

Effects of alkali-ion water on single species *Streptococcus mutans* biofilm

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Purpose: The aim of this study was to assess the efficacy of alkali-ion water (AW) on removal of *Streptococcus mutans* (*S. mutans*) single species biofilms from bovine enamel surfaces in vitro.

Materials and Methods: AW solutions were produced by electrolyzing tap water using a water electrolyzing device. *S. mutans* biofilms were formed on bovine enamel blocks in an oral biofilm reactor for 8 hours and 12 hours, respectively. AW solutions, AW-H (pH 11.5) and AW-L (pH 10.5), were tested for their efficacy in dissolving water insoluble glucans (WIG) from the above biofilms. Sodium hydroxide solution (NaOH pH 13.5) and mineral water (MW pH 7.5) were also compared as controls. Biofilms were tried to disintegrate employing two different methods; 1) assisted by a driving force (shaking) which was a simulation of gargling by an adult, and 2) incubation without applying any driving force to observe biofilm disintegration effect on self-penetration by the solutions on their sole chemical potentiality.

Results: One-way ANOVA and Tukey's HSD tests indicated that amount of dissolved glucan was significantly ($p < 0.05$) more in AW-H compared to MW and AW-H yielded significant differences ($p < 0.05$) in all tests with or without application of a driving force equivalent to NaOH. AW-L also dissolved more glucan than MW. Longer incubation period without any driving force dissolved more glucan by AW time dependently.

Conclusion: It was suggested that AW has the efficacy to remove a remarkable amount of biofilms by disintegrating glucans from the *S. mutans* artificial biofilms. (Int Chin J Dent 2009; 9: 55-60.)

Key Words: alkali-ion water, cariogenic biofilm, glucan, mutans streptococci, oral biofilm reactor.

Introduction

A major human health issue is dental caries which has been reported as a transmissible infectious disease in which mutans streptococci (MS) play major role. *Streptococcus mutans* (*S. mutans*), the leading member of mutans streptococci group, is the bacterial species most frequently implicated in dental caries.^{1,2} The organism produces glucosyltransferases (GTFs), which catalyze to synthesize glucans from dietary carbohydrate, especially sucrose. Glucans are one of central importance in adhesive interactions of *S. mutans* with the tooth surface and other oral bacteria, and contribute to the formation of the matrix of the dental plaque biofilms.³

In recent years, several commercially available functional waters are found to be remarkably useful in daily life and health care systems. One of those functional waters is well known as alkali-ion water (AW) because of its high alkalinity. It is usually produced by electrolyzing tap water or drinking water using a water electrolyzing device manufactured for household use. AW is widely used for drinking and cooking in Japan, particularly by the persons having stomachache for acid secretions. As popularity of using AW increased rapidly without showing any remarkable health hazard, the Japanese Ministry of Health, Labor and Welfare has officially recognized its usefulness in 2005.⁴ Other than improving the health condition of digestive tract AW found tastier as a drinking water compared to ordinary tap water.⁵ However, there might be several other health supportive properties of the AW remained unexplored, the high pH level of this functional water may contribute in controlling dental caries by removing plaque biofilms similarly as other high pH chemical reagents, like NaOH.^{6,7}

Therefore, the aim of this study was to investigate the efficacy of AW in removing *S. mutans* artificial biofilms by dissolving the glucans and detaching bacterial cells from tooth surfaces.

Materials and Methods

Solutions

Two different pH levels of AW (AW-H, pH 11.5 and AW-L, pH 10.5) were produced using a water electrolysis device (TK7705, Panasonic Electric Works Co., Ltd., Osaka, Japan). The chemical process that induces high concentrations of OH⁻ ion during electrolysis is shown in Fig. 1. Mineral water (MW, Crystal Geyser, Calistoga, CA, USA) was used as a control liquid with average pH level of 7.5. Sodium hydroxide (NaOH) 0.5 mol/L solution was used as positive control with pH level above 13.5.

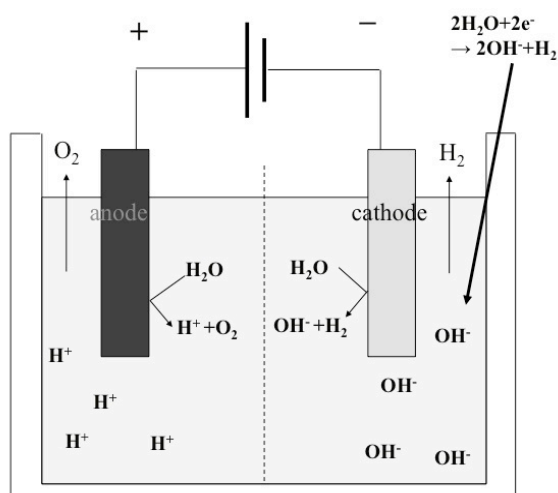


Fig. 1. Diagram represents the essential components of the water electrolysis device and the process of electro-chemistry to produce AW. Two electrodes were separated by a membrane in a water tank. With the initiation of water electrolysis ($\text{H}_2\text{O} \rightarrow 2\text{H}^+ + 1/2\text{O}_2 + 2\text{e}^-$) at the anode electrode acid water is produced. On reverse side (cathode side) AW is produced as water molecules splits and large quantity of OH⁻ ions are superadded in the water like; $2\text{H}_2\text{O} + 2\text{e}^- \rightarrow 2\text{OH}^- + \text{H}_2$.

Bovine enamel preparation as biofilm substrate

Square-shaped bovine enamel blocks (approximately 4.0x4.0x1.5 mm) were prepared as substrates to grow biofilms. The surface of each block was then ground flat using silicon-carbide (SiC) paper and uniformly polished with diamond pastes down to 3 μm.

Biofilms formation

The efficacy of AW on biofilm detachment was investigated using *S. mutans* MT8148 (*S. mutans*) a laboratory strain of cariogenic bacteria.⁸ A suspension of *S. mutans* in phosphate buffered saline (PBS) at OD₅₀₀=3.0 was prepared from a 16-hour fresh culture in brain heart infusion broth (BHI, Becton Dickinson, Sparks, MD, USA). The *S. mutans* biofilms were formed on the bovine enamel block surfaces assembled inside an oral biofilm reactor (OBR) chambers for 8 hours and 12 hours separately as described previously.^{6,9} In brief, eight coupons were placed on the same chamber-holder around a pH electrode and salivary pellicle was allowed to form using pooled sterile human saliva. Bacterial suspensions and other solutions were persistently pumped into each chamber so that a water-dome is formed on the specimen holder. The twin chambers of the OBR were operated simultaneously and recorded the pH continuously.

Disintegrating biofilms and measuring the dissolved glucan

1) Assisted by a driving force (shaking)

Each enamel block with artificial biofilms (of both 8-hour and 12-hour experiments) was removed from the holder and incubated in 1 mL of cool PBS. Loosely attached biofilms were removed by shaking at 5 Hz for 3

minutes using a TissueLyser (Qiagen/ Retsch, Hilden, Germany). Enamel blocks were then transferred carefully from the PBS to 1 mL of each liquid solution in separate microtubes (n=4 for each liquid). Subsequently, they were shaken three times with the TissueLyser at 10 Hz for 20 s with an interval of 1 minute and were transferred carefully from each liquid solution to 1 mL of 0.5 mol/L NaOH solution in order to separate water insoluble glucan (WIG) from the bacteria in the biofilm which was considered as the retained biofilms after shaking.

Each experimental solution containing detached biofilms was collected and centrifuged at 2,300 G for 10 minutes in order to separate the dissolved WIG from the bacteria embedded in the biofilms. The amount of dissolved WIG was analyzed by using the phenol-H₂SO₄ method and finally measured in a Biotrak II Plate reader (Biochrom, Cambridge, UK). The sum of the detached WIG and retained WIG was considered as the total amount of WIG for each coupon.

2) Inoculation without shaking

After *S. mutans* biofilm formation for 8 hours and 12 hours separately on bovine enamel blocks in OBR, the loosely attached biofilms were removed. Enamel blocks were then separately inoculated in NaOH, AW-H, AW-L and MW for 10 minutes, 2 hours, 5 hours, and 15 hours. Each experimental solution containing detached biofilms was collected and the dissolved glucan was measured separately in the same manner as described above.

Statistics

Data were analyzed by ANOVA, Tukey's HSD test ($p < 0.05$) and Dunnett T3 test ($p < 0.05$). The assumption of sphericity has been met by Mauchly's test of sphericity in case of the infiltration test. In each group four samples were used (n=4) and all experiments were repeated three times to assure reproducibility.

Results

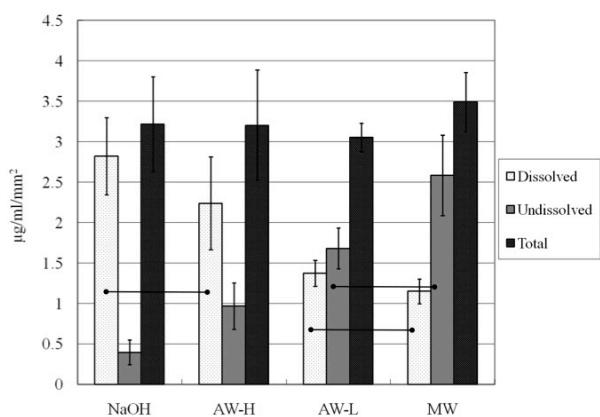


Fig. 2a

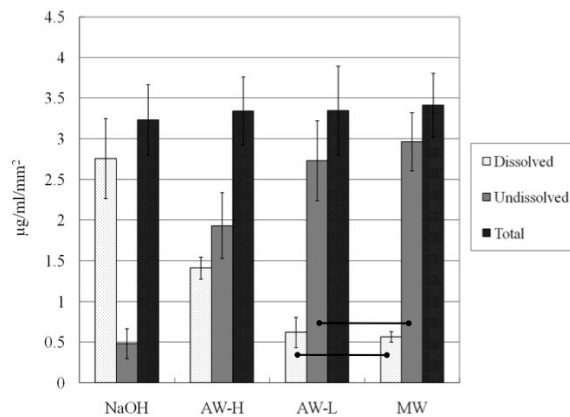


Fig. 2b

The amounts of glucan after treatment with the liquid solvents (NaOH, AW-H, AW-L, MW), each value is the mean \pm SD; (n = 4); (a) when a driving force was applied on 8-hour biofilms, (b) when a driving force was applied on 12-hour biofilms. The data for the amounts of glucan ($\mu\text{g}/\text{mL}/\text{mm}^2$) was analyzed by one-way analysis of variance (ANOVA), Tukey's HSD test and Dunnett's T3 test at 95%. Horizontal lines indicate no significant differences ($p > 0.05$), otherwise found significantly different.

The amount of dissolved glucan in *S. mutans* biofilms

1) Assisted by a driving force

8-hour

The amount of dissolved glucan detected significantly more in AW-H compared to MW and the amount of

retained glucan was significantly less in AW-H compared to MW as shown in the graph (Fig. 2a). However, there were no significant differences between MW and AW-L. As indicated, NaOH dissolved most of the glucan; 87.7% of total glucan; in comparison AW-H, AW-L, and MW dissolved 69.8%, 45.0%, and 32.9%, respectively.

12-hour

The amounts of dissolved glucan when biofilm were formed for 12 hours on the surface of the bovine enamel are shown in Fig. 2b. Almost the same results were recognized for 12 hours in glucan detachment relative to 8 hours of biofilm growth. Here also, NaOH dissolved most of the glucan (85.2% of total), compared to AW-H (42.2%), AW-L (18.5%), MW (16.5%). However, the percentages of dissolved glucan from 12-hour biofilms were less than from 8-hour biofilms in all four liquid solvents.

2) Incubation without shaking

8-hour

The percentages of dissolved glucan are shown when the enamel blocks with biofilms were incubated in liquid solvents for 10 minutes, 2 hours, 5 hours, and 15 hours (Fig. 3a). The graph indicates that glucan was dissolved gradually in all liquid solvents and the amounts of glucan were significantly ($p < 0.05$) more in AW-H with pH 11.5 compared to MW at each time point. AW-L, at pH 10.5, also dissolved more glucan than dissolved by MW.

12-hour

The percentages of dissolved glucan when the enamel blocks with 12-hour biofilms were incubated in liquid solvents for 10 minutes, 2 hours, 5 hours, and 15 hours are shown in Fig. 3b. The data indicate almost a similar relationship between the solutions in dissolving glucan as for 8 hours. AW-H dissolved significantly ($p < 0.05$) more glucans compared to MW, but at 15 hours time point only. Although the gaps between NaOH and AWs (H and L) remained wide at all time points AWs evidently dissolved more glucan than MW.

In both 8-hour and 12-hour biofilms increased amounts of glucan could be measured with the increase of incubation time in all solvents, but the difference between AW-H and MW widened further.

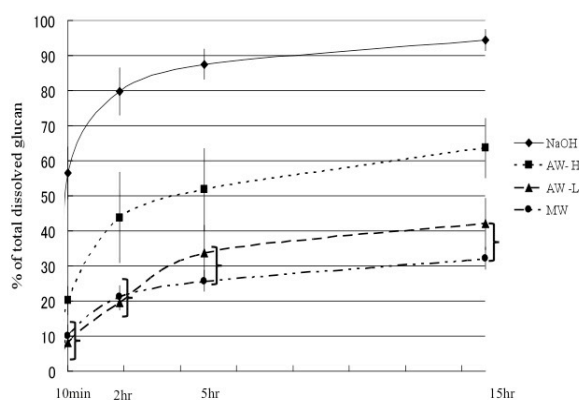


Fig. 3a

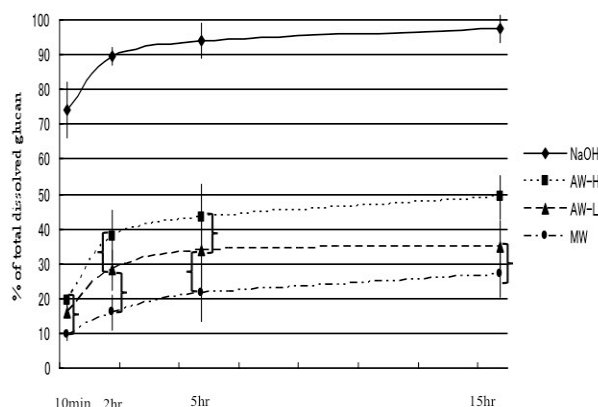


Fig. 3b

Fig. 3a. The amounts of dissolved glucan when the 8-hour biofilm was treated without assistance of any driving force.

Fig. 3b. The amounts of dissolved glucan when the 12-hour biofilm was treated without assistance of any driving force.

The data for the amounts of glucan (data expressed as % of total dissolved glucan) was analyzed by one-way analysis of variance (ANOVA), Tukey's HSD test and Dunnett's T3 test at 95%. Brackets indicate no significant differences ($p > 0.05$), otherwise found significantly different.

Discussion

In the present study, the efficacy of AW on *S. mutans* biofilm detachment was evaluated using an OBR. The result indicated that AW with pH level above 11.0 found significantly effective in disintegrating the glucan from the *S. mutans* biofilms. It was also understood that AW with pH level 10.5 effective in removing biofilms up to certain amount.

In any type of biofilm, whether in oral, natural or industrial environment, water and aqueous solutes represent most volume of the matrix. The dry material present is composed of a mixture of exopolysaccharides, proteins, salts and cell materials. Bacterial biofilms associated with surfaces are complex three-dimensional structures where bacteria are embedded in a matrix chiefly composed of extracellular polymeric substances (EPS).^{10,11}

S. mutans promotes tooth decay through the ability to adhere and accumulate in large numbers on the tooth surface, as well as produce and tolerate large amounts of acid. Biofilm formation occurs through the synthesis of water-soluble and water-insoluble glucans via the catalyzing activity of the glucosyltransferase enzymes (GTF).¹²⁻¹⁴ Glucan is believed to be the major factor contributing to the ability of *S. mutans* to adhere to the tooth surface and for aggregation of the bacterial cells within a biofilm. The ability of mutans streptococcal GTF to synthesize WIG from sucrose is an important virulence factor in initial caries development, potentially by increasing the colonization ability of infecting *S. mutans* and by influencing the diffusion characteristics of dental plaque.^{15,16} Since synthesis of WIG is one of the most important virulence properties of *S. mutans* that contributes to the development of mature biofilm, therefore the effects of AW of WIG disintegration were examined.

Alkali based compounds are commonly used to remove EPS produced by biofilm bacteria.¹⁷ Young et al.¹⁸ reported that treatment with NaOH can break H-bonds resulting in a partially opened triple-helix structure. In case of WIG that are synthesis by *S. mutans* also dissolved by alkaline solution.¹⁹ Freedman et al.²⁰ indicated that the water-insoluble, 1 N NaOH-soluble species were α -1,3-linkaged glucans and were cell associated. In some research reports it has been demonstrated that NaOH usually been used during analysis of glucans, including WIGs synthesized by *S. mutans*, because the strong alkaline chemical reagent is capable of completely dissolving all available glucans.^{19,21,22}

Shaking the enamel blocks by TissueLyser at a mild stroke of 10 Hz for 20 s three times with an interval of 1 minute each was a simulation of gargling by an adult. Which found to be most effective among several test conditions investigated so far (all data not shown). Results showed that longer incubation period without any stimuli or driving force dissolved more glucan, indicating that plenty of OH⁻ ions available in AW might have broken the glucan structures by slow etching reactions in a stimulation free condition. However, 12-hour glucans were not dissolved as same as 8-hours glucans, perhaps solidification of the biofilms has began by that time.

AW is used as drinking water that contains a high concentration of OH⁻ ion and it is produced by the electrolysis of ordinary tap water containing dissolved lactic acid and calcium. The mechanism of how it works was shown in Fig. 1. The Japanese Ministry of Health and Welfare has certified that alkali-ion electrolyzed water assists in the alleviation of gastrointestinal disorders, acidosis, chronic diarrhea, and poor digestion.^{23,24}

In the present study, biofilm formed with *S. mutans*, the results were almost same and the amounts of detached glucan were increased with a rise of pH. Furthermore, the amounts of bacteria (data not shown) were also showed the same tendency indicating bacteria in the biofilm were also detached from the enamel surface with glucan. Based on these results, it can be considered that the biofilm with pathogenic bacteria which produce glucan may be effectively detached by AW from enamel surfaces.

Therefore, it was suggested that glucans produced by *S. mutans* MT8148 was dissolved by the electrolyzed drinkable AW. In addition, it is expected that a similar effect may be acquired if AW is used intra-orally and would prevent plaque-biofilm accumulation on tooth surfaces on regular gargling with AW. Further studies from different viewpoints would help in understanding the unexplored potentials of this functional water, especially on caries prevention.

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