

Stabilized hypochlorous acid, a topical therapeutic strategy for *Trichomonas vaginalis* infection: An *in vitro* study

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ABSTRACT

Background: *Trichomonas vaginalis* (TV) parasitic protozoan is a widespread sexually transmitted infection. It is emphasized by the World Health Organization that the global prevalence of TV is rising. The parasite causes infections in the urogenital system in humans, and recently it was determined that it has a potential effect on both prostate and cervical cancer. Due to its being a significant public health problem, new treatment methods need to be developed to combat the parasite.

Objectives: Previous studies on the use of stabilized hypochlorous acid (HOCl) indicated that it has a potential microbicidal effect on different types of microorganisms. From this point, we investigated the effect of stabilized HOCl on TV *in vitro*.

Material and Methods: Direct microscopy for time-kill (TK) assay, and vital trypan blue assay were performed to determinate the effective dose (1/2 to 1/64 dilution) and time (0 to 90 min) of stabilized HOCl action on live TV. For negative controls, sterile PBS alone was added to TV and medium.

Results: The death of TV was dose and time dependent. Parasites were killed at a dilution of 1/2 and 1/4 at 0 min and for the rest of the time courses at these dilutions. Whereas, decreased effect was observed on TV viability with dilutions of 1/8, 1/16 and 1/32 with different time courses and there was no effect at a dilution of 1/64.

Conclusion: According to our findings, stabilized HOCl has an effect on TV viability and this is the first study on the effect of HOCl on TV.

Keywords: Stabilized hypochlorous acid, *Trichomonas vaginalis*, therapeutic agents.

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INTRODUCTION

Trichomonas vaginalis (TV) is a flagellated parasitic protozoan. It does not have a cyst form, but it has been reported that under certain circumstances it can be transformed to pseudocyst form^[1]. It is a widespread sexually transmitted parasite. It may cause a persistent infection in adult females involving the vagina, urethra and endocervix; and may cause prostatitis, epididymitis and decrease the motility of sperm cells in men^[2,3]. Recent studies showed that it may have a potential effect on both prostate and cervical cancer^[4,5]. The World Health Organization reported the high incidence rate of 276 million new cases each year^[6]. Nitroimidazole, metronidazole or tinidazole are treatment agents for TV and the increasing recurrence of infections due to resistance and side effects of these antibiotics encouraged scientists to find an alternative treatment^[7,8]. Recent studies propose the investigation of the effects of additional synthetic and natural products other than antibiotics against TV^[9,10].

Stabilized HOCl is a weak acid, naturally produced by the myeloperoxidase activation of neutrophils while undergoing phagocytosis^[11,12]. It reacts with various functional groups of proteins, carbohydrates, lipids and their bio-conjugates by oxidation, resulting in a powerful microbicidal effect that overcomes pathogens^[13,14]. *In vitro* synthesized stabilized HOCl has been known to demonstrate a powerful antimicrobial effect in wounds against numerous bacterial, viral, fungal pathogens and prions^[15,16]. Stabilized HOCl is naturally produced by the innate immune system. It eradicates microorganisms, reduces inflammation via inhibition of mast cell degranulation and inflammatory cytokines, reduces itching and pain, increases oxygenation of wound sites to improve healing, and breaks down biofilms^[17]. While it is effective against numerous microorganisms, there are limited studies evaluating its effect on parasites^[18-21]. Herein we aimed to investigate the effect of stabilized HOCl on TV *in vitro*, as an intro to its use as topical application in the form of vaginal douche or cream.

MATERIAL AND METHODS

This analytical descriptive study was conducted in Aydin Adnan Menderes University Faculty of Medicine Department of Parasitology, Turkey, between 2019 and 2020.

Reagents: Commercially available stabilized HOCl which is generated by reverse reaction of sodium hypochlorite and hydrogen peroxide was used. The composition of the solution used in this study was 218 ppm, pH 7.1, ORP 871 MV, stable for 24 months (NPS Biocidal, Istanbul, Turkey).

***T. vaginalis* culture:** Cryopreserved TV isolate was thawed and grown in a trypticase-yeast-extract-maltose (TYM) medium prepared as described by Ertabaklar *et al.*^[22]. Before assay, 6 ml of TYM medium was incubated at 37°C for three days. The number of parasites was counted in a hemocytometer counting chamber and adjusted to an average number of 8×10^5 flagellates/ml. The culture was centrifuged at 600 rpm for 5 min, and the supernatant was discarded.

Time kill (TK) assay: To resuspend the flagellates, 3 ml of sterile phosphate buffered saline (PBS) was added to the pellet. 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) were prepared in sterile PBS. Briefly, 100 μ l of stabilized HOCl dilutions were added into a 96-well microtiter plate, followed by the addition of 25 μ l of flagellates-PBS mixture and 75 μ l of TYM medium to reach a final number of 2×10^5 flagellates/well. For negative controls, sterile PBS alone with TV and medium were used. The plate was

incubated for 0, 10, 30, 60, 90 min at 37°C to investigate the TK effect of stabilized HOCl solution on the flagellates. Evaluation of the results was by: (1) Direct microscopy of each dilution; (2) Vital trypan blue assay to identify and count the dead forms of the flagellates (Fig.1 A and B); (3) Re-culture to verify the effective concentrations on the viability of the flagellates.

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

RESULTS

According to the TK assay, we determined the lethal effect of stabilized HOCl dilutions at various times by direct microscopy, vital trypan blue, and re-culture exclusion to determine the number of viable parasites (Figure 1A). Positivity was confirmed in TYM culture without stabilized HOCl (Fig. 1B). The lethal rate was 100% with trypan blue exclusion at all time intervals with 1/2 dilution, and at 90 min with 1/4 dilution. The lethal rate with 1/4 dilution was 82.3%, 94.33%, 98.66% and 99.33% at 0, 10, 30 and 60 min respectively. The effect of stabilized HOCl gradually decreased at a dilution of 1/8, 1/16 and 1/32 and the lethal effect increased with the pre-defined time intervals at these dilutions. There was no effect at a dilution of 1/64 (Table 1). These data indicated that stabilized HOCl has dose and time dependent effect on TV (Figures 2 and 3, respectively).

Table 1. Minimal parasitological concentrations and time kill of stabilized HOCl to *T. vaginalis*.

Dilutions		Time				
		0	10	30	60	90
Control	Mean	0.0	0.0	0.0	0.0	0.0
	SEM	0.0	0.0	0.0	0.0	0.0
1/2	Mean	100	100	100	100	100
	SEM	0	0	0	0	0
	<i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
1/4	Mean	82.300	94.330	98.667	99.333	100
	SEM	0.880	1.330	0.333	0.333	0.333
	<i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
1/8	Mean	14.000	37.000	47.000	52.000	56.333
	SEM	1.520	3.214	1.520	1.528	1.764
	<i>P</i> value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
1/16	Mean	0.0001	2.000	3.667	5.000	5.667
	SEM	0.0000	0.577	0.333	1.155	1.202
	<i>P</i> value	NS	NS	<0.0001	<0.0001	<0.0001
1/32	Mean	0.0001	0.0000	0.00001	1.667	2.667
	SEM	0.0000	0.0000	0.00000	0.882	0.333
	<i>P</i> value	NS	NS	NS	NS	NS
1/64	Mean	0.00001	0.00000	0.00001	0.00001	0.00001
	SEM	0	0	0	0	0
	<i>P</i> value	NS	NS	NS	NS	NS

NS: Not significant

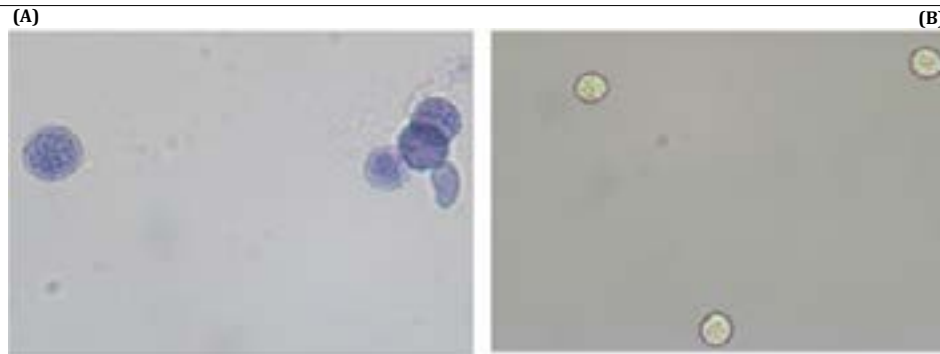


Fig. 1. (A) Effect of 1/2 dilution of stabilized HOCl on *T. vaginalis* at 0 min; dead forms of parasites stained with vital trypan blue. (B) Control live parasites in the absence of HOCl.

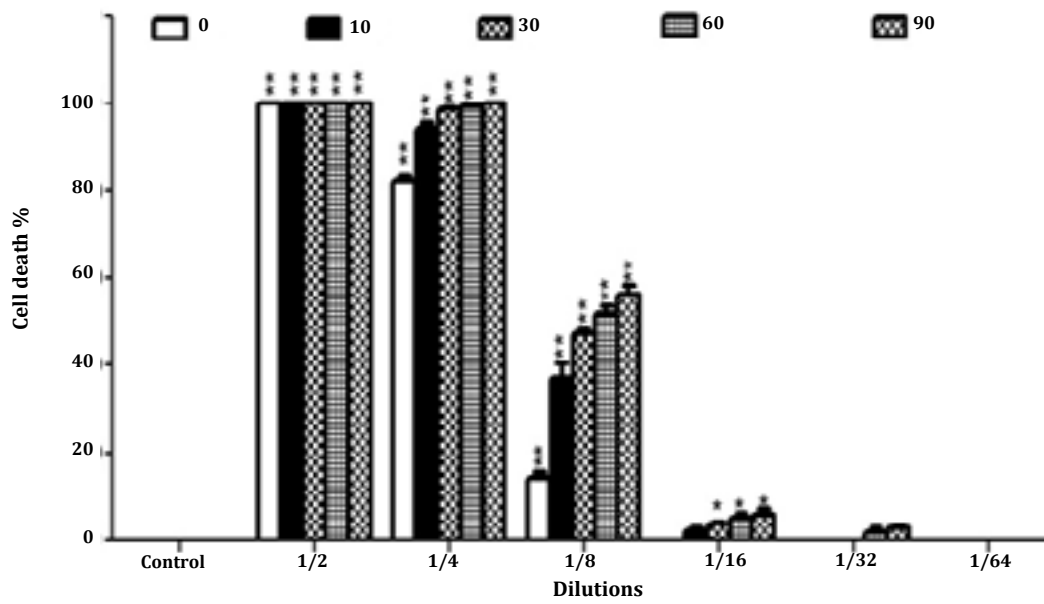


Fig 2. Effect of different dose responses to stabilized HOCl on *T. vaginalis*.

* $P < 0.001$, and ** $P < 0.0001$ significant decrease in viability of *T. vaginalis* in stabilized HOCl solution compared to the media control.

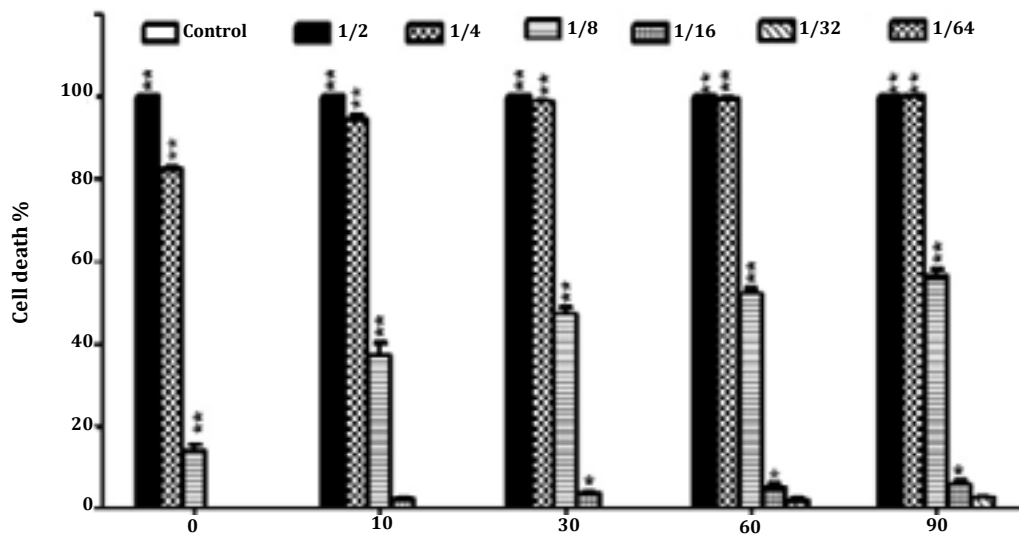


Fig 3. Effect of different time responses of stabilized HOCl on *T. vaginalis*.

* $P < 0.001$, and ** $P < 0.0001$ significant decrease in viability of *T. vaginalis* in stabilized HOCl solution compared to the media control.

DISCUSSION

Trichomonas vaginalis infection is the most common sexually transmitted disease worldwide. The disease is a basically curable sexually transmitted infection. In neglected female cases it may cause severe health consequences such as cervical cancer, infertility, HIV acquisition and adverse pregnancy outcomes leading to premature rupture of placental membranes in advanced cases^[23]. Trichomoniasis is generally asymptomatic in men, but recent studies indicate that TV has a potential effect on prostate cancer^[24].

The reported standard regimen for treatment of trichomoniasis is based on nitroimidazole-derived therapy, mainly metronidazole^[25]. The recommended oral dose for both men and women (pregnant/non-pregnant) is 2 g single dose, or a 7 day-dose therapy (400 or 500 mg twice daily for 7 days)^[26]. A meta-analysis of published comparisons reported that multidose metronidazole is superior to single-dose metronidazole for the treatment of trichomoniasis in a randomized controlled trial done in women with HIV infection^[27]. Also, trichomoniasis can be cured by the use of tinidazole^[28]. Oral or parenteral tinidazole and metronidazole have been approved for treatment in TV infections with cure rates of 92-100% and 85-95% respectively^[29,30]. Additionally, topical treatment, particularly vaginal douching in women is used for personal hygiene or aesthetic reasons, for preventing or treating an infection^[31]. A recent meta-analysis showed that no adverse outcomes or evidence of teratogenicity or mutagenic effects have been recorded for metronidazole used during pregnancy. But, it is still not clear whether metronidazole will have any adverse effect on pregnancy outcomes^[32]. Other *in vitro* studies indicated that 2.4–9.6% of isolates showed resistance to metronidazole^[33].

The high prevalence of TV, antibiotic resistance and limited tolerability, and toxicity to nitroimidazoles suggest that alternative treatment regimens are needed^[34,35]. An updated review provided comprehensive information on certain natural and synthetic compounds, and their modes of action^[36]. In recent studies, a wide variety of herbal compounds were tested for anti-trichomonal activity *in vitro*^[37-40]. Also inactivated *Lactobacillus acidophilus*, boric acid and other parasitic agents (benzimidazoles) were tested *in vitro* or *in vivo* against anti-trichomonal infections^[41-43]. In spite of the reported attempts for treatment with various compounds, an epidemiological study from South Korea reported that incidence of TV has increased^[44].

Besides of its use in recorded TV infections, vaginal douching using an antiseptic has been applied against pathogens associated with pelvic inflammatory disease, vaginitis, cervical cancer, low birth weight, preterm birth, sexually transmitted diseases, ectopic pregnancy,

and infertility^[45-47]. It is reported that douching with various products leads to deterioration of the vagina flora especially lactobacilli that prevent other potential pathogens from colonization or overgrowth^[48]. Topical vaginal medications and pessaries such as povidone iodine clotrimazole, paromomycin, furazolidone, used by women reduced the symptoms of trichomoniasis, while a two month course of intravaginal boric acid completely eradicated the infection^[49].

Cellular immunity plays a key role against harmful pathogens and creates chemicals such as reactive oxygen species. It activates neutrophils that produce hydrogen peroxide (H₂O₂), and the activated granule enzyme myeloperoxidase converts H₂O₂ into HOCl in the presence of Cl⁻ and H⁺^[50]. Commercial production of stabilized HOCl has become possible through electrolysis of sodium chloride brine. Accordingly it may be used against microorganisms causing loss of their intracellular contents, inhibition of protein synthesis and depressed DNA synthesis^[51,52]. Therefore, we aimed to test stabilized HOCl which is naturally produced in human, generated by the immune response as a bactericidal oxidant. Its eco-friendly property and ability to degrade the infectivity of prions and its active biocidal agent for wound care, encouraged us to study its effect on TV *in vitro*^[53-55].

In the literature, there is no *in vitro* study performed against TV using stabilized HOCl. Thus, ours is the first study to report that stabilized HOCl has time and dose dependent effect on TV, being effective at a dilution of 1/2 and 1/4 within 0-10 minutes. Whereas a dose and time dependent decrease was observed at dilutions of 1/8, 1/16 and 1/32; and no effect was observed at a dilution of 1/64.

Conclusion: This *in vitro* study supports the topical use of stabilized HOCl solution for its powerful and parasitocidal effect on TV trophozoites. Stabilized HOCl is widely used as an antiseptic in many fields due to its lethal effect on microorganisms. Foremost, this study has determined that stabilized HOCl can also be a highly effective agent in trichomoniasis. However, further studies need to be implemented to evaluate its *in vivo* application.

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