Evaluation of the effect of remineralization with strontium-doped nanohydroxyapatite with noncollagenous protein analog: Chitosan on the shear bond strength of resin composite to dentin – An in vitro study

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Abstract

Context: The resin–dentin interface is less durable, which reduces the longevity of tooth-colored restorations. To encounter this shortcoming, the use of nanotechnology to mimic biomineralization proves beneficial.

Aims: This study was conducted to evaluate the effect of remineralization with strontium-doped nanohydroxyapatite (Sr-nHAp) with chitosan on shear bond strength of resin composite to dentin.

Materials and Methods: Sixty five extracted human premolars were divided into five groups (n = 13) based on remineralization protocol as: Group A – 20% (Sr-nHAp) with chitosan, Group B – 10% (Sr-nHAp) with chitosan, Group C – 20% (Sr-nHAp) with simulated body fluid, Group D – 10% (Sr-nHAp) with simulated body fluid, and Group E – control. Following bonding, resin composite of specified dimension was built and was subjected to shear bond strength test after 24 h and 1 week using Universal Testing Machine, and mode of failure was assessed. ANOVA and paired sample t-test were used for analyzing the data, and the level of significance was set at 5%.

Results: The highest value of shear bond strength was obtained from Group A after aging for a week, and there is a significant increase in the value of all the groups as compared to the control group after 1 week of storage than 24 h.

Conclusions: Remineralization with Sr-nHAp and chitosan has positively improved the bond strength of resin to dentin at the end of 1 week.

Keywords: Biomimetic remineralization; chitosan; shear bond strength; strontium-doped nanohydroxyapatite powder

INTRODUCTION

Net mineral loss occurs due to an imbalance between demineralization and remineralization, which leads to dental caries. The approach to dental caries treatment has changed from “Extension from Prevention” to “Prevention of Extension.” Resin dentin bonds are always challenged by mechanical and chemical environment of oral cavity as they rely on organic part for mineralization. The most cited reasons for failure of adhesive restorations are loss of retention and marginal

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adaptation. Residual seeding crystals are absent in dentin, but present in enamel; therefore, in dentin, remineralization process becomes more difficult. The reason is due to the degradation and hydrolysis of resin ester linkages by water sorption and the activation of matrix metalloproteinases (MMP). Some strategies have been experimented with to prevent the degradation of resin–dentin bonds. They are: (a) Increasing the degree of conversion and esterase resistance of hydrophilic adhesives using hydrophilic photoinitiators, (b) Inhibition of collagenolytic enzymes using chlorhexidine, and (c) Use of cross-linking agents such as genipin and proanthocyanidin to silence the MMP and cathepsin activities. Therefore, the concept of biomimetic remineralization attempts to restore dentin to its original state replacing the water with the minerals using calcium and phosphate providing source and a noncollagenous protein analog.

Chitosan, which is a noncollagenous protein analog, has a protective effect on enamel and dentin against demineralization and enhances intrafibrillar mineralization by preventing nanohydroxyapatite aggregation by penetrating gap zones and forms hydroxyapatite crystals. However, limited studies are there on its effect on dentin remineralization. Its structure is similar to the extracellular matrix and it can be used to build collagen constructs, and the positively charged groups with terminal amino group make it an excellent antimicrobial agent. Nanodentistry has led to increase in the efficacy of biomimetic remineralization. Nanohydroxyapatite remineralizing agents have the ability to remineralize the tooth from within, but do not have adequate strength, have a high degree of crystallinity and low solubility. To improve its efficacy, calcium and phosphate or hydroxyl ions have been replaced with strontium, zinc, gold in various studies. Strontium element has both chemical and physical properties close to calcium; hence theoretically, it is able to replace Ca in hydroxyapatite. Sr at specific doses (1 and 2.5 mM) stimulates collagen formation, influences proliferation, odontogenic differentiation, and mineralization of human dental pulp cells. The calcium-sensing receptor – downstream pathways such as mitogen-activated protein kinase signaling pathway and Wnt/Beta-catenin signaling pathway are the mechanisms by which strontium regulates osteoblasts. Recently, it is concluded 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.

However, there is only a limited literature on evaluating the effect of combination of strontium-substituted nanohydroxyapatite and noncollagenous protein analog like chitosan on the shear bond strength of resin composite to dentin.

**MATERIALS AND METHODS**

**Synthesis of strontium-doped nanohydroxyapatite**

Coprecipitation technique was employed to synthesize strontium-doped nanohydroxyapatite (Sr-nHAp) samples. Strontium in the form of Sr(NO$_3$_)$_2$ (SDFL India) was added to calcium nitrate tetrahydrate (Ca(NO$_3$_)$_2$4H$_2$O) (SDFL India). The ratio of Ca + X/P was maintained at 1.67, where X = Sr$^{2+}$. The pH of the solution was adjusted to 11 using 30% ammonia solution, and diammonium hydrogen phosphate (NH$_4$H$_2$PO$_4$) (Merck India) was added drop-by-drop maintaining the pH at 11. The initial precipitate was transferred from beaker to centrifuging tubes and was centrifuged (REMI, Goregaon, Maharashtra, India), and washed twice with distilled water in to obtain the final precipitate which was stored in petri dish. Finally, the precipitate was dried for 20 h at 85°C in hot air oven, and was crushed using a mortar and pestle and sieved into fine fractions.

**Preparation of chitosan solution**

Chitosan solution with concentration of 2% was prepared by dissolving chitosan polymer in sufficient quantity of 1% acetic acid. The mixture was stirred for 24 h to obtain a perfectly transparent solution, and pH was adjusted to 4.5 by adding 1 M NaOH solution drop-by-drop.

**Preparation of simulated body fluid**

About 500 ml of distilled water was poured into 1 l polyethylene bottle and was stirred with a magnetic stirrer (REMI, Goregaon, Maharashtra, India), and the reagents were measured using a precision weighing balance (Saffron Electronics Scale, Gujarat, India) and dissolved one by one at 36.5°C in a water bath, and the pH was adjusted to 7.4 and was stored in a refrigerator at 5°C–10°C.

**Tooth sample preparation**

Sixty five extracted human premolars were sectioned perpendicular to long axis of tooth using a slow-speed diamond disc (Minitom, Struers, Copenhagen, Denmark) under water-cooling to obtain flat occlusal dentin surface. Specially fabricated customized aluminum jig (2 cm × 2 cm × 2 cm) was used for mounting of teeth using self-cured acrylic such that the teeth were embedded to the level of cementoenamel junction. 600-grit SiC paper (3M, India) under continuous water irrigation for 60 s was used to polish occlusal dentinal surface, acid etched for 15 s using 37% phosphoric acid (3M ESPE Adper™ Scotchbond™) and rinsed with water.

Following etching, they were divided into five groups (n = 13 in each group) based on remineralization protocol as:
Experimental protocol

Remineralizing paste application
In the ratio of 1:2, powder and liquid were mixed and applied on the dentin surface for 2 min, gently agitated, and rinsed off and dried with tissue paper.[11]

Application of bonding agent
3M™ ESPE™ Adper™ Single bond 2 Adhesive was applied using a microtip applicator brush for 15 s with gentle agitation, gently air-thinned for 5 s, and light-cured for 15 s using a light-emitting diode curing unit (Bluephase G2, Ivoclar – Vivadent Inc., Schaan, Liechtenstein).

Composite resin build-up
A transparent catheter tube of outer diameter ~4 mm, inner diameter ~2.5 mm, and height 3 mm was cut and then fixed manually on the prepared surface of each tooth. The nanocomposite resin (3M™ ESPE™ Filtek™ Z350 XT) was built-up in two 1.5 mm horizontal incremental layers, and each layer was photopolymerized for 20 s each, reaching 3 mm in total height.

Each group was divided into two subgroups according to storage time in distilled water at 37°C for 24 h (n = 6 in each group) and 1 week (n = 7 in each group) before being subjected to shear bond testing using universal testing machine (UTM).

Shear bond strength testing
Each specimen was horizontally secured with tightening screws to the lower fixed compartment of a UTM [Micro UTM Mecmesin (Slindfold, West Sussex, United Kingdom)], and data were recorded using computer software and were performed at a cross-head speed of 2 mm/min. Failure mode analysis was done using stereomicroscope (Leica stereomicroscope S6D [Wetzlar, Germany]) under magnification for all the samples. The criteria include:

- Cohesive failure – when a fracture allows a layer of adhesive to remain on both surfaces
- Adhesive failure – which occurs at interface of the adhesive and adherend
- Mixed failure – combination of both failures.

Statistical analysis
SPSS version 20. (IBM SPSS statistics (IBM corp. released 2011, IBM SPSS statistics, 2011, Chicago, US) was used to perform the statistical analysis. Inferential statistics like ANOVA was applied to compare the break scores among

| Groups | Minimum | Maximum | Mean±SD | P  |
|--------|---------|---------|---------|----|
| Group A | 19.23 | 25.92 | 21.73±2.35 | 0.001* |
| Group B | 16.72 | 21.27 | 18.51±1.83 |   |
| Group C | 10.11 | 19.56 | 14.38±3.93 |   |
| Group D | 10.43 | 21.57 | 15.31±4.60 |   |
| Group E | 22.53 | 41.08 | 30.57±7.02 |   |

*Significant. SD: Standard deviation

RESULTS

Powder X-ray diffraction analysis of the 10% and 20% strontium-doped nanohydroxyapatite powder
Here, all the major peaks matched with that of standard hydroxyapatite (PDF Card No. 09-432) [Figures 1 and 2]. Energy-dispersive analysis revealed that strontium was found to be present with an atomic weight of 3% in 20% Sr-nHAp compared to 1% from 10% Sr-nHAp. It was observed that when strontium concentration increases, there was a proportionate increase in the atomic weight from 1% to 3%.

Shear bond strength testing
At 24 h (n = 6 in each group)
The mean shear bond strength values obtained (in MPa) among the groups tested are given in Table 1. It is the highest for Group E, followed by Group A, Group B, Group D, and Group E (P = 0.001). Based on fracture pattern, the predominant failure was cohesive failure (37.1%), while mixed failure represents 31.4%, followed by adhesive failure (17.1%).

After 1 week (n = 7 in each group)
The mean shear bond strength values (in Mpa) among the groups tested are given in Table 2. It was the highest for Group A, followed by Group E, Group C, Group B, and Group D (P = 0.001). Distribution of the subjects based on fracture pattern showed predominant failure to be cohesive failure (62.9%), followed by mixed failure (31.4%) and adhesive failure (5.7%).

Comparison of the break scores between 24 h and 1 week within the group showed an increase in the

Table 1: Shear bond strength values after aging of specimens for 24 h (n=6)

| Groups | Minimum | Maximum | Mean±SD | P  |
|--------|---------|---------|---------|----|
| Group A | 19.23 | 25.92 | 21.73±2.35 | 0.001* |
| Group B | 16.72 | 21.27 | 18.51±1.83 |   |
| Group C | 10.11 | 19.56 | 14.38±3.93 |   |
| Group D | 10.43 | 21.57 | 15.31±4.60 |   |
| Group E | 22.53 | 41.08 | 30.57±7.02 |   |

*Significant. SD: Standard deviation

Table 2: Shear bond strength values after aging of specimens for 1 week (n=7)

| Groups | Minimum | Maximum | Mean±SD | P  |
|--------|---------|---------|---------|----|
| Group A | 47.40 | 60.29 | 53.82±4.73 | 0.001* |
| Group B | 25.12 | 36.98 | 31.10±4.86 |   |
| Group C | 30.54 | 50.89 | 37.62±8.32 |   |
| Group D | 25.82 | 33.82 | 29.45±3.29 |   |
| Group E | 30.57 | 48.85 | 37.68±6.27 |   |

*Significant. SD: Standard deviation
mean break scores from 24 h to 1 week in all the groups, with Group A showed the highest changes (mean diff −32.09), followed by Group C (mean diff −23.14), Group D (mean diff −14.14), Group B (mean diff −12.59), and Group E (mean diff −7.11).

DISCUSSION

The oral cavity environment is dynamic in nature. Under the combined challenges of enzymes, temperature and functional stresses, regions of incomplete resin infiltration within the dentin hybrid layer is susceptible to degradation, resulting in damage of interfacial integrity, reduction in bond strength, and ultimately failure of resin–dentin bonds.\textsuperscript{[12]} Therefore, there is a need to preserve its integrity by alternative methods.\textsuperscript{[13]} An attempt is made in this study to use Sr-nHAp and nonclinical panic (NCP) analog to evaluate its effect on the shear bond strength of composite resin to dentin.

Conventional remineralizing agents include fluorides, metastable calcium and phosphate ion-containing solutions or gels, which remineralize through top-down mineralization, in which remineralization occurs by epitaxial growth over existing seed crystallites.\textsuperscript{[14]} On the other hand, the bottom-up approach assembles materials from the nanoscopic scale, such as molecules and atoms, to form larger structures.\textsuperscript{[15]}

Disadvantages of nanohydroxyapatite are that it limits the availability of calcium and phosphate ions at the required sites.\textsuperscript{[16]} Thus, in the present study, to increase the solubility of nHAp, calcium was substituted with strontium by doping the HAP (10 and 20 mol%) using coprecipitation method. The substitution of large Sr\textsuperscript{2+} into small Ca\textsuperscript{2+} leads to denser atomic packing causing retardation of crystals growth, and the particle size of Sr-doped HA decreased as the concentration of Sr is increased as it is based on the bottom-up approach.\textsuperscript{[17]} The formulated powder was characterized using X-ray powder diffraction which showed the major peaks similar to that of nanohydroxyapatite (PDF Card No. 09-432).

Guided tissue remineralization represents a novel strategy in collagen biomineralization, which utilizes nanotechnology to achieve intrafibrillar and extrafibrillar remineralization of a collagen matrix.\textsuperscript{[18]} Thus, in the present study, chitosan was used as an NCP analog to stabilize the Sr-nHAp to bring about intrafibrillar remineralization as it can penetrate gap zones and forms hydroxyapatite crystals, which facilitates remineralization.\textsuperscript{[19]}

The bond strength testing method opted in this study is the shear bond strength testing, as true nature of adhesive strength of the materials at the interface is depicted by the shear bond strength.\textsuperscript{[20]}

The mean shear bond strength values are higher for Group E when specimens were aged for 24 h. The reason behind is due to the Adper\textsuperscript{™} Single Bond 2 Adhesive - total etch, visible-light activated dental bonding agent (Fifth Generation) whose shear bond strength is usually around 28.9 ± 1.8 Mpa (3M ESPE,2004). However, when the comparison was made between the chitosan-incorporated group (Group A – 20% (Sr-nHAp) with chitosan and Group B – 10% (Sr-nHAp) with chitosan) the SBF-incorporated group (Group C - 20% (Sr-nHAp) with simulated body fluid and Group D - 10% (Sr-nHAp) with simulated body fluid. This is in accordance with a study by Nivedita et al., in which the application of chitosan and proanthocyanidin improved the shear bond strength to dentin.\textsuperscript{[21]} As dentin pretreatment with chitosan, it improves the mechanical properties and resistance to enzymatic degradation and facilitates the intermolecular interaction between positive and negative charged units and results in cross-linking between chitosan and collagen.
The mean shear bond strength values are higher for Group A when specimens were aged for 1 week, and 20% Sr-nHAp-treated specimen recorded higher bond strength values. This could be due to the improved remineralizing potential of pastes containing a higher amount of strontium. Therefore, Group A and Group C – 20% (Sr-nHAp) have shown higher bond strength than 10% (Sr-nHAp) group (Group B and Group D). However, when the comparison is made between the chitosan-incorporated group (Group A and Group B) and SBF-incorporated group (Group C and Group D), it is seen that chitosan has shown a positive influence on bond strength more with 20% Sr-nHAp with chitosan (highest) and 20% Sr-nHAp with SBF has shown a positive influence on bond strength. However, the control group (Group E) did not show any statistically significant difference in the mean break scores after 1 week of storage than 24 h. There was only a slight increase from 30.57 ± 7.02 to 37.68 ± 6.27. However, when other groups are compared between 1 week of storage and 24 h, it is seen that there is a significant increase in the value of all the groups as compared to the control group.

The mode of failure was assessed using stereomicroscope -Leica Stereomicroscope S6D at ×3.2 and ×1.25. On comparison of failure mode analysis between 24 h and 1 week, it is seen that there is increase in an cohesive failure (25.8%) which makes it as the predominant failure, followed by mixed failure (31.4%) which is the same, and there is a decrease in adhesive failure rate after 1 week by 11.4%. This is in agreement with the study by Alagha stating that the frequency of the cohesive and mixed failure increase with fluorohydroxyapatite and nanohydroxyapatite than adhesive failure when the mode of failure pattern was assessed after 3 months for the microshear bond strength. Furthermore, similar results were found in a study by Kudva et al., in which the main type of failure observed was cohesive in biodentine, and it is also stated that if the predominant failure should be cohesive, followed by mixed and adhesive.

CONCLUSIONS

Within the limitations of the current study, remineralization with Sr-nHAp and chitosan has positively improved the bond strength of resin to dentin at the end of 1 week.

Therefore, the effect of longer aging periods on shear bond strength with the above remineralizing agents needs to be assessed in future studies. The study was done in a controlled laboratory environment mimicking the dynamic oral environment before being subjected to shear bond strength testing. In vivo studies are required to assess the long-term effect of remineralizing potential.

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Conflicts of interest

There are no conflicts of interest.

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