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].
Topical therapeutic strategy for trichomoniasis

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MATERIAL AND METHODS

This analytical descriptive study was conducted in Aydın 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., and 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 8x10⁵ 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 3 ml of sterile phosphate buffered saline (PBS) was added to the plate. 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 1A 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 parasitical concentrations and time kill of stabilized HOCl to _T. vaginalis_.

| Dilutions | 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  |
|           | P value | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| 1/2       | Mean | 100  | 100  | 100  | 100  | 100  |
|           | SEM  | 0    | 0    | 0    | 0    | 0    |
|           | P value | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| 1/4       | Mean | 82.300 | 94.330 | 98.667 | 90.000 | 100  |
|           | SEM  | 0.880 | 1.330 | 0.333 | 0.333 | 0.333 |
|           | P 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 |
|           | P 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 |
|           | P value | NS | NS | <0.0001 | <0.0001 | <0.0001 |
| 1/32      | Mean | 0.0001 | 0.0000 | 0.0001 | 1.667 | 2.667 |
|           | SEM  | 0.0000 | 0.0000 | 0.0000 | 0.882 | 0.333 |
|           | P 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    |
|           | P 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 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 metaanalysis 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 regiments are needed[29,35]. 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 (H$_2$O$_2$), and the activated granule enzyme myeloperoxidase converts H$_2$O$_2$ 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 parasiticidal 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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