



Original Research Article

Chitosan – A novel way to intervene in enamel demineralization - An *in vitro* study

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ABSTRACT

Keywords

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Acids have been implicated in enamel demineralization. The disadvantages of existing therapies have led to the search for better alternatives. Chitosan, a deacetylated product of chitin, with its antimicrobial and acid absorption properties, is expected to fill this void. To assess the acid protection competence of water soluble chitosan lactate on enamel against cariogenic acids, dietary acids and dental bleaching procedures. 105 teeth with sound enamel were divided into four groups, A, B, C and D. Group A (15 teeth) was taken as control. Groups B, C and D (30 each) were divided into equal subgroups B1, B2, C1, C2, D1 and D2 (15 teeth) and were subjected to cariogenic acid, acidic beverages and hydrogen peroxide bleaching respectively. Subgroups B2, C2 and D2 were pretreated with chitosan lactate. Post treatment, Vickers Hardness Number was measured. Teeth treated with chitosan lactate had higher mean VHN than those without chitosan lactate in all groups. Kruskal Wallis Test and post-hoc analysis revealed significant differences between subgroups in Group B. Chitosan lactate protects the enamel from demineralization and is safe to be incorporated into oral formulations

Introduction

The oral cavity is maintained in a healthy and balanced state by homeostatic mechanisms that regulate physiologic properties such as pH, temperature, salivary flow, viscosity, microflora, etc. Any disruptions in these mechanisms will initiate pathological processes, which may lead to various diseases that destroy the hard and soft tissues in the mouth.

Mature enamel is a non-living tissue, composed of mainly hydroxapatite crystals and cannot regenerate itself after substantial mineral loss (Sivapathasundaram and Raghu, 2009).

The pH of the oral cavity is normally maintained at a neutral to alkaline value of 7.0–7.5 through various buffering

mechanisms involving salivary proteins, bicarbonates, phosphates, and salivary flow. The protection competence of saliva is not sufficient to maintain the pH at an optimal level after exposure to acids, which may result in an increased risk of demineralization (Lamanda *et al.*, 2007).

The oral cavity pH tends towards acidic value due to the production of acids by cariogenic oral bacteria from food debris, dietary acids, fruits, carbonated beverages, salad dressings, endogenous acids etc. Hydrogen peroxide used in tooth bleaching renders the enamel weak (Ehlen *et al.*, 2008; Tredwin *et al.*, 2006).

Enamel demineralization is a major step for initiation of dental caries. Dental caries is the leading cause for loss of teeth, affecting between 50–60% of the Indian population (Moses *et al.*, 2011). The prevention of acid attack in the oral cavity is the most effective method in treating demineralization of teeth. Various treatment modalities and preventive methods have been explored to protect the tooth enamel from acid attack.

Antimicrobials like chlorhexidine, sugar substitutes such as erythritol, xylitol, etc., limit the production of acids, pyrophosphates and ZnO limit the attachment of *S. mutans* to the tooth surface, casein phosphopeptide, phosvitin, arginine and small peptides have also been used to increase the pH of the plaque as they accumulate on the surface and have a local buffering action.

Fluorides have been incorporated into toothpastes and water supplies to inhibit demineralization (Cai *et al.*, 2003, Featherstone *et al.*, 1990). These compounds, despite their widespread use, possess a high toxicological profile, develop microbial resistance, result in imbalance of

oral microflora, are expensive, depend on patient co-operation, are not easily available and require application by professionally trained individuals to achieve optimum results. These disadvantages make application of these products inefficient and cumbersome. Alternate substances which protect enamel and overcome the above drawbacks are necessary as demineralization is still a rampant problem.

The use of naturally occurring substances in dental applications has been in vogue in recent times and chitosan a deacetylated polymer of chitin is one such substance. Chitin, a natural polymer, is found in the cell walls of fungi and forms a major component of the exoskeletons of arthropods, such as the crustaceans (e.g. crab, lobster and shrimp), and the insects (e.g. ants, beetles, and butterflies) and of the beaks of cephalopods (e.g. squids, and octopi).

Chitosan is composed of 2-amino-2-desoxy-D glycopyranose interconnected by glycosidic bonds β -1,4 in variable proportions. It possesses highly reactive amino (-NH₂) and hydroxyl groups (OH). Chitosan is biocompatible, non-toxic, highly bioactive, biodegradable, selectively permeable, antimicrobial, anti-acid, forms gel and film, has ability to chelate and possess absorptive capacity. It is soluble in weak acids but is not water soluble, thus limiting its potential usage (Rinaudo, 2006).

Chitosan contains amino groups with a pKa value of 6.2–7 and it is considered as a strong base. Chitosan is soluble in dilute acidic solutions at pH below 6. It can also form water-soluble salts, such as chitosan acetate and chitosan lactate, with some aqueous inorganic or organic salts. The solubility of chitosan generally increases as pH decreases. This is because at low pH the amino groups of chitosan get protonated

resulting in a water soluble cationic polyelectrolyte. In these conditions the surface charge of chitosan is positive, allowing it to interact with negatively charged surfaces. However, if pH is higher than 6, the amines become deprotonated and lose their charge, resulting in a neutral insoluble polymer. At physiological pH 7.4 chitosan has low solubility in aqueous solutions. While chitosan is a weak base, pKa value of the glucosamine residue is in the range between 6.2 and 7.0. Therefore it is insoluble in neutral and alkaline solutions and needs to be in an acidic medium, allowing the amino groups to be positively charged. To overcome this problem, many water soluble chitosan derivatives have been developed by modifying the reactive functional groups of chitosan or by depolymerizing chitosan. Chitosan lactate dissolves in water since it is already in the form of a salt (Soares, 2011).

Chitosan has been used as an antimicrobial agent against various pathogens, including cariogenic and periodontal pathogens and possesses the ability to scavenge oxygen free radicals and enamel protected with acid soluble chitosan showed higher surface microhardness with Vickers test than enamel which were not treated with any chitosan (Arancibia *et al.*, 2013; Liu *et al.*, 2009; Arnaud *et al.*, 2010).

The advantages of water soluble chitosan lactate with its safe toxicological profile requires a systematic assessment of its protective effect on enamel from demineralization making it a potentially valuable agent in combating enamel demineralization and acid absorption.

Materials and Methods

Water soluble chitosan lactate gifted from Everest Biotech, Bengaluru, India was used for our study.

Surface microhardness testing

The mineral content of enamel can be quantified indirectly by measuring the surface microhardness (Lippert and Lynch, 2014). Hardness is tested by measuring the depth of penetration of a needle on the surface by a procedure known as Vickers method. Vickers hardness needle with a diamond shaped indenter was used to make indentations on the enamel surface of the teeth using a load of 30kg. The indentations were measured using a travelling microscope. The diagonals of the indentation were measured and the Vickers Hardness Number (VHN) of each specimen was calculated using a formula.

$$\text{VHN} = (1.854 \times \text{load}) / d^2$$

Where,

load = 30kg and

d = average length of the two diagonals.

Sample collection

105 dental caries free, human premolars extracted for orthodontic purposes were collected after ethical clearance and were maintained in an artificial saliva solution. Group A contained 15 teeth and was taken as a control. Groups B, C and D had 30 teeth each and were further divided into subgroups B1, B2, C1, C2, D1 and D2 of 15 teeth each.

Preparation of chitosan solutions

Chitosan lactate solution was prepared by dissolving 5g of chitosan lactate flakes in 10mL of water.

Enamel specimen preparation

All the teeth were sectioned and polished by a diamond disc using a Marathon™ -4

micromotor fitted with a Marathon™ H37L1 straight fissure handpiece to obtain flat polished enamel surfaces. Group A teeth were not subjected to any treatment.

Preparation of early artificial caries lesion

Group B specimens were divided into 2 subgroups of 15 each, B1 and B2. Teeth in subgroup B1 were exposed to a demineralization cycle with 1% acetic acid for one hour thrice daily for 5 days to cause demineralization without pretreatment with chitosan lactate solution. Teeth in subgroup B2 were pretreated with chitosan lactate solution thrice daily for 5 minutes for 5 days at an interval of 6 hours before exposure to each cycle of demineralization.

Demineralization by dietary acids

Group C specimens were divided into 2 subgroups of 15 each, C1 and C2. Teeth in subgroup C1 were exposed to a commercially available carbonated beverage for 10 minutes thrice daily for five days to cause demineralization without pretreatment with chitosan lactate solution. Subgroup C2 was pretreated with chitosan lactate solution thrice daily for 5 minutes for 5 days at an interval of 6 hours before each cycle of demineralization.

Bleaching of teeth

Group D specimens were divided into 2 subgroups of 15 each, D1 and D2. Teeth in subgroup D1 were exposed to bleaching with 6% hydrogen peroxide solution for 20 minutes thrice daily for 5 days without pretreatment with chitosan lactate solution. Teeth from subgroups D2 were pretreated with chitosan lactate solution thrice daily for 5 minutes for 5 days at an interval of 6 hours before each exposure to 6% hydrogen peroxide solution.

VHN measurement

All the 105 teeth sections were mounted on to self-cure acrylic (Fig. 1) to provide a base for surface microhardness testing. The surface microhardness of the enamel of all three groups was measured using Vickers hardness Tester Model VM-50, manufactured by Venus Instruments, Bangalore, Karnataka, India (Fig. 2).

The results were tabulated (Table 1) analyzed using Statistical Package for Social Sciences (SPSS) Version 15 for Windows.

Results and Discussion

Kruskal Wallis (K-W) test was used to analyze differences between groups. Under the significance of K-W test, post-hoc analysis was done to analyze the point of significance between the groups.

Group A, the control group, showed a mean VHN of 286.12.

Subgroups B1 and B2, had mean VHN values of 207.59, and 247.4, respectively. (Graph-1) Kruskal-Wallis test between Group A and subgroups B1 and B2 showed significant difference between the groups (P-value =0.0001 (<0.01)). Post-hoc analysis revealed statistically significant differences in hardness of the Group A (control), subgroup B1 and B2.

Subgroups, C1 and C2 had mean VHN values of 212.60 and 234.27 (Graph-2). Kruskal Wallis test between Group A and subgroups C1 and C2 showed significant difference between the [P-value =0.0001 (<0.01)]. Post-hoc analysis revealed statistically significant differences in hardness of the Group A (control), subgroup C1 and C2.

Subgroups D1 and D2 had mean hardness values of 241.69 and 260.13 (Graph-3). Kruskal Wallis test between Group A and subgroups D1 and D2 did not show significant differences between the groups. The mean hardness values of subgroups with chitosan were higher than the group without chitosan.

In physiological conditions (pH=7), saliva is supersaturated with calcium and phosphate ions, where demineralization is a slow process. The “critical pH” is the point where equilibrium exists and there is no mineral dissolution and no mineral precipitation. The critical pH of hydroxyapatite is around 5.5. The decrease in pH leads to demineralization. Due to the acids from the above sources, pH of the plaque falls below 5.5. This shifts the driving force within the tooth to mineral dissolution. The lower the pH, the higher the concentrations of calcium and phosphate required to reach saturation with respect to hydroxyapatite. As the pH decreases, positive hydrogen ions from the acid bind with negatively charged phosphate and hydroxyl ions from the hydroxyapatite (enamel). The ionic solution in the pellicle layer becomes unsaturated resulting in demineralization, which leads to the loss of calcium and phosphate ions from the hydroxyapatite (Mori *et al.*, 2012).

Arnaud *et al.* (2010) used two demineralizing solutions and remineralizing solutions and found that enamel protected with acid soluble chitosan showed a higher surface microhardness with Vickers test than enamel which were not treated with any chitosan (Arnaud *et al.*, 2010). Lee *et al.* (2012) found that Chitoclear™ adsorption onto an artificial saliva layer protects the hydroxyapatite surface by cross linking on the enamel surface and saliva (Lee *et al.*, 2012).

The mechanism of action of the protective effect of chitosan can be enumerated in many ways. Chitosan maintains the integrity and structure of the tooth as well as the oral cavity by inhibiting dissolution of hydroxyapatite by acids

- i) Maintains the pH of plaque above the critical level of enamel demineralisation. The organic anions in chitosan hinder the rate of acid dissolution of hydroxyapatite through rapid adsorption. The free amino (-NH₂) group in chitosan makes it highly reactive with dietary and cariogenic acids in the oral cavity and thereby reduces the acid and increase the pH to normal levels (Lee *et al.*, 2012; Shetty *et al.*, 2014).
- ii) The cross-linking of chitosan and saliva with the physical adsorption of chitosan onto saliva prevents acid erosion of the hydroxyapatite surface (Lee *et al.*, 2012).
- iii) The penetration of chitosan into the enamel as far as the dentino-enamel junction has been demonstrated by Arnaud *et al.* It has been postulated that chitosan may act as a mechanical barrier for the acid penetration in the enamel and interferes in the process of enamel demineralization by inhibiting the release of mineral element (Arnaud *et al.*, 2010).
- iv) Chitosans have been shown to scavenge free radicals (Liu *et al.*, 2009). Free radicals liberated by hydrogen peroxide bleaching damage the structure of enamel and these form weak spots for demineralization and hence initiate dental caries. Free radicals are also genotoxic. The use of chitosan can limit the free radicals post bleaching treatments.
- v) Chitosan is mucoadhesive which makes it highly bioavailable, ensuring

a long period of action after the time of application. It also potentiates the action of chlorhexidine and has been used in the controlled delivery of fluoride (De Carvalho *et al.*, 2011; Keegan *et al.*, 2012; Bae *et al.*, 2006; Pedro *et al.*, 2009; Andrews *et al.*, 2009).

- vi) Nano-complexes of phosphorylated chitosan and amorphous calcium phosphate have been shown to remineralize enamel subsurface lesions at a rate significantly higher than that of fluoride treatment (Zhang *et al.*, 2014).

In our study, water soluble chitosan lactate was found to be effective in protecting enamel from demineralization against cariogenic acid (group B), followed by the dietary acid (Group C) and finally, the dental bleaching procedure (Group D). The mean VHN was higher for the teeth protected by chitosan lactate than the

unprotected teeth in all groups.

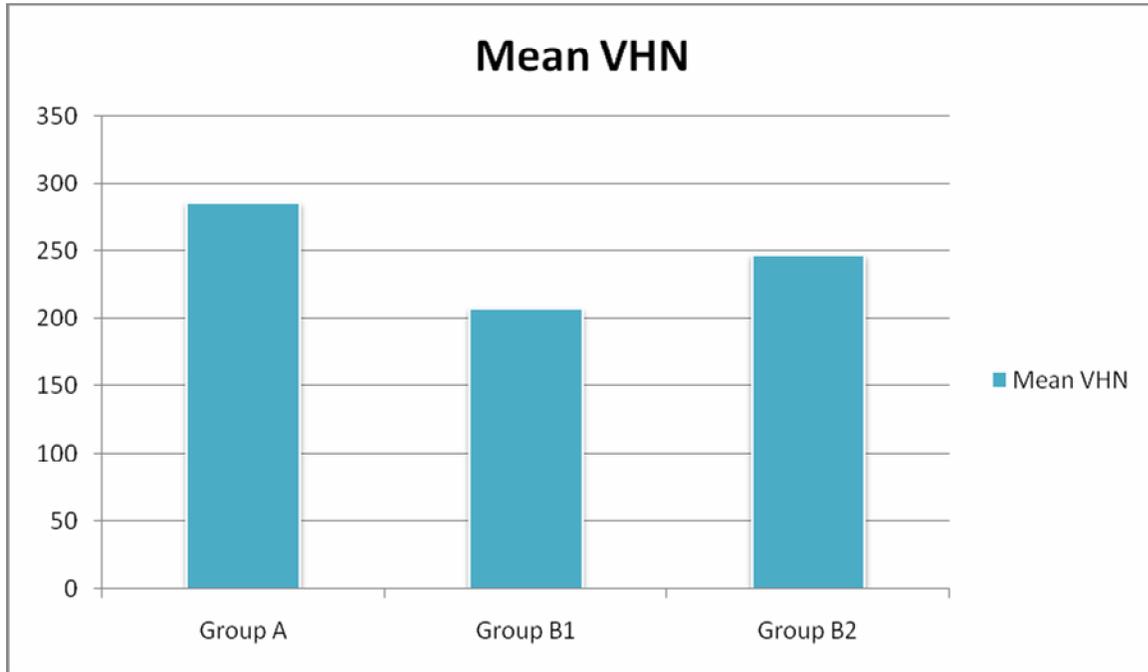
Chitosan lactate was most effective against acid cycling which mimicked the oral environment's pH cycle based on release of acids by bacteria after consumption of food. Their effects against dietary acids and bleaching agents were not statistically significant.

Apart from acid absorption and preventing demineralization of enamel, chitosan has a significant antimicrobial action against cariogenic and periodontal pathogens. In the field of medicine and dentistry, chitosan is used as an antioxidant, anti-bacterial, anti-acid, plaque inhibitor, carrier for controlled drug release, immune-enhancing effect, promotes osteogenesis, haemostasis, fat absorbent action, promotes healing of ulcers and wounds, etc. (Dai *et al.*, 2011; Farag AND Mohamed, 2013; Tan *et al.*, 2013).

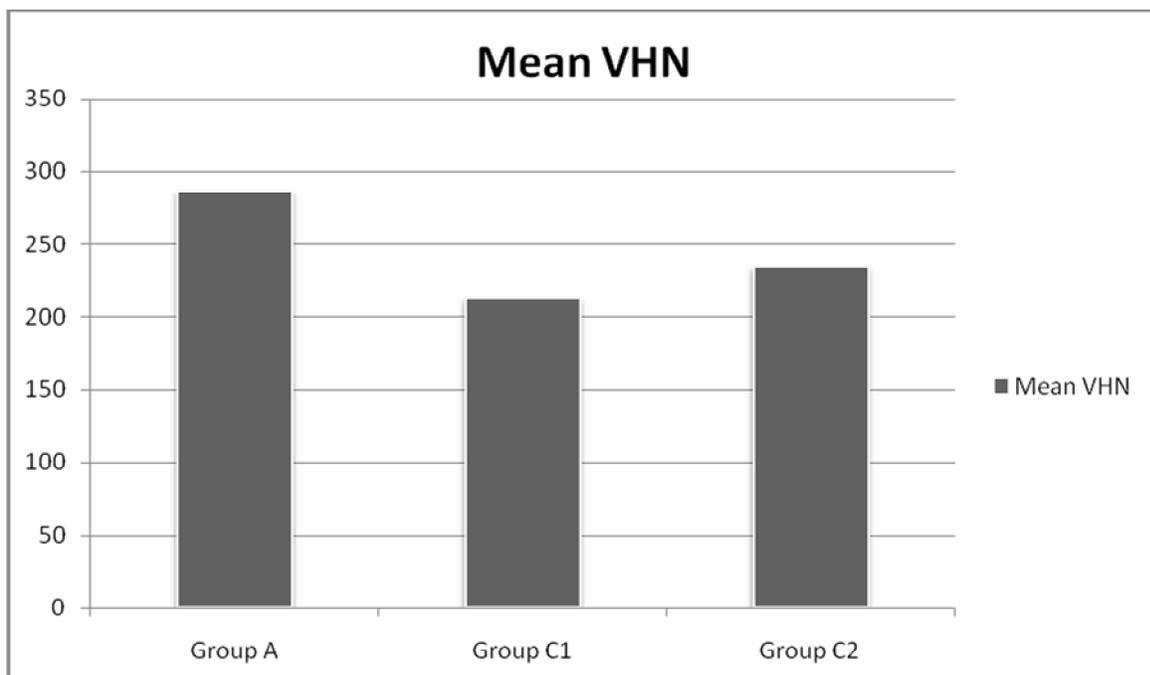
Table.1 Vickers Hardness Number (VHN) of the specimens in the different subgroups

S. No.	Group A	Group B1	Group B2	Group C1	Group C2	Group D1	Group D2
1	308	192.51	246.5	201.79	246.5	274.6	246.5
2	274	211.76	234.03	211.76	234.03	222.48	367.9
3	246.5	183.86	274.6	211.76	246.5	246.5	274.6
4	309	222.48	234.03	183.86	234.03	222.48	246.5
5	274.6	211.76	234.03	246.5	234.03	234.03	274.6
6	308	183.86	274.6	201.79	246.5	246.5	234.03
7	367.9	234.03	246.5	234.03	222.48	256.7	234.03
8	308	222.48	234.03	183.86	246.5	234.03	246.6
9	290.5	183.86	274.6	246.5	234.03	246.5	274.6
10	274.6	201.79	222.48	201.79	222.48	234.03	246.6
11	246.5	234.03	246.5	234.03	234.03	246.5	256.7
12	250.5	201.79	234.03	211.76	222.48	246.5	274.6
13	284.6	183.86	246.5	183.86	234.03	234.03	234.03
14	274.6	211.76	234.03	234.03	222.48	246.5	256.7
15	274.6	234.03	274.6	201.79	234.03	234.03	234.03

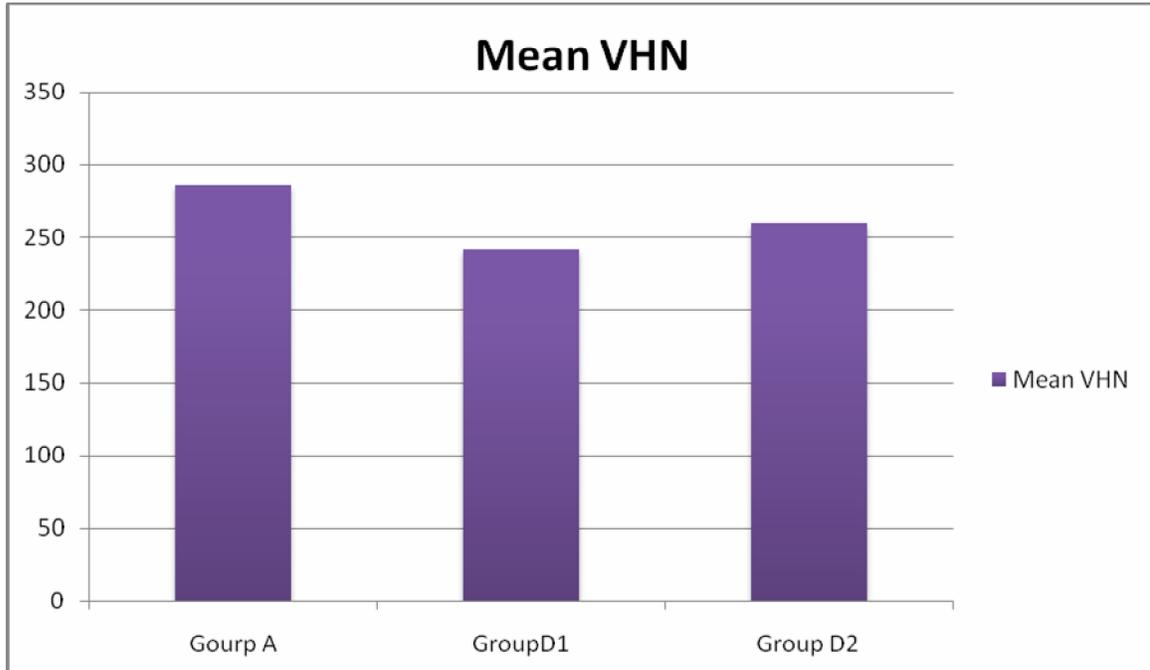
Graph.1 Comparison of mean VHN of Group A and Group B subgroups



Graph.2 Comparison of mean VHN of Group A and Group C subgroups (acidic beverages)



Graph.3 Comparison of mean VHN of Group A and Group D subgroups (bleaching)



Graph.4 Error graph of obtained data with 95% confidence interval

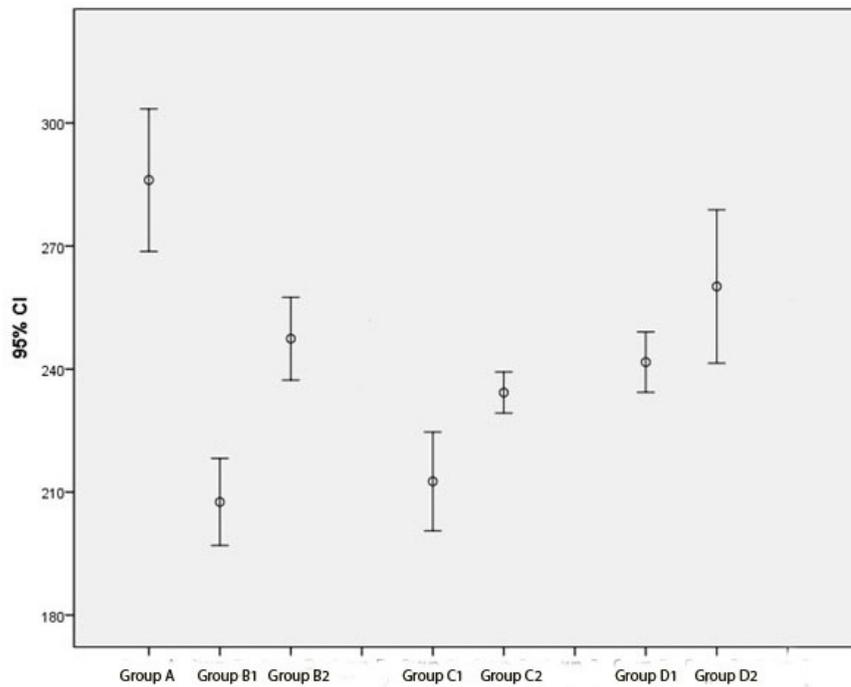
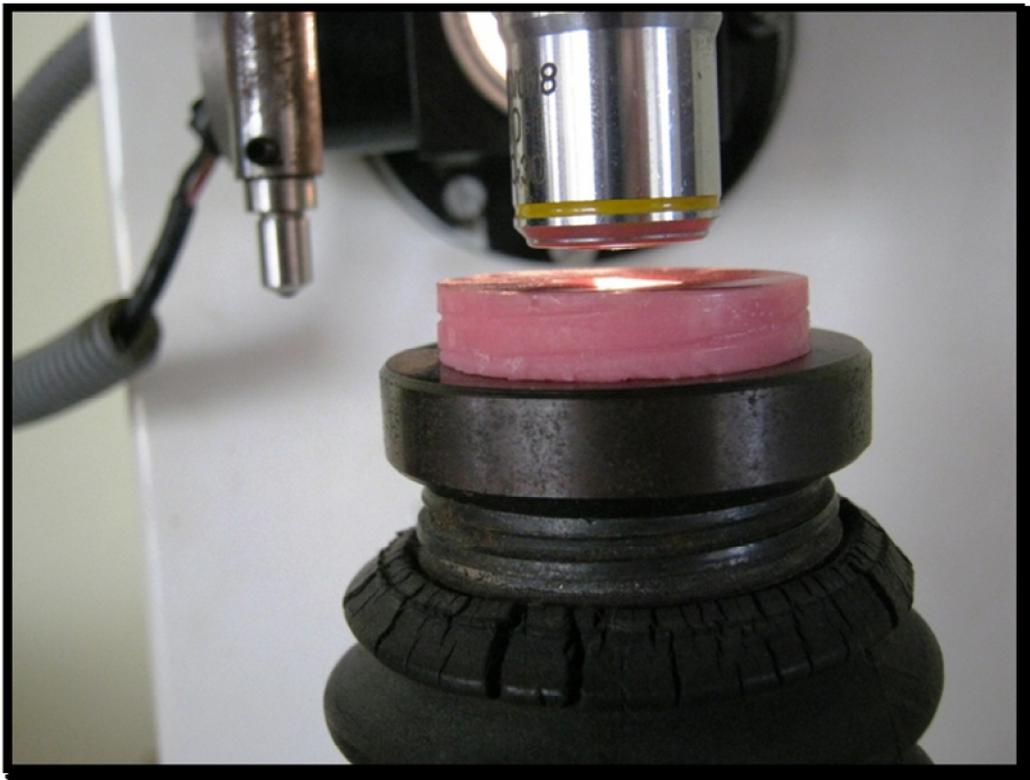


Figure.1 Tooth sections with flat enamel surfaces were mounted onto acrylic bases to measure hardness



Figure.2 Hardness of acrylic mounted enamel sections measured using Vickers Hardness tester



The acid adsorption and enamel protection competence along with the antibacterial activity, mucoadhesive nature, bioavailability, potentiating the action of chlorhexidine, controlled delivery of fluoride and ability of nano-complexes of phosphorylated chitosan and amorphous calcium phosphate to remineralize enamel subsurface lesions make it an effective, safe alternative to protect teeth and to treat the oral cavity against acid attack.

Chitosan products can be used in the oral cavity in formulations such as toothpastes, gels, mouth rinses, artificial saliva to protect teeth as it addresses multiple needs. It is a safe alternate for patients, sportspersons and healthy consumers. Chitosan formulations provide simple strategies to prevent oral health problems that require little or no additional time or monetary investment. This study shows that water soluble chitosan can be effectively used to protect the teeth and aid in preventing demineralization caused either by caries process, dietary acids or dental procedures.

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References

Andrews, G.P., Laverty, T.P., Jones, D.S. 2009. Mucoadhesive polymeric platforms for controlled drug delivery. *Eur. J. Pharm. Biopharm.*, 71: 505–518.

- Arancibia, R., Maturana, C., Silva, D., Tobar, N., Tapia, C., Salazar, J.C., Martínez, J., Smith, P.C. 2013. Effects of chitosan particles in periodontal pathogens and gingival fibroblasts. *J. Dent. Res.*, 92(8): 740–745.
- Arnaud, T.M.S. 2010. de Barros Neto, B., Diniz, F.B. Chitosan effect on dental enamel de-remineralization: An in vitro evaluation. *J. Dent.*, 38: 848–852.
- Bae, K., Jun, S., Lee, S., Paik, D., Kim, J. 2006. Effect of water-soluble reduced chitosan on Streptococcus mutans, plaque regrowth and biofilm vitality. *Clin. Oral Invest.*, 10: 102–107.
- Cai, F., Shen, P., Morgan, M.V., Reynolds, E.C. 2003. Remineralization of enamel subsurface lesions in situ by sugar - free lozenges containing casein phosphopeptide amorphous calcium phosphate. *Aust. Dent. J.*, 48(4): 240–243.
- Dai, T., Tanaka, M., Huang, Y.Y., Hamblin, M.R. 2011. Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. *Expert Rev. Anti. Infect. Ther.*, 9(7): 857–879.
- deCarvalho, M.M.S.G., Stamford, T.C.M., dos Santos, E.P., Tenório, P., Sampaio, F. 2011. Chitosan as an oral antimicrobial agent Science against microbial pathogens: communicating current research and technological advances. Badajoz, Formatex.
- Ehlen, L.A., Marshall, T.A., Qian, F., Wefel, J.S., Warren, J.J. 2008. Acidic beverages increase the risk of in vitro tooth erosion. *Nutr. Res.*, 28(5): 299–303.
- Farag, R.K., Mohamed, R.R. 2013. Synthesis and characterization of carboxymethyl chitosan nanogels for swelling studies and antimicrobial activity. *Molecules*, 18: 190–203.
- Featherstone, J.D., Glena, R., Shariati, M.,

- Shields, C.P. 1990. Dependence of in vitro demineralization of apatite and remineralization of dental enamel on fluoride concentration. *J. Dent. Res.*, 69: 620–625.
- Keegan, G.M., Smart, J.D., Ingram, M.J., Barnes, L., Burnett, G., Rees, G.D. 2012. Chitosan microparticles for the controlled delivery of fluoride. *J. Dent.*, 40: 229–240.
- Lamanda, A., Cheaib, Z., Turgut, M.D., Lussi, A. 2007. Protein buffering in model systems and in whole human saliva. *PLoS ONE*, 2(2): e263. doi: 10.1371/journal.pone.0000263.
- Lee, H.S., Tsai, S., Kuo, C.C., Bassant, A.W., Pepe-Mooney, B., Miska, D., Masters, J., Sullivan, R., Composto, R. 2012. Chitosan adsorption on hydroxyapatite and its role in preventing acid erosion. *J. Coll. Interface. Sci.*, 385: 235–224.
- Lippert, F., Lynch, R.J.M. 2014. Comparison of Knoop and Vickers surface microhardness and transverse microradiography for the study of early caries lesion formation in human and bovine enamel. *Arch. Oral Biol.*, 59(7): 704–710.
- Liu, H.T., Li, W.M., Xu, G., Li, X.Y., Bai, X.F., Wei, P., Yu, C., Du, Y.G. 2009. Chitosan oligosaccharides attenuate hydrogen peroxide induced stress injury in human umbilical vein endothelial cells. *Pharmacol. Res.*, 59(3): 167–175.
- Mori, F., Noriko H., Masayuki O. 2012. Effect of mastication on flow and properties of saliva. *APJD*, 12(1): 1–5
- Moses, J., Rangeeth, B.N., Gurunathan, D., et al. 2011. Prevalence of dental caries, socio-economic status and treatment needs among school children. *J. Clin. Diag. Res.*, 5(1): 146–151
- Pedro, A.S., Cabral-Albuquerque, E., Ferreira, D., Sarmiento, B. 2009. Chitosan: An option for development of essential oil delivery systems for oral cavity care? *Carbohydr. Polymers*, 76: 501–508.
- Rinaudo, M. 2006. Chitin and chitosan: properties and applications. *Prog. Polym. Sci.*, 31: 603–632.
- Shetty, S., Hegde, M.N., Bopanna, T.P. 2014. Enamel remineralization assessment after treatment with three different remineralizing agents using surface microhardness: An in vitro study. *J. Conserv. Dent.*, 17: 49–52
- Sivapathasundaram, B., Raghu, A.R. 2009. Dental caries. In: Rajendran, R., Sivapathasundaram, B. (Eds), *Shafer's textbook of oral pathology*. 6th edn. Elsevier, New Delhi. Pp. 412–452, 569–587
- Soares, H.T.F.C. 2011. Effect of chitosan lactate on the formation of chitosan-DNA particles: A physicochemical, haemocompatible and cytotoxic study. Ph.D Thesis, Universidade de Coimbra, Portugal.
- Tan, H., Ma, R., Lin, C., Liu, Z., Tang, T. 2013. Quaternized chitosan as an antimicrobial agent: Antimicrobial activity, mechanism of action and biomedical applications in orthopedics. *Int. J. Mol. Sci.*, 14: 1854–1869.
- Tredwin, C.J., et al. 2006. Hydrogen peroxide tooth-whitening (bleaching) products: Review of adverse effects and safety issues. *Brit. Dental J.*, 200(7): 371–376.
- Zhang, Xu, et al. 2014. Biomimetic remineralization of demineralized enamel with nano-complexes of phosphorylated chitosan and amorphous calcium phosphate. *J. Materials Sci.: Materials Med.*, Pp. 1–10.