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ORIGINAL ARTICLE

Evaluation of antibacterial efficiency of chitosan and chitosan nanoparticles on cariogenic streptococci: an *in vitro* study

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ABSTRACT

Background and Objectives: The most prevalent and worldwide oral disease is dental caries that affects a significant proportion of the world population. There are some classical approaches for control, prevention and treatment of this pathologic condition; however, the results are still not completely successful. Therefore new methods are needed for better management of this important challenge. Chitosan is a natural and non-toxic polysaccharide with many biological applications, particularly as an antimicrobial agent. Chitosan nanoparticle is a bioactive and environment friendly material with unique physicochemical properties. The aim of the present study was to investigate the antimicrobial effect of chitosan and nano-chitosan on the most important cariogenic streptococci.

Materials and Methods: For evaluation of antimicrobial effect of chitosan and nano-chitosan against oral streptococci broth micro-dilution method was carried out for four bacterial species; *Streptococcus mutans, Streptococcus sobrinus, Streptococcus sanguis* and *Streptococcus salivarius*. Also the effect of these materials on adhesion of above bacteria was evaluated. One-way ANOVA and post hoc Tukey test were used for statistical analysis.

Results: The MICs of chitosan for *S. mutans, S. sanguis, S. salivarius* and *S. sobrinus* were 1.25, 1.25, 0.625 and 0.625 mg/mL, respectively. The MIC of chitosan nanoparticle for *S. mutans, S. salivarius* and *S. sobrinus* was 0.625 mg/mL and for *S. sanguis* was 0.312 mg/mL. Chitosan and chitosan nanoparticles at a concentration of 5 mg/mL also reduced biofilm formation of *S. mutans* up to 92.5% and 93.4%, respectively.

Conclusion: The results of this study supported the use of chitosan and chitosan nanoparticles as antimicrobial agents against cariogenic Streptococci.

Keywords: Chitosan, Chitosan nanoparticles, Dental caries, Oral streptococci

INTRODUCTION

According to the World Health Organization reports, dental caries still remains a major health problem, especially among disadvantaged social groups worldwide (1). This disease affects both sexes, all

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races and age groups, and different social and economic classes. Dental caries causes pain and sadness, and it imposes a relatively high financial burden on families (2). There are about 700 different bacterial species in human oral microbiota. Cariogenic bacteria such as *S. mutans* and lactic acid bacterial species play an important role in the pathogenesis of dental caries. Both of them are able to grow in acidic conditions, such as lactic acid which is a characteristic organic product of sugar metabolism (3).

Antimicrobials such as ampicillin, chlorhexidine, quaternary ammonium compounds and metronidazole are used to prevent tooth decay, but they have several side effects such as tooth staining, diarrhea, increased calculus formation and changes in bowel flora (4). Therefore, new methods for prevention and probably better management of this important challenge are needed. In recent years, the use of natural nontoxic alternatives for the control and prevention of dental caries has emerged. Chitosan is a natural polymer with specific characteristics, including biodegradability, non-toxicity and antimicrobial activity, which has attracted great attention for some years (5). Prior studies have described the antimicrobial potential of chitin, chitosan and their derivatives during 1980-1990s (6). Some studies have shown that chitosan has antibacterial and anti-plaque actions and anti-adhesive properties against *S. mutans* and other streptococci (7–9). Chitosan nanoparticles have smaller size than chitosan and this property could make it unique. Nano-chitosan is a natural material with antibacterial effects (10–12) and gene transfer in artificial organs as controlled-release drug carrier; it also improves the strength and washability of textiles (13) and enhances the immunologic and protective efficacy of the DNA vaccine (14). Many studies have reported antibacterial characteristics of nano-chitosan (10, 12) but inadequate data are available on its effects against oral streptococci as the major cause of dental caries.

The aim of this study was to determine the anticariogenic effects of chitosan and nano-chitosan, especially its efficiency against oral streptococci.

MATERIALS AND METHODS

Test organisms. The antibacterial activity of the chitosan and nano chitosan was evaluated against four strains of bacteria which was purchased from Iranian Research Organization for Science and Technology (IROST) including: *Streptococcus mutans* (ATCC 35668), *Streptococcus sobrinus* (ATCC 27607), *Streptococcus sanguis* (PTCC1449) and *Streptococcus salivarius* (PTCC 1448).

All microorganisms were grown in Tryptic Soy Broth (TSB), Blood Agar and Mitis Salivarius Agar (MSA) at 37 °C in a atmosphere containing 5% CO2. Then, growths were confirmed using Gram staining, catalase test, optochin and bacitracin tests. The bacteria were stored at −80 °C until used in the study.

Preparation of chitosan and chitosan nanoparticles solution. Low molecular weight chitosan (>85% deacetylated) was purchased from Sigma- Aldrich (USA). Chitosan solution was prepared by dispersing it in 0.25% acetic acid solution (Merck, Germany).

Nanochitosan was prepared by Laboratory of Tarbiat Modarres University, Noor, Iran (15). Nanochitosan was dissolved in 1% acetic acid and for complete dissolution of its particles kept overnight under magnetic stirring then with distilled water to obtain the desired volume.

Determination of antibacterial activity. Bacterial suspensions with 1×10^8 bacteria were prepared from fresh cultures of bacteria in tryptic soy agar; (TSA; Quelab, Montreal, Canada) using sterile swabs they were cultured on the Muller-Hinton agar medium with 5% defibrinated sheep blood. Then, 6-mm-diameter wells were punched over the agar plates using sterile Pasteur pipettes. The bottoms of the wells were sealed by pouring a drop of molten MHA. Then 50 µL of the chitosan and nano-chitosan solutions were poured into the wells. These plates were kept at a temperature of 4 °C until the materials in the wells were completely diffused into the agar, and the plates were incubated anaerobically at 37 °C for 24 h. The diameter of the inhibition zone was measured (16).

Determination of MIC and MBC. For determination of minimum inhibitory concentration of chitosan and nano-chitosan solutions, micro-dilution broth method was used. Overnight, the streptococci cultures in tryptic soy broth (TSB; Quelab, Montreal, Canada) were adjusted to 10^6 colony-forming units (CFU/mL). Two-fold serial dilutions of chitosan and nano-chitosan solutions were prepared in broth to

give the final concentration of 0.039–5 mg/mL. In sterile 96-well plates, 100 µL of each dilution of chitosan and nano-chitosan solutions were placed into the well containing 100 µL of bacterial suspension. Triplicate samples were performed for each test concentration (17). Wells containing culture medium and bacteria were used as negative control. Turbidity measurements were made for all the wells after 24 h of incubation at 37 °C under anaerobic conditions. The growth of *Streptococci* was measured at 630 nm using a micro-plate reader (AWARENESS, Technology INC, Stat fax 2100).

The MBC (minimum bactericidal concentration) was defined as the lowest concentration of test compounds that did prevent any visible bacterial growth on the tryptic soy agar (TSA) plate after 24 h incubation at 37 °C under anaerobic condition (18).

Determination of anti-adhesion effect of chitosan and nanochitosan solution. To measure the anti-adhesion effect of chitosan and nano-chitosan solutions, the micro-titer plate method used as described previously by Di Giulio et al, with modifications (19). Briefly, overnight growth of bacteria in TSB with 1% sucrose were adjusted with the similar media to reach a concentration of 10^6 CFU/mL. Then 10 µL of different dilutions (serial dilutions) prepared from each dilution of chitosan and nano-chitosan were added to 1 mL of each bacterial suspension. Then 200 µL of them were transferred into each well of 96-well polystyrene plate. Blank wells contained only buffer and control wells contained bacteria without treatment. After 24 h of incubation at 37 °C at 5% CO_2, the wells were washed three times with 200 µL of sterile phosphate-buffered saline (PBS) to remove unattached cells. Then the adherent bacteria were stained for 5 min with 200 µL of 2% crystal violet. The stain was rinsed off by placing the plates under running tap water.

The level of formation of biofilm was evaluated after adding 200 µl of 33% (v/v) glacial acetic acid per well. This was done via measuring the absorbance of the solution at 492 nm by an ELISA reader. The anti-adhesion activity of chitosan and chitosan nanoparticle was determined and compared with the control.

Measurement of reduction percentage was calculated using equation:

$$\text{Percentage of reduction} = \frac{(C-B)-(T-B)}{(C-B)} \times 100$$

B = absorbance of blank, C = absorbance of control and T = absorbance of test (20).

Statistical analysis. The data were statistically analyzed by One way ANOVA and Tukey's post hoc tests on Graf Pad Prism5 software (GraphPad Software Inc., CA, USA). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Measurement of inhibition zone diameter. The means of three measurements of inhibition zone diameter of chitosan and chitosan nanoparticle for *Streptococcus mutans, S. salivarius, S. sobrinus* and *S. sanguis* are summarized in Table 1. The greatest zones of inhibition by the chitosan and chitosan nanoparticle were found at a dose of 5 mg/mL and a decrease in concentration resulted in a decrease in the zone of growth inhibition. The lowest zones of inhibition by the chitosan and chitosan nanoparticle were found at a dose of 1.25 mg/mL ($P<0.05$). The diameter of inhibition zone of chitosan nanoparticles was significantly more than that of chitosan with all the above-mentioned bacterial species.

Determination of MIC and MBC. MIC and

Table 1. The mean of diameters of inhibition zones of chitosan and nanochitosan against four oral streptococci

concentration mg/ml	Zone of inhibition (mm)							
	Streptococcus salivarius		*Streptococcus sanguis*		*Streptococcus sobrinus*		*Streptococcus mutans*	
	chitosan	nanochitosan	chitosan	nanochitosan	chitosan	nanochitosan	chitosan	nanochitosan
5	12.167	14.5	13.5	15.67	12	14	12.5	15.16
2.5	10.167	11.8	10.5	12.25	10	12	10.5	12
1.25	9	10.67	8.5	10.5	8.5	9.5	8.5	10

Table 2. Values of MIC and MBC for the four groups of oral streptocci bacteria.

Bacteria	MBC**(mg/ml)		MIC*(mg/ml)	
	chitosan	nanochitosan	chitosan	nanochitosan
Streptococcus mutans	1.25	.625	2.5	1.25
Streptococcus sobrinus	.625	.625	2.5	2.5
Streptococcus sanguis	1.25	.312	1.25	1.25
Streptococcus salivarius	.625	.625	1.25	2.5

MBC of chitosan and chitosan nanoparticles were determined, which are presented in Table 2. MIC of chitosan for *S. mutans* and *S. sanguis* was 1.25 mg/mL, with 0.625 mg/mL for *S. sobrinus* and *S. salivarius*. MIC of chitosan nanoparticle for *S. mutans, S. sobrinus* and *S. salivarius* was 0.625 mg/mL, with 0.312 mg/mL for *S. sanguis*, which was significantly less than that for the other three bacteria (P<0.05). MIC of nano-chitosan for *S. mutans* and *S. sanguis* was less than that of chitosan (P<0.05), indicating higher antibacterial activity. In relation to *S. sobrinus* and *S. salivarius* there were no significant differences between the MIC of chitosan and the MIC of chitosan nanoparticles (P>0.05). MBC of chitosan nanoparticle only for *S. mutans* was less than that of chitosan (P<0.05).

The inhibitory effects of different concentrations of chitosan and chitosan nanoparticle on the growth of the tested streptococci are shown in Fig. 1, which shows that in 1.25–5 mg/mL concentrations of chitosan, the growth of *S. mutans* and *S. sanguis* decreased >90% and in 0.625–5 mg/mL of chitosan the growth of *S. sobrinus* and *S. salivarius* decreased >90%. In addition, in 0.625–5 mg/mL concentrations of chitosan nanoparticles the growth of *S. mutans, S. sobrinus* and *S. salivarius* and in its 0.312–5 mg/mL concentration, the growth of *S. sanguis* decreased >90%. Decreases in concentrations of chitosan and chitosan nanoparticles resulted in increases in OD, indicating an increase in bacterial growth (P<0.05).

Determination of anti-adhesion effect of chitosan and nanochitosan solutions. The anti-adhesion effect of chitosan and nano-chitosan was evaluated for each tested *Streptococcus* species and the results are presented in Table 3 and Fig 2. The percentage of adherence reduction decreased with a decrease in the concentration of chitosan and chitosan nanoparticles from 5 to 1.25 mg/mL (P<0.05). The maximum effect of chitosan and nano-chitosan was observed on *S. mutans* with 92.5% and 93.4% reductions in adherence, respectively, at a concentration of 5 mg/mL (P<0.05). There were no significant differences in adherence levels between the study groups (P>0.05).

As shown in Fig. 2 , the different dilutions (1.25-5) of chitosan and chitosan nanoparticles significantly reduced biofilm formation compared to the control group (p <0.05).

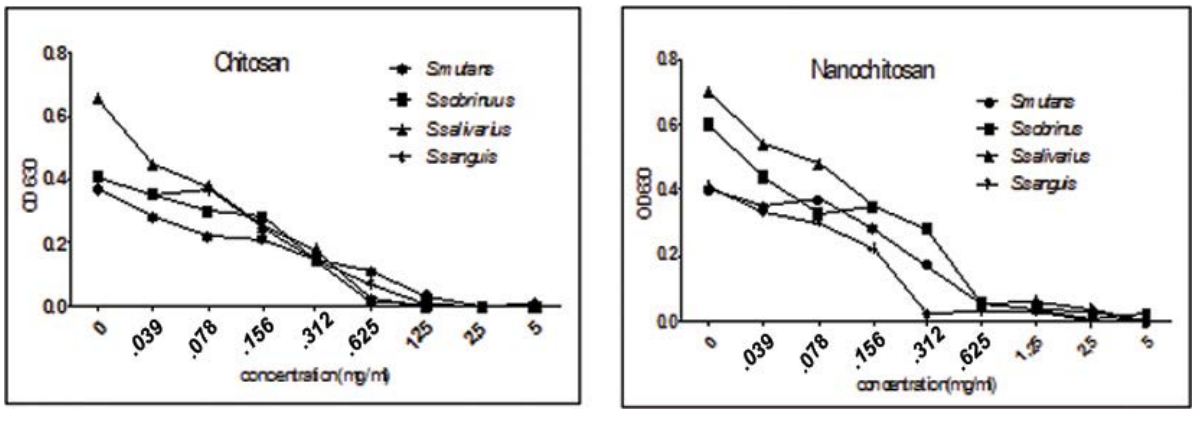

Fig. 1. The effects various concentrations of chitosan and nano chitosan on the growth of four oral *streptococci*

Table 3. Percentage of adherance reduction of various concentrations of chitosan and nanochitosan on oral *streptococci*

various concentrations of material mg/ml	Percentage of adherance reduction							
	Streptococcus salivarius		***Streptococcus sanguis***		***Streptococcus Sobrinus***		***Streptococcus mutans***	
	chitosan	nanochitosan	chitosan	nanochitosan	chitosan	nanochitosan	chitosan	nanochitosan
5	92.5	93.4	72.1	72.6	74.2	78.9	74.3	88.4
2.5	71.2	64.4	54.4	52.3	61.9	64.7	67.9	87.1
1.25	46.7	41.1	49.3	34.5	50.4	59.04	60.2	67.9

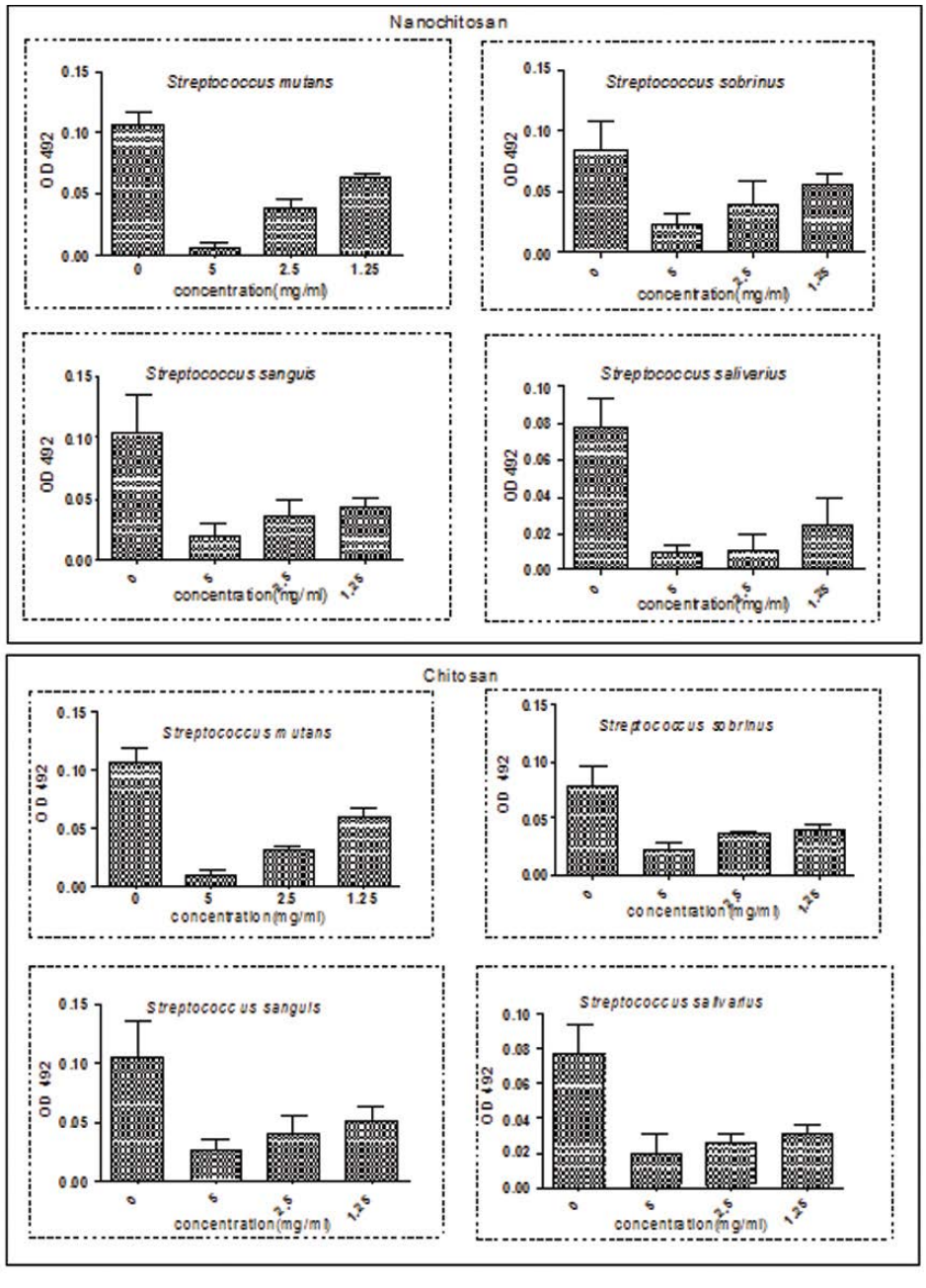

Fig 2. The effects various concentrations of chitosan and nano chitosan on adherence of four studied oral streptococci

DISCUSSION

Chitosan as a natural nontoxic biopolymer produced by partial deacetylation of chitin has numerous applications in food, pharmaceutical and chemical industries (21,16). In dentistry research, chitosan has been used due to its antibacterial characteristics for prevention of dental caries (22). Chitosan at nano-size has superior activities, including antimicrobial effects, drug, gene and/or vaccine delivery systems, and antitumor effect (10, 21, 23).

In this study, antimicrobial activity of chitosan and chitosan nanoparticles on four strains of cariogenic streptococci, including *S. mutans, S. sobrinus, S. sanguis* and *S. salivarius* was evaluated. The results showed that these substances have bacteriostatic or bactericidal and anti-adhesion effects, and they can reduce biofilm/plaque formation *in vitro*. In this study the effects of 1.25 mg/mL concentration of chitosan on growth reduction of >90% of *S. mutans* and *S. sanguis* and its 0.625 mg/mL concentration on growth reduction of >90% of *S. sobrinus* and *S. salivarius* were observed. Costa et al. (24) showed that chitosan mouthwash effectively reduced viable counts of *Streptococcus spp* and *Enterococcus spp* and it was safe with lower cytotoxicity than a commercial mouthwash.

In addition, it has been shown that the 0.626 mg/mL concentration of chitosan nanoparticles is able to decrease *S. mutans, S. sobrinus* and *S. salivarius* growth up to >90% and its 0.312 mg/mL concentration can reduce *S. sanguis* growth up to >90%.

In this study, the decreasing effect of chitosan and chitosan nanoparticles on adhesion of oral *Streptococci* was observed; therefore, chitosan decreased 92.5% of *S. mutans* adhesion and >70% that of *S. sobrinus, S. sanguis* and *S. salivarius.* Costa et al (25) compared mouthwashes containing chitosan with two commercial mouthwashes and found that only the chitosan mouthwash was capable of interfering with adherence, biofilm formation and mature biofilm of *S. mutans, L. acidophilus, E. faecium, C. albicans* and *P. intermedia.* In this study, antibacterial effect of different dilutions of chitosan against oral Streptococci was compared and it was found that with increasing the concentration of chitosan, anti-adhesion effect on tested bacteria increased, and 5 mg/mL concentration was found the most effective. These findings are consistent with the results of previous studies.

Sano et al. (26) reported that chitosan has an anti-plaque effect on *S. sobrinus* with saliva-treated hydroxyapatite and in another study (27) showed that chitosan rinse was effective in reducing plaque formation and *S. mutans* counts in saliva compared to the placebo rinse. Hayashi et al. (28) studied the impact of chewing gum containing chitosan on cariogenic bacteria and reported that oral bacteria in the group consuming chitosan chewing gum significantly decreased compared with the control group, and the count of *S. mutans* decreased 1 h after chewing the gum. Costa et al (29) reported that chitosan is capable of 94% reduction in *S. mutans* mature biofilms. Furthermore, it was shown that chitosan nanoparticle decreased 93.4%, 72.6%, 78.9% and 88.4% of adhesiveness of *S. mutans, S. sobrinus, S. sanguis* and *S. salivarius,* respectively, and a decrease in chitosan nanoparticle concentration resulted in an increase in their adhesion. Paz et al. (30) showed that chitosan nanoparticles prepared from low-MW chitosan induced >95% damage to *S. mutans* biofilms. Furthermore, Neilands et al. (31) reported that chitosan nanoparticles have the ability to hinder acid tolerance response (ATR) of adhered *S. mutans*. Several mechanisms are suggested for antimicrobial properties of chitosan, one of which is interaction between polycationic nature of chitosan with anion site in microbial cell membranes proteins, resulting in the leakage of some intracellular constituents (22, 32).

Comparison of chitosan and chitosan nanoparticles showed that inhibition zone for nano-chitosan was significantly higher than that of chitosan ($P<0.05$). Nano-chitosan has smaller size in comparison to chitosan and exhibits higher affinity for bacterial cells, which is probably responsible for its higher antibacterial activity (10). The MIC of nano-chitosan for *S. mutans* and *S. sanguis* and MBC of chitosan nanoparticle for *S. mutans* were less than those of chitosan ($P<0.05$). However, chitosan nanoparticles did not exhibit higher anti-adhesion property than chitosan on *S. mutans, S. sobrinus* and *S. sanguis* at a concentration of 5 mg/mL ($P>0.05$). Since there are few studies in this regard, further studies are needed to explain the findings exactly.

In conclusion, chitosan and nano-chitosan showed anti-growth and anti-adherence effects against cariogenic bacteria *in vitro.* The results indicated high potential of chitosan and nano-chitosan as anticariogenic agents, suggesting their potential application as dental biomaterials for prevention of dental caries.

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