Original Article

Anti-*Streptococcus mutans* property of a chitosan: Containing resin sealant

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Abstract

Objective: This study sought to assess the inhibitory effect of chitosan-containing sealants against *Streptococcus mutans*. **Materials and Methods:** The antibacterial activity of the resin sealant was evaluated by direct contact test following the addition of 0, 1, 2, 3, 4, and 5 wt% chitosan. At 3, 6, 9, 24 and 48 h, 1 and 3 months, 10 µl of the microbial suspension in contact with resin sealant was cultured to count the number of colonies. Data were analyzed by one-way one-way analysis of variance (ANOVA), repeated measures ANOVA, and Scheffe test. **Results:** The minimum inhibitory concentration of chitosan against *S. mutans* was 2 wt%. At 3 h, bacterial count in the presence of 2–5 wt% chitosan was significantly lower than that at 0 and 1 wt% ($P < 0.05$). However, this difference in bacterial count between 2 and 3 wt% chitosan and between 4 and 5 wt% chitosan was not significant. At 6 h, the difference in bacterial count between 3 and 4 wt% chitosan was not significant, whereas the remaining groups were significantly different in terms of bacterial count at this time ($P < 0.05$). At the remaining time points, significant differences were found between 2 wt% chitosan and higher concentrations ($P < 0.05$). **Conclusion:** Sealants containing 2–5 wt% chitosan show an antimicrobial property that is intensified by increasing the concentration of chitosan.

Key words: *Antimicrobial property, chitosan, direct contact test, resin sealant, Streptococcus mutans*

INTRODUCTION

Despite the improvement in knowledge about the cariogenic factors and methods of caries prevention, recurrent and secondary caries are still the main common reason for the replacement of resin restorations.[1,2] Sealants are resin materials used for the obstruction of occlusal pits and fissures.[3] Tooth decay is an infectious disease and mutans streptococci are the main organisms involved in initiation and development of caries. Therefore, by reducing the bacterial count at the resin–tooth interface, incidence of secondary caries may be decreased as well.[4] Thus, the incorporation of antimicrobial agents into resin dental materials may be effective for the prevention of secondary caries.

Fluoride and chlorhexidine are the most commonly incorporated antimicrobial agents into resin materials.[5] Although fluoride and chlorhexidine have strong antimicrobial properties initially, their release does not continue for a long time. Moreover, they change the mechanical properties of resin materials and significantly decrease their bonding strength.[5-7] In the

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recent years, chitosan has been the focus of attention due to its natural organic nature, biocompatibility, non-toxicity, and bactericidal properties.[8-11]

Chitosan with the chemical formula $(C_6H_{11}O_4N)^n$ is a natural biopolymer derived from the shells of shrimp and other crustaceans through deacetylation of chitin. Next to cellulose, chitin is the second most abundant organic polymer on earth.[8] Due to a positive charge, chitosan adheres to the bacterial cell wall and cell membrane and can have both bacteriostatic and bactericidal effects.[11,12] The maximum effect of this material is on gram-positive bacteria such as *Streptococcus sanguis*, *Streptococcus mutans*, *Streptococcus mitus*, *Streptococcus salivarius*, and yeasts.[13] Chitosan is available in different forms such as powder, paste, film, porous scaffolds, fiber, etc.[14,15] Besides the low cost of chitosan microparticles, they have some other favorable characteristics and applications such as prevention of demineralization,[9,16] prevention of plaque and biofilm formation, stimulation of salivary secretion,[12] antitumor activity, hemostatic properties, enhancing wound recovery, antihypertensive properties, reducing serum cholesterol,[8] drug delivery system,[17-19] coating of implants, bone tissue engineering,[20,21] blood vessel repair,[22] and nerve repair.[23,24] Under physiological and biological conditions, this material does not stimulate the immune system. It is easily absorbed by the human body, reacts with body fluids, and its physical and chemical properties are easily adjustable.[25,26]

In the present study, we added 1, 2, 3, 4, and 5 wt% chitosan to resin-based sealants and assessed the antibacterial properties of the compounds against *S. mutans*. We also determined the minimum inhibitory concentration of chitosan against *S. mutans*.

MATERIALS AND METHODS

Specimen preparation

This experimental study was conducted on six groups of the resin sealant containing 0, 1, 2, 3, 4, and 5 wt% chitosan. For higher precision, three specimens were prepared from each group (n = 18). Chitosan-containing sealant was prepared in a dark room under red light to avoid early polymerization of resin. For the preparation of sealants with different concentrations of chitosan, first a sterile hourglass was placed on a laboratory scale and adjusted to zero. Then, the sealant tube (Clinpro; 3M ESPE, Saint Paul, MN, USA) was squeezed out into the hourglass and after weight determination (about 1.5 g), the desired amount of chitosan (Sigma Aldrich Chemical Co., Saint Louis, MO, USA) was added to the sealant in hourglass to obtain 1, 2, 3, 4, and 5 wt% concentrations. The mixture was stirred manually with a plastic spatula for 15 min to evenly spread the chitosan particles in the sealant. One chitosan-free sealant group was considered as the control group.

Preparation of microtubes containing sealants

Using an insulin syringe, 200 µl of each sealant group was poured into 0.5 ml microtubes and after adapting the sealant to the internal walls of the microtube [Figure 1], it was light-cured using a light-curing unit (VALO; Ultradent, South Jordan, UT) with an intensity of 1000 mW/cm^2 which was determined by the radiometer. The tip of light-curing unit was placed on the wall of microtubes directly and the sealants were light-cured from two sides for 40 s. Thus, microtubes were filled with 200 µl of the sealant and 300 µl remained empty.

Direct contact test

Direct contact test was carried out to assess the antibacterial activity of the free surface of sealant specimens containing chitosan. A total of 10 µl of 0.5 McFarland standard suspension of *S. mutans* (about 10^7 bacteria) was poured into microtubes [Figure 2] and the microtubes were placed in a sterile environment under the hood for an hour. During this time, the bacteria were in direct contact with the free resin surface. Next, 300 µl of Brain Heart Infusion (BHI) culture medium (Merck, Darmstadt, Germany) was added to each microtube. The lid was completely closed and the dishes were placed in an incubator at 37°C. At 3, 6, 9, 24, and 48 h, 1 and 3 months, 10 µl of the suspension in each microtube was cultured on solid culture medium and after 24 h of storage at 37°C, the

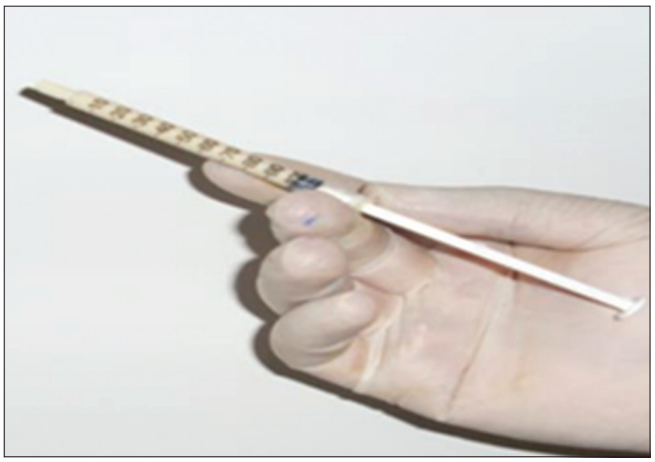

Figure 1: Insulin syringe containing chitosan resin sealant

number of grown colonies was counted by a digital colony counter [Figure 3]. It should be mentioned that since the baseline number of bacteria in each dish and the volume of suspension were known, the reduction of bacterial count is indicative of the antibacterial activity of the resin. The mean and standard deviation (SD) of bacterial count were calculated for each experimental group and statistical analyses were performed.

Statistical analysis

The obtained raw data were statistically analyzed using SPSS Version 19 by one-way analysis of variance (ANOVA) and repeated measures ANOVA. If the difference was significant, Scheffe test was also used for pair wise comparison. $P < 0.05$ was considered statistically significant.

RESULTS

Mean (SD) of bacterial count assessed at 3, 6, 9, 24, and 48 h, 1 and 3 months for the resin groups are presented in Table 1.

Repeated measures ANOVA showed that the bacterial count did not change in the group containing 0 and 1 wt% chitosan. In the remaining experimental groups, the level of bacteria significantly decreased in 1 month. But in 3 months, despite the high antimicrobial activity compared to the control group, the bacterial count increased, compared to the corresponding value in 1 month. Also, the difference between groups in terms of bacterial count at different time points was statistically significant ($P < 0.001$).

DISCUSSION

This study sought to assess the anti-*S. mutans* activity of sealants containing 1–5 wt% chitosan and the minimum inhibitory concentration of chitosan against *S. mutans* at different time points was also determined. Chitosan is an organic substance with antimicrobial properties was used in combination with a sealant. Chitosan used in this study had low molecular weight and high deacetylation degree (DD) (75–85%). The antimicrobial activity of chitosan is related to its molecular weight and DD, and it has been shown that chitosan with low molecular weight and high DD has better antimicrobial activity.[12] Our obtained results demonstrated that the resin sealant containing chitosan significantly inhibited

Figure 2: 0.5 McFarland microbial suspension

Figure 3: Colony counter

Table 1: Mean bacterial count (CFU/ml) at different time points

wt% chitosan	Time							*P*
	3 h	6 h	9 h	24 h	48 h	1 month	3 months	
2%	2540.0± 69.282[a]	2466.67± 57.73[a]	2458.33± 52.042[a]	2443.33± 51.316[a]	2523.33± 68.069[a]	1716.67± 04.083[a]	2020.67± 20.33[a]	0.015
3%	2490.00± 36.056[b]	1526.67± 64.291[b]	1550.00± 86.603[b]	1440.00± 36.056[b]	1413.33± 32.146[b]	1206.67± 11.547[b]	1373.33± 25.166[b]	0.008
4%	1503.33± 55.076[b]	1516.67± 28.868[b]	978.33± 22.5[c]	871.67± 12.583[c]	836.33± 6.506[c]	659.00± 85.294[c]	782.00± 33.956[c]	0.007
5%	1497.33± 16.166[b]	985.67± 12.897[c]	698.00± 37.041[d]	599.33± 9.018[d]	563.67± 12.342[d]	250.67± 9.452[d]	361.67± 17.559[d]	0.006
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	

[a,b,c,d]*Similar letters indicate no significant difference between the two groups at the level of α=0.05. CFU=Colony forming unit*

the growth of *S. mutans*, compared to the chitosan-free control group. By increasing the concentration of chitosan from 2 to 5 wt%, a significant reduction occurred in the bacterial count, which is in concordance with the results of studies by Hayashi *et al*.,[12] Mohire and Yadav,[27] Mahapoka *et al*.,[28] Chen and Chung,[29] Uraz *et al*.,[30] and Elsaka and Elnaghy.[31]

Chitosan weight percent in the resin sealant groups under study and the selected time points were chosen according to a study by Mahapoka *et al.* who evaluated the anti-*S. mutans* activity of chitosan whiskers in combination with sealant.[28] Mahapoka *et al.* used 1, 1.5, 2, and 2.5% chitosan whiskers and showed that 1% and 1.5% concentrations had a significantly less antimicrobial activity than higher concentrations.[28] In the present study, the antimicrobial activity of higher concentrations (3, 4, and 5 wt%) of chitosan was also evaluated. The compound showed its antibacterial activity at 3 h and it continued for 3 months, which was the longest period evaluated in our study. The highest anti-*S. mutans* activity of each concentration of chitosan in this study was observed after 1 month and showed a decreasing trend thereafter (1–3 months).

According to Mahapoka *et al.*,[28] further studies are required to assess whether the chitosan incorporated into sealants has long-term antibacterial effects. So, in this study, the effect of time (maximum of 3 months) was assessed and it was found that although the antibacterial activity of chitosan gradually declined after 1 month, this effect lasted for 3 months.

Chen and Chung[29] evaluated the antimicrobial effect of water-soluble chitosan against *S. mutans* by direct contact test and confirmed that water-soluble chitosan had significant antibacterial properties. They stated that acid-soluble chitosan in lower pH had better antimicrobial activity and this effect decreased by increasing the pH, whereas water-soluble chitosan in the pH range 5–8 had a wider antibacterial effect.[29] The chitosan oligomers used in our study were water soluble as well.[12]

In our study, the bacteria continued to proliferate at all time points under study following their exposure to the resin surface in the culture medium in resin groups containing 0 and 1 wt% chitosan, but the bacterial count decreased in groups containing 2, 3, 4, and 5 wt% chitosan. In other words, in the presence of 1 wt% chitosan, bacterial growth inhibition was not significant and the bacteria continued to grow and proliferate, similar to the results of Mahapoka *et al.*[28] Their results showed that the greatest antibacterial effect was observed at 1 and 3 months; which is attributed to the fact that the bacteria were exposed to chitosan for a longer time.

The antibacterial activity of chitosan can be attributed to its polycationic property. Like chitosan, various homopolymers or peptides containing polycationic amino acids show antimicrobial activity by the same mechanism. However, their antimicrobial agents need to be investigated further against oral pathogens.[32]

Chitosan has one primary amine group and because of having a free NH_3^+, it is clearly a cationic biomaterial. Cationic materials exert their antimicrobial effect by degrading the cell wall structure and the cell membrane of bacteria. The cell membrane of bacteria is surrounded by a cell wall composed of peptidoglycan layers that are *per se* made of *N*-acetylglucosamine, *N*-acetylmuramic acid, and d and l amino acids which link the positively charged amine groups of chitosan oligomers to glycine in the peptidoglycan structure. Thus, this material disrupts the cell wall and exposes the cell membrane to osmotic shock. Consequently, cytoplasmic contents are extruded and cell death occurs. Chitosan oligomers in the oral environment can have bactericidal and/or bacteriostatic properties.[8] Mohire and Yadav[27] noticed a significant reduction in the bacterial count on using a chitosan-based herbal toothpaste and attributed this finding to both the physical characteristics of chitosan such as mucosal adhesion and its chemical interference with bacterial cell wall.

In summary, incorporation of chitosan into a resin sealant caused short-term and long-term anti-*S. mutans* activity. Further studies are recommended to assess the physical and chemical properties of chitosan-containing sealants.

CONCLUSION

Fissure sealants containing 2–5 wt% chitosan showed anti-*S. mutans* activity in direct contact test. By increasing the concentration of chitosan, bacterial count significantly decreased.

The highest anti-*S. mutans* activity was noted at 1 and 3 months (based on the experimental group).

The minimum inhibitory concentration of chitosan for its anti-*S. mutans* activity was 2 wt%.

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Conflicts of interest

There are no conflicts of interest.

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