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The Anti-microbial Activity of Electrolysed Oxidizing Water against Microorganisms relevant in Veterinary Medicine

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With 1 figure and 1 table

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Summary

Standards of the German Association of Veterinary Medicine (DVG) for the evaluation of chemical disinfectants were used to assess the anti-microbial efficacy of electrolysed oxidizing water (EOW). *Enterococcus faecium*, *Mycobacterium avium* subspecies *avium*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* were exposed to anode EOW (pH, 3.0 ± 0.1; oxidation-reduction potential (ORP), +1100 ± 50 mV; free chlorine, 400 ± 20 mg/l Cl_2) and combined EOW (7 : 3 anode : cathode, v/v; pH, 8.3 ± 0.1; ORP, 930–950 mV; free chlorine, 271 ± 20 mg/l Cl_2). In water of standardized hardness (WSH), all bacterial strains were completely inactivated by a 30 min exposure to maximum 10.0% anode EOW (~40.0 mg/l Cl_2) or 50.0% combined EOW (~135.5 mg/l Cl_2). The sensitivity ranking order for anode EOW to the bacterial test strains was *P. mirabilis* > *S. aureus* > *M. avium* ssp. *avium* > *E. faecium* > *P. aeruginosa*. *P. mirabilis* and *S. aureus* decreased to undetectable levels after 5 min of exposure to 7.5% anode EOW (~30.0 mg/l Cl_2). *Candida albicans* was completely inactivated by a 5-min exposure to 5.0% anode EOW. Both, anode and combined EOW exhibited no anti-microbial activities in standardized nutrient broth or after addition of 20.0% bovine serum to the WSH. Further research is necessary to evaluate the efficacy of EOW as a disinfectant under operating conditions in animal production facilities.

Introduction

Electrolysed oxidizing water (EOW) has been attracting attention as a disinfectant because of its strong microbicidal activities on a broad variety of bacterial pathogens including mycobacteria as well as bacterial endospores (Tanaka et al., 1996; Selkon et al., 1999; Venkitanarayanan et al., 1999; Loshon et al., 2001). Moreover, EOW appeared to have an inactivative potential against bacterial endotoxin, exerts fungicidal activity, and is described to inactivate bacterial or fungal toxins probably by break down of such hazardous substances into non-harmful components (Suzuki et al., 2002a,b).

Electrolysed oxidizing water is generated by electrolysis of a dilute aqueous solution of sodium chloride (NaCl) in an electrolysis chamber where the anode (+ electrode) and cathode (- electrode) are separated by a cationic membrane to form two compartments (Kumon, 1997; Fig. 1). The anode acidic EOW has a low pH of 2–3, a high oxidation-reduction potential (ORP) > 1000 mV, and contains relative concentrations of chlorine (Cl_2), hypochlorous acid (HOCl) and hypochlorite (OCl^-; Sharma and Demirci, 2003). The alkaline solution from the cathode (often referred to as electrolysed reducing water) has a high pH and low ORP (Kumon, 1997). The physical properties and chemical composition of EOW varies dependent on concentration of NaCl, amperage level, time of electrolysis or water flow rate (Kiura et al., 2002; Hsu, 2003; Nakajima et al., 2004).

A variety of applications of EOW in agriculture, medicine, and food sanitation have been described so far (Sharma and Demirci, 2003). Because of the reported broad spectrum of microbicidal activities, EOW is proving to be of considerable interest as a disinfectant in animal husbandry and veterinary medicine. However, before any approval can be given to apply EOW as an anti-microbial treatment of e.g. food animal husbandry facilities, candidate substances must be thoroughly evaluated. In the present study, the procedures specified by the German Association of Veterinary Medicine (Deutsche Veterinärmedizinische Gesellschaft, DVG; http://www.dvg.net) and by the German Institute of Standardisation (Deutsches Institut für Normung, DIN; http://www2.din.de) for the evaluation of chemical disinfectants were used to assess the microbicidal efficacy of EOW.

Materials and Methods

Electrolysed oxidizing water

Electrolysed oxidizing water was generated with the Stericold® electrolysis device (Biostel® Schweiz AG, Buttikon, Switzerland). The principle of the apparatus is given in Fig. 1. The generator was connected to a water faucet. A 0.1% solution of NaCl in tap water automatically provided from a continuous supply of a saturated aqueous NaCl solution was pumped into the electrolytic cell at a rate of 1.3 l/min. Under these conditions, the 0.1% NaCl solution was electrolysed at room temperature for 46 s. The current passing through the electrolysis cell and the voltage between the electrodes was set at 12 A and 10 V respectively. Electrolysed solutions from the electrodes were automatically provided at separate outlets. The generator was allowed to run for 10 min before collecting solutions for analyses so that the amperage level was stabilized to the set value of 12 A. The electrolysed solutions were collected in sterile containers and were used within 30 min for

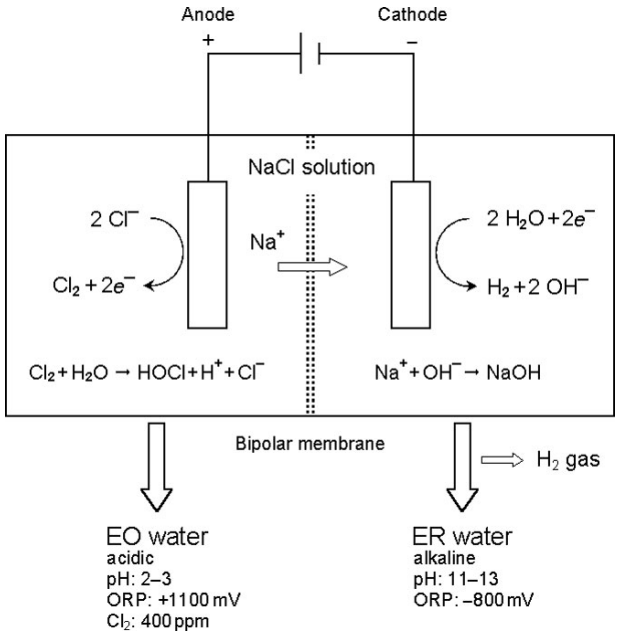

Fig. 1. Basic principle of the electrolysis chamber. The anode (+) and cathode (−) are separated by a bipolar membrane which inhibits the migration of hydroxyl ions (OH^-) from the cathode to the anode. The overall chemical reaction during operation of electric current is: $NaCl + H_2O \rightarrow NaOH + \frac{1}{2}Cl_2 + \frac{1}{2}H_2$. The principle anode and cathode reactions are given in the figure. At the anode chlorine evolves which hydrolyses rapidly into hypochlorous acid (HOCl) according to the equation given in the figure. At the cathode, the H^+ ions from H_2O are reduced to hydrogen gas, leaving the hydroxyl ion (OH^-) in the catholyte. The Na^+ ion is free to migrate from the anolytic chamber into the catholytic chamber and to join the OH^- ion (EO, electrolysed oxidizing; ER, electrolysed reducing).

microbiological studies. Samples for the determination of physical and chemical properties of electrolysed solutions were collected simultaneously and analysed immediately. The pH was determined by using a digital pH meter (Metrohm AG, Herisau, Switzerland) according to established standards (Anonymous, 1999). The ORP was measured with an ORP meter (SenTix ORP Redox, WTW Wissenschaftlich Technische Werkstätte, Weilheim, Germany). Total available chlorine was determined by iodometric titration with sodiumthiosulphate. Iodine is formed by oxidation of iodide by chlorine (Aieta et al., 1984). Electrolysed oxidizing water from the anode was characterized by the following parameters: pH, 3.0 ± 0.1; ORP, +1100 ± 50 mV; free chlorine concentration, 400 ± 20 mg/l Cl_2). Analogous values for electrolysed water from the cathode were a pH of 11–13 and an ORP of −850 ± 50 mV. Anode EOW and combined EOW (7 : 3 anode : cathode, v/v; pH, 8.3 ± 0.1; ORP, 930–950 mV; free chlorine, 271 ± 20 mg/l Cl_2) were subjected to analyses of anti-microbial activities.

Microorganisms

The following test strains were used in accordance with the proposed guidelines of the DVG for the testing of disinfectants in livestock husbandry (Anonymous, 2000); *Enterococcus* (*E.*) *faecium* Kulmbach strain 2 (DSM 2918), *Mycobacterium* (*M.*) *avium* subspecies (ssp.) *avium* (ATCC 15769), *Proteus* (*P.*) *mirabilis* (ATCC 14153), *Pseudomonas* (*P.*) *aeruginosa* (ATCC 15442) and *Staphylococcus* (*S.*) *aureus* (ATCC 6538). *Candida* (*C.*) *albicans* (ATCC 10231) was used as the yeast representative. Test strains were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSMZ, Braunschweig, Germany). Growth conditions for the microorganisms followed recommendations of the supplier (http://www.dsmz.de/dsmzhome.htm). Prior to testing, each strain was subcultured at least twice onto suitable media to ensure purity and viability. The standardization of inocula from Gram-positive and Gram-negative bacteria followed guideline procedures provided by the DVG (Anonymous, 2000). To ensure that the final inoculum concentrations routinely obtained approximate these defaults, CFU/ml of inocula prepared from different strains were measured periodically by serial dilution and spread plate counting on trypticase soy agar (TSA) according to DIN 10161 (Anonymous, 1984; Foster and Johnstone, 1986). *Mycobacterium avium* ssp. *avium* was grown on commercially available agar slants of Loewenstein-Jensen Medium (LJM, TV102C, Oxoid, Basel, Switzerland). When sufficient growth was encountered at the 3-week reading of pure cultures, colonies were harvested in sterile saline (0.9 M NaCl in Aqua dest.) and were vigorously vortexed. The mycobacterial suspension was adjusted to McFarland turbidity standard 2 representing approximately 1.0×10^8–1.0×10^9 CFU/ml. *Candida albicans* was subcultured at least twice onto TSA supplemented with 2.0% dextrose. Colonies from pure cultures were harvested in sterile saline and were vigorously vortexed. The yeast suspension was clarified from massive clumps of yeast cells and agar residues by filtration through columns consisting of 10-ml syringes filled with sterile glass wool (Jürgens, Hannover, Germany) and was adjusted to the turbidity of a 2 McFarland standard ($\sim 10^8$–10^9 cells/ml).

Bacteriostatic and fungistatic efficacy

The bacteriostatic efficacy of anode and combined EOW was determined to the bacteria *E. faecium*, *S. aureus*, *P. mirabilis*, and *P. aeruginosa*. A 5.0 ml volume of each full strength EOW as well as from a 50.0%, 25.0%, 10.0% and 1.0% solution of each EOW in sterile water of standardized hardness (WSH: Aqua bidest. containing 2.42 mM $CaCl_2$ and 0.61 mM $MgSO_4$) was transferred to separate, sterile screw-cap tubes containing 5.0 ml of double concentrated trypticase soy broth (TSB) resulting in final concentration (f.c.) of 50.0%, 25.0%, 12.5%, 5.0%, and 0.5%. To each tube, 0.1 ml of a 1 : 10-diluted inoculum (equivalent to $\sim 1.0 \times 10^7 - 1.0 \times 10^8$CFU/ml) was then added. Tubes were vortexed for 10 s to ensure homogenization, and were incubated for 72 h at 37°C. Growth controls were incorporated for each test and consisted of a 1:1 mixture of sterile WSH with double concentrated TSB and of a bacterial inoculum as described above. Testing of growth inhibition of bacteria was carried out by using aqueous phenol (1.0, 0.50, 0.25, 0.10, and 0.01% f.c. in WSH) instead of EOW dilutions. The fungistatic efficacy of both EOWs was determined accordingly using *C. albicans* as the test strain, TSB supplemented with 2.0% dextrose as the growth medium, and an incubation of 96 h at 37°C; 3.0% formalin and its dilutions in WSH as mentioned above were used as inhibition controls of *C. albicans*. All tests were performed at least in duplicate. Growth of bacteria or fungi resulted in a visible clouding of the

culture medium. The lowest concentration of EOW that inhibited visible growth was recorded as the minimum bacteriostatic or fungistatic concentration.

Bactericidal and fungicidal efficacy

Test strains to determine the bactericidal and fungicidal efficacy were *E. faecium*, *M. avium* ssp. *avium*, *P. mirabilis*, *P. aeruginosa*, *S. aureus* , and *C. albicans*. The following concentrations of EOW solutions in WSH were used: anode EOW, 50.0%, 25.0%, 10.0%, 7.5%, 5.0% and 1.0% (f.c.); combined EOW, 80.0%, 50.0%, 25.0%, 20.0%, 10.0%, 5.0% and 1.0% (f.c.). A volume of 10.0 ml of each EOW dilution was given to separate sterile tubes. To each tube 0.1 ml of standardized bacterial and yeast inoculum was added. After vortexing, samples were incubated at 22°C (ambient temperature) for 5, 15, 30 and 60 min. Water of standardized hardness was used as the growth control. Inactivation controls consisted of 1.0% (f.c.) phenol for inhibition of Gram-negative and Gram-positive bacteria and 3.0% (f.c.) formalin for *C. albicans* and *M. avium* ssp. *avium*. At each time point, a 0.1 ml aliquot from each sample and each control was transferred into a sterile tube containing 10.0 ml of appropriate culture medium (bacteria, TSB; *C. albicans*, TSB + 2.0% dextrose). Samples were incubated at 37°C for 72 h (bacteria) or 96 h (*C. albicans*). In the testing of *M. avium* ssp. *avium*, 0.1 ml aliquots were inoculated onto LJM. Agar slants were incubated at 37°C for 4 weeks. Growth of surviving bacteria or yeasts was indicated either by visible clouding of the broth culture medium or growth of characteristic *M. avium* ssp. *avium* colonies on LJM. The identity of growing microorganisms was confirmed by random checks using standard laboratory procedures (Quinn et al., 1994). To evaluate the interference of organic matter (protein) with the microbicidal activity of EOWs, inactivation tests were performed with the f.c. of EOWs as given above and a final protein load of 20.0% bovine serum (Gibco^{TM}, Invitrogen AG, Basel, Switzerland). All tests were performed at least in duplicate.

Results

Bacteriostatic and fungistatic efficacy

Bacteriostatic and fungistatic effects were evaluated against *E. faecium*, *P. mirabilis*, *P. aeruginosa*, *S. aureus*, and *C. albicans*, respectively by exposure of standardized inocula of microorganisms to a range of anode and combined EOW concentrations in a defined nutrient broth. Seventy-two or 96 h of exposure to a f.c. of maximum 50% anode or combined EOW [equivalent to (~) 200 and 135 mg/l Cl_2, respectively] did not lead to any bacteriostatic or fungistatic efficacy.

Bactericidal and fungicidal efficacy

Bactericidal and fungicidal activities were evaluated by exposure of standardized inocula of the microorganisms to a range of EOW concentrations in a defined aqueous system devoid of organic matter. Data are shown in Table 1. At ~22°C (ambient temperature) all microorganisms were more effective inactivated by anode EOW than by combined EOW. In general, all bacterial strains were completely inactivated by anode EOW concentrations of maximum 10.0% (≈40.0 mg/l Cl_2) after maximum 30 min of exposure. By comparison, the bactericidal activity of combined EOW was less efficient; here a minimum concentration of 50.0% (~135.5 mg/l Cl_2) was required for a complete inactivation within 30 min of exposure. Employing

Table 1. Bactericidal and fungicidal efficacy of anode electrolysed oxidizing water (EOW) and combined EOW

		Final concentration of anode EOW (vol%)						Final concentration of combined EOW (vol%)						
Test microorganism	Exposition time (min)	1	5	7.5	10	25	50	1	5	10	20	25	50	80
Staphylococcus aureus	5	+	+	−	−	−	−	+	+	+	+	+	−	−
	15	+	+	−	−	−	−	+	+	+	+	+	−	−
	30	+	+	−	−	−	−	+	+	+	+	+	−	−
	60	+	+	−	−	−	−	+	+	+	+	+	−	−
Proteus mirabilis	5	+	+	−	−	−	−	+	+	+	+	+	+	−
	15	+	+	−	−	−	−	+	+	+	+	+	−	−
	30	+	−	−	−	−	−	+	+	+	+	−	−	−
	60	+	−	−	−	−	−	+	+	+	+	−	−	−
Mycobacterium avium ssp. *avium*	5	+	+	+	+	−	−	+	+	+	+	+	+	+
	15	+	+	+	−	−	−	+	+	+	+	+	+	+
	30	+	+	−	−	−	−	+	+	+	+	+	−	−
	60	+	+	−	−	−	−	+	+	+	−	−	−	−
Enterococcus faecium	5	+	+	+	+	−	−	+	+	+	+	+	−	−
	15	+	+	+	−	−	−	+	+	+	+	+	−	−
	30	+	+	+	−	−	−	+	+	+	+	+	−	−
	60	+	+	−	−	−	−	+	+	+	+	+	−	−
Pseudomonas aeruginosa	5	+	+	+	+	−	−	+	+	+	+	+	+	−
	15	+	+	+	+	−	−	+	+	+	+	+	−	−
	30	+	+	+	−	−	−	+	+	+	+	+	−	−
	60	+	+	−	−	−	−	+	+	+	+	+	−	−
Candida albicans	5	+	−	−	−	−	−	+	+	+	+	+	−	−
	15	+	−	−	−	−	−	+	−	−	−	−	−	−
	30	+	−	−	−	−	−	+	−	−	−	−	−	−
	60	+	−	−	−	−	−	+	−	−	−	−	−	−

+, Positive for *in vitro* growth; −, negative for *in vitro* growth.

inactivation values from Table 1, a sensitivity ranking order for anode EOW to the bacterial test strains is as follows: *P. mirabilis* > *S. aureus* > *M. avium* ssp. *avium* > *E. faecium* > *P. aeruginosa*. Test strains of *P. mirabilis* and *S. aureus* decreased to undetectable levels after 5 min of exposure to 7.5% anode EOW (~30.0 mg/l Cl_2). Comparatively, the Gram-positive bacterium *E. faecium* and the Gram-negative bacterium *P. aeruginosa* exhibited a greater resistance to the bactericidal activities of anode EOW as a concentration of 25.0% (≈100.0 mg/l Cl_2) was required for a complete bactericidal effect within 5 min of exposure. *Mycobacterium avium* ssp. *avium* was completely inactivated by 15 min of exposure to 10.0% anode EOW whereas a 5-fold concentration of 50.0% and an extended exposure of 30 min was required to achieve complete inactivation in combined EOW. The yeast representative *C. albicans* was highly susceptible to microbicidal activities of anode EOW with complete inactivation after 5 min of exposure to a concentration of 5.0%. *Candida albicans* was less susceptible to fungicidial activities of combined EOW. Here a minimum concentration of 50.0% was required for complete inactivation after 5 min of exposure (Table 1). Microbicidal activities of EOWs against vegetative cells of bacteria and yeasts were completely abolished after addition of 20.0% bovine serum to the defined aqueous system.

Discussion

The microbicidal properties of anode EOW are determined by its physical and chemical properties, such as low pH (~3), high ORP (+1100 mV), and large concentrations of chlorine (Cl_2, in the present case ~400 mg/l). In aqueous solutions, Cl_2 hydrolyses rapidly into hypochlorous acid (HOCl; Fig. 1). HOCl is one of the most germicidal chlorine compounds which are by definition oxidizing agents. The oxidizing capacity of the HOCl is equal to the extremely active molecular Cl_2. The availability of HOCl is primarily a function of the pH, which establishes the amount of dissociation of HOCl to a hydrogen ion (H^+) and a hypochlorite ion (OCl^-; White, 1999). In general, the concentration of HOCl increases significantly as the pH decreases: at a pH above 9 and a temperature of 20°C, there is only marginal disinfecting activity as at this pH level ~96% of the free available chlorine consists of the OCl^- ion, which is a relatively poor disinfectant. This is in agreement with our finding that in comparison with anode EOW the combined EOW consisting of 70% anode and 30% cathode EOW (pH 8.3) lacked strengths of disinfecting efficacy. By way of explanation; at a pH of 8.3 and a temperature of 20°C, the percentage of undissociated HOCl is 16.1% (White, 1999). However, at a pH below 5, the percentage of undissociated HOCl in a chlorine solution is ~99.7%. Thus, the large amount of HOCl on account of the low pH of ~3 is considered the chief factor of the disinfecting efficacy of EOW (White, 1999; Vorobjeva et al., 2004). Because of the water-like structure, the low molecular weight, and the electrical neutrality, HOCl molecules can easily diffuse through the bacterial cell wall into the cytoplasm (White, 1999; Len et al., 2000; Kiura et al., 2002). The bactericidal mechanism is probably the irreversible oxidation of cytoplasmic enzymes in particular in the inner membrane and peripheral cytoplasm (Matsunaga et al., 1984). Whether hydrogen peroxide (H_2O_2), ozone (O_3) and chlorine dioxide (ClO_2) contribute to the microbicidal activity of EOW remains controversial, as these substances were not detected in freshly prepared EOW (Gordon et al., 1999; Vorobjeva et al., 2004).

As was confirmed in our study, anode EOW renders a strong bactericidal activity against Gram-positive and Gram-negative bacteria as well as against mycobacteria. However, marked differences in the sensitivity for anode EOW between the bacterial test strains were found. The Gram-negative bacterium *P. mirabilis* and the Gram-positive bacterium *S. aureus* were more susceptible to the bactericidal activity of anode EOW water than the Gram-negative *P. aeruginosa* and the Gram-positive *E. faecium*. The former agents were completely inactivated by 5-min exposure to 7.5% anode EOW (~30 mg/l Cl_2) whereas the latter required a 5-min exposure to 25% (~100 mg/l Cl_2, i.e. 3.3-fold concentration) for complete inactivation. *Mycobacterium avium* ssp. *avium*, a representative of the medically important *Mycobacterium* group which includes amongst others the veterinary significant agent of paratuberculosis (*M. avium* ssp. *paratuberculosis*) in ruminants, exhibited intermediate susceptibility with complete inactivation by 15-min exposure to 10% EOW (~40 mg/l Cl_2). The highest germicidal activity of anode EOW was measured against the yeast representative *C. albicans*. Here a complete inactivation was obtained by a 5-min exposure to 5% EOW (~ 20.0 mg/l Cl_2) and these results agree with a recent report on the relative susceptibility of *C. albicans* for antifungal activities of EO solutions (Balch et al., 2000). Overall, in a defined aqueous system containing 10% anode EOW (~40.0 mg/l Cl_2), complete sacrificing of vegetative forms of all test organisms was achieved within 15–30 min of exposition.

It is well established that nitrogenous compounds like proteins are chlorine-consuming, that means organic nitrogen (N) will react instantaneously with the free available chlorine residual (HOCl) to form non-germicidal N-chloro-compounds (White, 1999). Thus, proteins exert a significant chlorine demand thereby reducing the disinfection effectiveness of the total chlorine residual. This was particularly true in our analyses of the bacteriostatic and fungistatic efficacy of EOWs using a peptide-containing nutrient broth according to DVG guidelines (Anonymous, 2000). In these experiments, no bactericstatic or fungistatic effectiveness was seen, and it is obvious that the organic N has depleted the disinfection activity. Consistently, the microbicidal activities of EOWs were also completely abolished in our study after addition of 20% BSA to the test solutions. Indeed, the rapid inactivation of the anti-microbial activity following contact with organic matter is the main disadvantage of the application of EOWs (Nakae and Inaba, 2000; Shimmura et al., 2000). Thus, to ensure the efficacy of disinfection procedures using EOW, a thorough pre-cleaning to minimize the organic load present is essential. Obviously, further studies are required to establish the applicability and safety of EOWs in livestock husbandry. Field trials have to be carried out to determine the effectiveness of EOWs in particular under adverse conditions such as the presence of heavy organic soiling and low temperatures. Moreover, as reactions of HOCl and OCl^- with organic substances are certain to form potentially cytotoxic and genotoxic chlorinated disinfection by-products like trihalomethanes or chlorohydroxyfuranones (Knasmüller et al., 1996; Gibbons and Laha, 1999), the safety of EOWs has to be ascertained prior to application e.g. in continuous disinfection of drinking water in food animal production facilities.

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