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Advances in Microbiology, Infectious Diseases and Public Health

Gianfranco Donelli *Editor*

# Advances in Microbiology, Infectious Diseases and Public Health

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# **Advances in Experimental Medicine and Biology**

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## **Advances in Microbiology, Infectious Diseases and Public Health**

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Gianfranco Donelli  
Editor

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# The Efficacy of an Electrolysed Water Formulation on Biofilms

Anne-Marie Salisbury and Steven L. Percival

## Abstract

Electrolysed water is a basic process whereby an electric current is passed through deionised water containing a low concentration of sodium chloride in an electrolysis chamber, which results in a more complex chemistry resulting in the production of a strong bactericidal and fungicidal solution at the anode. This microbicidal solution contains hypochlorous acid that is fast-acting and environmentally safe, as upon bacterial killing, the equilibrium shifts from hypochlorous acid back to salt and water. Other antimicrobial agents produced in this process include sodium hypochlorite and chlorine. The use of electrolysed water formulations in wound care to control wound bioburden is underway. However, there is limited evidence of the efficacy of electrolysed water on the control of biofilms, which are renowned for their tolerance to a variety of antimicrobials. Therefore this study aimed to assess a new electrolysed water formulation on in vitro *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Results showed that the electrolysed water formulation effectively reduced biofilm in all models following a 15 min contact time. Microbial cell counts confirmed the reduction biofilm bacteria. Additional cytotoxicity using

L929 fibroblasts confirmed that a 50% and 25% dilution of the electrolysed water formulation was non-cytotoxic to cells. In conclusion, this study has confirmed that the application of a new electrolysed water product effectively removed biofilm after a short exposure time. The use of this technology as a wound cleanser may help to control existing biofilms in complicated, non-healing wounds.

## Keywords

Antimicrobial · Biofilms · Electrolysed water · Hypochlorous acid · Wounds

## 1 Introduction

Electrolysis of deionised water containing a low concentration of sodium chloride (0.1%) in an electrolysis chamber whereby the anode and cathode electrodes are separated by a diaphragm has been shown to impart strong bactericidal, fungicidal and virucidal properties to the water collected from the anode. Much research surrounding the bactericidal, fungicidal and virucidal properties of electrolysed water has been investigated in dentistry and the food industry (Huang et al. 2008; Horiba et al. 1999).

By subjecting the electrodes to direct current voltages, negatively charged ions such as chloride and hydroxide in the diluted salt solution move to the anode to give up electrons and become oxygen

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gas, chlorine gas, hypochlorite ions, hypochlorous acid, hydrochloric acid and a small concentration of sodium hypochlorite (by-product), whilst positively charged ions such as hydrogen and sodium move to the cathode to take up electrons and become hydrogen gas and sodium hydroxide. Therefore, the production of two types of water occurs simultaneously: (1) electrolysed water (EW) (from the anode side) with a low pH (2.3–2.7), high oxidation–reduction potential (ORP) (>1000 mV), high dissolved oxygen concentration and free chlorine (between 10 and 80 ppm) and (2) EW (from the cathode) with a high pH (10.0–11.5), high dissolved hydrogen and low ORP (–800 to –900 mV). EW with a high ORP is often referred to in the literature as acidic electrolysed water or electrolysed oxidising water, whereas the EW with a low ORP is referred to as electrolysed-reduced water (Hsu 2005).

The control of bioburden in open wounds is considered to be of great importance in the promotion of wound healing and is thought to reduce the risk of slow wound closure (Wolcott et al. 2010; Edwards and Harding 2004). The development of microbial biofilms within chronic wounds has been reported (James et al. 2008; Dowd et al. 2008; Kirketerp-Møller et al. 2008). Biofilms are complex societies of microorganisms that attach to a surface and surround themselves with extracellular polymeric substances and have increased tolerance to antimicrobials (Donlan 2002). Therefore as a wound care strategy, the control of these biofilms should be investigated. Electrolysed water has already been reported to have positive effects on wound healing (Sen et al. 2002). In vivo studies have shown that the use of acidic or neutral electrolysed water also increases wound healing in full-thickness cutaneous wounds in rats, potentially via keratinocyte and fibroblast migration (Yahagi et al. 2000).

Therefore the aim of this study was to assess the antimicrobial and anti-biofilm efficacy of a new formulation of electrolysed water against microorganisms associated with complicated chronic wounds.

## 2 Method

### 2.1 Microorganisms

The microorganisms *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* NCTC 10662 were employed in all studies.

### 2.2 Test Solution

The electrolysed water used in this study was produced by passing an electric current through deionised water. Product was collected from the Anode chamber and consisted of Hypochlorous acid, Hypochlorite, Hydroperoxyl, Hydroxide, Hydrogen peroxide, Chlorine and Singlet Oxygen, with the 2 main constituents being Sodium hypochlorite and Hypochlorous acid.

### 2.3 Planktonic Time-Kill Studies

This test followed the 10 mL sample size procedure outlined in the American Society for Testing and Materials (ASTM) E2783–11. An overnight culture was set up by inoculating 10 mL tryptone soya broth (TSB) (Oxoid, Hampshire, UK) with a single colony and incubating it at 37 °C and 125 rpm overnight. The following day, the overnight culture was adjusted to 0.5 McFarland ( $\sim 1 \times 10^8$  CFU/mL) before adding 0.1–10 mL ( $\sim 1 \times 10^6$  CFU/mL) of the test antimicrobial. Inoculated samples were incubated at 37 °C (shaken at 125 rpm) for 1 min, 5 min and 24 h. For each time point, a 1 mL sample was taken and diluted 1:10 in the dilution blank. The procedure was performed in triplicate. A blank control (no antimicrobial) was included containing a 0.85% sodium chloride solution. Bacterial enumeration was performed by counting bacterial colonies using the spot-plate technique, which was performed in triplicate. The  $\log_{10}$  of each

sample was subtracted from the  $\log_{10}$  value of the blank control to determine the  $\log_{10}$  reduction value. Percentage kill was then calculated using the following formula:

$$\%kill = (1 - 10^{-\text{Log reduction value}}) \times 100$$

## 2.4 CDC Biofilm Bioreactor Model

The experimental procedure for the CDC biofilm reactor studies followed an adapted version of the ASTM E2871–13, Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in the CDC Biofilm Reactor Using Single Tube Method.

TSB was inoculated with bacteria to a concentration of  $1 \times 10^8$  CFU/ml, which was determined by optical density and total viable counts.

Each CDC reactor contains eight polypropylene rods designed to hold three coupons. In this experiment, polycarbonate coupons were used. The CDC reactor was sterilised before aseptically adding 300 ml of sterile TSB through the inoculation port. Following this, 1 ml of the previously prepared  $10^8$  CFU/ml inoculum was then added to the reactor. The reactor was placed on a magnetic stir plate. The rotation speed was set to  $125 \pm 5$  rpm. The CDC reactor was operated in batch mode at room temperature ( $21 \pm 2$  °C) for 48 h (48 h biofilms).

Following incubation, the rods containing the polycarbonate coupons were removed and rinsed in sterile 0.85% sodium chloride solution to remove planktonic cells. Each coupon was released from the rods into individual sterile 50 ml centrifuge tubes. Each coupon was then treated with 3 mL of the electrolysed water. Treatments of the coupons were performed in duplicate. Antimicrobials used included the electrolysed water at concentrations of 100%, 75%, 50% and 25%. The coupons were then incubated at 37 °C for 5, 15 or 60 min contact times. Following incubation, the antimicrobial was diluted in 27 ml of sterile water. Each 50 ml centrifuge tube containing the coupon and neutraliser was mixed using a vortex for 30 s, followed by sonication for 10 min. The

disaggregated biofilm samples were sampled and serially diluted on tryptone soya agar (TSA) (Oxoid, Hampshire, UK) for bacterial enumeration using the spot-plate technique, which was performed in triplicate. Biofilm density was calculated as  $\log_{10}$  density for each coupon. The  $\log_{10}$  density of each coupon was subtracted from the  $\log_{10}$  density of the untreated control coupon to determine the  $\log_{10}$  reduction value of each treated biofilm. Percentage kill was then calculated using the following formula:

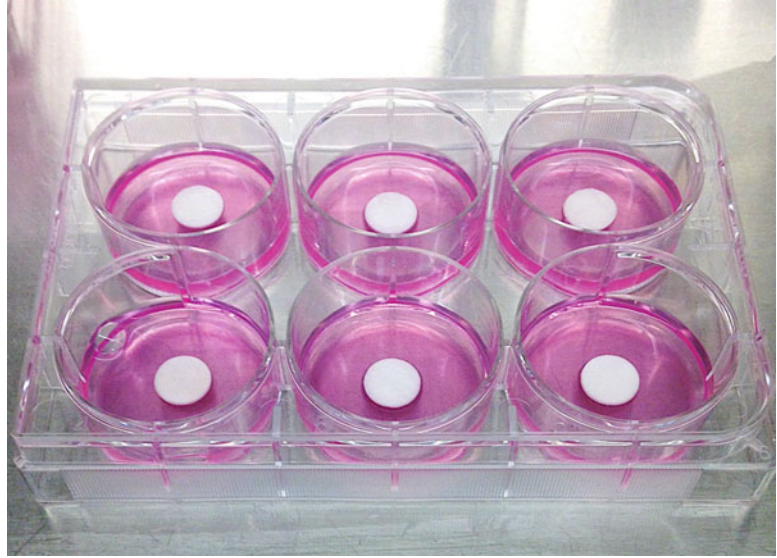
$$\%kill = (1 - 10^{-\text{Log reduction value}}) \times 100$$

## 2.5 Cytotoxicity Testing

Indirect cytotoxicity tests performed were in accordance with the international standard ISO 10993–5 Biological Evaluation of Medical Devices – Part 5: Tests for in vitro cytotoxicity. To supplement the guidance in ISO 10993–5, the ASTM 895–11 Standard Test Method for Agar Diffusion Cell Culture Screen for Cytotoxicity was also used. Several negative control samples were used including Dulbecco's Modified Eagle's Medium (DMEM) supplemented with foetal bovine serum (10%) and penicillin-streptomycin (100 units/ml), phosphate-buffered saline and distilled water. For a positive control of cytotoxicity, sodium dodecyl sulphate (SDS) was used at 0.05, 0.10, 0.15 and 0.2 mg/ml.

L929 fibroblast cell cultures were examined microscopically, and any cell monolayers that were not of correct confluency or showed signs of granulation or sloughing were rejected. A 3% solution of agar (suitable for cell culture) was made and autoclaved for 15 min at 121 °C. The autoclaved agar was put into a 45 °C water bath and allowed to cool to 45 °C. DMEM was warmed to 45 °C in a water bath. Equal volumes of the DMEM and 3% Nobel agar were mixed and allowed to cool to approximately 39 °C. The medium from all acceptable cultures was removed and replaced with 2 ml of agar medium. The cultures were placed on a flat surface to solidify at room temperature. Sterile, 10 mm filter discs were saturated in test solution or control

**Fig. 1** Image of the indirect cytotoxicity experiment using the agar diffusion assay



solution (0.85% sodium chloride solution) before being placed in each dish in contact with the agar surface (Fig. 1). Triplicate cultures for each test material and both positive and negative controls were performed. All cultures were incubated for  $24 \pm 1$  h. The outline of the specimen was marked on the bottom of the culture dish with a permanent marker, and then the specimen was removed. Two millilitres of 0.01% neutral red solution was added to each dish and incubated for 1 h. Following incubation, the neutral red solution was removed, and each culture was examined microscopically under and around each control and test specimen. The cell culture was deemed to show a cytotoxic effect if microscopic examination revealed malformation, degeneration, sloughing or lysis of the cells within the zone or a moderate-to-severe reduction in cell-layer density. The zone index measures the clear zone in which cells do not stain with neutral red. The lysis index measures the number of cells affected within the zone of toxicity.

## 2.6 Statistics

Statistical analysis was performed using GraphPad Prism 7 software. Statistical comparisons between microbiological mean log values were performed using the two-way ANOVA.

## 3 Results

### 3.1 Efficacy of the Electrolysed Water on Planktonic Microorganisms

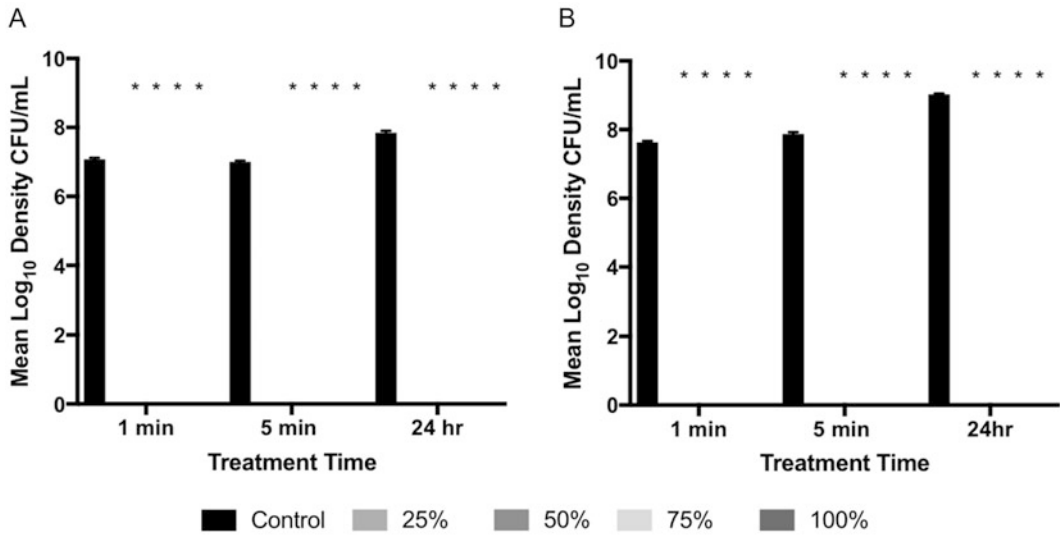
Upon bacterial enumeration, no colonies of either *S. aureus* or *P. aeruginosa* were detected following antimicrobial treatment with all dilutions of the electrolysed water at treatment times of 1 min, 5 min and 24 h (Fig. 2a, b) ( $p = <0.0001$ ).

### 3.2 Efficacy of the Electrolysed Water on Biofilms

The use of the CDC model showed that the electrolysed water at 100, 75, 50 and 25% concentration could significantly reduce *S. aureus* and *P. aeruginosa* biofilms after 5 min contact time (Fig. 3a, b) ( $p < 0.0001$ ).

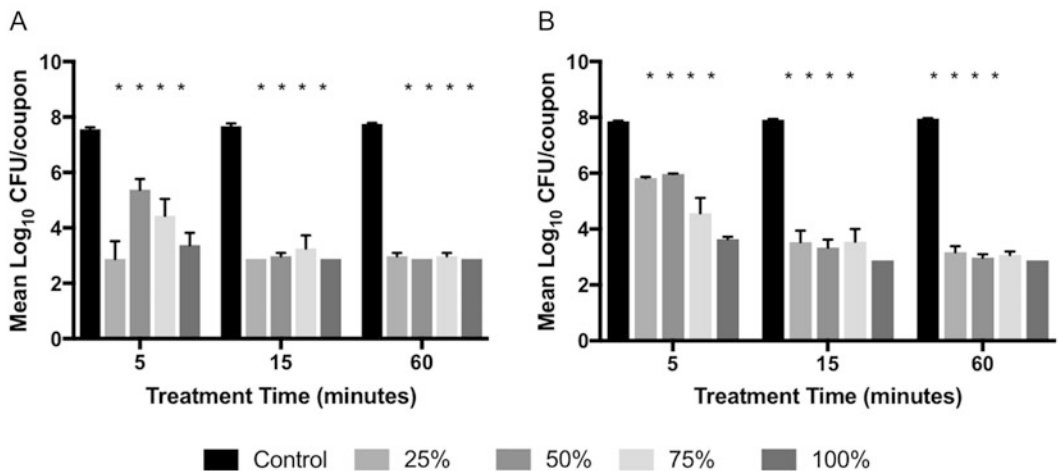
### 3.3 Cytotoxicity of the Electrolysed Water

The agar diffusion method allows for the qualitative assessment of cytotoxicity. According to ISO



**Fig. 2** Log density of *S. aureus* (a) and *P. aeruginosa* (b) in the electrolysed water time-kill study. Microbial cultures were exposed to the electrolysed water at 100%, 75%, 50% and 25% concentration for 1 min, 5 min and 24 h. Samples were performed in triplicate. The negative control treatment was a 0.85% sodium chloride solution.

Mean log density values were obtained and the standard deviation (SD) calculated. Error bars represent SD. All treatment groups showed a significant decrease in bacterial log<sub>10</sub> density in comparison with the control group ( $p < 0.0001$ )



**Fig. 3** Mean log<sub>10</sub> density of *S. aureus* (a) and *P. aeruginosa* (b) biofilm in the CDC model. Biofilms were treated with the electrolysed water test solution at 25%, 50%, 75% and 100% concentration. For the control, coupons containing biofilm were treated with a 0.85%

sodium chloride solution. The experiment was performed in triplicate. Error bars represent the standard deviation of the mean log<sub>10</sub> CFU/coupon. All treatment groups showed a significant decrease in bacterial log<sub>10</sub> density in comparison with the control group ( $p < 0.0001$ )

**Table 1** Reactivity grades for zone index

Zone index	Description of zone	Reactivity
0	No detectable zone around or under specimen	None
1	Zone limited to area under specimen	Slight/mild
2	Zone extends less than 0.5 cm beyond specimen	Moderate
3	Zone extends 0.5–0.1 cm beyond specimen	Moderate
4	Zone extends greater than 1.0 cm beyond specimen but does not involve entire dish	Severe

**Table 2** Qualitative lysis description

Lysis index	Description of zone	Reactivity
0	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth	None
1	Not more than 20% of zone shows rounded cells, loosely attached and without intracytoplasmic granules or show changes in morphology	Slight
2	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable	Mild
3	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable	Moderate
4	Nearly complete destruction of the cell layers	Severe

**Table 3** Zone index and lysis index for indirect cytotoxicity test

Sample	Zone index	Interpretation	Lysis index	Interpretation
DMEM (negative control)	0	Non-cytotoxic	0	Non-cytotoxic
Water	0	Non-cytotoxic	0	Non-cytotoxic
Phenol	4	Cytotoxic	3	Cytotoxic
Electrolysed water 100%	3	Cytotoxic	3	Cytotoxic
Electrolysed water 75%	3	Cytotoxic	1	Non-cytotoxic
Electrolysed water 50%	2	Non-cytotoxic	1	Non-cytotoxic
Electrolysed water 25%	2	Non-cytotoxic	1	Non-cytotoxic

Zone index measures the clear zone in which cells do not stain with neutral red. The lysis index measures the number of cells affected within the zone of toxicity. All samples were tested in triplicate

10993-5, an achievement of a numerical grade greater than 2 (Tables 1 and 2) is considered a cytotoxic effect. The evaluation of the electrolysed water showed that 100% and 75% of the electrolysed water was cytotoxic to cells. The electrolysed water at 50% and 25% was not cytotoxic to cells (Table 3).

## 4 Discussion

The use of salt water electrolysis has been used to produce sanitising agents and cleaning agents but has also been utilised to produce hydrogen energy as

a potential alternative energy source (Meier 2014). Within this study, the antibacterial and anti-biofilm properties of electrolysed water were assessed in a series of in vitro assays. It was shown that the electrolysed water formulation could kill *S. aureus* and *P. aeruginosa* within 1 min of exposure in planktonic studies. In veterinary medicine, it has been shown that dilute hypochlorous acid solutions are bactericidal against *S. aureus* and *Escherichia coli*, after 1 h exposure (Ramey and Kinde 2015). However, the study failed to evaluate shorter contact times. Another study has demonstrated complete kill of *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Aeromonas hydrophila* and