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The Effect of Mouthwash Containing Chitosan/Zinc Oxide Nanoparticles on Surface Hardness and Color Change of Bleached Enamel

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ABSTRACT

Introduction: This study compared the effects of mouthwashes containing chitosan/zinc oxide nanoparticles, 0.2% chlorhexidine, 1.5% hydrogen peroxide (Colgate), and distilled water on the color and microhardness of bleached enamel.

Materials and Methods: Sixty bovine incisors were randomly divided into four groups (n = 15). After polishing and tea staining for six days, baseline color and microhardness were recorded using the CIELAB system and Vickers test. The enamel surfaces were bleached three times with 40% hydrogen peroxide. After ten days, measurements were repeated. The bleached samples were then immersed in 5 ml of each mouthwash twice daily for 14 days. The synthetic mouthwash contained 1% chitosan and 500 µg/ml zinc oxide nanoparticles. Color and microhardness were re-evaluated, and data were analyzed using the Kruskal–Wallis and post-hoc tests ($\alpha = 0.05$).

Results: Significant color differences were observed between the mouthwash groups and water ($p < 0.05$), with no significant difference between chlorhexidine and the nanoparticle mouthwash. Both the nanoparticle and Colgate mouthwashes similarly increased enamel microhardness, while chlorhexidine caused a slight decrease.

Conclusion: Mouthwash containing chitosan and zinc oxide nanoparticles improved bleached enamel microhardness without affecting color stability compared to 0.2% chlorhexidine.

Keywords: Chitosan; Mouthwashes; Nanoparticles; Tooth bleaching; Zinc oxide; Enamel; Remineralization.

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Introduction

Cosmetic dentistry has quickly become a major component of current restorative dental practice and tooth whitening is among the most sought-after aesthetic services available [1,2]. Tooth bleaching has changed the way that people think about their smiles, as bleaching adds a cosmetic modifier to current restorative treatment options, rather than an invasive procedure (assessment of teeth that are discolored; i.e. both vital and non-vital teeth) [3-5]. Rather than obtaining correction with non-cosmetic treatment, like a patient getting a veneer or crown, where the patient has to undergo considerable tooth preparation, the bleaching procedure gives an innovative approach to obtaining peri-oral aesthetic benefits that is non-invasive, relative ease of use, and high patient satisfaction [2,4,6,7]. Bleaching products, including gels, mouthwashes, and strips are both readily available and accessible to consumers, due to their innovative delivery systems, high satisfaction, and cost. Although others exist, such as Colgate Optic White and Whitens and Brightens (it is in mouth rinse format; [8]], the most common products for bleaching are based on hydrogen peroxide (HP). The office bleaching with high concentrations of hydrogen peroxide (up to 40% concentration) offers the advantages of immediate results, control over application, and less chance of irritation to soft tissues [8]. Nonetheless, the increased popularity of bleaching has raised serious concerns regarding what the secondary side effects may be, particularly on the enamel surface properties. The bleaching process includes the diffusion of peroxide agents (e.g., hydrogen peroxide or carbamide peroxide) through the tooth where these react with the tooth structure to produce reactive oxygen species (ROS). This ROS then breaks down organic chromophores and converts larger light-absorbing molecules into smaller, less pigmented molecules [4,6].

Bleaching can be an effective procedure in dentistry, although there can be some temporary enamel surface changes such as decreased microhardness and increased surface roughness. Although the enamel may return to a natural state over time via remineralization in saliva or remineralizing toothpaste, this process could be slow and uncomfortable [4,6]. Several different agents for remineralization have been studied for enamel remineralization due to bleaching, including sodium fluoride (NaF) and casein phosphopeptide-amorphous calcium phosphate (CPP/ACP) [4,9]. CPP/ACP is a milk-based protein complex that can aid in enamel remineralization and prevention of white spot lesions, but whether or not it affects the microhardness of bleached enamel was unclear [6,8]. Fluoride has been heavily studied in unethical ways to allow the formation of calcium fluoride and fluorapatite on the surface of enamel for remineralization and returning microhardness [8]. Recent advances in nanotechnology have given rise to new methods for post-bleaching enamel maintenance [10,11]. Zinc oxide (ZnO) nanoparticles is one potential agent that has recently gained much scientific interest, largely due to its antimicrobial and remineralization potential. ZnO nanoparticles can boost enamel remineralization and further have more impact on surface roughness than traditional agents when added into oral care products [12-14]. Chitosan, after being derived from chitin, as a natural-based polysaccharide, gained a lot of interest in preventive and conservative dentistry due to its unique properties. Chitosan is commonly used in preventive dentistry as an antimicrobial agent in mouthwashes and toothpaste to prevent biofilm accumulation and lessen cariogenic bacteria such as Streptococcus mutans [15-17]. Chitosan aids in remineralization of both enamel and dentin, stimulating deposition of calcium and phosphate, and has been combined with nanoparticles, including hydroxyapatite or zinc oxide [18].

In conservative dentistry, the use of chitosan enhances the performance of bonding agents in that they increase the bonding strength and antibacterial activity of dental adhesives at the interface of the tooth and restoration. It has also been studied as a pulp-capping agent or liner because it promotes the activity of odontoblasts and pulp tissue repair due to its biocompatibility and bioactive properties [15,18]. Chitosan-based solutions can also be used in endodontic irrigation because of their effective disinfection of root canals without damaging the periapical tissues. Other applications include chitosan nanoparticles acting as nanocarriers of targeted drugs, such as antimicrobials or remineralizing ions and bioactive restorative materials with self-healing and antibacterial properties [19-21].

Various studies have studied the use and application of chitosan and ZnO nanoparticles in dentistry, specifically to remineralize enamel and protect its surface [10, 17]. However, a few more critical limitations present in the current literature require further exploration. Although the microhardness improvements were studied by Nagasaki et al. (2023) and Elshehawy et al. (2020), they failed to evaluate color stability - another important aesthetic consideration in bleaching treatment - at the same time. Though promising in vitro results have been obtained from various combinations of chitosan with ZnO [19,20], the currently unknown concentration ratios for the mouthwashes represent a markedly significant translational gap. Not only that, though, but all evidence as it stands comes from pure in vitro studies [6,12] and calls for future validation in clinical trials. Recognizing the shortcomings of studies conducted before this research, we plan to assess in detail the influence of a novel mouthwash, containing chitosan/zinc oxide nanoparticles, on the surface hardness and color stability of bleached enamel. We hope to fill the gaps that resulted from the prior research and provide a more effective, more patient-friendly method for the care of post-bleaching enamel in dental and aesthetic terms.

Materials and Methods

Preparation of 50 ml mouthwash containing 1% nano chitosan and 500 mcg/ml nano zinc oxide: 500 mg of nano chitosan powder (Iranian nano materials pioneers, Iran) and 25 mg of nano zinc oxide powder (Iranian nano materials pioneers, Iran) were added separately in 20 ml of deionized water and left for one hour. The stirrer was kept at 30°C. Then they were placed in a sonicator bath at a temperature of 40 for 10 minutes. After dissolution, they were added together and 50 mg of sodium saccharin dissolved in 5 ml of deionized water was added to their total. Finally, pH was adjusted to 5.5 with the help of NHCL. After adjusting pH, the solution was brought to a volume of 50 cc and stored in the refrigerator. The final composition of mouthwash is shown in Table 1.

Preparation of samples

Sixty (60) extracted cow centrals were stored in distilled water at room temperature. To create an enamel surface, first, a trimmer separated the crown of each tooth from the root and each sample was mounted in self-curing acrylic resin in such a way that the surface of tooth was parallel to the horizon. To create a smoother enamel surface, 800 to 1200-grit sandpaper was used along with water flow (Figure 1-A).Then, they were randomly divided into 4 groups of 15.

- Group 1: to Colloidal solution containing chitosan/zinc oxide nanoparticles.
- Group 2: Colgate mouthwash containing 1.5% hydrogen peroxide (Colgate-Palmolive, USA).
- Group 3: 0.2% chlorhexidine mouthwash (CHX) (Vi-One, Iran) (positive control).
- Group 4: Distilled water (Sabalan Co., Iran) (negative control).

Staining and initial examination

For staining, the teeth were immersed in tea bags for 6 days and kept in an incubator (Fine TechSSl-202, China) (Figure 1-B). After confirming the coloration, colorimetry was performed on the parts that had no surface stain. Primary colorimetry and initial microhardness tests were performed on the teeth. A spectrophotometer (UV-BT-770/770 PC, Canada) was used to evaluate the color of the buccal enamel surface (T1, initial examination) based on the CIELAB L *a* and b color system. Vickers microhardness measurement was performed by a microhardness-measuring device (Koopa, Iran) under a force of 0.49 newtons (50 grams) with a time of 15 seconds from the surface of the samples. Each sample will undergo the hardness test three times, with a force effect distance of 5 mm in each step, in order to improve accuracy. The average outcome of the three tests will be provided.

Bleaching and secondary examination

Immediately, the bleaching process was carried out by 40% hydrogen peroxide (Opalescence boost, USA) based on the manufacturer's recommendation. This process was done three times and each time for 15 minutes, on the enamel surfaces. To eliminate the bleaching dehydration effect, the samples were placed in distilled water in a 37°C incubator for 10 days, and then re-evaluation of colorimetry and hardness (T2, second examination) was performed on them.

Immersion in mouthwashes and the third examination

In the next step, the bleached samples were exposed to mouthwashes. Hence, each enamel sample was immersed in 5 ml of mouthwash for 2 minutes twice a day for 14 days and placed in a shaker (Behsan, Iran) at room temperature under 100 rpm. After each immersion, the samples were rinsed with distilled water for 10 seconds and retained in it until the next cycle. Upon completion of the cycles, color and hardness measurements (T3, third inspection) were conducted once again.

Statistical methods and sample size

The description of data was done using appropriate statistical tables and graphs, and analysis of variance with repeated measures or its non-parametric equivalent was used to analyze the data. In all tests, a significance level of 5% was considered.

To determine the sample size, Puangpanboot et al.'s study was used [22]. Based on it, the mean and standard deviation of percentage changes in control and chitosan groups were 21.85±8.31 and 34.94±15.13, respectively. Considering alpha equal to 0.05 and beta equal to 0.2, the sample size in this study is equal to:

$$n=\frac{(z_{1-\frac{a}{2}}-z_{1-\beta})^2(S_2^{\,2}+S_2^2)}{(\bar{x}_1-\bar{x}_2)^2}=\frac{(1.96+0.84)^2(8.31^2+15.13^2)}{(21.85-34.94)^2}=15$$

Based on this formula, the sample size in each group was calculated to be 15.

Ethics committee authorization code (Blinded for Review)

This in-vitro study used extracted bovine incisors obtained from a certified slaughterhouse. No human participants or live animals were involved. Therefore, ethical approval was not required. Further ethical details have been removed and blinded for the double-blind peer-review process.

Results

This study evaluated the effects of a mouthwash containing chitosan/zinc oxide nanoparticles on surface microhardness (H) and color changes in bleached enamel, comparing it with positive controls (1.1% hydrogen peroxide [Colgate] and 0.2% chlorhexidine) and negative control (water). Descriptive statistics for color change parameters (ΔE, L, a, b*) across examination intervals (1⟶3 and 2⟶3) for all four groups are presented in Table 2. Key findings include:

- The greatest reduction in a* values (red-green axis) at both 1⟶3 (a_{13}) and 2⟶3 (a_{23}) intervals was observed in the Colgate group, while water showed the smallest decrease.

- For L* values (lightness), Colgate showed the most significant increase during both follow-up periods (L_{13} and L_{23}).

- Changes in b* values (yellow-blue axis) at 1⟶3 (b13) were nearly identical among Colgate, chitosan, and water groups, with chlorhexidine showing minimal reduction.

- At 2⟶3 (b_{23}), chitosan and Colgate groups exhibited increased b* values, whereas chlorhexidine and water showed decreases. The magnitude of change was comparable between chlorhexidine and Colgate, both being less pronounced than water and chitosan treatments. The Shapiro-Wilk test was employed to assess data normality. Results indicated non-normal distribution of ΔE values across all three measurement comparisons (baseline vs. second, baseline vs. third, and second vs. third measurements; $p = 0.00$ for all). Consequently, non-parametric tests were utilized for subsequent data analysis.

- **Color Change Analysis (ΔE)**

Kruskal-Wallis tests showed statistically significant differences in ΔE values among the four treatment groups for both second-to-third (ΔE_{23}, $p = 0.00$) and first-to-third (ΔE_{13}, $p = 0.03$) measurement intervals. However, no significant difference was observed between first and second measurements (ΔE_{12}, $p = 0.36$). In comparison to all other groups, Colgate mouthwash exhibited significantly distinct ΔE changes ($p < 0.05$) in post hoc pairwise comparisons. It is important to note that no significant differences were observed between the experimental chitosan/zinc oxide nanoparticle mouthwash and 0.2% chlorhexidine at any time interval ($p > 0.05$).

- **CIELAB Color Parameters (L, a, b*)**

Analysis of individual CIELAB components using the Kruskal-Wallis test revealed significant differences among three mouthwashes and water controls in bleached enamel for second-to-third measurements of all three parameters (a_{23}, b_{23}, and L_{23}; $p = 0.00$ for each). Significant variations were observed between first and third measurements for a* (a_{13}, $p = 0.01$) and b* (b_{13}, $p = 0.00$) values, via L* values (L_{13}) showed no significant change ($p = 0.07$). Notably, the chitosan/zinc oxide nanoparticle mouthwash and Colgate treatment increased L* (lightness) and b* (yellowness) values while reducing a* (redness), whereas chlorhexidine decreased all three colorimetric parameters.

- **Microhardness Assessment**

Kruskal-Wallis test demonstrated statistically significant differences in microhardness changes among the four treatment groups across all examination intervals (HV_{12}: $p = 0.03$; HV_{23}: $p = 0.01$; HV_{13}: $p= 0.045$). Both chitosan/zinc oxide nanoparticle-containing mouthwash and Colgate mouthwash produced approximately equivalent increases in bleached enamel microhardness, while the chlorhexidine mouthwash resulted in a slight but measurable decrease in surface hardness.

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Table 1. The final composition of nano-chitosan and zinc oxide mouthwash ingredients.

composition	content
Nano zinc oxide (ZnO 500 mcg/ml) Molecular weight: 81.37 Particle size: 10-30 nm Purity: 99.8% Color: white Crystal Phase: single crystal Morphology: nearly spherical SSA: 20-60 m2/g True Density: 5.606 g/cm3	25 mg
Sodium saccharin –(Na-saccharin 0.1%)	50 mg
Nanochitosan 1% Purity : ≥99% APS: 50 nm Molecular formula: C6H11NO4 Molecular weight: 161 g/mol Form: Powder Specific Gravity: 1.4 Color: White	500 mg
HCL	to the amount required to adjust Ph
distilled water	up to 50 cc

Table 2. Descriptive table of changes in variables E, L, a and b in the time intervals between initial Staining, Immersion in mouthwashes (1 and 3), and Bleaching, Immersion in mouthwashes (2 and 3): Mean (standard deviation).

Time periods	1,3				2,3			
Groups \ Variables	ΔE	L	a	b	ΔE	L	a	b
Water	3.85 (0.98)	10.02 (1.62)	-6.75 (0.99)	-2.94 (2.60)	12.64 (3.66)	1.01 (1.33)	-0.98 (0.52)	-3.30 (1.01)
ChX	3.05 (0.88)	9.84 (1.36)	-7.49 (0.93)	-0.81 (1.82)	12.61 (2.74)	-0.97 (1.56)	-1.44 (0.40)	-1.84 (1.16)
Colgate	5.57 (2.22)	14.29 (2.20)	-9.30 (1.64)	-3.00 (2.04)	17.60 (6.30)	3.94 (2.72)	-2.52 (0.87)	1.90 (1.64)
chitosan	3.47 (1.76)	9.11 (1.23)	-8.12 (0.87)	-2.90 (1.89)	12.83 (3.13)	1.32 (1.52)	-1.03 (1.62)	2.55 (1.01)

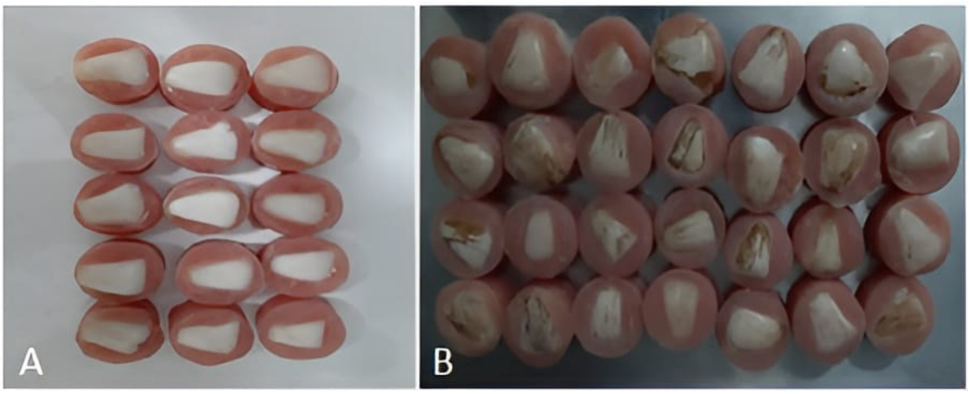

Figure 1. Preparation and staining of specimens; (A) Prepared specimens before staining. (B) Teeth immediately after staining in tea.

Discussion

This research looked at how well a mouthwash made with chitosan and zinc oxide nanoparticles may strengthen bleached enamel and keep its color from fading. There was a 7- to 10-day pause after bleaching to get rid of any confusing effects that may have transpired due to temporary whitening artifacts caused by dehydration. The chitosan/ZnO combo worked better than chlorhexidine and mars, and it rendered the color much more stable (ΔE, $p < 0.05$). It worked just as well as Colgate's hydrogen peroxide. Chitosan may stick to metal ions that speed up oxidation, including iron, and ZnO nanoparticles may bend light, which inhibits pigment from re-oxidizing [14,17].

Chlorhexidine, on the other hand, had greater ΔE values, which makes sense since it is known to alter the color of surfaces and take minerals off of them [6]. Microhardness tests showed that both Colgate and chitosan/ZnO mouthwashes caused a lot of enamel to remineralize ($p < 0.05$). This is because ZnO promotes the growth of hydroxyapatite crystals [13] and chitosan adheres minerals to cells [18]. On the other hand, chlorhexidine didn't seem to make things any softer. This might be because it generated acidic byproducts. The results show that chitosan/ZnO nanoparticles might be utilized as a medication after bleaching to fix both cosmetic and structural problems at the same time.

Color Stability (ΔE)

The ΔE values showed that the Colgate, chlorhexidine, and water mouthwash, as well as the chitosan/zinc oxide mouthwash, changed color a lot. The ΔE values for Colgate mouthwash and chitosan/zinc oxide mouthwash were the lowest. This means that the color didn't alter much after they were bleached. This shows that the color of both types of mouthwash remained the same after they were bleached. The values from the second and third measures (ΔE23 and ΔE13) are very different from the readings from the first and third measures ($p < 0.05$). In other words, the mouthwash with chitosan and zinc oxide works. These comparisons likewise didn't support the null hypothesis, which means that the changes that were noticed were clinically significant. This was consistent with more recent studies by Mousa et al., (2023) and Elminofy et al., (2024), who stated that chlorhexidine generally causes surface staining, which also has limited ability for color stability. The lack of significant differences between the chitosan/zinc oxide mouthwash and chlorhexidine in some intervals ($p > 0.05$) suggests that the former can achieve comparable or superior results without the adverse effects associated with chlorhexidine.

CIELAB L *a* and b color system

The Colgate mouthwash and the chitosan/zinc oxide mouthwash both made the L* (lightness) and b* (yellowness) values much higher while making the a* (redness) values lower. In addition, other studies have shown that the remineralization of enamel by zinc oxide nanoparticles with chitosan would seem to promote whitening by a combination of their optical and bioactive properties (Nagasaki et al., 2023; Qu et al., 2023). The significant differences in L, a, and b* values between the second and third measurements (L23, a23, b23) ($p < 0.05$) further highlighted the effectiveness of the chitosan/zinc oxide mouthwash in maintaining color stability. Chlorhexidine, on the other hand, lowered all three color parameters: L*, a*, and b*. Some investigations have shown that this could be because it tends to leave marks on surfaces (Kutuk et al., 2018; Memarpour et al., 2019). The findings are similar to those of Mousa et al., (2023), which showed that chitosan-based products kept their color better than regular antibacterial agents.

Microhardness

The microhardness analysis revealed that the chitosan/zinc oxide mouthwash and Colgate mouthwash significantly increased enamel microhardness after bleaching ($p < 0.05$). Chitosan and zinc oxide nanoparticles may assist calcium and phosphate ions attach to the enamel surface [13,17,20], which is a beneficial thing. The fact that the null hypothesis was not accepted when looking at changes in microhardness (HV12, HV23, and HV13) showed that the chitosan/zinc oxide mouthwash functioned effectively. Chlorhexidine, on the other hand, led to a slight reduction in microhardness, likely in terms of its acidic nature and potential to demineralize enamel. These findings align with previous research indicating that chlorhexidine may compromise enamel integrity over time [8,9].

In contrast, the chitosan/zinc oxide mouthwash not only prevented demineralization but also enhanced enamel microhardness, making it a superior choice for post-bleaching care. The study's results were in line with other breakthroughs in dental care that used nanotechnology. Magalhães et al., (2022) observed that nanoparticles and self-assembly peptides made the enamel tougher and rougher when they were mixed. Unlike earlier studies, our study evaluates both microhardness and color stability, providing a more ho-

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listic knowledge of the effects of chitosan/zinc oxide mouthwash. The results showed that the chitosan/zinc oxide nanoparticle mouthwash performed just as well as regular treatments after bleaching. It helps a lot of people who want both cosmetic and functional results that last, since it may improve both microhardness and color stability. It's a better choice than chlorhexidine since it doesn't harm the enamel or leave stains. Even though the outcomes were quite favorable, the study properly limited itself as short-term and focused. The in vitro model, however acceptable scientifically, may not offer full soundness to clinical oral environments. Furthermore, the evaluation period was set up more to evaluate immediate post-bleaching effects than the long-term consequences. Such methodological choices allowed definite determination of the primary endpoints (microhardness and color stability) and further merit the need for clinical trials with longer observation periods in the future.

Conclusion

In conclusion, our study reveals that mouthwashes with chitosan/zinc oxide nanoparticles may make enamel stronger and longer-lasting in color after bleaching than mouthwashes without these nanoparticles, such as chlorhexidine. Chitosan and zinc oxide nanoparticles work together to form a mouthwash that is great for your teeth when you bleach them. It helps in two ways: it looks good and it works. In the future, researchers may do clinical trials to see whether these results can be confirmed and to uncover additional ways this material might be utilized in preventive and restorative dentistry.

Conflict of Interest

There is no conflict of interest to declare.

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