Comparative evaluation of remineralization potential of nanohydroxyapatite crystals, bioactive glass, casein phosphopeptide-amorphous calcium phosphate, and fluoride on initial enamel lesion (scanning electron microscope analysis) – An in vitro study

RD Geeta, Saritha Vallabhaneni¹, Kainath Fatima²

Conservative Dentistry and Endodontics, ITS Dental College and Hospital, Muradnagar, Uttar Pradesh, ¹Conservative Dentistry and Endodontics, PMNM Dental College and Hospital, Bagalkot, ²Conservative Dentistry and Endodontics, SB Patil Dental College and Hospital, Bidar, Karnataka, India

Abstract

Aim: The aim of this in vitro study was to evaluate and compare the remineralization potential of four different remineralizing agents, i.e., nanohydroxyapatite crystals, bioactive glass, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), and fluoride on initial enamel lesion.

Materials and Methods: Sixty human maxillary central incisors were used in the present study. Samples were randomly divided into four groups (n = 15). Group 1: nanohydroxyapatite-containing dentifrice (Acclaim); Group 2: bioactive glass containing dentifrice (SHY-NM); Group 3: CPP-ACP-containing dentifrice; and Group 4: fluoride-containing dentifrice. Baseline microhardness was checked, followed by immersion of teeth samples in demineralizing and remineralizing solution. This was followed by a pH cycle of 10 days. Data were analyzed using ANOVA and Bonferroni method. After this, scanning electron microscopic analysis was done to evaluate remineralization.

Results: Statistical analysis of data was conducted using ANOVA, and multiple comparisons within groups were done using the Bonferroni method (post hoc tests). The decision criterion was to reject the null hypothesis if P < 0.05. If there was a significant difference between the groups, multiple comparisons (post hoc test) using the Bonferroni test were carried out.

Conclusion: There is a significant difference in mean microhardness between the groups after remineralization. The mean value was found to be highest for nanohydroxyapatite, bioactive glass, CPP-ACP, and fluoride in descending order.

Keywords: Bioactive glass; caries; nanohydroxyapatite; remineralization; scanning electron microscope

INTRODUCTION

Dental caries in enamel is unique among diseases as enamel is both acellular and avascular.¹

Address for correspondence:
Dr. RD Geeta,
ITS Dental College and Hospital, Muradnagar, Uttar Pradesh, India.
E-mail: rdgeeta88@gmail.com

Date of submission: 17.02.2020
Review completed: 17.08.2020
Date of acceptance: 19.08.2020
Published: 04.12.2020

The current consensus in caries management is that they should be detected at the earliest to prevent surgical intervention. Over the past few decades, fluoride was used in its various forms, which resulted in a widespread reduction in caries due to its extensive use in the form of a toothpaste. In recent years, other
remineralizing agents have come up, which helps in remodeling the hydroxyapatite crystals and promote remineralization along with reducing the apatite crystal dissolution.[2] Some of these remineralizing agents are nanohydroxyapatite, bioactive glass, and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP).

MATERIALS AND METHODS

A total of sixty human maxillary central incisors were collected. Teeth were cleaned thoroughly to remove any deposits. Samples were decoronated 1 mm below the cementoenamel junction with a diamond disc [Figure 1]. The crowns were subjected to removal of 200 µm of surface enamel on the labial aspect to eliminate variability in samples. A 4 × 4 mm working window was marked on the labial surface of all samples, which was considered for remineralization, and the rest of the area of the crown was covered with nail varnish. Samples were randomly divided into four groups (n = 15). Group 1: nanohydroxyapatite-containing dentifrice; Group 2: bioactive glass-containing dentifrice; Group 3: CPP-ACP-containing dentifrice; and Group 4: fluoride-containing dentifrice.

Baseline surface microhardness measurement

Baseline surface microhardness (B-SMH) was checked with Vickers microhardness testing machine for all tooth samples in the area of the working window. The indentations were made with Vickers microhardness testing machine at the rate of 25 g load for 5 s.[3]

Preparation of demineralizing solution

The buffered demineralizing solution was prepared using analytical grade chemicals and deionized water. The demineralizing solution contained 2.2 mM calcium chloride, 2.2 mM sodium phosphate, and 0.05 M acetic acid; the pH was adjusted with 1 M potassium hydroxide to 4.4.

Preparation of artificial carious lesion

Samples were kept in demineralizing solution for 96 h to produce an artificial carious lesion in enamel. Demineralizing surface microhardness was checked with Vickers testing machine similar to B-SMH.

Preparation of remineralizing solution

The remineralizing solution was prepared with 1.5 mM calcium chloride, 0.9 mM sodium, and 0.15 M potassium chloride, which had a pH of 7.0.

Toothpaste preparation

Dentifrice supernatant was prepared by suspending 12 g of the respective dentifrice in 36 ml deionized water to create a 1:3 dilution. The suspensions were thoroughly mixed and then centrifuged at 3500 rpm for 20 min at room temperature, once daily before starting pH cycling.

The pH cycling model

The specimens were placed in a pH cycling system on a cylindrical beaker for 10 days.

- 1 min brushing with the dentifrice
- 3 h in demineralizing solution
- 2 h in remineralizing solution
- 1 min brushing with the dentifrice
- 3 h in demineralizing solution
- 1 min brushing with the dentifrice and overnight in remineralizing solution.

Scanning electron microscopic analysis

After the pH cycling for 10 days, scanning electron microscopic (SEM) analysis was done to evaluate remineralization [Figure 2].

RESULTS

The present study compared the remineralizing potential of four agents, i.e., nanohydroxyapatite-containing dentifrice, bioactive glass-containing dentifrice, CPP-ACP-containing dentifrice, and fluoride-containing dentifrice on initial enamel caries [Figure 3].

There is a significant difference in mean microhardness between the groups after remineralization, which was found to be statistically significant at $P < 0.05$, indicating

Figure 1: Decoronated samples
changes in the mineralization of teeth samples as shown in Figure 1 and Table 1.

**DISCUSSION**

Dental caries is one of the causes of tooth loss for all human beings across age and gender. It is a highly prevalent multifactorial disease and although in most developed countries. The signs of caries process cover a continuum from the first molecular changes in apatite crystals of the tooth, to a visible white spot lesion, to dentin involvement, and eventual cavitation.

Progression through these stages requires a continual imbalance between pathological and protective factors that result in the dissolution of apatite crystals.

Remineralization of white spot lesions and carious lesions may be possible with a variety of currently available agents containing nanohydroxyapatite, fluoride, bioavailable calcium phosphate, CPP-ACP, and self-assembling peptide.

Instead of using the traditional pH cycling method, a modified version was utilized in our study, which included a 3 h demineralization cycle twice a day, with one 2 h and one intervening overnight remineralization cycle, respectively. To replicate early morning, midday, and before bedtime toothbrushing, toothpaste was applied thrice daily. Brushing time of 1 min was utilized in the present study to replicate real-life situations similar to a study done by Kumar et al.

| Points                  | Mean | Std.Dv. | Mean Diff. | SD Diff. | % of change | Paired t | P  |
|-------------------------|------|---------|------------|----------|-------------|----------|----|
| Fluoride                |      |         |            |          |             |          |    |
| Baseline                | 236.54 | 75.60   |            |          |             |          |    |
| After demineralization  | 196.81 | 63.54   | 39.73      | 96.29    | 16.80       | 1.5981   | 0.1323 |
| Bioactive               |      |         |            |          |             |          |    |
| Baseline                | 236.54 | 75.60   |            |          |             |          |    |
| After demineralization  | 201.86 | 38.77   | 34.68      | 80.29    | 14.66       | 1.6729   | 0.1165 |
| Cpp-ACP                 |      |         |            |          |             |          |    |
| Baseline                | 219.70 | 81.07   |            |          |             |          |    |
| After demineralization  | 233.84 | 77.01   | -14.14     | 94.44    | -6.44       | -0.5799  | 0.5712 |
| Bioactive               |      |         |            |          |             |          |    |
| Baseline                | 235.46 | 74.46   |            |          |             |          |    |
| After demineralization  | 226.77 | 53.50   | 8.69       | 115.28   | 3.69        | 0.2921   | 0.7745 |
| Nano                    |      |         |            |          |             |          |    |
| Baseline                | 230.39 | 74.75   |            |          |             |          |    |
| After demineralization  | 329.82 | 69.75   |           |          |            |          |    |

Figure 2: Scanning electron microscopic images (a) nanohydroxyapatite, (b) bioactive glass, (c) casein phosphopeptide-amorphous calcium phosphate, (d) fluoride

Figure 3: Comparison of four groups with respect to baseline, after demineralization, and after remineralization

Table 1: Comparison of baseline, after demineralization and after re-mineralization scores in four groups by paired t test
similar results, wherein CPP-ACP was better than fluoride. SEM utilized in the study is the most recent technology that will reveal distinct coatings of hydroxyapatite crystals deposited on the treated enamel surface. This technology has been used by many investigators such as Narayana et al., Swarup and Rao, and Huang et al. For SEM, samples were thoroughly vacuum dried and gold sputtered, and then images of the surface enamel were recorded at various magnification.

Nanohydroxyapatite also acts as a filler because it repairs small holes and depressions on enamel surface, a function enhanced by the small size of the particles that compose it.

By penetrating the enamel pores, nanohydroxyapatite crystals act as a template in the precipitation process and promote crystal integrity and growth.

Moreover, nanohydroxyapatite is hydrophilic and has a greater surface area than conventional hydroxyapatite crystals, so they have better wettability and form a thin layer on enamel surface that bonds to tooth structure.

The ACLAIM toothpaste used in this study contains the synthetic biomimetic hydroxyapatite. The function of this hydroxyapatite is to protect the teeth with the creation of a new layer of synthetic enamel around the tooth.

In our study, nanohydroxyapatite has proved to be a better remineralizing agent, shown by the highest mean hardness value when measured with Vickers microhardness testing machine.

This has been supported by a similar study conducted by alaudin SS et al. who compared nanohydroxyapatite with CPP-ACP and fluoride varnish and showed that the surface microhardness value for nanohydroxyapatite was higher than the other groups.

Various studies conducted by Kim et al. also evaluated the effect of nanohydroxyapatite on remineralization using Vickers hardness number and SEM to evaluate enamel surface and found similar results.

Bioactive glass used in the study was invented by Dr. Larry Hench in the 1960s. It acts as a biomimetic mineralizer matching the body’s own mineralizing traits and also affecting cell signals, thereby benefitting the restoration of tissue structure and function.

Bioglass in an aqueous environment immediately begins surface reactions in three phases – leaching, exchange of cations and network dissolution of SiO₂, and precipitation of calcium-phosphate forming an apatite layer.

SHY-NM used in the study has been demonstrated to show fine particulate bioactive glasses incorporated into an aqueous dentifrice, which has the ability to clinically reduce the tooth hypersensitivity through the occlusion of dentinal tubules by the formation of carbonated hydroxyapatite layer.

A study conducted by investigated the remineralization potential of bioactive glass and CPP-ACP using SEM and found that bioactive glass seals the fissures formed after demineralization more intimately, and the deposits are more angular, whereas those of CPP-ACP are smaller and amorphous which is in accordance with our study.

The result of the current study revealed a considerable amount of increase in surface microhardness of the enamel achieved after treatment with SHY-NM, which contains bioactive glass as shown by the mean hardness value of 278.79. Furthermore, P = 0.0021 showed that the result is significant when compared to CPP-ACP and fluoride.

CPP-ACP was patented in the United Nations in 1991. Casein is present in various dairy products such as milk, milk concentrates, and cheese.

CPP-ACP is a new remineralization technology based on phosphopeptide from milk protein casein. The casein phosphopeptide contains multiple phosphoryl sequences with the ability to stabilize calcium phosphate in nanocomplex in solutions such as ACP. Through their multiple phosphoryl sequences, CPP binds to ACP in a metastable solution preventing the dissolution of calcium and phosphate ions.

The CPP-ACP also acts as a reservoir of bioavailable calcium and phosphate and maintains the solution supersaturated, thus facilitating remineralization.

In a study sodium fluoride showed higher remineralization potential of CPP-ACP crème. Variation in results may be due to the variable duration of pH cycling.

In the present study, statistical analysis by ANOVA and post hoc showed that the remineralizing potential of CPP-ACP is higher than fluoride but not significant at P < 0.05.

Fluoride becomes incorporated into the tooth structure, and hydroxyl group of the hydroxyapatite is replaced by fluoride. The mechanism by which fluoride increases caries resistance may be from both systemic and topical applications.

Brown et al. believed that the working mode of fluoride was due to change in solubility after incorporation in enamel, but only 10% of fluoride substitutes hydroxyl group in the apatite of surface enamel which cannot be considered the only factor causing cariostatic effect.

Results by statistical analysis revealed lowest mean microhardness for fluoride-containing dentifrices showing that the other three
dentifrices have better remineralizing capacity than fluoride. This may be due to the fact that fluoride acts predominately during the growth period of dentition rather than after development.

**CONCLUSION**

- All experimental groups have remineralizing potential as shown by increase in microhardness of the respective groups when compared to their baseline values
- Nanohydroxyapatite-containing dentifrice has highest remineralizing potential followed by bioactive glass, CPP-ACP, and fluoride
- Fluoride has the least remineralizing potential as compared to other groups, used in the study
- SEM analysis has confirmed the formation of hydroxyapatite crystals in all the groups.

**Financial support and sponsorship**
Nil.

**Conflicts of interest**
There are no conflicts of interest.

**REFERENCES**

1. Li L, Pan H, Tao J, Xu X, Mao C, Gu X, Tang R. Repair of enamel by using hydroxyapatite nanoparticles as the building blocks. J Mater Chem 2008;18:4079-84.
2. Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC. New approaches to enhanced remineralization of tooth enamel. J Dent Res 2010;89:1187-97.
3. Linton JL. Quantitative measurements of remineralization of incipient caries. Am J Orthod Dentofacial Orthop 1996;110:590-7.
4. Mehta AB, Kumari V, Jose R, Izadikhah V. Remineralization potential of bioactive glass and casein phosphopeptide-amorphous calcium phosphate on initial carious lesion: An in-vitro pH-cycling study. J Conserv Dent 2014;17:3-7.
5. Mozizadeh M, Moayedi S. Anticariogenic effect of amorphous calcium phosphate stabilized by casein phosphopeptide – A review. Res J Biol Sci 2009;4:132-6.
6. Kumar VL, Ilthagarun A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: An in vitro study. Aust Dent J 2008;53:34-40.
7. Naveena P, Nagaratna C, Sakunthala BK. Remineralizing agents then and now—an update. Dentistry 2014;4:1-6.
8. Narayana SS, Deepa VK, Ahamed S, Sathish ES, Meyappan R, Sateesh Kumar KS. Remineralization efficiency of bioactive glass on artificially induced carious lesion- An in vitro study. J Indian Soc Pedod Prev Dent 2014;32:19-25.
9. Swarup JS, Rao A. Enamel surface remineralization: Using synthetic nanohydroxyapatite. Contemp Clin Dent 2012;3:433-6.
10. Huang SB, Gao SS, Yu HY. Effect of nanohydroxyapatite concentration on remineralization of initial enamel lesion in vitro. Biomed Mater 2009;4:1-5.
11. Jayarajan J, Janardhanan P, Jayakumar P, Deepika. Efficacy of CPP-ACP and CPP-ACPF on enamel remineralization An in vitro study using scanning electron microscope and DIAGNOdent. Indian J Dent Res 2011;22:77-82.
12. Timmaiah G, Shetty P, Shetty NB, Natrajan S, Thomas AT. Comparative analysis of the remineralization potential of CPP-ACP with Fluoride, Tricalcium Phosphate and Nanohydroxyapatite using SEM/EDX-An In vitro study. J Clin Exp Dent 2019;11:1120-6.
13. Pepia E, Besharat KL, Falagia G, Tenore G, Migliau G. Nanohydroxyapatite and its application in preventive, restorative and regenerative dentistry; a review of literature. Ann Stomatol 2014;5:108-14.
14. Najibfard K, Ramalingam K, Chedieu I, Amechi BT. Remineralization of early caries by a nanohydroxyapatite. J Clin Dent 2011;22:139-45.
15. Amasech BT, Abdul Azeez PA, Alshareif D.O, et al. Comparative efficacy of hydroxyapatite and fluoride toothpaste for prevention and remineralization of dental caries in children. BJD 2019;18:5.
16. Kim MY, Kwon HK, Choi CH, Kim BI. Combined effects of Nano-Hydroxyapatite and NaF on remineralization of early caries lesion. KEM 2007;330:1347-50.
17. Jeong SH, Jang SQ, Kim KN, Kwon HK, Park YD, Kim BI. Remineralization potential of new toothpaste containing Nano-Hydroxyapatite. KEM 2006;309:537-40.
18. Prabhakar AR, Arali V. Comparison of remineralizing effects of sodium fluoride and bioactive glass using bioerodible gel system. J Dent Res Dent Clin Dent Prospects 2009;3:117-21.
19. Zhou C, Zhang D, Bai Y, Li S. Casein phosphopeptide-amorphous calcium phosphate remineralization of primary teeth early enamel lesions. J Dent Res 2014;42:12-9.
20. Wu G, Liu X, Hou Y. Analysis of the effect of CPP-ACP tooth mousse on enamel remineralization by circularly polarized light. Angle Orthod 2010;80:933-8.
21. Reynolds EC. Remineralization of enamel subsurface lesions by casein phosphopeptide – Stabilized calcium phosphate solutions. J Dent Res 1997;76:1587-95.
22. Cochrane NJ, Reynolds EC. Calcium phosphopeptides-mechanisms of action and evidence for clinical efficacy. Adv Dent Res 2012;24:41-7.
23. Peter S. Essentials of Preventive and Community Dentistry. 4th ed. New Delhi: Arya Medi Publishing House, 2009.
24. Lata S, Varghese NO, Varughese JM. Remineralization potential of fluoride and amorphous calcium phosphate casein phosphopeptide on enamel lesions: An in vitro comparative evaluation. J Conserv Dent 2010;13:42-6.
25. Brown WE, Gregory TM, Chow LC. Effects of fluoride on enamel solubility and cariostasis. Caries Res 1977;11 Suppl 1:118-41.