

## A Comparative *In Vitro* Study of the Effects of Irsha and Chlorhexidine Mouthwashes and Acyclovir on HSV-1

<sup>1</sup>Hooman Ebrahimi <sup>1</sup>Sara Pourshahidi <sup>2</sup>Niloofar Sahraeian \*<sup>3</sup>Mohammad Motamedifar

<sup>1</sup>Assistant Professor, Dept. of Oral Medicine, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

<sup>2</sup>Undergraduate Student, Student Research Committee, School of Dentistry, Shiraz University of Medical Sciences, Shiraz, Iran.

\*<sup>3</sup>Professor, Dept. of Bacteriology and Virology, HIV/Aids Research Center (SHARC), School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran. E-mail: motamedm@gmail.com

### Abstract

**Objective:** Being able to cause disease in human, herpes simplex viruses (HSVs) clinically demonstrate themselves as intra-oral, extra-oral or recurrent lesions. The existing acyclovir has the selective anti-herpetic drug to control HSV infections. Due to emerged resistance to this drug and limitations of using it in especial situations, there is a need for alternative treatments such as available mouthwashes. This study aimed to compare two mouthwashes (Irsha and Chlorhexidine) with Acyclovir on HSV-1 *in vitro*.

**Methods:** In this experimental research, we used MTT (Thiazolyl Blue Tetrazolium Bromide) colorimetric test to determine the cytotoxicity level of three solutions consecutively, antiseptic and nonalcoholic Irsha mouth wash (blue-colored), chlorhexidine 0.2% mouthwash, and acyclovir and absorbed wavelengths were recorded by Eliza Reader. After infecting the cells with different dilutions of HSV-1 in different concentrations of Irsha and chlorhexidine mouthwashes, we analyzed their antiherpetic effects on Vero cells. By using suitable statistical tests in version 15 of SPSS the results were then analyzed.

**Results:** The results showed that in the concentrations of 0.38% for Irsha and 0.003% for chlorhexidine these mouthwashes kill 50% of Vero cells (CC<sub>50</sub>). After determining CC<sub>50</sub>, we detected the antiviral effects of Irsha and chlorhexidine mouthwashes and acyclovir solutions. We observed a significant difference between 0.5% concentration of Irsha mouthwash and other concentrations of it. The least logarithm of virus titration was observed in 0.002% concentration of chlorhexidine mouthwash. Both tested acyclovir concentrations (1250  $\mu\text{g}^r/\text{Mililiter}$  and 2500  $\mu\text{g}/\text{mL}^{\mu\text{g}^r}/\text{Mililiter}$ ) had a similar effect on decreasing virus titre

**Conclusion:** According to our results, anti-herpetic effect of Irsha is less than chlorhexidine and anti-herpetic effect of Acyclovir and Chlorhexidine is stronger than Irsha.

**Key words:** Chlorhexidine, Irsha, HSV-1, Mouthwash.

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### Introduction:

Oral mucosa and its peripheral tissues are suitable places for recurrent viral infections (1). Herpes simplex virus (HSV) belongs to the group of DNA viruses which have the ability to develop disease in human. A gamut of diseases including Keratoconjunctivitis, gingivostomatitis, encephalitis, genital disease, and neonatal infections are caused by herpes simplex (2).

Type 1 Herpes simplex virus (HSV-1) is classically related to developing oral and guttural lesions and causes recurrent attacks of what is called “cold sore” (2). Following the initial infection, HSV-1 remains latent in trigeminal neural ganglion (3, 4). Surgical manipulations on trigeminal peripheral nerves like operations on oro-facial injuries and dentistry activities can reactivate HSV-1 (3, 5, 6). Furthermore, stress, both in acute and chronic

forms, is related to increasing serum antibody titration against herpes simplex viruses (7, 8). Reactivating the virus, clinical demonstrations may show themselves as extra oral lesions, intra-oral lesions or asymptomatic (3, 4). Acyclovir is an effective antiviral drug for treating HSV infections. It is a nucleoside analog which becomes monophosphorus by means of thymidine kinase and then cell kinases turns it into triphosphate. Aiding HSV polymerase, Acyclovir triphosphate effectively attaches itself inside DNA virus and stymies the elongation of the chain (2). In recent years, following an increase in mutated cases, resistant to acyclovir due to loss of thymidine kinase in viral mutants, the application of alternative treatments using mouthwashes has gained more popularity (9). Using mouthwashes as a dentistry treatment has a long history (10). The first references of mouthwashes are found in Chinese medicine and Ayurveda (2700 B.C.) used for gingivitis treatment. Then following mechanical methods of oral cleansing, using mouthwashes prevailed among Greeks and Romans higher class and Hippocrates (11). Chlorhexidine and Irsha are typical mouthwashes in Iran's market. Irsha is a product of Shafa pharmaceuticals company that contains antimicrobial compounds as menthol, (Eucalyptol) thymol, methyl salicylate and benzoic acid. In some studies *in vivo* effect of (Yellow) Irsha mouthwash on oral microbial flora has been investigated (12, 13). There are also *in vitro* studies comparing antimicrobial and cytotoxic effects of Irsha with other mouthwashes (10, 14) Chlorhexidine is the first and most common mouthwash and its effects on lessening plaque and gingivitis to some extent has been studied (15-17). Recently, several studies have been performed on anti-HSV-1 effect of chlorhexidine (18, 19). In addition to good antimicrobial and antifungal effects, chlorhexidine has important side effects like changing the color of teeth, causing allergy and even anaphylactic shock, acute respiratory

distress syndrome (ARDS), teratogenic effects on embryo, and cytotoxicity (20-23). So far, there is no study on the effects of non-alcoholic antiseptic Irsha (blue colored) on HSV-1. In this study we aimed to compare the anti-herpetic effect of this type of mouthwash with 0.2% chlorhexidine vero cell line.

## Methods:

This is an experimental study carried out *in vitro*. HSV-1 was isolated from the lip lesions of a patient suffering Recurrent Herpes Labialis and then confirmed by using anti-HSV-1 guinea antiserum (24). Vero cells (fibroblast cell of kidney of African green monkey) are suitable for analyzing cytopathic effects of herpes simplex viruses (25) and were prepared according to the following standard method.

First, Vero cells in DMEM (Dulbecco's Modified Eagle Medium) [containing 7% fetal calf serum, 14% sodium bicarbonate, 100 (units/mL) penicillin, 100 µg/mL streptomycin sulfate, and 0.25 (µg/mL) Amphotericin B] was grown in a sterile flask, at 37 °C in 5% CO<sub>2</sub> incubator.

After 2-3 days, a monolayer cell was formed in the flask and observed by inverted microscope. Then DMEM was poured out from the flask and attached Vero monolayer cells in the flask rinsed by PBS (Phosphate-Buffered Saline). Then 1 mL of trypsin-versene solution was warmed up in the 37 °C incubator and added to monolayer cell inside the flask to detach Vero cells from the flask and each other, and after two minutes it was pulled out to this solution.

With a flick of palms, the cell layer was detached, and then 1-2 mL fresh DMEM containing 7% FBS (Fetal Bovine Serum) was added to the flask. 1-2 mL of DMEM was repeatedly pipette to make Vero cells into a suspension state. The amount of augmentation of DMEM containing 7% serum should be enough for Vero cells to reach a number of  $1.5-2 \times 10^5$

cells in each mL of DMEM (26).

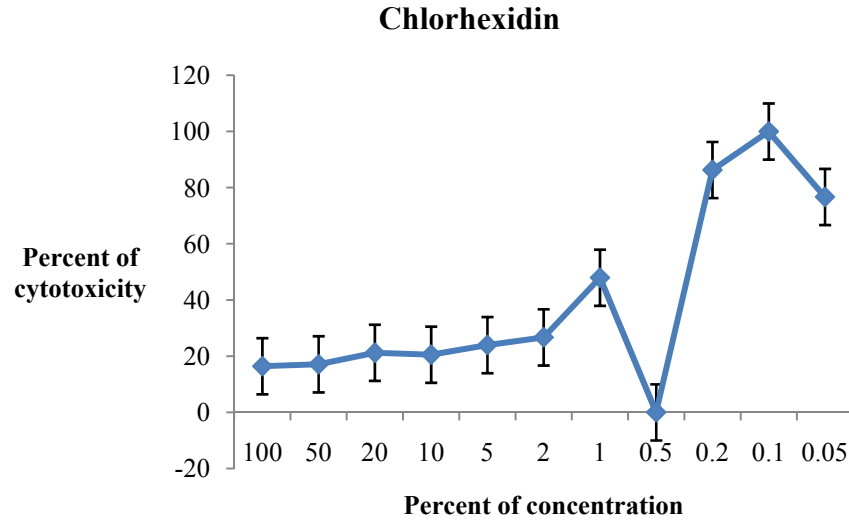
To determine the cytotoxicity of Irsha (Shafa Pharmaceutical Co, Tehran, Iran) and chlorhexidine 0.2% (Donyaye Behdasht, Tehran, Iran) mouthwashes along with Acyclovir on Vero cells, first 1 mL of the suspension of Vero cells was added to each well of the 96 well plate and the plate was placed in the incubator. The cells grew for 2 days in 7% DMEM to form a cell monolayer. Then the medium was plucked from the wells and the monolayer cell was washed by PBS. Then 1 mL of the medium containing 0.2% fetal calf serum and different concentrations of Irsha (0.05, 0.5, 0.2, 0.1, 2, 1, 5, 10, 20, 50 and 100%) was added to each well of a sterile 96 well plate. This step was done for chlorhexidine too (with percentages of concentrations as follows: 0.0004, 0.0002, 0.0001, 0.004, 0.002, 0.001, 0.04, 0.02, 0.01, 0.2 and 0.1%). Used concentrations of Acyclovir were 1250 and 2500 µg/mL. The plate was placed in the incubator for 48 hours, and then the medium was plucked out of the wells. To determine the cytotoxicity, the MTT colorimetric test was applied. Using this method, Formosan dyes turned into violet color, so we were able to assess the percentage of the living cells (cells enumeration), and also determine the cytotoxicity of the drugs (10). During this procedure living cells were stained. 50λ MTT was added to each well and the plate was placed in the incubator for 2 hours. Then MTT was removed from the wells and the solvent of 50λ DMSO (Dimethyl Sulfoxide) was added to each well. During the 30 minutes of incubation, the DMSO solved the Formosan dyes and the violet color appeared. The intensity of light absorption was recorded by Eliza Reader with a wavelength of 450-630 nanometers in test wells, positive and negative controls twice and then 50% cytotoxic concentration (CC<sub>50</sub>) was shown in a diagram.

To determine the effects of Irsha and Chlorhexidine mouthwashes in compare with

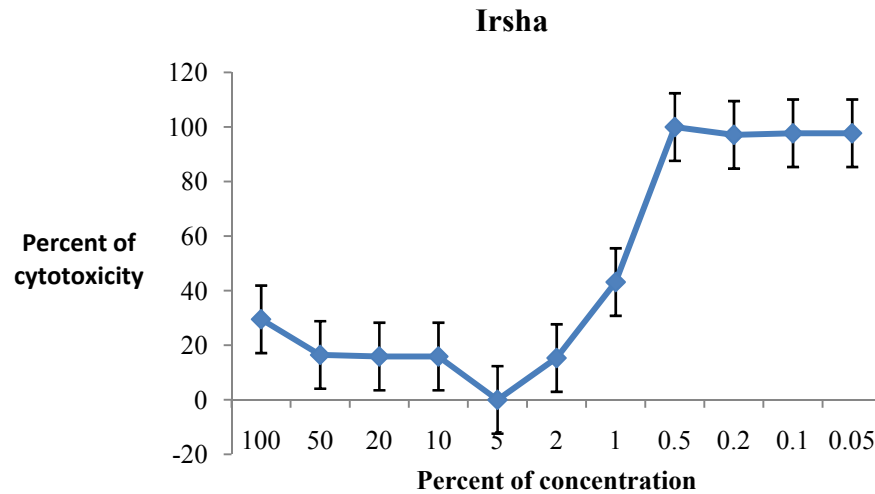
acyclovir effect on HSV-1, culture media were removed from Vero cell cultures in 96 well plate ; 200λ of different virus dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ ) was added to the wells. For each concentration of the virus, 4 wells were considered. Then the plate was placed in the incubator for 45 minutes for virus absorption (required for cells to absorb the virus). Then the plate was brought out of the incubator. Then, different concentrations of Irsha (0.5%, 0.2% and 0.1%) and Chlorhexidine (0.004%, 0.002%, and 0.001%) and Acyclovir (1250, 2500 µg/ml were added to different plates as mentioned above. The plates were placed in the incubator for a period of 4 days, in 37 °C, and 5% CO<sub>2</sub>. The plates were studied under inverted microscope, and the wells were marked positively or negatively according to presence or absence of cytopathic effects (morphologic changes like ballooning). Then the ultimate titration of the virus (TCID<sub>50</sub>) was calculated by the method of Karber (18, 27, 28) All the stages of the experiment were repeated and the results were reported as mean (standard deviation). The anti-HSV results statistically analyzed as the logarithm of the ultimate virus titres. To analyse the effect of mouthwashes, one-way ANOVA test was applied. Another statistical test applied was non-parametric Kruskal-Wallis test. Tukey's HSD test was used as the post-hoc test. All of the statistical analyses were done by using SPSS (version 15).

## Results:

Results of cytotoxicity assays showed that none of the used concentrations of acyclovir was toxic for the Vero cells, so Acyclovir is safe for vero cells. CC<sub>50</sub> for Chlorhexidine was detected to be 0.003% , and that for Irsha was 0.38%. So higer concentrations can led to more than 50% of cell death (Diagrams 1, 2).



**Diagram 1- Cytotoxicity of different concentrations of Chlorhexidine mouthwash**



**Diagram 2- Cytotoxicity of different concentrations of Irsha mouthwash**

After the absorption of virus into cells, effects of Irsha and Chlorhexidine on HSV-1 were studied. The results showed that both mouthwashes have inhibition effects at studied concentrations. The concentration of 0.004% of chlorhexidine was toxic for the cells and it was impossible to study the titre of the virus. The minimum logarithm of the virus titre for chlorhexidine mouthwash was observed at a concentration of 0.002%. For Irsha, the maximum and the minimum logarithm of the virus titre was observed at concentrations

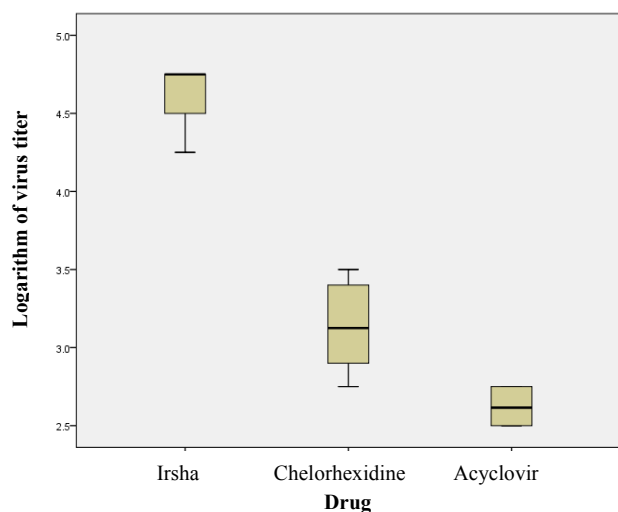
of 0.1% , and of 0.5% respectively. As presented in Table 1, also the lowest and the highest virus titres were detected after treating Vero cells with 1250 µg/mL of Acyclovir and 0.1% of Irsha mouthwash respectively.

According to the result of one-way ANOVA (Tukey post Hoc HSD), there was no significant difference between the concentrations of 0.1 and 0.2 of Irsha mouthwash ( $p=0.918$ ). There was a statically significant difference between the concentration of 0.5 % of Irsha with each of

0.2% and 0.1% concentrations of this mouthwash ( $p=0.002$ ). Both concentrations used for acyclovir had similar effects on decreasing the logarithm of virus titration and no statically significant difference was observed between these two concentrations of Acyclovir ( $p=0.918$ ) (Diagram 3).

**Table 1- Logarithm of HSV-1 titre (M(SD)) in solutions contain diferent concentrations of diferent drugs after virus entry to the cell according to concentration**

Drug	Concentration	Logarithm of virus titre
Irsha	0.5 percent	4.35 (0.132)
	0.2 percent	4.70 (0.086)
	0.1 percent	4.71 (0.069)
Chlorhexidine	0.004 percent	Undetectable
	0.002 percent	2.88 (0.125)
	0.001 percent	3.38 (0.125)
Acyclovir	1250 $\mu$ g/ml	2.61 (0.125)
	2500 $\mu$ g/ml	2.62 (0.125)
Control		5.35 (0.132)



**Diagram 3- Diagram of Box plot, effect of drugs on logarithm of virus titre (Y axis) (from left to right: Irsha, Chlorhexidine, Acyclovir)**

### Discussion:

Thanks to the development of antiviral treatments, the use of available material for treating or imposing changes leading to recovery from illness has been taken into consideration

(1). Mouthwashes are amongst drugs which do not require prescription by a dentist and can be used as home remedies to increase the level of oral hygiene. To assure the non-toxicity for the cells of oral tissues like epithelial tissues, first the cytotoxicity of the mouthwashes was studied. AS we mentioned in the results, acyclovir is safe for Vero cells (fibroblast) at the studied concentrations but chlorhexidine is toxic at concentrations above 0.003%. Irsha mouth wash, is toxic at concentrations above 0.38%. According to Figure 3, compared to Irsha and chlorhexidine, acyclovir has the highest efficiency in decreasing logarithm of virus titre and chlorhexidine is more effective compared to Irsha. Cytotoxicity of chlorhexidine has been considered in some studies. In a study by Baqui at the dilution of  $1/10 \frac{1}{10}$  or  $1/100 \frac{1}{100}$ , of chlorhexidine had the cytotoxicity effects on MT-2 and Vero cells (28). In another study by Hashemipour *et al.* using MTT colorimetric method, it had been shown that chlorhexidine has less cytotoxicity on J774A.1 cells (mouse macrophage cell lines), human oral carcinoma, HepG2 (liver hepatocellular cells), Osteosarcoma, and MRF (human gum fibroblast) compared to other mouthwashes in that study (10). In a study by Pourshahidi *et al.*, chlorhexidine was reported cytotoxic after 5 minutes (18). The difference in concentrations used for tests, time of juxtaposition of cells to chlorhexidine, and statistical tests can be the probable reasons for the different results. In a study by Zarei *et al.*, the cytotoxicity of Irsha was compared to Listerine and it was determined that there was no statistically significant difference between different concentrations of both mouthwashes (14).

The results of antiherpetic effect of studied mouthwashes in compare with Acyclovir showed that at applied concentrations, acyclovir, was more effective on decreasing the logarithm of virus titer than 0.002% concentration of chlorhexidine. Also, the results revealed that, the

concentration of 0.004% of chlorhexidine is cytotoxic, it was impossible to measure the virus titer. These differences may have been due to different functions of mouthwashes in compare with acyclovir. Acyclovir is activated by thymidine kinase and stymies the reproduction of virus (9). There are a number of *in vitro* and sometimes both *in vitro* and *in vivo* studies available for comparing mouthwashes (28, 10, 29). In a study by Pourshahidi and others using quintal method, the antiherpetic effect of chlorhexidine, acyclovir and Persica has been reported before and after HSV-1 infection of the cells (18). Our findings indicate that Irsha has an antiherpetic effect, despite the fact that acyclovir and chlorhexidine have better efficiency compared to that for Irsha. So, still, Acyclovir might be an optimum antiherpetic agent in mouthwashes for the treatment or preventive purposes. Further investigations on the mechanism of the observed anti HSV-1 effects of the studied mouthwashes is still suggested. The advantage of quintal method, used in the present study is simplicity and reproducibility for assessing antiviral effects of any agent. In

addition, compared to other methods like PCR (Polymerase Chain Reaction), it is more available and economic

### **Conclusion:**

The results of the present experiments proved the less cytotoxicity of Irsha comparing with Chlorhexidine. However, since Irsha showed less anti-herpetic effect than Chlorhexidine and acyclovir in Vero cells, using higher non-cytotoxic concentrations of Irsha (>0.1) is required for exerting its anti-HSV-1 activity.

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### **Conflict of Interest: “None Declared”**

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