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RESEARCH ARTICLE

Study of Anti-Herpetic Activity of a Soft Dosage form With Acyclovir and Miramistin

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Abstract

The aim of the present work is to study the anti-herpetic action of the soft dosage form with acyclovir and miramistin on the model of herpes simplex virus. Using in vitro methods were studied cytotoxic concentration of substances, effective concentration, index of selectivity of substances and disinfectant action. Non-germinal guinea pigs were divided into three groups. Group I was infected with herpes virus, group II was infected with herpes virus and treated with the soft dosage form with acyclovir (5 g / 100 g) and miramistin (0.5 g / 100 g) and group III was infected with herpes virus and treated with 5 % Zovirax cream. It has been proven that the soft dosage form with acyclovir and miramistin is an active inhibitor of the reproduction of herpes virus type 2.

Keywords: *Acyclovir, Miramistin, Herpes viral diseases, Soft dosage form.*

Introduction

Today, the problem of the treatment of herpes virus infections is a relevant task of modern medicine and pharmacy. Due to that it is advisable to creation new combined drugs for the treatment of the above-mentioned pathology. Currently, the combined drugs are leading among pharmacotherapeutic agents, including the treatment of herpetic infections, which require complex treatment. Choice of drug combination allows to expand the range of action of the drug and the complex influence on the disease, enhance the activity of the every ingredient, as well to improve tolerability and reduce side effects [1-5].

Nowadays one of the most urgent medical and social problems are high morbidity for herpetic infections [6]. Long chronic process leads to a negative immune reorganization of the body [7-10]. There is a development of secondary immune deficiency, inhibition of cellular immune responses, reduction of nonspecific protection of the body, etc [11, 12]. The spread of this pathology is caused by: the diversity of clinical forms; impossibility of complete elimination virus from the body; the need for combined therapy; development of the virus resistance against drugs; evolution mechanisms that contribute to the survival of the virus as a result of modification of the host's immune response [13, 14].

According to the etiology, pathogenesis, clinical symptoms, drugs for the treatment of herpetic diseases can be divided into 3 groups: anti-herpetic chemotherapeutics (abnormal nucleosides) -acyclovir, valacyclovir (valtrex), penciclovir (vectavir), famciclovir (famvir), ganciclovir (tsimeven); inductors of interferons - thiloron (amixin), neovir, cycloferon; immunomodulators – alpizarin, immunofan, lycopid, polyoxidonium [15, 16].

Miramistin was chosen to develop a combined drug with acyclovir («Infamed», Russia). The drug is active against various pathogenic microorganisms, including viruses, fungi, bacteria and protozoa.

The local immunostimulating effect of the drug is provided by activating the function of phagocytic cells (phagocytes and macrophages) [17]. In the aspect of the above, the purpose of this work was to study the anti-herpetic action of the soft dosage form with acyclovir and miramistin on the model of herpes simplex virus.

Materials and Methods

Drugs

Herpes simplex virus type 2 (HSV-2) a strain obtained from the Museum of Viruse-s Institute of Virology. The virus was maintained by serial passages in Vero cell culture. The infectious titre for cytopathogenic action in cell culture was 5.5-9.0 lg tissue cytopathic action$_{50}$ / 0.1 ml. The virus was stored at 70°C prior to the start of experimental studies. Vero is a creeping line of culture of the kidneys of the green monkey.

Experimental Animals

18 males of non-germinal guinea pigs weighing 250-300 g were used in this work while studying; they were obtained from the nursery "Glevakha", where they were kept under standard vivarium conditions. The study of anti-herpetic activity of substances and the soft dosage form on their basis was carried out on the base of the laboratory of experimental chemotherapy of viral infections of the Ukraine State Institution "Institute of Epidemiology and Infectious Diseases named after L.V. Gromashevsky "(Kiev). The studies were carried out in accordance with the National General Ethical Principles of Animal Experiments (Ukraine, 2001) based on the provisions of Vertebrate Animals for Experimental and Other Scientific Purposes (Strasbourg, 1986).

In Vitro Methods

Determination of the Cytotoxic Concentration of Substances (CC_{50})

Different cell cultures Vero were used to determine the substance CC_{50}. While experiments at least ten rows of wells in cell culture plates were used for each dilution of substances in the nutrient medium. Cell culture plates were incubated at 37°C with a feed of 5 % CO_2 for 5 days. Observations of experimental and control cultures were conducted daily to determine the presence or absence of cytopathogenic action (CPA).

The degree of cytotoxic concentration of substances was determined by changing the cell morphology (rounding, shrinkage of cells, exclusion from the surface of the wells degenerate) on the 4+ plus system from + to ++++:

"-" -Complete absence of cell degeneration;

"+" -Not more than 25 % affected (protection of monolayer from antiviral drugs by 75 %);

"++"-Not more than 50 % of the cell monolayer is affected;

"+++"-Not more than 75 % of the cell monolayer is affected;

"++++"- Complete degeneration of the cell monolayer.

By CC_{50}, the substance received the largest amount that did not cause cellular degeneration.

Determination of Effective Concentration (EC_{50})

The EC_{50} is the minimum concentration of substances that inhibits the development of a virus-specific toxic cytopathogenic action (TCA) by 50 % and infectious titre of the virus by at least 2 lg. To determine the ED_{50}, a test dose of 100 TCA_{50} / 0.1 ml was added to the culture medium of Vero cells and incubated for 60 minutes at 37°C. After adsorption of the virus into the cells the residues were removed, the cells were washed with nutrient medium, and then substances in different concentrations were introduced into the support medium (RPMI-1640 + 2 % fetal serum).

The absence of cytopathogenic action in the experiment (in treated cultures) as it was present in the control, as well as the reduction of the infectious titer in the treated cultures, in the presence of it in the control and the difference in the infectious titers in the experiment compared with the control of the virus, at least 2 lg allowed the establishment of the EC_{50} substances.

Determination of the Index of Selectivity (IS) of Substances

IS was determined by establishing the ratio of CC_{50} to the EC_{50}, which is the minimum amount of the drug, which inhibits the development of the virus-specific cytopathogenic action by 50%.

Study of Anti-herpetic Activity of Substances Acyclovir + Miramistin and Miramistin on Extracellular Virus (Disinfectant Action)

Vero cell culture was used in order to study the antiviral activity of the substances. Cells were grown in droplets on a medium RPMI-1640 + 10 % fetal serum at a temperature of 37°C in a thermostat with CO_2 feed. To study the disinfectant antiviral activity of substances, miramisin and miramistin + acyclovir, daily culture cultures of Vero cells with a solid monolayer of cells were taken. The growth medium was poured into the cell, the test substance was added to the cell monolayer at different concentrations after 1 hour of contact with the herpes virus in a dose of 1000 TCA_{50}.

The cultures were incubated in a thermostat with CO_2 feed for 5 days, each day controlling the reproduction of the virus through the cytopathic effect of HSV on Vero cells by microscopy compared to control cultures where the cell monolayer was infected with the herpes virus. After 3 days, the culture medium was collected from the wells of droplets and it determined the infectious titre in each sample at the introduction of the substances.

***In Vivo* Methods**

The study of Anti-herpetic Activity of the Soft Dosage Form *In Vivo*. Infection Model Marennikova S. S. [18]

Guinea pigs were infected with a virus-containing liquid with an infectious titer of 6.0 lg of TCA_{50} / ml. The viral fluid was applied to the pre-scarified genital skin. Scarification was performed using a surgical lanceolate after the animals were anesthetized with ether. The size of the scarification area was 4-7 mm^2. The virus-containing liquid was applied by pipette immediately after scarification (followed by rubbing).Clinical symptoms of experimental herpes genitalis were recorded every day before treatment and were observed throughout the period of the disease.

Criteria for assessing the severity of the infectious process were the area and the degree of specific lesions, the presence of edema, hyperemia, rash, selection. The maximum severity of each sign was 4 points. Observation of the animals was carried out for 10 days. The effectiveness of the drug was evaluated at the peak of the development of the pathological process: reduction of the severity of clinical manifestations, reduction of the duration of the disease, index of therapeutic effect (ITE) in experimental groups compared with the control. Each group consisted of 6 animals.

Statistical Analysis

Statistical calculations were maintained using MS Excel 2010 and StatSoft Statistics 10.
P value < 0.05 was considered as statistically significant.

Results and Discussion

For the cytotoxic concentration of compounds it was considered the largest dose that did not cause degeneration of cells.

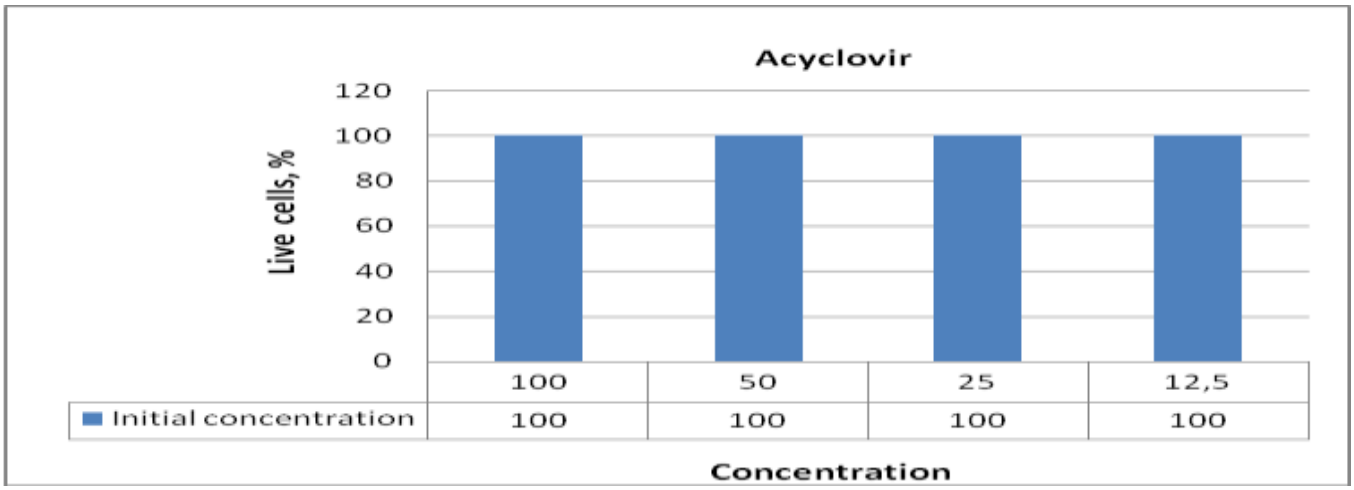

Fig. 1: Definition of CC_{50} compounds of acyclovir

CC_{50} compounds of acyclovir at a concentration of 5 mg / ml was greater than 100 mkg / ml (Figure 1).

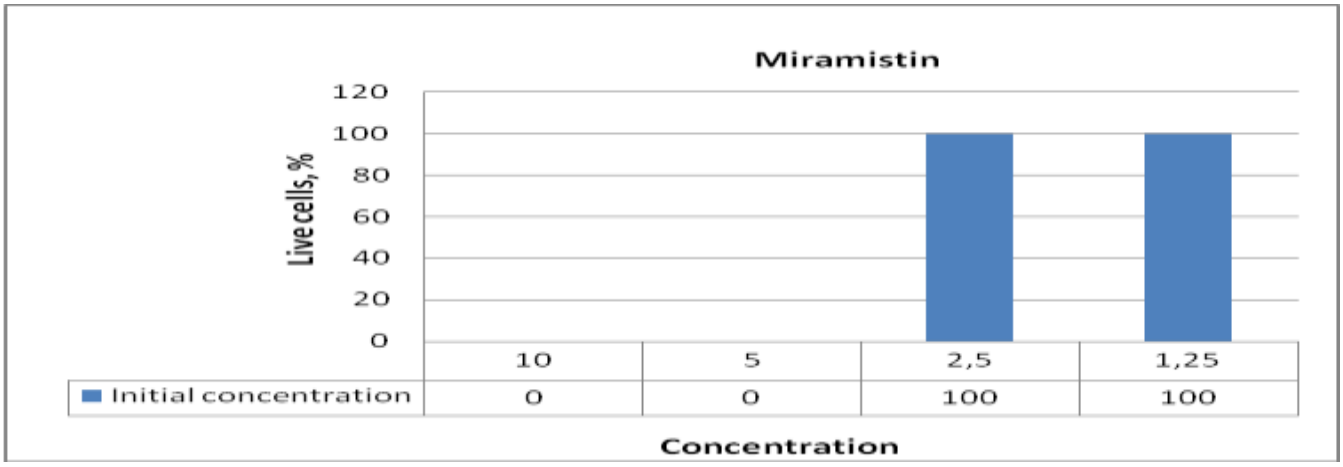

Fig. 2: Definition of CC_{50} compounds of miramistin

CC_{50} compounds of miramistin at a concentration of 0.5 mg / ml was greater than 3.8 mkg / ml (Figure 2).

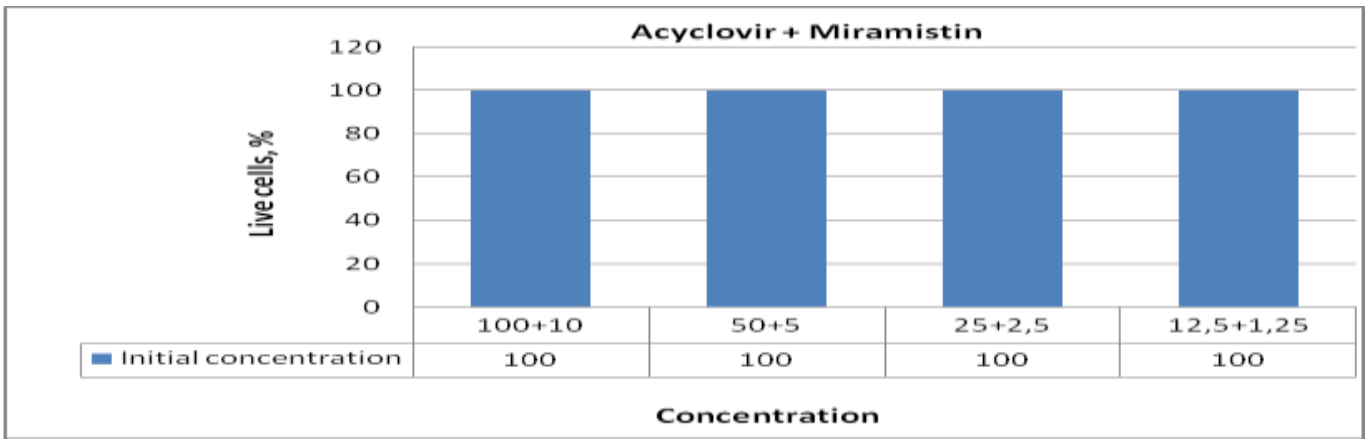

Fig. 3: Definition of CC_{50} compounds of acyclovir + miramistin mixtures

CC_{50} compounds of acyclovir at a concentration of 5 mg / ml + miramistin at a concentration of 0.5 mg / ml was greater than acyclovir + miramistin mixtures at a concentration acyclovir 100 mkg / ml + miramistin 10 mkg / ml (Figure 3).To study the minimum active concentration of substances were used: 1) acyclovir – at a concentration of 5 mg / ml; 2) miramistin 0.5 mg / ml and 3) acyclovir + miramistin mixtures (1:1) (Figure 4).

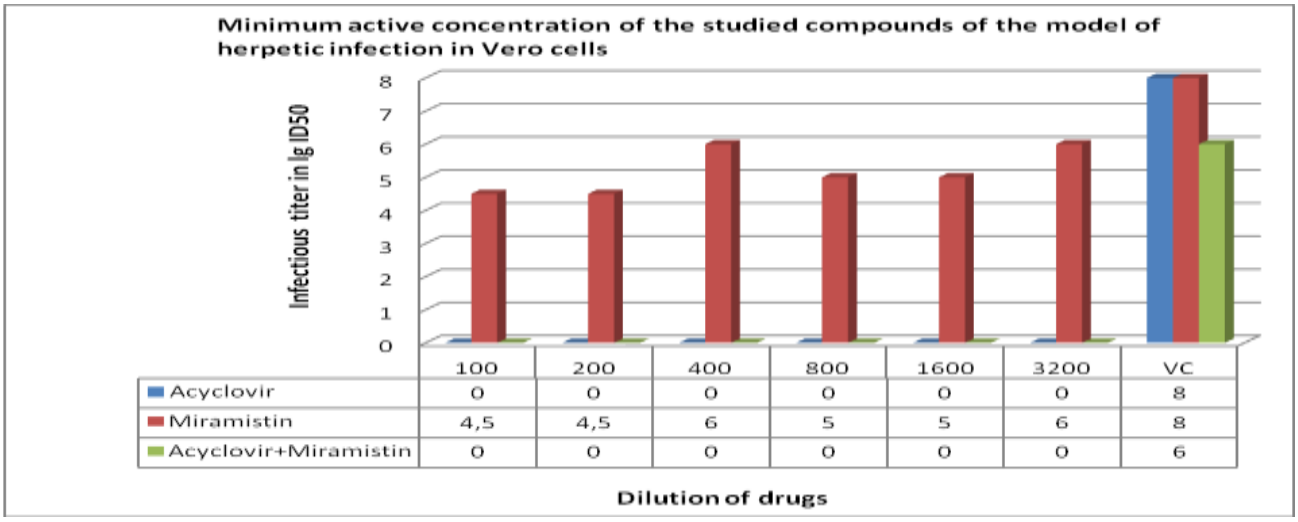

Fig. 4: Minimum active concentration of the studied compounds of the model of herpetic infection in Vero cells

Note. VC-Virus control.

EC_{50} for miramistin is 0.15 mkg / ml (1:3200) (Figure 4).

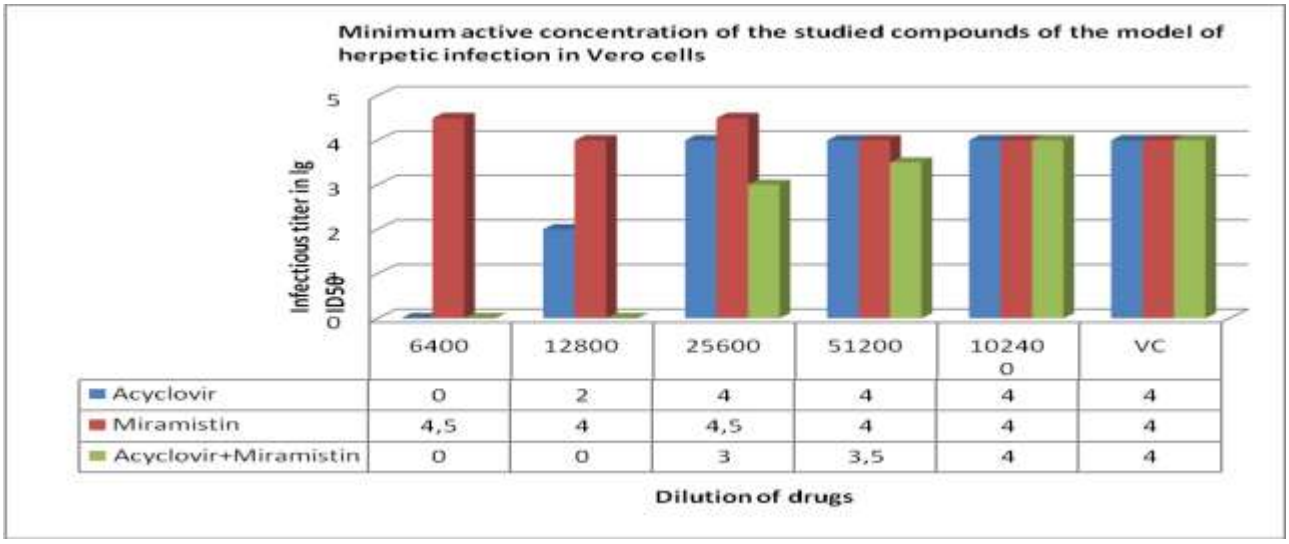

Fig. 5: Minimum active concentration of the studied compounds of the model of herpetic infection in Vero cells

Note. VC-Virus control.
EC_{50} for acyclovir is 0.39 mkg / ml (1:12800) and acyclovir + miramistin–0.39 + 0.03 mkg / ml (1:12800) (Figure 5).

Table 1: Selectivity index of the studied compounds of acyclovir, miramistin and mixtures of compounds acyclovir + miramistin (1:1)

The name of the substance	CC_{50}, mkg/ml	EC_{50}, mkg/ml	IS
Acyclovir	100	0.39	256.41
Miramistin	3.8	0.15	25.3
Acyclovir + miramistin	100+10	0.39+0.03	256+333

As can be seen from the table. 1, IS of acyclovir, miramistin and acyclovir + miramistin are 256.41, 25.3 and 256 + 333 respectively. That is, all active substances exhibit anti-herpetic activity, most expressed in acyclovir.The cytopathic effect of HSV on cells is morphologically manifested itself in the formation of symplast or rounded cells in conjunction with the proliferation and the emergence of giant multicore cells (Figure 6).

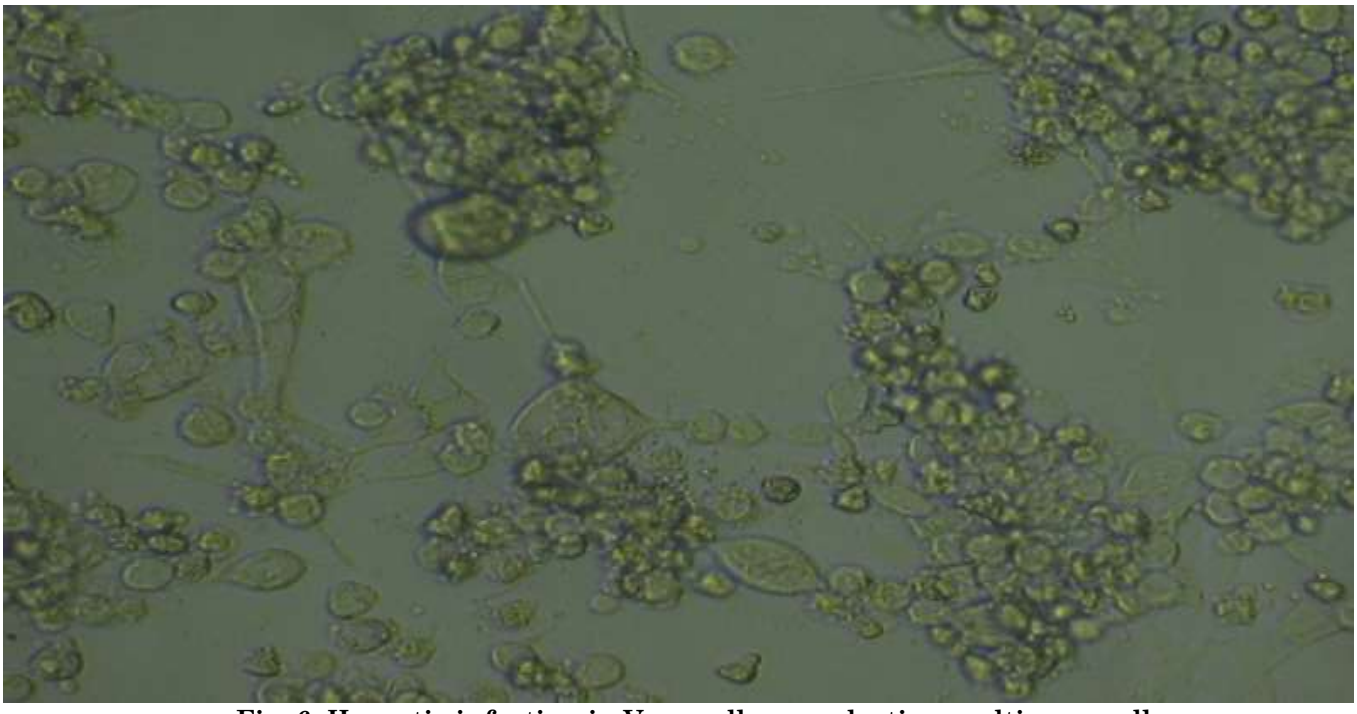

Fig. 6: Herpetic infection in Vero cells: symplastics, multi-core cells

In determining the action of drug substances on the extracellular herpes virus, the following results were obtained (Figure 7, 8).

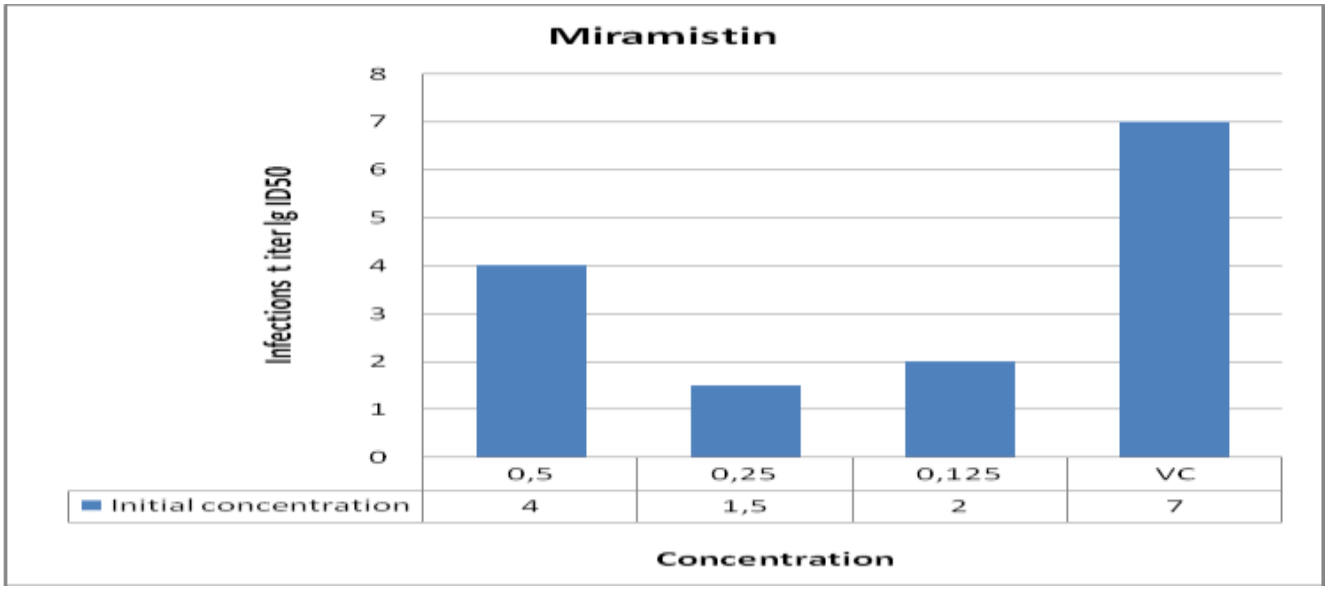

Fig. 7: ED_{50} of miramistin in relation to herpes virus

Note. VC-Virus control.
ED_{50} of miramistin at a concentration of 0.5 mg / ml was at breeding 1:1000.

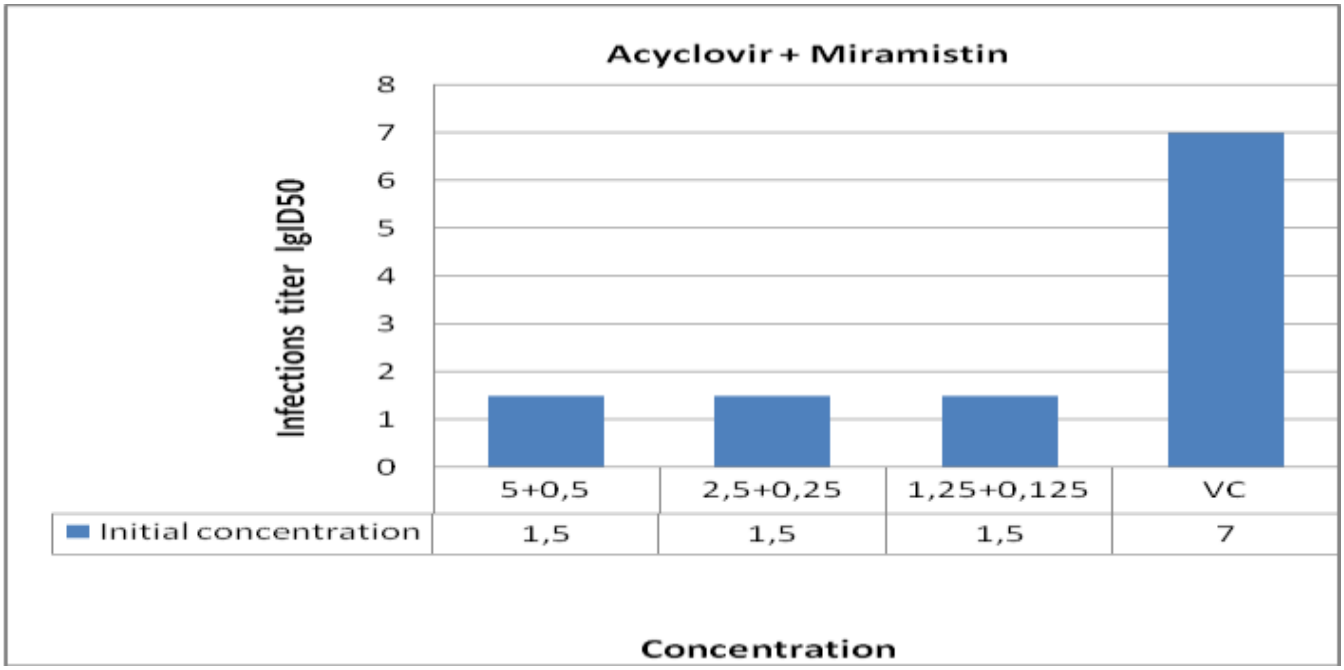

Fig. 8: ED_{50} of acyclovir + miramistin in relation to herpes virus

Note. VC-Virus control.

According to the results of the studies the substance miramistin and acyclovir + miramistin were effective inhibitors of the reproduction of extracellular herpes virus type 2.The antiviral activity of the soft dosage form was investigated on the model of genital herpes in guinea pigs. Treatment with use of the soft dosage form of miramisin with acyclovir began 24 hours after infecting animals. In the experiment there were 3 groups of animals:

- Animals infected with herpes virus only.
- Animals that were infected with herpes virus and treated with a soft dosage form with acyclovir and miramistin.
- Animals infected with herpes virus and treated with 5 % Zovirax cream.
- Criteria for assessing the severity of the infectious process were the area and the degree of specific lesions, the presence of edema, hyperemia, rash, selection. The maximum severity of each sign was 4 points.
- The following results were obtained in the study of the effectiveness of drugs on the genital herpes model in guinea pigs (Table 2).

Table 2: Effectiveness of drugs on the model of genital herpes in guinea pigs

Impact	Duration of the disease, (days)	Certainty, P	Reduction of symptoms, bales	Index of therapeutic action, %
Herpes virus	10		68.0	
Soft dosage form acyclovir (5g / 100g) and miramistin (0.5g / 100g)	1	< 0.05	4.0	94.2
Cream Zovirax 5 % acyclovir	3	< 0.05	24.0	64.7

As can be seen from the results of the research, the use of the soft dosage dosage form acyclovir + miramistin reduces the severity of symptoms to 4.0 points, which corresponds to a therapeutic index of 94.2 % and significantly reduces the duration of the disease to 1 day.

The closest to the proposed drug by the action and composition of the components is the drug Zovirax, which contains acyclovir, propyleneglycol, paraffin white soft, mineral oil, alcohol cetostearyl, arlacel 165, poloxamer, sodium lauryl phosphate, dimethicone, purified water. The drug is indicated for the treatment of infections of the lips and face caused by the herpes simplex virus.

Application of the referent drug cream Zovirax 5 % has reduced the symptoms to 24.0 points, the therapeutic effect is 64.7 %, the duration of the disease in animals – 3 days, which is statistically reliable for all parameters. The results of the conducted studies prove significantly greater effectiveness of the studied drug in the form of the soft dosage form compared with the reference drug cream Zovirax 5 %.

Conclusion

The created the soft dosage form with acyclovir and miramistin is an active inhibitor of the reproduction of the herpes virus type 2 with a disinfectant effect and is an effective therapeutic agent with an index of therapeutic effect 94.2 and a duration of symptoms of herpes for 1 day.

The research was carried out using an experimental model of herpes infection of the genitals in guinea pigs.Lokal treatment of virus diseases is more effective is the use of combination drugs, they have higher clinical efficiency and less side effects, are also more accessible to the public.

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