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ESTIMATION OF DIRECT INFLUENCE OF UKRAIN PREPARATION ON INFLUENZA VIRUSES AND THE BACTERIA *E.COLI* AND *S. AUREUS*

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Summary: *Ukrain is a semisynthetic drug with immunomodulatory properties derived from* Chelidonium majus *L. alkaloids and thiophosphoric acid. It acts selectively in a lytic way on cancer cells. Its protective properties have been shown in mice infected by influenza viruses. In this paper, the studies made on the estimation of the direct activity of Ukrain preparation on viruses and bacteria* E. coli *and* S. aureus *are described. Viruses of different haemagglutination titres were incubated with different concentrations of the preparation during period of 1, 2 and 24 h. Afterwards the samples were collected and used for the infection of the allantoic cavity obtained from 10-day-old hen embryos. A second method was based on the introduction of the Ukrain preparation into the allantoic cavity of embryos before infection with influenza viruses and after the infection of embryos. In both the described methods, the embryos were incubated within 48 hours. Then the presence of influenza viruses in allantoic fluid was estimated using a haemagglutination reaction with 30% hen blood cells. The influence of the preparation on hen embryo was also studied. In order to estimate the antibacterial activity the following procedure was used. To the preparation diluted with the growth medium from 500 µg/ml to 1 µg/ml a definite amount of the bacteria* S. aureus *or* E. coli *was added, and after 24 and 48 h of incubation at 37°C the results were read off. In the second method, the bacteria were added to 1, 10, 100 and 500 µg of the preparation in 1.0 ml of 0.85% NaCl, and after 1, 2 and 24 of incubation at room temperature the samples were collected and inoculated on solid Mueller-Hinton medium. The presence of bacterial growth or medium turbidity after 24 and 48 h of incubation was taken as a positive result. Our studies have revealed that the above mentioned preparation does not exert any negative influence on hen embryos that could make it difficult to estimate replication of influenza viruses. This preparation did not show any direct influence on the inactivation of influenza viruses and the bacteria* E. coli *and* S. aureus.

Introduction

The Ukrain preparation is an alkaloid derivative obtained from the celandine *Chelidonium majus* conjugated with thiophosphoric acid. This compound permeates through cancer-cell membrane, showing cytotoxic or cytolytic action (4, 6). It also exhibits immunomodulating properties (2, 6). It seems that the main modulation point of action of this preparation is the stimulation of the activity of

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thymus-dependent cells (mostly cytotoxic ones) and the natural-killer (NK) cells (2, 5).

Our previous investigations (1) showed the protective influence of the Ukrain preparation on mice infected by lethal doses of influenza viruses. The question is whether this influence was the result of stimulation of the cytotoxic activity in the immune cells; or perhaps it was another mechanism causing this protective effect, e.g. other workers point to the disturbance of metabolism exerted by Ukrain on the cells infected by influenza viruses (3). In an attempt to extend the explanation of the action of this preparation on viral and/or bacterial infections, we have made our studies on the direct anti-viral and antibacterial action of the Ukrain preparation.

Materials and methods

Influenza viruses of the APR8/HON1/34 strain were cultured on 10-day-old hen embryos. In order to achieve this, the egg-shells were disinfected with ethanol. Then the shell was punctured with a medical syringe, and into the allantoic cavity we introduced 0.1 ml of diluted 1:1000 allantoic fluid containing influenza viruses of haemagglutination titre 1:1280. Earlier, using a microscopic lamp, the positions of the air chamber and embryo had been determined. The outlet was then glued up with sterile paraffin and the embryos were placed in an incubator and incubated for 48 h.

After this time, the embryos were taken out from the incubator, cooled and the shells disinfected with ethanol. Then the air chambers were removed and the allantoic fluid transferred to a sterile vessel. Finally, the haemagglutination titre of the viruses was determined and found to be 1:2560. This fluid was stored in the refrigerator and used for further experiments.

The Ukrain preparation of the series 290614 produced by the Viennese firm Nowicky Pharma was used in a range of doses. In the *in-vitro* investigations, the following doses were applied: 1, 10, 100 and 500 µg/ml, but for the *in-vivo* studies the doses used were 1, 10 and 100 µg/ml.

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In the first stage of the experiment, the influence of the preparation on hen embryo was estimated. For this purpose 7- and 10-day embryos were employed. After the determination of the embryo position as well as the air chamber by means of a microscope lamp, the shell was disinfected, the embryo punctured and then the Ukrain preparation (100 µg/0.1 ml) was introduced into the allantoic cavities of three one-day-old embryos and three 10-day-old embryos. Other embryos (three one-day-old and three 10-day-old) received Ukrain in the same doses but applied to the chorioallantoic membrane (Table I). After sealing with sterile paraffin, the embryos were incubated until the hatching time of normal chickens.

Table I *Direct influence of Ukrain preparation on hen embryo*

Preparation dose	Age of the embryo	Site of administration	Number of embryos employed	Effect
100 µg/embryo	7 days	allantoic cavity	3	chicken-in 21st /22nd/ day of incubation
		chorioallantoic membrane	3	
	10 days	allantoic cavity	3	
		chorioallantoic membrane	3	

Bacteria. For our investigation, *E. coli* derived from current clinical material and the strain 209 P of *S. aureus* were employed. Bacteria were cultivated on Mueller-Hinton fluid medium. The culture of bacteria, 18 h old, was centrifuged, washed twice with 0.85% NaCl and from this material the suspension No.1 according to McFarland's scale was prepared.

Estimation of antibacterial activity of Ukrain preparation in vitro.

A. In two series of test tubes containing 1 ml of Mueller-Hinton fluid medium, the preparation

Table II *Antibacterial activity of Ukrain preparation*

A

Concentration of Ukrain preparation in µg/ml		0		500		250		125		62,5		31,25		15,62		7,81		3,9		1,95		0,98	
Time of incubation in hours		24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48	24	48
Bacterial growth	E. coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

B

Concentration of Ukrain preparation in µg/ml	0						1						10						100						500					
Bacteria	*E. coli*			*S. aureus*			*E. coli*			*S. aureus*			*E. coli*			*S. aureus*			*E. coli*			*S. aureus*			*E. coli*			*S. aureus*		
Time of incubation in hours	1	2	24	1	2	24	1	2	24	1	2	24	1	2	24	1	2	24	1	2	24	1	2	24	1	2	24	1	2	24
Presence of bacterial growth	In all the test tubes the presence of bacterial growth.																													

Ukrain was diluted 1:1. The initial concentration was 500 µg/ml and the final concentration amounted to 1 µg/ml (0.98 mg/ml). Then to each test tube 0.04 ml of a previously prepared suspension of *S. aureus* or *E. coli* was added. After 24 and 48 h of incubation at 37°C, the result was read. Turbidity of the medium was taken as a good result.

B. The activity of the preparation was also estimated by another method. For this purpose, the preparation was diluted in fluid Mueller-Hinton medium so that 1 ml respectively contained

Table III *Influence of Ukrain preparation on influenza viruses* in vitro.

Influenza viruses in haemagglutinating units	10^1					10^2					10^3				
Ukrain preparation in µg/ml	0	1	10	100	500	0	1	10	100	500	0	1	10	100	500
Incubation time in hours	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24	1 2 24
Presence of virus replication	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

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Table IV *Influence of Ukrain preparation on influenza viruses* in vivo

Preparation dose, µg	0	1		10		100	
Time of applying the preparation		-24h -2h +2h	-24h	-24h -2h +2h	-24h	-24h -2h +2h	-24h
Virus dose in haemagglutinating units	10^1	10^1	10^1	10^1	10^1	10^1	10^1
Incubation time in hours	48	48	48	48	48	48	48
Presence of replication	+	+	+	+	+	+	+

and 1, 10, 100 and 500 µg Ukrain. To each of these dilutions 0,04 ml of previously prepared bacteria (according to McFarland's scale) was added. Then, after 1, 2 and 24 h of incubation at room temperature, 0.1-ml samples of the solution were taken and inoculated on solid Mueller-Hinton medium by the method of superficial smear. The result was read after 24- and 48-h incubation at 37°C. The test tubes containing only bacterial medium and 0.04 ml of the above-mentioned bacteria were taken as controls.

Estimation of the effect of Ukrain preparation on influenza viruses in vitro. To three series of test tubes, containing in 1 ml of allantoic fluid (diluted with 0.85% NaCl) an amount of influenza viruses corresponding to 10^1, 10^2 and 10^3 haemagglutinating units, the Ukrain preparation was added in such a dose as to achieve the concentrations of 1, 10, 100 and 500 µg/ml. From these test tubes after 1, 2 and 24 h of incubation at room temperature, 0.1 ml samples of virus suspension were taken and introduced into the allantoic cavity of 10-day-old hen embryos. For one determination, 3 embryos were used. The embryos were incubated during 48 h at 37°C and then the presence of influenza virus replication was determined by the haemagglutination method. The result was considered as positive if the presence of viruses was found in only one of the three embryos. Haemagglutination was carried out using 30% cock erythrocytes. Controls were the test tubes containing the above-mentioned haemagglutinating amounts of influenza viruses with added 0.85% NaCl corresponding in quantity to the Ukrain preparation.

Estimation of the influence of Ukrain preparation on influenza viruses in vivo. For this purpose, 10-day-old hen embryos were used. The experiment was carried out by two methods. In the first method, 1, 10 and 100 µg/0.1 ml of Ukrain preparation were introduced into the allantoic cavity of embryos only once in 24 h before infection and then the embryos were infected with the dose of 10^1 haemagglutinating units of influenza viruses. For each concentration of Ukrain preparation 5 embryos were used. After 48 h of incubation at 37°C the allantoic fluid was drawn from every embryo, and the presence of influenza viruses was estimated by the agglutination method. In the second method, the Ukrain preparation was introduced into the allantoic cavity of 10-day-old hen embryos using the same doses as above, but the preparation was given at 24 and 2 h before the

infection of embryos and 2 h after their infection with 10^1 haemagglutinating units of influenza viruses. After 48-h incubation at 37°C, the allantoic fluid was drawn and the presence of influenza viruses was determined by the haemagglutination method. Embryos infected with a dose of 10^1 haemagglutinating units of influenza viruses served as controls.

Results and discussion

This paper describes a succession of tests to try to explain the mechanism of action of Ukrain preparation. Its protective action in experimental infections of viruses in mice, observed in a previous investigation (1), was the result of the influence of this preparation on the animal immune system.

The planned program of investigation required either confirmation or exclusion of the direct influence of the Ukrain preparation on infection factors, viral or bacterial ones. This may be carried out *in vitro* by putting a range of concentrations into contact with bacteria and viruses. In this case we used the methods which have proved useful for other preparation batches already checked in our laboratory. The lack of inactivating properties found for the present preparation batch in doses ranging from to 500 µg/ml being tested *in vitro* and on hen embryos is sufficient for an equivocal estimation of its activity. The model of experiments was enriched by the use of different doses of viruses as well as differentation of inactivation times at room temperature (Tables III and IV).

By using 10-day-old hen embryos we have eliminated the influence of the host immune system. The embryo as a very young individual has not yet developed its immune system which could participate in antiviral activity. The lack of this system allowed a complete estimation of direct antiviral effectiveness of the Ukrain preparation. The production of normal chickens after their embryos had been incubated with 100 µg of Ukrain precludes any toxic influence of this preparation on the embryo that could disturb the replication of influenza viruses which it has contacted (Table I).

With respect to the bacteria *E. coli* and *S. aureus*, we have not observed any inactivating influence of Ukrain preparation dependent on its dose or the time of action. In all the samples studied, an increase in the amount of bacteria similar to an increase in the controls was observed. The preparation did not show any direct inactivating influence on the bacteria employed as the object of investigation (Table II).

Therefore the present stage of investigations excludes a direct inactivating effect of this preparation on influenza viruses and the bacteria *E. coli* and *S. aureus*. Taking into account earlier reports, our studies confirm in an indirect way another mechanism of anti-infectious action of Ukrain preparation in the infected macroorganism. It is probable that the indirect influence is exerted through the stimulation of some elements of the host immune system due to a secondary destruction of microorganisms or cells infected by these microorganisms.

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