DRAFT MANUSCRIPT FOR DISCUSSION
PRELIMINARY DATA: NOT FOR PUBLICATION

21 June 1998

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Thanks Carlton

Evaluation of electrically superoxidised water against tropical pathogens

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Abstract

There is a World-wide need for new water purification technology. Laboratory experiments have suggested that electrically treated, super-oxidised water rapidly kills some bacteria and viruses. We have therefore tested its activity against important causes of human water-borne disease. We found that superoxidised water is active against *Vibrio cholerae*, *Salmonella paratyphi* and cholera phage viruses. A 2% solution completely sterilised solutions of 100,000,000 pathogens per milliliter. The presence of contaminating protein attenuated the disinfecting activity but the concentrations of protein we found in sewage were overcome by increasing the concentration of superoxidised water. Drinking water collected from impure sources in shanty towns was found to be contaminated with pathogenic coliform bacteria in 53% of cases but addition of superoxidised water to a final concentration of 2% killed all pathogenic bacteria. These results suggest that superoxidised water effectively kills several tropical pathogens and has potential value in the purification of contaminated drinking water in developing countries.

Introduction

Clean drinking water is a fundamental human need. Approximately 1.2 billion people World-wide are regularly forced to drink water contaminated by pathogens. It is estimated that more than three-quarters of all infections in the developing world result from water contamination and that more than 24,000 children die every day from infectious diarrhoea alone. Disinfection of drinking water by chlorination is of limited utility in many developing countries because of cost, toxicity and limited activity against waterborne parasites. The protozoan parasite *Cryptosporidium parvum* may even survive standard water purification procedures in wealthy Nations, leading to outbreaks of diarrhoeal disease. There is therefore an urgent need for new water purification technologies which are more effective against parasitic cysts and which are safe and inexpensive enough for use in poorer countries.

Treatment of salinated water with an electrical current passed through rare metal electrodes produces superoxidised water. This has been shown in laboratory experiments to kill several viral, bacterial and mycobacterial species and physically disrupt oocysts of *Cryptosporidium parvum*. Superoxidised water is being used in British and North American hospitals for sterilising endoscopes and other medical equipment.

We became interested in the potential utility of superoxidised water for purifying drinking water in developing countries because it is non-toxic and the equipment required for its production is portable and inexpensive: table salt and electricity are the only running expenses. We have therefore tested the activity of superoxidised water against several important bacterial, viral, and protozoal water-born pathogens: *Vibrio cholerae* and *Salmonella paratyphi*, cholera phage and *Giardia duodenalis*. The presence of organic matter reduces the effectiveness of disinfectants by coating microorganisms and preventing penetration of chemical germicides, or by directly inactivating germicides (Manual of Clinical Microbiology, 5th ed., p.194). We therefore also studied the effect of controlled concentrations of protein contamination. These experiments gave encouraging results so we prospectively studied the efficacy of superoxidised water for the purification of contaminated drinking water sampled from a Peruvian shantytown.

Material and Methods

Superoxidized water

Superoxidised water with a pH 5.0-6.0 and redox potential 1.030-1,050mV was produced using a bench-top Sterilox 350 unit (Willowbank Medical Ltd, Berkshire, England). Tap water had a flow rate greater than 0.7L/min and the device used 8-10A electrical current. Superoxidised water was diluted in distilled water and used within 4 hours of production.

Bacterial studies

Vibrio cholerae serotype 01 El Tor Ogawa and *Salmonella paratyphi* 04:H, I were isolated from patients' faeces and used as test bacteria. The bacteria were cultured in peptone broth (Difco Laboratories, Detroit, MI, USA) and tryptone soya broth (TSA, Difco Laboratories) respectively for 24h at 37°C. They were then quantified by logarithmic dilution and culture for 24h at 37°C on selective media: thiosulphate citrate bile sucrose agar (TCBS, Difco Laboratories), for *V. cholerae* and MacConkey agar (Difco Laboratories) for *S. paratyphi*. A standard curve of optical density to colony forming units was derived, allowing dilution of cultures to 8 +/- 0.5 $x10^8$ cfu/mL.

To determine bactericidal activity, 10^8 bacteria suspended in 1mL PBS was added to 9 mL of superoxidised water diluted in distilled water to the following concentrations: 100% (pure superoxised water), 50%, 10%, 5%, 2%, 1%, 0.1%, and 0% (control). Following mixing by vortexing for 5 seconds and 5 inversions, these tubes were left at room temperature for 5 minutes. To limit exposure time, the bacteria were then pelleted by centrifugation at 5000 rpm for 3min. The pellet was washed twice and the resuspended in PBS. The number of surviving bacteria were quantified by logarithmic dilution and 24h culture on selective media.

Viral studies

Cholera phage isolated from sewage during a Peruvian cholera epidemic were cultured for 24h at 37°C in Lennox broth (LB, Sigma Chemical Co., St. Louis, MO, USA) with 0.1% maltose and *V.cholerae* MAK 757 bacteria. They were then purified by centrifugation at 2,000 rpm for 5min and 0.2µm micropore filtration. Cholera phage was quantified by culture in *V. cholerae* MAK 737 in LB broth and 0.7% agarose (Gibco BRL, Grand Island, NY, USA) which was plated on TSA for 24h at 37°C.

A volume of 1 mL of PBS containing 4 x 10^4 vp/mL was mixed by vortexing and 5 inversions in 9 mL of superoxidised water diluted in distilled water to the following concentrations: 100%, 50%, 10%, 5%, 2%, 1%, and 0% (control). Exposure time was then limited by the addition of a high concentration of protein: after 5 minutes, 9 volumes of LB broth and an aliquot cultured to quantify surviving viral phage.

Protozoal studies

Giardia duodenalis cysts were isolated from faecal specimens obtained from the Hospital del Nino in Lima. Stool samples were first dissolved in distilled water and then filtered through cheeseclothe before centrifugation at 3000 rpm for 15 minutes. The pellet was resuspended in distilled water and 5 mL aliquots were mixed with 40 mL of 1M sucrose solution and centrifuged again. The cysts were then removed with a Pasteur pipette, washed and resuspended in PBS. Quantification was performed utilizing a light microscope.

1 mL aliquots of 10^3 cysts/mL suspension of *G. duodenalis* were exposed to 9 mL of superoxidised water diluted in distilled water to concentrations of 100%, 50%, 10%, 5%, 2%, 1% and 0% (control) for 1 hour. After centrifugation at 4200 rpm for 5 minutes, the pellet washed with distilled water and resuspended to 1 mL in distilled water. 0.5 ml aliquots of the treated samples were mixed with 0.5ml of 1% eosin, and this was centrifuged for 3 minutes. 0.5ml of eosin sobrenatant was discarded and the rest was vortexed. 15 ul was examined with a light microscope, and the number of clear and stained cysts noted. Red stained cysts were assumed to be inviable, and the unstained clear cysts viable.

Protein contamination studies

To determine the effect of protein contamination, the bacterial and viral experiments were repeated with suspensions of bacteria and virus in PBS containing 10%, 5%, and 1% solutions of bovine serum albumin.

Effect of storage time on functioning of superoxidised water

The effect of storage time on the functioning of superoxidised water was also examined. Superoxidised water was produced and then stored for 0, 6, 12, 24, 48, and 72 hours. Using the bacterial studies protocol described above, 10^4 and 10^8 *S. paratyphi* bacteria were suspended in 1 mL aliquots of PBS and then exposed to 9 mL of aged superoxidised water diluted to 2% concentration in distilled water. After 5 minutes of exposure time, the number of surviving bacteria were quantified by logarithmic dilution and 24h culture on selective media.

Field trial for disinfection of drinking data

70 drinking water samples of 50 mL were collected from different households in Las Pampas de San Juan de Miraflores, a shantytown on the outskirts of Lima where 77% of homes lack running water. Water is commonly stored in large containers for household use, and is frequently contaminated with human pathogens. The source, storage time and storage conditions of each sample was recorded.

5 ml aliquots were mixed with 5%, 2% and 1% superoxidised water or a control solution of distilled water. After standing for 5 minutes, 0.1 mL aliquots were cultured at 37°C on TSB and McConkey agars. After 24h the number and colour of colonies was recorded.

Results

Table 1 shows the activity of superoxidised water against concentrated suspensions of water-borne pathogens. 1% solutions of superoxidised water achieved complete killing of *V. cholera* and cholera phage but only partially killed *Salmonella paratyphi* which required a 2% solution of superoxidised water for complete killing. *Giardia duodenalis* was more resistant, requiring a 10% solution of superoxidised water and a 1 hour exposure time for complete killing.

Organism	Number of pathogens	Percentage superoxidised water required for complete killing
Vibrio cholerae	10^8 bacteria/ml	1 % (5 minutes exposure)
Salmonella paratyphi	10^8 bacteria/ml	2 % (5 minutes exposure)
Cholera phage	4 X 10^4 viruses/ml	1% (5 minutes exposure)
Giardia duodenalis	10^3 cysts/ml	10% (1 hour exposure)

Table 1: percentage superoxidised water required to kill water-borne pathogens

Cysticidal activity of superoxidised water

Effect of protein contamination on superoxidised water functioning

Table 2, 3 and 4 show the effect of bovine serum albumin on the disinfectant activity of superoxidised water. No significant killing is not shaded, partial killing is faintly shaded and complete killing is darkly shaded. Protein contamination was therefore inhibitory but 50% superoxidised water was sufficient to sterilise concentrated bacteria in the presence of 5% protein.

Concentration of superoxidised water	No protein	1% protein	5% protein	10% protein
0%	<1	<1	<1	<1
0.1%	<1	<1	<1	<1
1%	>7	<1	<1	<1
10%	>7	>7	<2	<1
50%	>7	>7	>7	<2
100%	>7	>7	>7	>7

Table 2: number of log units of Vibrio cholerae bacteria killed by superoxidised water in the presence of contaminating protein.

Concentration of superoxidised water	No protein	1% protein	5% protein	10% protein
0%	<1	<1	<1	<1
0.1%	<1	<1	<1	<1
2%	>7	<1	<1	<1
20%	>7	>7	<2	<1
50%	>7	>7	>7	<2
100%	>7	>7	>7	<2

Table 3: number of log units of Salmonella paratyphi bacteria killed by superoxidised water in the presence of contaminating protein.

Concentration of superoxidised water	No protein	1% protein	5% protein	10% protein
0%	<1	<1	<1	<1
1%	>4	<1	<1	<1
2%	>4	>4	<1	<1
5%	>4	>4	>4	<2
10%	>4	>4	>4	>4
50%	>4	>4	>4	>4

Table 4: number of log units of cholera phage viruses killed by superoxidised water in the presence of contaminating protein.

Effect of storage time on functioning of superoxidised water

Table 5 shows the effect of storage time on the disinfectant activity of superoxidised water. No significant killing is not shaded, partial killing is faintly shaded and complete killing is darkly shaded. As shown, superoxidised water maintains complete disinfectant activity for up to 12 hours after production.

Time of storage of superoxidised water	10^8 bacteria treated	10^4 bacteria treated
0 hours	>7	>3
6 hours	>7	>3
12 hours	>7	>3
24 hours	<1	<1
48 hours	<1	<1
72 hours	<1	<1

Table 5: number of log units of Salmonella paratyphi bacteria killed by superoxidised water in the presence of contaminating protein

Field trial of superoxidised water for disinfection of drinking water

70 drinking water samples were collected and Table 6 shows the results of bacterial culture and the disinfectant effect of superoxidised water. 1% superoxidised water killed all coliform bacteria in all but one samples and 2% superoxidised water achieved complete sterility.

Concentration of superoxidised water	Proportion of samples with any bacteria present	Proportion of samples with coliforms present
0%	87%	53%
1%	2.6%	1.4%
2%	0%	0%
5%	0%	0%

Table 6: effect of superoxidised water on drinking water samples.

Discussion

In this study, a 2% solution of superoxidised water disinfected concentrated *V. cholerae*, *S. paratyphi* and cholera phage in the laboratory. In a field trial, 2% superoxidised water also killed coliform bacteria in drinking water samples collected from a shanty town.

Produced by a portable electrochemical device from tap water and salt, superoxidised water is colourless and odourless but has a high oxidation-reduction potential. Chemical and spectrophotometric analyses indicates that the main product of electrolysis is hypochlorous acid (HCLO), but the solution also contains a variety of radicals, including ozone and hydrogen peroxide. At pH 5.5, the HCLO content of freshly prepared superoxidised water is 144ppm (U. of Birmingham report,The Composition of Products from a Willowbank Double Tube Electrolyser). However, the concentration of all these compounds except sodium chloride in 100% superoxidised water are within the WHO recommendations for drinking water quality. By diluting superoxidised water to 5%, the concentration of sodium chloride also falls to acceptable levels. Preliminary investigations of the mechanism of action suggests that the high redox potential allows the dilute chemical solution to work in a hyperactive manner.

Superoxidized water is a powerful disinfectant, with bactericidal activity superior to 0.1% chlorhexidine and 0.02% povidone iodine, and similar to 80% ethanol (1996 *Journal of Hospital Infection* article). In contrast to these agents, superoxidised water is relatively non-toxic. No toxicity resulted from superoxidised water injection in day-old ducklings and experiments with rabbits revealed no evidence of gastrointestinal, ocular or cutaneous injury from instillation of 100% superoxidised water. (Huntingdon Life Sciences report, University of Sydney/Dept of Infectious Diseases report). Further toxicity data would be required before use in drinking water may be considered.

The presence of protein attenuated the disinfectant activity but this was largely overcome by increasing the concentration of superoxidised water. This inhibition of activity by organic material is also seen with other disinfectants. For comparison, samples of turbid contaminated water was collected from a sewage treatment center in Lima, and analyzed for protein content using the Bradford Assay. In all samples, the protein content was found to be less than 2μg/mL, or 0.2% protein. The concentrations of protein we tested were, therefore, much higher than heavily contaminated drinking water.

The field test of superoxidised water in Las Pampas de San Juan de Miraflores, a shantytown in Lima, studied the level of water contamination and the minimum amount of superoxidised water needed to disinfect water. Coliform bacteria, an indicator of faecal contamination, were found in 53% of household water supplies. It should be noted that drinking water in the United Kingdom is required to be free from coliform bacteria but usually contains non-pathogenic bacteria. The addition of 2% superoxidised water killed all coliform bacteria but tests were conducted with small volumes. Disinfection of large water containers may require a higher concentration of superoxidised water to overcome incomplete mixing.

These results suggest that superoxidised water could theoretically provide clean drinking water for communities with severely limited resources. It is simple to produce, low in cost and is an effective disinfectant against the tropical pathogens tested. At the time of this study, numerous Peruvian communities were displaced or cut off from clean water supply as a result of the El Nino phenomena. In refugee situations such as these, portable electrolysers could conceivably be utilized to purify contaminated drinking water although the our finding that disinfectant activity was lost between 12 and 24 hours of production would necessitate the production of superoxidised water close to the point of use. These results suggest that superoxidised water has potential utility for water treatment in developing countries and warrant further investigation of its spectrum of action and safety for human consumption.

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