Original Article

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In vitro bactericidal activity against periodontopathic bacteria by electrolyzed ion-reduced water

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ABSTRACT: As typical periodontopathic bacteria, *Porphyromonas gingivalis* (*P. gingivalis*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) were exposed to electrolyzed ion-reduced water (ERI) and ERI containing 1% sodium carboxymethylcellulose (CMC-Na) (ERI-1% CMC-Na), and the time course of their bactericidal action was evaluated. More than 99% of each bacteria species were killed after exposure to each solution for 15 sec. In addition, 1% CMC-Na, which was added to prolong bactericidal action, did not affect the bactericidal action of ERI. Its bactericidal action was concentration-dependent. No viable *P. gingivalis* bacteria were observed at a concentration of 15% of the undiluted solution and no viable *A. actinomycetemcomitans* bacteria were observed at a concentration of 50%, indicating differences in the bactericidal action of ERI for the two bacteria species. These results suggest that ERI may be extremely useful in preventing and treating periodontal diseases.

Keywords: Electrolyzed ion-reduced water, functional electrolyzed alkaline water, functional electrolyzed water, periodontopathic bacteria, bactericidal activity

1. Introduction

The electrolysis of water containing a small amount of salt or tap water produces a form of water that is useful and is generally known as functional electrolyzed water. Various types of functional water can be produced depending on the conditions of electrolysis, and these types of water are classified into two major categories. One is functional electrolyzed acidic water, which is generated at the anode and is used mainly for sterilization. The other is functional electrolyzed alkaline water, which is generated at the cathode and is used mainly for drinking and washing (*1,2*). Many studies have examined electrolyzed acid water, revealing that this water has marked bactericidal activity and is safe and environmentally friendly. Therefore, this water has been used in various fields (*3,4*) such as washing food (*5,6*) and medicine (*7,8*). In dentistry in particular, functional electrolyzed acidic water is used to wash dental materials and as a mouthwash (*4,9*). However, there are problems with using functional electrolyzed acidic water in dentistry, such as the decrease in bactericidal strength due to organic matter such as blood and saliva and the corrosion of dental instruments (*10*).

Functional electrolyzed alkaline water is used as drinking water and as a supplement to sustain/promote health and treatment. This water also has cleaning action and has been reported to be useful in washing dental cutting instruments and the oral cavity (*4*). However, few studies have examined functional electrolyzed alkaline water, and its usefulness has not been adequately confirmed.

The current authors have conducted several studies on electrolyzed ion-reduced water (ERI) (*11-19*). The resulting ERI, which was produced by using the general method of producing functional electrolyzed water, is physically electron-rich water obtained after the application of electric current/voltage to water containing a small amount of salt using a special diaphragm system. This water has cleansing, deodorizing, antimicrobial, and anti-dust action because its potent alkalinity and negatively-charged ions detach and remove dirt and bacteria that cause odor (*11*). ERI prevents oxidation, and, therefore, also has rust-preventing and anti-septic action. In addition, stable emulsions of various types of oil can be prepared using ERI with no emulsifiers, demonstrating its emulsifying action (*12*). Capitalizing on these properties, ERI is widely used as a cleansing agent in various industrial products (*11*). It is also reported to have burn-healing action and an effect on atopic dermatitis (*16-19*).

To investigate the clinical use of this strong bactericidal action of ERI in dentistry, the current study evaluated the bactericidal action of ERI on periodontopathic bacteria.

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2. Materials and Methods

2.1. *Materials*

Porphyromonas gingivalis (*P. gingivalis*) JCM8528 and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) JCM12985 were purchased from Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan (*20*). Modified general anaerobic medium (GAM) broth and agar media were purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. AnaeroPack® Kenki (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) was used for anaerobic culture. S-100® (A. I. System Product Corp., Kasugai, Aichi, Japan) was used as the ERI. Sodium carboxymethylcellulose (CMC-Na) was purchased from Kanto Chemical Co., Inc., Tokyo, Japan. All reagents were of special grade.

2.2. *Preparation of ERI and ERI containing 1% CMC-Na (ERI-1% CMC-Na)*

ERI was produced using an ERI generator (A. I. System Product Corp.), and ERI was used in experiments immediately after its production. At 20°C, this ERI had a pH of 12.0-12.4, oxidation reduction potential of −344 mV, specific gravity of 1.002, and an osmolarity of 100 mOsm (corresponding to a 0.3% NaCl solution). An ERI solution containing 1% CMC-Na was prepared. Controls were a 0.3% NaCl solution with the same osmolarity as that of the ERI and a 0.3% NaCl solution containing 1% CMC-Na.

2.3. *Bacteria strains*

The bacteria strains used were *P. gingivalis* JCM8528 and *A. actinomycetemcomitans* JCM12985.

2.4. *Bacterial culture and preparation*

P. gingivalis and *A. actinomycetemcomitans* were cultured in GAM broth medium, and an AnaeroPack® Kenki was added as a disposable O_2-absorbing and CO_2-generating agent to an anaerobic jar kept under anaerobic conditions at 37°C for 4 days.

After culture, bacteria were collected by centrifugation at 10,000 × g for 10 min, washed with phosphate-buffered saline (PBS) and diluted with PBS, and subjected to measurement with a UV-visible spectrophotometer V-630 (JASCO Corp., Tokyo, Japan) at 540 nm. A bacterial suspension with an optical density of about 0.3 (viable cell count: 10^8/mL) was prepared.

2.5. *Bactericidal action of ERI and ERI-1% CMC-Na*

With the *P. gingivalis* samples, 0.1 mL of bacterial suspension (10^8/mL) was added to 10 mL of ERI or ERI-1% CMC-Na and stirred. Then, 10 μL aliquots were added to 1 mL of GAM broth medium after 15, 30, and 60 sec. In addition, 0.1 mL of the mixture was added to GAM agar medium. These procedures were performed aerobically at room temperature, followed by anaerobic culture in an anaerobic jar using an AnaeroPack® Kenki at 37°C for 2-5 days. Colony-forming units/mL (CFU/mL; viable cell count) were determined and the CFU/mL was compared to the value after bacterial exposure to 0.3% NaCl solution or that containing 1% CMC-Na as the controls.

With the *A. actinomycetemcomitans* samples, a similar experiment was performed using GAM broth and agar medium.

2.6. *Bactericidal action of diluted ERI*

With the *P. gingivalis* samples, a bacterial suspension (10^8/mL) was added to 10 mL of ERI diluted to concentrations of 50, 25, 15, 10, and 5% (undiluted ERI-1% CMC-Na), and 10 μL was added to 1 mL of GAM broth medium. In addition, 0.1 mL of this was added to GAM agar. All of these procedures were performed aerobically at room temperature. Subsequently, culture was continued in an anaerobic box using an AnaeroPack® Kenki at 37°C for 2-5 days, and CFU/mL was determined.

With the *A. actinomycetemcomitans* samples, a similar experiment was performed using GAM broth and agar medium.

2.7. *Dependence on the pH of ERI*

The pH of ERI was adjusted with hydrochloric acid using the pH/Ion Meter F-53 (Horiba, Ltd., Japan) as a combination electrode. Each bacteria species was cultured using the above methods, and CFU/mL was determined.

2.8. *Influence of the pH of ERI or NaCl solution*

The pH of ERI was adjusted using the above method. The pH of NaCl solution was adjusted using a pH meter after the addition of NaOH solution to 0.3% NaCl. Each bacteria species was cultured using the above method, and CFU/mL was determined.

3. Results

Two typical periodontopathic bacteria species (*P. gingivalis* and *A. actinomycetemcomitans*) were added to ERI and ERI-1% CMC-Na, and the time course of their bactericidal action was evaluated. The results are shown in Figure 1. As shown in Figures 1A and 1B, the viable cell count (CFU/mL) did not change after exposure to the controls of 0.3% NaCl or 1% CMC-Na even after 60 sec, whereas more than 99 and 100% of each bacteria species were killed after exposure to ERI or ERI-1% CMC-Na for 15 and 30 sec, respectively.

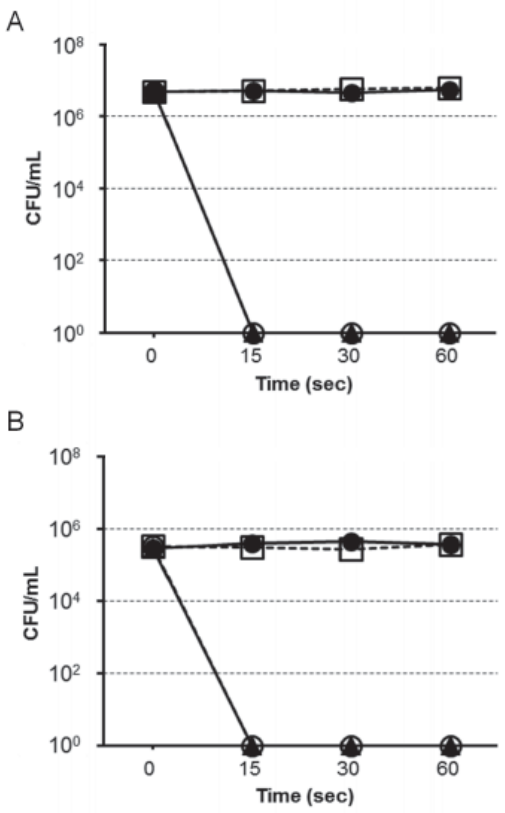

Figure 1. Bactericidal action of ERI or ERI-1% CMC-Na on *P. gingivalis* (A) and *A. actinomycetemcomitans* (B). Control (0.3% NaCl): solid line (●), ERI: solid line (▲), Control-1 (0.3% NaCl + 1% CMC-Na): dashed line (□), ERI-1% CMC-Na (ERI + 1% CMC-Na): dashed line (○). ERI: electrolyzed ion-reduced water; CMC-Na: sodium carboxymethylcellulose.

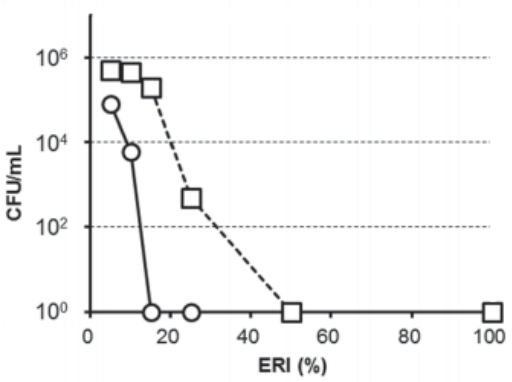

Figure 2. Bactericidal action on *P. gingivalis* and *A. actinomycetemcomitans* by ERI concentration. *P. gingivalis*: solid line (○), *A. actinomycetemcomitans*: dashed line (□).

The results of evaluating the concentration dependence of the effects of ERI on the two periodontopathic bacteria species are shown in Figure 2. The bactericidal action of ERI was concentration-dependent. Viable *P. gingivalis* bacteria were observed after exposure to ERI diluted with 0.3% NaCl to a concentration of 10%, but no viable bacteria were observed at an ERI concentration of 15%. Similarly, viable *A. actinomycetemcomitans* bacteria were present at an ERI concentration of 25% but absent at a concentration of 50%.

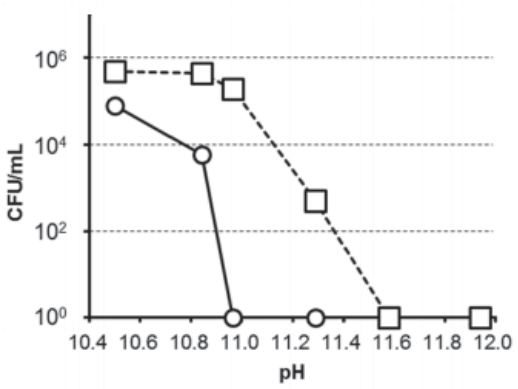

Figure 3. Bactericidal action of ERI on *P. gingivalis* and *A. actinomycetemcomitans* by dependence on pH. *P. gingivalis*: solid line (○), *A. actinomycetemcomitans*: dashed line (□).

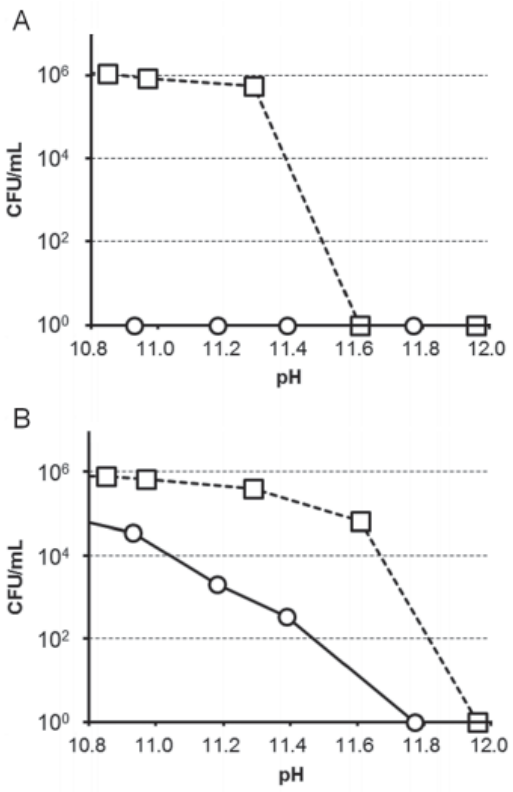

Figure 4. Bactericidal action on *P. gingivalis* (A) and *A. actinomycetemcomitans* (B) in terms of pH changes. ERI + HCl: solid line (○), 0.3% NaCl + NaOH: dashed line (□).

Results regarding the effect that a decrease in the pH of ERI had on its bactericidal action are shown in Figure 3. No viable *P. gingivalis* bacteria were observed at a pH ≥ 11.0. Bactericidal action on *A. actinomycetemcomitans* bacteria was observed at a pH ≥ 11.6.

Bactericidal action was compared when the pH of 0.3% NaCl was adjusted by adding dilute NaOH solution and that of ERI was adjusted by adding dilute HCl solution. The results are shown in Figure 4. With ERI, viable *P. gingivalis* bacteria were observed at a pH ≥ 10.8. With 0.3% NaCl, viable *P. gingivalis* bacteria were present at a pH of 11.3 but absent at a pH of 11.6 (Figure 4A). With ERI, viable *A. actinomycetemcomitans*

bacteria were present at a pH of 11.4 but absent at a pH of 11.8. With 0.3% NaCl, viable bacteria were present at a pH of 11.6 but absent at a pH of 12.0 (Figure 4B).

4. Discussion

Two periodontopathic bacteria species were exposed to ERI and ERI-1% CMC-Na, and the time course of their bactericidal action was evaluated (Figure 1). Results indicated no difference in the bactericidal action of ERI and ERI-1% CMC-Na, confirming that the addition of 1% CMC-Na had no effect on the bactericidal action of ERI. In this study, CMC-Na was added based on a study on electrolyzed acidic gels by Kase *et al.* (*21*). In their study, a glycerin solution containing salt was electrolyzed, and electrolyzed acidic water in gel form was produced to prolong the bactericidal action of electrolyzed strongly acidic water. Since ERI does not have prolonged bactericidal action when used by itself in the oral cavity, CMC-Na was added to provide viscosity in order to prolong bactericidal action. As shown in Figures 1A and 1B, ERI and ERI-1% CMC-Na had bactericidal action persisting for several dozen seconds, so the bactericidal action of mouthwashes with antimicrobial agents such as chlorine dioxide, cetylpyridinium chloride, chlorhexidine, and triclosan (*9*) or toothpastes containing those agents should help with treatment to prevent periodontal disease.

Evaluation of the concentration dependence of the bactericidal action of ERI indicated that ERI had bactericidal action on *P. gingivalis* at a concentration of 10% of undiluted ERI and on *A. actinomycetemcomitans* at a concentration of 25% (Figure 2). These results suggest differences in the bactericidal action of ERI for these bacteria species.

Evaluation of the effect that a decrease in the pH of ERI had on bactericidal action indicated that ERI had decreased bactericidal action on *P. gingivalis* at a pH ≤ 11.0 and on *A. actinomycetemcomitans* at a pH ≤ 11.6 (Figure 3). Therefore, bactericidal action of ERI and a dilute NaOH solution (Figure 4) was compared in a more limited pH range (pH 10.8-12.0). No viable *P. gingivalis* bacteria were observed with ERI at a pH ≥ 10.9 and dilute NaOH solution at a pH ≥ 11.6 (Figure 4A). No viable *A. actinomycetemcomitans* bacteria were observed with ERI at a pH of 11.8 and dilute NaOH solution at a pH of 12.0 (Figure 4B). These results suggest that the bactericidal action of ERI is due to not only to hydroxide ions (OH^-) but also to its low oxidation reduction potential (−344 mV).

Lee and Choi (*9*) reported that the antibacterial activity of electrolyzed water (pH 8.4) is presumably due to 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. The aforementioned results and the current results suggest that the antibacterial activity of ERI on two types of bacteria is due to the synergistic effect of a very high negative oxidation-reduction potential (−344 mV) and hydroxyl radicals (OH^-). This may prove extremely useful to the prevention and treatment of periodontal diseases through daily oral care, such as rinsing the mouth out with ERI and/or brushing the teeth with ERI-1% CMC-Na.

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