Original Article

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Antibacterial and Anti-biofilm Effects of Chitosan Nanoparticles on *Streptococcus Mutans* Isolates

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Abstract

Background: Dental caries is an infectious disease caused by bacterial colonization and biofilm formation. *Streptococcus mutans* (*S. mutans*) is mainly responsible for dental caries development. Considering the side effects of synthetic antibacterial agents, attempts are ongoing to find antimicrobial agents with minimal or no side effects for preventing dental caries. Based on the reported antibacterial activity of chitosan, this *in vitro* study aimed to assess the antibacterial and anti-biofilm effects of chitosan nanoparticles on *S. mutans* clinical isolates.

Methods: *S. mutans* isolates were isolated from supragingival plaque and carious lesions of patients by standard biochemical tests and Polymerase Chain Reaction (PCR) of the *gtfB* gene. The antibacterial activity and Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) of chitosan nanoparticles against *S. mutans* was evaluated by the agar well-plate and broth micro-dilution test, respectively. Also, the effect of chitosan nanoparticles on biofilm formation was evaluated using micro-titer plate method. Data were analyzed using ANOVA.

Results: Fifteen *S. mutans* isolates were collected from patients. The chitosan nanoparticles synthesized had a diameter of 20–30 *nm*. The chitosan nanoparticles showed antibacterial activity against *S. mutans* isolates. MICs and MBCs ranged from 0.625-2.5 *µg/ml* and 1.25-5 *µg/ml*, respectively. All isolates evaluated in this study were biofilm-forming and 5 of these produced a strong biofilm. The chitosan nanoparticles inhibited biofilm formation at 0.75 *µg/ml* concentration.

Conclusion: Chitosan nanoparticles had antibacterial and anti-biofilm activity on *S. mutans* clinical isolates. This study suggests the potential of chitosan nanoparticles as antimicrobial agents against cariogenic Streptococci.

Keywords: Biofilm, Chitosan, Dental caries, Nanoparticle, *Streptococcus mutans*

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Introduction

Based on the World Health Organization reports, dental caries still remains a main health problem, especially among poor social groups (1). Dental caries is a multifactorial, sugar- and biofilm-dependent disease that initiates decalcification of tooth structure and degradation of the organic matrix (2,3). Cariogenic bacteria such as *Streptococcus mutans* (*S. mutans*) play an important role in the pathogenesis of dental caries. This bacterial species is a gram-positive cocci that is a normal inhabitant of the oral cavity and is a key participant to the formation of Extracellular Polysaccharides (EPS) matrix in dental biofilms (3,4). Dental biofilm production is a biological process mediated by the adhesion of oral planktonic bacteria to dental surfaces and proliferation (5).

Due to its multifactorial etiology, treatment of oral dental biofilm-related disease is complicated. In addition, biofilms are composed of more than 90% EPS that make biofilms more resistant to antimicrobial substances due to their limited diffusion to access microorganism cells (6).

Today, several antimicrobial substances including metronidazole, chlorhexidine, and quaternary ammonium compounds are used for the deletion of cariogenic microorganisms and the prevention of dental caries, but they have side effects such as increasing calculus formation, staining, and causing diarrhea by changing the gastrointestinal normal microbial flora (1,5,7). Thus, new strategies for prevention of dental biofilm-related disease are required. One of the strategies that have been investigated widely is by using nanoparticles. Nanoparticles are proven to have superior penetration ability, effective antimicrobial activity, and cost effective, compared to treatment with naturally derived anti-biofilm agents (6).

Chitosan is a linear cationic polysaccharide with optimal biocompatibility and biodegradability. It is non-toxic and has no immunological effects. It is abundant in nature as a biopolymer, and has been used for treatment of neural diseases, rheumatism, and cerebrovascular accident (8,9). Antibacterial properties of chitosan nanoparticles have been previously documented (1,5-7). The exact mechanism of the antimicrobial activity of chitosan and its derivatives has yet to be fully understood. However, some theories have been proposed in this respect. According to one suggested theory, positively charged chitosan molecules interact with the negatively charged bacterial cell membrane, and lead to leakage of proteins and other intracellular components of the bacteria. Also, chitosan acts as a chelating agent, binds to metals, and inhibits the microbial growth as such (10).

Advances in nanotechnology have enabled the production of dental materials with unique properties. Considering the reported antibacterial activity of chitosan nanoparticles, this study aimed to assess the antibacterial and anti-biofilm effects of chitosan nanoparticles on *S. mutans* clinical isolates.

Materials and Methods

Bacterial strains

The study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RIDS.REC.1396.594).

This *in vitro*, experimental study was conducted on standard strain *S. mutans* (ATCC25175) purchased from the Iranian Industrial Bacterial Collection and 15 clinical isolates of *S. mutans* collected from the supragingival plaque and carious lesions of patients presenting to the dental clinic of Shahid Beheshti Dental School. Collected microbial samples were transferred to the microbiology laboratory of the university in thioglycolate broth medium. Then, the samples were cultured on Mitis Salivarius Agar medium, and incubated at 37°*C* in presence of 5% CO_2, 10% H_2 and 80% N_2 for 48 *hr* (11). The obtained colonies were evaluated by conventional biochemistry tests including Gram-staining, catalase test, oxidase test, mannose fermentation test, and mannitol salt agar test.

Polymerase chain reaction

The obtained isolates were also confirmed by Polymerase Chain Reaction (PCR) of the *gtfB* gene amplification using forward: 5′-ACTACACTTT CGGGTGGCTTGG- 3′ and reverse: 5′ CAGTATAA GCGCCAGTTTCATC- 3′ primers. DNA extraction was done by the boiling method as described previously. The PCR reaction was prepared in a final volume of 20 *µl*, containing 10 *µl* Mastermix (Ampliqon, Denmark), 0.5 *µl* of each primer (10 *pM*), 5 *µl* (50 ng) DNA template and 4 *µl* distilled water.

Then, the PCR assay was carried out as follows: an initial denaturation at 94℃ for 5 *min*, followed by 30 cycles at 94℃ for 30 s, 55℃ for 30 *s*, 72℃ for 30 *s*, and a final extension at 72℃ for 5 *min* (12). The PCR distilled water and *S. mutans* strain (ATCC25175) were used as the negative and positive control, respectively.

Preparation of chitosan nanoparticles

Chitosan with 95% purity was purchased from Sigma Aldrich (Sigma Aldrich, USA). Low molecular weight chitosan (500,000 D) was used in this study. The 10 *mg/ml* stock solution of chitosan was prepared as follows: 2 *g* of chitosan was added to 100 *ml* of distilled water; 2 *ml* acetic acid was also added, and the mixture was stirred by a magnetic stirrer for 24 *hr*. Next, the volume of the solution was increased to 200 *ml* with distilled water and the pH was adjusted at 5 by adding NaOH. This stock solution was utilized to prepare 320 *μg/ml* concentration of chitosan nanoparticles. Also, Transmission Electron Microscopy (TEM) was used to determine the chitosan nanoparticles' size and shape.

Antibacterial activity determination

Bacterial suspensions (1×10^8 CFU) were cultured in Muller-Hinton agar medium with 5% sheep blood. Then, using sterile Pasteur pipettes, wells were created over the culture plates. Next, 100 *ml* of chitosan nanoparticles was added to into the wells. The plates were then incubated 37℃ for 24 *hr*. To ensure the accuracy of testing, it was repeated 3 times for each bacterial isolates (1). The diameter of the growth inhibition zones for the 15 isolates was measured and means value was reported. Acetic acid without chitosan nanoparticles served as the negative control. The *S. mutans* strain (ATCC25175) was positive control.

MIC and MBC determination

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of chitosan against *S. mutans* isolates were determined by broth microdilution assay according to the CLSI guidelines. The stock solution of chitosan nanoparticles was prepared with a concentration of 320 *μg/ml*. Next, 100 *μl* Mueller Hinton broth was added to each well of a 96-well plate. After that 100 *μl* of the chitosan were added to first well, and two-fold serial dilutions concentrations ranging from 160 to 1.25 *mg/ml* were made. A suspension with a turbidity of 0.5 McFarland standard (~1.5 x 10^8 Colony-Forming Units [CFU]/*ml*) was prepared in Phosphate-Buffered Saline (PBS) and was subsequently diluted 1:20. Then, 10 *μl* was added to each well. Then, the plates were incubated for 24 *hr* at 37℃ under anaerobic conditions. Acetic acid without chitosan served as the negative control. Quality control was done under similar conditions to those of the experiment using *S. mutans* (ATCC25175). For MIC determination, the plates were read for Optical Density (OD) under PLATE reader at 600 *nm*.

The last well that did not have turbidity indicated the MIC, to determination of the MBC, 20 *μl* of MIC, 2MIC and 4MIC well were cultured on MHA medium and incubated at 37℃ for 18 *hr*. and the least concentration with the colony growth not over 0.1% compared to the initial concentration was considered as the MBC value.

Effect of chitosan nanoparticles on biofilm formation

Effect of chitosan nanoparticles on biofilm formation was evaluated using micro-titer plate method. Tryptic Soy Broth (TSB) supplemented with 1% (w/v) sucrose was used for biofilm formation assay in this study. All 15 clinical isolates and the control strain were cultured in TSB and incubated overnight at 37℃. Two to three colonies of the fresh culture of bacteria were cultured in sterile tubes containing 10 *ml* of TSB and incubated at 37℃ in a shaker incubator operating at 200 *rpm* for 15-18 *hr*. The OD of each liquid culture was adjusted using fresh medium with an OD of 0.1 at 600 *nm* wavelength (6).

Next, 100 *μL* of the microbial suspension was added to 100 *μL* and served as the control. Then, 100 *μL* of the microbial suspension along with 100 *μL* of chitosan nanoparticles was cultured on a 96-well plate to assess its effect on biofilm formation and incubated overnight at 37℃. The overlaying medium was removed from the wells, and the microorganisms were rinsed with phosphate buffer three times. Biofilm-forming bacteria adhering to the walls and bottom of the plate were fixed with methanol for 15 *min*. Next, the plate was exposed to air upside down to dry. The fixed biofilm layer at the bottom and on

the walls of the plate was stained with 200 *µL* of 1% crystal violet aqueous solution for 15 *min*. The dye was then discarded, and the biofilm was rinsed with phosphate buffer three times. The plate was dried at room temperature and the dye absorbed by the biofilm was rinsed off with 200 *µL* of 33% acetic acid, and the OD of each well was read with an ELISA Reader (BiotTek, UK) at 570 *nm* wavelength. The medium without bacteria served as the negative control. Each experiment was performed in triplicate (6,13).

The OD values were categorized as follows:

- ODcut = ODavg of negative control+3×standard deviation (SD) of ODs of negative control.

OD ≤ODcut = Non-biofilm

ODcut <OD ≤2×ODcut = Weak biofilm

2×ODcut <OD ≤4×ODcut = Moderate biofilm

OD >4×ODcut = Strong biofilm.

Statistical analysis

Data were analyzed using SPSS software version 20. The difference between OD values and Mean were compared using one-way analysis of variance (ANOVA), and the significance level in the tests was considered 0.05.

Results

PCR results indicated that all 15 obtained isolates carried the *gtfB* biofilm formation gene (517 *bp*) and confirmed as *S. mutans* (Figure 1). Figure 2 showed an image of chitosan nanoparticles by TEM. The diameter of the synthesized chitosan nanoparticle ranged between 20 and 30 *nm*. Also, the chitosan nanoparticle shape was nearly spherical with a smooth surface. The well-plate technique indicated that the growth inhibition zone for the clinical isolates ranged from 18 to 21 *mm* (mean value of 19 *mm*).

MICs and MBCs of the chitosan nanoparticles against *S. mutans* isolates are presented in table 1. MICs and MBCs ranged from 0.625-2.5 *µg/ml* and 1.25-5 *µg/ml*, respectively. Twelve (80%) of the isolates had MIC 1.25 *µg/ml* and MBC 2.5 *µg/ml*.

Six isolates (40%) showed moderate biofilm formation, 4 isolates (26.7%) indicated weak biofilm formation while 5 isolates (33.3%) formed a strong biofilm. Among these 5 isolates, chitosan nanoparticles in 0.75 *µg/ml* concentration inhibited the biofilm formation.

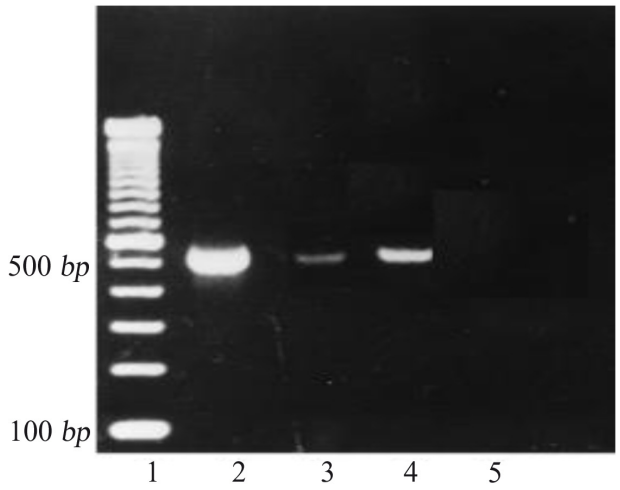

Figure 1. PCR product of the gtfB biofilm formation gene: (1) ladder, (2) positive control, (3 and 4) specimens, (5) negative control.

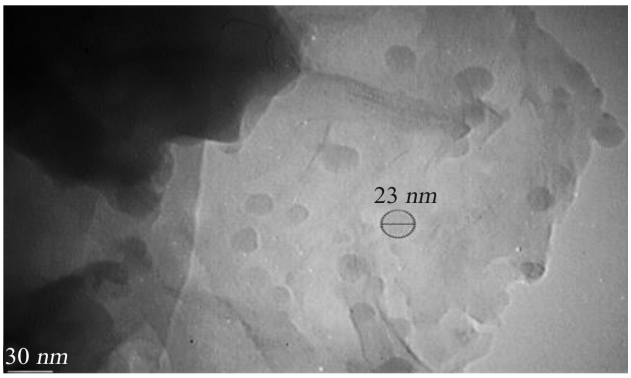

Figure 2. The figure shows that chitosan nanoparticles have a particle size below 30 *nm* in TEM image (scale bar 30 *nm*).

Discussion

Considering the side effects of synthetic antibacterial agents, attempts are ongoing to find antimicrobial agents with minimal or no side effects for preventing dental caries. According to the reported antibacterial activity of chitosan, this study aimed to assess the antibacterial and anti-biofilm effects of chitosan nanoparticles on *S. mutans* isolates. Our results demonstrate that chitosan nanoparticles have antibacterial effect, and that it can reduce biofilm formation *in vitro*. The results showed a MIC of 0.625-2.25 *µg/ml* and MBC of 1.25-5 *µg/ml* for chitosan nanoparticles against *S. mutans* isolates. Also, 80% of isolates had MIC of 1.25 *mg/ml*. This finding is consistent with the result of Aliasghari *et al*'s study (1) that reported an MIC of chitosan nanoparticles for

Table 1. The MIC, MBC and biofilm formation of chitosan nanoparticles among *S. mutans* isolates

Sample	MIC (*µg/ml*)	MBC (*µg/ml*)	Biofilm
1	1.25	2.5	Weak
2	1.25	2.5	Strong
3	1.25	2.5	Weak
4	2.5	5	Moderate
5	1.25	2.5	Strong
6	1.25	2.5	Moderate
7	1.25	2.5	Strong
8	1.25	2.5	Moderate
9	0.62	1.25	Weak
10	1.25	2.5	Moderate
11	0.62	1.25	Moderate
12	1.25	2.5	Moderate
13	1.25	2.5	Weak
14	1.25	2.5	Strong
15	1.25	2.5	Strong

S. mutans of 1.25 *µg/ml*. But Khoshmaram *et al* (14) found an MIC of 0.114 *mg/ml* that was higher than our results

On the other hand, results indicated that 5 isolates formed strong biofilm. Chitosan nanoparticles at 0.75 *µg/ml* concentration inhibited biofilm formation by these isolates. These results supported the results of Costa *et al* (15). Divya *et al* (16) evaluated the antimicrobial activity of chitosan nanoparticles against *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* by calculation of MIC. They reported that chitosan nanoparticles had antimicrobial activity against all the tested microorganisms. They also assessed the anti-biofilm activity of chitosan nanoparticles using ELISA and Congo red agar test. They confirmed the anti-biofilm effects of chitosan nanoparticles. Fujiwara *et al* (17) evaluated the effect of pH and polymerization rate of chitosan on inhibition of *S. mutans*. They evaluated chitosan polymer, oligomer, and monomer at three pH levels and found that water-soluble chitosan directly inhibited the proliferation of standard strain *S. mutans* even at a pH of 6.5 without causing tooth surface degradation. In the present study, the pH was 5, and the results indicated the antibacterial effects of chitosan even in more acidic conditions than that in the study by Fujiwara *et al* (18). Sarasam *et al* (19) demonstrated that chitosan scaffolds had antibacterial activity against *S. mutans* and *Actinomyces actinomycetemcomitans*. They showed that chitosan inhibited bacterial adhesion, and prevented biofilm formation, which is in harmony with the present findings. Mirhashemi *et al* (20) reported that biofilm formation and proliferation of *S. mutans* significantly decreased due to the effect of chitosan nanoparticles, findings that agree with the present results. Also, Rajabnia *et al* (21) demonstrated that chitosan-containing sealants had antibacterial effects on *S. mutans* that were intensified by increasing the concentration of chitosan. They concluded that addition of 500 *µg/ml* of chitosan to a mouthwash eliminated 99% of *S. mutans* bacteria after 5 *s*. Their findings are in agreement with the present results and highlight the high potential of chitosan for use in the composition of mouthwashes (22). Kim and Shin (23) pointed to the inhibitory effect of chitosan incorporated in resin composites on *S. mutans*. They concluded that all chitosan-containing resin composites had inhibitory activity; however, addition of chitosan caused some unfavorable changes in the mechanical properties of some resin composites. Their results regarding the antimicrobial activity of chitosan were in line with the present results. Chávez de Paz *et al* (24) evaluated the effect of molecular weight of chitosan nanoparticles on *S. mutans*. They showed that chitosan nanoparticle complexes with high molecular weight had lower antimicrobial activity than complexes with lower molecular weights. Considering the low molecular weight of particles used in the present study (50000 D), our findings supported the results of Chávez de Paz *et al* (24).

Future studies are required to assess the effect of chitosan on human cell lines to assess its biocompatibility in greater detail.

Conclusion

Chitosan nanoparticles have antibacterial and anti-biofilm activity on *S. mutans* clinical isolates. This

study suggests the potential of chitosan nanoparticles as antimicrobial agents against cariogenic Streptococci.

Acknowledgements

The study was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RIDS.REC.1396.594).

Conflict of Interest

Not applicable.

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