Abstract: The effects of bittern water (BW), obtained from the ocean floor, on cariogenic bacteria and saliva secretion were examined. *Streptococcus mutans* was mixed with BW for 1, 3, 5, 10, and 20 min to explore the bactericidal effects of BW against cariogenic bacteria. Bacterial viability was calculated by counting the number of colony-forming units on Brain Heart Infusion agar plates. The results indicated a bacterial viability of more than 35% even after 20 min of incubation. Subsequently, the effects of BW on saliva secretion and the salivary concentration of secretory IgA (sIgA) were examined. Gargling with BW significantly augmented saliva secretion. Although the sIgA concentration was reduced, the total sIgA secreted into saliva was increased significantly. Our findings indicate that the use of BW may be a new strategy for the treatment of various oral diseases, including dry mouth.

Keywords: bittern water; saliva; secretory IgA; *S. mutans*.

Introduction

Absorption of essential trace elements from foods into the body is essential for maintenance of homeostasis (1). Bittern is obtained by evaporating halite from sea water, and contains various essential trace elements. It is used for a variety of purposes including preparation of the traditional Japanese food, *tofu* (2). Bittern water (BW) is generated from seawater close to the ocean floor, and contains magnesium chloride (MgCl₂) and sodium chloride (NaCl). Although magnesium (the main constituent) is important as a co-factor for various enzymes (3), the direct effects of this ion on microorganisms or the human body are not well documented.

The oral cavity is soaked with saliva, which is produced by the salivary glands (4). The main antibody in saliva, secretory IgA (sIgA), is composed of an IgA dimer, a joining (J) chain and a secretory component (SC), and plays a major role in protecting the oral mucosa (5,6). The two major diseases of the oral cavity, dental caries and periodontitis, are initiated by bacterial infections (7). The most important pathogenic bacteria involved in dental caries is *Streptococcus mutans*, which decalcifies hard tooth tissue with metabolically produced organic acid (8). The protective role of sIgA against *S. mutans* is well documented (9).

In the present study, we attempted to evaluate the
bactericidal effect of BW, and its effect on sIgA secretion into saliva.

Materials and Methods

Reagents

BW was obtained from Ako Kasei Co., Ltd. (Ako, Japan). SureBlue/TMB Peroxidase substrate was purchased from SeraCare Life Sciences (Milford, MA, USA).

Evaluation of the anti-bactericidal effect

*S. mutans* was obtained from the American Type Culture Collection (ATCC) and cultured in Brain Heart Infusion (BHI) medium. The bacterial solution (10 μL) was mixed with 1 mL of BW, and incubated for 1, 3, 5, 10 or 20 min at room temperature (RT). After incubation, the samples (100 μL) were plated onto BHI agar plates and incubated for 48 h at 37°C; subsequently, the numbers of colony-forming units were counted. *S. mutans* treated with phosphate-buffered saline (PBS) was plated in the same way as above and used as a control.

Saliva collection and enzyme-linked immunosorbent assay (ELISA)

The study was approved by the Ethical Committee of Nihon University School of Dentistry (EP16D019) and all experiments were performed in accordance with legal requirements. Saliva samples were collected from ten volunteers, and the details are summarized in Table 1. The samples were diluted with PBS (×3,000) and subjected to ELISA. Rabbit anti-human secretory IgA (Binding Site, Birmingham, UK) was diluted with PBS (×2,000) and coated onto 96-well plates (50 μL) for 18 h at 4°C. After the plates had been blocked with 1% bovine serum albumin-PBS for 2 h, the saliva samples (50 μL) were added and incubated for 1 h at RT. The plates were then washed three times, and further incubated with 50 μL of horseradish peroxidase conjugated rabbit anti-human IgA antibody (DAKO, Tokyo, Japan) for 1 h. After washing, Sure Blue (50 μL) was added to the wells and the plates were incubated for another 30 min. Subsequently, the reaction was stopped and absorbance was measured on a microplate reader model 3550 (Bio Rad, Tokyo, Japan).

Statistical analysis

One-way ANOVA was used for all statistical analyses. Results are presented as mean ± standard deviation (SD) values. Differences at *P* < 0.05 were considered to be statistically significant.

Results

Anti-bactericidal effect of bittern water

To explore the bactericidal effect of BW, *S. mutans* was treated with the liquid for 1, 3, 5, 10, and 20 min. The mixture was plated onto BHI agar plates and the numbers of colonies were counted. The colony count for the untreated control was set at 100%, based on which the viability of each sample was calculated (Fig. 1). The viability was found to decrease as the period of treatment increased (70, 45, 52, 37 and 36% for 1, 3, 5, 10 and 20 min, respectively). Even after 20 min of treatment, viability remained at more than 35%. These results indicated that bittern water has a relatively weak bactericidal effect.

Effect of bittern water on sIgA secretion

To examine the effect of BW on saliva secretion, total saliva was collected 3 min before and after gargling with 25 mL of BW. Sample collection was performed according to the experimental schedule shown in Fig. 2. The mean

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Table 1 Information about the volunteers

PH: past history.

Fig. 1 Bactericidal effect of bittern water (BW). *S. mutans* (1 × 10⁶/10 μL BHI) was mixed with BW (1 mL) and incubated for 1, 3, 5, 10, and 20 min at RT. After incubation, 100 μL of the mixture was plated onto BHI agar plates. The plates were incubated at 37°C for 48 h and then the colonies on the plates were counted. An untreated sample was used as a control and the colony count observed for this sample was set at 100%.
The total volume collected was 1.3 mL before gargling (Fig. 3). Two minutes after gargling, the saliva volume had increased to 2.83 mL. However, the total saliva volume was slightly reduced (1.47 mL) 1 h after gargling (Fig. 3). sIgA concentration was measured by ELISA using the collected saliva samples. In a resting state, the mean sIgA concentration was 2.27 pg/mL, which was reduced to 1.84 pg/mL after 2 min and increased to 2.37 pg/mL after 1 h (Fig. 4).

**Total sIgA**

The total sIgA can be calculated by multiplying the sIgA concentration by the total saliva volume (Fig. 5). Our data indicated that gargling with BW significantly increased the total amount of sIgA from 3.0 to 5.3 ng. Nevertheless, this increase was observed only after 2 min of gargling; the sIgA amount was reduced to 3.59 ng 1 h after gargling (Fig. 5).

**Discussion**

Various effects of Mg$^{2+}$ on bacteria have been reported; however, only magnesium oxide (MgO) has been shown to be effective against several bacterial species. In the present study, BW was demonstrated to have a very weak bactericidal effect against *S. mutans*. An approximately 60% reduction in microorganism cell viability was observed after 20 min of treatment with BW. This finding may be of clinical relevance for the development of effective anti-cariogenic Mg$^{2+}$-based bacterial agents.

In order to adapt to their surrounding environment, bacteria develop a two-component sensor/regulator environmental sensing system involving two indispensable factors: a membrane-bound histidine kinase and a response regulator (10). Although this system is common in gram-negative bacteria, a recent study has identified the same system in Group A *Streptococcus* (11), where it acts to repress the target genes thereby reducing its virulence. Intriguingly, the environmental Mg$^{2+}$ ion concentration has been shown to be a potent and specific stimulus for this system (11).

Salivary lysozyme is known to inhibit *S. mutans* glucose fermentation, which results in growth inhibition (12,13). Moreover, a low concentration of magnesium reduces the inhibitory effect of lysozyme on glucose fermentation (12). In the present study, *S. mutans* was
incubated alone with BW. Therefore, the inhibitory effect of BW on lysozyme should be examined in future studies.

The secretion of saliva is regulated by sympathetic and parasympathetic nerves (14). Generally, sympathetic nerve stimulation evokes secretion of protein-rich saliva, whereas parasympathetic nerve stimulation evokes an increase in the volume of secreted saliva. In the present study, gargling with BW significantly increased the secretion of saliva, thus implying a role of parasympathetic nerve stimulation. Bitter taste stimulation is always accompanied by vigorous salivation in the rat (15). Moreover, in rats that have been chronically decerebrated (at the precollicular level), the parabrachial nucleus, including the so-called taste area, and the ventral part of the parabrachial nucleus have been shown to be responsible for this increase in salivation.

Although the sIgA concentration was reduced after gargling, the total amount of sIgA secreted was increased significantly. Interplay between salivary epithelial cells and plasma cells in the salivary gland stroma is necessary for sIgA secretion (16). In plasma cells, two IgA molecules are dimerized with the J-chain and secreted; the secreted dimeric IgA (dIgA) is captured by the polymeric immunoglobulin receptor (pIgR) expressed on the basolateral membrane of salivary acinic or ductal cells. The dIgA-pIgR complex is transcytosed on the apical side of the cells and cleaved by unknown enzymes. The extracellular part of pIgR is known as the SC and is secreted as a component of sIgA. To increase the total amount of sIgA, the following reactions should be augmented. 1) IgA dimerization, 2) dIgA secretion, 3) pIgA expression or capture, 4) release from intracellular storage, or 5) extracellular cleavage and release of pIgR. The mechanisms underlying the augmentation of sIgA secretion need to be investigated further.

In the present study, BW was demonstrated to enhance total sIgA secretion via an increase in salivation. Our findings may aid in the development of a novel strategy using BW for the treatment of various diseases such as dry mouth.

Conflict of interest
The authors have no conflict of interest to declare.

References