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Antibacterial Effect of Electrolyzed Water on Oral Bacteria

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This study investigated the antibacterial effect of electrolyzed water on oral bacteria both *in vitro* and *in vivo*. Tap water was electrolyzed in a water vessel using platinum cell technology. The electrolyzed tap water (called Puri-water) was put in contact with five major periodontopathogens or toothbrushes contaminated with these bacteria for 30 sec. In addition, Puri-water was used as a mouthwash for 30 sec in 16 subjects and the antibacterial effect on salivary bacteria was evaluated. Puri-water significantly reduced the growth of all periodontopathogens in culture and on toothbrushes, and that of aerobic and anaerobic bacteria in saliva, when compared to the effect of tap water. It also significantly reduced mutans streptococci growing on mitis salivarius-bacitracin agar. Our results demonstrate that the electrolyzed tap water is effective as a mouthwash and for toothbrush disinfection.

***Keywords*:** electrolyzed water, antibacterial activity, periodontopathogens, salivary bacteria, mouthwash

Dental plaque control is the primary measure to prevent major oral diseases like caries, gingivitis, and periodontitis. It can be achieved by mechanical methods alone such as tooth brushing and flossing. Mouthwashes are widely used and a huge market has developed during the last decade. Commercial mouthwashes have more or less complicated formulas, and most of them contain antimicrobial agents such as chlorhexidine, triclosan, cetylpyridinium chloride, $ZnCl_2$, chlorine dioxide, and cationic peptides (Renton-Harper *et al*., 1996; Barnett, 2003; Kim *et al*., 2003). While these substances have marked antibacterial effects, mouthwashes that do not disturb the normal oral ecosystem but that can reduce plaque significantly are preferred for daily use.

Caries-causing bacteria are primarily mutans streptococci including *Streptococcus mutans* and *Streptococcus sobrinus* in humans. Major periodontopathogens are gram-negative and anaerobic bacteria, and some of them are highly proteolytic and cause malodor (Socransky and Haffajee, 2005; Feng and Weinberg, 2006). Although selective antibacterial agents against these bacteria are not available, overall reduction in the number of these bacteria will contribute to the prevention of caries and periodontitis.

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The purpose of this study was to evaluate the antibacterial effect of electrolyzed tap water (Puri-water) on five major periodontopathogens cultured in vitro, on toothbrushes contaminated with the periodontopathogens, and on salivary bacteria. Puri-water caused a significant inhibition of the growth of oral bacteria compared to tap water.

Materials and Methods

Production of electrolyzed water (Puri-water)

Tap water of drinking water quality (pH 7.3, 0.76 ppm of residual chlorine) was subjected to electrolysis (30 V of DC/300 mA) for 2 min at ambient temperature using an electrolysis apparatus equipped with platinum electrodes (280 × 120 × 90 mm, 650 g, Sciacua™, Puri Co., Korea). The apparatus was a single-cell chamber without a separation between the cathode and anode regions. Puri-water was used within 2 min of its preparation in all experiments. The pH of Puri-water measured immediately after electrolysis was about 8.4. The temperature of the water increased by less than 1°C after electrolysis.

Bacteria and culture

The five major periodontopathogens included in this study were *Actinobacillus actinomycetemcomitans* (ATCC 33384), *Fusobacterium nucleatum* (ATCC 25586), *Porphyromonas gingivalis* (ATCC 53978),

Prevotella intermedia (ATCC 49046), and *Treponema denticola* (ATCC 33521). *A. actinomycetemcomitans* was cultured in brain heart infusion (BHI, Becton Dickinson, USA) broth. *F. nucleatum, P. gingivalis*, and *P. intermedia* were cultured in BHI broth supplemented with 5 μg/ml of hemin and 0.1 μg/ml of menadione. *T. denticola* was cultured in OMIZ-Pat broth (Wyss *et al*., 1996). Bacteria were cultured at 37°C anaerobically (5% H_2, 10% CO_2, 85% N_2) and harvested at the late exponential phase for the analysis.

Evaluation of the antibacterial activity of Puri-water on in vitro cultured periodontopathogens

Periodontopathogens cultured as described above were harvested and their density adjusted so that their optical density at 660 nm (OD_{660}) was 0.5. One milliliter of each bacteria were centrifuged for 10 min at 3,000 × g and the supernatants were discarded. The pellets were resuspended in 1 ml of Puri-water or tap water, vortexed for 30 sec, serially diluted 10-fold with each bacterial medium, and immediately plated onto BHI agar plates (for *A. actinomycetemcomitans*) or BHI agar plates supplemented with 5 μg/ml of hemin and 0.1 μg/ml of menadione (for *F. nucleatum*, *P. gingivalis*, and *P. intermedia*) using a spiral plater system (Autoplate® 4000, Spiral Biotech, USA). Colony forming units (CFUs) were counted after cultivation. *T. denticola* was inoculated in 5 ml of OMIZ-Pat broth after contact with Puri-water or tap water for 30 sec and the change of OD_{660} of the culture was checked for 7-day incubation. CFU or OD_{660} was compared to the control value for each bacterial culture medium.

To test whether the antibacterial effect of Puri-water could be attributed to the elevated pH, tap water was adjusted to pH 8.4 with NaOH and evaluated for its antibacterial activity against cultured bacteria.

Evaluation of the antibacterial activity of Puri-water on toothbrushes contaminated with periodontopathogens

New commercially available synthetic toothbrushes were inoculated with each periodontopathogen by immersing for 2 h in 50 ml bacterial cultures that were adjusted to an OD_{660} of 0.5. The toothbrushes were then washed for 30 sec by hand with 100 ml of Puri-water or 100 ml of tap water. The wash solutions were inoculated on BHI agar plates or in OMIZ-Pat broth as described above. The plates and the broth cultures were incubated anaerobically at 37°C for 3-7 days, and CFU or OD_{660} was determined. In order to evaluate the number of bacteria remaining on the toothbrushes after the first washing with Puri-water or tap water, the toothbrushes were washed again in PBS for 30 sec and this PBS was inoculated on the medium. Seven brushes were used for each bacterium per experiment and the experiment was repeated three times.

Collection of saliva

Saliva was collected from a total of 16 participants (5 males, 11 females). All the participants were volunteers attending the Graduate School of Dentistry, Seoul National University, Seoul, Korea. The age of the participants ranged between 24 and 32 years. Written consent to participate in the study was obtained from all participants. They were instructed to maintain their oral health as usual during the study, to clean their teeth at 6 p.m. the day before the sampling and to drink only water from then until the sampling next morning. Saliva was collected for 5 min after washing the mouth with 10 ml of tap water. One week later, the same procedure for sampling saliva was performed except that the mouth was washed with 10 ml of Puri-water.

Evaluation of the antibacterial activity of Puri-water on bacteria in saliva

Saliva samples were immediately processed for bacterial culture. Saliva was vortexed and 1 ml of saliva was mixed with 9 ml of PBS. The mixture was serially diluted 10-fold with PBS and inoculated onto blood agar plates (5% sheep blood) with a spiral plater system. The plates were incubated aerobically for 3 days and anaerobically for 10 days at 37°C. For mutans streptococci, diluted saliva was plated on mitis salivarius-bacitracin (MSB) agar, a selective medium, and incubated aerobically for 7 days. CFUs were counted after cultivation.

Statistical analysis

The number of living bacteria in culture after exposure to Puri-water and tap water was compared using analysis of variance (ANOVA) and Student's t-test. The statistical difference between salivary bacteria after exposure to Puri-water and after exposure to tap water was evaluated by correlation using the SPSS 12.0 statistical package. Statistical significance was determined at the $P < 0.05$ level.

Results

Antibacterial effect of Puri-water on periodontopathogens

The antibacterial activity of Puri-water against five major periodontopathogens was evaluated. As shown in Fig. 1A, Puri-water reduced the bacterial counts to 12.6-15.4% for *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *P. gingivalis*. In contrast, tap water did not inhibit bacterial growth. Growth of *T. denticola* was not observed during 7-day incubation after exposure

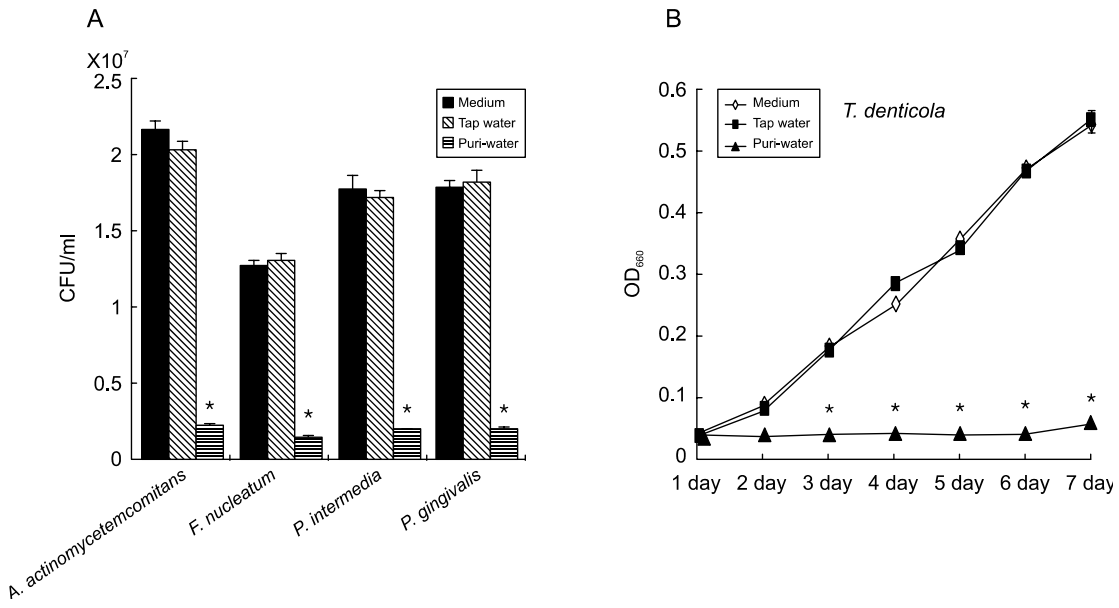

Fig. 1. Growth inhibition of periodontopathogens by Puri-water. Five periodontopathogens were cultured, exposed to Puri-water or tap water for 30 sec, and inoculated on BHI agar medium (A) or in OMIZ-Pat broth medium (B). CFU (A) or OD_{660} (B) was determined after culturing, and compared with the control values that were obtained with each bacterial culture medium. *Significant difference ($P < 0.05$) compared to the control values.

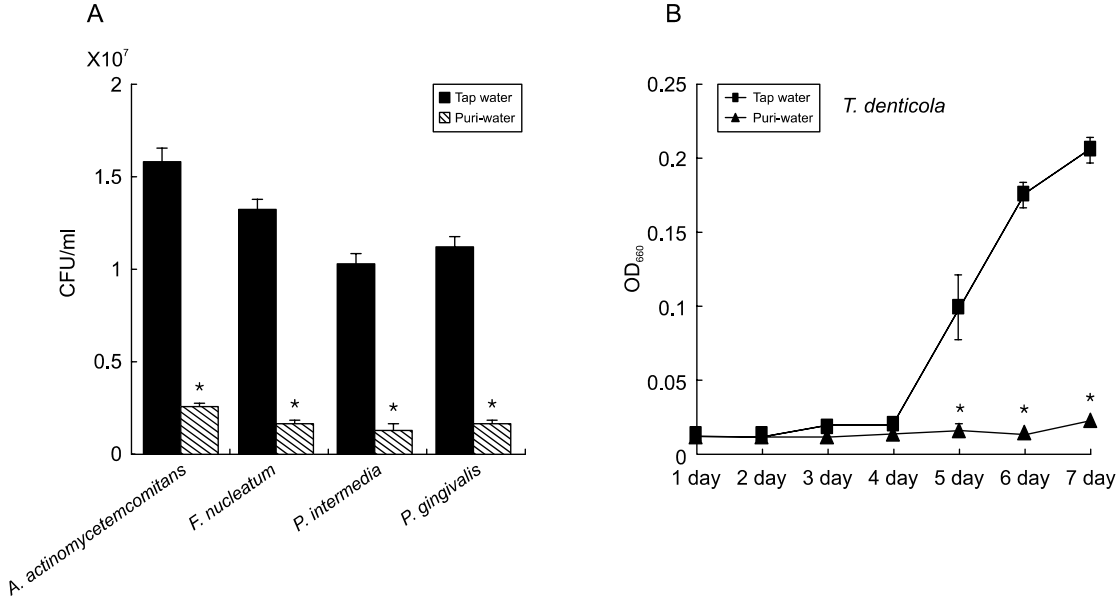

Fig. 2. Bacteria in wash solution of toothbrushes with Puri-water. Toothbrushes were contaminated with periodontopathogens (OD_{660} = 0.5), and washed with Puri-water or tap water. CFU was counted on BHI agar medium (A) or OD_{660} was measured in OMIZ-Pat broth medium (B) after culturing the wash solution. For each bacterium, seven brushes were used and the experiments were repeated three times. *Significant difference ($P < 0.05$) compared to the values that were obtained after exposure to tap water.

to Puri-water, while it was not affected by tap water (Fig. 1B). Tap water adjusted to pH 8.4 did not show antibacterial activity (data not shown).

Bacteria on toothbrushes were also significantly reduced by Puri-water, when both the bacteria in the wash solutions and the bacteria remaining on the toothbrushes were assessed. *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *P. gingivalis* remaining in the Puri-water wash ranged between 11.0 and 12.4% of the amount remaining in the tap water wash

(Fig. 2A). The growth of *T. denticola* was not observed in the Puri-water wash during 7-day incubation, whereas the growth of *T. denticola* was observed in the tap water wash after 4 days (Fig. 2B). The number of bacteria remaining on toothbrushes after washing with Puri-water was about 50% of that after washing with tap water (Fig. 3). The growth of *T. denticola* was not observed for 7-day incubation in both cases (data not shown).

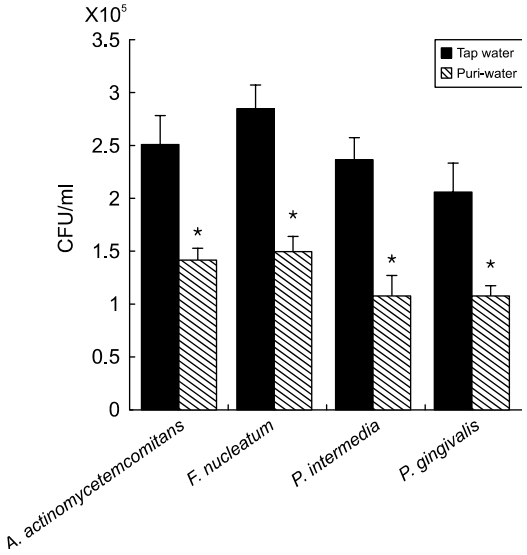

Fig. 3. Residual bacteria on toothbrushes after washing with Puri-water. Toothbrushes washed with Puri-water or tap water (as described in Fig. 2) were washed again in PBS for 30 sec and CFU in PBS was determined after culturing on BHI agar medium. For each bacterium, seven brushes were used and the experiments were repeated three times. The representative data are shown. *Significant differences ($P < 0.05$) compared to the control values that were obtained after exposure to tap water.

Antibacterial effect of Puri-water on salivary bacteria

We evaluated the antibacterial effect of Puri-water on aerobes and anaerobes in saliva. We also evaluated the antibacterial effect of Puri-water on mutans streptococci using a selective medium. Saliva samples were obtained from 16 subjects after they washed their mouths for 30 sec with Puri-water or tap water, and bacterial growth was assessed on blood agar or MSB agar plates. As shown in Fig. 4, aerobes and anaerobes in saliva were significantly reduced after washing with Puri-water compared to those after washing with tap water in the same subjects ($P < 0.05$). Significant reduction of mutans streptococci was also observed in all participants. The numbers of aerobic and anaerobic bacteria, and mutans streptococci in saliva after washing with Puri-water were 1.7±0.6%, 34.4±14.1%, and 21.0±8.7%, respectively, of those after washing with tap-water.

Discussion

Tooth brushing is a normal oral hygiene practice and toothpastes possess some plaque inhibitory activity. However, many people use mouthwashes which give adjunctive benefits to oral health and to halitosis (Barnett, 2003). Most mouthwashes are antiseptic and their antiplaque effect has been demonstrated. However, daily use of antiseptics could disturb the health of the oral ecosystem, affecting the quality of colonizing bacteria (Kononen, 2000).

In this study, we demonstrated the antibacterial

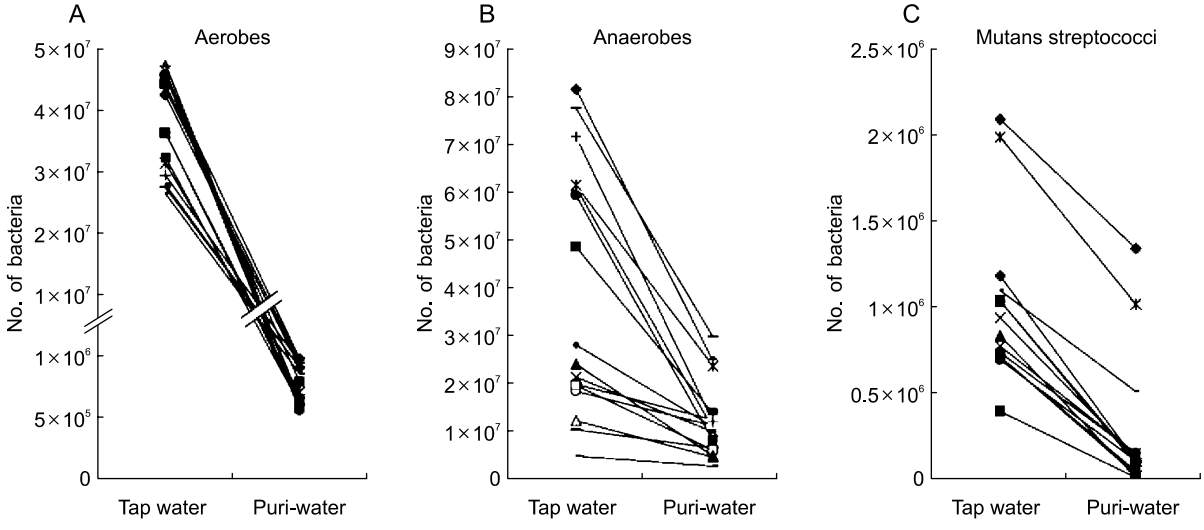

Fig. 4. Growth inhibition of salivary bacteria by Puri-water. Saliva was collected from 16 participants after washing the mouth with 10 ml of Puri-water or tap water. Saliva was inoculated on blood agar (A, B) or MSB agar plates (C), and incubated aerobically (A, C) or anaerobically (B). CFU was counted on the agar medium.

effect of Puri-water, a kind of electrolyzed water, both in vitro and in vivo. Compared to tap water, Puri-water showed significant inhibition of the growth of aerobes, anaerobes and mutans streptococci in saliva as well as five periodontopathogens in culture. Also toothbrushes contaminated with cultured periodontopathogens were significantly decontaminated by Puri-water. The number of bacteria both in the wash solution and remaining on the toothbrush after washing was significantly lower when the toothbrushes were washed with Puri-water compared to washing with tap water. The microbial load on toothbrushes might have a significant impact on the transmission of pathogens (Nelson *et al*., 2000; Sato *et al*., 2005). Surviving bacteria on toothbrushes 48 h after brushing with toothpaste have been observed, although some toothpastes did reduce cariogenic and periodontopathogenic bacteria significantly (Quirynen *et al*., 2003).

Electrolyzed water (EW) can be generated by the electrolysis of aqueous NaCl solution in a electrolytic cell that has a separation membrane between the cathode and anode (Tanaka *et al*., 1996). Acidic EW from the anode side has been demonstrated to exert antibacterial activity and has been used in medical and food applications (Tanaka *et al.*, 1996; Nakagawara *et al*., 1998; Miyamoto *et al*., 1999; Park *et al*., 2002; Koseki *et al*., 2004). Tanaka *et al*.(1996) reported that superoxidized water, a strong acidic solution (pH 2.3-2.7) with a high oxidation-reduction potential, which was prepared by electrolysis of tap water in the presence of 0.1% NaCl, showed antibacterial activity against a variety of gram-positive and gram-negative bacteria. Its bactericidal activity was superior to that of 0.1% chlorhexidine, the leading oral antiseptic. Acidic EW was effective for the prevention of infection with methicillin resistant *Staphylococcus aureus* (Miyamoto *et al*., 1999), and for killing *Campylobacter jejuni* on chicken (Park *et al*., 2002) and *Escherichia coli* O157:H7 on lettuce (Koseki *et al*., 2004). With respect to oral bacteria, there is one report on the inhibitory effect of acidic EW on dental plaque formation (Itoh *et al*., 1996). The mechanism of the bactericidal effect of acidic EW has been proposed to be the combined action of hydrogen ion concentration, high oxidation-reduction potential and the presence of extremely bactericidal hypochlorous acid (HOCl) (Tanaka *et al*., 1996; Park *et al*., 2002). Nakagawara *et al*. (1998) showed that the bactericidal activity was correlated to the concentration of hypochlorous acid in acidic EW.

Puri-water is not an acidic EW. Its pH was weakly alkaline, but this characteristic did not influence the antibacterial effect of Puri-water. The antibacterial effect of Puri-water diminished with time and disappeared after 2 h. The antibacterial activity of Puri-water is likely to be based on the combined action of short-lived reactive oxygen species (ROS) such as singlet oxygen, superoxide free radicals (O_2^-), and hydroxyl radicals (OH^-), and free chlorine. It is unlikely that hypochlorous acid was generated in a significant amount since Puri-water had a pH of 8.4. Nakajima *et al*. (2004) reported that electrolyzed tap water without the addition of NaCl had bactericidal activity against contaminated bacteria in tap water. They suggested that free chlorine is a bactericidal substance and showed time- and current-dependent increase of free chlorine concentration in electrolyzed tap water. The maximum concentration of free chlorine changed from less than 1 ppm to 30 ppm in 30 min.

In summary, we demonstrated that electrolyzed tap water markedly inhibited the growth of salivary bacteria as well as cultured periodontopathogens. It can be made easily in a small scale and could be useful for daily oral hygiene if used as a mouthwash and for toothbrush washing. It may be especially useful for people wearing orthodontic apparatus as well as physically and mentally handicapped people.

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