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COMPARATIVE EFFECTS OF CHITOSAN, CHLORHEXIDINE, AND CHITOSAN-CHLORHEXIDINE MOUTHWASHES ON THE ENAMEL COLOR OF NATURAL TEETH

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Abstract

Introduction: Chitosan mouthwash has higher antibacterial effects with lower cytotoxicity than many other mouthwashes. For the first time, the present study compared the enamel discoloration of natural teeth by using chitosan, chlorhexidine, and chitosan-chlorhexidine mouthwashes.

Method: In this laboratory study, 45 premolar teeth extracted for orthodontic reasons without structural defects, cracks, restorations and observable caries were randomly divided into three groups (Chitosan, Chlorhexidine, and Chitosan-Chlorhexidine) of 15 each. First, prophylaxis pastes and plastic rubbers were used to clean the surfaces of the teeth; then, a spectrophotometer was used to measure the initial color of the buccal surfaces of the teeth. Then the first-, second-, and third-group of teeth were immersed in the 0.5% chitosan solution, 0.2% chlorhexidine solution, and the combined 0.5% chitosan and 0.2% chlorhexidine solution, respectively, for 24 h. Then the color of buccal surfaces of the teeth were again measured by the spectrophotometer, and discoloration (ΔE) was calculated. Statistical analysis was performed by SPSS.26 software, ANOVA analysis of variance and Bonferroni post hoc tests.

Results: In the chitosan group, discoloration (3.14±1.86) was significantly higher than those in the chitosan-chlorhexidine group (1.74±0.88) and the chlorhexidine group (1.78±0.58); however, the latter two groups experienced similar discoloration.

Conclusion: Chitosan-chlorhexidine mouthwash revealed dental discoloration effects similar to those of chlorhexidine.

Keywords: chitosan mouthwash, chlorhexidine, enamel discoloration

Introduction

Dental caries and plaque-related gingival diseases are among the most prevalent diseases in human societies and effects of microbial plaques on causing such diseases are widely known [1, 2]. The main method to remove plaque is mechanical methods; however, using chemical methods such as mouthwashes as complementary methods can help improving the control of plaques in the patients [3, 4]. A mouthwash is an effective mechanism in controlling plaques chemically. Due to problems and limitations caused by the mechanical control of dental plaques, researchers are nowadays paying more attention to using chemical mouthwashes and their effects on dental health [5]. Chlorhexidine (CHX) is considered a golden standard to chemically control bacterial plaques [6]. This mouthwash is used for treating gingivitis, periodontitis, mild pericoronitis, denture stomatitis, the symptomatic treatment of canker sores (aphthous ulcer), reducing secondary infection and pain following acute necrotic periodontitis and herpetic ulcers, reducing bacteremia after oral surgeries, treating oral fungal lesions including leukoplakia, preventing the recurrence of gingival hypertrophy in patients taking phenytoin, preventing gingival hyperplasia in pregnancy, preparing surgeons' hands and patients' skin, rinsing during the periodontal abscess drainage, cleaning root canals, preventing mucositis during radiation therapy, and helping people who cannot brush their teeth [7].

Chlorhexidine has cationic molecules and has large-scale anti-bacterial properties and low toxicity. Based on the dosage, this mouthwash can be bactericidal or bacteriostatic [8]. The anti-bacterial properties of this medication come from its positive charge and strong affinity to bind to and react with the cellular walls of bacteria and their degradation, causing the loss of the wall uniformity and the increased permeability of bacteria, and finally their lyses [9]. Chlorhexidine also has long-term complications, including the brownish discoloration of the teeth, some restorative materials and the back surface of the tongue, the changing of taste and flavor senses, oral mucosa erosion, the sense of burning, the swelling of one or both parotid glands, and the increased accumulation of calculus [10,11].

Today, chlorhexidine has received much attention due to its strong anti-plaque and anti-bacterial effects. However, its complications have limited this mouthwash as a permanent mechanism of controlling plaques; for this, it is highly required to find an appropriate alternative to this mouthwash with similar anti-plaque properties with no or less complications [12, 13].

Chitin is a biopolymer composed of N-Acetyl-D-glucosamine units. This biopolymer is the second most abundant biopolymer in nature after cellulose [14, 15]. Various sources of chitin include marine invertebrates, shells of crustaceans such as shrimp and crab, algae, Mollusca, some kinds of fungal walls and insect cuticles [16, 17]. The low solubility of chitin in water and many organic solvents caused by large and strong hydrogen bonds and the high levels of acetyl groups in their structures is one of the most important factors limiting the consumption of this biopolymer; however, despite these limitations, chitin and its derivatives are widely applied [18, 20].

Chitosan is a completely or partially deacetylated derivative of chitin that can have different deacetylation degrees (DD) [21, 22]. Because of the lower number of acetyl groups and the presence of free amine groups along the polymer chain of chitosan, and due to the C-2 amine functional group present in its structural units which can have reaction with the hydrogen protons of acids, the resulting cations reduce the hydrogen bonds between chitosan molecules, and cause substance solubility in such weak acids as acetic acid, hydrochloric acid, and formic acid [23]. Because of this property, chitosan has a special status. Chitin and the resulting chitosan have great biocompatibility, desirable biodegradability, low toxicity, anti-bacterial, and anti-allergic properties [24-29]. These substances are widely used in tissue engineering, commercial applications in medicine, cosmetics and health care products, such as optic lenses, toothpastes, chewing gum, mouthwashes, as restorative materials, and nowadays they are using for producing Nano-fibers [14, 30-36]. Chitin and its derivatives are cheap and accessible in nature. The previous studies have indicated that chitosan is characterized by various anti-microbial properties, including bactericidal and bacteriostatic effects, and the prevention of bio-film formation [37-39].

The different molecular weights of chitosan reveal different anti-bacterial and solubility properties [40]. Chitosan of a low molecular weight has more solubility and can easily penetrate bacterial cells, while chitosan of a high molecular weight can bind to the negatively-charged cellular wall of bacteria and serve as a chelating agent [41, 42].

Sano et al.'s study (2011) investigated the effects of four different compounds of chitosan in reducing bacteria attachment to tooth surfaces, concluding that chitosan of low molecular weights had the highest effects. According to the findings, the 0.5% chitosan solution of a low molecular weight had reduced bacteria attachment to the tooth surface within 3 hours as much as 50 ppm chlorhexidine [43].

These special characteristics have made this substance be widely used in different dentistry branches and in various forms such as mouthwashes [44, 45]. Over the past years, mouthwashes containing chitosan have been considered as an option for replacing chlorhexidine, and are widely examined in different studies. Meanwhile, the effects of chitosan mouthwashes in reducing plaques and microorganisms have been compared with the effects of chlorhexidine and other mouthwashes [35].

Studies by Baskaran et al. (2021) and also Nair et al. (2017) demonstrated chitosan mouthwashes as possible alternatives to chlorhexidine as anti-bacterial mouthwashes [9, 47].

Studies by Vilasan et al. (2020), Mhaske et al. (2018) and Decker et al. (2005) indicated that mouthwashes containing a combination of chlorhexidine and chitosan could produce more anti-plaque properties than those containing each of these substances alone [48-50].

Concerning mouthwash safety, Costa et al. (2014) concluded that chitosan mouthwashes were safe, effective and a natural alternative to many available mouthwashes. Moreover, they demonstrated that this kind of mouthwash did not produce Geno toxicity, and its cytotoxicity was also lower than that of other mouthwashes in the market [51].

The anti-microbial properties of the chitosan-containing mouthwashes have been examined in different studies. However, the complications of these mouthwashes such as dental discoloration have not been investigated. On the other hand, the anti-microbial properties of chlorhexidine mouthwashes and the resulting discolorations are clear to all. In This study for investigating whether or not chitosan-containing mouthwash could be an alternative to Chlorhexidine due to its anti-microbial effects and less dental discoloration, dental discoloration following the use of Chitosan, Chlorhexidine and Chitosan-Chlorhexidine is compared.

Materials and procedures:

This study was an experimental study. As many as 45 premolar teeth extracted for orthodontic reasons were kept in distilled water at room temperature until experiments started. The teeth had no structural defects, cracks, restorations, observable caries, and were randomly placed in three groups (Chitosan, Chlorhexidine, and Chitosan-Chlorhexidine) of 15 each.

Twenty-four hours before the experiment, prophylaxis pastes and plastic rubbers were used to clean the surfaces of the teeth, as running water was used to rinse them all. Then the teeth were placed in distilled water at room temperature again. prepared chitosan powder (the chitosan powder of a low molecular weight (M.W>100 kDa), high deacetylation degree (DD>90%) made by the Alborz Rayan Nano Equipment Factory, Karaj, Iran) was dissolved in 1% acetic acid to yield 0.5% chitosan.

Chlorhexidine mouthwash (0.2% chlorhexidine mouthwash made by the Rozhin Cosmetics factory, Tabriz, Iran) was also prepared. Then a combined 0.5% chitosan and 0.2% chlorhexidine solution of similar amounts of both mouthwashes was prepared.

A spectrophotometer device (Handy Dental SpectroShade Device (Type: 71.3000 Verona, Italy)) was used to measure the initial color of the buccal surfaces of tooth enamels by applying the color model of CIELAB (Commission Internationale de l'Eclairage L*a* and b*) under natural room light and temperature. The device was regularly calibrated to evaluate the teeth colors, and L_1, a_1, and b_1 values for all the teeth of the three groups were obtained. After the initial color was measured, the first-, second-, and third-group teeth were respectively immersed in the 0.5% chitosan solution, 0.2% chlorhexidine mouthwash, and the combined 0.5% chitosan and 0.2% chlorhexidine solution for 24 hours. After this time, the teeth were rinsed with running water for a minute and dried with cotton rolls. Within this time interval, the tooth colors were again measured by the spectrophotometer under the natural light and at room temperature, and L_2, a_2, and b_2 values were recorded for all the teeth in the three groups. The device calibration was regularly performed during the examination. In the end, the teeth discoloration (ΔE) was calculated using the following formula for all the teeth in the three groups separately [52].

$\Delta E = [(\Delta L*2) + (\Delta a*2) + (\Delta b*2)]^{½}$

The resulting data were reported based on descriptive indices of mean, standard deviation, and minimum and maximum values. To investigate data normality, the Shapiro-Wilk Test was used. Because the normality test was met, one-way analysis of variance was used to compare the discoloration of the teeth of each group under the effects of the mouthwashes used. Given the significance of ANOVA test, Bonferroni post-hoc test was used to pairwise compare the groups. The significance level in one-way analysis of variance was P-value<0.05, while in the post hoc test using Bonferroni adjustment it was considered P-value $<\frac{0.05}{3} \simeq 0.02$. Also, statistical analyses were carried out by SPSS026 software.

Results

After determining the quantified discoloration (ΔE) of all 45 samples, the obtained data for all three groups were separately reported based on descriptive indices of mean, standard deviation, and minimum and maximum values. To investigate data normality, the Shapiro-Wilk Test was used. Because the normality test was met, one-way analysis of variance was used to compare the discoloration of the teeth of each group under the effects of the mouthwashes used. The significance level in one-way analysis of variance was P-value<0.05.

Table 1 gives statistical results and descriptive statistics of natural teeth discoloration in different groups of mouthwashes.

According to the data, there was a significant difference in the mean discoloration of the different groups (P-value=0.004). In fact, using all the mouthwashes of chitosan, chitosan-chlorhexidine, and chlorhexidine caused the natural teeth to discolor, as the discoloration in all three groups was significant. Because ANOVA Test was significant, Bonferroni post-hoc test was used to pairwise compare the groups. The significance level of the post-hoc test was found to be P-value $<\frac{0.05}{3} \simeq 0.02$, using the Bonferroni adjustment.

Table 2gives Bonferroni test results for the pairwise comparison of the three groups under study.

Table 1: Natural teeth discoloration in different groups

	Mean	**S.D**	**95% Confidence Interval for Mean**		**Minimum**	**Maximum**	***P-value**
			Lower Bound	**Upper Bound**			
Chitosan	3.14	1.86	2.11	4.17	0.36	6.90	0.004
Chitosan-Chlorhexidine	1.74	0.88	1.25	2.22	0.59	3.09	
Chlorhexidine	1.78	0.58	1.46	2.11	0.82	2.68	

S.D: Standard Deviation, *One-Way ANOVA

Table 2: Bonferroni test results for the pairwise comparison of the groups under study

Group (I-J)	**Mean Difference (I-J)**	**Std. Error**	**P-value**	**95% Confidence Interval**	
				Lower Bound	**Upper Bound**
Chitosan & Chitosan-Chlorhexidine	1.41	0.45	0.010	0.28	2.53
Chitosan & Chlorhexidine	1.36	0.45	0.013	0.24	2.49

Chitosan-Chlorhexidine & Chlorhexidine	-0.04	0.45	0.999	-1.17	1.08

S.E: Standard Error

According to Table 2 data, discoloration in the chitosan-group teeth (3.14±1.86) was significantly different from that of the chitosan-chlorhexidine group (1.74±0.88) (P-value=0.010).

Also, discoloration in the chitosan-group teeth (3.14±1.86) was significantly different from that of the chlorhexidine group (1.78±0.58) (P-value=0.013).

However, discoloration in the chitosan-chlorhexidine group teeth (1.74±0.88) was not significantly different from that of the chlorhexidine group (1.78±0.58) (P-value=0.999).

In sum, the highest discoloration occurred in the chitosan mouthwash-group teeth (3.14±1.86), which had a significant difference from that of chitosan-chlorhexidine (1.74±0.88) and chlorhexidine (1.78±0.58) mouthwash solutions; however, no significant difference of discoloration was noted between the chitosan-chlorhexidine and chlorhexidine teeth groups.

The following diagram 1 illustrates the mean discoloration of each of the studied groups.

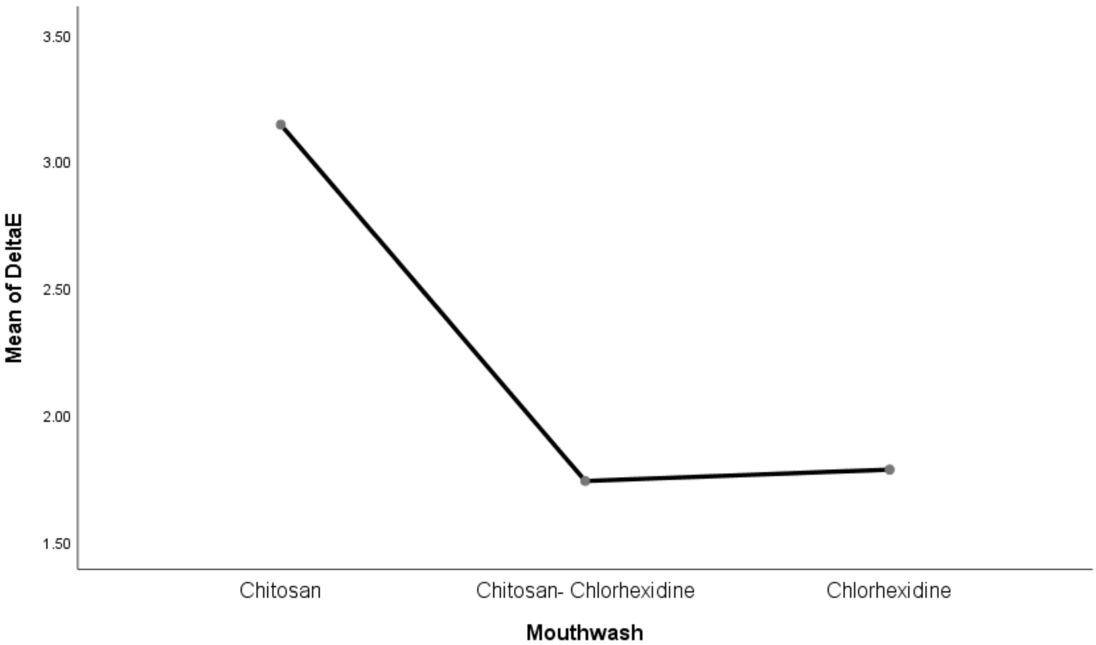

Diagram 1: Mean discoloration in the chitosan, chitosan-chlorhexidine and chlorhexidine solutions

As illustrated by the diagram, the mean discoloration shows a significant difference between chitosan and chitosan-chlorhexidine, and also between chitosan and chlorhexidine teeth groups.

Discussion

Currently, mechanical cleaning is the most important and widely-used method to control upper gingival plaques, and is very much effective in regions where access to plaque deposits is possible [53]. The tools that are useful to mechanically control the upper gingival plaque include brushes (electrical or manual), dental floss, toothpick, and interdental brushes. Motivations and skills required to use these products may be beyond patients' ability to observe hygienic principles [54]. In a group of people, like the disabled people, observing mouth and tooth care is a major problem; thus, finding an alternative method to control the plaque is critical and substances to chemically control the plaque should become available [55]. Today, mouthwashes serve as auxiliary tools and play a major role alongside the mechanical methods of plaque removal in controlling upper gingival plaques and gingivitis [13]. Chlorhexidine is an anti-plaqu

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e and anti-bacterial mouthwash that fights bacteria, candida, and some viral types such as HIV and hepatitis [11, 56-58]. This mouthwash prevents the formation and accumulation of bacterial plaques and the progress of periodontal diseases [56, 59-61]. Based on these properties, this mouthwash is one of the most common anti-microbial mouthwashes which is widely used in dentistry [62]. Apart from its advantages, chlorhexidine also has various complications such as the discoloration of tooth enamels, tongue and restorations, oral mucosa erosion, changes with the sense of taste, and accumulation of calculus, which making use of this mouthwash difficult.

The results of the present study also demonstrated dental discoloration caused by consuming chlorhexidine.

Here, three mechanisms have been proposed for dental discoloration caused by chlorohexidine [68,69].

1. The most common dental discoloration mechanism by chlorhexidine is caused by the interference between di-cationic chlorhexidine and chromogenic substances available in food stuffs [10, 62, and 62]. In other words, a major part of discoloration is caused by the deposition of chromogenic anions of food regimens on absorbed chlorhexidine cations. [66,67].
2. Another mechanism is the formation of pigmented sulfides caused by the reaction with iron and tin; chlorhexidine denatures proteins and resulted sulfide groups bond to metals which can result in discoloration [68].
3. The third mechanism pertains to non-enzymatic reactions that cause discoloration into brown (Maillard Reactions).

The severity of teeth discoloration, following using chlorhexidine, depends on the concentration of mouthwashes and its amount and duration of use [57,69].

For most of the patients who look for dental treatments, the appearance of dental system is a major cause of worry. Because most dental discoloration occurs in the frontal region of the mouth, it is natural for patients to express concerns about the complications caused by taking chlorhexidine.

Due to the complications of chlorhexidine, it cannot be used in the long-term, and cannot serve as an appropriate mouthwash for the children under 6. In addition, the alcohol in this substance is a cause of concern [70,11]. As a result, the application of a mouthwash with anti-plaque and anti-bacterial effects similar to those of chlorhexidine, albeit with lower complications, has received the attention of dentists [71]. The chitosan-containing mouthwashes have also the same goal.

In 2021, Yawilat et al. investigated the effects of chitosan mouthwash of different molecular weights against streptococcus mutans, along with chlorhexidine. The findings indicated that chitosan mouthwash of low and high molecular weights with similar deacetylation degrees enjoyed similar anti-bacterial and anti-biofilm properties against S. mutans. According to this study, chlorhexidine mouthwash, compared to chitosan mouthwash, had managed to significantly reduce the planktonic S. mutans; however, chitosan mouthwash had reduced the formation of mature biofilm more than chlorhexidine mouthwash and prevented the formation of new biofilms. Therefore, chitosan mouthwash may be used as an alternative to prevent dental caries, as it has low toxicity, and more compatibility with the environment, compared to chlorhexidine [46].

Baskaran et al. (2021) did a triple-blind randomized clinical trial to compare the effects of 2% chitosan mouthwash and 0.2% chlorhexidine mouthwash on reducing oral plaque among under-20 adolescents. Following plaque index examinations, the results suggested that both mouthwashes had significantly reduced oral plaques, though no significant difference was noted between the results of the two types of mouthwashes. This investigation proposed chitosan mouthwash as an appropriate alternative for people who suffer from chlorhexidine mouthwash complications [47].

Aparna et al. (2019) studied the effects of chitosan mouthwash on the formation of plaque biofilm and its antibacterial activities on the amount of the streptococcus and lactobacillus bacteria in the saliva. The findings revealed that according to the plaque index, the antibacterial effects of the mouthwash were significant even in a short period (14 days), as a significant reduction of the bacteria present in saliva indicated the anti-bacterial activity of this mouthwash. This study proposed chitosan, and especially its solvent in water type, to be an appropriate substance in new formulations of oral products because of its anti-plaque and anti-bacterial features [72].

The anti-microbial function of the chitosan and chlorhexidine mouthwash combination has also been examined in different studies.

In a systematic review study, Pandiyan et al. (2022) investigated the efficacy of chitosan and chlorhexidine mouthwashes on dental plaque and gingival inflammation. Randomized clinical trials were studied to compare the effects of chitosan and chlorhexidine mouthwashes on the accumulation of dental plaque and gingival inflammation. The studied variables were the plaque index, the gingival index, the gingival bleeding index, and the colony-forming unit (CFU/ml). In the end, three studies were investigated; their findings concluded that although using chitosan and chlorhexidine mouthwashes had separately caused a significant reduction in plaque accumulation, gingival inflammation, and colony-forming units, but all three studies had reported a combination of both substances was more effective [73].

Vilasan et al. (2020) compared the anti-plaque effects of the mouthwash containing chlorhexidine and chitosan with chlorhexidine and chitosan mouthwashes alone. The findings revealed that the mouthwashes containing both substances had more antiplaque properties than the ones containing each of them alone [49].

Mhaske et al. (2018) compared the microbiological and clinical effects of the mouthwash containing chlorhexidine and chitosan with chlorhexidine and chitosan mouthwashes alone. This study indicated that the mouthwash containing both substances had more anti-plaque effects [51].

Because studies in this area have not yet examined the complications of mouthwashes containing chitosan, this present study aimed to investigate and compare the discoloration of the natural tooth enamels under the effects of chitosan, chlorhexidine, and chitosan-chlorhexidine mouthwashes.

The findings of the present study showed that discoloration in the chitosan group (3.14±1.86) was significantly higher than that in the chlorhexidine-chitosan group (1.74±0.88) and in the chlorhexidine group (1.78±0.58); though the two groups of chitosan-chlorhexidine and chlorhexidine revealed similar discoloration.

Chitosan-caused dental discoloration can be attributed to its bio-adhesive properties. Chitosan is a bio-adhesive substance that adheres to living tissues, and easily attaches to negatively-charged surfaces; for this, it has great anti-microbial and anti-fungal properties [74, 75].One of the proposed mechanisms for the anti-bacterial properties of chitosan is that the interaction between positively-charged chitosan and the negatively-charged bacterial cell changes the permeability of bacterial cells, and as a consequence causes the leakage of intracellular content and the cell death [74,76]. The bio-adhesive properties of substances are likely to cause discoloration, and this can be a reason for chitosan mouthwash discoloring.

Although the present study used chitosan of a low molecular weight, chitosan molecules have a higher molecular weight compared to other substances [34], which along with its high viscosity can contribute to its deposition on the dental surfaces and its discoloration.

It should be borne in mind that chitosan performs well in acidic environments, because amine functional groups present in the chitosan molecular chains absorb hydrogen protons in the acidic environments and form positively charged polyelectrolytes which increase the substance’s bonding to negatively-charged surfaces, such as dental enamel, soft tissues, and cellular membranes [77,78]. In This study dissolving the chitosan powder in 1% acetic acid created an acidic environment, which could be one of the reasons for the dental discoloration.

Another possible reason for enamel discoloration caused by using chitosan mouthwash could be its remineralization property [79]. Studies have indicated that dental discoloration can cause by remineralizing compounds. Chitosan is also a compound with a remineralization effect on dental enamel [80].

Moreover, due to its polycationic nature, chitosan can, as a coagulant and chelating agent, entrap colloidal particles, organic substances, and heavy metal ions; for this, it is widely used in wastewater treatment. Amine functional groups and hydroxyl groups in chitosan molecular chains also affect the selective chelating of some metals, especially transition metals and rare earth elements [81]. With this feature Weltrowski et al. (1996) used chitosan derivatives in an acidic environment to remove metal ions from wastewater [82]. In 2008, a complete report on using chitosan to remove color from aqueous solutions was published [83]. According to a study in 2016, chitosan mixed with activated carbon and resin could reduce or remove bacteria, iron ions, and chlorine molecules in the urban drinking water [84]. Chitosan can reduce total solids and heavy metals in liquids. Today, even a new generation of chitin and chitosan membranes are available and widely used in dialysis, hemodialysis, osmosis, and reverse osmosis devices, and are used to purify water. Besides affinity with metal ions, chitosan has also affinity with colors due to its cationic features in acidic environments. The reason why chitosan chelates with colors and metals is the ionic exchange and electrostatic absorption [85]. As a result, chitosan and its derivatives can absorb pigments present in food, which is why it is used in food industries. Chitin and chitosan are widely used in compounds separation using chromatography [86]. The presence of free amine functional groups and also hydroxyl groups in chitosan makes it a useful substance for separation procedures and as an absorbent. Recently, chitosan and its derivatives also serve as appropriate candidates to prepare scaffolds in tissue engineering, due to their cationic and absorbent nature [87]. Chitosan properties of high absorption and affinity can contribute to the discoloration of dental enamel.

Chitosan is characterized by the formation of film, mucosal adhesiveness, polyelectrolyte behavior, the formation of salts, adhesion and chemical reactivity, all of which, along with the above-mentioned features, could be involved in teeth discoloration.

Moreover, the present study noted that chitosan mouthwash could alone cause more discoloration than combined mouthwashes. This suggests that combining chitosan with chlorhexidine can reduce the said properties and cause less discoloration.

According to this study combination of chitosan and Chlorhexidine mouthwash had Less discoloration compared to the chitosan mouthwash, and similar discoloration to chlorhexidine. This property as well as the higher anti-bacterial effects in comparison with chitosan mouthwash and Chlorhexidine mouthwash separately have all suggested acceptable clinical performance of this combination. [48, 50, 73].

Conclusion

This study found that chitosan mouthwash had caused more discoloration compared to chitosan-chlorhexidine and chlorhexidine mouthwashes. Thus, in its current form, chitosan mouthwash can cause dental discoloration and compromise the beauty of patients' teeth, if used for the long term. Although chitosan mouthwashes are becoming more common as anti-microbial solutions these days, they reveal more dental discoloration even compared to chlorhexidine mouthwash and for this, the available form of this mouthwash in dental field, should be used with care.

On the other hand, as suggested by the study, dental discoloration caused by chitosan-chlorhexidine mouthwash was similar to chlorhexidine mouthwash. Recent studies indicated that chlorhexidine-chitosan mouthwash had more anti-bacterial and higher anti-plaque effects compared to chlorhexidine mouthwash [46, 48, 70]. Consistent with the findings, we could be hopeful that chitosan-chlorhexidine mouthwash could be used as an alternative with more anti-plaque and anti-bacterial properties, while producing less or similar complications of dental discoloration as compared to chlorhexidine. More researches are needed to investigate dental discoloration following the consumption of this mouthwash, and also its other complications should be addressed so that it can be used as an alternative to chlorhexidine.

Finding other mouthwashes with acceptable anti-plaque and antimicrobial effects and the least complications should be the focus of future researches in this field.

Suggestions

This study simply used teeth extracted for orthodontic reasons, and did not specify the age and gender conditions of patients. The study was an in vitro study, which is possible to fail to reconstruct conditions of patients' mouths.

To eliminate these limitations and make more comprehensive investigations, the followings are proposed:

- Dental discoloration under the effects of using chitosan-chlorhexidine and chitosan mouthwashes is suggested to be examined in longer follow-ups.
- Studies to compare the effects of chlorhexidine and chitosan mouthwashes of various concentrations on dental discoloration are suggested.
- different types of chitosan from molecular-weights and deacetylation aspects should be studied.

- Other possible complications of chitosan-chlorhexidine mouthwashes such as the increased calculus formation on teeth need to be studied.
- The accurate cause of dental discoloration following the use of chitosan mouthwashes should be investigated.
- it would be better if in vivo studies study the discoloration caused by chitosan-chlorhexidine mouthwash so that more clinical condition simulations in comparison with in vitro conditions could help investigating the role of the mouth environment and the presence of saliva and possible interferences between mouthwashes and saliva in dental discoloration.
- The effects and complications of chitosan and chitosan-chlorhexidine mouthwashes in edentulous patients should be investigated, such as discoloration of dentures after using these mouthwashes.

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