

## Neutral Electrolyzed Water for Prevention of Dental Caries

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### Abstract

Gargle solution has typically been used for the prevention of oral infectious disease such as dental caries and periodontitis. However, the use of most gargle solutions is controversial in application for children because some gargle solutions have harmful side effects. Electrolyzed water is generated by passed an electric current and has antimicrobial activity. The purpose of this study was to investigate and compare the efficacy of electrolyzed water in various conditions for eliminating cariogenic bacteria.

Electrolyzed water was generated by a platinum electrode in the presence of sodium chloride at various concentrations. *Streptococcus mutans* and *Streptococcus sobrinus* were cultivated into a brain heart infusion broth. After harvesting planktonic bacteria, the pellets were treated with the electrolyzed water and commercial gargle solutions and plated on a mitis-salivarius agar plate. Also, the anti-biofilm activity of the electrolyzed water and commercial gargle solutions was investigated after biofilm formation of *S. mutans* and *S. sobrinus*. The bacteria in the biofilm were plated onto a mitis-salivarius agar plate. The plates were incubated, and the colony forming unit was measured.

The electrolyzed water containing sodium chloride showed significant antibacterial activity against *S. mutans* and *S. sobrinus* as well as some gargle solutions. Furthermore, the electrolyzed water had more disruptive effect on the biofilm of *S. mutans* and *S. sobrinus* and killed more bacteria in the biofilm than commercial gargle solutions.

The results demonstrate that electrolyzed water may be a useful gargle solution for prevention of dental caries.

**Key words :** Anti-cariogenic effects, Electrolyzed water, *Streptococcus mutans*, *Streptococcus sobrinus*, Biofilm

### I . Introduction

The characteristics of *Streptococcus mutans* and *S. sobrinus* are acid production and aciduricity<sup>1,2)</sup>. Since this characteristics, they play a central role in the induction of dental caries<sup>3)</sup>, and *S. mutans* and *S. sobrinus* have been called cariogenic bacteria. Especially, compared to planktonic bacteria, their biofilm is associated more with

dental caries due to acid production in limited space<sup>4)</sup>. Also, the biofilm of *S. mutans* and *S. sobrinus* is more resistant for antimicrobial agents than planktonic bacteria because of glucan<sup>5)</sup>. Glucan is an exopolysaccharide of *S. mutans* and *S. sobrinus* and plays a barrier role in cariogenic biofilm<sup>6)</sup>. Therefore, glucan inhibits the penetration of antimicrobial agents into biofilm. Chemical gargle solutions are used to prevent infectious diseases

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such as caries and periodontitis in oral cavity<sup>7,8)</sup>. Since some gargle solutions contain alcohol, their use in oral health has been controversial<sup>9-11)</sup>. Therefore, dental gargle solution for the prevention of caries is not recommended for children.

Electrolyzed water (EW) is generated by the decomposition of water (H<sub>2</sub>O) into oxygen (O<sub>2</sub>) and hydrogen gas (H<sub>2</sub>) by an electric current being passed through the water. The characteristics of EW was differ according to the type of electrodes, such as silver and platinum, and the type of molecules added such as citric acid and sodium chloride. Also, the physical properties of EW vary according to the presence or absence of a membrane in the chamber<sup>12)</sup>. Several types of EW such as acidic, basic, and neutral water have been used in many fields such as industry, agriculture, and food because of its disinfecting effects<sup>12,13)</sup>. Recently, neutral electrolyzed water (NEW) was tried to apply to the medical and dental areas, as it is harmless to biological tissues<sup>14-16)</sup>. Also, NEW has anti-cancer, anti-oxidative and anti-bacterial effects<sup>17-19)</sup>. Acidic electrolyzed water has bactericidal effect on *Listeria monocytogenes* and *Escherichia coli*<sup>13)</sup>. Basic electrolyzed water affects oral hygiene including bactericidal activity, biofilm removal and bacterial growth inhibition<sup>19)</sup>. However, acidic and basic electrolyzed water were not sure of harmfulness due to their acidic and basic condition.

The purpose of this study was to investigate the antimicrobial effects of NEW on cariogenic bacteria and their biofilm, and to compare the antimicrobial activity of NEW and commercial gargle solutions.

## II. Materials and Methods

### 1. Bacterial species and cultivation

*S. mutans* ATCC 25175 and *S. sobrinus* ATCC 33478 were used in this study and cultivated by brain heart infusion broth (BHI)(BD bioscience, Sparks, MD, USA). In order to form biofilm, *S. mutans* and *S. sobrinus* were cultivated in BHI broth containing 2% sucrose according to the methods of Lee *et al.*<sup>3)</sup>.

### 2. Production of electrolyzed water

Two types of EW were used in this study and in order to investigate its antibacterial activity. EW was generated from an electrolyzing distilled water containing 0.05%

and 0.15% sodium chloride in an undivided anode chamber and cathode chamber. Also, the platinum electrodes were placed in the chamber horizontally. The pH and oxidation reduction potential (ORP) level of the EW was measured by a pH meter (Thermo Fisher Scientific, Waltham, MA, USA). The free available chlorine species (HClO and ClO<sup>-</sup>) were determined using N,N-diethyl-p-phenylenediamine (Sigma-Aldrich Co. St. Louis, MO, USA)<sup>20)</sup>. The free chlorine, pH and ORP of the EW were analyzed within 10 min after electrolysis.

### 3. Antimicrobial efficacy of the EW against cariogenic bacteria

*S. mutans* and *S. sobrinus* were cultivated in BHI broth for 36 h and counted by a bacterial counting chamber (Marienfeld, Lauda-Königshofen, Germany). The bacterial density was adjusted to  $1 \times 10^7$  cells/mL with BHI broth. Each bacterial suspensions (1 mL) was harvested by centrifugation at  $4,000 \times g$  for 10 min and then washed by phosphate buffered saline (PBS). The bacterial pellets were treated with 1 mL of the EW and commercial gargle solutions (alcohol- or fluoride-containing gargle) for 30 sec or 1 min, and then immediately added to 1 mL of BHI broth. The samples were diluted serially 10 fold to 10<sup>5</sup>, each diluted suspension was spread on an agar plate and incubated at 37°C until the colonies could be counted.

### 4. Comparison of biofilm biomass and viable bacteria

For biofilm formation, a plastic coverslip (SPL bioscience, Gyeonggi-do) was coated with saliva according to the methods of Lee *et al.*<sup>21)</sup>. *S. mutans* and *S. sobrinus* were inoculated into BHI broth including 2% sucrose and dispensed on the saliva-coated cover slip in a 12-well polystyrene plate (SPL bioscience). The plates were incubated at 37°C for 72 h, and the media were changed with fresh BHI broth including 2% sucrose each day. The biofilms were washed twice with PBS to remove planktonic bacteria and treated with the EW or commercial gargle solutions for various durations. The EW and commercial gargle solution were immediately aspirated by suction pump. Each biofilm was stained with 0.5% crystal violet and washed three times with PBS. Absolute alcohol was added to dissolve the crystal violet on the bacteria in biofilm, and the supernatant was transferred into a 96-well polystyrene plate (SPL bio-

science). The optical density of the supernatant was measured at a wavelength of 590 nm by a microplate reader (Biotek, Winooski, VT, USA). In order to analyze the viable bacteria in the biofilm, electrolyzed water-treated biofilms were disrupted mechanically, and weak sonication was performed (output 3W) to homogenize the bacteria. The bacterial suspensions were serially diluted from 10<sup>3</sup> to 10<sup>6</sup> with BHI broth, and the diluted suspensions (50 μL) were plated on Mitis-salivarius agar (BD bioscience). The plates were incubated at 37°C for 48 h, and the colony forming units of each bacteria on each plate were counted.

### 5. Statistical analysis

The differences between the control and the samples were analyzed by Krusical-wallis and Mann-Whitney test using SPSS 21 (IBM, Armonk, NY, USA). P-values less than 0.05 were considered statistically significant.

## III. Results

### 1. Physical property of the electrolyzed water

When distilled water in the presence or absence of sodium chloride at the concentration of 0.05% and

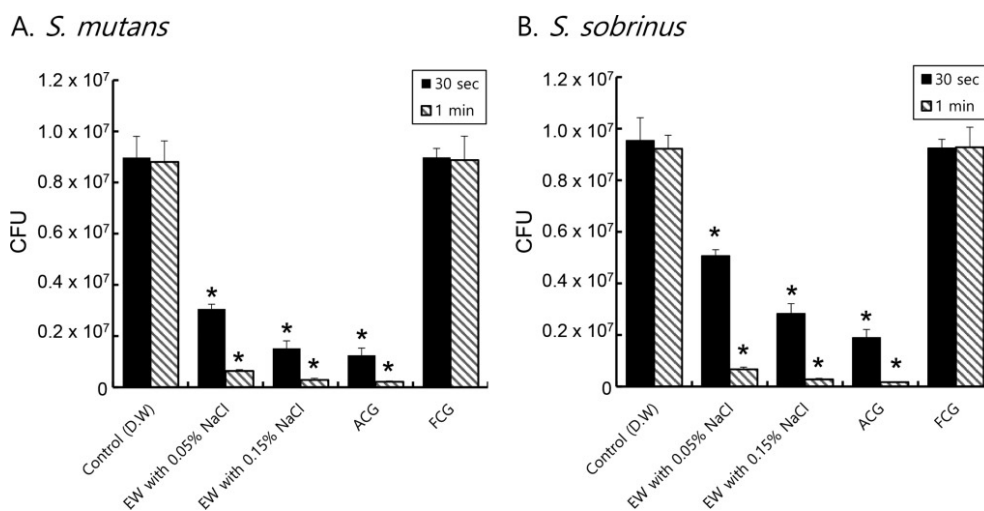
0.15% was by an passed electric current through the platinum electrode, the level of pH was neutral, and did not change. However, the concentration of free chlorine was increased in a dose-dependent manner (Table 1). The concentration of free chlorine indicated hypochlorous acid (HOCl) and hypochlorite ion (OCl<sup>-</sup>).

### 2. Antimicrobial activity of the electrolyzed water

The antimicrobial activity of the generated EW was compared with two commercial gargle solutions, including an alcohol-containing gargle for adults and a fluoride-containing gargle for children. The EW from the water including sodium chloride showed significant antimicrobial activity against *S. mutans* and *S. sobrinus* (Fig. 1). Furthermore, the sodium chloride-containing EW has more antimicrobial activity compared to the gargle solution for children.

**Table 1.** Physicochemical properties of the electrolyzed water

Electrodes	pH	ORP (mV)	Free Chlorine (ppm)
Distilled water	7.32 ± 0.07	342 ± 16	0 or N.D
0.05% NaCl	7.21 ± 0.16	764 ± 26	163 ± 21
0.15% NaCl	7.43 ± 0.23	752 ± 34	389 ± 37

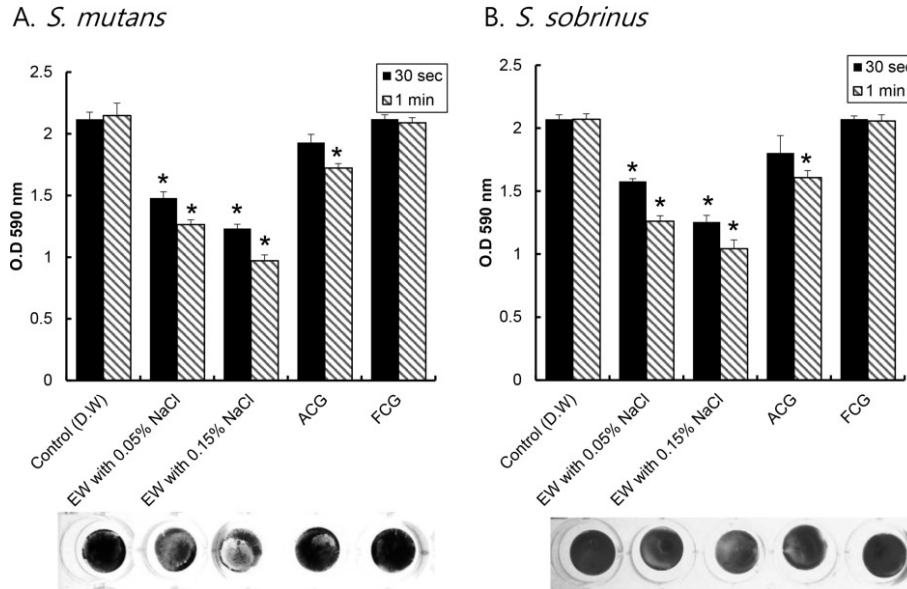


**Fig. 1.** Investigation of antimicrobial activity against *S. mutans* and *S. sobrinus*. The bacteria (1 × 10<sup>7</sup> CFU/ml) were harvested by centrifugation after cultivation in BHI broth and treated with alcohol-containing gargle (ACG), fluoride-containing gargle (FCG) and the EW from distilled water including 0.05% or 0.15% NaCl for 30 sec and 1 min. The count of *S. mutans* (A) and *S. sobrinus* were plated on mitis-salivarius agar and counted colonies. The tests were performed duplicate in three independent experiments.

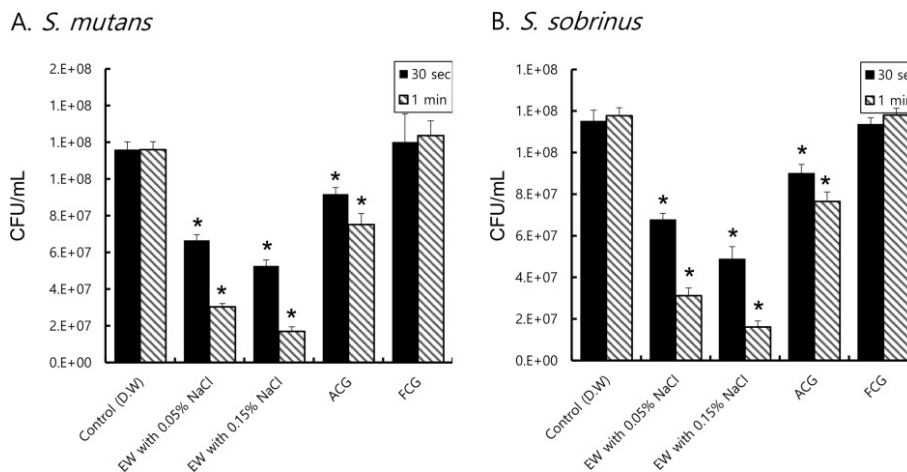
### 3. Effect of the electrolyzed water on biofilm of *S. mutans* and *S. sobrinus*

Mature biofilm of *S. mutans* and *S. sobrinus* was formed and treated with the EW and commercial gargle solutions. Fluoride-containing gargle as a gargle for children did not affect the biofilm of *S. mutans* and *S. sobrinus*. However, the EW from the water including sodium

chloride and the alcohol-containing gargle significantly disrupted the biofilm (Fig. 2). Furthermore, the count of *S. mutans* and *S. sobrinus* in the biofilm was decreased by both the EW and the alcohol-containing gargle (Fig. 3). Interestingly, the EW disrupted more the biofilm and killed more the cariogenic bacteria in the biofilm than the alcohol-containing gargle.



**Fig. 2.** Disruptive effect of the electrolyzed water on *S. mutans* and *S. sobrinus* biofilm. The biofilm was formed on saliva-coated plastic slip using BHI broth supplemented with 2% sucrose and treated with alcohol-containing gargle (ACG), fluoride-containing gargle (FCG) and the electrolyzed water from distilled water including 0.05% or 0.15% NaCl for 30 sec and 1 min. After removing the supernatants, the biofilm was stained with crystal violet and washed with PBS. The biofilm was detained with alcohol, and the optical density of destained supernatant was measured at a wavelength of 590 nm by a microplate reader. The tests were performed duplicate in three independent experiments.



**Fig. 3.** Analysis of live bacteria in the biofilm. *S. mutans* and *S. sobrinus* biofilm were formed on 12-well polystyrene plate using BHI broth including sucrose for 72h. The biofilm was treated with alcohol-containing gargle (ACG), fluoride-containing gargle (FCG) and the EW from distilled water including 0.05% or 0.15% NaCl for 30 sec and 1 min. After suction of the supernatant, the biofilm was disrupted mechanically by a scraper and homogenized. The suspension of biofilm was plated on MS agar and the colony forming unit was counted. The examination were carried out duplicate in three independent experiments.

#### IV. Discussion

Dental caries as tooth decay is the most prevalent infectious disease in children and is associated with *S. mutans* and *S. sobrinus*<sup>22)</sup>. Gargle solutions have been used to prevent caries in daily life. However, general gargle solutions contain chemicals including alcohol to kill oral bacteria. Therefore, the use of gargle solutions for oral health has been controversial because of their harmful side effects<sup>23,24)</sup>. Eventually, the gargle solution is reluctant to use in children. In this study, EW was investigated antimicrobial activity against *S. mutans* and *S. sobrinus* and compared with the conventional gargle solutions. Also, the EW was evaluated for its potential to replace conventional gargle solutions.

The EW was generated by an electric current by passing through it platinum electrodes in the presence of sodium chloride. This EW showed neutral pH and contained free chlorines such as hypochlorous acid (HOCl) and hypochlorite ion (OCl<sup>-</sup>). When the EW was generated using silver electrodes, the level of pH was neutral. However, the level of free chlorines was barely detected. The free chlorines in aqueous solution have antimicrobial activity<sup>25)</sup>. The bactericidal mechanisms of free chlorines are to react with the bacteria surface components and to impair intracellular components. In current study, these characteristics of the EW from NaCl-containing water was examined antimicrobial and anti-biofilm activity against *S. mutans* and *S. sobrinus*. Although the antimicrobial activity of the EW was weaker than a commercial gargle solution with alcohol, it showed stronger antimicrobial activity compared to the gargle solution for children.

Interestingly, although the EW exhibited weaker antimicrobial effect on planktonic *S. mutans* and *S. sobrinus* than the commercial gargle solution, it was more effective in destroying the biofilm of *S. mutans* and *S. sobrinus* than the commercial gargle solution. On the basis of biofilm disruption, the EW destroys the surface barrier of the biofilm, penetrates into the biofilm, and then reacts with each bacterium. Since the ability of commercial gargle solution to destroy the biofilm is weak, the solution might not penetrate deeply into the biofilm. *S. mutans* and *S. sobrinus* produce abundant glucan from sucrose, and the glucan is a barrier of biofilm, protecting the bacteria in the biofilm and forming limited space<sup>26,27)</sup>. Finally, the level of pH was rapidly decreased in the biofilm because *S. mutans* and *S. sobrinus* can produce

lactic acid in a limited space and under conventional conditions. Therefore, dental caries is related more to the biofilm of cariogenic bacteria than to planktonic bacteria<sup>21)</sup>.

The NEW is effective in removing cariogenic biofilm as thoroughly as the commercial gargle solutions and showed antimicrobial activity against *S. mutans* and *S. sobrinus*. Furthermore, no harmful effects have been reported with the use of neutral EW. Therefore, the neutral EW from NaCl-containing water may be a candidate for the prevention of dental caries.

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국문초록

## 기존 구강청결제를 대체할 수 있는 치아우식 예방을 위한 전기분해수

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치아우식은 *Streptococcus mutans*와 *S. sobrinus*가 주요 원인균이며, 이를 예방하기 위해서 구강 청결제를 사용한다. 그러나 구강청결제의 부작용 및 해로움으로 인하여 소아에게 권장하기에 주의를 요한다. 전기 분해수는 물을 전극을 통해서 전류를 흘려주어 생성되는 용액으로 항균력을 갖는다. 본 연구에서는 전기 분해수를 구강 청결제 대용으로 사용하기 위해서 치아 우식원성 세균 및 바이오 필름에 대한 항균력을 조사하였다.

염화나트륨이 포함된 증류수를 백금전극을 이용하여 전기 분해수를 생성하였다. 전기분해 수소수의 물리화학적 성분을 분석하고 *S. mutans* 및 *S. sobrinus* 배양액을 원심분리하여 세균을 얻고 전기 분해수, 알코올 및 불소가 포함된 구강 청결제를 처리하고 살아있는 세균 수를 배양을 통해서 조사하였다. 또한 치아우식균의 바이오 필름을 형성시킨 후, 전기 분해수 및 구강 청결제를 처리하여 바이오 필름 제거능을 크리스탈 바이올렛 염색을 이용하여 분석하고, 바이오 필름 내 살아있는 세균 수의 양은 배양을 통하여 분석하였다.

전기분해수는 높은 농도의 자유염소 (free chlorine)을 포함하고 중성 폐하를 보였다. 전기 분해수는 유의하게 *S. mutans* 및 *S. sobrinus*에 대해서 항균력을 보였으며, 이 세균들의 바이오 필름 제거능 및 바이오 필름 내 세균에 대해서도 항균력을 보였다. 전기 분해수는 부유세균에 대해서 알코올이 포함된 구강청결제보다 약한 항균력을 보였지만, 바이오 필름 세균에 대해서는 더 강한 항균력을 나타냈다.

이러한 결과를 바탕으로 중성 전기 분해수는 치아우식 예방을 위한 기존 구강 청결제의 대체 용액으로 사용이 가능할 것으로 사료된다.

**주요어:** 항우식 효과, 전기분해수, *Streptococcus mutans*, *Streptococcus sobrinus*, 바이오필름

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