Effect of chitosan application on the decreased enamel demineralization process in vitro (surface damage test)

N F Irfani, H A Gunawan* and L R Amir

Departement of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta 10430, Indonesia

*E-mail: atjiek@ui.ac.id

Abstract. Chitosan’s amino and hydroxyl groups may react with acid ions, increasing environmental pH level, and thereby interfering with the enamel demineralization process. We analyzed enamel surface damage after chitosan application in an acidic environment and evaluated its mechanism of action. Eleven postextraction teeth were divided into baseline, negative control, acid+chitosan application, and chitosan application groups. The environmental pH level increased and enamel surface damage decreased after chitosan application in an acidic environment compared to the negative control group. Therefore, chitosan interfered with the enamel demineralization process in vitro.

1. Introduction

Tooth caries is a pathological process of dental tissue destruction caused by organic acids produced by carbohydrate fermentation by bacteria in dental plaque. In Indonesia, caries remains the most common oral health problem. The prevalence of caries and delayed, missing, and filled teeth (DMF-T) tends to increase every decade with 70% of caries found in early stages [1–3]. Organic acids will diffuse into enamel and dentine pores, beginning the process of teeth demineralization which may form a cavity if allowed to persist. Inside the oral cavity, there is a dynamic process termed the demineralization and remineralization cycle, that influence tooth surface integrity, which continues as long as bacteria, carbohydrates, and saliva are present. Caries results when there is an imbalance between the demineralization and remineralization [4].

Besides caries, another process causing dissolution of teeth mineral substances is tooth erosion. Tooth erosion is another form demineralization, due to chemical substance that does not involve bacteria. Loss of enamel surface structure due to erosion occurs rapidly, in contrast to subsurface demineralization in the relatively slow caries process. The acid that causes tooth surface erosion can be extrinsic (from outside the body, such as acidic foods, drinks, and drugs) or intrinsic (from the body, such as chronic regurgitation, belching, and vomiting) [5].

Over the years, serious methods to prevent demineralization have been developed. Topical and systemic fluoridation can increase enamel durability from demineralization. Unfortunately, fluorine, which is very widely used, has high toxicity, and is dangerous if used excessively. Current systemic fluoridation is no longer used because it is difficult to measure the dose and period of administration, possibly leading to excessive fluorine intake and fluorosis in the teeth and bone [3]. Other preventive methods, such as low-carb diet, low-acid foods, oral hygiene and mouth maintenance, and use of a
chlorhexidine antibacterial agent, will limit the production of organic acids by bacteria to prevent demineralization [6,7].

The use of natural ingredients in dentistry is being studied increasingly. Chitosan is a deacetylation product of chitin (a natural polymer of polysaccharides found in many crustacean animal shells). It has wide potential for medical and biological application because it is biocompatible, biodegradable, nontoxic, antimicrobial, and can bind to acids [6–10]. Chitosan is retained in teeth structure by inhibiting hydroxyapatite dissolution due to acids. The amino (NH2) and hydroxyl (OH) groups in chitosan are suspected to be reactive to acid (H+) in the oral cavity, thus, increasing the pH of the oral cavity [6].

Several studies on the effect of chitosan on tooth enamel demineralization have been performed. Arnaud et al. [8], Schlueter et al. [11], and Visveswaraih et al. [6] revealed that chitosan inhibited demineralization of tooth enamel exposed to acids through enamel surface hardness testing. However, to date, to our knowledge, no study has proven the acid absorption mechanism by chitosan in the oral cavity to inhibit the enamel demineralization process. Therefore, we attempted to prove the effect of chitosan application on decreasing the enamel demineralization process by observing the mechanism of increasing pH and comparing surface structure damage to enamel in an acidic environment in teeth treated with and without chitosan.

2. Materials and Methods

The samples in this study were 11 postextracted teeth that were caries-free, abrasion-free, and clinically erosion-free. The chitosan was purchased from Biotech Surindo Cirebon (Jawa Barat, Indonesia) and had a >90% deacetylation degree.

The study consisted of two stages: (1) a pH change test of the demineralization solution due to chitosan and (2) an enamel surface damage test in an acidic environment treated with chitosan. In stage 1, the sample solutions (Aquadem; Wilhelm Werner GmbH, Leverkusen, Germany; and chitosan solution 2.5 mg/mL) were prepared by first dissolving 250 g chitosan powder into 100 mL 1% acetic acid using a magnetic stirrer for 24 hours, and the pH was measured. We then prepared demineralization solutions with 10% phosphoric acid only, 10% phosphoric acid mixture and Aquadem (volume ratio 1:1), and 10% phosphoric acid mixture and chitosan solution (volume ratio 1:1).

In phase 2, the enamel specimens were immersed into four different sample solutions: (1) baseline group (20 mL Aquadem), (2) negative control group (10 mL 10% phosphoric acid solution + 10 mL Aquadem), (3) treated group 1 (10 mL 10% phosphoric acid and 10 mL chitosan solutions), and (4) treated group 2 (10 mL chitosan solution and 10 mL Aquadem). Before immersion, the enamel specimens were ground with 1000 and 1500 grit sandpaper until their surfaces were dulled, then covered with nail polish leaving a 5 × 5 mm area on the buccal surface. The immersion covered the entire 5 × 5 mm area and the hanging wires were not immersed. The immersion duration was 6 hours. Following immersion, the specimens were removed from the solutions and the pH of all the solutions was measured. All four specimens were cut using a carborandum disc to obtain the crown portion. Thereafter, each specimen underwent a carbon coating process to obtain good emission reflection. Two electron micrographs were obtained from each specimen. Then, the surface damage of one specimen from each sample group was tested using a scanning electron microscope (SEM) with ×500 magnification, 5 kV ray voltage, and 8–19 mm working distance. The SEM analysis was done by five blinded reviewers and scored from 1 to 5 [12–14]:

- Score 1: Normal, no damage, intact surface.
- Score 2: Little damage (e.g., spreading dots), not changing the overall surface image.
- Score 3: Little damage, irregular surface with shallow basin.
- Score 4: Significant damage, complete loss of surface structure, visible crystal fractures.
- Score 5: Significant damage, loss of surface structures with deep hollows, irregular crystalline fractures.
The categorical data obtained were assessed with the Kruskal–Wallis and Mann–Whitney nonparametric tests to determine any statistically significant differences between groups with a significance value of 0.025.

3. Results

3.1. pH alteration test of demineralization solution

The pH was measured before and after enamel specimen immersion in all specimens groups.

![pH level of sample solutions](image)

**Figure 1.** pH level of sample solutions.

The pH level in treated group 1 was higher than that in the negative control group with or without enamel. The pH level after immersion in all groups was higher than that before immersion.

3.2. Enamel Test on the enamel specimens to assess surface damage test

3.2.1. Visual analysis. Enamel specimens immersed in 10% phosphoric acid solution for 6 hours were evaluated for surface damage using SEM at ×500 magnification.

![Electron micrographs of baseline specimen (Aquadem treatment), ×500 magnification. Visible surface of the enamel was intact with no visible demineralization.](image)

**Figure 2.** A, B. Electron micrographs of baseline specimen (Aquadem treatment), ×500 magnification. Visible surface of the enamel was intact with no visible demineralization.
Figure 3. A, B. Electron micrographs of negative control specimens (phosphoric acid treatment and Aquadem), ×500 times magnification. Overall surface damage due to demineralization. There were crystal fractures. X, Y, and Z areas were more damaged than the surrounding areas. The deep basin showed the depth of the damage; the basin was visible at some point and not throughout.

Figure 4. A, B. Electron micrographs of treated group 1 (phosphoric acid + chitosan), ×500 magnification. Visible demineralization is seen throughout the entire surface. There were crystal fractures (*) and erosion of enamel prisms (**).

Figure 5. A, B: Electron micrographs of treated group 2 (chitosan + Aquadem), ×500 magnification. There was discontinuity on the enamel surface.
In Figures 2 to 5, treated group 1 (phosphoric acid + chitosan; Fig. 4) showed less damage than the negative control specimen (Fig. 3). Meanwhile, little damage was detected in the chitosan specimen (treated group 2, chitosan + Aquadem; Fig. 5) compared to the baseline enamel surface (Fig. 2). Thus, the order of surface changes of enamel from the most damaged to the most intact was: negative control specimens, treated group 1, treated group 2, and baseline specimens.

3.2.2. Blinded Reviewer Scoring.

| Score | Group          | Group            | Group            |
|-------|----------------|------------------|------------------|
|       | Baseline       | Negative Control | Treated Group 1  | Treated Group 2  |
| 1     | 10             | 0                | 0                | 1                |
| 2     | 0              | 0                | 1                | 6                |
| 3     | 0              | 0                | 3                | 3                |
| 4     | 0              | 4                | 4                | 0                |
| 5     | 0              | 6                | 2                | 0                |

Electron micrograph scoring data analysis revealed significant differences in enamel surface damage between the groups (\( P < 0.025 \), Kruskal–Wallis test), and differences in enamel surface damage between the baseline and negative control (\( P < 0.025 \)) groups, baseline and treated groups 1 (\( P < 0.025 \)) and 2 (\( P < 0.025 \)), negative control group and treated groups 1 (\( P < 0.025 \), all Mann–Whitney U tests).

4. Discussion

4.1. Increase in pH of acid environment

The increase in pH of the acid environment by chitosan was evident in this study. The pH value of treated group 1 (10% phosphoric acid + chitosan) was higher than that of treated group 2 (10% phosphoric acid solution + Aquadem). This occurred with solutions before and after immersion of enamel for 6 hours, indicating that chitosan caused an increase in pH of acidic solutions.

The pH value in all groups after immersion was higher compared to before immersion. This is most likely due to the binding of H+ in the acid solution by the PO4 and OH in the enamel, so that the H+ ions decreased. According to Delvar et al. [15], the increase in pH of the demineralization solution over time can be due to increase in the the amount of the hydroxyapatite dissolution. The increased pH in negative control solutions (0.385) was higher than that in treated groups 1 (0.272) and 2 (0.253), indicating that there was more enamel dissolution in the negative control than in the treated groups. Therefore, chitosan had shown the ability to inhibit the enamel demineralization process in acidic conditions.

In treated group 2, the pH increased due to the acidic chitosan solution, which also resulted in enamel dissolution, causing a decrease in H+ concentration of the solution. In the baseline group, the pH of Aquadem after enamel immersion had increased to 0.124 because the water lowers the surface tension of the surrounding object so that the water molecules (H₂O) enter the object. The hydrogen
atom is smaller than the oxygen atom, so it will enter the objects first. Therefore, the level of H+ in Aquadem will decrease and the pH will increase.

The negative control group and treated group 1 demonstrated the difference in ability between Aquadem and chitosan to increase the pH of the environment. Chitosan had a greater ability than Aquadem. Chitosan has NH2 and OH, which are reactive to H+, so that the H+ level decreases and the pH of the environment increases.

As described above, the H+ ions in the demineralization solution also can bind to PO4 and OH in the enamel, resulting in the dissolution of the enamel. However, the presence of NH2 and OH in chitosan, which also bind H+, cause competition between chitosan and enamel in binding to H+ ions, so that the enamel dissolution will be inhibited [6,9,16].

No statistical analysis test was conducted on the pH alteration of the demineralization solution because demonstrating the significant presence or absence of differences between sample groups was not necessary. This section of the study only aimed to observe the ability of chitosan to increase the pH of demineralization solutions as its working mechanism to inhibit the enamel demineralization process.

4.2. Reduction on the enamel surface damage

Inhibition of the demineralization process, resulting in decreased solubility of enamel, was demonstrated as enamel structure surface damage using SEM. Figure 3 shows decreased enamel surface damage in the acidic environment of chitosan-treated specimens and Table 1 shows the scoring results by blinded reviewers. The enamel surface treated with chitosan in the acidic environment was more protected from damage than the untreated enamel surfaces in the negative control group (Fig. 2), thus illustrating the magnitude of the demineralization process that occurred in the enamel. Our results indicated that chitosan application had a protective effect on enamel that could inhibit the demineralization process caused by 10% phosphoric acid.

In this study, the chitosan solution was made by dissolving chitosan powder in 1% acetic acid for 24 hours according to the method of Arnaud et al. [8]. This caused an acidic pH (3.321) of the chitosan solution, so that despite having a protective effect on enamel, this chitosan solution unfortunately also had an enamel demineralizing ability, even though it was not as much as the 10% phosphoric acid solution (pH 0.751). The effect of chitosan solution on the enamel surface is shown in Figure 3, where the discontinuity appeared on the enamel surface compared to the baseline specimen (Fig. 1), in which the surface was intact. The enamel surface damage treated with chitosan and Aquadem was not as severe as that in the chitosan plus phosphoric acid specimens.

Our results were consistent with those of Ruan et al. [17], who proved that the enamel surface treated with chitosan-amelogenin hydrogel before and after demineralization with the pH cycling method showed less surface damage than untreated enamel. They concluded that chitosan-amelogenin hydrogel showed potential as a preventive and restorative agent in teeth enamel.

In addition to Ruan’s research, there are several other studies also support our results. Arnaud et al. [8] proved that enamel treated with chitosan and then immersed in a demineralization solution showed higher surface hardness and lower phosphorus loss rates than untreated enamels. Schlueter et al. [11] also proved that chitosan added to toothpaste increased the antierosion effect. Visveswaraiah et al. [6] revealed that water-soluble chitosan can be an effective material to protect teeth and prevent demineralization due to caries, acidic foods, and teeth whitening procedures.

The decrease in the solubility of enamel treated with chitosan in an acidic environment can be explained by the increased pH of the demineralization solution caused by chitosan. Another possibility is that chitosan in a positively charged acidic situation will coat the negatively charged enamel surface. The NH2 of the chitosan in an acidic situation will bind to H+ to form NH3+, making chitosan positively charged and so it can come into contact with a negatively charged enamel surface [6,8].

Our results showed that chitosan was able to prevent enamel surface damage caused by demineralization due to 10% phosphoric acid in vitro. However, the application form of chitosan should be considered, because if the solvent was acetic acid, the chitosan actually would demineralize
the previously intact enamel. Therefore, we suggest using water-soluble chitosan with neutral pH for further in vivo laboratory research. Water-soluble chitosan is not marketed currently in Indonesia so future investigators will need to import or produce water-soluble chitosan by modifying chemical structures, reducing deacetylation degrees, reducing molecular weights, or degrading chitosan polymer chains [18–23].

5. Conclusion
It can be concluded that chitosan had the ability to inhibit the process of demineralization in vitro, increase the pH of an in vitro acidic environment, and prevent the enamel surface damage in an acidic environment.

6. References
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