Evaluation of remineralization potential and cytotoxicity of a novel strontium-doped nanohydroxyapatite paste: An in vitro study

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Abstract

Background: The focus of caries management has shifted to the early detection of caries and noninvasive methods of management of incipient lesions with novel remineralizing agents.

Aim: The aim of this study is to evaluate and compare the remineralization potential of a novel laboratory synthesized strontium-doped nanohydroxyapatite (SrHAp) paste to a commercially available regular dentifrice.

Materials and Methods: Sixty enamel specimens (4 mm × 4 mm × 1 mm) were divided into two groups based on the type of dentifrice applied: Group I – regular toothpaste and Group II – SrHAp paste. Calcium/phosphorous ratio of all sound specimens was evaluated using Scanning Electron Microscopy-Energy Dispersive X-ray analysis. Samples in both groups were subjected to demineralization, and the calcium/phosphorous ratio was analyzed. The samples were then subjected to remineralization using the specific agents in each group, and the mean calcium–phosphorus ratio was assessed. Cytotoxic evaluation of both pastes was done by direct microscopic observation and MTT assay.

Statistical Analysis: Comparison of mean calcium and phosphorous values of sound enamel, demineralized, and remineralized specimen in Groups I and II was done using the one-way ANOVA and Tukeys post hoc test. Intergroup comparison after remineralization was done using the Student’s t-test.

Results and Conclusion: Group II showed higher remineralization potential than Group I and was statistically significant. Cytotoxicity of novel paste was less compared to the regular toothpaste. SrHAp showed better remineralization than regular toothpaste and can be considered for enamel repair in incipient carious lesions.

Keywords: Cytotoxic evaluation; remineralization; scanning electron microscopy-energy dispersive X-ray; strontium-doped nanohydroxyapatite

INTRODUCTION

Dental caries is one of the significant public health problems and we continue to search for ways to reduce the risk of caries in patients. The focus of caries management has shifted to the early detection of caries and noninvasive methods of management of incipient lesions with novel remineralizing agents.[1]

White-spot lesions are the earliest phase of the caries process and are reversible. The mechanical and crystallographic studies on white-spot lesions revealed...
around 10% loss in mineral content, making the area softer and prone to enamel caries.\textsuperscript{[3]} Noncavitated lesions as well as caries extending up to the dentinoenamel junction can be arrested. This is possible if the cariogenic challenges of the specific microenvironment are sufficiently controlled or/and if therapeutic agents are applied for tissue healing.\textsuperscript{[3]}

Recently, nanoparticles of hydroxyapatite (nHAp) with the same chemical composition as of tooth enamel \( \text{Ca}_9(\text{PO}_4)_{6}(\text{OH})_2 \), has been used for remineralization. Nanohydroxyapatite (nHAp) shows higher \( \text{Ca}^{2+} \) ion release rates and superior functional properties due to uniform grain size.\textsuperscript{[4]} Fluorides which are considered as a gold standard for remineralization cause hypermineralization of the surface layers and fail to strengthen the teeth from within, while nHAp induces mineralization from within the teeth along with the natural therapy of saliva.\textsuperscript{[5]}

However, the main drawbacks of nHAp are lack of strength, brittleness, high degree of crystallinity, and low solubility at neutral pH.\textsuperscript{[6]} Many researchers have tried replacing calcium (\( \text{Ca}^{2+} \)) ion with strontium ion (\( \text{Sr}^{2+} \)) in varied ratios to increase the acid reactivity of apatite, improvement in solubility, and increased fluoride release successfully.\textsuperscript{[7–10]} Hence, strontium-doped nHAp (SrnHAp) may be beneficial for inducing enamel repair and remineralization.

Evaluation of remineralization will require the measurement of even small changes in a tooth’s mineral content. Scanning electron microscopy (SEM) along with energy dispersive X-ray analysis (EDX) is a microanalytical technique that is employed to quantitatively estimate the amounts of mineral in a given tooth sample.\textsuperscript{[11]} SEM helps to assess the topographic changes of the enamel surface.

The present study evaluates and compares the remineralization potential of a novel laboratory synthesized nanohydroxyapatite paste containing SrnHAp to a commercially available regular dentifrice. Toxicology assessment was also done to correlate its possibility for future application.

**MATERIALS AND METHODS**

**Preparation of enamel specimens**

Sixty intact premolars, extracted for orthodontic reasons were cleaned and used for the study after approval from the Institutional Ethical Committee.

Sixty enamel specimens of 4 mm × 4 mm × 1 mm size were prepared from the buccal surfaces of the teeth by sectioning. The samples were divided into two groups of thirty specimens each. Group I (control group) in which the samples was treated with commercially available regular toothpaste and Group II treated with the novel SrnHAp paste.

All sound enamel samples were subject to SEM to assess the surface topography and EDX analysis to evaluate the mean calcium and phosphorus values.

**Preparation of strontium-doped nanohydroxyapatite paste**

SrnHAp was prepared by co-precipitation method which is described as follows.\textsuperscript{[12]} 50 mL of the solution containing calcium nitrate tetrahydrate (0.57M) and strontium nitrate (0.18M) in 1:1 ratio was prepared. After stirring the solution for 15 min, 50 mL of aqueous solution of ammonium dihydrogen orthophosphate (0.45M) was added drop wise. The \( \text{pH} \) was maintained at \( \geq 10 \) by adding ammonia at a temperature of 80°C. The suspension obtained was washed with distilled water, centrifuged at 5500 rpm for 30 min and lyophilized. The fine powder of 25 mol\% SrnHAp thus obtained was used for the preparation of the paste.

100 g of the novel paste was prepared. The paste was constituted in the following proportion using 25 mol \% SrnHAp 50% by weight (wt), sorbitol, and glycerine as humectant 30% by wt, sodium lauryl sulphate as detergent 2% by wt, sodium alginate as binder 3% by wt, and de-ionized water 15% by wt.\textsuperscript{[13,14]} [Table 1]. All the dry ingredients were finely powdered in a mortar and pestle and mixed with glycerine and deionized water until a paste like consistency was obtained. It was then assimilated into a tube labeled novel SrnHAp paste and used for the experiment.

**Demineralization of the samples**

The samples in both the groups were subjected to demineralization with freshly prepared McInnes’s demineralizing solution (1 ml of 36% hydrochloric acid, 1 ml of 30% hydrogen peroxide, and 0.2 ml of anesthetic ether in the ratio of 5:5:1).\textsuperscript{[15]} The demineralizing solution was applied to the surface of enamel samples using a cotton applicator for 5 min. It was then washed under running tap water, damped dry with absorbent paper, and then stored in artificial saliva for 24 h to prevent dehydration. After 24 h interval, the demineralization cycle was repeated in the same manner as described above. All the demineralized specimen were washed in running water, damped dry, and subjected to SEM-EDX analysis. The mean calcium and phosphorus content of demineralized specimen was recorded.

**Table 1: Table of ingredients (strontium-doped nanohydroxyapatite paste)**

| Ingredients (/100 g) | Quantity (w/w) | Role |
|----------------------|----------------|------|
| 25 mol% SrnHAp       | 50             | Remineralizing agent and abrasive |
| Sorbitol             | 14             | Humectant |
| Glycerine            | 16             |       |
| Sodium lauryl sulphate | 2               | Detergent |
| Sodium alginate      | 3              | Binder |
| Deionized water      | 15             | Vehicle |

SrnHAp: Strontium-doped nanohydroxyapatite
Remineralization of the samples
The demineralized specimens were subjected to remineralization with, regular dentifrice (Colgate Strong Teeth Anticavity Toothpaste) in Group I and the laboratory synthesized SrnHAp paste in Group II. All the samples were brushed using a motorized toothbrush for 3 min twice daily (12 h interval), rinsed with running tap water, and stored in artificial saliva for 28 consecutive days. The samples were then subjected to SEM-EDX analysis to evaluate the mean calcium and phosphorus content after remineralization.

Cytotoxic evaluation of the novel laboratory synthesized strontium-doped nanohydroxyapatite dentifrice

Cells seeding in 96 well plates
The L929 (Fibroblast) cell line was cultured in 25 cm² tissue culture flask with Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine, sodium bicarbonate (Merck, Germany), and antibiotic solution containing: Penicillin (100U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany). The viability of cells was evaluated by direct observation of cells by inverted-phase contrast microscope and followed by MTT assay method.

After 24 h incubation period, the sample content in wells was removed, and 30 µl of reconstituted MTT solution was added to all test and control wells, the plate was gently shaken well, then incubated at 37°C for 4 h. After the incubation period, the supernatant was removed and 100 µl of MTT solubilization solution (dimethyl sulphoxide) was added. The wells were mixed gently to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm.

Statistical analysis
The comparison of mean calcium and phosphorous values of sound enamel, demineralized and remineralized specimen in Groups I and II was done using the one-way ANOVA and Tukeys post hoc test. Intergroup comparison of Group I and Group II after remineralization was done using the Student’s t-test.

RESULTS

Comparative analysis
The mean calcium and phosphorus values of sound enamel specimen, demineralized as well as remineralized specimen in Group I (regular toothpaste) are given in Tables 1 and 2. Following remineralization with the regular toothpaste, the mean calcium and phosphorus values obtained were lesser than that of sound enamel specimens and were statistically significant (P = 0.000) [Table 2 and Graph 1].

Table 2: Group I - mean calcium phosphorus values (scanning electron microscopy-energy dispersive X-ray analysis)

| Group     | Mean calcium | Mean phosphorus |
|-----------|--------------|-----------------|
| Baseline (B) | 65.32±0.53   | 21.05±0.76      |
| Demineralized (D) | 55.15±0.47 | 15.77±0.57      |
| Remineralized (R) | 55.14±0.47 | 15.77±0.57      |
| P         | 0.000        | 0.000           |
| Post hoc test | B > D = R   | B > D = R       |

Table 3: Group II - mean calcium phosphorus values (scanning electron microscopy-energy dispersive X-ray analysis)

| Group II | Mean calcium | Mean phosphorus |
|----------|--------------|-----------------|
| Baseline (B) | 65.25±0.59   | 20.76±0.81      |
| Demineralized (D) | 55.03±0.59 | 15.59±0.48      |
| Remineralized (R) | 65.4±0.86    | 20.5±0.76       |
| P         | 0.000        | 0.000           |
| Post hoc test | B = R > D   | B = R > D       |
SrnHAp paste showed higher calcium and phosphorus values (65.48 ± 0.53 and 21.05 ± 0.76, respectively) after remineralization than the regular dentifrice (55.24 ± 0.49 and 15.76 ± 0.54, respectively) and was statistically significant ($P < 0.001$) [Table 4 and Graph 3].

SEM images of the sound enamel specimens in Group I and II showed smooth surfaces [Figure 1]. After demineralization, specimens showed an uneven and irregular surface with porosities [Figure 2]. Following remineralization, Group I specimens showed open pores with few mineral crystals [Figure 3]. Group II showed smoother surface with more mineral deposition [Figure 4].

**Toxicology assessment**

Percentage viability assessment of cells treated with both regular and SrnHAp pastes was done at different dilutions by comparing the optical density of the samples with that of the untreated controls. At all dilutions, the percentage viability of cells was greater with SrnHAp paste compared to the regular toothpaste [Table 5 and Figure 5].

LC50 Value (ED50PLUS is a pharmacological analysis tool in the form of a Microsoft Excel worksheet, which allows you to create and analyse dose-response curves. It works on Windows 95/98/Me/NT/2000 operating system.)

- Regular Paste – 193.921 µg/mL
- SrnHAp Paste – 202.62 µg/mL.

The LC50 values for the SrnHAp paste are higher than the regular toothpaste, which shows it is less toxic than regular tooth paste.

**DISCUSSION**

Many remineralization agents have been developed to control demineralization and promote remineralization of incipient carious lesions. These include fluorides, calcium supplements as well as hydroxyapatite (HA) itself. Limitations of nHAp include large crystal size, questionable biocompatibility, lack of strength, brittleness, the high degree of crystallinity and low solubility at neutral pH requiring an acidic pH to dissolve. Moreover, the strength of nHAp was not enough to provide adequate protection for the enamel surface leaving it rough as before and further amenable to plaque accumulation and acid attack from microbial. In a study by Krishnan et al., strontium was doped in different concentrations to nHAp to overcome this problem. It is reported that the incorporation of strontium (Sr) for Calcium (Ca) in HA allows the formation of a pure but nonstoichiometric HA with low (Sr + Ca)/P ratio. This partial replacement of Ca\(^{2+}\) ions by Sr\(^{2+}\) ions in HAp matrix is responsible for the increase in solubility and increases the acid reactivity. It is found that Sr-Hap (above 10 mol% substitution) is a more soluble material and its increased bioactivity, due to Sr\(^{2+}\) release, makes it more desirable in vivo.
Krishnan et al. also stated that the increase in crystallinity and reduced particle size of 25% Sr-nHAp favors diffusion through small incipient carious lesions and white-spot areas making it the material of choice for enamel repair.\[12\] However, the strontium-doped HA used was in a solution form, and its application on tooth surface could be difficult. Hence, a thicker material in paste form incorporating the SrnHAp in its formulation will be beneficial because of its better retention on the tooth surface and easy application.

The present study evaluated the remineralization potential and cytotoxicity of a novel SrnHAp incorporated paste.

| Sample concentration (µg/mL) | OD value I | OD value II | OD value III | Average OD | Percentage viability |
|-----------------------------|------------|-------------|--------------|-------------|----------------------|
| Control (untreated cells)   | 0.5535     | 0.6474      | 0.5508       | 0.5839      | 100.00               |
| Sample code: Regular paste  |            |             |              |             |                      |
| 6.25                        | 0.5415     | 0.5436      | 0.5442       | 0.5431      | 93.01                |
| 12.5                        | 0.5258     | 0.5289      | 0.5273       | 0.5273      | 90.31                |
| 25                          | 0.4621     | 0.4537      | 0.4604       | 0.4587      | 78.56                |
| 50                          | 0.4408     | 0.4315      | 0.4326       | 0.4350      | 74.49                |
| 100                         | 0.4281     | 0.4257      | 0.4174       | 0.4237      | 72.57                |

Cytotoxic evaluation: The percentage of growth inhibition was calculated using the formula: Percent of viability = \(\frac{\text{Mean OD samples} \times 100}{\text{Mean OD of control group}}\). OD: Optical density, SrnHAp: Strontium doped nanohydroxyapatite

The caries demineralization process was simulated by using McInnes solution on the enamel specimen. Demineralization with McInnes bleaching solution shows a significant reduction in microhardness of enamel.\[19\] All the samples were kept in artificial saliva to simulate the oral environment.

Mean calcium and phosphorus values were determined using EDX analysis. EDX is considered as the “gold standard” for the determination of mineral loss or gain in experimentally induced initial carious lesions. It provides a precise quantitative measurement of the mineral...
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It is a micro-analytical technique used along with SEM. SEM analysis is the structural part, and EDX analysis is the elemental part at an ultrastructural level. The topographic changes of the enamel layer that is in sound enamel, demineralized, and remineralized specimen were assessed with SEM. After remineralization with the novel SrnHAp paste, the calcium and phosphorus values were almost similar to that of natural teeth. The SrnHAp paste showed a higher remineralization potential compared to the regular toothpaste and was statistically significant. The SEM images of Group II specimen treated with SrnHAp paste showed a smoother surface with almost complete obliteration of surface pores compared to Group I, which showed an open porous structure. SrnHAp paste appears to have better remineralization potential than the regular dentifrice, and this can be attributed to the reduced particle size, which helps to penetrate the pores and reduced crystal size which increases the solubility.

Similar results were observed in a study by Krishnan et al. in which SrnHAp in solution was found to be superior to casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) cream and nHAp toothpaste for repair of demineralized enamel surface. The study concluded that the presence of strontium increased dissolving capacity of the material and improved retention on the tooth surface making it a better choice than CPP-ACP and novamin for remineralization or repair of enamel.

Evaluation of cytotoxicity of the novel toothpaste was done by direct microscopic observation and MTT assay. The percentage viability assessment of the cells with the SrnHAp paste, exhibited nontoxic nature of the material. Uninterrupted cellular growth was seen with the paste when added at different dilutions. The fibroblastic growth promotion was much more prominent in SrnHAp paste compared to the regular dentifrice [Figure 5]. LC50 values were higher for the SrnHAp paste. The novel paste showed improved cell viability and reduced cytotoxicity compared to the regular dentifrice as evidenced through MTT assay and LC50 values.

The improved remineralization potential and reduced cytotoxicity make this novel SrnHAp paste a suitable agent for remineralization of incipient caries and white-spot lesions.

However, further evaluation with different concentrations of SrnHAp and assessment of other characteristics is required before the application of the paste clinically.

**CONCLUSION**

Within the limitations of this study, it can be concluded that SrnHAp paste showed better remineralization potential and favorable surface changes in enamel compared to the regular dentifrice. The cytotoxic evaluation showed the nontoxic nature of the paste. The novel SrnHAp paste can be considered for enamel repair and remineralization in incipient caries and white-spot lesions.

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**Conflicts of interest**

There are no conflicts of interest.
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