ANTIBACTERIAL ACTIVITY AND ANTI-BIOFILM EFFECT OF CHITOSAN AGAINST STRAINS OF *STREPTOCOCCUS MUTANS* ISOLATED IN DENTAL PLAQUE

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Streptococcus mutans **is the major cause of dental plaque and is often associated with biofilm formation. The aim of this study is to evaluate the activity of a hydrosoluble derivative of chitosan against** *S. mutans* **biofilms** *in vitro* **and** *in vivo*. **Strains of** *S. mutans* **were isolated from the dental plaque of 84 patients enrolled in the study. The antibacterial activity of chitosan was determined by broth microdilutions. The effect of chitosan at different concentrations and exposure times on** *S. mutans* **biofilms at different phases of development was assessed by a clinical study using the classical "4-day plaque regrowth" experiment in adult volunteers. The MIC values of chitosan were between 0.5 and 2 g/L. Compared to distilled water, the chitosan solution significantly decreased the vitality of plaque microflora (p≤0.05). Chlorhexidine, used as a positive control, reduced vitality even further. The results showed that** *S. mutans* **in the adhesion phase (4 h) was completely inhibited by chitosan at any concentration (0.1, 0.2, 0.5XMIC) or exposure time investigated (1, 15, 30, 60 min), while** *S. mutans* **at successive stages of accumulation (12-24 h) was inhibited only by higher concentrations and longer exposure times. These data confirm the effective action of chitosan against** *S. mutans* **biofilms.**

S. mutans, described for the first time by Clarke (1), is a Gram-positive facultative anaerobic bacterium frequently found in the oral cavity. It is considered the greatest cariogenic agent among all oral streptococci (2). Its significant contribution to the cariogenic process occurs through the metabolism of sucrose to lactic acid (3-4). The acidic environment created at the dental level as a result of this process causes a high demineralization of the tooth, which then becomes susceptible to caries. *S. mutans* is also one of the microorganisms with the most receptors for adhesion to the dental surface. *S. mutans* utilizes sucrose to produce an extracellular, dextran-based polysaccharide that strongly enhances the formation of plaque, as described by the following reaction:

n sucrose -----> $(glucose)_n$ + n fructose.

While the sucrose is the only sugar that *S. mutans* can use to form strongly adhesive polysaccharides, other sugars, such as glucose, fructose, and lactose, may be metabolized to lactic acid. The combination of plaque and acid is the cause of caries (5). Since there is a significant correlation between the cariogenic process and the presence of *S. mutans* in plaque, limiting this bacterium's presence plays an important role in preventing caries. Mechanical procedures to keep the dental surfaces clean and local chemotherapy

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are possible methods of limitation. Nowadays, various substances, such as plant alkaloids, biguanides, essential oils, fluorinated amines, and triclosan, are used to control plaque formation. However, the frequent use of these substances can lead to negative changes in the oral microflora that favours resistant strains (6). Chlorhexidine, the best-known and most widely-used anti-plaque agent, does not appear to be free from side effects, and therefore does not fall within the definition of an exemplary substance proposed by Brecx (7). According to this definition, such a substance should clinically benefit plaque control and must have anti-bacterial properties, a good ability to stay in the oral environment, an anti-adhesive effect, and no side effects (7). Chitosan is a co-polymer of glucosamine and N-acetylglucosamine deacetylate and is a derivative of chitin. Its anti-bacterial activity against *S. mutans* and other microorganisms present in the oral cavity makes it a very promising anti-plaque agent (8-10). The low molecular weight chitosan inhibits the absorption of *S. mutans* on hydroxyapatite and seems to have bioadhesive properties, increasing chitosan's retention time on the surface of the oral mucosa (11). Decker *et al.* reported the synergistic anti-plaque effect of chlorhexidine and chitosan on the basis of the adhesive property of chitosan (12). Despite these proven properties, the application of chitosan by mouth rinses or dentifrices is limited by its insolubility in water. Recently, many studies have been conducted to increase its solubility so as to broaden its spectrum of applications (8, 13-14). Water-soluble derivatives of chitosan, obtained by replacing N-alkyl groups with disaccharides, have displayed significant anti-bacterial activity against *Escherichia coli* and *Staphylococcus aureus* (9). However, there are few studies concerned with the direct evaluation of dental anti-bacterial properties of chitosan derivatives (10). The purpose of this study is to examine the activity of a water-soluble derivative of chitosan, and to evaluate its anti-adhesive and anti-biofilm activities against *S. mutans* strains isolated from dental plaque.

MATERIALS AND METHODS

Reagents

A 1% solution (pH 6.5) of a water-soluble derivative of chitosan (M.W. between 3,000 and 5,000) obtained by reduction of chitosan with acetic anhydride, with a degree of deacetylation $\geq$ 75%, was used. Distilled water and a solution of 0.1% chlorhexidine digluconate were used as negative and positive controls, respectively.

Bacterial Strains

Strains of *S. mutans* were isolated from the dental plaque of 84 adult subjects enrolled in the study. The isolation was performed by passing a sterile swab over the dental arch, previously dried of saliva by a jet of compressed air. The samples were transferred to a tube containing 0.15 M saline solution. Afterwards, 1:10 serial dilutions were made and 25 μL of each was inoculated into sucrose agar medium containing bacitracin and incubated at 37°C for 48 h in a jar with a controlled atmosphere (5% carbon dioxide). The morphology, dimension and haemolysis of colonies for each isolate were observed by means of a stereomicroscope. Candidate *S. mutans* strains were isolated and subjected to molecular identification by PCR using primers for *S. mutans* *gtf*B (15).

Determination of the Minimum Inhibitory Concentration (MIC)

For each isolate, cells were grown in a Brain Heart Infusion broth supplemented with 3% (w/v) sucrose (BHI-S) in 5% CO_2. The cultures were centrifuged and resuspended in fresh liquid medium to a final concentration of 5×10^5 CFU/mL. Three different conditions were tested: medium alone or medium supplemented with different concentration of either chitosan (0.06-64 g/L) or chlorhexidine. Chitosan was prepared in distilled sterile water. All tests were run in triplicate. After inoculation in the appropriate test condition, cultures were incubated for 48 h in CO_2, and turbidity was measured spectrophotometrically at a wavelength of 600 nm. The MIC was defined as the lowest dilution of the test molecule that restricted growth to below 0.05 units of optical density at 660 nm.

Clinical study of biofilm vitality by the "4-day plaque regrowth" test

Eighty-four volunteers, 54 men and 30 women aged 28 to 52 years, were enrolled in the study and subjected to a preliminary prophylactic treatment with toothpaste and a toothbrush for a week before 6 weeks of tests. At the beginning of each week of testing, a dental cleaning by professional staff was performed. The following substances were assigned: chitosan (final concentration 1%), chlorhexidine digluconate (final concentration 0.1%), and distilled water. In subsequent days, the participants of the study were urged to avoid other mechanical hygiene measures and were asked to use the mouthwash solution twice a day (the first use supervised, the other performed at home). Each week of testing was followed by 10 days of washing in order to remove any
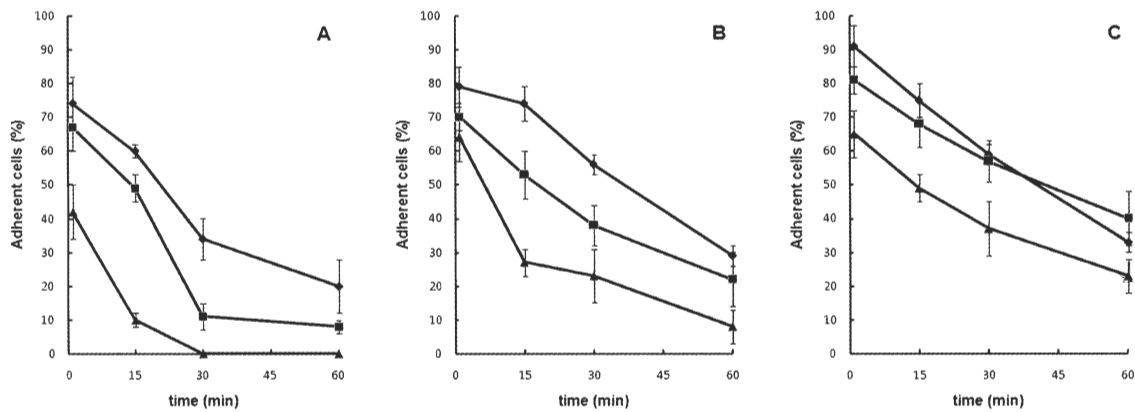
Fig. 1. *Effect of chitosan at different concentrations and exposure time on S. mutans biofilm at different phases of growth. Mean values are expressed in percentage (±SD) of absorbance of cells in treated wells compared with that in untreated wells (considered to be 100%). (**A**) Accumulation phase at 12 h; (**B**) Early accumulation plateau at 20 h; (**C**) Accumulation plateau at 24 h. Adhesion measurements (4 h) are not shown because biofilm was completely removed at this phase.*

Table I. *Viability (V.I.) determined on plaque samples taken at day 22.*

	V.I.1		p value (chitosan vs D.W./CLX)	V.I.2		p value (chitosan vs D.W./CLX)
	average	SD		average	SD	
Distilled Water (D.W.)	46.81	6.0		59.2	7.1	
Chitosan	29.82	10.2	0.025/0.010	38.9	9.4	0.05/0.001
Chlorhexidine (CLX)	16.90	5.12	<0.001/-	21.8	7.9	<0.001/-

trace of the tested products. During monitoring visits, any side effects were recorded.

In order to establish the vitality index (V.I.), samples of overgingival plaque were taken from the surfaces of teeth 16 and 46 (V.I.1) on days 8, 22, and 36, using a probe, and from teeth 26 and 36 (V.I.2) on days 11, 25, and 39. Samples were deposited on a slide and stained directly with fluorescein diacetate, which colors live microorganisms green, and ethidium bromide, which stains dead bacteria red. The samples were examined and the vitality of the biofilm determined using a fluorescent microscope with a grid to count the number of live bacteria. Using 105 boxes at 400x magnification we calculated the percentage of viable bacteria (i.e. the Vitality Index, V.I.).

In vitro formation and quantification of the biofilm

The *in vitro* formation of *S. mutans* biofilms was examined on commercially-available microtiter plates with 96 wells conditioned with a solution of artificial saliva. *S. mutans* cells at the appropriate density were added to each well containing BHI-S. The plates were incubated at 37°C without shaking. Usually three main phases are recognized during *S. mutans* biofilm formation in BHI (16): adherence (0-4 h), active accumulation (4-20 h), and a slow accumulation plateau (after 20 h). In our study, we chose to dissect the formation of biofilms into four phases: adherence (4 h), active accumulation (12 h), early accumulation plateau (20 h), and accumulation plateau (24 h). Biofilms at different stages of development

were obtained from five out of the eighty-four clinical isolates, with MIC values of 1g/L. They were then treated with chitosan solutions at final concentrations of 0.1, 0.2, and 0.5 times the MIC value. The exposure times were 1, 15, 30, and 60 minutes. The amount of biofilm formation was calculated using the crystal violet method described recently (17-18). The wells, covered with biofilm, were shaken vigorously to remove non-adhered bacteria. The adhered bacteria were washed twice with PBS (pH7.0) and air-dried. Each well was subsequently stained with a solution of crystal violet, washed twice with distilled sterile water, and destained with 95% ethanol. After bleaching, the upper solvent phase was transferred to a new well for quantization using an ELISA spectrophotometer (595 nm wavelength). The activity of chitosan and chlorhexidine was calculated as a percentage of the total biofilm mass formed in the absence of treatment. The experiment was repeated three times. Plates without chitosan were used as a negative control and a solution of chlorhexidine digluconate was used as a positive control.

Statistical analysis

Statistical analysis was performed using the software program Statgraphic Centurion XV (StatPoint Inc.). The differences between groups were analysed using a paired *t* test.

RESULTS

The MIC of chitosan deacetylate was evaluated for 84 different isolates of *S. mutans* from a defined set of patients especially enrolled for this study. The MIC values ranged from 0.5 g/L to 2 g/L. Clinical trials conducted to evaluate the anti-adhesive and anti-biofilm effects of chitosan were performed on 84 patients. The trials had excellent compliance by all participants and no significant registered side effects. With distilled water as the mouth-rinsing liquid the viability (V.I.) of *S. mutans* in samples of plaque from teeth 16/46 (V.I.1) and teeth 26/36 (V.I.2) was 46.81% and 59.2%, respectively (Table I). Rinsing with chitosan resulted in a vitality of 29.82% for V.I.1 and 38.9% for V.I.2. With the solution of chlorhexidine gluconate the V.I. values were 16.90% and 21.8%. The results of the experiment assessing the effect of chitosan deacetylate at different concentrations and with different exposure times on biofilms are shown in Fig. 1. The biofilms were produced from five different isolates and were monitored at different stages of development. The results show that the ability of chitosan to remove

the biofilm depends on the concentration, exposure time, and the development phase of the biofilm. A similar result is obtained when the biofilm is exposed to chlorhexidine (data not shown). It appears that it is important to completely remove the biofilm in the initial phase of adhesion, regardless of the concentration and exposure time of the treatment. In the second phase (the active accumulation phase), complete removal of the biofilm can be achieved with the maximum concentration with an exposure of at least 30 min. In two of the stages (the beginning of the plateau phase and the accumulation phase), none of the concentrations used were able to completely remove the biofilm, regardless of the exposure time. In general, however, it was observed that a 60-minute exposure was able to significantly reduce the biofilm, even after it had progressed into later stages of development.

DISCUSSION

All the tested strains of *S. mutans* isolated from patients showed a high susceptibility to chitosan. This result demonstrates the greater susceptibility of *S. mutans* to chitosan, compared to other *Streptococcus* species such as *S. sanguinis*, which is the primary caries agent. Only after plaque formation does *S. mutans* become the predominant species. However, during oral hygiene procedures, it is impossible to expose *S. mutans* to a constant concentration of chitosan, especially over a long period of time. This is also the difficulty for determining the MIC. Experience suggests that an exposure time of 5-10 min prevents the growth of *S. mutans* best. Moreover, the use of a 3-4% concentration seems to be most appropriate, due to the chemical, physical and organoleptic properties of chitosan. This is supported by data reported in the literature, which confirms that compounds such as chlorhexidine have an anti-bacterial activity quite similar to that of the derivative we examined (19). The techniques used to determine the viability of flora constituent plaque (V.I.) after treatment demonstrates the bactericidal effects of the chitosan derivative. The V.I. value obtained was significantly lower than that of distilled water, demonstrating the definite advantage of this treatment. A similar result was obtained by Bae at al. (20), who found a stronger effect (about

20%) of treatment with distilled water and similar antibacterial effects of chitosan and chlorhexidine.

Currently, few studies address the anti-biofilm action of oral anti-bacterial agents (21). Our results clearly show that exposure of *S. mutans* biofilms to chitosan removes a high percentage of the biofilm, greater than that removed in the absence of treatment. This is the case for biofilms in the initial plateau phase as well as in the accumulation phase. Therefore, the tested water-soluble derivative of chitosan is an excellent candidate for preventing the formation of plaque and reducing total *S. mutans* viability in the mouth. Furthermore, we present evidence that routine oral hygiene practices prevent plaque formation and might limit the onset of diseases related to plaque, such as caries and periodontitis. Ultimately, the derivative of chitosan studied here is shown to have powerful activity against *S. mutans* biofilms.

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