Remineralizing effect of zinc reinforced synthetic nano-hydroxyapatite on caries-like lesion in human permanent teeth - An in vitro study

Maheswari Elumalai, Sri Sakthi Doraikannan*, Meignana Arumugham Indiran, Pradeep Kumar Rathinavelu

ABSTRACT

Introduction: Dental caries is among the most common chronic disease worldwide. A number of research projects are now focusing on the development of remineralizing agents to cease the progression of initial dental caries. Synthetic nano-hydroxyapatite (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. Zinc is a trace element of valuable importance in the oral cavity. Zinc and synthetic NHA has the potential to reduce demineralization and promote remineralization of incipient caries lesion. Aim: This study aims to evaluate and compare the remineralizing effect of synthetic NHA and zinc reinforced synthetic NHA on dental caries-like lesion in extracted human permanent teeth. Materials and Methods: In this in vitro study, 21 maxillary premolars extracted for orthodontic reasons were included in the study. Synthetic NHA was synthesized using calcium hydroxide and orthophosphoric acid. There are three groups, the first group for demineralized teeth specimen, the second group for 10% synthetic NHA solution, and the third group - zinc reinforced hydroxyapatite solution as treatment agents. Enamel window formation of 4 mm (length) × 4 mm (width) was created in teeth specimen. Initial enamel lesion was prepared with an acidic buffer followed by tooth specimens are subjected to pH cycling. Analysis of caries lesion depth after demineralization and remineralization of tooth specimens was done using polarized light microscope. Results: The mean dental caries lesion depth in demineralized solution group was 160 ± 32.6. In this study, the mean dental caries lesion depth in 10% synthetic NHA solution group and zinc reinforced synthetic NHA was 135.49 µm and 111.78 µm. Conclusion: Synthetic NHA has a remineralizing effect due to deposition of calcium phosphate ions in the porosities of demineralized area. The remineralizing potential of zinc reinforced synthetic NHA was found to be higher than synthetic NHA. Zinc reinforced synthetic NHA showed an additive remineralizing potential compared to synthetic NHA.

KEY WORDS: Demineralization, Lesion depth, Nano-hydroxyapatite, pH cycling, Zinc

INTRODUCTION

Dental caries is among the most common chronic diseases worldwide.[1] Dental caries is a microbial disease which affects all ages of population irrespective of their socioeconomic status. The pathogenesis of dental caries involves a complex phenomenon. Restoration of carious lesions through 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.[2] In the 20th century, the world is witnessing many revolutions in the medical treatment and care. There is increasing awareness of the value of good health. Good health cannot be attained without good oral hygiene. It is said that mouth is the mirror of body and it is the gateway to good health. The field of dentistry plays a major role in the maintenance and development of oral hygiene.[3] In earlier times, herbs are used in dentistry for prevention and curative purpose of dental caries. The main advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance.[4] Nowadays, a newer treatment approach for dental caries involve nanotechnology. Nanoparticles are small inert particles which possess superior bioactivity.
Science of biomaterials made giant strides for the past few decades and has been recognized as a separate specialty in the field of medicine and dentistry.\cite{5} Nanotechnology is the science which deals with the physical, chemical, and biological properties of structures and their components with dimensions in a nanoscale.\cite{9} Poorly soluble, poorly absorbed, and labile biologically active substances are remodeled to promising deliverable drugs through the recent advancements of nanotechnology.\cite{7} Many conservative treatment procedures for dental caries involve sacrificing tooth structure. Tooth structure once destroyed, it will not remineralized by natural biological process. The physiological integrity of tooth structure is hampered majorly by the loss of dentin. Restorative strategies have been continuously developing to repair and replace lost tooth structures.\cite{8}

Restorative materials have been relied on to restore the teeth affected with dental caries. The most commonly used was glass ionomer cement. The origin of this glass ionomer cement is the inclusion of nanofillers, which constitute up to two-thirds of the filler content. The presence of nanosized glass particles and clusters of nanosilica particles in this material has been claimed to improve its physical properties.\cite{9} Dental caries restorative procedure includes removal of caries-affected tooth part and restored with a restorative cement. Kumar conducted a study on Papacarie; he stated that the chemomechanical caries removal is a non-invasive technique for caries removal which aims at the elimination of infected dentin.\cite{10}

Hydroxyapatite is the mineral crystalline form of calcium and phosphorus found in the enamel, dentin, cementum, and bone. It is widely used in biology, medicine, and dentistry due to its optimal characteristics such as similarity to the mineral structure of hard tissue, biocompatibility, and low solubility.\cite{11} Tooth structure is made of hydroxyapatite. Hydroxyapatite crystal lattice contains voids in the structure and the tooth structure gets modified in the presence of other minerals such as zinc, strontium, and fluoride.

Nano-hydroxyapatite (NHA) particles have unique properties such as higher solubility, higher surface energy, and optimal biocompatibility compared to the typical hydroxyapatite. Moreover, it has been reported that NHA particles have superior bioactivity to larger crystals.\cite{12} 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.\cite{13} Synthetic NHA was synthesized by various methods such as wet precipitation method and sol-gel method, and it proved to be equivalent in properties to natural hydroxyapatite.

In several literature, an increasing number of reports have shown that NHA has the potential to remineralize caries-like lesion following addition to toothpastes, mouthwashes, etc.\cite{14-16} Some studies have also reported that a 4% (wt%) nano-HA liquid suspension had good potential to remineralize incipient caries lesions.\cite{17} 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.\cite{19} For remineralization of tooth structure, various remineralizing agents are incorporated in dental cement, dentifrices, and mouthwashes to facilitate remineralization of white spot lesions, incipient dental caries lesion, and tooth structure lost due to wasting diseases.

Zinc (Zn) is a trace element of valuable importance in the oral cavity, it is naturally present at various sites such as dental plaque, dental hard tissues, and saliva. Hanke investigated the effect of zinc on oral bacteria among varied consortia.\cite{20}

Finney et al. reported the broad-spectrum activity of zinc.\cite{21} Zinc has antimicrobial properties against different bacteria such as Streptococcus mutans.\cite{22} Zinc is incorporated in mouthwashes to prevent biofilm formation and cure gingivitis problem.

The outer layer of enamel contains higher content of zinc, whereas it is present in low concentration in subsurface. It exhibits the similar pattern to that of fluoride which is also present in lower concentration in deeper layers of enamel. Concentration of zinc varies from 430 ppm to 2100 ppm depending on the different layers of tooth. It has been reported that zinc in low concentrations has the capability to reduce dissolution and promote remineralization under caries simulating conditions.\cite{23} It has been reported that the zinc content in enamel increases during post-eruptive phase of the tooth suggesting that the tooth surface acquires more minerals from the oral fluids, similar to that of fluoride uptake.\cite{22} Zinc-doped synthetic NHA is used in various fields of dentistry such as for bone formation in maxillofacial surgical procedures and as bone graft material in dental implant treatment procedures.

Studies with synthetic hydroxyapatite showed that zinc is readily acquired by the apatite as it competes with calcium and can attain position on the apatite crystal making hydroxyapatite resistant to the acid dissolution. Thus, it has the potential to influence both demineralization and remineralization. Hence, the aim of the study was to evaluate and compare the remineralizing effect of synthetic NHA and zinc reinforced NHA on caries-like lesion in human permanent teeth.
MATERIALS AND METHODS

Preparation of Nano-HAp
Synthetic NHA was synthesized by wet chemical precipitation method. An aqueous solution of 0.5 m calcium hydroxide was mixed with 0.3 m orthophosphoric acid (Sigma-Aldrich) drop by drop until the pH reaches 12.5. The mixture was kept for stirring for 24 h. The mixture was then centrifuged at 6000 rpm for 15 min. The precipitate was rinsed with distilled water and then dried at 100°C for 7 h. Finally, synthetic nano-hydroxyapatite crystals was obtained [Figure 1].

Characterization
Crystalline phases were identified by means of an X-ray diffractometer (XRD). Crystalline phases detected in the patterns were identified by comparison to standard patterns from the ICDD (International Centre for Diffraction Data) - PDF. All XRD patterns show diffraction peaks characteristics of hydroxyapatite present in standards and literature [Figure 2].

Study Methodology
In this in vitro study, 21 fully erupted sound maxillary premolar extracted for orthodontic reasons were included in the study. Exclusion criteria include teeth with dental caries, endodontically treated teeth, teeth affected with fluorosis, teeth with enamel defects, fractured teeth, and teeth affected with wasting diseases such as attrition, abrasion, and erosion. Inclusion criteria include sound maxillary premolar. Teeth extracted for orthodontic reasons were collected from the Department of Oral Surgery, Saveetha Dental College, Chennai. All the teeth were then microscopically examined for caries and other possible microcracks or defects. The specimen that was not fulfilling the inclusion criteria was rejected while those fulfilling the criteria were stored in 10% formalin solution until further use. Ethical clearance was obtained from the Institutional Ethical Board, Saveetha University.

Specimen preparation and enamel window formation: Each tooth was sectioned buccolingually in a vertical

Figure 1: Synthetic nano-hydroxyapatite crystals

Figure 2: X-ray diffractometer pattern analysis of synthetic nano-hydroxyapatite
The roots of the teeth were then cut using a water cool saw. All the 21 teeth specimens were subjected to enamel window formation. The buccal surface of each tooth was coated with acid-resistant nail varnish (Revlon Corp., NY, USA) leaving a window of 4 mm × 4 mm [Figure 3]. This was done to limit the area of demineralization followed by remineralization only in the window area. The teeth were then placed in distilled water until further use.

Preparation of solution: The buffered de-/re-

mineralizing solutions were prepared using analytical grade chemicals and deionized water.

a. Demineralizing solution
It was prepared using the following chemicals:
2.2 mm calcium chloride, 2.2 mm sodium phosphate, 0.05 m acetic acid, and 1 m potassium hydroxide to adjust the pH to 4.4.

b. Remineralizing solution
It was prepared using the following chemicals:
1.5 mm calcium chloride, 0.9 mm sodium phosphate, and 0.15 m potassium chloride which had a pH of 7.

c. Therapeutic agents
The therapeutic agents used are demineralized solution (Group 1), 10% solution of synthetic NHA (Group 2) and 10% solution of zinc reinforced synthetic NHA (Group 3).

Preparation of early artificial caries lesion: Early artificial caries lesion was produced in the enamel, according to 10 Cate and Duijsters. Each specimen was immersed in 8 ml of demineralization solution for 72 h at 37°C [Figure 4]. After artificial caries preparation, size of demineralized area and lesion depth of the enamel blocks were measured.

pH-cycling model: The pH-cycling models are used to stimulate the dynamics of caries formation by inducing demineralization and remineralization cycles.[27] After pH cycling process [Flowchart 1], the teeth specimens were sectioned using hard tissue microtome (Leica SP 1600), to obtain 200µm thickness of teeth specimen. [Figure 5]. Each section obtained was analyzed for depth of the lesion under polarized light microscope (Olympus BX 51) [Figures 6-8]. The lesion depth was measured from the surface of the tooth to the maximum depth using the Image J software (Java-based image processing program). The mean and standard deviation (SD) of the lesion depth from each group was obtained and statistical analysis was done using independent t-test.

RESULTS
The mean lesion depth in demineralized solution group was 160 ± 32.6. The mean lesion depth in 10% synthetic hydroxyapatite solution and zinc reinforced synthetic hydroxyapatite was 135.49 µm and 111.78 µm. The mean lesion depth of the teeth specimens ranged from 111.78 to 160.27 µm and the SD ranged from 32.6 to 38.3 µm. Table 1 shows the intergroup comparison of the mean and SD values of Group 2 (135.49 µm) and Group 3 (111.78 µm). p-value obtained was significant at 5% level. Zinc reinforced synthetic hydroxyapatite group illustrates the least mean lesion depth score (111.78 µm). Table 2 depicts the intergroup comparison of dental caries lesion depth of synthetic NHA and zinc reinforced synthetic NHA after demineralization and remineralization. The mean lesion depth in 10% synthetic hydroxyapatite solution and zinc reinforced synthetic hydroxyapatite was 135.49 µm and 111.78 µm. The difference in mean lesion depth between demineralized solution group and 10% synthetic hydroxyapatite was found to be statistically significant (P = 0.02). The difference in mean lesion depth between demineralized solution group and zinc reinforced synthetic hydroxyapatite was found to be statistically significant (P = 0.01).

DISCUSSION
The basic mechanism of remineralization involves the diffusion of calcium and phosphate ions from saliva and other topical sources to build a hypermineralized, acid-resistant, and fluorapatite-like layer on the existing crystal remnants which act as remineralization nuclei. Remineralization of white spot lesions may be possible
with a variety of currently available agents such as fluoride, casein phosphopeptide-amorphous calcium phosphate, and bioavailable calcium phosphate. This concept bridges the traditional gap between prevention and surgical procedures.[27] In the current study, NHA was directly selected as a remineralizing agent, and an *in vitro* pH-cycling model was used to evaluate the effect of nano-HA concentrations and zinc reinforced synthetic NHA on the initial enamel caries lesions. Since the surface area

### Flowchart 1: Step-by-step procedure (pH cycling)

**Table 1: Intergroup comparison of dental caries lesion depth after remineralization with synthetic hydroxyapatite and zinc reinforced synthetic hydroxyapatite**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Mean lesion depth in µm</th>
<th>SD</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% synthetic hydroxyapatite solution</td>
<td>7</td>
<td>135.49</td>
<td>4.93</td>
<td>0.02*</td>
</tr>
<tr>
<td>Zinc reinforced synthetic hydroxyapatite solution</td>
<td>7</td>
<td>111.78</td>
<td>4.66</td>
<td></td>
</tr>
</tbody>
</table>

Independent *t*-test. *t*-value=−9.03. *P*<0.05 considered as statistically significant, SD: Standard deviation

**Table 2: Intergroup comparison of dental caries lesion depth of synthetic hydroxyapatite and zinc reinforced synthetic hydroxyapatite after demineralization and remineralization**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean lesion depth in µm±SD</th>
<th><em>t</em>-value</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>After demineralization</td>
<td>After remineralization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% synthetic hydroxyapatite solution</td>
<td>161±4.57</td>
<td>135.49±4.93</td>
<td>10.11</td>
</tr>
<tr>
<td>Zinc reinforced synthetic hydroxyapatite solution</td>
<td>161±4.57</td>
<td>111.78±4.66</td>
<td>12.85</td>
</tr>
</tbody>
</table>

Independent *t*-test. *P*<0.05 considered as statistically significant, SD: Standard deviation
and proportion of atomicity increase with decreasing particle size, nano-HA has bioactive and biocompatible properties.\cite{28} Incipient caries lesions are more porous than sound enamel, which allows a greater penetration of solution ion constituents and a larger surface area for subsequent reaction with enamel mineral.\cite{29} These factors increased the potential of nano-HA to directly fill up defects and micropores on demineralized teeth. If nano-HA penetrates the enamel pores, nano-HA will act as a template in the precipitation process and will continuously attract a large amount of $\text{Ca}^{2+}$ and $\text{PO}_4^{3-}$ from the remineralization solution to the enamel surface to fill the vacant positions of the enamel calcium crystals. This, in turn, will promote crystal integrity and growth.\cite{30}

In the current study, the mean lesion depth obtained after remineralization with 10% synthetic NHA analyzed by polarized light microscope was 135.49 $\mu$m. In contrast, a study done by Rajan \textit{et al.}\cite{31} found the mean lesion depth of 1185.14 $\mu$m with Remin Pro dentifrice containing NHA. Reason for higher lesion depth in comparative study could be the different pH-cycling model and they have used dentifrice.

Najibfard \textit{et al.}\cite{11} 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 synthetic NHA instead of NHA toothpaste. Haghgoo \textit{et al.}\cite{32} showed that immersion of teeth with erosive lesions in NHA solution increased their microhardness. However, we evaluated the effect of NHA solution on initial carious lesions while they evaluated the effect of NHA solution on erosive lesions.

In most of the studies, polarized light microscope was used for qualitative assessment of lesion and microradiography as a quantitative measure of lesion mineralization as it shows more highly demineralized areas of the lesions as radiolucent areas and mineralized area as a radiopaque surface layer.\cite{33} However, polarized light microscope can give a high degree of differentiation between demineralized area and normal area of tooth sample. This differentiation is better than microradiography.\cite{34} In our study, as we
were concerned with lesion depth measurement, the polarized light microscope was supposed to be the best method of choice.

In the current study, the mean lesion depth obtained after remineralization with zinc reinforced synthetic NHA analyzed by polarized light microscope was 111.78 µm. In a dissimilar study done by Poggio et al., found the mean enamel microhardness value of 313.5 with dentifrice containing zinc-hydroxyapatite (Zn-HAp). In the comparative study, they have done microhardness test as outcome analysis, whereas in the present study, we have assessed lesion depth using polarized light microscope. However, we evaluated the effect of zinc-NHA solution on initial carious lesions while they evaluated the effect of zinc-NHA solution on erosive lesions. Polarisized light microscope was used to assess the lesion depth because the histological features of dentin and enamel can be visualized better due to its birefringence property, which is not well appreciated in a transmitted light microscope.

The solubility of carbonated and non-carbonated apatite is reduced after the incorporation of zinc. It also reduces the acid reactivity of the apatite which subsequently may reduce the crystalline disorder. Larger crystals precipitating out with very few defects may occur. The combination of fluoride and zinc proved to be more effective in reducing the structural disorder of HAp which is due to incorporation of carbonate. In the present study, the mean lesion depth in demineralized group was found to be 160.3 µm. The intergroup comparison between demineralized specimen group and 10% synthetic hydroxyapatite group was found to be statistically significant (P = 0.02). However, the intergroup comparison between demineralized group and zinc reinforced synthetic NHA was marginally higher with P = 0.01.

The prevention of dental caries has long been considered as an important task for the health profession. Scientific research continues to make progress in identifying the best practices for diagnosing, treating, and preventing dental caries. To assess the magnitude of the preventive task, it is necessary to know the extent and severity of the disease.

Thus, the initial carious lesions can be treated non-invasively by remineralization with calcium, phosphate, and fluoride to restore the strength and esthetic appearance and to increase the resistance to future acid challenge. However, this in vitro study had certain limitations such as difficulty in simulating the oral environment, lower level of salivary proteins, lack of bacteria in the artificial saliva solution used, control over the salivary flow rate, and a harsher acidogenic challenges used in a shorter period of time.

**CONCLUSION**

NHA had the potential to remineralize initial enamel caries lesions under dynamic pH-cycling conditions. A suspension of 10% NHA appeared to be the optimal concentration for remineralization of early enamel caries. NHA of proper concentration could, therefore, be beneficial in promoting remineralization with regular daily usage. Zinc is substituted with synthetic NHA because zinc ions have been described as a crystallization inhibitor and have, therefore, been used in toothpaste as anti-calculus agents in addition to their use as an antibacterial active. Hence, the current study shows the additive remineralizing potential of zinc reinforced synthetic NHA on initial enamel lesions. This newer remineralizing agent proved to be beneficial and when incorporated in toothpaste, mouth rinse, varnish, and pit and fissure sealant will promote remineralization process to cease the progression of initial dental caries. Dental caries still remains to be a major public health problem worldwide. Hence, prevention and control of dental caries helps to improve the quality of life of the people.

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