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RESEARCH ARTICLE

Effects of Bamboo Salt with Sodium Fluoride on the Prevention of Dental Caries

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Background: Dental caries is one of several prevalent oral diseases caused by dental plaque biofilms. This study evaluated the anti-cariogenic effects of a bamboo salt (BS) and sodium fluoride (NaF) mixture on oral bacteria.

Methods: The effects of several mixtures of NaF and BS on acid production, growth, and adhesion to glass beads of *Streptococcus mutans*, and their anti-cariogenic properties were investigated. The growth of *S. mutans* was measured according to optical density at 3, 6, 9, 12, 15, 18, and 24 hours after treatment using spectrophotometry at a wavelength of 600 nm, while pH was measured using a pH meter. Adhesion of *S. mutans* was measured according to the weight of glass beads from each group before and after incubation. Gene expression was measured using real-time polymerase chain reaction. Acid production and growth patterns of *S. mutans* were compared using repeated measures analysis of variance, followed by Scheffe's post-hoc test. The Kruskal-Wallis test was used to compare adhesion, followed by the Mann-Whitney test. Gene expression in the experimental and control samples was compared using the Student's t-test.

Results: Growth, acid production, and adhesion of *S. mutans* were inhibited in all experimental groups. Expression of *gft* and fructosyltransferase in *S. mutans* was inhibited in all groups. A mixture of NaF and BS significantly reduced growth, acid production, adhesion, and gene expression of *S. mutans* compared with the other groups.

Conclusion: Results of the present study demonstrated that a mixture of NaF and BS was useful as a mouth rinse in preventing dental caries.

Key Words: Bamboo salt, Dental caries, Sodium fluoride, *Streptococcus mutans*

Introduction

Dental caries is defined by dissolution of the tooth surface by acid produced from cariogenic microorganisms, especially *Streptococcus mutans*[1]. One significant characteristic of *S. mutans* in caries development is its ability to adhere to the tooth surface. *S. mutans* produces extracellular glucosyltransferase (*gtf*B, *gtf*C, *gtf*D, and fructosyltransferase (*ftf*) in the presence of sucrose, which are important in the formation of plaque and in the processes leading to dental caries[2]. To prevent dental caries and periodontal disease, it is essential to inhibit the formation of dental plaque on tooth surfaces. In this context, antimicrobial agents could serve as a valuable complement to mechanical plaque removal.

Bamboo salt (BS) is a Korean traditional salt. It is prepared by packing bay salt in bamboo, then baking it nine times in high heat using pine firewood. Through this process, the impurities in the bay salts are removed while its inorganic contents, including calcium, potassium, copper and zinc ions, and alkalinity, are increased compared to sun-dried salts (SDS)[3]. BS is known to have therapeutic effects for ailments including viral and bacterial infections, and inflammatory disorders[4]. The remineralization effects of BS on incipient artificial enamel caries have also been described[5]. Thus, BS can be considered a

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useful anti-microbial agent for the inhibition of dental plaque.

Sodium fluoride (NaF) is considered to play an important role in the prevention of dental caries, primarily due to its remineralization effect on decalcified tooth surfaces. Another advantage of fluoride is its ability to decrease acid formation in dental plaque, especially by *S. mutans*. Several studies investigating the effect of mouth rinses of diverse chemical composition(s) demonstrated that compound substances of NaF and other chemical agents are able to inhibit the metabolic activity of micro-organisms in the dental biofilm[6-8]. The combination of BS and NaF is used as a component of dentifrice for the prevention of dental caries in Korea. A previous report described the remineralization effect of the combination of BS and NaF[5]; however, no study has determined its anti-microbial activity and mechanism of action in the inhibition of *S. mutans*, which is a pathogenic oral micro-organism. As such, studies are needed to evaluate the anti-microbial activity and the mechanism of the anti-microbial effect of the combination of BS and NaF on *S. mutans*.

The objective of the present study, therefore, was to investigate the anti-microbial effect of NaF and BS, and whether NaF and BS can modify the cariogenic properties of *S. mutans*, including growth, acid production, and adhesion.

Materials and Methods

1. Preparation of solution

BS was provided by Insanga Food Inc. (Insanga Bamboo salt 9; Insanga, Hamyang, South Korea). BS (3% and 5%) was used in this study, and has previously been shown to inhibit the growth of *S. mutans* effectively[9]. NaF was purchased from Sigma-Aldrich (St. Louis, MO, USA). NaF (0.02% and 0.05%) was used in this study as a mouth rinse solution.

2. Measurement of *Streptococcus mutans* growth and acid production

S. mutans, American Type Culture Collection accession number 31,989, was used in this study. *S. mutans* was inoculated in brain heart infusion (BHI; Difco, Detroit, MI, USA) broth and incubated at 37°C for 24 hours. The optical density (OD; i.e., absorbance) of each culture was then measured at a wavelength of 600 nm to calculate the number of *S. mutans* to use in the experiments (1.6×10^8 colony forming units/ml).

BHI broth containing 10% glucose and different concentrations of NaF and BS mixture (3% BS, 0.02% NaF, 0.05% NaF, 0.02% NaF+3% BS, and 0.05% NaF+3% BS) was prepared. The growth of *S. mutans* was measured at 3, 6, 9, 12, 15, 18, and 24 hours using spectrophotometry (EZ Read 400; Biochrom, Cambridge, UK) according to OD at a wavelength of 600 nm (OD_{600}). pH was measured at 2, 4, 8, 12, 16, 20, and 24 hours using a pH meter (920A; Thermo Orion, Beverly, MA, USA).

3. Analysis of adherence properties

BHI agar broth containing 10% glucose and different concentrations of NaF and BS mixture (3% BS, 0.02% NaF, 0.05% NaF, 0.02% NaF+3% BS, and 0.05% NaF+3% BS) was prepared. The weight of glass beads for each group was measured, and then 5 ml of experimental solution was poured onto the beads in each group. The mixture was incubated at 37°C for 24 hours and the experimental solution was removed, followed by addition of 75% ethanol to the plate. After drying, the weight of the glass beads was measured[10]. The experiment was performed in triplicate.

Table 1. Nucleotide Sequence of RT-PCR Primers for Genes

Gene description	Direction	Nucleotide sequence (5'-3')
*gtf*B	Forward	AGCAATGCAGCCATCTACAAAT
	Reverse	ACGAACTTTGCCGTTATTGTCA
*gtf*C	Forward	GGTTTAACGTCAAAATTAGCTGTATTAGC
	Reverse	GGTTTAACGTCAAAATTAGCTGTATTAGC
*gtf*D	Forward	CACAGGCAAAAGCTGAATTAACA
	Reverse	GAATGGCCGCTAAGTCAACAG
ftf	Forward	AATCCCTATCAACCTCGACTGC
	Reverse	GCCTTTCTCCTGCAACCAAATC
16s rRNA	Forward	CCTACGGGAGGCAGCAGTAG
	Reverse	CAACAGAGCTTTACGATCCGAAA

RT-PCR: reverse transcription polymerase chain reaction, *ftf*: fructosyltransferase.

4. Measurement of bacterial gene expression using real-time polymerase chain reaction

Total RNA was purified using a commercially available kit (RNeasy Mini Kit; Qiagen, Venlo, The Netherlands). The primer sequences are provided in Table 1. Polymerase chain reaction conditions included an initial denaturation at 95°C for 15 minutes, followed by 40-cycle amplification consisting of denaturation at 94°C for 15 seconds and primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The expression levels of *gtf*B, *gtf*C, *gtf*D, and *ftf* were normalized using the 16S ribosomal RNA gene as an internal standard.

5. Statistical analysis

Data analysis was performed using SPSS ver. 18.0 (IBM Corp., Armonk, NY, USA). Repeated measures ANOVA was used for acid production and growth patterns of *S. mutans*, followed by Scheffe's post-hoc test. Adhesion was analyzed using the Kruskal-Wallis test, followed by the Mann-Whitney test. Differences in gene expression in the experimental and control samples were analyzed using the Student's t-test.

Table 2. Change of pH in Culture Medium after the Incubation

Time (h)	Group*					
	Control (BHI)[a]	3% BS[d]	0.02% NaF[b]	0.05% NaF[c]	0.02% NaF+3%BS[e]	0.05% NaF+3%BS[e]
0	7.18±0.00	7.35±0.00	7.20±0.01	7.18±0.00	7.33±0.01	7.35±0.00
2	7.18±0.00	7.35±0.00	7.16±0.00	7.15±0.00	7.31±0.01	7.31±0.01
4	7.09±0.00	7.26±0.00	7.14±0.00	7.15±0.00	7.29±0.00	7.28±0.00
8	7.01±0.00	7.25±0.00	7.08±0.00	7.15±0.00	7.24±0.00	7.25±0.01
12	6.71±0.01	7.24±0.00	6.95±0.01	7.15±0.00	7.24±0.00	7.24±0.01
16	5.30±0.00	7.21±0.03	6.82±0.00	7.12±0.01	7.25±0.01	7.26±0.00
20	4.30±0.00	7.08±0.00	6.71±0.00	7.08±0.01	7.18±0.00	7.22±0.02
24	4.18±0.01	7.08±0.01	6.58±0.01	7.08±0.01	7.18±0.00	7.21±0.01

Values are presented as mean±standard deviation.
BHI: brain heart infusion, BS: bamboo salt, NaF: sodium fluoride.
*$p < 0.01$, by repeated measures ANOVA.
[a,b,c,d,e]The same letter indicates no significant difference by Scheffe.

Table 3. Inhibitory Effects of BS and NaF on the *Streptococcus mutans* Growth

Time (h)	Group*					
	Control (BHI)[a]	3% BS[b]	0.02% NaF[b]	0.05% NaF[c]	0.02% NaF+3%BS[c]	0.05% NaF+3%BS[d]
0	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.00
3	0.06±0.00	0.06±0.00	0.06±0.00	0.07±0.01	0.06±0.00	0.06±0.00
6	0.07±0.00	0.07±0.00	0.07±0.00	0.07±0.00	0.07±0.00	0.06±0.00
9	0.20±0.00	0.10±0.00	0.15±0.00	0.09±0.01	0.09±0.00	0.06±0.00
12	0.31±0.00	0.22±0.01	0.22±0.00	0.11±0.00	0.13±0.00	0.07±0.00
15	0.29±0.01	0.24±0.00	0.22±0.00	0.14±0.01	0.16±0.00	0.07±0.00
18	0.28±0.02	0.24±0.01	0.21±0.00	0.14±0.01	0.16±0.00	0.07±0.00
21	0.28±0.00	0.21±0.02	0.20±0.00	0.14±0.01	0.15±0.00	0.07±0.00
24	0.28±0.00	0.22±0.00	0.19±0.00	0.14±0.00	0.15±0.01	0.07±0.00

Values are presented as mean±standard deviation.
BHI: brain heart infusion, BS: bamboo salt, NaF: sodium fluoride.
*$p < 0.01$, by repeated measures ANOVA.
[a,b,c,d]The same letter indicates no significant difference by Scheffe.

Results

1. Effects of NaF and bamboo salt on the acidogenicity of *Streptococcus mutans*

After 24 hours, the pH value of all experimental groups was >6.5 and exhibited significantly increased values compared with the control group. pH reduction was lower in the 0.02% NaF+3% BS and 0.05% NaF+3% BS groups compared with the other groups ($p<0.01$) (Table 2).

2. Inhibitory effects of NaF and bamboo salt on the growth of *Streptococcus mutans*

After 24 hours, the growth of *S. mutans* was inhibited by 50% and 75% compared with that of the control group when treated with 0.02% NaF+3% BS and 0.05% NaF+3% BS, respectively. The inhibitory rates were 25% and 50%, when treated with 0.02% NaF and 0.05% NaF, respectively ($p<0.01$) (Table 3).

3. NaF and bamboo salt inhibited *Streptococcus mutans* adhesion to glass beads

The effect of NaF and BS on the adhesion of *S. mutans* to glass beads was examined. The combination of 0.05% NaF+3% BS significantly inhibited the adhesion of *S. mutans* (Table 4).

4. Expression of *gtf* and *ftf*

The expression of *gtf* and *ftf* was decreased in the NaF and BS group compared with the control group (Fig. 1). Messenger RNA expression of *gtf*B, *gtf*C, *gtf*D, and *ftf* was decreased in the NaF and BS group compared with the control group.

Discussion

Dental caries is caused by bacteria in dental biofilms. Pathogenic bacteria can adhere to tooth surfaces using an extracellular polymer termed the “glycocalyx”, leading to biofilm formation[2]. Because *S. mutans* has been established as the primary causative factor of dental caries, inhibiting *S. mutans* adhesion to the tooth surface could be one major approach to the prevention of dental caries[11]. In recent years, several anti-caries agents, such as NaF, tea polyphenols, triclosan and chlorhexidine, have been widely studied for preventing dental caries through the inhibition of biofilm formation[12-14]. In this study, we evaluated the

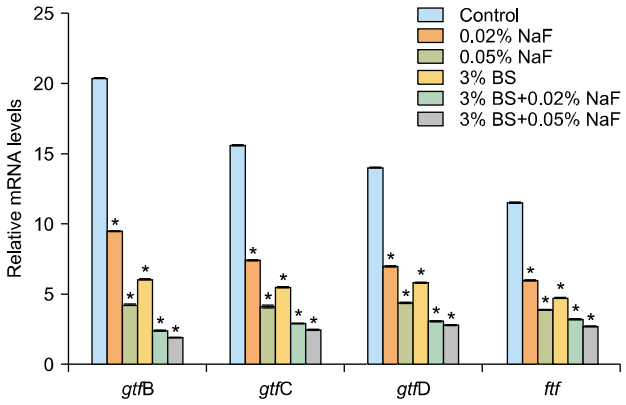

Fig. 1. Effects of bamboo salt (BS) and sodium fluoride (NaF) on *gtf* and fructosyltransferase (*ftf*) expression in *Streptococcus mutans*. *p <0.05.

Table 4. The Adhesion of *Streptococcus mutans* to Glass Beads

Group	Weight of glass beads (mg)		Δm* (after-before)
	Before	After	
Control (BHI)	813.92±29.54	815.01±29.53	1.08±0.09[a]
0.02% NaF	803.27±70.86	803.93±70.83	0.66±0.14[b]
0.05% NaF	818.38±79.48	818.85±70.83	0.46±0.19[c]
3% BS	807.88±66.29	808.45±66.33	0.56±0.19[bc]
0.02% NaF+3% BS	805.08±41.88	805.43±41.97	0.35±0.17[cd]
0.05% NaF+3% BS	774.70±56.59	774.95±56.56	0.25±0.07[d]

Values are presented as mean±standard deviation.
BHI: brain heart infusion, NaF: sodium fluoride, BS: bamboo salt.
*$p<0.05$, by Kruskal-Wallis test.
[a,b,c,d]The same letter indicates no significant difference by Mann-Whitney test.

anti-microbial effect of NaF and BS, and whether a mixture of NaF and BS could modify the cariogenic properties of *S. mutans* for prevention of dental caries was investigated.

BS has been known to have therapeutic effects on dental caries, inflammation, and viral infectious diseases[3,5]. The majority of table salts in Korea are purified salt (PS) and SDS. However, the quality of SDS is not adequately pure possibly due to seawater contamination, and PS is known to have a different component of biological electrolytes[15]. These facts have led to increasing use of "health-promoting" salts, such as BS, by the general population. Incipient artificial enamel lesions treated with BS exhibited increased surface hardness and mineral levels. The mixture of NaF and BS resulted in significantly increased remineralization effects on artificial enamel caries[5].

Fluoride interferes with bacterial metabolism and inhibits bacterial growth[16]. This study also found that fluoride reduced *S. mutans* growth and acid production. Although the results demonstrated the inhibitory effect of NaF treatment on *S. mutans*, it is indefinite whether the treatments physiologically affected the function of other bacterial-related factors. As such, further studies are required to investigate bacterial interactions among dental biofilm microorganisms when NaF and BS are administered. This may lead to a deeper understanding of the bacterial community involved in dental caries pathology[17].

One important factor inducing dental caries is acidity. The pH differences in BS- and NaF-treated *S. mutans* cultures were compared with those grown in BHI. The pH of BS-treated samples was similar to that of 0.05% NaF-treated bacterial cells; however, the mixture of NaF and BS led to a slight decrease in pH compared with samples treated with BS or NaF alone. It has been reported that the pH of BS can influence anti-inflammatory activity[3]. The BS and NaF mixture also resulted in a reduction of acid production. Thus, a combination of BS and NaF would result in increased anti-cariogenic activity.

In this study, it was found that various cariogenic conditions, such as acidogenicity and bacterial adhesion, were inhibited by treatment with NaF and BS. The NaF and BS group inhibited *S. mutans* growth and adhesion. Especially, the group with mixture of BS and NaF exhibited inhibitory effects that were more effective on the growth and adhesion of *S. mutans* than the BS, NaF, and control groups did. The mixture suppressed the expression of *S. mutans* biofilm-related *gtf*B, *gtf*C, *gtf*D, and *ftf* genes (Fig. 1). Among them, the expression of *gtf*B demonstrated the greatest reduction in the 0.05% NaF+3% BS mixture. *gtf*B is an important virulence gene associated with the pathogenesis of biofilm formation and dental caries[18]. More detailed gene expression profiling, however, is needed for a deeper understanding of the molecular mechanisms involved in the synthesis of biofilms in the presence of NaF and BS.

The 0.02% NaF+3% BS mixture was found to effect the activity of *S. mutans* by four routes: reduced adhesion of *S. mutans* to glass beads after incubation; reduced growth of *S. mutans*; repressed acid production by *S. mutans*; and modulation of the expression of specific genes including *gtf*B, *gtf*C, *gtf*D, and *ftf*. These findings demonstrate that a combination of NaF and BS can inhibit the cariogenic activity of *S. mutans*, and that a mouth rinse consisting of this mixture may be useful in preventing dental caries.

Notes

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Ethical approval

This project does not require IRB review because it is an experimental paper using commercially available microorganisms.

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