STABILISED HYPOCHLOROUS ACID: A NEW THERAPEUTIC STRATEGY AGAINST DANGEROUS PARASITIC EYE INFECTION AGENT *Acanthamoeba* spp.

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Abstract. *Acanthamoeba* species are protozoan parasites that cause serious diseases in human. It is significant to investigate new treatment agents to combat the parasite which is very difficult to treat. The recent studies about stabilised hypochlorous acid indicated that with a rapid killing effect on different types of microorganisms, it could be used as a treatment agent for *Acanthamoeba* infections. Thus, we aimed to investigate the effect of stabilised hypochlorous acid on *Acanthamoeba*. Stabilised hypochlorous acid at serial dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 was used in different time course. The direct microscopy for time-kill assay and Trypan blue assay were used to determine the effective dilutions and time exposure of stabilised hypochlorous acid to live form of parasite. We determined that the effect of stabilised hypochlorous acid was reached at 96 and 84% (dilution of 1/2 and 1/4, respectively) at the end of 90 min. The effect of stabilised hypochlorous acid was dose- and time-dependent. Stabilised hypochlorous acid solution could be used for eye infections due to the its safety feature. Since its effective feature at 1/2 and 1/4 dilutions within 0–10 min, it could be a new treatment agent to combat the parasite and its infections.

Keywords: stabilised hypochlorous acid, *Acanthamoeba*, eye infections, biofilm.

AIMS AND BACKGROUND

*Acanthamoeba* is a parasite that is widely distributed in the environment. *Acanthamoeba* has two stages in its life cycle, an active trophozoite stage and a dormant cyst stage. *Acanthamoeba* species, which are found in protozoans known as free living amoebas (FLA), are widely available in nature. FLA have shown to survive in a variety of environments throughout the world such as soil, lakes, swimming pools, spas, drinking water, treatment pools. FLA are reported to be opportunistic pathogens not only in humans, but also in fish and in different species of animals

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(dog, rabbit, turkey, cattle, etc.). FLA infections were detected in a large number of fish during 1970s, in the USA.

Acanthamoeba species can cause serious infections in humans. As an opportunistic pathogen, the parasite can cause subacute granulomatous amoebic encephalitis (GAE) which is known to be effective, especially in immunodeficiency patients. Some Acanthamoeba species also cause skin and sinus infections in people with AIDS and immunodeficiency. Among the infections caused by Acanthamoeba species, the most common clinical presentation is reported to be chronic keratitis, which can result in corneal pain and blindness in healthy individuals.

Since the existence of both forms of Acanthamoeba and cyst form may be highly resistant to therapy, a combination therapy generally used. Surgical interventions are often required due to the failure of treatment with chemotherapeutic agents. Although the use of different chemotherapeutic against Acanthamoeba, delay in treatment of keratitis may result in blindness. The failure of medical agents to affect all forms of parasites and the toxic effects of effective agents on the cornea, suggest that new agents are needed.

Hypochlorous acid (HOCl) is a weak acid produced by activated neutrophils during oxidative burst. In the oxidative burst pathway, the superoxide produced by nicotinamide adenine dinucleotide phosphate oxidase reacts to produce free radicals such as H₂O₂. Myeloperoxidase then produces hypochlorous acid. HOCl has the potential to cause tissue damage, but the risk is reduced when the nonessential amino acid taurine scavenges and converts HOCl to other chemicals.

HOCl leads to cell death with various effects such as oxidation of sulphhydroly enzymes and amino acids, ring chlorination of amino acids, loss of intracellular contents, decreased uptake of nutrients, inhibition of protein synthesis, decreased oxygen uptake, oxidation of respiratory components, decreased adenosine triphosphate production, breaks in DNA, and depressed DNA synthesis.

Stabilised hypochlorous acid has powerful and rapid killing effect on different types of microorganisms. In previous studies it has been demonstrated that it has antibiofilm and microbicidal effects on microorganisms within the biofilm. Stabilised HOCl is a non-toxic and proliferative effect on human cells. With these properties it is widely used as a wound care solution. Although, there are many studies with bacteria, virus and fungi, there is no study about whether stabilised HOCl may effect on the parasites or not. Thus, we investigated its effect on Acanthamoeba for the first time.

EXPERIMENTAL

Reagents. HOCl is generated from sodium hypochlorite and hydrogen peroxide reverse electrochemical reaction. The concentration used in this study was 218 ppm,
pH 7.1, Oxidation reduction potential (ORP) 871 MV and its stability were 24 months (NPS Biosidal, Istanbul, Turkey).

**Microorganism strains and growth.** *Acanthamoeba castellani* was grown in the standard broth that is PYG medium (Peptone-Yeast extract-Glucose) in glass tubes at 37°C for three days before assay. The day of assay, quantity of parasites was counted with Trypan blue that is $2 \times 10^5$. The culture was centrifuged at 600 rpm for 5 min, and the supernatant was removed. 2 ml of PBS solutions were added in a pellet that included with the parasite.

*Time kill (TK) assay:* Since the stabilised HOCl solution inactivated with organic materials and HOCl solution with serial dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 (109, 55, 22.5, 11, 5.5, and 2.75 ppm, respectively) was prepared in sterile phosphate buffered saline (PBS) solution. Briefly, 100 µl of HOCl dilutions were added into wells of 96-well polystyrene F-bottom microtiter plate. Then, 25 µl of mixture that includes parasite-PBS were added into the wells of HOCl dilutions. After that, reagents 75 µl of PYG medium were added. Sterile PBS and medium was used as a control. The same quantity of parasite was also added. The plate was incubated for 0, 10, 30, 60, 90 min at 37°C to investigate the effect of stabilised HOCl solution (HOCl) on the parasite and: (1) the direct microscopy for time-kill assay was used to determinate the effective dilutions; (2) the Trypan blue assay was used to compare death and live form of parasite; (3) viability confirmed with the transfer of HOCl treated *Acanthamoeba* into the wells with the medium and viability test was performed with trypan blue staining.

**Statistical analysis.** All experiments were repeated three times and each experimental and control condition assayed in triplicate. Analysis of variance (ANOVA) was used to compare the mean responses among experimental and control groups. A value of $p$ below 0.05 was considered statistically significant.

**RESULTS AND DISCUSSION**

**Minimum parasidal concentration and time kill.** In this study, we determined the time and dose response of HOCl on *Acanthamoeba* spp. In 1/2 dilution mean killing rate was 83.6, 89.3, 94, 96 and 96.6% in 0, 10, 30, 60 and 90 min, respectively. Also in 1/4 dilution mean killing rate was similar with 1/2 dilutions and it was 72.6, 79, 86.6, 83.6 and 84% in 0, 10, 30, 60 and 90 min, respectively. In 1/8 and 1/16 dilution killing rate decreased significantly. In 1/32 dilution, killing rate was significantly higher in 10, 30, 60, and 90 min compared to control, but there were no differences in 1/64 dilution and 0 min of 1/32 dilution compares to control (Table 1). These data indicated that HOCl has dose (Fig. 1) and time-dependent (Fig. 2) effect on *Acanthamoeba* (Fig. 3).
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na – statistically not significant.
Fig. 1. Effect of different doses of HOCl on *Acanthamoeba*: * no significant changes in stabilised HOCl solution treated *Acanthamoeba* compared to the media control at $p > 0.05$

Fig. 2. Effect of different exposure times of HOCl on *Acanthamoeba*: * no significant changes in stabilised HOCl solution treated *Acanthamoeba* compared to the media control at $p > 0.05$

Fig. 3. Control group; live form of the parasite – A; effect of 1/2 dilution stabilised hypochlorous acid observed on *Acanthamoeba* at 0 min; dead forms of the parasite stained with Trypan blue – B; In the same dilution, the cystic form of the parasite that is dying and the live forms – C
*Acanthamoeba* species are free living organisms, but under unfavourable conditions they form cysts and cyst forms are resistant to most of the anti-parasitic and high level disinfectants. Also, they have a great medical importance because they have a large living area and they are causative agents of serious infections such as keratitis and encephalitis.

Hypochlorous is a weak acid produced during the oxidative burst by active neutrophils. It has been shown that the stabilised HOCl solution had rapid and powerful effects on different microorganisms such as viruses, bacteria and fungus. Also, it has been shown that HOCl has anti-biofilm and killing effect on microorganism within the biofilm. Moreover, it is non-toxic and favourable effects on the human cells promote its use on mucosal surfaces. With these properties stabilised HOCl widely using in infected or non-infected wounds.

Since, natural water sources, ponds, air conditioning filters, lens solutions, etc. are sources of contamination, it is important for effective sterilisation of the potentially contaminated area. For that reason it is necessary to develop protective compounds that can prevent parasitic transmission as well as investigate effective medicinal agents for combating this parasite. Biofilms are known to play an important role in the pathogenesis of *Acanthamoeba* keratitis. Interestingly the biofilm formation on contact lenses, increasing binding affinity of *Acanthamoeba* as well as growth, but there is no evidence of biofilm production of *Acanthamoeba*. It has been shown that HOCl had antibiofilm effect and killing effect on microorganisms within the biofilm. This property of HOCl gets an edge over in preventing and treatment of contact lens associated *Acanthamoeba* keratitis. Since most of the disinfectant agent used for contact lenses failed to effective decontamination, successful anti-parasitic and anti-biofilm effect of HOCl make its protective effect superior to other disinfectants. Moreover, most of these disinfecting solutions and chemotherapeutic agents are found toxic to the eye, HOCl non-toxic and favourable effects on the human cells.

Early diagnosis and aggressive medical treatment are important in *Acanthamoeba* keratitis. Other therapeutic approaches such as epithelial debris (removal of organisms) and penetrating keratoplasty may increase the efficacy of medical treatment. There is no single chemotherapeutic agent that is effective against all genotypes and different forms of parasites.

**CONCLUSIONS**

In this study, we showed that HOCl has rapid and effective killing effect on *Acanthamoeba* spp. Since the current treatment strategies and antiparasitic drugs have several problems such as resistance, toxicity and complications, new treatment agents or methods are needed. With these problems HOCl may be a new therapeutic
strategy in *Acanthamoeba* keratitis with rapid and effective killing, anti-biofilm effect, non-toxic to human cell properties.

REFERENCES


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