

# Possible involvement of reactive oxygen species in biological effects of acid electrolyzed water

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## 論文内容の要旨

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 <sup>1-7</sup>. It is also widely used for the disinfection of medical instruments such as dialyzers <sup>8</sup>, endoscopes <sup>9</sup>, and dentures <sup>10</sup> because of its potent antimicrobial potential <sup>11-15</sup>.

The antimicrobial activity of AEW might be the result of the combined effects of the oxidation–reduction potential (ORP) and pH <sup>16</sup>. The characteristic values of AEW, such as low pH (2.7 or lower) and high ORP (+1100 mV or higher), deviate from the tolerable range for microbial growth (pH 3–10, ORP +900– 400 mV) proposed by Becking *et al* <sup>17</sup>.

In addition, AEW has potent oxidative power in relation to its antimicrobial potential, and hydroxyl radical ( $\cdot\text{OH}$ ), an oxygen radical species, is often suggested as a putative active ingredient for AEW antimicrobial activity <sup>18</sup>.

The purpose of my study is to examine the presence of reactive oxygen species (ROS) in AEW in relation to its biological effects including antimicrobial activity and cytotoxicity.

Studies conducted so far are summarized below.

### **Determination of reactive oxygen species (ROS) in AEW**

The aim of the study was to examine if ROS such as  $\cdot\text{OH}$  are present in AEW. The oxygen radicals in AEW prepared under different conditions were determined using an electron spin resonance (ESR) technique. A signal from 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)-OH, an adduct of DMPO and  $\cdot\text{OH}$ , was detected in AEW prepared

by double or triple electrolyses of 1% NaCl but not of 0.1% NaCl solution. In addition, the presence of hydrogen peroxide as a proposed source of  $\cdot\text{OH}$  was also examined using a combination of ESR and a Fenton reaction. The DMPO-OH signal was clearly detected, even in AEW prepared by single electrolysis of 0.1% NaCl solution, when ferrous sulfate was added to induce a Fenton reaction, indicating the presence of hydrogen peroxide in the AEW. Since sodium formate, a  $\cdot\text{OH}$  scavenger, did not affect the bactericidal activity of AEW, it is concluded that the radical unlikely contributes to the antimicrobial activity of AEW, although a small amount of the radical is produced from hydrogen peroxide. Dimethyl sulfoxide, the other hydroxyl radical scavenger used in the present study, canceled the bactericidal activity of AEW, accompanied by complete depletion of free available chlorine, suggesting that HClO is probably a major contributor to the antimicrobial activity. In conclusion, although hydrogen peroxide is present in AEW as a source of  $\cdot\text{OH}$ , the antimicrobial activity of AEW does not depend on the radicals.

## **2. Involvement of ROS in the inhibition of microbial growth by AEW**

The lag of bacterial and fungal regrowth after a short-term exposure to antimicrobial agents is known as the postantibiotic effect and postantifungal effect (PAFE), respectively <sup>19-24</sup>. I evaluated the PAFE-like activity of AEW against *Candida albicans* under sublethal conditions by exposing *C. albicans* to dilute AEW. The growth of *C. albicans* after a short-term exposure to dilute AEW was evaluated in broth and on agar culture. The involvement of ROS in the PAFE was examined by flow cytometric analysis with hydroxyphenyl fluorescein (HPF) as a fluorescence probe. The dilute AEW exerted PAFE-like activity against *C. albicans*. ROS were produced in the cells treated with AEW diluted 16 times or fewer. The increase in HPF fluorescence after treatment with dilute AEW was cancelled by dimethyl sulfoxide, a  $\cdot\text{OH}$  scavenger. From these results, it would be expected that the ROS, especially  $\cdot\text{OH}$ , produced in the *C. albicans* cells treated with sublethal dilutions of AEW could exert PAFE-like activity against the fungal cells.

### 3. Involvement of ROS in the cytotoxic effect of AEW

I have shown that short-term treatment with dilute AEW inhibits the growth of *C. albicans* via intracellular ROS formation. Thus, I speculated that cytotoxic effect of AEW would also be mediated by intracellular ROS formation. Although several studies have been conducted to examine toxicity of AEW *in vitro* and *in vivo*, the cytotoxic mechanism of AEW has never been verified. The purpose of the 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 ROS assayed by a cell-permeable probe (2',7'-dichlorodihydrofluorescein diacetate) 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,  $\cdot\text{OH}$  because the increase in intracellular ROS was attenuated by post-treatment with dimethyl sulfoxide, a  $\cdot\text{OH}$  scavenger, and with the antioxidant polyphenol, proanthocyanidin.

### 4. Conclusion

HClO penetrating through the cell membrane of not only microorganisms but mammalian cells would be converted to  $\cdot\text{OH}$  that causes oxidative cellular damage. The  $\cdot\text{OH}$  may be formed as follows: 1) electron leakage from the aerobic respiration in the AEW-treated cells would reduce oxygen molecules ( $\text{O}_2$ ) to  $\text{O}_2^{\cdot-}$ , and then the reaction between HClO and  $\text{O}_2^{\cdot-}$  generates  $\cdot\text{OH}$  as in the following way,  $\text{HClO} + \text{O}_2^{\cdot-} \rightarrow \cdot\text{OH} + \text{O}_2 + \text{Cl}^-$  (except that the cases of anaerobes), 2)  $\text{H}_2\text{O}_2$ , which arises from the dismutation of  $\text{O}_2^{\cdot-}$  or is penetrated through the cell membrane, and trace metals such as ferrous ions would generate  $\cdot\text{OH}$  as in the following way,  $\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{OH}^- + \text{Fe}^{3+}$  (a Fenton-like reaction), and 3) the one-electron reduction of HClO in the presence of ferrous ion would produce  $\cdot\text{OH}$  as in the following way,  $\text{HClO} + \text{Fe}^{2+} \rightarrow \cdot\text{OH} + \text{Cl}^- + \text{Fe}^{3+}$ .

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