Brief Report

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Antiviral effect of electrolyzed reduced water on SARS-CoV-2

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SUMMARY The inhibitory activity of electrolyzed reduced water (ERW) against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which is the etiological agent responsible for coronavirus disease 2019 (COVID-19), was tested *in vitro* on Vero E6 cells using a plaque assay. Infectious virus titers of cells treated with ERW 100%, 50% and 33.3% solutions and phosphate buffered saline (PBS, negative control) and exposed to the virus suspension for 60 seconds were 2.25, 2.65, 3.21 and 7.38, respectively. ERW has a high pH and low surface tension. It is considered that the alkaline property of ERW breaks down phospholipids and proteins of envelopes. The role of pH and reducibility on the virucidal effect of ERW should be further evaluated. This study provides a foundation for utilizing ERW as an effective antiviral aqueous solution in a variety of applications.

Keywords Electrolyzed reduced water, alkaline electrolyzed water, reducibility, SARS-CoV-2, antiviral activity

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent responsible for Coronavirus disease 2019 (COVID-19). The global pandemic of COVID-19 has led to an urgent need to develop antiviral agents. Disinfectants, such as benzalkonium chloride and ethyl alcohol-based formulations, have sufficient antiviral activity but also cytotoxicity (*1*). In terms of safety, low cytotoxicity of disinfectants is important.

Electrolyzed water is generated through water electrolysis containing sodium chloride or potassium chloride. At the anode, acidic electrolyzed water is obtained and this has a low pH, high oxidation-reduction potential (ORP) and high concentrations of dissolved oxygen and free chlorine. At the cathode, alkaline electrolyzed water is obtained and this has a high pH, low ORP and a high concentration of ionized alkali metals such as sodium and potassium. It is known that acidic electrolyzed water has marked bactericidal activity and is regarded as a non-environmental hazard; thus, it is widely used as a disinfectant for foods (*2*). Several studies have shown that acidic electrolyzed water has antiviral effects against some kinds of viruses including SARS-CoV-2 (*3-6*). However, hypochlorous acid produced as acidic electrolyzed water (effective chlorine concentration: 100 ppm) has been reported to be insufficiently effective against SARS-CoV-2 under protein loading conditions (*7*). In addition, it is known that acidic electrolyzed water is cytotoxic (*8*), whereas alkaline electrolyzed water has low cytotoxicity compared to acidic water (*9*) and is safe to drink (*10*). Several studies have shown that alkaline electrolyzed water has antibacterial activity against some bacteria (*11,12*) and antiviral effects against some viruses (*13*). However, there have been no reports on the anti-SARS-CoV-2 effect of alkaline electrolyzed water. Therefore, this study aimed to evaluate the virucidal effects of alkaline electrolyzed water against SARS-CoV-2. Okajima *et al.* performed bactericidal tests against periodontopathic bacteria (*14*) and found anti-inflammatory and healing effects in thermal wounds (*15*) using electrolyzed reduced water (ERW, product name "S-100™", supplied by A. I. System Products Co.). Although ERW is characterized by alkalinity, its composition differs from that of the general alkaline electrolytic water described above. ERW is produced by electrolysis of water containing natural salt at high voltage, resulting in a weakly basic liquid containing 0.3% inorganic salts, such as sodium, potassium, calcium, magnesium, chlorine, silicon and phosphorus (*16*), with a pending alkali reserve value equivalent to 0.1 g/100 mL. In this study, the antiviral effect of ERW (classified as one of the alkaline electrolytic waters) on SARS-CoV-2 was evaluated.

2. Materials and Methods

2.1. Materials

The JPN/TY/WK-521 strain of SARS-CoV-2 was provided by National Institute of Inflectional Disease, Japan. Vero E6 cells (VeroE6/TMPRSS2; #JCRB1819) were purchased from the Japan collection of Research Bioresources: JCRB1819. Dulbecco's modified Eagle's medium (DMEM, low-glucose; SIGMA, #D6046), Minimum Essential Medium Eagle (EMEM; SIGMA, #M4655), Fetal Bovine Serum (FBS; SIGMA, #173012) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Soya Casein Digest Lecithin Polysorbate Broth (SCDLP; Bouillon medium, #E-MC72), was purchased from Eiken Chemical Co., Ltd. (Tokyo, Japan). Costar® 6 Well Clear TC-Treated Multiple Well Plates, Bulk Packed, Sterile (Product #3506), were purchased from Corning (NY, USA). Minimum Essential Medium Eagle (modified) with Earle's salt (EMEM; MP Biomedicals Catalog #1010122), was purchased from MP Biomedicals (CA, USA). ERW for evaluation of this study was provided by A. I. System Products Corp. (Aichi, Japan). In this study, the antiviral activity of 100%, 50% and 33.3% ERW was evaluated. The physical properties of ERW, purified water and sodium hydroxide solution at each dilution rate are shown in Table 1. The pH and biological antioxidant potential (BAP) values in the table were measured with a pH meter (HI9916N; Hanna Instruments Japan Co., Ltd., Chiba, Japan) and Hand spectrophotometer (FREE carrio duo; Wismerll Co., Ltd., Tokyo, Japan), respectively. BAP is a method for quantifying the reducing power of a reaction from an oxidized state (Fe^{3+}) to a reduced state (Fe^{2+}) (*17*).

2.2. Methods

2.2.1. Preparation of virus suspension

SARS-CoV-2 was added to the Vero E6 cells and incubated with EMEM at 37°C. The cell suspension was transferred to a conical tube and centrifuged at 1,000 × g for 15 min at 4°C. The supernatant was used as a test virus suspension.

Table 1. Characteristics of purified water, sodium hydroxide solution and ERW.

Items	pH at 25°C	BAP/μmol/L
Purified Water	5.7	258
NaOH aq.	12.1	267
ERW 100%	12.1	4,905
ERW 50%	11.8	2,469
ERW 33.3%	11.7	1,724

2.2.2. Cytotoxicity test

Cytotoxicity test was performed to confirm that ERW had no cytotoxic effect on Vero E6 cells. EMEM (0.1 mL) was added to 1.9 mL of ERW; 0.1 mL of this mixture was then added to 0.9 mL of inactivating agent (SCDLP: DMEM including 2% FBS = 1:9) and then stirred well. This was repeated to prepare a 10-fold dilution of the series from 10^{-1}-10^{-4}. Cytotoxicity was determined using a plaque assay (see 2.2.5. for details).

2.2.3. Susceptibility of cells to the virus

This test was performed to confirm the susceptibility of cells to the virus. EMEM (0.1 mL) was added to 1.9 mL of ERW and then stirred well; 0.5 mL of this was then mixed well with 4.5 mL of inactivating agent (SCDLP: DMEM including 2% FBS = 1:9) at room temperature. This was repeated to prepare a 10-fold dilution of the series from 10^{-1}-10^{-4}. Virus suspension, prepared at 4-6 × 10^4 PFU/mL, was added at a rate of 1% to each dilution and incubated at room temperature for 10 min. As a negative control, cells were treated with phosphate buffered saline (PBS). Susceptibility of cells to virus was determined by a plaque assay.

2.2.4. Anti-SARS-CoV-2 effect of ERW

Virus suspension (0.1 mL) was added to 1.9 mL of ERW 100%, 50% or 33.3% ERW solutions (diluted with purified water), and then mixtures were stirred sufficiently. After being kept at 25°C for 20 seconds, 60 seconds or 5 minutes, 0.5 mL of each mixture was added to 4.5 mL of inactivating agent (SCDLP: DMEM including 2% FBS = 1:9) and stirred well. The prepared solution was used as the test solution. Using this test solution, various 10-fold diluted solutions of the series from 10^{-1}-10^{-4} were prepared as shown in Figure 1. As a negative control, cells were treated with PBS. The anti-SARS-CoV-2 effect of ERW was determined using a plaque assay.

2.2.5. Plaque assay

Monolayer Vero E6 cells were cultured in a six-well plate and growth medium was removed from the plate. A serial dilution of the virus-containing supernatants (test solution) was inoculated into the Vero E6 cell monolayer. The cells were then incubated for viral adsorption in a 37°C 5% CO_2 incubator and the test solutions were spread over cells every 15 minutes. After cleaning the cell surface with EMEM, cells were overlaid with 3 mL/well of EMEM including 2% FBS. Cells were then incubated for a further 40-48 h. After fixing the cells, plaque phenotypes were visualized by staining with methylene blue. Plaque numbers were counted as plaque-forming units per 0.1 mL (PFU/0.1 mL).

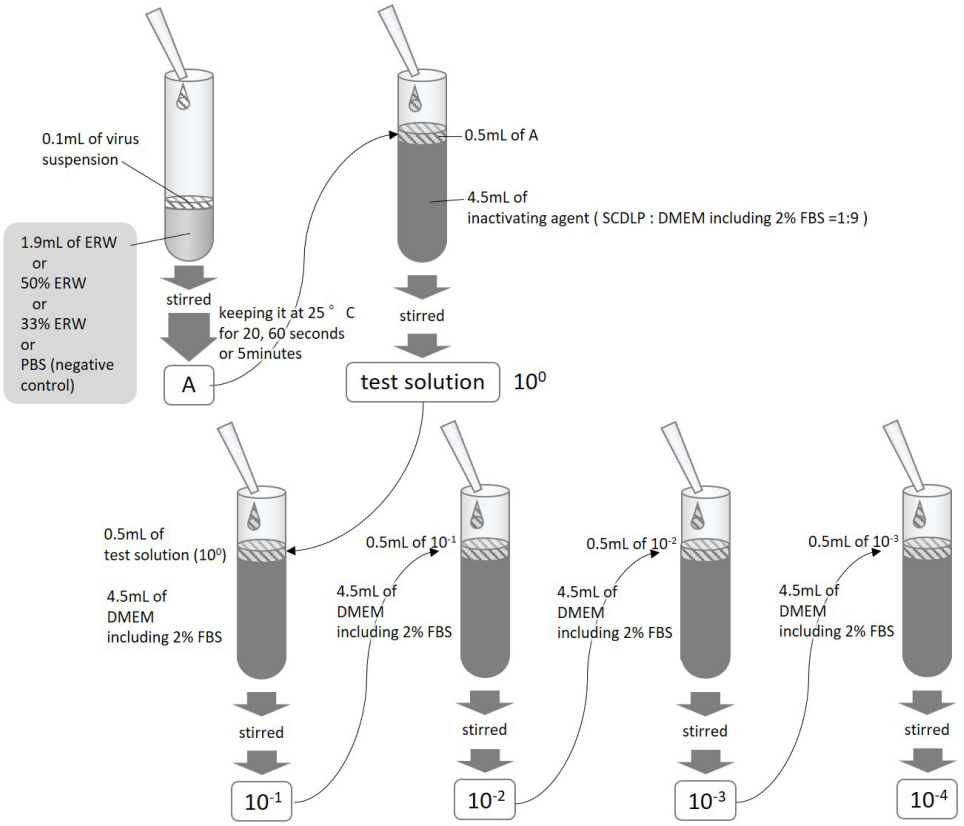

Figure 1. Schematic diagram of dilution method.

2.2.6. Statistical analysis

For each experimental result, significant differences were analyzed using the paired *t*-test. The results are expressed as the mean ± standard deviation. When $p \leq 0.05$ and $p \leq 0.01$, there was a difference at a significance level of 0.05 and 0.01, respectively.

3. Results and Discussion

In the experiment to investigate if ERW has cytotoxic effects on Vero E6 cells (experiment 2.2.1), results showed that ERW 100%, 50% and 33.3% solutions had no cytotoxicity in test conditions. In addition, we confirmed the susceptibility of cells to the virus (experiment 2.2.2). Infectious virus titers in cells exposed to ERW 100%, 50% and 33.3% solutions and PBS were 2.59, 2.60, 2.62 and 2.62 PFU/mL (Log10), respectively. The virus infectivity titer was successfully measured without influence of the sample by diluting the test solution 10-fold with an inactivating agent (SCDLP: DMEM including 2% FBS = 1:9).

To investigate whether ERW has a potential as an anti-SARS-CoV-2 agent, SARS-CoV-2-infected Vero E6 cells were treated for 20 seconds, 60 seconds or 5 minutes with various dilutions of ERW. The results demonstrated that ERW inhibited SARS-CoV-2 replication, whereas virus replication was observed in the negative control (Figure 2). Infectious virus titers in cells treated with ERW 100%, 50% and 33.3% solutions and PBS and exposed to the virus suspension for 60 seconds were 2.25, 2.65, 3.21 and 7.38 PFU/mL (Log10), respectively (Figure 3).

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus. The virus envelope is typically derived from portions of the host cell membranes (phospholipids and proteins), but include some viral glycoproteins. It is considered that the inoculation of ERW breaks down phospholipids and proteins of envelopes. ERW not only has high pH, but also high reducibility, which is not found in NaOH aqueous solutions. Previously, the antiviral activity of NaOH solutions has been reported (*18*), and in the case of SARS-CoV, it took 1-2 hours to reduce more than 5 log10 at pH > 12 (*19,20*). In addition, some reducing agents have been reported to inactivate Sindbis virus and Mayaro virus within 3 hours by cleaving the disulfide bonds of the viral membrane proteins (*21*). In such studies, high pH and high reducibility have antiviral effects but do not lead to inactivation in a short time. On the other hand, ERW, which has both properties, showed antiviral activity of more than 4 log10 in 20 seconds at 100% concentration of pH = 12.1, BAP = 4,905 μmol/L and more than 3 log10 in 60 seconds at 33.3% concentration of pH = 11.3, BAP = 1,724 μmol/L. From these results, it is considered

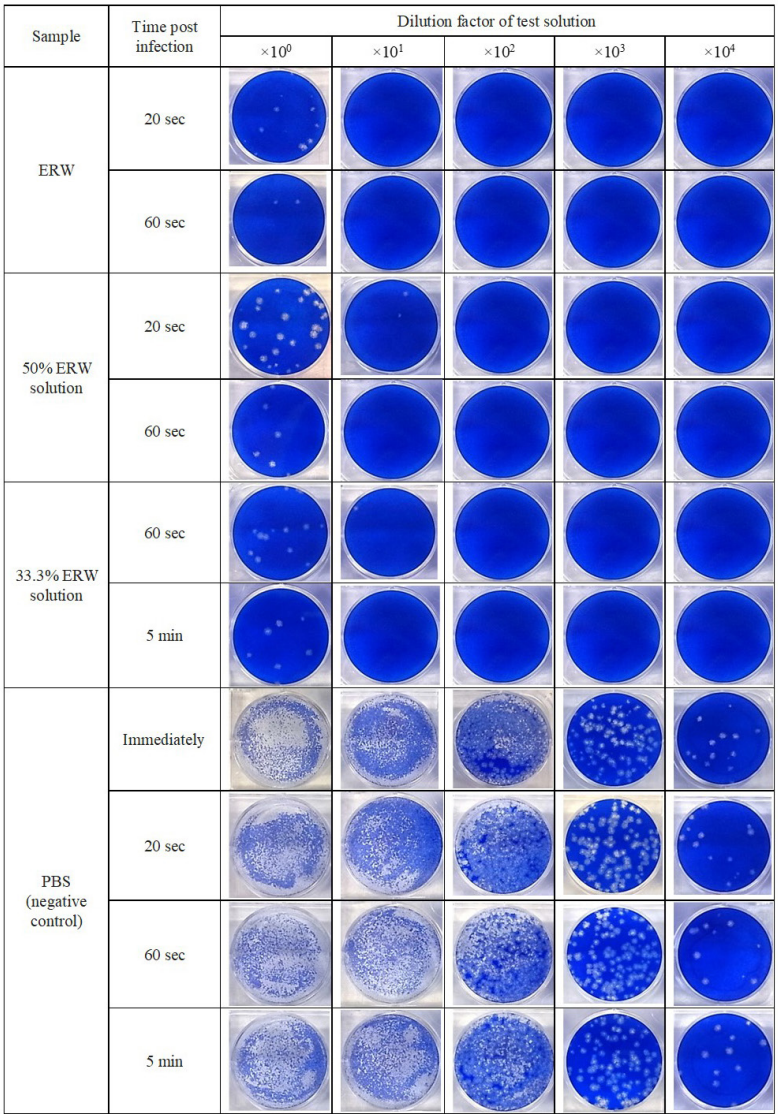

Figure 2. Photographs of the plaque assay for counting SARS-CoV-2. These are the results of photographing a petri dish from above after culturing the virus in each condition. The white spots in the petri dish are called "plaques" and represent the state of cytopathic caused by the virus infection. If there is a large amount of virus infection, the plaques will overlap. In order to avoid it, the test solution is diluted after stopping the drug reaction until the plaques can be counted correctly. The viral titer is calculated from the number of plaques counted, the dilution factor and the virus concentration in the test solution (plaque assay).

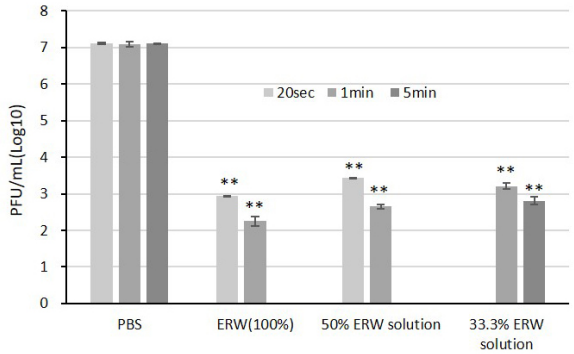

Figure 3. Infectious virus titers in cells treated with various concentration of ERW solutions and PBS. Infectious virus titers (PFU/mL (Log10)) of SARS-CoV-2 of cells mixed with ERW (100%), 50% ERW solution, 33.3% ERW solution and PBS as negative control and exposed to virus suspension for 20 seconds, 1 minute or 5 minutes (mean ± S.D., $n = 3$). $^{**}p < 0.01$ (*t*-test *vs.* PBS).

that the antiviral mechanism of ERW is attributed to the synergistic effect of pH and reducibility properties, which resulted in instantaneous antiviral action. The role of pH and reducibility on the antiviral effect of ERW should be further evaluated.

In conclusion, this study demonstrated the anti-SARS-CoV-2 activity of ERW using a plaque assay. Potent anti-SARS-CoV-2 activities, together with the lack of cytotoxic effects, support the further development of ERW as a monotherapy or in combination with other effective agents against SARS-CoV-2 infection.

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Conflict of Interest: The electrolyzed reduced water (ERW, product name S-100) used in this study was manufactured by A. I. System products, Corp. Yoshinao Okajima, Masahiro Okajima and Mitsuo Ikeda are employees of A. I. System products, Corp.

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