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The method of activation of animal cells cultures in vitro for the reproduction of viruses

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Abstract.

Blood serum, protein hydrolysates of animal or vegetable origin and other growth stimulating factors, vitamins and antioxidants are known to be used as activators to increase growth promoting activity of cells in vitro. A high biological activity of natural biopolymers – chitin and chitosan obtained from crustaceans and insects respectively has already been proved. The objective of the research is to investigate how the use of apisan – a natural biopolymer, which contributes to a high reproduction of viruses, being used in the production of viruses-vaccines, increases the cultivation of animalcells in vitro by stimulating their growth and proliferation.The produce (50-70 mg/ml) was added to the growth promotion medium of cells cultures to study the stimulating properties of sells growth. In testing apisan as a potential metabolism activator, growth promotion media 199, MEM, DMEM and Lactalbumin Hydrolysate were used in combination with blood serum of mature cattle and fetuses of cows in 10% concentration, antibiotics and glutamine.Proliferation index (PI) calculated in the conventional way was used as a criterion to assess growth stimulation (activation) of apisan.The research aiming to investigate the impact of natural biopolymer chitosan of bee apisan on the MDBK cells metabolism revealed that the growth promotion medium containing apisan in concentration of 50.0-70.0 mg/l stimulates the maximal growth and cells development of the testing culture: the concentration of cells in the medium 48 hours later (logarithmic phase) amounted to 8.8±3.5 and 9.1±0.35 respectively by the PI 2.7-2.9. The level of type 1 (IBR) herpesvirus enrichment and parainfluenza-3 (PIV-3) virus in the MDBK cell culture, containing chitosan as a stimulating activator, exceeded target values by 1.9 and 1.20 respectively. Thus, the introduction of apisan into the growth promotion medium of natural biopolymer – apisan stimulates the proliferation growth and sensitivity of cell culture to the test viruses and their reproduction level.

Keywords: cell culture, biopolymer, chitosan, apisan, hydrolysate, virus, cytotoxic activity.

INTRODUCTION.

Cell cultivation practice shows that blood serum, protein hydrolysates of animal [1] and vegetable [2] origin are used as activators to increase growth promoting activity of cells in vitro.

General disadvantage of these methods is that animal and plant raw materials are expensive, and it is technologically difficult to get them; in addition, growth promotion media may be contaminated by microorganisms and prions inherent in blood serum, hydrolysates of animals' tissues and organs.

Natural biopolymers chitin and chitosan obtained from crustaceans (chitosan) and insects (apisan) penetrating into the microorganism's cells and interacting with ribosomes stimulate proteins and ferments synthezing ribonucleic acid, thus reinforcing the growth and development of cells [3].

As a result of a large body of research, antioxidant, antibacterial, antiviral, regenerating, immune stimulating, antitoxic and some other factors of chitosan have been discovered [4].

Chitosan has manifested itself as an effective radioprotector, sorbent of toxins and heavy metals in the body, immune modulator in veterinary and in other fields. More than 70 fields have been known so far in its usage. According to Japanese researchers, chitosan is the substance of the 21st century [5].

A cuticle containing chitin is a potential source of apian chitosan. Dead bees, which do not survive in low winter temperatures and fall down on the hive bottom, are the source for chitin and chitosan. Their loss in summer is more important, though it is more difficult to register it than in winter as bees die outside their hives.

The unique chitosan's qualities such as biocompatibility, its easy digestion, nontoxicity, metabolism stimulation, bactericidal activity and the fact that it contains proteins, carbohydrates, amino acids, macro- and microelements and other substances arouse interest to it of not only pharmacologists, therapists, gerontologists, physiologists but also of biotechnologists [6].

The ways to activate cell metabolism in animals in vivo by using the apian products (honey, propolis, bee-bread) which stimulate activity and increase the content of the immune system cells (T- B- lymphocytes, splenocytes, thrombocytes, myelocytes [7], T- and B-systems of animal body during stimulation by compositional forms with apian products have been described in some researches [8].

MATERIAL AND METHODS.

Growth promotion media 199, MEM, DMEM and Lactalbumin Hydrolysate in combination with the blood serum of mature cattle and fetuses of cows in a 10% - concentration, antibiotics (benzilpenicillin of sodium salt, streptomycin sulphate, kanamycin sulphate, amphotericin B in doses of 100 U/ml) and glutamine were used to grow cell culture.

Tripsinversen (1:3) was used to take the cells off the glass during their transfer. The cells were incubated in culture flasks under the temperature of 37^{0}C and the atmosphere of 5% CO_2. Sterility control of the cell culture was performed by seeding of cell suspension on meat infusion agar-agar, meat infusion broth, Sabouraud's medium, Czapek's

medium, Kitt-Tarozzi medium, transport nutritional medium-agar and transport nutritional medium-broth.

L.P. Dyakonov's common methodology was used to defrost ampules with sell suspension after its drawing out from the cryobank. Then, the cells were cultivated by the use of common R. Adams methodology under standard conditions, monolayer, in the thermostat under the temperature of 37ºC and the seed concentration of 40,000-80,000 kl/ml.

We got apisan by the irradiation of chitin-containing material in "Puma" gamma-apparatus at a dose of 10 Gr and gamma exposure rate of 3.13×10^{-5}Kl/kg. Hydrolysis of chitin (radiolysis) was performed in the presence of a 30%-solution of perchlorate ($HClO_4$) in 4:1 proportion. The produce of radiolysis was flushed by distillated water till the neutral meaning of pH. Then, the analysis of chemical contain followed, after that the concentration of the dry substance in the hydrolyzate was determined which fluctuated within the limits of 12-15 mg/ml. The substance was poured into ampules for lyophilization. One ampule contained 30±0.5 mg of apisan.

To determine the concentration of apisan in the growth medium, promoting an optimal growth of cell cultures, the ampules were opened and introduced to the growth medium at a dose of 6.28; 12.5, 25.0, 50.0, 70.0, 90, 100.0, 110, 120, 130mg/ml. MEM was used as a growth medium which was bottled and apisan was added into it at a dose of 6.25-130.0 mg/l. The media with apisan as a biological supplement were seeded by the cell culture of MDBK line with the seed concentration of 40,000 kl/ml. MEM containing 20 % of cows' blood serum as a stimulator of cells growth was used as a control growth medium. Proliferation index calculated on the basis of a common method served as a criterion of growth stimulation (activation) of the activator (apisan).

RESULTS AND DISCUSSION.

The influence of apisan – an MDBK natural biopolymer chitosan of bees and its content in the growth medium on cells metabolism has been determined during experiments (Table 1).

It follows from the table that the growth medium containing apisan in the concentration limits between 50.0-70.0 mg/l, contributes to the maximal growth and development of cells in MDBK test-culture, during which the cell concentration of cells in cultural medium 48 hours later (logarithmic phase) equaled to 8.8±0.35 and 9.1± 0.35 relatively and proliferation index of 2.7-2.9 relatively.

A further growth of biopolymer concentration (90, 100, 120, 150 mg/ml) did not lead to the growth of cell concentration in the cultural medium and proliferation index.

To find an optimal concentration of perchlorate ($HClO_4$), aiming at getting apisan, different concentrations of the reagent ($HClO_4$) were used: 10, 15, 2 0, 25, 30, 35, 40 %, into which a hydrolysable chitin containing material, deprotonated by sodium hydroxide, was introduced. Then radiolysis was performed by irradiation in the gamma apparatus at a doze of 10.0 Gr. The produce of radiolysis was flushed by distillated water till the neutral meaning of pH followed by testing apisan's growth stimulating activity. Apisan produces were added into growth promoting media which were inoculated by the test culture cells (MBDK).

The experiment results of apisan's growth stimulating activity, which was obtained by radiolysis at the presence of different concentrations of the reagent ($HClO_4$), are given in Table 2.

Table 1 – Influence of apisan on the growth and development of cells belonging to MDBK line

Concentration of apisan in the growth medium, mg/l	Concentration of cells ($x10^5$) and proliferation index depending on the time of cultivation, h					
	24		48		72	
	КК	ИП	КК	ИП	КК	ИП
6.25	1.8±0.21	0.8	3.5 ±0.43	1.9	2.5±0.29	1.7
12.50	2.9±0.19	1.0	5.0±0.29	2.1	2.7±0.33	1.8
25.00	3.5±0.73	1.3	8.8±0.55	2.9	2.9±0.35	1.9
50.00	3.6±0.35	1.4	9.0±0.017	3.1	2.9±0.19	1.9
70.0	3.6±0.29	1.5	9.0±0.35	3.2	2.8±0.41	1.8
90.00	3.6±0.25	1.5	9.0±0.41	3.2	2.7±0.23	1.7
100.00	3.6±0.33	1.5	9.0±0.39	3.2	2.7±0.17	1.6
120.0	3.6±0.35	1.5	9.0±0.70	3.2	2.7±0.45	1.6
130	3.6±0.41	1.5	9.0±0.77	3.2	2.6±0.25	1.5

Table 2 – Optimal concentration of the reagent ($HClO_4$)

Variant	The reagent ($HClO_4$) concentration in solution, %	Proliferation index within the time (h)		
		24	48	72
I	10	0.3	1.6	1.5
II	15	0.6	1.9	1.6
III	20	0.9	2.5	1.7
IV	25	1.0	2.8	1.8
V	30	1.6	3.1	1.9
VI	35	1.5	3.0	1.8
VII	40	1.4	2.9	1.7

Table 3 – Optimal correlation of the $HClO_4$ reagent and chitin containing material in the process of substrate radiolysis

Variant	The reagent ($HClO_4$) concentration in the solution, %	Proliferation index within the time (h)		
		24	48	72
I	1:1	0.3	1.5	1.5
II	2:1	0.5	1.9	1.3
III	3:1	0.4	2.7	1.6
IV	4:1	1.5	3.1	1.8
V	5:1	1.4	2.9	1.7
VI	7:1	1.3	1.6	1.5
VII	10:1	1.2	1.3	1.1

Table 4 - Reproduction of IBR and PIV-3 viruses on the line of MDBK cells cultures, incubated in apisan containing and serum media Eagle's MEM

Growth medium	Virus titre, lg TCD 50/ml	
	IBR	PIV-3
MEM + apisan	6.9±0.3	6.7±0.5
MEM +20% blood serum of cattle + 100 Units/ml antibiotics – penicillin, streptomycin, kanamycin(testing)	5.8±0.1	5.6±0.3

It follows from the table that a 30% -concentration of the reagent ($HClO_4$) in the solution promotes a complete apisan's radiolysis and proliferating activity of MDBK cells culture.

To find an optimal correlation of the $HClO_4$ reagent and chitin in the substrate's radiolysis a 30%-solution of $HClO_4$ was introduced into the hydrolysable substrate - a chitin containing material in the following proportion: 1:1, 1:2, 1:3, 1:4, 2:1, 3:2, 4:1, 5:1; after that all the material was irradiated by gamma rays at a dose of 10.0 Gr, and the variants of the hydrolyzate were introduced into growth promoting media, inoculated by the test culture cells (MBDK).The results are given in Table 3.

From Tables 2 and 3 it follows that an optimal dose of irradiation of chitin containing material is the dose of 10.0 Gr (Table 2); an optimal $HClO_4$ reagent concentration for the hydrolysis (radiolysis) of chitin – 30 % under an optimal correlation of chitin and the reagent in the proportion 4:1 (Table 3). Any changes of the abovementioned parameters of apisan hydrolysate lead to the reduction of the proliferation index of MDBK cells culture.

For the comparative assessment of growth promoting activity of the two types of apisan: CO_2-extracted and the radio-extracted one both of them were introduced into growth promoting media at a dose of 50-70 mg/ml, inoculated by the cells of MDBK test-cultures with the density of 40,000 kl/ml; they were grown within the time of 72 hours, after what the proliferation index was determined. CO_2-extracted apisan had been solved in a 1%-acetic acid as it does not dissolve in water.

The results of comparative experiments on the assessment of growth promoting activity of the two types of apisan show that apisan produced on the basis of a common method (CO_2- extraction), does not have a growth stimulating activity because its proliferation index was by 2.37 times lower than of the radio-extracted one, which is caused by the use of high temperature (150^0C) in the process of chitin extraction and the presence of acetic acid in apisan.

To determine the sensitivity of MDBK cell cultures to viruses MEM, containing apisan, was contaminated by infectious rhinotracheitis (IBR) and PIV-3 viruses; the cultures were taken after the formation of a monolayer.

Five consecutive passages of each virus have been carried out during the experiment. The method of titration, commonly used in virology, was used to determine the layer of viruses.

The results of the research on the reproduction of IBR and PIV-3 viruses by the cells of MDBK line in apisan containing and testing serum media are presented in Table 4.

Data given in the table show that the greatest IBR and PIV-3 virus mass was obtained when apisan was used as a growth stimulator in the growth medium MEM containing MDBK cells line. It should be noted that IBR virus proliferates much better than PIV-3 virus and its titer was 1.19 times higher than control values, while the titer of PIV-3 virus exceeded them by 1.2 times.

Thus, the introduction of apisan into the growth medium cultivation with cultures of animals' cells leads to the significant increase (by 3 times) of cells proliferating activity and allows avoiding the use of deficient components – growth factors.

CONCLUSION.

Chitosan, having unique properties such as biocompatibility, digestibility, nontoxicity, metabolism stimulating, bactericidal action; the fact that it contains proteins, carbohydrates, amino acids, macro- and microelements, etc. is of growing interest not only for pharmacologists, therapists, gerontologists, physiologists but also for biotechnologists. Using previous research data we obtained the natural biopolymer – apisan and proved its growth stimulating properties. Our research results show that its introduction into the growth medium stimulates proliferation and sensitivity of cells cultures to PIV-3 virus and type I herpesvirus (IBR) and their reproduction level.

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