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Journal of Microbiological Methods 49 (2002) 285–293

Journal of Microbiological Methods

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Bactericidal activity of electrolyzed acid water from solution containing sodium chloride at low concentration, in comparison with that at high concentration

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Received 20 August 2001; received in revised form 15 November 2001; accepted 13 December 2001

Abstract

Electrolyzed strong acid water (ESW) containing free chlorine at various concentrations is becoming to be available in clinical settings as a disinfectant. ESW is prepared by electrolysis of a NaCl solution, and has a corrosive activity against medical instruments. Although lower concentrations of NaCl and free chlorine are desired to eliminate corrosion, the germicidal effect of ESW with low NaCl and free-chlorine concentrations (ESW-L) has not been fully clarified. In this study, we demonstrated that ESW-L possesses bactericidal activity against *Mycobacteria* and spores of *Bacillus subtilis*. The effect was slightly weaker than that of ESW containing higher NaCl and free-chlorine concentrations (ESW-H), but acceptable as a disinfectant. To clarify the mechanism of the bactericidal activity, we investigated ESW-L-treated *Pseudomonas aeruginosa* by transmission electron microscopy, a bacterial enzyme assay and restriction fragment length polymorphism pattern (RFLP) assay. Since the bacterium, whose growth was completely inhibited by ESW-L, revealed the inactivation of cytoplasmic enzyme, blebs and breaks in its outer membrane and remained complete RFLP of DNA, damage of the outer membrane and inactivation of cytoplasmic enzyme are the important determinants of the bactericidal activity. © 2002 Elsevier Science B.V. All rights reserved.

1. Introduction

In many institutions, glutaraldehyde has been used for the disinfection of medical instruments. Although aldehydes have been proved disinfective against many pathogens (Hanson, 1990; Jeng et al., 1987; Reynolds et al., 1992; Russell, 1994), insufficient rinsing of aldehydes may cause bloody diarrhea and abdominal cramps following endoscopy in clinics (Durante et al., 1992). Aldehydes were also demonstrated to be cytotoxic and genotoxic to cultured human cells (St.-Clair et al., 1991; Sun et al., 1990). This chemical is toxic not only to patients and clinical staff but also the environment.

For the substitute for aldehydes, chlorination is one of the most effective methods used in the medical field. Sodium hypochlorite has been demonstrated to be effective in the disinfection of almost all pathogens (Rutala and Weber, 1997). Electrolyzed NaCl solution, which contains free chlorine, is considered to be ef-

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PII: S0167-7012(01)00385-2

fective for disinfection. In the 1990s, electrolyzed NaCl solutions containing high free-chlorine concentrations have been investigated from the viewpoint of clinical application in Japan. At present, two types of electrolyzed solutions are available: electrolyzed weak acid water (EWW) and electrolyzed strong acid water (ESW). EWW is obtained by the electrolysis of a NaCl solution in a single well. ESW is generated by the electrolysis of a NaCl solution using positive and negative electrodes in wells separated by a cationic membrane, and is obtained from the well of the positive electrode (Kumon, 1997). Electrolyzed NaCl solutions containing low and high free-chlorine concentrations are abbreviated as ESW-L and ESW-H, respectively. ESW-H is strongly effective against many human pathogens (Abe et al., 1994; Iwasawa and Nakamura, 1996; Iwasawa et al., 1993) including *Bacillus cereus* (Iwasawa et al., 1993) and *Mycobacterium tuberculosis* (Iwasawa and Nakamura, 1993). However, ESW-H has a corrosive activity against medical instruments. Moreover, ESW-H contains high concentrations of free chlorine and its byproducts may have cytotoxicity and genotoxicity, as Knasmuller et al. (1996) and Daniel et al. (1993) reported. Recently, to minimize its corrosive activity and toxicities, ESW was prepared by the electrolysis of a solution containing low NaCl concentrations (ESW-L), and was confirmed to be effective against blood borne pathogenic viruses (Morita et al., 2000; Tagawa et al., 2000). ESW-L is suggested to be suitable for application in clinical setting such as disinfection of endoscopes (Tagawa et al., 2000; Tsuji et al., 2000). Although ESW-L has been studied as a disinfectant for bacteria (Tsuji et al., 2000), studies on the bactericidal effect of ESW-L and its disinfective mechanism have not been extensively carried out. In this study, we compared ESW-L and ESW-H in terms of their disinfective potential against *Mycobacteria* and *Bacillus subtilis*, and discussed the mechanism of their bactericidal activity against *Pseudomonas aeruginosa*.

2. Materials and methods

2.1. Electrolyzed strong acid water (ESW)

ESW was prepared in an electrolyzing apparatus (CLEANTOP WM-1, Kaigen, Osaka, Japan). The principle of the apparatus is presented in Fig. 1. The apparatus consists of two wells separated by a cationic membrane (Nafion 450, Dupont, NY, USA), with positive and negative electrodes installed in each well (Fig. 1). To prepare ESW-L, 10 l of 0.05% NaCl in tap water was electrolyzed for 45 min at room temperature using a 3 A current. ESW-L was obtained from the well with the positive electrode. To obtain ESW-H, 10 l of 0.3% NaCl solution in tap water was electrolyzed for 25 min at room temperature using a 6 A current in a modified apparatus.

Oxidation reduction potential (ORP) and pH were measured using an electrometer (HM-14P, Toa Electronics, Tokyo, Japan) equipped with an ORP sensor (PLS-2019P, Toa Electronics), and a pH sensor (GST-2419C, Toa Electronics). Free-chlorine content was

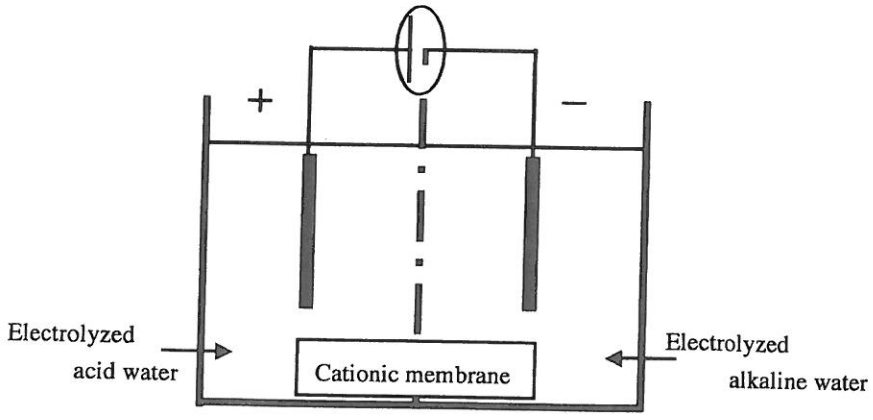


Fig. 1. Principle of electrolysis apparatus. The apparatus consists of two wells separated by a cationic membrane. Positive and negative electrodes were installed in each well. ESW-L and alkaline water were obtained from the positive and negative wells, respectively.

measured with a chlorine meter (CL2-2Z, Kasahara Chemical Instruments, Saitama, Japan).

2.2. Bacteria and culture

P. aeruginosa was cultured overnight. *B. subtilis* cells were cultured for 3 days to allow spore formation in heart infusion broth (Difco Lab., Detroit, USA) and washed three times in physiological saline. The bacterial cells were resuspended in saline at McFarland 0.01–1. *Mycobacterium bovis* (BCG, Tokyo strain) and a clinical isolate of *M. tuberculosis* were cultured in 3% Ogawa slants for 6 weeks at 37 °C, respectively. The bacterial colonies were minced, suspended in saline and allow to stand for 10 min to remove massive clumps of bacterial colonies. The supernatant of the bacterial suspension was collected and adjusted to McFarland #1.

2.3. Experimental design

One volume of bacterial suspension was mixed with 9 volumes of ESW, and incubated for the designated time. After incubation, pH of the mixtures with ESW-L and ESW-H was neutralized by adding 7 and 3.5 volumes of alkaline water from each electrolysis, respectively. The free chlorine of the mixtures was inactivated by adding 17 and 20.5 volumes of 3% bovine serum albumin (BSA) in phosphate-buffered saline, respectively.

The final mixtures of *P. aeruginosa* and *B. subtilis* were cultured to determine the colony forming units of residual viable bacteria. To determine viable cells of *Mycobacteria*, 0.1 ml each of the mixtures of *M. bovis* and *M. tuberculosis* was inoculated into 3% Ogawa slants, cultured for 8 weeks at 37 °C and observed for visible colonies. The results from the mixtures with ESW-L and ESW-H were compared with those from unelectrolyzed 0.05% and 0.3% NaCl solutions.

2.4. Electron microscopy

The cells of *P. aeruginosa* were mixed with ESW for 5 min at room temperature, and pH of the mixtures was neutralized and free chlorine was inactivated with alkaline water and BSA solution, respectively. The cells were pelleted and fixed with 2% glutaraldehyde in cacodylate buffer (0.05 M, pH 7.4) containing 0.05% (w/v) ruthenium red. The pellets were washed with cacodylate buffer (0.05 M, pH 7.4) and postfixed with 1% osmium tetroxide in the same buffer containing 0.05% (w/v) ruthenium red. The fixed pellets were embedded

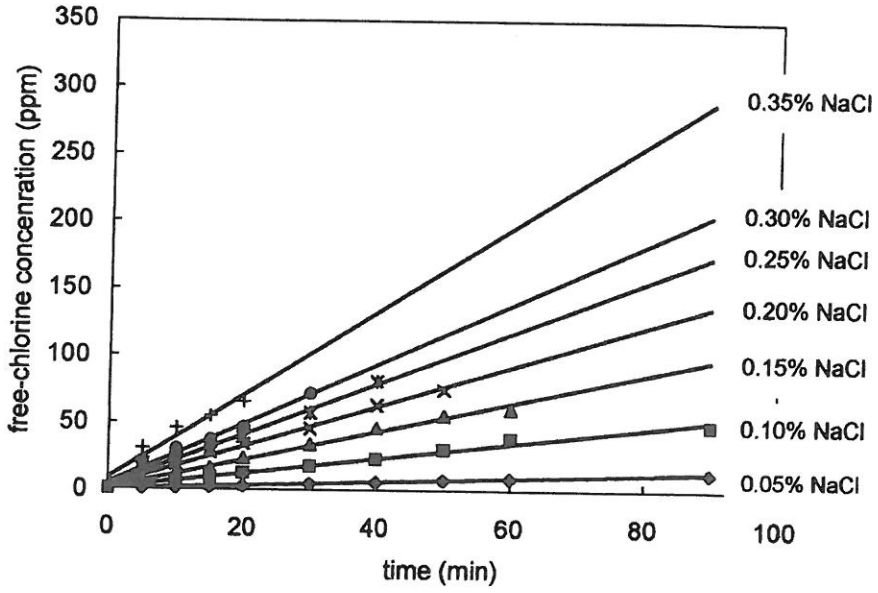


Fig. 2. Concentration of free chlorine in electrolyzed solution containing various concentrations of NaCl. Electrolysis of 0.05% NaCl resulted in a time-dependent increase in free-chlorine concentration.

Table 1
Free-chlorine concentration, pH and ORP of electrolyzed and unelectrolyzed NaCl solutions with or without neutralization

	Electrolyzed		Unelectrolyzed	
	0.05% NaCl	0.3% NaCl	0.05% NaCl	0.3% NaCl
Free chlorine (ppm)				
Fresh	4.95 ± 0.22	49.00 ± 1.25	0.44 ± 0.12	0.44 ± 0.08
Neutralized	0.05 ± 0.03	0.06 ± 0.03	0.04 ± 0.01	0.03 ± 0.01
pH				
Fresh	2.32 ± 0.07	2.16 ± 0.10	7.51 ± 0.17	7.47 ± 0.12
Neutralized	6.83 ± 0.09	6.78 ± 0.18	6.74 ± 0.02	6.72 ± 0.01
ORP (mV)				
Fresh	1131 ± 26	1153 ± 7	690 ± 23	710 ± 16
Neutralized	295 ± 195	304 ± 191	462 ± 68	473 ± 54

in Epoxy resin and cut in ultrathin sections. The sections were doubly stained with 4% uranyl acetate and lead citrate, and observed under an electron microscope (H-7100, Hitachi, Tokyo, Japan). *P. aeruginosa* cells, which were untreated or treated with unelectrolyzed NaCl solutions, were observed in the same manner.

2.5. Pulsed field gel electrophoresis of bacterial DNA

The DNA of *P. aeruginosa* was extracted by using a DNA extraction kit (CHEF Bacterial Genomic DNA Plug Kit, BioRad, Hercules, CA, USA). In brief, 5×10^7 bacterial cells were embedded in a plug of 1% Clean Cut Agarose (BioRad). The plug was treated with lysozyme at 37 °C for 2 h and then with proteinase K at 56 °C for 16 h. The plug, which was treated with a restriction enzyme (*Spe I*), was set in a 1% Pulsed Field Certified Agarose (BioRad) and linearly electrophoresed at 6 V/cm and switched time ramped from 50 to 90 s for 24 h in 0.5 × TBE buffer.

2.6. Detection of nitrate reductase activity

Activity of bacterial nitrate reductase was detected by using Api 20 NE system (Japan bioMerieux, Tokyo, Japan) with modified method. In brief, ESW-L-treated *P. aeruginosa* cells were resuspended in physiological saline at McFarland #2, applied to Api 20 NE plate, and the plate was incubated for 1 h at 37 °C. The activity of nitrate reductase was detected by colorization of the NO3 well.

3. Results

3.1. Concentration of free chlorine in electrolyzed apparatus

To obtain ESW containing 5 or 50 ppm free chlorine, changes in the concentration of free chlorine in the electrolysis apparatus were determined. The concentrations of free chlorine increased with the time of electrolysis and concentration of NaCl (Fig. 2). In the subsequent experiments, ESWs with NaCl and chlorine at commonly used concentrations were applied. That is, ESWs containing 5 and 50 ppm free

Table 2
Effect of electrolyzed solutions of 0.05% and 0.3% NaCl on growth of *B. subtilis*

Incubation time (min)	Growth (CFU/ml at contact)							
	High-density bacterial suspension[a]				Low-density bacterial suspension[b]			
	Unelectrolyzed		Electrolyzed		Unelectrolyzed		Electrolyzed	
	0.05%	0.3%	0.05%	0.3%	0.05%	0.3%	0.05%	0.3%
5	N.T.	N.T.	1800	0	N.T.	N.T.	27	0
10	N.T.	N.T.	1520	0	N.T.	N.T.	15	0
20	N.T.	N.T.	620	0	N.T.	N.T.	10	0
30	N.T.	N.T.	320	0	N.T.	N.T.	2	0
60	>10,000	>10,000	20	0	73	107	0	0
Original suspension[c]	5.4×10^6		5.4×10^6		176		176	

[a] High-density bacterial suspensions were treated with electrolyzed and unelectrolyzed NaCl solutions.
[b] Low-density bacterial suspensions were treated with electrolyzed and unelectrolyzed NaCl solutions.
[c] CFU of the original suspension in the assay was calculated from CFU of the original bacterial suspension.

Table 3
Effect of electrolyzed solutions of 0.05% and 0.3% NaCl on growth of *Mycobacteria*

Incubation time (min)	Growth (negative/positive)[a]							
	M. bovis (BCG)				*M. tuberculosis* (clinical strain)			
	Unelectrolyzed		Electrolyzed		Unelectrolyzed		Electrolyzed	
	0.05%	0.3%	0.05%	0.3%	0.05%	0.3%	0.05%	0.3%
5	N.T.	N.T.	+&+	−&−	N.T.	N.T.	+&+	−&−
10	N.T.	N.T.	−&+	−&−	N.T.	N.T.	+&+	−&−
20	N.T.	N.T.	−&−	−&−	N.T.	N.T.	−&+	−&−
30	N.T.	N.T.	−&−	−&−	N.T.	N.T.	−&−	−&−
60	+	+	−&−	−&−	+	+	−&−	−&−
Original suspension[b]	+		+		+		+	

[a] +: positive for culture with visible colony; −: negative for culture without visible colony. Duplicate results are represented.
[b] The original bacterial suspensions were inoculated to the Ogawa medium and cultured for 8 weeks.

chlorine as well as 0.05% and 0.3% NaCl, respectively, were compared in terms of their bactericidal activity. The coefficient of variations (CV) was smaller than 0.05 in each measurement.

3.2. Neutralization of pH and inactivation of free chlorine

The free-chlorine concentration, pH and ORP of ESW-L, ESW-H and unelectrolyzed solutions containing 0.05% and 0.3% NaCl were measured, and the results are shown in Table 1. The electrolysis resulted in an increase in the concentration of free chlorine, an increase in ORP and a decrease in pH in 0.05% and 0.3% NaCl solutions. The concentration of free chlorine of the electrolyzed solution of 0.3% NaCl was 10 fold greater than that of 0.05% NaCl. The pH and ORP were not significantly different between ESW-L and ESW-H used in this study. The addition of alkaline water from a negative electrode-installed well and BSA to electrolyzed solutions resulted in a decrease of the free-chlorine concentration and ORP, and neutralization of pH. We confirmed that the addition of alkaline water and BSA solution was sufficient to inactivate free chlorine, neutralize pH and decrease ORP; thus, we used this process in subsequent experiments.

3.3. Comparison of bactericidal effect of ESW

In order to clarify the bactericidal activity of ESW against highly disinfectant-resistant microorganism, the sensitivity of *B. subtilis* cells incubated its disinfectant-resistant spores was examined. The results are shown in Table 2. ESW-H completely inhibited the growth of 5.4×10^6 CFU/ml *B. subtilis* within 5 min. The inhibition of the growth proceeded in a time-dependent manner, and it was also observed in the bacterial suspension mixed with ESW-L but complete inhibition was not observed. To confirm the disinfective potential of the ESW-L, the bacterial suspension at a low cell density was mixed with the solution. The solution completely inhibited the growth of the bacterium within 60 min. Unelectrolyzed solution of 0.05% and 0.3% NaCl did not inhibit the growth of the bacterium.

Table 4
Effect of electrolyzed solutions of 0.05% and 0.3% NaCl on growth of *P. aeruginosa*

Contact time (min)	Growth (CFU/ml)[a]			
	Unelectrolyzed		Electrolyzed	
	0.05%	0.3%	0.05%	0.3%
5	N.T.	N.T.	0	0
10	N.T.	N.T.	0	0
20	N.T.	N.T.	0	0
30	N.T.	N.T.	0	0
60	>10,000	>10,000	0	0
Original suspension[b]	6.3×10^6		6.3×10^6	

[a] Duplicate experiments on *P. aeruginosa* were performed, and almost identical results were obtained.
[b] CFU of the original suspension in the assay was calculated from CFU of the original bacterial suspension.

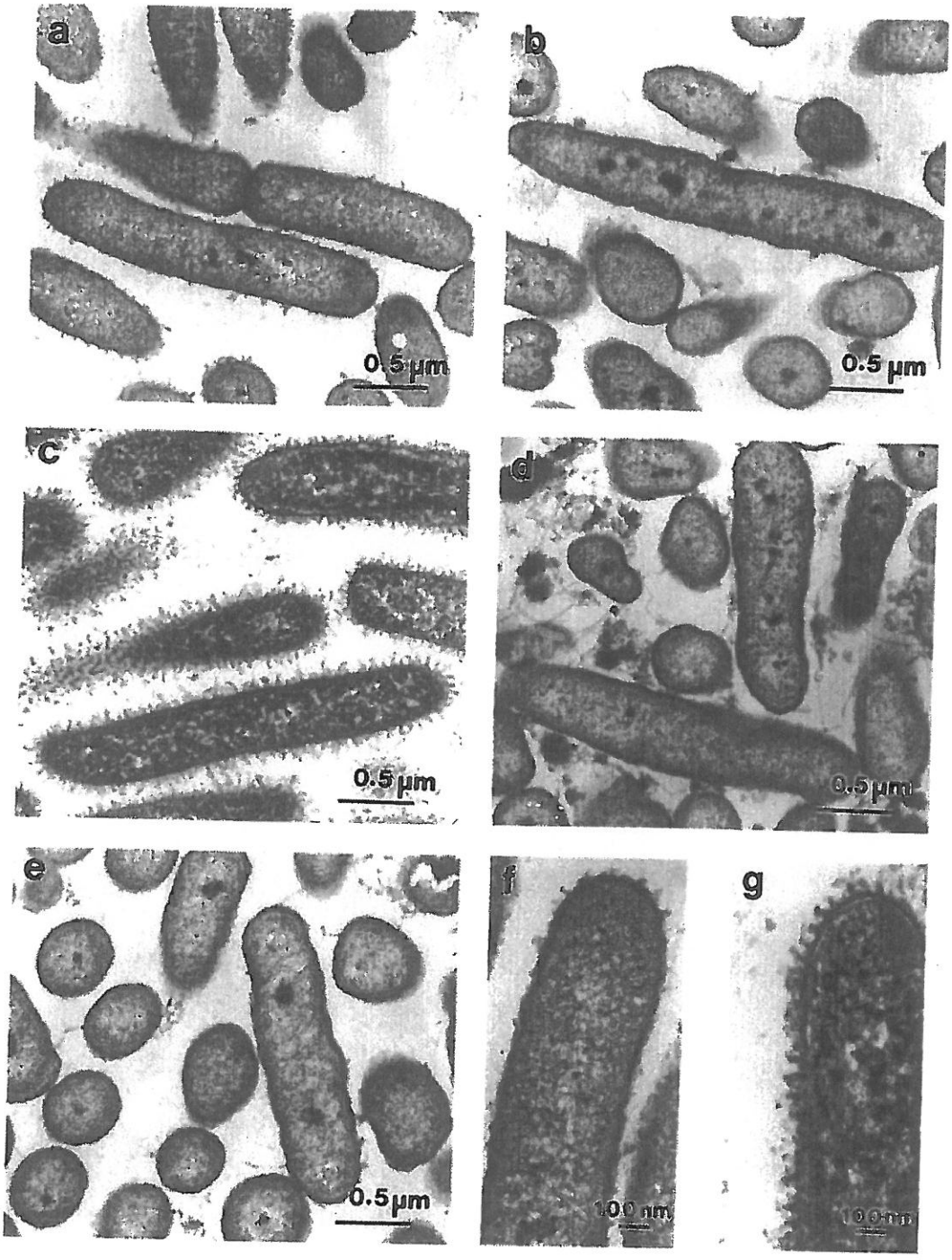


Fig. 3. Representative electron micrographs of *P. aeruginosa* was treated with ESW-L and ESW-H. *P. aeruginosa* was treated with electrolyzed and unelectrolyzed solutions containing 0.05% and 0.3% NaCl for 5 min, and examined by electron microscopy. Bacterial cells, which were treated with electrolyzed solution containing 0.05% NaCl (ESW-L) (a), unelectrolyzed solution containing 0.05% NaCl (b), electrolyzed solution containing 0.3% NaCl (ESW-H) (c) and unelectrolyzed solution containing 0.3% NaCl (d), and without any treatment (e) were shown. ESW-L slightly breaks the outer membrane of the bacterial cell (f) and the cells treated with ESW-H exhibited numerous breaks in the membrane (g).

To clarify the disinfective potential against *Mycobacteria*, the sensitivity of *M. tuberculosis* and *M. bovis* to ESW was examined. The growth of both *M. bovis* and *M. tuberculosis* was inhibited by ESW-H within 5 min, and by ESW-L within 20 and 30 min, respectively (Table 3). When treated with ESW-L, the inhibition proceeded in a time-dependent manner. Unelectrolyzed solutions with 0.05% and 0.3% NaCl did not inhibit the growth of the bacteria.

3.4. Bactericidal mechanism of ESW

Since *Mycobacteria* and spores of *B. subtilis* are difficult to examine by various assays, we used *P. aeruginosa* in the study of bactericidal mechanism.

To clarify the bactericidal activity of ESW against common bacteria, the sensitivity of *P. aeruginosa* to ESW was examined. The growth of the bacterium was completely inhibited by the contact with both ESW-H and ESW-L within 5 min (Table 4). Unelectrolyzed solution with 0.05% and 0.3% NaCl did not inhibit the growth of the bacterium.

To analyze the mechanism of the bactericidal effect, morphological changes of *P. aeruginosa*, whose growth was completely inhibited, were examined by electron microscopy. The bacterial outer membrane formed breaks and blebs when contacted with ESW-L (Fig. 3a and f) but not with unelectrolyzed 0.05% NaCl solution (Fig. 3b). The membrane formed more breaks and blebs when contacted with ESW-H (Fig. 3c and g), but not with unelectrolyzed 0.3% NaCl solution (Fig. 3d). The diameter of the bleb is approximately 28 ± 7 nm. The intact outer and inner membranes of the bacterium, which was not contacted with ESW and the unelectrolyzed solutions of NaCl were observed (Fig. 3e). These findings suggest that a higher free-chlorine concentration increases the number of blebs and breaks in the membranes.

To clarify whether the effect of ESW reached deep into the bacterial cells, the presence of chromosomal DNA was detected by RFLP assay. No band was detected in the lane of bacterial sample treated with ESW-H, and weak bands were detected in that treated with ESW-L (Fig. 4). Strong bands were detected in the lanes of bacterial samples treated with unelectrolyzed NaCl solutions.

In well of Api 20 NE, *P. aeruginosa* which was treated with 0.05% NaCl changed its color to pink, but ESW-L-treated bacteria did not. Since the change of color was induced by adding zinc powder, nitrate reductase in the bacteria was considered to be inactivated by ESW-L.

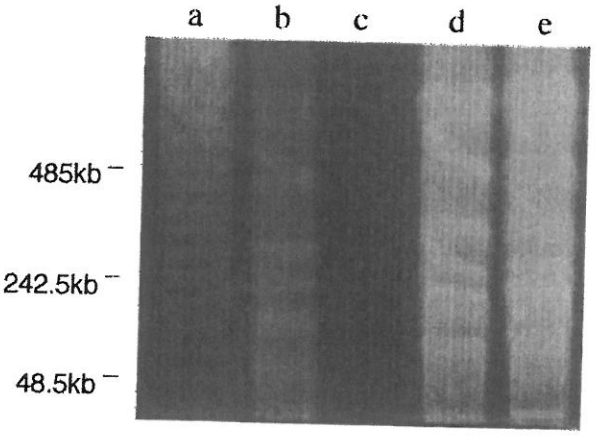


Fig. 4. Restriction fragment length polymorphism pattern (RFLP) assay for *P. aeruginosa* treated with ESW-L and ESW-H. *P. aeruginosa* was treated with ESW-L and ESW-H for 5 min, and examined by RFLP. Lane a: 48.5 kb ladder; lane b: treated with ESW-L; lane c: treated with ESW-H; lane d: treated with unelectrolyzed 0.05% NaCl; lane e: treated with unelectrolyzed 0.3% NaCl.

4. Discussion

ESW-L was compared with ESW-H in terms of ORP, pH, concentration of free chlorine and disinfective potential. The only significant differences were found in the concentration of free chlorine and disinfective potential. We postulate that the different concentrations of free chlorine are the reason for the difference in bactericidal effects between ESW-L and ESW-H. Strong bactericidal effects were observed in *B. subtilis*, *M. tuberclosis* and *M. bovis* treated with ESW-H, and weak bactericidal effects in those treated with ESW-L. We concluded that ESW-L is potentially effective disinfectant against those bacteria.

In the treatment of *P. aeruginosa*, both ESW-H and ESW-L revealed a strong bactericidal effect. Iwasawa and Nakamura (1995) demonstrated that ESW-H completely destroyed the cells of *P. aeruginosa* but did not show the destruction in detail. We confirmed that the cell wall of Gram-negative bacteria is one of the targets of ESW based on the morphological findings in the ESW-treated *P. aeruginosa*. Although

ESW-L resulted in a smaller number of breaks and blebs on the cell wall than ESW-H did, and incomplete degradation of chromosomal DNA, the treatment with ESW-L was sufficient to kill the bacterium. Since ESW-L inactivated an enzyme of the bacterium, the water probably breaks the cell wall, penetrates into the cytoplasm and damaged various inner proteins of the bacterium. The breaks of the cell wall may induce the release of endotoxin. Since ESW, however, appeared to have an inactivative potential against an endotoxin (Fujiwara et al., 1996), the released endotoxin could be inactivated.

Although sodium hypochlorite forms free chlorine at a high pH and can be used as a disinfectant, it is difficult to adjust the pH and concentration of free-chlorine. Electrolysis of NaCl solution generates H^+, O_2, O_3^- and free chlorine such as HClO, Cl_2 and Cl^-. An electrolyzing apparatus produces ESW with a stable pH and free-chlorine concentration.This is great advantage of ESW to sodium hypochlorite. The problem with ESW-L, however, is its inactivation following contact with contaminants such as proteins. There are two strategies for solving this problem. The first is the use of ESW-H, which contains higher concentration of free chlorine. In this study, we demonstrated that stronger bactericidal effects on *B. subtilis* and *Mycobacteria* were observed as a result of treatment with ESW-H than that with ESW-L. ESW-H, however, corrodes instruments due to high concentration of free chlorine. Secondary, to eliminate corrosion, a stream of fresh ESW-L may be beneficial to disinfect a material. Recently, a new system for disinfecting an endoscope has been developed by the application of this concept. The system provides fresh ESW-L to an endoscope, restores the water in an electrolyzing apparatus, re-electrolyzes the water, and supplies the refreshed ESW-L to the endoscope. Tsuji et al. (2000) demonstrated that ESW-L in the system eliminated bacteria, which contaminated endoscopes, and their clinical trials of disinfection of an endoscope were successful. Our study provides scientific evidence that supports their findings.

Acknowledgements

This study was performed under the Industry–University Cooperation between Osaka Medical College and Kaigen. We appreciate Ms. Hanada for the excellent preparation of the experiments and Ms. Matsumura for the preparation of this manuscript.

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