OPEM

Oriental Pharmacy and Experimental Medicine 2003 **3(1)**, 40-45

Effect of Bamboo salt-pro on carries-inducing properties of *Streptococcus mutans*

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SUMMARY

We studied the effect of Bamboo salt-pro on the growth and acid production of *S. mutans*. The growth of *S.mutans* was reduced by the presence of the Bamboo salt-pro (1 mg/ml) and NaCl (1 mg/ml) significantly, and the positive control group (1% of NaF) also exhibited antibacterial activity significantly. Bamboo salt-pro (1 mg/ml) reduced the rate of acid production by *S. mutans*. Bamboo salt alone did not demonstrate such a reduction in acid production at the concentration of 1 mg/ml. The inhibitory action of Bamboo salt-pro on acid production was found at a concentration of 1 mg/ml, but bamboo salt alone was not at a concentration of 1 mg/ml. In addition, we investigated the anti-inflammatory effect of Bamboo salt-pro on human mast cell line HMC-1. Bamboo salt-pro (0.1 and 0.01 mg/ml) inhibited significantly the secretion of inflammatory cytokine, tumor necrosis factor-a with 59.47±0.15%, 51.98±0.16% respectively. Our results suggest that Bamboo salt-pro importantly contributes to the prevention or treatment of periodontitis and other oral diseases and inflammatory diseases.

Key Words: Bamboo salt-pro; S. mutans; Periodontitis; Tumor necrosis factor-α

INTRODUCTION

Dental caries and periodontal disease are the most common chronic diseases in the dental fields (Shani *et al*., 2000; Oh *et al*., 2002). Because of increasing sugar consumption and extension of average human life, these diseases are widely spread all over the world as the most typical cause for a person to lose a tooth. Dental caries is a disease in which the hard tissues of tooth are gradually destroyed by bacteria (Hamada and Torii, 1980). Periodontal disease is a chronic disease that soft tissues and alveolar bone around the teeth are destroyed by host inflammatory response (Schwartz *et al*., 1997). It causes the bleeding in the gingiva, mobility of the teeth, and ultimately losing the teeth. Dental caries and periodontal disease are mainly caused by the dental plaque, and a lot of bacteria inhabit in the dental plaque. The bacteria in dental plaque metabolize carbohydrates to form organic acids, which destroy the hard tissues of tooth and result in dental caries, and the periodontal disease is caused by the inflammatory response of human body on the bacteria (Twetman and Lindqvist, 1985; Schwartz *et al*., 1997). *Streptococcus mutans* (*S. mutans*) is the most important bacteria in the formation of dental plaque and dental caries (Wiater *et al*., 1999). It is a G (+) factultative anaerobic bacteria which is settled in the dental plaque. *S. mutans* metabolizes the carbohydrates contained in the foods and releases the organic acids, which demineralize tooth surface (Twetman and Lindqvist, 1985; Kohler *et al*., 1995).

Previous reports have shown that the antibiotics such as penicillin and erythromycin are effective in inhibiting *S. mutans* for the prevention of dental caries and periodontal disease, but they are not used in dental clinics due to the development of antibiotic resistance for long-term use (Namba *et al*.,

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1982). The fluoride compounds also have been used in the control of the dental plaque (Guha-Chowdhury *et al*., 1995), but it show the cytotoxicity if it is used at the concentration over 80 ppm (Jeng *et al*., 1998).

In addition, several mouth rinses have been developed as the agents for prevention of dental caries and periodontal disease (Marsh, 1993; Pan *et al*., 1999), but the fact that dental caries and periodontal disease are still major causes of tooth lose is the evidence that these agents are not sufficiently effective. Therefore, development of more effective, practical, and safe preventive agents for dental caries and periodontal disease are needed.

Previous reports have shown that several natural products are candidate substances that could be used as the preventive agents for dental caries and periodontal disease (Namba *et al*., 1982; Wu-Yuan *et al*., 1988). Bamboo salt-pro has been used in traditional folk medicine for treatment of dental caries and periodontal disease, and is still used as the tooth pastes and mouth rinse agents in Korea. However, there is no scientific evidence about the effect of Bamboo salt-pro on dental caries and periodontal disease.

Cytokines produced in response to plaque bacteria clearly play a key role in the periodontal diseases (Fletcher *et al*., 1997). Tumor necrosis factor-a (TNF-a) is a pleiotropic cytokine capable of altering physiological and immunological sequelae as well as mediating the pathophysiological responses of various disease conditions (Lederer *et al*., 1995). TNF-a seems to be a target in therapy of inflammatory and immune diseases (Choi *et al*., 2001). Human mast cell line (HMC-1) is known to release pro-inflammatory cytokines such as TNF-α, IL-1α and β, and IL-6 (Grabbe *et al*., 1994).

This paper deals with an anti-bacterial, anti-inflammatory effect of Bamboo salt-pro. We investigated whether Bamboo salt-pro inhibits the growth and acid production of *S. mutans* and secretion of pro-inflammatory cytokine TNF-á from mast cells.

MATERIALS AND METHODS

Preparation of extract

Bamboo salt-pro was composed of Bamboo salt (9 times processing at very high temperature with bay salt, bamboo, pine tree firewood, and yellow earth etc.) 55%, pine resin 30%, xylitol 14%, propolis 1%. Powdered Bamboo salt-pro was solubilized in DMSO and diluted in PBS, filtered through 0.45 mm filter, and kept at 4°C.

Reagents

Brain heart infusion (BHI) broth, Mitis Salivarius agar, and phenol red broth were purchased from Difco Laboratories (Detroit, MI, USA). NaF and glucose were obtained from Sigma (St Louis, MO, USA). Cell culture medium, Iscoves Modified Dulbeccos Media (IMDM) was purchased from Gibco BRL (Grand Island, NY USA). PMA, avidin-peroxidase, 2,2'-azio-bis(3-ethylbenzthiazoline-6-sulfonic acid), 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) and other reagents were obtained from Sigma (St. Louis, MO, USA). Anti-human TNF-α antibody (Ab), biotinylated anti-human TNF-α Aβ, and recombinant human TNF-α were purchased from R&D Systems (Minneapolis, MN).

Bacterial growth inhibition assay

The bacterial growth was determined by using a modification method described previously (Matsumoto *et al*., 1999; Choi *et al*., 2001). The growth of *S. mutans* ATCC 25175 was examined at 37°C in test tubes containing 0.95 ml of BHI broth containing 1% glucose. These tubes were inoculated with 0.1 ml of an overnight culture grown in BHI broth, and incubated at 37°C for 24h. Optical Density (OD) of cells was measured spectrophotometrically at 550 nm. The cell suspensions were removed from tubes, and subjected to ten-fold serial dilutions. Aliquots (100 ml) were spread on Mitis Salivarius Agar (Difco Laboratories) plates, incubated at 37°C for 2 days, and colony forming unit (CFU) was counted. 1% NaF was used as a positive control.

Acid production Assay

To examine the effect of Bamboo salt-pro on acid production by *S. mutans*, acid production assay was determined by using a modification method described previously (Matusumoto *et al*., 1999). The filter-sterilized Bamboo salt compounds were added to 0.95 ml of phenol red broth (Difco Laboratories) containing 1% glucose, which were

then inoculated with 0.05 ml of seed culture of *S. mutans* ATCC 25175. The cultures were incubated at 37°C for 24h, and pH of the cultures was determined using a pH meter (Corning Inc., Corning, NY, USA)

Culture of HMC-1 cells

Human leukemic cell line HMC-1 cells were grown in IMDM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10^{-5} M monothioglycerol and 10% heat-inactivated FBS at 37°C in 5% CO_2 and 95% humidity. Cells were treated Bamboo salt-pro for 30 min prior to stimulation with 20 nM PMA plus 1mM A23187 and incubated at 37°C for 6h.

Assay of TNF-α secretion

TNF-α secretion was measured by modification of an enzyme-linked immunosorbent assay (ELISA) as described previously (Scuderi *et al*., 1986). HMC-1 cells were cultured with IMDM plus 10% FBS and resuspended in Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6Mm glucose, 0.1% bovine serum albumin). The cells were sensitized with PMA (20 nM) plus A23187 (1 μM) for 6 h in the absence or presence of Bamboo salt-pro. The ELISA was sensitive to TNF-α concentrations in the medium above 0.01 ng/ml. For ELISA, was performed by coating 96-well plates (Nunc, Denmark) were coated with 6.25 ng/well of murine monoclonal antibody with specificity for murine TNF-α. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the standard curve, rTNF-α was added to serum previously determined to be negative for endogenous TNF-α. After exposure to the medium, the assay plates were exposed sequentially to biotinylated anti-human TNF-α, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) tablets as substrates. Optical density readings were made within 10 min of the addition of the substrate on a Titertek Multiscan (Flow Laboratories) with a 405 nm filter. Appropriate specificity controls were included.

% Inhibition=(a-b)×100/a

where 'a' is cytokine secretion without Bamboo salt-pro and b is cytokine secretion with Bamboo salt-pro.

Statistical analysis

Each datum represents the meanSEM of the different experiments under the same conditions. The Student¢s *t*-test was used to make a statistical comparison between the groups. The P value less than 0.05 was considered as significant.

RESULTS

Inhibitory effect of Bamboo salt-pro on growth of *S. mutans*

Initially, we investigated antimicrobial activity of Bamboo salt-pro against *S. mutans*. Table 1 shows inhibitory effect of Bamboo salt-pro on OD of bacterial cultures. The growth of *S. mutans* was reduced by the presence of the Bamboo salt-pro (1 mg/ml), NaCl (1 mg/ml) significantly, and positive control group (1% of NaF) also exhibited antibacterial activity. However, the bamboo salt alone did not exhibit antibacterial activity significantly. These results were confirmed by the results expressed as CFU (Table 2).

Table 1. Effect of Bamboo salt-pro on growth of *S. mutans*

Treatment	OD	% of Control
Control	0.5870±0.007	-
Bamboo salt-pro (1 mg/ml)	0.0560±0.005[b]	9.51±3.13[b]
Bamboo salt (1 mg/ml)	0.5835±0.006	99.30±1.00
NaCl (1 mg/ml)	0.3590±0.007[a]	61.97±1.39[a]
NaF (1%)	0.0041±0.005[b]	0.70±0.78[b]

Optical density at 540 nm, values are expressed as the mean±SD, (n=10). [a]$p<0.01$, [b]$p<0.001$

Table 2. Effect of Bamboo salt-pro on CFU of *S. mutans*

Treatment	CFU / ml	% of control
Control	$1.60\times10^{8}\pm0.26$	-
Bamboo salt-pro (1 mg / ml)	0 ± 0.00^{b}	0 ± 0.00^{b}
Bamboo salt (1 mg / ml)	$1.44\times10^{7}\pm0.08^{b}$	8.990 ± 0.55^{b}
NaCl (1 mg / ml)	$1.03\times10^{5}\pm3.42^{b}$	0.063 ± 0.02^{b}
NaF (1%)	$2.20\times10\pm8.16^{b}$	0.014 ± 0.00005^{b}

Colony forming unit, values are expressed as the mean±SD, (n=10). $^{b}p<0.001$

Inhibitory effect of Bamboo salt-pro on acid production by *S. mutans*

To determine whether Bamboo salt-pro inhibits organic acid production in *S. mutans*, the cells were treated with Bamboo salt-pro (1 mg / ml), NaCl (1 mg / ml) and pH was measured. The effect of Bamboo salt-pro on acid production was presented in Table 3. The decrease of pH was significantly inhibited in the presence of Bamboo salt-pro (1 mg / ml) compared to the control group. The decrease of pH was also inhibited in the presence of positive control group (1% of NaF), but the bamboo salt alone did not demonstrate the inhibitory activity. The inhibitory action of Bamboo salt-pro on acid production was found at a concentration of 1 mg / ml, but bamboo salt alone was not at a concentration of 1 mg / ml.

Inhibitory effect of Bamboo salt-pro on inflammatory cytokines secretion from HMC-1 cells

We finally examined the inhibitory effect of Bamboo salt-pro on the PMA plus A23187-stimulated secretion of TNF-α from HMC-1 cells. Culture supernatants were assayed for TNF-α level by ELISA method. As shown in Table 4, Bamboo salt-pro inhibited the secretion of TNF-α in PMA plus A23187-stimulated HMC-1 cells. We also examined Bamboo salt-pro-induced cytotoxicity. MTT assay showed that Bamboo salt-pro did not show cytotoxicity of against HMC-1 cells in a concentration 0.1-0.01 mg / ml (data not shown).

Table 3. Effect of Bamboo salt-pro on acid production by *S. mutans*

Treatment	pH
Control	3.98±0.09
Bamboo salt-pro (1 mg / ml)	7.11 ± 0.07^{b}
Bamboo salt (1 mg / ml)	3.94±0.10
NaCl (1 mg / ml)	3.96±0.11
NaF (1%)	7.03 ± 0.02^{b}

Values are expressed as the mean±SD, (n=10). $^{b}p<0.001$

Table 4. Inhibitory effect of Bamboo salt-pro on PMA plus A23187-stimulated TNF-α secretion from HMC-1 cells

Treatment	Concentration (mg / ml)	Inhibition (%)
Saline	-	-
Bamboo salt-pro	0.01	51.98 ± 0.16^{c}
	0.1	59.47 ± 0.15^{c}
Bamboo salt	1	67.04 ± 0.08^{c}

PMA plus A23187-stimulated HMC-1 cells (3×10^{5}) were incubated for 6 h in the absence or presence of Bamboo salt-pro or purple bamboo salt. TNF-α secreted into the medium are presented as the mean±SEM of three independent experiments.
$^{c}P<0.05$: Significantly different from the saline value.

DISCUSSION

Bamboo salt, components of Bamboo salt-pro, is a specially treated salt according to the traditional recipe using normal salt and bamboo in Korea. It is known to have various therapeutic effects on diseases such as inflammations, viral disease, diabetes, circulation organ disorder and cancer etc (Kim *et al*., 1993; Min *et al*., 1995; Yang *et al*., 1999; Huh *et al*., 2001). Pine resin, a component of Bamboo salt-pro, used for the therapy of burns, wound, and purulent and inflammatory diseases (Simbirtsev *et al*., 2002). Xylitol, a components of Bamboo salt-pro has been successfully used for the treatment of periodontitis and other oral diseases (Jannesson *et al*., 2000). The level of *S. mutans* in the oral cavity is reduced by frequent xylitol exposure, and several clinical studies indicate that the addition of xylitol to chewing gums can contribute to prevention of dental caries (Birkhed *et al*., 1994). Propolis, components of Bamboo salt-pro, has been widely

used in Brazil folk medicine. Propolis and its phenolic constituents were shown to exhibit a variety of pharmacological properities including antiproliferative activity in human tumor cells and anti-inflammatory, antibacterial, antiviral, immunomodulatory, antioxidant and antiprotozoan activities (Starzyk *et al.*, 1977; Grunberger *et al.*, 1988; Grange and Davey, 1990; Krol *et al.*, 1990; Scheller *et al.*, 1990; Dobrowolski *et al.*, 1991; Dimov *et al.*, 1992; Guarini *et al.*, 1992; Serkedjieva *et al.*, 1992; Steinberg *et al.*, 1996; Eley, 1999). During the last decade, new therapeutic substances such as bamboo salt, pine resin and xilitol have been added to dentifrice, in order to improve its clinical effect against periodontal diseases and dental caries. But its mechanism of action still remains unknown. In the present study, we investigated antimicrobial activity of Bamboo salt-pro against *S. mutans*. Our results showed that Bamboo salt-pro inhibited growth and acid production of *S. mutans*. Table 1 showed that the growth of *S. mutans* was reduced by the presence of the Bamboo salt-pro (1 mg/ml), NaCl (1 mg/ml) significantly, and the positive control group (1% of NaF) also antibacterial activity. However, bamboo salt alone did not exhibit antibacterial activity significantly. These results were confirmed by the results expressed as CFU (Table 2). The inhibitory effect of Bamboo salt-pro on acid production was presented in Table 3. The decrease of pH was significantly inhibited in the presence of Bamboo salt-pro compared to the control group (1% of NaF), but the bamboo salt alone did not demonstrate the inhibitory activity.

We examined the effects of Bamboo salt-pro on TNF-α secretion from HMC-1 cells because TNF-α has powerful inflammatory effects and is released by activated mast cells. We have demonstrated that Bamboo salt-pro inhibited secretion of pro-inflammatory cytokine TNF-a in PMA plus A23187-stimulated HMC-1 cells. This result showed that Bamboo salt-pro had effective anti-inflammatory activity.

In conclusion, we demonstrated that the inhibitory effect of the Bamboo salt-pro on the growth and acid production of *S. mutans* as well as secretion of TNF-α from HMC-1 cells. But, bamboo salt alone did not demonstrate the inhibitory activity on growth and acid production of *S. mutans*. Thus the application of Bamboo salt-pro on a daily basis can be considered useful materials for the prevention of dental caries and inflammation.

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