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ORIGINAL ARTICLE

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Effect of water-soluble reduced chitosan on *Streptococcus mutans*, plaque regrowth and biofilm vitality

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Abstract The purpose of the present study was to examine the effects of a newly developed water-soluble reduced chitosan on *Streptococcus mutans*, plaque regrowth, and biofilm vitality. A 1.0%, water-soluble reduced chitosan, with pH ranging from 6.0 to 6.5, molecular weights between 3,000 and 5,000 Da, and 70% degree of deacetylation, was used. To determine antibacterial and antiplaque potency of chitosan, minimal inhibitory concentrations (MICs) for *S. mutansand S. sanguinis* (formerly *S. sanguis*), short-term exposure to *S. mutans*, and clinical trial of plaque regrowth and biofilm vitality were conducted. Twelve dental students volunteered to participate in the 6-week, double blind, randomized clinical trial using the classical 4-day plaque regrowth design. The MIC of water-soluble reduced chitosan for *S. mutans* was 1.25 g/l. While the cells exposed to distilled water (DW) grew rapidly, with a maximum turbidity reached by 16 h postinoculation, *S. mutans* exposed to chitosan (5.0 g/l) exhibited a substantial delay in growth and reached a maximum turbidity by 32 h postinoculation. The chitosan solution reduced the plaque index and the vitality of the plaque flora significantly when compared to DW, but this was less than the reductions found with the positive control of 0.1% chlorhexidine solution. The water-soluble reduced chitosan exhibited potent antibacterial effect on *S. mutans*, and displayed a significant antibacterial and plaque-reducing action during the 4-day plaque regrowth.

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Introduction

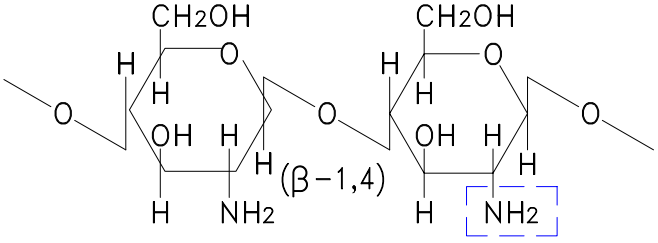
A clear relationship exists between the number of dental caries and *Streptococcus mutans* inhabiting the plaque [18, 29]. For this reason, plaque control plays a significant role in the prevention of caries. Both mechanical tooth cleansing procedures and local chemotherapeutics are used for this purpose. Until now, many substances have been tested for plaque control including, plant alkaloids, bisbiguanides, essential oil, amine fluoride, triclosan, and etc. [20, 30]. The most thoroughly tested and effective antiplaque agent known today is chlorhexidine, which has been used for more than three decades [4, 31], but chlorhexidine is restricted to short-term use because of some reversible local side effects [1, 12]. Brecx [7] proposed that effective substances, which provide clinical benefits for plaque control, should have antimicrobial effect on pathogenic bacteria, significant substantivity in the oral environment, and inhibiting effect on the adhesive properties of bacteria but no side effect.

Chitosan, a natural polysaccharide derived from chitin by *N*-deacetylation, is an interesting candidate in this respect. Chitosan has an in vitro antibacterial effect on *S. mutans* [8, 13], *Actinobacillus actinomycetemcomitans* [8], and *Porphyromonas gingivalis* [15]. It was also reported that low-molecular-weight chitosan prevents the adsorption of *S mutans* to hydroxyapatite [35, 36]. Moreover, chitosan has been proposed as a bioadhesive polymer that provides an extended retention time on the oral mucosa [22]. Decker et al. [10] reported the synergistic antiplaque effect of chlorhexidine/chitosan combination, based on the bioadhesive property of chitosan.

Despite these potent antibacterial and antiplaque properties, the application of chitosan as a chemical agent for mouthwashes or dentifrices was limited because of its insolubility in water and incompatibility with mouthwash

or dentifrice formulation. In the previous studies, several investigations have been carried out to increase its solubility in water to broaden its application [14, 34, 37]. Water-soluble *N*-alkylated disaccharide chitosan derivatives showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* [39]. However, few studies on the antibacterial effect of water-soluble chitosan have been made in the dental field [13].

We developed water-soluble reduced chitosan through reduction process using acetic anhydride. The water-soluble chitosan used in the present study has a structural formula as follows.



The purpose of the present study was to examine the effects of newly developed water-soluble reduced chitosan on *S. mutans*, plaque regrowth, and biofilm vitality.

Materials and methods

Bacteriological tests

Test agents

Water-soluble reduced chitosan (Youngchito, Korea), obtained from chitin extracted from red crab shells, was dissolved in distilled water (10 g/l). This 1.0% chitosan, with pH ranging from 6.0 to 6.5, molecular weights between 3,000 and 5,000 Da, and 70% degree of deacetylation, was used in the present study. The negative control was distilled water (DW), and the positive control was 0.1% chlorhexidine digluconate (CHX; Corsodyl, Germany).

Bacterial strains and growth condition

S. mutans (ACTT 25175) and *Streptococcus sanguinis* (ACTT 10556) were used. The streptococci were cultured in Trypticase Soy Broth (BD, USA) supplemented 0.5% Yeast-Extract (TSB-YE) in a 5% CO_2, 95% air incubator (Forma Scientific, USA) at 37°C.

Determination of minimal inhibitory concentrations

Microorganisms were cultured as described above. Cells were harvested by centrifugation (5,856×*g*, 4°C, 15 min) and diluted in fresh media to obtain starting assay concentrations of 5×10^5 cfu/ml. Estimates of viable cell concentration were ascertained by reference to predetermined standard curves equating absorbance (660 nm) to log colony-forming units per milliliter (cfu/ml). Appropriate dilutions were made to obtain starting assay concentrations of 5×10^5 cfu/ml. Dilution plating was performed using a spiral plating system to determine the actual number of viable cells in the inocula. Chitosan was serially diluted (twofold increments) in sterile distilled water. The components of the assay were: (1) 2.0 ml 2× strength growth medium, (2) 2.0 ml diluted chitosan or DW (control), and (3) 50 μl microorganism inoculum. Each dilution of chitosan and the controls were assayed in triplicate. The assay tubes were incubated under appropriate atmospheric conditions for 48 h. Culture turbidity was assayed by spectrophotometer (UV-1201, Shimadzu, Japan). The minimal inhibitory concentration (MIC) was defined as the lowest dilution of chitosan that restricted growth to a level ≤0.05 absorbance at 660 nm.

Short-term exposure to test agents [11]

S. mutans was cultured in TSB-YE in 5% CO_2 at 37°C for 24 h. The cells were harvested by centrifugation (5,856×*g*, 4°C, 15 min) and diluted in fresh media to obtain starting assay concentrations of 5×10^5 cfu/ml. Estimates of viable cell concentration were ascertained by reference to predetermined standard curves equating absorbance (660 nm) to log colony-forming units per milliliter. Five hundred microliters of chitosan and chlorhexidine were added to achieve a twofold and an eightfold dilution, respectively, in 500 μl of 2× strength growth media. Negative control cells received 500 μl of DW in 500 μl 2× strength growth media. Ten microliters of *S. mutans* suspension was inoculated in the media. The suspensions were incubated for 5 min under appropriate atmospheric conditions. The cells were then harvested again by centrifugation and resuspended in 50 ml of fresh media. The culture tubes were incubated under identical conditions as above. At different time intervals, samples were removed and measured in a spectrophotometer at 660 nm. Each sample was assayed in triplicate.

Clinical study

The clinical study consisted of a 6-week, double blind, randomized, clinical trial using the classical 4-day plaque regrowth design [3, 5]. This study was approved by the Institutional Review Board of Pusan National University (IRB of PNU).

Study population

Based on informed and written consent (Declaration of Helsinki, 2000), 12 dental students of PNU (South Korea) volunteered to participate in this study. Criteria for exclusion were the use of antibiotics during the last 3 months or other antibacterial medicaments that could

Table 1 Study design and schedule

		Test cycle 1			Test cycle 2			Test cycle 3		
Investigational events	Day 0	Day 7	Day 8	Day 11	Day 21	Day 22	Day 25	Day 35	Day 36	Day 39
Dental status	x									
Personal data	x									
Medical history	x									
Professional toothcleaning	x	x		x	x		x	x		x
Plaque index (PI)			x	x		x	x		x	x
Vitality (VF)			x	x		x	x		x	x
Side effects		x	x	x	x	x	x	x	x	x
Gingival index	x	x		x	x		x	x		x

have affected plaque growth, less than 20 teeth available for evaluation, fixed or removable orthodontic appliances, partial dentures, known allergy against test agents, pregnancy, and an age<18 years.

Study design

Table 1 shows the flowchart of the study. After exact anamnesis, all volunteers received a professional prophylaxis (day 0), a toothpaste (Bukwang, Korea), and a toothbrush (Yechiwon, Korea), which they were obliged to use during a 1-week hygiene phase before the test weeks and during the washout times. At the beginning of every test week (days 7, 21, and 35), a further professional tooth cleaning was conducted as well as the supply of the test products in a randomized, 3×4 latin square design [26, 27]. For the following four test days, the participants had to refrain from all mechanical oral hygiene measures. Flossing and chewing gums were not allowed, as well. Instead, every volunteer had to rinse with 15 ml of the allocated mouthrinse solution twice daily (in the morning and the evening after meals) for 1 min. The first rinse was supervised; the following eight ones were conducted at home. After every test week, there was a 10-day washout period to eliminate the antimicrobials, in which the subjects could follow their personal oral hygiene measures (also flossing) with the allocated toothpaste. The use of antibiotics or other antibacterial medicaments had to be indicated and led to exclusion from the study. On every visit, side effects or adverse events were recorded.

Clinical evaluation

Plaque index (PI) [33] was assessed on days 8, 22, and 35 from three surfaces (mesiobuccal, midbuccal, and distobuccal) of teeth 14–17 and 44–47 (PI 1) and on days 11, 25, and 39 from the same surfaces of teeth 24–27 and 34–37 (PI 2) to avoid disturbance of plaque regrowth for the estimation of the other parameter. Additionally, the gingival index (GI) [21] was recorded at the gingival margin of all teeth on days 7, 11, 21, 25, 35, and 39 as a control parameter for the gingival health of the subjects during the whole test period (Table 1). GI was not used to measure the antiplaque effect and biofilm vitality.

Microbiological evaluation

On days 8, 22, and 36 (about 2 h after the morning rinse) supragingival plaque samples were taken from buccal surfaces of teeth 16 and 46 (vital fluorescence, VF 1) and from teeth 26 and 36 on days 11, 25, and 39 (VF 2) with a probe (PCP 11, Hu-Friedy, Germany). After mounting them on a glass slide, the samples were immediately vital stained with fluorescein diacetate, which gave a green color to the living microorganisms and ethidium bromide, which introduced a red color into the nucleic acids of the dead bacteria [24, 25]. The samples were examined by one investigator, with the intraindividual variation always remaining below 5%. The vitality of the ex vivo biofilm was recorded with a fluorescence microscope using a counting grid [6, 25]. Total of 105 small squares analyzed for each sample at a magnification of 400× under a

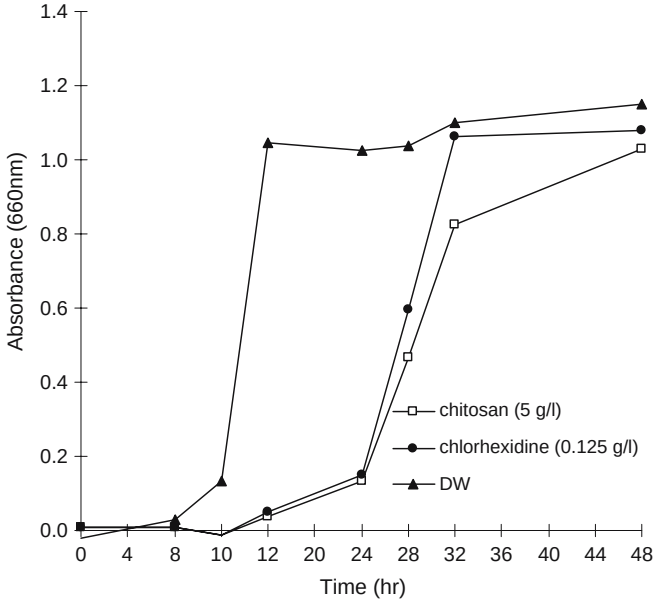


Fig. 1 Short-term (5 min) exposure of *S. mutans* to water-soluble reduced chitosan

Table 2 Mean plaque index after 24 (PI 1) and 96 h (PI 2)

	PI 1		*p* values compared to DW/CHX	PI 2		*p* values compared to DW/CHX
	Mean	SD		Mean	SD	
DW	0.89	0.32		2.39	0.45	
Chitosan	0.65	0.29	0.014/0.002	1.85	0.74	0.006/<0.001
CHX	0.32	0.29	<0.001/–	0.75	0.65	<0.001/–

DW Distilled water, *CHX* chlorhexidine digluconate

fluorescence microscope (BX-41, Olympus, Japan). The percentage of vital bacteria in the entire sample (VF) was calculated.

Statistical evaluation

After completion of the study statistical analysis was performed using the software program Statistical Package of Social Science/SPSS 11.0. Mean values and standard deviations of the PI and VF were calculated. The differences of PI and VF between groups were analyzed using paired *t* test. Bonferrroni adjustments were not conducted [28].

Results

Bacteriological tests

The MIC of water-soluble reduced chitosan for *S. mutans* and *S. sanguinis* was 1.25 g/l and 10.0 g/l, respectively. *S. mutans* tended to be more susceptible to chitosan than *S. sanguinis*. The results of the short-term exposure experiments with *S. mutans* are shown in Fig. 1. While the negative control cells (exposed to DW) grew rapidly with a maximum turbidity reaching 16 h postinoculation, *S. mutans* exposed to chitosan (5.0 g/l) or chlorhexidine (0.125 g/l) exhibited a substantial delay in growth and reached a maximum turbidity by 32 h postinoculation.

Clinical study

The 12 volunteers (nine men and three women) with a mean age of 24 years (range 21 to 30 years) completed the study. The compliance estimated by measuring the used solutions in the returned bottles was very good. No side effects occured during the study in any of the groups. The mean GI was less than 0.2 during the three test cycles, so that healthy gingival conditions during the clinical trial can be assumed. Both the chitosan and chlorhexidine solution were active to reduce PI and VF compared to distilled water (Tables 2 and 3).

The chitosan solution reduced PI after 24 h to 0.65 (p=0.014, compared to DW) and after 96 h to 1.85 (p=0.006), while the CHX solution reached a PI of 0.32 (p<0.001) and 0.75 (p<0.001), respectively. The VF of the plaque biofilm was reduced after 24 h from 36.9% (DW) to 27.1% (VF1; p=0.025) and after 96 h from 49.4 to 40.9% (VF2; p=0.041), while the CHX solution reached a VF of 13.9% (p<0.001) and 20.9% (p<0.001), respectively.

Discussion

Determining the MIC of an antimicrobial is a classical method of assessing the potency of a compound. The MIC value obtained for chitosan with *S. mutans* was about two times less than the value reported in previous researches [35, 36]. While measuring MIC, it yielded valuable information on the susceptibility of *S. mutans* to chitosan. It can be argued then that *S. mutans* would never be exposed to a constant concentration of chitosan in a mouthrinse for a long period of time such as in the MIC. Hence, the present study showed that chitosan solution (5 g/l) and chlorhexidine (0.125 g/l) exhibited the same patterns of delay of growth. Therefore, it can be suggested that a one-time, 5-min exposure to chitosan had a major impact on the ability of *S. mutans* to grow.

Chlorhexidine MIC for *S. mutans* have been reported to be no more than 1 mg/l [16], which is thousands times lower than chitosan MIC measured in the present study. In a 5-min exposure study, chitosan at a concentration of 5 g/l, which is 40 times higher than the concentration of CHX (0.125 g/l) showed the same patterns of delayed growth. Based on such results of in vitro study, the optimal concentration of chitosan solution was determined be

Table 3 Mean vitality after 24 h (VF 1) and 96 h (VF 2)

	VF 1		*p* values compared to DW/CHX	VF 2		*p* values compared to DW/CHX
	Mean	SD		Mean	SD	
DW	36.88	6.86		49.37	6.79	
Chitosan	27.10	13.66	0.025/0.010	40.91	11.18	0.041/0.001
CHX	13.87	3.74	<0.001/–	20.86	10.57	<0.001/–

DW Distilled water, *CHX* chlorhexidine digluconate

4.0%, which is 40 times higher in concentration than CHX used widely in clinical dental practice, but finally we used 1.0% chitosan solution in the clinical trial considering mechanical properties such as viscosity and taste.

The antibacterial and antiplaque effect of chitosan solution in the clinical trial was assessed with the relative changes of plaque index and the percentage of vital microorganisms in plaque biofilm after rinsing. The experimental design of this study had already been used and validated in several other short-time plaque regrowth studies [5, 23, 26, 27].

The test agent, water-soluble reduced chitosan, clearly showed a clear inhibition of the plaque regrowth compared to DW. Other plaque indices, requiring a staining of plaque, such as the Turesky [38] modification of the Quigley and Hein plaque index, could not be used due to influence on the further regrowth during the next test days.

The vital fluorescence technique offers the opportunity to differentiate between dead and vital bacteria and to prove the bactericidal effect of a mouthrinse in vivo [7, 24, 25]. The VF value of chitosan was significantly lower than that of DW. Chitosan solution seems to possess bactericidal effects.

Placebo DW showed lower VF during 4 days of rinsing than that in the previous study [3]. These results are presumably caused mainly by differences in the criteria used by the examiners between the two studies but partly by dietary differences between Germans and Koreans. Korean meals comprise rice and various side dishes. Of these, a main side dish is Kimchi, and lots of garlic, green onions, and peppers were used in other side dishes. These things have frequently been reported to have considerable antimicrobial effects [2, 9, 17, 19].

The postulated mechanism of action may consist of enzyme inactivation, chelation of essential metal ions, and formation of polyelectrolyte complexes with bacterial surface compounds [36]. An ionic interaction between the cations due to the amino groups of chitosan and anionic parts of bacterial cell wall, such as phospholipids and carboxylic acids, has also been proposed as the mechanism for the antimicrobial activity of chitosan [32]. Further studies were required to explain the mechanism of the antibacterial activity of water-soluble reduced chitosan. The long-term effect of water-soluble reduced chitosan on caries and the synergistic effect between water-soluble reduced chitosan and fluoride or other chemical agents for caries prevention need to be analyzed.

It can be concluded that the water-soluble reduced chitosan exhibited potent antibacterial effect on *S. mutans*, and it displayed a significant antibacterial and plaque-reducing action during 4-day plaque regrowth.

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