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Comparative evaluation of surface micro hardness and morphology of enamel remineralization using chitosan hydrogel and APF gel: An *in vitro* study

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Abstract
Biocompatible chitosan hydrogel shows promise as a biomaterial for the prevention and treatment of defective enamel over Fluoride application. Thirty three human anterior extracted teeth were selected for study and divided into three groups: Group I: Control group, teeth etched with 37% phosphoric acid for 30 seconds. Group II: Teeth etched with 37% phosphoric acid for 30 seconds, coated with APF gel, then incubated at 37ºC in artificial saliva solution for 10 days. Group III: Teeth etched with 37% phosphoric acid for 30 seconds, coated with bonding agent and chitosan hydrogel, then incubated at 37ºC in artificial saliva solution for 10 days. After remineralization, enamel surfaces observed with scanning electron microscope for induced structural changes on enamel surface and surface micro hardness was evaluated. Results showed statistically significant difference (P<0.001) between all three groups. APF showed highest micro hardness of demineralized enamel although Chitosan hydrogel showed the biomimetic remineralization of enamel.

Keywords: APF gel, biomimetic enamel remineralization, chitosan hydrogel, enamel micro hardness, scanning electron microscopy

Introduction
Enamel rods possess exclusive morphological structure and distinct mechanical properties. They are composed of highly organized architectural hydroxyapatite crystals. The protein-mediated process of mineralization as well as ameoloblastic activity are critical for achieving such specifically organized structures [1]. However, a cellular nature of mature enamel devoid them of any self-regenerative potential.

The first topographic manifestation of dental enamel caries are the white spot lesions (WSL). These WSL are made up of a porous lesion body and a relatively intact surface layer which are results of re-precipitation of dissolved phosphate ions and calcium ions. These ions are retained partially in the overlying dental plaque biofilm as well as saliva [2].

Remineralization of the enamel is a significant topic of discussion in the arena of dental material science and dentistry with regards to the treatment of white spot lesions (WSL) and erosion. It is a well-known fact that remineralization can be initiated at the early stages of WSL to halt the progress of lesions [3]. There are various in-office and over the counter products available to be used for remineralization procedure of dental enamel such as fluoride dentifrice, fluoride varnish, amorphous calcium phosphate (ACP), etc. Though fluoride applications are one of the most common treatment options for WSL; studies have shown that topical fluoride application is not a perfect treatment approach for remineralization of enamel. Also, when amorphous calcium phosphate was used, the newly formed crystalline phases such as octacalcium phosphate or apatitic products were irregularly arranged after treatment with these agents [4]. In recent years, another treatment modality opted for early carious lesions is the application of Nano- sized hydroxyapatite. Different in-vitro techniques have been applied to prepare this nano-sized hydroxyapatite (nHA) such as the sol–gel method, electrochemical deposition, hydrothermal crystallization, chemo-mechanical process, crystallization under magnetic field, wet precipitation method etc. However, these techniques commonly require specific temperature, highly acidic condition or involving harmful reagents, high pressure [5].

These approaches are also associated with lengthy and complex processes combined with the need for expensive equipment [6]. Chitosan is the N-deacetylated derivative of chitin which is soluble in dilute acids like acetic acid, formic acid, etc. Chitosan has found wide application in biomedical fields like drug delivery, wound dressing, as a tissue replacement material, etc. due its biocompatibility and non-toxicity [7]. Furthermore, chitosan hydrogel prepared by the polymer dilution in acetic acid can be used as a preventive and therapeutic material for cavity providing antimicrobial properties [8,9]. Therefore, regeneration of enamel with chitosan as biomimetic material has been recently proposed which is inspired by the molecular mechanism of organic-matrix mediated bio mineralization. Also manufacturing of this new found material might also found to be cost effective. In order to focus upon, physical and chemical properties of chitosan as demineralizing agent of enamel, this study aimed to compare surface micro hardness and morphology of enamel remineralization using Chitosan Hydrogel and APF gel.

Materials and methods

Preparation of chitosan hydrogel [10]

0.25 gram of chitosan powder was added in 25 mL of acetic acid solution followed by stirring at 80°C overnight for complete dissolution of the power in acetic acid. Later, this chitosan solution was cooled gradually at room temperature. After cooling of chitosan solution, the pH of the solution was adjusted by adding 1 M NaOH solution drop by drop till pH reached 6.5 and the chitosan hydrogel was formed.

Preparation of samples

The study protocol was approved by the institutional ethical committee (388-A, 07/08/19). A total number of thirty-three intact extracted maxillary anterior human teeth were collected for the study. These teeth were cleaned using ultrasonic scaler and stored in artificial saliva. Written consent was taken from the patients for the use of their extracted teeth for scientific research. Enamel slabs were cut from the buccal surfaces of each tooth. Cutting and grinding procedures were performed under sufficient water flow and the slabs, sized 3mm*3mm*1 mm, were mounted in acrylic resin for checking the surface microhardness and morphology of enamel remineralization of each groups as follows:-

Group I: Control group, n=11

Samples were etched with 37% phosphoric acid for 30 seconds, followed by incubation at 37ºC in artificial saliva solution for 10 days.

Group II: n=11

Samples were etched with 37% phosphoric acid for 30 seconds, coated with APF gel, followed by incubation at 37ºC in artificial saliva solution for 10 days.

Group III: n=11

Samples were etched with 37% phosphoric acid for 30 seconds, coated with bonding agent followed by light curing and chitosan hydrogel application, followed by incubation at 37ºC in artificial saliva solution for 10 days.

From each group, total of ten samples were assessed for micro hardness values of enamel surface, while one sample is used for observing the morphological changes after remineralization procedures. Micro hardness of the samples was tested using Micro Vicker's hardness machine (Prisma, Germany) at 100 gm for five seconds and morphology was assessed by Scanning electron microscopy (NCL, Pune) at 1000X, 2000X and 5000X after metallic coating of the samples at 20,000 kV.

Results

Values of the surface enamel micro hardness was collected in terms of Vicker's Hardness Number (VHN) as shown in (table 1) and results were obtained by using student paired t test for analysis between two groups and correlation test for comparison of all three groups with the help of IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. The level of significance was set at $P<0.05$.

Assessment of micro hardness

Group I showed the least mean value of surface micro hardness of demineralized enamel of 266.8 VHN. While group II showed maximum mean surface micro hardness of 404.6 VHN followed by group III which showed mean surface micro hardness of 345.6 VHN as mentioned in table 1. All the three groups showed statistically significant difference between them ($P<0.001$) as shown in table 2. Although Group II and Group III found to have statistically significant difference between them, correlation test showed that these groups shared most comparable results (0.366) as shown in table 3.

Assessment of morphology

Scanning electron microscopy (SEM) at 1000X, 2000X and 5000X was used for assessment of morphological changes at the enamel surfaces after remineralization procedures. SEM images of control group showed that the surface of etched enamel surface had number of porous defects resembling demineralization. Also there was no typical integrity to the enamel surface (Figure 1A). Two different patterns of demineralization could be specified from the images obtained (Figures 1B and 1C). Some areas showed loss of enamel rod peripheries with intact rod cores leading to widening in inter-prismatic distance while some areas showed microporosities due to loss of rod cores and relatively intact peripheries.

The surfaces of enamel treated with APF (group II) remained relatively dense and intact compared with other groups. The enamel surfaces in the group treated with APF revealed very minimal inter-crystalline space with the crystals deposited irregularly and without any orientation to crystals (Figures 1D, 1E, 1F).

Samples of group III which were demineralized using chitosan hydrogel showed a superior and uniform re-establishment of surface integrity in contrast to other groups. Particular feature observed in this group was reduction in porosities and dense layer of remineralization surface. The deposited crystals on the enamel surface found to be homogeneous (Figure 1G). Because of such well-organized crystal growth at the surfaces; widened inter-prismatic distance and micro porosities observed as in control group were absent. Newly deposited rod like structures found to be perpendicular to the enamel surface mimicking natural enamel rods (Figures 1H and 1I). These deposited nano-crystals when observed at higher magnification revealed inter-relations between adjacent rods indicative of strong attraction between them.

Tables and Figures

Table 1: Surface enamel microhardness of samples in terms of Vicker's Hardness Number (VHN)

Sr. No.	Group I: Control group (in VHN)	Group II: APF group (in VHN)	Group III: Chitosan hydrogel group (in VHN)
1.	263	426	378
2.	254	378	297
3.	279	413	339
4.	293	418	363
5.	272	431	348
6.	266	408	305
7.	290	394	334
8.	297	385	368
9.	268	392	353
10.	256	401	371
Mean	266.8	404.6	345.6

Table 2: Student t-paired tests of surface microhardness of remineralized enamel in groups

Groups	Mean	S.D.	Standard error mean	95% Confidence interval of the difference		T	*P*
				Upper	Lower		
Group I and Group II	-130.800	23.203	7.338	-147.399	-114.201	-17.286	<0.001
Group I and Group III	-71.800	27.377	8.657	-91.384	-52.216	-8.293	<0.001
Group II and Group III	59.000	26.592	8.409	39.978	78.022	7.016	<0.001

*S.D.: Standard deviation

Table 3: Correlation (Pearson) test showing interrelation of surface microhardness for comparison of the groups

	Group I	Group II	Group III
Group I	1	0.014	0.281
Group II	0.014	1	0.366
Group III	0.281	0.366	1

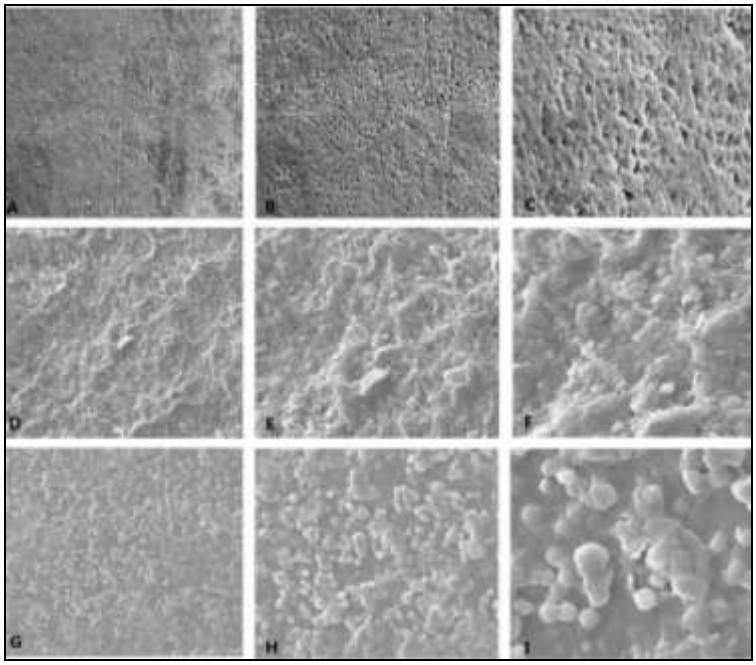

Fig 1: SEM photographs of control group at 1000x (A), 2000x (B), 5000x (C), SEM photographs of APF group at 1000x (D), 2000x (E), 5000x (F) and SEM photographs of Chitosan hydrogel group at 1000x (G), 2000x (H), 5000x (I), respectively

Discussion

Enamel remineralization are a well-accepted concept for repairing enamel defects like WSLs for which different treatment modalities such as fluoride varnish, fluoride dentifrice, amorphous phosphate, nano-sized hydroxyapatite, etc. have been developed. In this present study, assessment of enamel remineralization has been conducted using conventional application of fluoride gel i.e. APF gel and Chitosan hydrogel. Application of chitosan hydrogel is a recent approach to tissue engineering concept to regenerate instead of repair for bio-remineralization of enamel.

In the present study, it was observed that APF gel i.e. fluoride gel showed highest values of microhardness of remineralized enamel surfaces. Although Chitosan Hydrogel was not able to produce as good values of microhardness of enamel surfaces when compared to APF gel, newly established microhardness values of enamel tissue after application of both the remineralizing agents were comparable and significantly greater than that on control group. Similar results were found by Yönel N *el al.* [11] where remineralization solutions,

SnF_2/NaF and Sn/F/chitosan reduced enamel tissue loss by a greater extent between demineralization procedure than that of control group. Striking results found at morphological study of demineralized enamel tissue with the help of SEM, where chitosan hydrogel showed biomimetic remineralization of enamel as found by Ibrahim I *et al.* [10]. Which attributes to the fact that chitosan is able to help in the biomineralisation process of enamel tissue and controls the mineral crystallites through the molecular interaction.

In this study, agents were used in gel form since hydrogels are an optimal versatile growth media for crystals, [12] as well as easy to handle and quite mimicking the initial formation of enamel hydroxyapatite in nature that occurs in a unique gel-like organic matrix. Several studies have shown that fluoride application to initial carious lesion leads to surface remineralization [13-15]. It is well known fact that applied fluoride gel incorporates into enamel crystals, thereby forming a fluoroapatite-like mineral that improves the strength of the enamel to resist an acid challenge. Following application of fluoride, loosely bound fluoride or CaF_2 is formed on the enamel surface or in caries lesions [16]. CaF_2, produced by pellicle proteins, is the major product formed when enamel is treated with topical fluoride at neutral pH. This CaF_2 layer then dissolves, releasing fluoride to react with calcium and phosphate ions. [17] The top product of those reactions could also be Fluoroapatite. Fluoroapatite inhibits demineralization and enhances the remineralization of crystals and microhardness [18]. This explains highest microhardness of enamel after application of APF gel i.e. Group II. However, fluoride has the potential to cause fluorosis while overused, and a few fluoride product like silver Diaminefluoride may cause black staining of the carious lesion [19]. Therefore, the choice, effective no-fluoride anti-caries agents got to be explored. Also, limitation of those therapies is that remineralization takes place predominantly on the lesion surface. This surface precipitation is probably going to fill superficial pores and block pathways to the lesion body, giving rise to a restriction in complete lesion consolidation [20, 21]. According to Wang *et al.*, when chitosan comes into contact with acetic acid, carboxylic groups in acetic acid dissociate and mix with amino groups from chitosan by electrostatic attraction causing calcium cations from saliva are released rapidly then chelated by acetic acid [22]. Therefore, chitosan within the lesion subsurface enhances the delivery of calcium cations. Additionally, chitosan attracts phosphate ions from saliva through electrostatic forces of NH_3^+ groups. This explains why chitosan pre-treatment only caused significant greater remineralization and microhardness in group III than group I. In this study, the utilization of dental adhesive agent which acted purely as a template before chitosan application was beneficial for two different perspectives. First was as Kun Tian *et al.* [23] stated that the positively charged-NH_2 of the chitosan hydrogel gets attracted towards phosphate ions on the dental adhesive agent. This leads to the orientation of chitosan molecules, perpendicular to the substrate and parallel to each other. Also the functional carboxyl that's present on the surfaces of chitosan, is responsible for effective spontaneous apatite nucleation, in an ordered orientation over the surface. While second was phosphate ions on dental adhesive agent can intake calcium and phosphorus from the synthetic saliva solution, through the porous chitosan hydrogel, which contains many water (up to 96 wt%), providing space for calcium or phosphate ions to spontaneously diffuse into the lesion [24].

Although further studies are required so as to extend surface microhardness after application of Chitosan Hydrogel with help of certain materials also to mimic oral environment as biofilms also are said to affect the remineralization procedures [25]. Despite of these facts, use of Chitosan Hydrogel as demineralizing agent of enamel tissue certainly proved to be effective treatment modality.

Conclusion

Within the limitations of the study, it can be concluded that, fluoride gel and chitosan hydrogel were able to produce remineralization of enamel tissue. Highest value of microhardness of the demineralized enamel tissue was found after application of fluoride gel. Microhardness of the remineralized enamel was correlatable when APF gel and chitosan hydrogel were compared indicating further uses in clinical trials. Most importantly, chitosan hydrogel showed the biomimetic remineralization of the enamel, which gives hope for the enamel tissue regeneration in the modern era of the preventive dentistry.

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