidic buffer solution. Deionized water was used as the control group, and 10% NHA and 3% and 10% ES powders in deionized water were tested as therapeutic agents. The surface microhardness of the samples was measured by a Vickers hardness tester before and after demineralization and after the application of therapeutic agents in a 7-day pH-cycling model. The data were analyzed using repeated measures analysis of variance.

Results: Microhardness significantly decreased in all samples after demineralization and significantly increased after exposure to therapeutic solutions. Microhardness of the samples was not significantly different among the therapeutic groups after pH cycling.

Conclusion: Based on the results of this study, ES can be used as a remineralization agent in enamel caries-like lesions.

Key Words: Eggshell, nano-hydroxyapatite, permanent tooth, remineralization, surface microhardness

Introduction

Dental caries is among the most common chronic diseases worldwide.[1] Restoration of carious lesions via tooth preparation and application of restorative material is a repetitive and irreversible cycle which increases the size of restoration and results in further loss of tooth structure over time. In the past two decades, knowledge about the etiology of tooth decay and factors responsible for its development has greatly enhanced.[2,3] Formation of incipient enamel caries is a reversible process where periods of progression alternates with periods of remineralization.[4] Under optimal conditions, the process of remineralization dominates demineralization and arrests the caries.[5]

Fluoride is one of the most effective remineralizing agents for caries prevention.[6] However, some concerns exist with regard to the extensive use of fluoride. Chronic exposure to low levels of fluoride can cause gastrointestinal, urogenital, and respiratory problems in normal individuals.[7] On the other hand, the prevalence of dental fluorosis has increased noticeably in non-fluoridated areas and to a lesser extent in optimally fluoridated areas.[7-9] Moreover, fluoride ions alone cannot completely remineralize carious lesions.[1,3] Formation of each fluorapatite molecule requires calcium and phosphate in addition to fluoride ions.[10] Thus, it is imperative to find an efficient, safe alternative to fluoride to completely prevent caries and remineralize the incipient enamel lesions.

Hydroxyapatite is a natural substance with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$; due to its favorable properties such as similarities to the mineral phase of the human hard tissues, biocompatibility and low solubility in humid environments, it has extensive applications in medicine and dentistry.[11] Nano-hydroxyapatite (NHA) particles have unique properties such as higher solubility, higher surface energy, and optimal biocompatibility compared to the typical hydroxyapatite.[12] Moreover, it has been reported that NHA particles have superior bioactivity to larger crystals.[13] Synthetic NHA has the same physicochemical properties as those of apatite in the enamel. It shows strong affinity to the tooth and can strongly adsorb on enamel surfaces.[14] A previous study evaluated the effect of NHA solution on erosive lesions and showed that enamel microhardness which had been decreased following erosion, significantly increased after exposure to NHA solution.[15] Another study compared NHA and sodium fluoride (NaF) mouthwash for remineralization of incipient caries and showed that NHA remineralized the incipient carious lesions similar to NaF mouthwash.[16] An *in situ* study evaluated the efficacy of NHA toothpaste for remineralization of carious lesions and prevention of demineralization and reported that NHA toothpaste was as effective as fluoride toothpaste for this purpose.[17]

Eggshell (ES) is a rich source of calcium.[18] It contains 94% calcium carbonate, 1% calcium phosphate, 1% magnesium carbonate, and 4% organic matter.[19] ES contains not only calcium but also other elements such as fluoride and strontium -despite the low concentration- and have a positive effect on bone and dental metabolism.[20,21] Researchers recently synthesized hydroxyapatite with excellent properties using ES.[22,23] In 1992, ES powder was registered as a medicinal and pharmaceutical agent. Biomin H® is a natural, rich source of calcium. In addition to calcium and phosphorous, it contains other elements and organic materials as well which boost the efficacy of calcium. 1 g of Biomin H® contains 370 mg calcium, 0.6 mg phosphorous, and 5 mg magnesium and other elements.[24]

In two separate animal and human studies, ES was used as a bone substitute for treatment of maxillofacial defects. The results showed that ES was biocompatible, cost-effective, and safe and can be used as an alternative to bone grafts for regeneration of bone defects.[23,25]

In a review article on the most important biological and clinical aspects of ES, it was shown that ES had antirachitic effects; it stimulates chondrocyte differentiation and cartilage growth. Moreover, it had positive efficacy for treatment of osteoporosis.[24]

No previous study has evaluated the application of ES for prevention of dental caries. Considering the important minerals present in the composition of ES and their confirmed efficacy for regeneration of bone defects.[23,25] We sought to assess the efficacy of ES for caries prevention in comparison with NHA, which is a calcium phosphate compound with well-recognized remineralizing potential for incipient caries. The aim of this study was to compare the efficacy of different concentrations of ES in distilled water with that of NHA for remineralization of enamel caries-like lesions in permanent teeth.

Materials and Methods

This *in vitro* study was conducted on 47 human third molar teeth.

Preparation of solutions

Demineralizing solution

The demineralizing solution used to induce subsurface carious lesions and in the pH-cycling model was a pH 4.6 and contained 1.4 mM calcium in the form of $Ca(NO_3)_2$, 0.9 mM phosphorus in the form of KH_2PO_4, 0.1 M acetate buffer in the form of acetic acid, and 0.03 ppm fluoride in the form of NaF.[26]

Remineralizing solution

The remineralizing solution used in pH-cycling model had a pH of 7 and contained 1.5 mM calcium in the form of $Ca(NO_3)_2$, 0.9 mM phosphorus in the form of KH_2PO_4, 0.1 M tris buffer (hydroxymethyl aminomethane), and 0.05 ppm fluoride in the form of NaF.[26]

Therapeutic agents

The therapeutic agents used included 10% solution of NHA, which was prepared by dissolving NHA powder (Shel Co., USA) in sterile, deionized water and 3% and 10% solutions of ES, which were prepared by dissolving Biomin H® (Biomin H®, Biomin A.S., Cifer, Slovak Republic) in sterile deionized water.

Preparation of the teeth

A total of 47 surgically extracted human impacted third molars which had no enamel cracks, erosion, or hypoplastic lesions were collected and stored in saline until the experiment. After cleaning, the teeth were mounted in a mold containing polyester and the external enamel surface was polished using rotary discs and silicon carbide abrasive papers for evaluation of surface microhardness (SMH). The teeth were then randomly divided into three groups of 15 and a control group of two samples using a table of random numbers.

Baseline SMH measurement

After polishing, baseline SMH1 of the teeth was measured by a Vickers microhardness tester (Type M, No g 5025, Shimadzu® Corporation, Japan) using 25 g load for 5 s.

Induction of early artificial carious lesions

To induce early artificial carious lesions, each tooth was immersed in 40 ml of the demineralizing solution at 37°C for 72 h without stirring.[11] After 72 h, SMH of the teeth was measured again with the same device and under the same load (SMH2).

The pH-cycling model

The pH-cycling models are used to simulate the dynamics of caries formation by inducing demineralization and remineralization cycles. In these models, enamel and dentin are exposed to demineralizing and remineralizing agents.[27] The pH-cycling model used in the current study included immersion of samples in the therapeutic solutions for 12 min.[28] For instance, the 15 samples in group one were immersed in 10% NHA solution; the 15 samples in group two were immersed in 3% ES solution, and the 15 samples in group three were immersed in 10% ES solution. Next, the samples were removed from the solutions and rinsed with deionized water for 5 s to eliminate the excess material. Next, to simulate daily acidic challenge in the oral environment, the teeth were immersed in the demineralizing solution for 3 h and after rinsing (as described above), they were immersed in the remineralizing agent for the rest of 24 h. This cycle was repeated for 7 days; the therapeutic solutions were refreshed daily while the demineralizing and remineralizing solutions were refreshed once every 3 days. The two samples in the control group were immersed in deionized water for the entire 7-day period. After completion of the 7-day pH-cycling period, SMH of the samples was measured again by the same device under the same load as described earlier (SMH3).

The data were analyzed by SPSS version 16 using repeated measures analysis of variance (ANOVA) and Bonferroni analysis. The 95% confidence interval was calculated, and level of significance was set at $P < 0.05$.

Results

The mean and standard deviation of SMH values in the groups are shown in Table 1. 15 samples were evaluated in each group; however, due to errors during SMH measurement, two samples in the NHA group were excluded from the study.

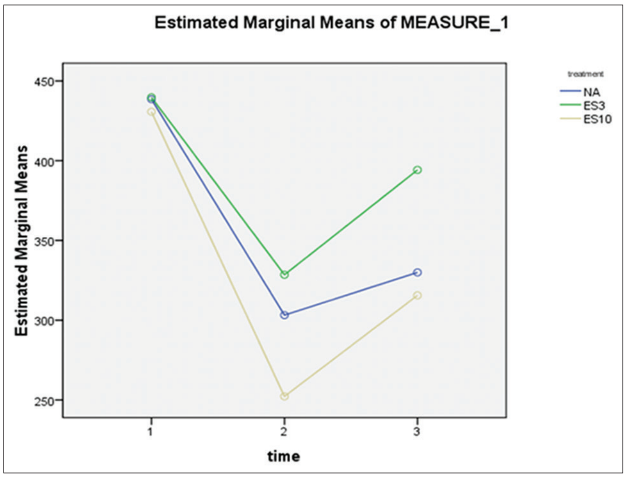
Repeated measures ANOVA revealed no significant difference in SMH changes at the three-time points among the three groups ($P = 0.087$). SMH showed significant changes over time in all three groups ($P < 0.001$). Pairwise comparison of the microhardness at each time point revealed that in all three groups, microhardness at the second time point significantly decreased compared to the baseline ($P < 0.001$) and significantly increased thereafter in all three groups ($P < 0.001$; Graph 1).

Microhardness of the samples in the control group did not change after immersion in deionized water compared to the second time point.

Table 1: The mean microhardness values of the groups before the induction of caries, after the induction of caries and after pH cycling.

Treatment	Mean	Standard deviation	*N*
SMH1			
NA	438.54	73.376	13
ES3	439.73	37.456	15
ES10	430.67	74.493	15
Total	436.21	62.232	43
SMH2			
NA	303.15	75.927	13
ES3	328.47	80.583	15
ES10	252.20	97.016	15
Total	294.21	89.572	43
SMH3			
NA	329.92	118.349	13
ES3	394.20	67.561	15
ES10	315.53	85.982	15
Total	347.33	96.057	43

SMH: Surface microhardness, NHA: Nano-hydroxyapatite, ES3: 3% eggshell, ES10: 10% eggshell



Graph 1: The interaction effect of time and microhardness in nano-hydroxyapatite (NA), 3% eggshell (ES3), and 10% eggshell (ES10) groups.

Discussion

Dental caries is one of the most common chronic diseases of the childhood.[29] However, incipient (non-cavitated) enamel carious lesions are reversible given that a supersaturated state of calcium and phosphorous ions is maintained adjacent to the enamel.[4,5] This study sought to assess and compare the efficacy of 10% NHA, 3% ES, and 10% ES for remineralization of enamel simulated caries in permanent teeth. The results showed that SMH of the samples in the three groups was not significantly different at the three measurement time points. The microhardness of the samples at baseline was not significantly different which indicates the random distribution of the samples in the three groups. The microhardness of the samples at the second measurement time point was not significantly different either which indicates that the efficacy of therapeutic agents could be well compared to the samples in the three groups. The microhardness of the samples at the third measurement time point was not significantly different either which indicates that all three solutions namely 10% NHA, 3% ES, and 10% ES had similar efficacy for remineralization of enamel simulated carious lesions. Furthermore, changes in microhardness during the experiment were significant in all three groups. In other words, in all groups, microhardness significantly decreased following exposure to demineralizing solution while it significantly increased following pH cycling; this indicates the efficacy of all three remineralizing agents for the remineralization of incipient enamel caries-like lesions.

The optimal efficacy of NHA solution for remineralization of initial carious lesions shown in the current study was in line with the results of previous studies. Haghgoo *et al.*[15] showed that immersion of teeth with erosive lesions in NHA solution increased their microhardness which is in agreement with the current findings. However, we evaluated the effect of NHA solution on initial carious lesions while they evaluated the effect of NHA solution on erosive lesions.

Najibfard *et al.*[17] in their study demonstrated the similar efficacy of NHA and NaF toothpaste for remineralization of carious lesions and prevention of demineralization; the difference between our study and theirs was in that we used pure NHA instead of NHA toothpaste.

The efficacy of ES solution for remineralization of incident carious lesions has not been previously investigated, and the authors did not find any similar study for the comparison of results. However, this result was expected because ES is a rich source of calcium, phosphorus, and other minerals. On the other hand, since the efficacy of 3% and 10% ES solutions for remineralization of enamel caries-like lesions was similar to that of 10% NHA solution as well as the fact that the efficacy of the 10% NHA solution has been confirmed in previous studies,[15-17] it may be concluded that ES solution can also be used as a remineralizing agent and an adjunct or even an alternative to fluoride and NHA.

Following the application of remineralizing agents such as NHA and ES on enamel caries-like lesions, it appears that mineral ions diffused into the superficial layer obstruct the surface porosities; further diffusion of minerals is limited after reaching a plateau. This explains the lack of a significant difference in remineralization of lesions following the use of 3% and 10% ES solutions in our study. Similarly, previous studies showed an increase in remineralization by an increase in the concentration of NHA by up to 10%; however, further increase over 10% caused no significant change in remineralization.[11] On the other hand, since no significant difference was noted in remineralization between 3% and 10% ES groups and both showed similar efficacy for remineralization of simulate enamel caries, it appears that 3% is the ideal concentration of ES powder in distilled water for this purpose. The remineralizing effect of ES solution on simulated enamel caries may be of benefit for preventive dentistry given that this effect is proven in future clinical studies.

To simulate carious lesions, *in vitro*, *in vivo*, animal, and clinical demineralization models may be used. Ten Cate and Marsh believe that to confirm the efficacy of suggested non-fluoride caries-preventive agents for remineralization of carious lesions, their mechanism of action, optimal concentration, and possible shortcomings must be primarily studied in simple laboratory studies before conduction of *in vivo* studies and clinical trials.[30] Moreover, it would be difficult, if not impossible, to control for all the confounding factors in clinical studies such as diet, differences in flow and composition of saliva, patient cooperation, and interpretation of results.[31] For this reason, we designed a pH-cycling model for caries simulation *in vitro*; by doing so, we exclusively evaluated the efficacy of ES and NHA for remineralization of artificially induced carious lesions in simulated oral conditions in the absence of the confounding factors that are normally present in the oral environment.

To simulate subsurface carious lesions *in vitro* while maintaining the superficial layer, weak organic acids such as lactic or acetic acid are used in the composition of the demineralizing agent.[31,32] Furthermore, the presence of calcium and phosphate,[33] as well as fluoride[34] in the solution, helps preserve the superficial later while enhancing mineral loss from the subsurface layer. For this reason, to artificially create incipient enamel lesions, a demineralizing agent containing calcium, phosphate, acetic acid (weak acid), and small amount (0.03 ppm) of fluoride was used. The presence of small amount of fluoride in the demineralizing and remineralizing agents preserves the superficial layer while not interfering with the response to therapeutic agents. It can also simulate the concentration of fluoride in the oral environment following tooth brushing with a fluoridated toothpaste or use of a fluoride-containing mouthwash or even fluoride in the saliva of a person that drinks fluoridated water.[35]

Microhardness testing is a reliable method extensively used for assessment of remineralization of the tooth surface.[27,36]

Conclusion

Based on the results of this study, ES solution can be used as a remineralizing agent for incipient enamel carious lesions. This natural substance is as effective as NHA for remineralization of enamel simulated caries.

Suggestions

Future studies are required to assess the efficacy of ES solution for remineralization of incipient enamel caries *in situ*.

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