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ORIGINAL ARTICLE

Efficacy of chitosan-based chewing gum on reducing salivary *S. mutans* counts and salivary pH: a randomised clinical trial

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ABSTRACT

Objective: To determine chitosan-based chewing gum role on reducing salivary *S. mutans* counts and salivary pH.

Materials and Methods: The present double-blind randomised clinical trial with the trial registration number of IRCT20190724044319N1 was conducted on 36 dental students. The volunteers were, randomly, divided into two groups ($n = 18$) including: G1: intervention group (chitosan chewing gum) and G2: control group (placebo chewing gum). Each participant was given eight pieces of the chewing gum, and was asked to chew each gum piece for 5 min and this was repeated for eight times. Their Saliva was collected before and after chewing gums and the number of *S. mutans* colonies and salivary pH were determined. Data were analysed using SPSS (ver.21) and independent student *t* test. *p* Value less than .05 was set as significant.

Results: There was significant difference between two groups for the number of salivary *S. mutans* colonies ($3.31*10^5$ in the intervention group compared to $13.94*10^5$ in the Control group) ($p < .001$). The salivary pH evaluation showed that salivary pH mean value in intervention group was not significant in compared with control group ($p = .17$). However, the chitosan chewing gum led to an increase in salivary pH by 0.17, which was statistically significant ($p = .01$).

Conclusion: Results of this study showed that chitosan chewing gum has a positive effect on the reduction of numbers of salivary *S. mutans* colonies but had no considerable effect on the increase of salivary pH.

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Introduction

Dental caries is an infectious disease that begins with the acid produced by the metabolism of bacteria present in the dental plaque [1]. Tooth decay is believed to be an infectious disease of microbial origin that is caused by several types of bacteria in the mouth. One of the most common bacteria involved in dental caries is *Streptococcus mutans* (*S. mutans*), which is capable of producing a large amount of acid and is highly tolerant under acidic conditions.

There are various methods for plaque control and caries prevention including mechanical and chemical methods such as using toothbrush and toothpaste, dental floss, interdental toothbrushes and mouthwashes [2,3]. Until now, the most accepted plaque control mechanism is the mechanical harvesting of bacterial plaque biofilm, which is performed to remove plaque and microorganisms, but is not sufficient alone, and the best and most successful plaque control program is the simultaneous application of mechanical and it is chemical. Chewing gums are one of the most common delivery systems for plaque components [4,5].

Studies have shown that chewing gum is effective in reducing caries and plaque control [6,7]. There are, also, many claims about the properties of chewing gum to clear food debris and plaque from tooth surfaces, stimulate salivary flow, increase salivary and plaque pH, reduce gingivitis and periodontitis. Generally, these factors depend on the ingredients used in the gum [8]. Numerous clinical studies have been conducted on the rinsing property of different chewing gums, indicating a relationship between the effects and composition of gum [9]. Stimulation of the chewing gum system after each meal increases salivary gland activity and the saliva resulting from this stimulation introduces more ions, including acid neutralising bicarbonate, calcium and phosphorus [10]. Incorporation of various elements to the gum plays an important role in preventive dentistry [11].

Chitosan is a chitin-derived polysaccharide and is a natural polymer found in the outer skeleton of arthropods and crustaceans and insect skin. Chitosan has a mucus binding and antibacterial activity. The positive charge of chitosan facilitates its attachment to the bacterial cell wall and consequently results in the bacteriostatic and bacteriocidal property of the material [12]. It has been reported that chitosan has an inhibitory effect on S. *mutans*. Previous studies showed that chitosan interferes with *S. mutans* binding and

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primary biofilm formation. In addition, chitosan led to a significant decrease in the survival rate of mature biofilms [13,14].

Nowadays, there is a growing interest in the use of natural products in the prevention of diseases. In this regard, chitosan has also been widely used as a natural substance due to its non-toxicity and biocompatibility in the pharmaceutical and food industries. It is clear that chewing gum can play more effective role than mouthwash and toothpaste due to the gradual release of substances and longer contact with the mucosa [15,16]. Therefore, the present study was designed to determine the antibacterial effect of Iranian chewing gum containing chitosan and its effect on salivary pH. As a result, if it has an effect on reducing the number of *S. mutans* bacteria, it can be widely used as a gum to help prevent dental caries.

Material & methods

Study design

The present study is a double-blind, parallel design, randomised controlled trial which is performed by Department of Operative Dentistry at the Dental school of Hamadan university of medical sciences, Hamadan, Iran. The protocol of the study was approved by the Ethics Committee of Hamadan University of Medical Sciences (No. IR.UMSHA.REC.1398.353). Detailed informed consent form was obtained from all the participants. The trial registration number in Iranian Registry of Clinical Trials (IRCT) is IRCT20190724044319N1.

The sample size was calculated based on this formula

$$n = \frac{\left(Z_{\square-\frac{\alpha}{\gamma}} + Z_{\square-\beta}\right)^{\gamma}\left(\delta_{\square}^{\gamma} + \delta_{\gamma}^{\gamma}\right)}{\left(\mu_{\square} - \mu_{\gamma}\right)^{\gamma}}$$

and the mean numbers of S. *mutans* in chitosan and control group was considered based on the previous studies, which were 23.3 ± 15.9 and 37.1 ± 19.6, respectively [17]. The required precision of the estimate (d) was set at 10%, power was set as 80% and the Confidence Interval was 95%.

In this study, 36 dental students were selected in the Hamadan University of medical sciences with the age range 18–30 years (average age 22.74 ± 1.69). The volunteers with good to moderate oral hygiene status with at least 24 healthy teeth (6 teeth per quadrant) who were willing to participate in the study were evaluated. Smokers, subjects using mouthwashes, people who have had antibiotics for the previous 30 days, participants with no current periodontitis (no sites of probing pocket depth $\geq$5 mm or attachment loss of $\geq$2 mm, apart from gingival recession, oral mucosal allergy to the toothpaste, systemic diseases, or with dentures in their mouths were excluded.

The method of sampling was convenience non-probability method. The volunteers randomly were assigned to intervention and control groups using randomised permuted block design to receive either 1: 1 chitosan or placebo chewing gum and we considered block size of 4. In this study, the researcher and the participations were no aware of the content of the package.

Chewing gum formulation

Chitosan, flavouring agents, sweeteners, and co-adjuvant excipient amounts were added to the gum-base as reported in Table 1. Both chitosan and placebo chewing gums were sugar-free gums. In order to produce placebo chewing gum, as mentioned above, the materials listed in Table 1 were mixed, and then to make chitosan chewing gum, chitosan was added, in addition to the ingredients listed for placebo chewing gum.

Gum base, flavouring agents, sweeteners were mixed in a laboratory mixer suitable for mixing small batches of powders or dry granules. Preferably high intensity and not cariogenic sweeteners were selected. At this point, chewing gums, with their weighed amounts of different mixtures, were produced (Figure 1) by progressively filling the die of a single-punch tableting machine and were then compressed at room temperature.

Cytotoxicity measurement of the gums

The Vero cell line was cultured in RPMI (Rosewell park memorial Institute) with 10% FBS serum (Fetal Bovine Serum). For cell toxicity evaluation, 10,000 cells were placed in each well of 96 cells, and in the next day, 5% and 10% of each solutions were added to each well (each concentration as a group). Vero cells were also considered as the control group. After 72 h, the MTT solution was added to each well and the plate placed in a 37 °C incubator for 4 h. To obtain the survival rates, the absorption was read by the ELISA device at a wavelength of 570 nm. To investigate the effect

Table 1. Composition (%) of 2.5 g of chewing gum.

Ingredients	Chitosan chewing Gum	Placebo chewing gum
Gum-base	30	35
Sorbitol	33.35	38.35
Maltitol	8	8
Mannitol	8	8
Xylitol	8	8
Liquid flavouring agent	1.4	1.4
Powdered flavouring agent	1	1
Chitosan	10	0
Acesulfame K	0.25	0.25

Figure 1. Experimental chewing gums produced, weighing 2.5 g and composed as described in Table 1.

of cellular toxicity of samples, their hydro-alcoholic extract was prepared using an ultrasonic bath.

Study procedure

Design and objectives of the study were explained to the participants and written informed consent was obtained. Participations were asked to brush their teeth for one hour before collecting saliva, and then did not eat, drink or chew gum.

Before using the chewing gums, 3 mL non –stimulating saliva was collected from each participant at 9 am, in order to count the number of *S. mutans* colonies and measure its pH in a sterile container. The saliva sample was stored in an ice chamber and then sent to the Microbiology Laboratory within one hour to prevent the growth of other microorganisms.

Then the first group received placebo chewing gum and the second group received 10% chitosan-containing chewing gum ($n = 18$). In total, each participations was given eight pieces of 2.5 grams gum, and were asked to chew each gum for 5 min and then rest for 5 min, and repeat this process for eight times. In general, each person chewed gum for 40 min and rested for 40 min between chewing's times [17].

The patient was not allowed to consume water and food during chewing gum as well as during rest between chewing gums and only had to use gums. Immediately after consuming eight pieces of chewing gum, the volunteers' saliva was again collected and sent to the Microbiology Laboratory to count the number of *S. mutans* colonies.

Microbial analysis

For each saliva sample Serial 10-fold dilutions was made using sterile normal saline from each sample and then diluted saliva was transferred into plates containing 20 mL of Mitis-salivarius bacitracin agar (MSBA) [15]. The plates were incubated in a CO_2 incubator at 37° C for 48 h. Finally, the number of *S. mutans* colonies was counted and for each participant, the inverse of the volume of diluted saliva transferred to the plate by the sampler and the inverse of the prepared dilution coefficient were multiplied and the number of colonies per millilitre of saliva was determined (CFU/ml).

Salivary pH evaluation

Salivary pH was measured using a calibrated digital pH metre. The electrode was placed in the sample and the pH was recorded to two decimal places [18,19].

Statistical analysis

Data were analysed by using SPSS (version 23) (SPSS Inc., Illinois, USA). Salivary pH and salivary *S. mutans* were compared between the two groups before and after the intervention by independent student t test. Paired t-test was used to compare the salivary pH and salivary *S. mutans* within groups before and after the intervention. p Value less than .05 was considered as significant.

Results

Cytotoxicity evaluation

The Mann-Whitney test was used to examine cellular toxicity and to compare the percentage of cell survival in each time interval, in each group compared to the control group. According to the results obtained at concentrations of 5% and 10% in 72 h, there is no significant difference compared to the control group ($p > .05$).

Salivary pH evaluation

A total of 36 participants (18 in the intervention group and 18 in the control group) were evaluated. The age range of the subjects was 18–30 years.

There was a significant difference between the two groups in terms of gender, with 12 (66.67%) of participations in the chitosan group were female, while in the control group 6 (33.33%) were female ($p = .046$).

Table 2, compares the salivary pH values between two groups before and after the intervention. Figure 2 presents the amount of increase in salivary pH value in the two groups after the intervention. As shown, there was a significant difference between the two groups in terms of salivary pH (6.47 ± 0.44 in the chitosan group compared to 6.94 ± 0.51 in control group, $p = .005$). Also the difference between two groups after the intervention was significant (6.64 ± 0.41 in the chitosan group compared to 7.0 ± 0.45 in the control group, $p = .017$). There was no significant difference between the two groups in terms of salivary pH changes (salivary pH after - salivary pH before) ($p = .17$). This, indicating that after controlling the baseline values, there was no significant difference in salivary pH change between groups. However in the chitosan group the salivary pH amount was significantly increased after intervention ($p = .01$).

The number of salivary mutans colonies evaluation

Table 3 and Figure 3 present comparison of the number of salivary *S. mutans* colonies in the two groups before the intervention. As observed, there was no significant difference

Table 2. Comparison of salivary pH in the two study groups before and after intervention.

	Before intervention Mean (SD)	After intervention Mean (SD)	Change score Mean (SD)	p Value*
Chitosan group	6.47 (0.44)	6.64 (0.44)	0.17 (0.24)	.01
Control group	6.94 (0.51)	7.00 (0.45)	0.05 (0.24)	.33
p Value**	.005	.017	.17	

*Paired t test, **independent student t test.

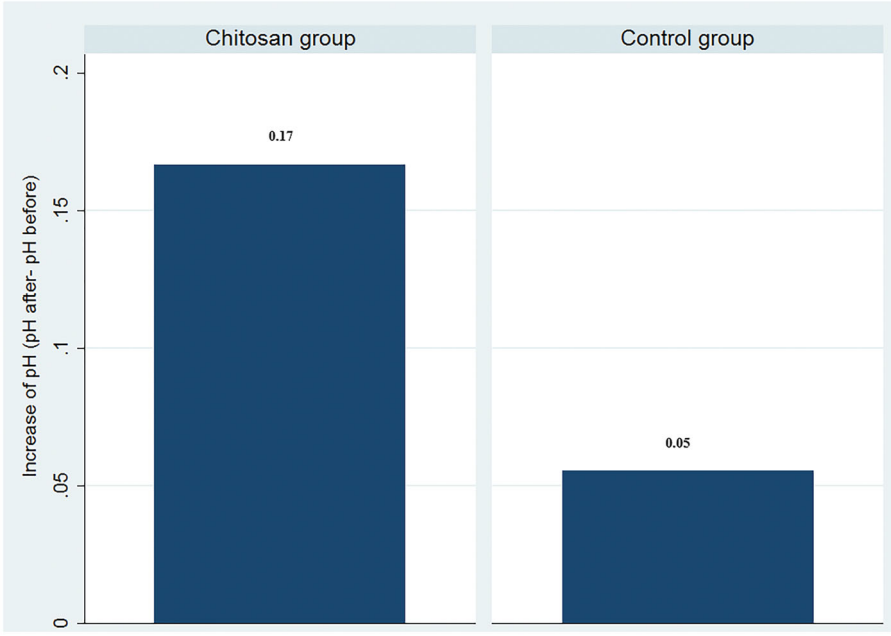

Figure 2. Amount of increase in salivary pH value in the two groups after the intervention.

Table 3. Comparison of salivary *S. mutans* colonies in the two groups before and after the intervention.

	Before intervention Mean (SD)	After intervention Mean (SD)	Change score Mean (SD)	*p* Value*
Intervention group	$15.93*10^5$ $(6.91*10^5)$	$3.31*10^5$ $(4.04*10^5)$	$-12.62*10^5$ $(3.97*10^5)$	<.001
Control group	$14.42*10^5$ $(6.29*10^5)$	$13.94*10^5$ $(6.25*10^5)$	$-0.47*10^5$ $(0.27*10^5)$	<.001
p Value**	.5	<.001	<.001	

*Paired *t* test, ** independent student *t* test.

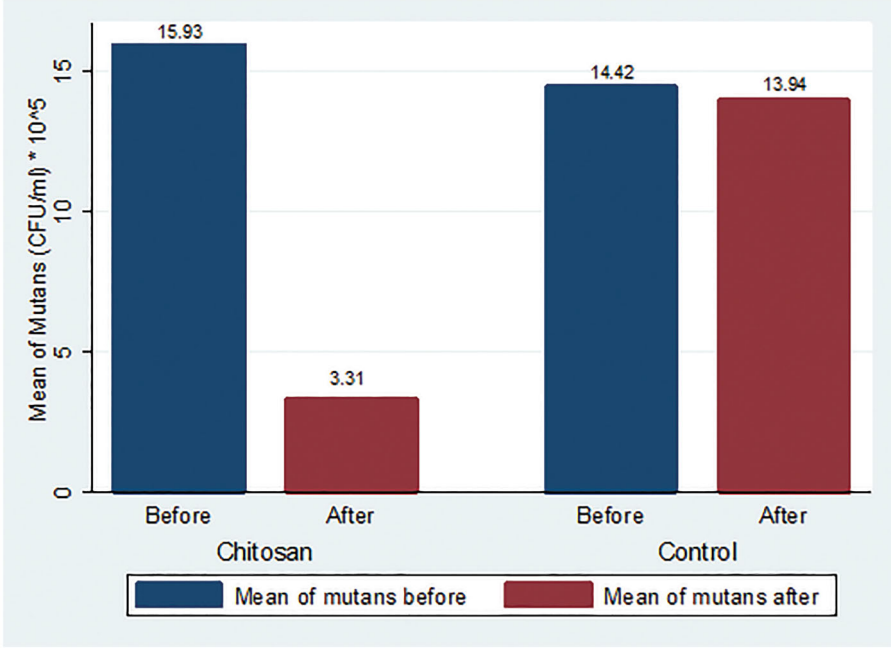

Figure 3. Salivary *S. mutans* colonies in the two groups before and after the intervention.

between the two groups in terms of salivary *S. mutans* colonies ($15.93*10^5$ in the intervention group compared with $14.42*10^5$ in the control group, $p = .5$). But after intervention, the difference between the two groups was statistically significant ($3.31*10^5$) in the intervention group compared to $13.94*10^5$ in the control group, $p < .001$). The results also showed that in the intervention group the number of salivary mutans colonies decreased by $12.61*10^5$ which this difference was statistically significant ($p < .001$). Whereas in the control group the number of salivary mutans colonies decreased by $0.47*10^5$ which was also statistically significant ($p < .001$).

Discussion

The present study evaluated the effect of chitosan chewing gum on the number of *S. mutans* colonies and salivary pH as a randomised clinical trial. The results of the present study showed that chewing chitosan-containing chewing gum led to a five-fold reduction in the number of *S. mutans* and showed a significant reduction effect on *S. mutans* colonies, also chewing gum, regardless of its composition, is associated with a slight increase in salivary pH. The cytotoxicity of the gum was, also, measured in the laboratory before the clinical trial and after the assurance of its non-toxicity, the gums were administered to the volunteers.

The study of Hayashi et al., evaluated the effect of chitosan as chewing gum on cariogenic bacteria in a clinical study. The results of this study showed that oral chewing gum containing chitosan significantly decreased oral bacteria [17]. Therefore, these results were consistent with our findings. In our study, salivary pH was also measured.

Some previous studies have evaluated the antibacterial effect of chitosan in other products. The results of the study by Achmad et al. indicated that toothpaste containing 2.5% and 5% chitosan resulted in a decrease in the number of *S. mutans* colonies [20]. Costa et al. showed that the chitosan mouthwash reduced oral bacteria including *S. mutans* [14]. In Wassel and Khattab study, it was found that the chitosan nanoparticle varnish had more inhibitory effects on *S. mutans* than the sodium fluoride varnish [21].

Some studies have previously reported various mechanisms for the antimicrobial properties of chitosan [22–24]. One of these mechanism is interaction between polycationic nature of chitosan with anion site in cellular membrane's proteins of microorganism, subsequently, resulting in the leakage of some intracellular components [25,26]. In other words, Chitosan has amino bases and is a typical cationic biomaterial through its free ($-NH_{3+}$) that can easily attach to the cell wall of bacteria, especially gram-positive bacteria (which contain acidic amino acids such as thioic acid and isoglutamate on their cell wall) [27]. This means that the positive charge of chitosan provides a tight attachment to the cell wall of the bacteria, which subsequently forms the openings of the cell wall. This phenomenon results in bacterial cell lysis and releases of cellular components, and ultimately leads to cell death [28]. In general, the antibacterial effects of chitosan depend on different factors. It has been shown that the chitosan is more effective on gram-positive bacteria such as *S. mutans* because it has no outer membrane in its cell wall [29]. Aliasghari et al. reported that chitosan and chitosan nanoparticles, in addition to inhibitory effects on the growth of Streptococcus, prevent their attachment to surfaces. Thus they prevent biofilm formation [30].

A slight decrease was also observed in the number of *S. mutans* colonies in the control group, because the chewing gums have the potential of stimulating of salivary flow, and hence the increase of salivary flow leads to bacterial wash out of the oral environment and a decrease in number of bacteria such as S. *mutans* [31,32]. The use of chewing gum can cause a decrease in the dental caries through mechanical mechanisms such as washing out the bacteria, oral clearance of food particles and neutralisation of dental plaque acids by increasing the salivary flow [33].

Another finding of the present study showed that chitosan-containing chewing gum leads to a slight increase in salivary pH (0.17), and the placebo gum also caused a slight increase in salivary pH (0.05), however this increase was not statistically significant. This increase in salivary pH can be attribute to salivary bicarbonate ion, which is proportional to salivary flow [34]. A study by Vantipalli *et al.* showed that two types of commercial sugar free gum resulted in an increase in salivary pH, which was consistent with the findings of Dawes and Kubieniec, Polland et al. and Markovic et al. But the third commercial chewing gum, which was the sugared chewing gum resulted in a decrease in saliva pH after 30 min of chewing [35]. A study by Ferrazzano et al. showed that chewing gum containing Quercetin increased salivary pH, but the changes were not statistically significant [15]. Saliva has buffering systems, which resist against changes in pH. Bicarbonate is the main buffering component in the saliva that is responsible for neutralising acids. Bicarbonate concentration in the stimulated saliva was reported to be approximately 12 times higher than that of the unstimulated saliva [36,37].

In general, this study was different from previous studies on the use of chitosan in the form of gum and its different methodology. So far, Hayashi et al. study [17] is the only study that has used chitosan as a chewing gum and generally few studies have been performed clinically on the properties and effects of chitosan in various ways on oral bacteria.

One of the limitations of the present study was that the participants in the present study had a good oral hygiene and those with very poor oral hygiene and rampant caries were not included. Also, chewing gums was used for a short-term period (1 day) due to the restriction of people's diet and the lack of possibility of monitoring the appropriate use of chewing gums.

It is recommended to investigate and design a study with the use of chewing gum and its effects on the reduction of the number of *S. mutans* colonies over long term time. In this study, we used a constant concentration of chitosan in chewing gum, and therefore, it is suggested to conduct future studies to investigate the effect of various concentrations of chitosan in the chewing gum and its influences on populations with high dental caries rates. It is also recommended to conduct further studies on the effect of chitosan chewing gum on the biofilm producing ability of the cariogenic bacteria.

Conclusion

This research aimed at determining the role of chitosan containing chewing gum on reducing the salivary *S. mutans* counts and salivary pH. The chitosan chewing gum and placebo chewing gum were produced in laboratory, and then used by 36 participants to investigate their effects on the reduction of salivary *S. mutans* counts and salivary pH change. Given the limitations of this study, the results

showed that the use of chitosan containing chewing gum significantly reduced the number of *S. mutans* colonies in saliva but had no considerable effect on the increase of salivary pH. It should be noted that the placebo chewing gum also reduced the number of *S. mutans* colonies, however, this effect was much less than that of the chitosan chewing gum. According to the results, no effect was observed on the increase of salivary pH. It can be concluded that chitosan containing chewing gum can be used as effective and efficient measure for the prevention of dental caries.

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Author contributions

Z. Khamverdi was the supervisor for planning and contributed to the manuscript revision. F. Farhadian designed the study and collected data, interpreted the results and wrote the manuscript. S. Khazaei was the statistical advisor and contributed to the analysis of results and data and manuscript revision. M. Adabi was the advisor of microbial analysis. All authors gave approval for the final submitted version.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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