Involvement of reactive oxygen species in the cytotoxic effect of acid-electrolyzed water

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ABSTRACT — Acid-electrolyzed water (AEW) is commonly used as a disinfectant in the agricultural and medical fields. Although several studies have been conducted to examine its toxicity in vitro and in vivo, the cytotoxic mechanism of AEW has never been verified. The purpose of the present study was to elucidate the underlying mechanism by which AEW exerts its in vitro cytotoxic effect. Mouse fibroblasts treated with AEW experienced dilution rate-dependent cytotoxic effects in the 100% confluent phase as well as in the mitotic phase. The levels of intracellular reactive oxygen species (ROS) increased significantly in fully-confluent cells treated with undiluted and four times diluted AEW. In both of these treatments, cytotoxicity was also observed. It is thus concluded that the in vitro cytotoxicity of AEW is attributable to increased intracellular ROS. Additionally, the ROS responsible for these effects appears to be, at least in part, hydroxyl radical because the increase in intracellular ROS was attenuated by post-treatment with dimethyl sulfoxide, a hydroxyl radical scavenger, and with the antioxidant polyphenol, proanthocyanidin.

Key words: Acid-electrolyzed water, Mouse fibroblast, Cytotoxicity, Reactive oxygen species

INTRODUCTION

Acid-electrolyzed water (AEW) is produced by the electrolysis of dilute sodium chloride (NaCl) or potassium chloride solution on the anode side of an instrument where the anode and cathode are separated by an ion-permeable diaphragm. AEW is commonly used in the agricultural and medical fields as a disinfectant for farm and food hygiene (Fabrizio and Cutter, 2004; Northcutt et al., 2007; Guentzel et al., 2008; Cao et al., 2009; Park et al., 2009; Lu et al., 2010; Rahman et al., 2010, 2011). It is also widely used for the disinfection of medical instruments such as dialyzers (Tanaka et al., 2000), endoscopes (Lee et al., 2004), and dentures (Nagamatsu et al., 2001) because of its potent antimicrobial potential (Kiura et al., 2002; Sharma and Demirci, 2003; Nisola et al., 2011; Rodriguez-Garcia et al., 2011; Feliciano et al., 2012). AEW has potent oxidative power in relation to its antimicrobial potential. The hydroxyl radical (‘OH), an oxygen radical species, has been suggested as the putative active agent responsible for AEW antimicrobial activity (Hotta, 2000). However, our previous study demonstrated that hydrogen peroxide (H$_2$O$_2$) is present in AEW and may be a source of `OH, but that the antimicrobial activity of AEW does not depend on the radicals existing in AEW (Mokudai et al., 2012). To date, the antimicrobial activity of AEW has been thought to derive from hypochlorous acid (HClO), which has a low pH and a high oxidation-reduction potential (ORP) (Al-Haq et al., 2005). Some studies have also suggested that HClO, as an undisassociated form of chlorine, penetrates microbial cell membranes and subsequently achieves its antimicrobial action through the oxidation of key metabolic enzymes (Albrich et al., 1986; Barrette et al., 1989; Hurst et al., 1991).

Several studies have been conducted for a safety evaluation of AEW. Treatment of mouse fibroblasts with serial two-fold dilutions of AEW with distilled water resulted in cytotoxicity comparable to that of acidic hypochlorite solution (Okubo et al., 1999). Another study demonstrated that AEW was cytotoxic in human pulp cells in vitro but the cytotoxicity was mild when compared with that of sodium hypochlorite solution (Gomi et al., 2010). However, the mechanism by which AEW exerts its cytotoxic effect has not been discussed in these studies. In vivo, AEW has low toxicity, presumably owing to the presence of organic materials such as proteins and amino acids that
rapidly transform free available chlorine in AEW into
N-chloro compounds (Oomori et al., 2000). For instance,
experiments were conducted in mice given free access to
AEW as drinking water. No abnormal findings or meas-
urements were observed in terms of visual inspection of
the oral cavity, histopathological tests, or measurements
of surface enamel roughness of teeth, perhaps because of
rapid neutralization of chlorine in the AEW by saliva and
protein (Morita et al., 2011). Furthermore, peritoneal irri-
gation of experimental perforated peritonitis with AEW in
rats showed no adverse effect (Kubota et al., 2009).
In immune cells such as neutrophils and monocytes,
myeloperoxidase catalyzes the formation of HOCl, which
may then be responsible for transition metal-independent
hydroxyl radical (‘OH) generation (Ramos et al., 1992).
It could thus be postulated that the bactericidal effect of
AEW was also induced by intracellular ‘OH, although
there is no clear evidence for the existence of intracellular
’OH. The purpose of this study was to elucidate wheth-
er the underlying mechanism by which AEW exerts its in
vivo cytotoxic effect is dependent on intracellular reactive
oxygen species (ROS) such as ‘OH.

MATERIALS AND METHODS

Preparation of AEW
NaCl solution (0.1% (w/v)) was electrolyzed for
15 min using a batch-type electrolyzed water generator
(ALTRON MINI AL-700A; Altec Corporation, Nagano,
Japan) at a regular AC voltage of 100 V and a rated cur-
rent of 0.6 A. The characteristic values of the resultant
AEW were determined using a pH/ORP meter (SG2;
Mettler-Toledo, LLC, Columbus, OH, USA) for pH
and ORP, and a residual chlorine meter (HI196771C;
Hanna Instruments Japan, Tokyo, Japan) for residual
chloride concentrations. The pH, ORP, and residual chlo-
ride concentration of the undiluted AEW were 2.2-2.7,
≥ 1100 mV, and 50-60 mg/L, respectively. Two-fold serial
dilutions of AEW were then prepared with pure water.
In some experiments, the concentrations of free avail-
able chlorine, including Cl2, HClO, and ClO–, in AEW
were determined by the N,N'-diethyl-p-phenylenedi-
amine (DPD) standard method (Oomori et al., 2000). The
H2O2 concentrations in AEW were also determined by the
colorimetric method based on the peroxide-mediated oxida-
tion of Fe2+ followed by the reaction of Fe3+ with xyle-
nol orange (Jiang et al., 1990).

Cell culture and assay for viable cells
3T3-L1 mouse fibroblasts were purchased from DS
Pharma Biomedical Co., Ltd. (Osaka, Japan). Dulbecco’s
Modified Eagle Medium (DMEM, Thermo Fisher
Scientific, Waltham, MA, USA) containing 10% fetal
bovine serum (Thermo Fisher Scientific), 100 U/mL of
penicillin (Wako Pure Chemicals, Osaka, Japan), and
0.1 mg/mL of streptomycin (Wako Pure Chemicals) was
used as a medium for cell culture. An aliquot (100 μL) of
the cell suspension (2 × 104 cells/mL) was placed in each
well of a 96 well-culture plate. The plates were incubated
at 37°C in humidified 5% CO2 for 23-25 hr to reach sub-
confluence (20-30%) or 4 days to achieve 100% con-
fluence. In the experiment with sub-confluent cells, cells
were washed with phosphate-buffered saline (PBS, pH 7.4) then
treated with the two-fold serial dilutions of AEW (100 μL /
well) for 30 sec before being washed with fresh medium.
The cells were incubated in culture medium for a further
24 hr before determining cell viability by the methyl thia-
zolyl tetrazolium (MTT) assay, in which an insoluble for-
mazan converted from MTT was colorimetrically quanti-
ﬁed at 595 nm using a microplate reader (FilterMax F5;
Molecular Devices, Sunnyvale, CA, USA). The MTT
assay was performed using a TACS® MTT Cell Prolif-
eration Assay kit (Treivgen Inc., Gaithersburg, MD, USA).
In the experiment with 100% confluent cells, cells were
treated exactly as for the sub-confluent cells, except that
the number of viable cells was determined by MTT assay
immediately after washing (i.e., no 24 hr incubation). In
both experiments, cells treated with pure water instead
of serial dilutions of AEW were used as the corresponding
controls. To examine the effect of low osmotic stress by
exposure of cells to pure water for 30 sec, phosphate
buffered saline (PBS, pH 7.4)-treated group was added
to the experiment with sub-confluent cells. In the group,
cells were similarly treated with PBS for 30 sec. In addi-
tion, to confirm if low pH affect the cell viability, 0.01 M
NaH2PO4 solution (pH was adjusted to 2.5 by adding 1 M
HCl) with 0.15 M NaCl was prepared as an isotonic solu-
tion with low pH, and effect of the solution on the cell
viability was similarly examined in sub-confluent cells.

Effect of bovine serum albumin (BSA) on
AEW-induced cytotoxicity
The bactericidal activity of AEW is attenuated in the
presence of organic materials such as proteins and ami-
no acids through quick transformation of free available
chlorine into N-chloro compounds (Oomori et al., 2000).
We therefore tested the effect of BSA (Wako Pure
Chemicals) on the AEW-induced cytotoxicity. Sub-con-
fluent cells, plated as for the cytotoxicity assay described
above, were washed with PBS and then treated with undi-
luted AEW supplemented with 12.5-100 μg/mL of BSA
(100 μL/well) for 60 sec before washing with fresh medi-
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um and incubating for a further 26 hr in fresh medium. Cell viability was then assessed by MTT assay. Additionally, free available chlorine and \( \text{H}_2\text{O}_2 \) concentrations in the culture medium were determined. Control cells were treated with pure water instead of AEW for 60 sec and, after washing, were incubated in fresh medium.

**Intracellular reactive oxygen species (ROS) determination**

Cells at 100% confluence were washed twice with PBS and then treated with four-fold serial dilutions of AEW for 30 sec, then washed twice more with PBS. Intracellular ROS were determined immediately using an Oxiselect™ Intracellular ROS Assay kit (Cell Biolabs, Inc., San Diego, CA, USA) in accordance with the protocol provided by the manufacturer. In this assay, a cell-permeable probe (2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)) is dissolved in serum-free medium, diffuses into cells and is deacetylated to a nonfluorescent product, 2',7'-dichlorodihydrofluorescein (DCFH) by cellular esterases. In the presence of cytosolic ROS, DCFH is oxidized to a highly fluorescent, 2',7'-dichlorodihydrofluorescein (DCF). In brief, after washing with PBS, 100 \( \mu \text{L} \) of 1 mM DCFH-DA dissolved in serum-free DMEM was added to each well followed by incubation at 37°C in humidified 5% \( \text{CO}_2 \) for 1 hr. After washing twice with PBS, 100 \( \mu \text{L} \) of serum-free DMEM and 100 \( \mu \text{L} \) of 2X Cell Lysis Buffer (provided by the kit) were added to each well followed by mixing thoroughly and incubation for 5 min. Then 150 \( \mu \text{L} \) of the mixture was transferred to each well of a black 96 well culture plate for reading fluorescence at the excitation and emission wavelengths of 485 and 535 nm by using the microplate reader (FilterMax F5). In the experiment in which the effect of dimethyl sulfoxide (DMSO), a \( \cdot \)OH scavenger, and proanthocyanidin (Leucoselect®, Indena S.p.A., Milan, Italy), an antioxidant polyphenol, were examined, cells at 100% confluence were washed twice with PBS and treated with undiluted or four-times diluted AEW for 30 sec before being washed twice with PBS. Immediately after washing, 100 \( \mu \text{L} \) of 1 mM DCFH-DA containing 140 mM DMSO or 1 mg/mL of proanthocyanidin were added to each well (100 \( \mu \text{L/well} \)) and incubated for a further 1 hr. Intracellular ROS were then determined by the Oxiselect™ technique as described above. In some specimens cultured in chamber slides and similarly treated with AEW, fluorescence microscopy was performed using a fluorescence microscope (Leica DM 4500 B; Leica Microsystems, Wetzlar, Germany). Control cells treated with pure water instead of serial dilutions of AEW were used as controls. Regarding the timing of DCFH-DA load to determine intracellular ROS, desired timing was before the AEW treatment. However, since we confirmed that both DMSO and proanthocyanidin reduced free available chlorine in AEW to below the detection limit, DCFH-DA load with these scavengers could not be conducted before the AEW treatment.

**Statistical analysis**

The experiments investigating the cytotoxicity of two-fold serial dilutions of AEW, the cytotoxicity of AEW in the presence of BSA, and measuring the intracellular ROS induced by four-fold serial dilutions of AEW were statistically compared with their respective control groups by Dunnett’s multiple comparison test. The statistical significance of differences in the cytotoxicity of pure water and the low pH isotonic solution, and the intracellular ROS induced by AEW in the presence of either DMSO or proanthocyanidin were assessed by the Tukey-Kramer multiple comparison test. \( P \) values of less than 0.05 (\( P < 0.05 \)) were considered to be significant.

**RESULTS AND DISCUSSION**

Figure 1a shows the cytotoxic effect of two-fold serial dilutions of AEW on cells in the mitotic phase (i.e., sub-confluent cells). When cells in the mitotic phase were treated with two-fold serial dilutions of AEW for 30 sec, cell viability (compared with control) was significantly reduced at 24 hr after treatment with AEW preparations diluted four times or less. Viability of cells treated with undiluted AEW was only ~20% of that in the control. Figure 1b shows the effect of pure water used as the control in Fig. 1a and the low pH isotonic solution on cells in the mitotic phase. Regarding the effect of low osmolality by exposing cells to pure water, cell viability was reduced to approximately 65% of that in the PBS treated group showing that exposure of pure water even for only 30 sec exerted cytotoxic effect in some degree. As for the effect of low pH on the cell viability, since the isotonic solution with pH 2.5 showed no significant effect on the cell viability as compared with that in the PBS-treated group, low pH seemed to exert no toxic effect at least under the condition of 30 sec exposure.

Figure 2 shows that BSA protects against the cytotoxic effect of AEW. BSA reduced the levels of both free available chlorine and \( \text{H}_2\text{O}_2 \) (Fig. 2a). Furthermore, the cytotoxicity of undiluted AEW (applied for 60 sec) was reduced in a dose-dependent manner by BSA, up to ~80% in the presence of 100 \( \mu \text{g/mL} \) BSA (Fig. 2b).

Figure 3 shows the cytotoxic effect of two-fold serial dilutions of AEW on fully confluent cells. When treated
for 30 sec, there was no observable cytotoxic effect when the AEW was diluted by four times or more. A significant reduction in viability was observed in cells treated with undiluted and two-times diluted AEW, but nevertheless remained at ~60 and ~80% of control, respectively.

Figure 4a shows a fluorescence microscopy image of ROS production in the 100% confluent cells treated with undiluted AEW for 30 sec, in which DCF fluorescence can be clearly observed. The intracellular ROS level in these 100% confluent cells after treatment with four-fold serial dilutions of AEW for 30 sec was also determined. A significant increase in intracellular ROS was observed in cells treated with AEW diluted four times, and a dramatic increase (~10-fold, vs. control) was seen in cells treated with undiluted AEW (Fig. 4b). As shown in Fig. 5, this increased intracellular ROS level induced by AEW was significantly reduced by the application of 140 mM DMSO or 1 mg/mL of proanthocyanidin in combination with 1 mM DCFH-DA for 1 hr. Increased ROS levels induced by a 1/4 dilution of AEW were reduced
back to baseline by both DMSO and proanthocyanidin (Fig. 5a). Similarly, DMSO and proanthocyanidin both produced significant (but not total) reductions in the ROS levels induced by undiluted AEW (Fig. 5b). It was confirmed that 140 mM DMSO and 1 mg/mL of proanthocyanidin were not themselves cytotoxic. Since it seemed to be important to confirm if the ROS scavengers could ameliorate cytotoxic effect of AEW, we examined the effect of DMSO on the viability of the cells exposed to AEW. However, post-treatment with DMSO failed to rescue the cells treated with AEW (data not shown). It was presumed that dead cells suffered from lethal effect dur-
The cytotoxicity of AEW is caused by intracellular ROS, presumably \( \cdot \text{OH} \), derived from HClO penetrating through the cell membrane. These results suggest that the cytotoxicity of AEW is caused by intracellular ROS, presumably \( \cdot \text{OH} \), derived from HClO penetrating through the cell membrane. To confirm which ROS are involved in the cytotoxic effect of AEW and to elucidate the mechanism of ROS formation, further study should be required.

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**Conflict of interest**—— The authors declare that there is no conflict of interest.

**REFERENCES**


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