In Vitro Comparative Study on Antiherpetic Effect of Chlorhexidine and Persica Mouthwashes with Acyclovir

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Abstract: *Introduction*: Recurrent intraoral herpes in one of the common oral diseases that causes painful ulcers and viral shedding. The question was if chlorhexidine and persica mouthwashes has antiviral effects comparing to acyclovir.

Materials and Methods: In this experimental study, virucidal effects of both mouthwashes were examined, before and after HSV-1 infection of the vero cells, in the presence of various concentrations (½,¼,⅛) of mouthwashes applied at different time intervals(0.5,1,5 minutes), by using quantal assays then were compared with acyclovir. The data were analyzed by one way and two way ANOVA.

Results: Before inoculation, both mouthwashes showed virucidal effects on HSV-1, at all concentrations and persica showed more virucidal effect than chlorhexidine and acyclovir (p=0.0001,p= 0.04, respectively). After virus inoculation although persica and chlorhexidine indicated antiviral effect, this already were so far less than that of acyclovir which caused more significant reduction on virus titer (p=0.0001).

Conclusion: Because of the better direct anti-HSV effect of the herbal mouthwash, persica, and its less side effects than chlorhexidine, it can be used for reduction of oral fluid contamination caused by viral shedding and also reduction of infectivity of oral ulcers.

Keyword: Herbal mouthwash, chemical mouthwash, acyclovir, HSV1, ulcer oral.

INTRODUCTION

Herpes simplex is the most common contagious infective virus in human that causes various diseases such as primary gingivostomatitis, recurrent herpes labialis, ocular and genital infection, encephalitis, pneumonitis,… . [1].

Acyclovir (ACV) as a selective and potent antiherpetic agent, is widely used for treatment of HSV infection but increasing resistance to ACV and its nephrotoxicity are the major concerns in the management of HSV infection [1, 2]. The antimicrobial monthrinses are an important part of comprehensive dental treatments, for both prophylactic and therapeutic purposes. Chlorhexidine (CHX) and persica are two commonly used monthrinses in Iran. CHX is a safe and effective agent for prevention of dental plaque formation that inhibits a variety of microorganism such as gram-positive, gram-negative facultative aerobic and anaerobic bacteria and also fungi [4].

There are several *in vitro* and *in vivo* studies on CHX's antiviral activity against HSV-1.Park *et al.* have reported that CHX not only inhibited the replication of HSV-1 *in vitro*, but also prevented the development of the virally-induced cutaneous lesion by inhibiting viral replication [4].

Baqui *et al.* in 2001 evaluated antiviral effects of various common mouth rinses and indicated antiherpetic effect of CHX up to ¼ dilution [3]. The adverse effects of the CHX's prolonged usage are calculus formation, permanent dental discoloration, taste changes and cell toxicity that limits its widely usage as a routine mouthwash [5, 6].

Persica, a herbal mouthrinse, is an alcoholic extract of the plant *Miswak* which is composed of several definite constituents as *Salvadora persica* 30%, *Achillea milefolium* 25%, *Menta spicata* 40%. It has antibacterial, anti inflammatory and analgesic effects [7].

Salehi *et al.* in 2006 compared antibacterial effect of CHX and persica on *streptococcus mutans* and showed similar effect of both mouthwashes on colony reduction with lower side effects of persica [8]. Several studies have also reported antibacterial effects of *Salvadora persica* [3, 9, 10]. Other studies have shown some positive effects of persica on the prevention and the healing period of the oral recurrent aphthous ulcers, however from the best of our knowledge there is no study on the antiviral effect of persica [11].

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Recurrent Intra oral Herpes (RIH), a form of HSV-1 infection, causes single or clusters of painful ulcers with more incidence and severity and increased frequency in immunocompromised patients compared with normal hosts. Treatment resistance to ACV also occurs more frequently in these patients [1, 2].

Since there has been no topical treatment regimen introduced for control of RIH on a theoretical basis, finding mouthwashes with an experimentally-proven antiherpetic effect and little adverse effects can be useful for prevention and treatment of these lesions [4].

On the other hand, asymptomatic viral shedding in saliva poses the risk of cross-infection *via* bio-aerosols produced during dental practice, so such mouth washes can potentially reduce the risk of contamination and prevent the spread of herpetic infection during dental treatment.

This study is aimed to comparatively investigate the potential *in vitro* antiherpetic effect of CHX and persica, in an attempt to introduce an available and safe mouthwash with proven antiherpetic action for ongoing clinical trials in the future.

MATERIALS AND METHODS

Virus Stock

HSV-1 isolated from lip lesions of a patient and was confirmed by neutralization test using guinea pig anti-HSV-1 serum (NIH, USA) [12].

Cell Culture and Cytotoxicity Assays

Vero cells line (Cell Bank of Pasteur Institute, Tehran, Iran), for assessing cytopathic effect of HSV, prepared by the following method, confluent Vero cells were grown in Dulbecoo's modified Eagle's growth medium (DMEM) (Sigma, USA) containing 5% fetal bovine serum (FBS) (Gibco, Germanay), 0.14% Sodium bicarbonate, 100 U/Ml penicillin, 100 Mg/Ml streptomycin sulphate and 0.25 Mg/Ml amphotericin B [13-15].

Grown Vero cell monolayers in sterile 24-well plates (NUNC, Denmark) were washed twice with PBs, increasing concentrations of both month washes (0.12,0.25,0.50%) and added to each well. After 5 minute, 30 minute, 1 hour and 24 hour. The cells were stained with trepan blue and cytotoxic concentration 50% (CC50) was determined by the regression curve [14, 15].

Antiviral Effect Assays

Various concentrations of mouth rinses (12.5, 25, 50%) were prepared in DMEM containing %2 FBs (maintenance medium). After washing with PBS, cell monolayers in 24 well plates (Nunc, Denmark) were exposed to different increasing concentrations of mouthwashes for 0.5, 1, 5 minutes, for one hr prior to infection, one hr after infection, with100 $TCID_{50}$ HSV-1 virus [3, 5]. Appropriate mixtures of SV concentrations and virus (100TCID50) were also incubated for 0.5, 1, 5 minutes, at room temperature before adding to cell monlayers.

Control samples consisted virus-infected untreated monolayer and treated cells with acyclovir 1250 Mg/ML. Following the incubation period at 37°C under 5% CO_2 for four days, the contents of each series of wells were pooled and stored at -70°C along with their corresponding controls for subsequent infectivity titration.

Quantal method (determination of tissue culture infected dose 50%, $TCID_{50}$) was used for virus titrationin 96 well plates (NUNC, Denmark). Cell in 96 well plates were evaluated under inverted microscope for presence of cytopathic effect (CPE) and ultimately viral titration were determined by Karber method [15,16]. Experiments were performed at least twice in quadruplicate.

Statistical Analysis

The mean number of virus titration of two different experiments was compared with one way analysis of variance (ANOVA) using spss version 16. Then for evaluation effect of type of drug with effect of concentration and time, two way ANOVA was used.

RESULTS

The cytotoxicity assay showed that, acyclovir in the used concentration was safe for the cells, but the CHX for the all periods (more than 5 min.) was toxic and CC_{50} of persica at 5 and 30 min were determined as 20% and 14% respectively but no viable cells had been detected at 1 and 24 hrs.

CHX and persica, one hour before the Vero cell infection with HSV-1, showed inhibitory effect at various concentrations and periods applied. But after infection of Vero cells with the virus, both mouthwashes inhibited HSV-1 with the dilutions up to 1:4 (25%) (Tables **1** and **2**).

Table 1: AntiHSV-1 Effect of CHX and Persica Before Inoculation of Vero Cells with HSV-1 in Comparison with ACV

Time			Concentration	Type of drug
5	1	0.5		
2.00±.35	2.75±0.35	1.87±0.17	1/2	persica
2.00±.70	2.37±1.23	1.87±0.53	1/4	
1.87 ±53	2.12±1.59	2.00±0.70	1/8	
2.75±0.35	2.62±0.17	2.75±0.35	1/2	CHX
3.50±0.00	3.12±0.17	3.50±0.00	1/4	
3.75±0.35	3.50±0.00	3.50±0.00	1/8	
2.50±0.00	2.87±0.17	2.87±0.17		acyclovir
4.60	4.60	4.60		Negative control

Table 2: AntiHSV-1 Effect of CHX and Persica After Inoculation of Vero Cells with HSV-1 in Comparison with ACV

Time			Concentration	Type of drug
5	1	0.5		
0.75 ± 0.35	0.75 ± 0.35	1 ± 0	1/2	persica
0.75 ± 0.35	4.63 ± 0.18	5.25 ± 0.35*	1/4	
4.88 ± 0.18*	5.13 ± 0.18*	5.13 ± 0.18*	1/8	
0.75 ± 0.35	3.38 ± 0.18	4.125± 0.18	1/2	CHX
2.63 ± 0.18	4.125 ± 0.18	4.5 ± 0	1/4	
3.125 ± 0.18	5.25 ± 0.35*	5.25 ± 0.35*	1/8	
0.25 ± 0	0.5 ± 0	0.25 ± 0		acyclovir
5.35	5.35	5.35		Negative control

*Not significant.

Before the Vero cell infection, persica led to a lower virus titre than CHX and acyclovir (P=0.0001, P=0.04 respectively) and so it had more inhibitory effect on HSV-1, however after Vero cell infection with HSV-1, acyclovir had more inhibition of viral replication than both other mouthwashes (Figure **1**).

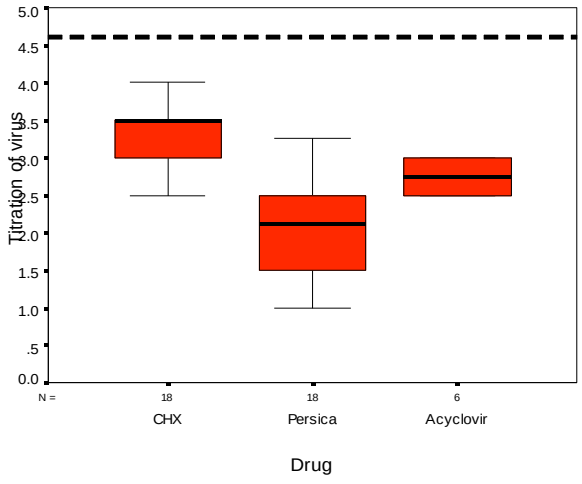

Figure 1: BOX PLOT of virus titration before inoculation at different solution.
(--- indicate virus titration in control group and ▬ in BOX indicate mean).

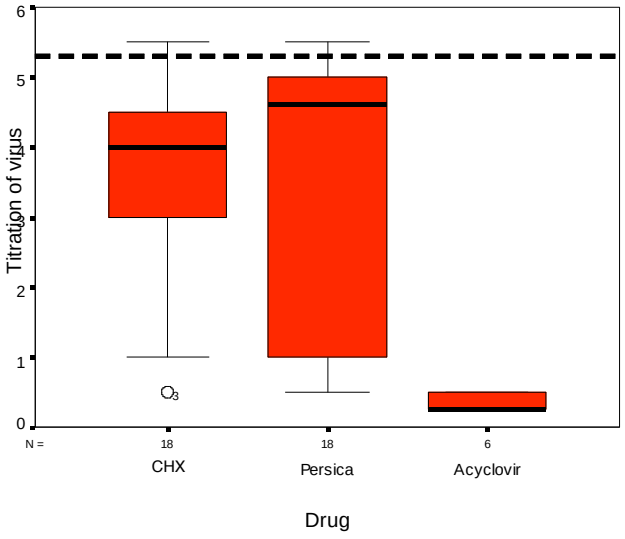

Figure 2: BOX PLOT of virus titration after inoculation at different solution.
(--- indicate virus titration in control group and ▬ in BOX indicate mean).

No significant differences between all treatments were observed when various concentrations were applied before viral infection (P>0.05) however after inoculation, concentration of mouthwash had significant

effect on viral titers. Before inoculation, time of cell exposures to the mouthwashes had no significant effect on viral titration (P>0.05) but after cell inoculation it indicated a significant effect.

DISCUSSION

Mouthwashes are widely used for the prevention and treatment of various oral and dental diseases. RIH is one of the most common and debilitating oral diseases [1]. There is no standard topical treatment for control of RIH so we studied the persica and CHX as available mouthwashes to determine their antiviral activity and compare them with each other along with acyclovir as an classic antiherpetic drug.

Mouthwashes, in addition to antiseptic effect, may also be toxic for epithelium and connective tissue cells, so firstly, we examined the potential cytotoxicity of the CHX and persica. In contrast to the CHX that was toxic for all cell lines, the persica indicated safety at concentrations up to 1:4 (25%) and times lower than 5 min.

Some studies did not report any toxic effect from the CHX on the Vero cells, whereas others reported such an effect in the dilutions up to 12.5% (1:8) [2, 7, 17-19].

Until the present time only one study evaluated the cytotoxicity of the persica which reported its toxic effect at the concentrations more than 0.5% [7].

The difference between the finding of that study and our results may lie in the varieties in factors such as the exposure times, tests implied for viable cell assay and the percentage of FBS used in the tissue culture.

In order to determine the mode of antiherpetic activity of CHX and persica, Vero cells were treated with various concentrations of mouth washes before and after HSV-1 adsorption. The results of the present study revealed that before the cell inoculation, both mouthwashes had rapid inhibitory effect, for 30 second and in the dilutions up to 1:8 (0.12% concentration) with a comparatively better antiherpetic effect in the persica than CHX, whereas, after inoculation, acyclovir indicated best antiherpetic effect with no significant difference between the mouthwashes. Such a paradoxical results from the two different methods can be explained by the different modes of antiviral activity of the agents. Since acyclovir inhibits the DNA polymerase of the virus while activated by the viral thymidine kinase, it exerts the antiherpetic effect mostly after the inoculation of virus, however persica indicated better antiviral effect before infection of the cells with HSV-1. So such an inhibitory effect might be mostly through its direct inhibition on the free virions, by blocking fusion of the viral envelope with the cell membrane and prevention of virus attachment to vero cells [12, 13, 20].

CHX indicated relatively similar results in both methods.That might be attributed to direct virucidal effect and also inhibition of viral replication as reported by the studies of Bernstein, Baqui and Park and their colleagues [2, 4, 5, 21]. Our results on CHX were consistent with the findings of those studies, but there is not such a report available for the persica.

The results of this study for optimal direct effect of persica on free virions, can be suggestive for utilizing this mouthwash in reducing the viral contamination of oral fluids (For at least 30 second after oral rinse) and decreasing the risk of viral cross contamiation in close personal contacts or aerosols produced during dental treatment, in asymptomatic viral shedders and also reducing the infectivity of herpetic ulcers. Moreover, persica might be considered as a new option for treating resistant HSV-1 due to its action being independant of the cellular thymidine Kinase.

This herbal mouthwash does not have the adverse effects of CHX and also it is alcohol-free so it has no risk for the use in pregnant women.

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There is no conflict of interest in this study.

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