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of the Institute of Plant Biology and Biotechnology

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APPLICATION OF ERYTHROCYTES' GHOSTS FOR TARGETED DELIVERY OF IMMUNE MODULATING PEPTIDES INTO LIVER

Abstract. The overall aim of this research is to investigate a possibility of using erythrocytes' ghosts (pharmacocytes) for liver-targeted delivery of immune modulating peptide pegylated IFN α–2b. We have applied different methods of drug encapsulation: hypo-osmotic hemolysis method, hypotonic pre-swelling method and dialysis method. Dialysis method was found to be the most effective for drug encapsulation into erythrocytes' ghosts, and effectiveness of encapsulation increased with the lower doses of IFN α–2b. For the pharmacokinetic study, single IV injections of either free or encapsulated IFN α–2b were made and the concentration of IFN α–2b in serum samples and tissue homogenates were determined. Encapsulation in erythrocytes' ghosts improved pharmacokinetic profiles of IFN α–2b by increasing the half-life, reducing its clearance, and increasing the deposition of the drug in the liver and spleen – the organs abundant in cells of the reticuloendothelial system. These data support the hypothesis that the erythrocytes' ghosts are effective drug carriers for liver-targeted delivery of immune modulating peptides.

Keywords: targeted delivery, viral hepatitis, erythrocytes' ghosts, pegylated IFN α–2b.

Introduction. Hepatitis B and C are potentially life-threatening infectious diseases that are widespread in all industrialized countries, including Kazakhstan. According to WHO, nowadays two billion people are infected with hepatitis B virus (HBV) worldwide (http://www.who.int/mediacentre/factsheets/fs204/en/), and about 150 million people are infected with hepatitis C virus (HCV) (http://www.who.int/mediacentre/factsheets/fs164/en/). A chronic infection that leads to a high risk of developing cirrhosis and / or liver cancer develop in 20% of infected persons. Every year, about 600 000 people die of the effects of hepatitis B, and more than 350 000 people die of the effects of hepatitis C. While there are many ways the treatment of acute forms of viral hepatitis, the disease remains a clinical challenge. HBV and HCV treatment requires prolonged systemic (oral and parenteral) administration of antiviral agents and immunostimulating drugs, which often causes serious side effects, which lead to premature termination of the therapy and the development of viral resistance [1].

Currently existing methods of treatment of HBV consist of the use of immunomodulators (IM) in combination with nucleoside analogues (NA) [2-4]. IM comprise interferon alpha (IFN-α), interferon alpha 2b (IFN α-2b), pegylated IFN α-2b and thymosin α1 (Tα1). The aim of IM therapy is to control amplification of T lymphocytes HBV / HCV specific immune response, which help the immune system to protect human from viral infection. NA is directed to inhibition of reverse transcriptase activity / DNA polymerase. The most common treatment of patients with HCV is the use of pegylated IFN-2a in combination with ribavirin [5-8].

Efficacy of anti-HBV and anti-HCV drugs mostly depends on their pharmacokinetics, in particular, their distribution and accumulation in the liver. From this point of view, a targeted delivery of drugs to the liver is one of the most promising approaches for improving the results of treatment of viral hepatitis [3, 9]. On the one hand, it can significantly reduce the toxicity of drugs to the lower therapeutic doses and

low concentrations in the blood. On the other hand, the targeted delivery of drugs may enhance their therapeutic effect due to the higher concentrations in the diseased liver [10].

Earlier it was reported that during the introduction of some chemotherapeutic agents and antibiotics encapsulated in erythrocyte ghosts, there was an increase in the accumulation of drugs in the liver and spleen [11]. Such increased accumulation is associated with the life cycle of red blood cells. Typically, they are circulating in the peripheral blood for 120 days, and after aging and damaging, red blood cells are detected, phagocytosed and destroyed by tissue macrophages mainly in the liver, spleen and bone marrow [12-14]. On this basis, it was assumed that the erythrocyte ghosts can be effective transport systems for targeted delivery of antiviral drugs for the treatment of acute and chronic hepatitis. In this study, we studied erythrocyte ghosts as carriers of pegylated interferon α- 2b (pegylated IFN α-2b) for targeted delivery to the liver.

Materials and methods of research. Pegylated IFN α-2b (brand name PegIntron® (Schering - Plough)), lyophilized powder for preparation of solution for injection and solvent into the pen injector (redipen) containing 100 mcg of pegylated IFN α-2b, 0.5 ml solution for injection.

Encapsulation of drugs in the FE using method of hyposmotic hemolysis. In order to add drug to autologous EG, 5.0 ml of blood was taken from a vein into a sterile tube containing heparin (25 U/ml) in sterile conditions. The tube was capped with sterile stopper and centrifuged at 3000 rev/min for 5 minutes (1057 g). The supernatant was removed with a sterile pipette. The three tubes were filled by 0.9 ml of erythrocyte sediment. 4.5 ml of saline was added to the erythrocyte sediment and centrifuged under the same conditions to wash the erythrocytes. The procedure was repeated twice. 5 fold volume of distilled water cooled to 0°C was added to the erythrocyte sediment. The resulting suspension was thoroughly stirred and then centrifuged at 8000 rev/min for 20 minutes at 4°C. The supernatant was drained. 10 mcl of required concentration and 90 mcl of distilled water were added to 0.9 ml EG drug. The resulting suspension was incubated for 15-20 minutes at 4°C. Subsequently, 1/9 part of the volume (111 mcl) of 10% sodium chloride was added to this mixture, the suspension was mixed thoroughly and placed in an incubator for 20-30 min at 37°C. After incubation the mixture was centrifuged at 10 minutes, 1000 g.

Cells were washed three times with phosphate buffered saline, and then they were incubated for 30 minutes at 37°C for drug-erythrocyte dissociation. The sample was centrifuged for 10 minutes at 1000 g, the supernatant was removed. To estimate the amount of irreversibly bound drug EG was lysed with distilled water. To this end, two volumes of distilled water were added to 1 volume of EG.

Encapsulation of drugs in EG using method of hypotonic pre-swelling. In order to introduction the drugs in FE by hypotonic pre-swelling method, the model developed by Rechsteiner MC [15] with some modification of Hamidi M. was used [16, 17]. For this purpose, 10 ml of heparinized blood was centrifuged at 1000 g for 10 minutes. The supernatant was removed and sediment erythrocytes were washed 3 times with phosphate buffer. Subsequently, 4 ml of a hypotonic solution of sodium chloride (0.65%) were added to 1 ml of the washed erythrocytes and mixed gently. The mixture was centrifuged at 600 g for 5 minutes and the supernatant was removed. 100 mcl of drug solution dissolved in distilled water or phosphate buffer was added to 0.9 ml of the obtained erythrocytes. For the successful incorporation of drug, 4 ml of a hypertonic sodium chloride solution (1.1%) were added to 1 ml of the resulting suspension. After incubation for 30 minutes at 37°C, the mixture was centrifuged at 1000 g for 10 minutes. Then, 10 ml of phosphate buffer was added to obtained EG, thoroughly mixed and left for 30 minutes at 37°C, then centrifuged for 10 minutes at 1000 g. This procedure was performed three times. To estimate the amount of irreversibly bound drug EG was lysed with distilled water – two volumes of distilled water was added to EG to 1 volume.

Encapsulation of drugs in EG using dialysis method. Venous whole blood samples in a tube with heparin or EDTA has been unscrewed in centrifuge for 10 min at 1900 g, +4C˚. Plasma and white blood cells were aspirated, and the sediment erythrocytes were washed twice as followings. A solution of Hepes (10 mM Hepes, 154 mM NaCl, 5 mM glucose, pH 7.4) was added to the erythrocytes so that the final volume was 14 ml. The resulting solution was centrifuged for 10 minutes at 3000 rev/min, 4˚C, and the supernatant was removed. During centrifugation, samples of studying drug were prepared in the required concentrations, 2 mM ATP – 0.121 g and 3 mM – 0,0092g were added to diazine buffer (15 mM $NaHCO_3$, 15 mM NaH_2PO_4, 20 mM glucose, 4 mM $MgCl_2$). Meanwhile, the dialysis sacs in the required amounts were thoroughly hydrogenated in 50 ml tubes using dialysis buffer (without ATP and GSH).

It should be noted that the dialysis sac was always hydrogenated, and throughout the process it was in the same 50 ml tube with dialysis buffer without ATP and GSH. A tight knot was tied at one end of the sac, 900 mcl of red blood cells and 100 l mcl of the sample were in the sac. The content has been constricted to the knotted end of the dialysis sac, and at the other end, 3 tight knots were tied, leaving a little air. 50 ml of dialysis buffer with ATP and GSH were transferred to new tubes each of 50 ml, the number of new tubes determined by quantity of studied drug concentrations. Dialysis sacs were placed into each tube. Dialysis was carried out in a rotator for 1 hour at a temperature of 4˚C. After dialysis, the obtained EG was transferred from the dialysis sacs into new 15 ml tube. The tubes were incubated for 5 min at 37˚C. For EG membrane sealing, 100 mcl of PIGPA solution (5 mM adenosine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM Na_3PO_4, 100 mM glucose, 12% (m/w) NaCl) was added to each tube. The content has been thoroughly mixed, after there was a change of EG color, which indicates the successful restoration of the membranes. The tubes were incubated for 30 min at 37˚C. Next, the samples were centrifuged for 10 minutes at 500 g, 23˚C, and the supernatant was carefully removed. The EG was washed 2 times using Hepes solution (2 volume of solution to 1 volume of the obtained EG) in each tube. The tubes were centrifuged for 10 min at 5500 g, 23˚C, and the supernatant was carefully removed. The procedure was repeated one more time. Obtained EG was stored no longer than 24 hours at + 4˚C. To estimate the amount of irreversibly bound drug EG was lysed with distilled water – two volumes of distilled water were added to EG 1 volume.

Enzyme immunoassay. The content of pegylated IFN α-2b in erythrocyte samples was measured by using equipment (Abcam) for the quantitative determination of pegylated molecules in the plasma, serum, cell cultures and tissues.

Experimental design in vivo. In order to study the dynamics of content of pegylated IF 2b, lab white mongrel male rats weighing 180-200 g were used. The animals were divided into 2 groups: group 1 received pegylated IFN α-2b at a concentration of 20 mcg/kg i.v.; 2 group received erythrocyte ghost with encapsulated pegylated IFN α-2b at a concentration of 20 mcg/kg. The animals of both groups were taken from the experiment after 15 minutes, 1 hour, 3 hours, 6 hours, 12 hours, 24 hours, 36 hours, 48 hours. In all animals, the internal organs were removed, and the blood was taken. The content of pegylated IFN α-2b was determined using a commercial EIA Abcam kit. Total amount of animals in the experiment were 80. The recovered bodies were subjected to homogenization and the samples were frozen at -80. Pegintron content by EIA (Abcam) was studied in liver homogenates.

Pharmacokinetic studies. For the mathematical modeling of pharmacokinetic processes Borgia 1.03 program was used. The received data characterizing the level of concentration of IFN α-2b in serum after a single intravenous administration of free drug and erythrocyte ghost, approximated within one compartment pharmacokinetic model.

Results of research and their discussion. Three methods of preparation of erythrocyte ghosts (EG) for encapsulation of the drug were used, such as the hypo-osmotic hemolysis method, hypotonic preswelling method and dialysis method. For obtaining EG with IFN α-2b we was used lyophilized powder (Schering – Plough), containing 100 mg of active substance. In order to determine the appropriate concentration of the drug for its encapsulation in EG, three different concentrations of IFN α-2b: 10 mcg/ml, 50 mcg/ml and 200 mcg/ml were included in erythrocytes. It was shown that a dialysis method used to incorporate IFN α-2b in erythrocyte ghosts, is suitable method that does not destruct the morphology of erythrocytes and their contents in 1 ml (Figure 1).

In order to study the parameters of IFN α-2b inclusion, we determined the content of IFN α-2b by enzyme immunoassay in the dialysis water and the supernatant (not included preparation), flush water (reversibly bound fraction of drug) in the composition of erythrocytes ghost (irreversibly bound fraction of drug) (Table 1).

The Table shows that the IFN α-2b has a relatively high degree of inclusion in erythrocyte ghosts, thus, inclusion efficiency of IFN α-2b was increased using lower drug concentrations. In this connection, it should be concluded that the concentration of 10 mcg/ml was suitable for carrying out further work on assessing the dynamics of the content of pegylated IFN α-2b in the blood and internal organs of laboratory animals during intravenously administration in erythrocytes ghost.

Pharmacokinetic curves of changes in the concentration of IFN α-2b in the blood of rats after a single intravenous administration of free drug are shown in Figure 2. Since the "concentration - time" curve is a

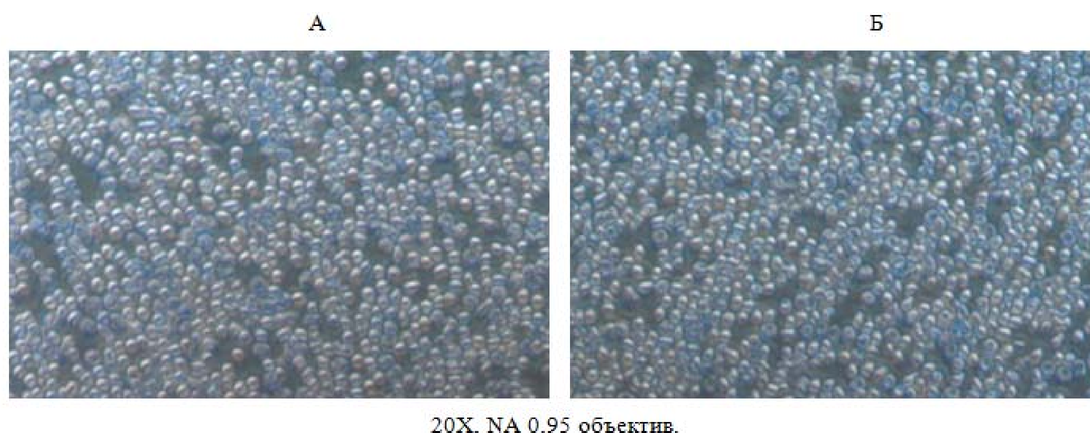


Figure 1 – The micrographs of erythrocytes before (A)
and after the inclusion of pegylated IFN α-2b using dialysis method (Б)

Table 1 – Analysis of the effectiveness of the inclusion of IFN α-2b in the erythrocytes ghost

Distribution parameters	IFN α–2b content		
	200 mcg/ml	50 mcg/ml	10 mcg/ml
The degree of bounding (%)	31,9	57,4	77,6
Irreversible bound (%)	60,2	67,1	96,5
The degree of dissociation (%)	39,8	32,9	3,5
Concentration in ghosts (%)	19,2	38,6	74,9
The degree of recovery of erythrocytes (%)	57	27	82

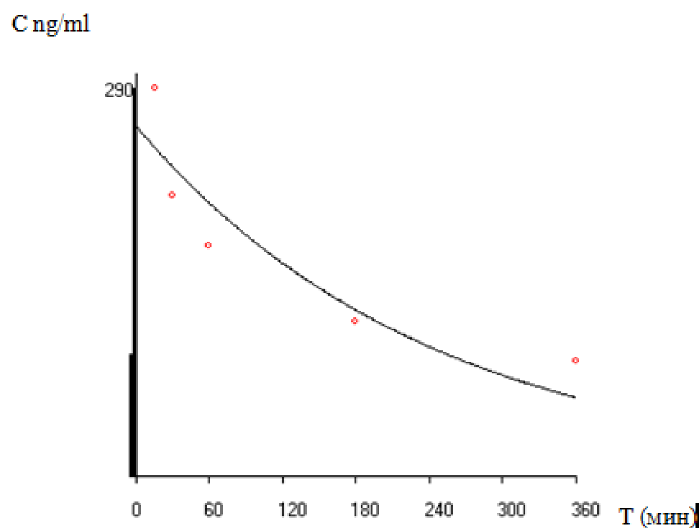


Figure 2 – "Concentration - time" curve in half-life coordinates of IFN α-2b in rats after a single intravenous administration of free drug at a concentration of 10,000 pg/ml of body weight

monotone decreasing curve, we used one compartment pharmacokinetic model with no absorption. Relatively long half-life ($T_{1/2}$) – 167 minutes was determined, which is associated with low total clearance (C_{el}) (Table 2). A low apparent volume of distribution (V – 3,8ml) shows a slight move of the studying substance from the central chamber into peripheral one and holds the main part of the drug in the extra-cellular fluid. Taking into account the two-phase curve, one compartment pharmacokinetic model with resorption was used.

Table 2 – Pharmacokinetic parameters of free IFN α-2b encapsulated in EG after single intravenous administration to rats

Parameter, dimension	Free IFN α–2b	IFN α-2b encapsulated in EG
Half-absorbtion period, min	–	13.096
T $_{1/2}$ half-life periof, min	167.270	373.988
Resorption constant	–	0.05292
K el Removal constant	0.00414	0.00185
Vss Volume of distribution, ml	3.82	14.6
CL tot Clearance, ml / min	0.002	0.001
AUC 0-∞ Integral area	63117.5	133047.8

Pharmacokinetic curves of changes in the concentration of IFN α-2b in the blood of rats after single intravenous administration of the drug encapsulated in EG are presented in Figure 3. It was shown that at intravenous injection of IFN α-2b deposited in erythrocytes, the half-life ($T_{1/2}$) and integral area under the concentration-time curve compared to the pharmacokinetics of free drug doubles, and accordingly, the elimination constant (C_{el}) and clearance decreases; it uniquely determines the possibility of a more prolonged preservation of drug in the blood and the prolongation of its effect (Table 2). The increase in distribution volume index (V) may be indicative of increasing the ability of a substance to pass from the depot created in the blood to peripheral tissues

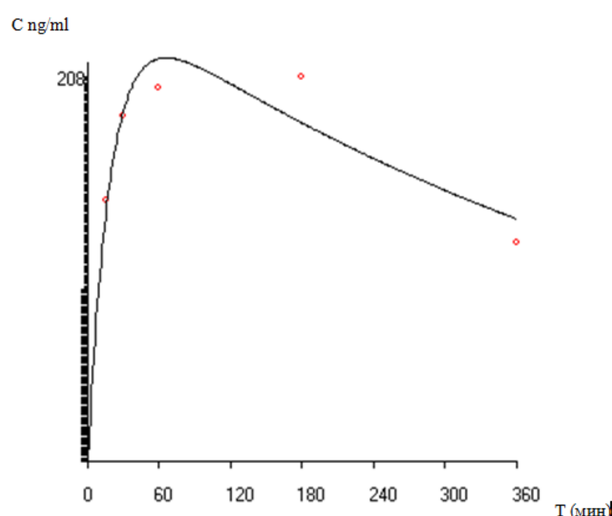
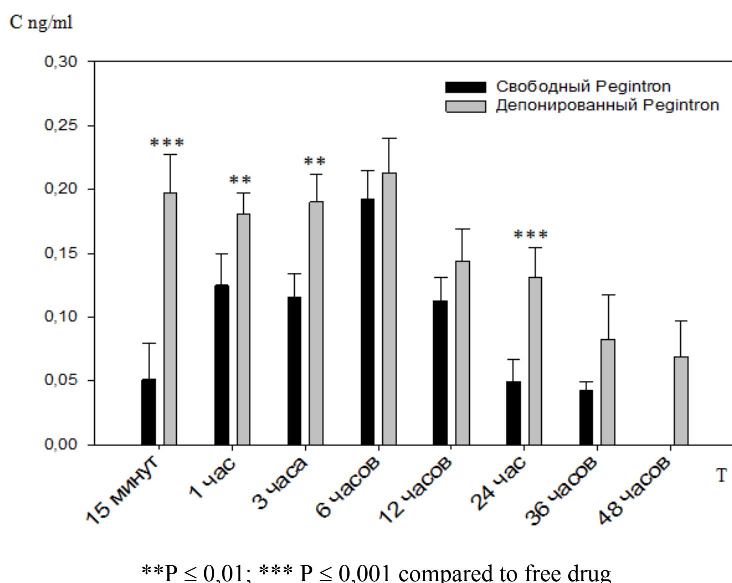


Figure 3 – "Concentration - time" curve semilogarithmic IFN α- 2b in rats
after a single intravenous administration of the drug encapsulated in ESP at a concentration 10,000 pg / ml of body weight

The results of studies of pegylated IF 2b dynamics in the liver have shown a statistically significant increase of IFN α-2b in the homogenate of liver tissue of laboratory animals at a single intravenous administration of pegylated IFN α-2b in the form of erythrocytes ghosts, as compared to control (free drug) after 15 minutes of administration, and this dynamic is continued throughout the experiment (Figure 4). Thus, the presence of IFN α-2b was recorded even 48 hours after administration, while in the livers of animals treated with injections of the drug in free form, we used in the liver samples was impossible by IFA to determine IFN α-2b.

At 24 hours after a single intravenous administration of free and deposited in EG IFN α-2b we revealed some amount of the drug and in other peripheral tissues. The results are given in Table 3. As can be seen from the data, during administration of free drug, the highest concentration of IFN α-2b is defined in kidney tissue homogenates, indicating that the final stage of the elimination process. Further, the distribution of the drug goes to the liver, spleen, adipose tissue, heart and lungs. During administration of IFN α-2b in the composition of the EG, the main deposit location in the body becomes the liver and spleen. Some increase in the activity of IFN α-2b fixed in the lungs, subcutaneous fat and heart was not statistically firm.



**P ≤ 0,01; *** P ≤ 0,001 compared to free drug

Figure 4 – The content of IFN α - 2b in the liver of laboratory rats (ng/mg of total protein)

Table 3 – The concentration level of IFN α-2b in peripheral rat tissues at 24 hours after intravenous administration (ng/mg of total protein)

Tissue homogenate	Free drug	The drug deposited in erythrocytes
Liver	0,049 ± 0,017	0,13 ± 0,02***
Kidneys	0,15 ± 0,046	0,08 ±0,01
Spleen	0,025 ± 0,01	0,09 ± 0,02*
Heart	0,018 ± 0,003	0,025 ± 0, 004
Lungs	0, 022 ± 0,006	0,06 ± 0,03
Adipose tissue	0,023 ± 0,002	0,035 ± 0,004
*p < 0.05, ***p < 0.001.		

Thus, the results of the study allow us to conclude that EG provides delay elimination of IFN α-2b and cause its redistribution in the body with maximum accumulation in the tissues of the liver and spleen, which are the organs that contain the largest number of cells of the reticuloendothelial system. The results support the hypothesis that the lysis of EG with antivirals and immunomodulators in reticuloendothelium, allows local release of these substances into the liver parenchyma.

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ИММУНОРЕТТЕУШІ ПЕПТИДТЕРДІ БАУЫРҒА МАҚСАТТЫ БАҒЫТТАП ЖЕТКІЗУ МАҚСАТЫНДА ЭРИТРОЦИТТЕРДІҢ ҚАПШЫҒЫН ҚОЛДАНУ

Аннотация. Зерттеудің мақсаты – ИФН α–2b пегилирленген иммунореттегіш пептидін бауырға мақсаттап, бағыттап жеткізу мақсатында эритроциттердің қапшығын қолданудың мүмкіндігін зерттеу болып табылады. Зерттеу барысында инкапсуляциялаудың түрлі әдістері қолданылды:гипоосмостық гемолиз әдісі, гипотондық пресвеллинг әдісі және диализ әдісі қолданылды. Жүргізілген зерттеулердің нәтижесіне сүйене отырып, дәрілік препаратты эритроцит қапшығына жүктеуде ең тиімді әдіс болып диализ әдісі таңдалып алынды. Осы әдіс негізінде төмен концентрацияда ИФН α–2b фармакоциттерге жүктелуі жақсы жүрді. Дәрілік препараттың фармакокинетикасын зерттеу мақсатында, бос формадағы және фармакоциттерге жүктелген ИФН α–2b зертханалық жануарларға инъекция жасалынып, белгілі уақыт өлшемімен жануарлардың ішкі ағзаларының гомогенаттары мен қан сарысуындағы препараттың концентрациясы өлшенді. Эритроциттердің қапшығына ИФН α–2b инкапсуляциялаудың төмендегідей артықшылықтары болатыны көрсетілді: инпаксуляциялау жолымен препатарттың фармакокинетикасы жақсарды, препараттық клиренсі төмендеді, басқа ішкі ағзаларға таратылуы азайды. Алынған нәтижелер, иммунореттеуші пептидтермен толықтырылған эритроцит қапшықтарының бауырға дәрілік заттарды бағыттап жеткізуде тиімді жүйе гипотезасын растады.

Тірек сөздер: мақсатты бағыттап жеткізу, вирустық гепатит, эритроцит қапшықтары, пегилирленген интерферон α– 2b.

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ПРИМЕНЕНИЕ ТЕНЕЙ ЭРИТРОЦИТОВ ДЛЯ ЦЕЛЕНАПРАВЛЕННОЙ ДОСТАВКИ ИММУНОРЕГУЛЯТОРНЫХ ПЕПТИДОВ В ПЕЧЕНЬ

Аннотация. Цель исследования – изучение возможности использования теней эритроцитов (фармакоцитов) в качестве носителей иммунорегуляторного пептида пегилированного ИФН α–2b для целенаправленной доставки в печень. В ходе исследования были использованы различные методы инкапсуляции лекарственных препаратов: метод гипоосмического гемолиза, метод гипотонического пресвеллинга и диализный метод. В результате проведенных исследований диализный метод был признан наиболее оптимальным методом загрузки лекарственных препаратов в фармакоциты; при этом эффективность включения ИФН α–2b увеличивалась с использованием более низких концентраций. Для изучения фармакокинетики производили разовую внутривенную инъеккцию свободного или инкапсулированнорго в фармакоциты ИФН α–2b и измеряли концентрацию препарата в сыворотке крови и гомогенатах внутренних органов. Было показано, что включение ИФН α–2b в тени эритроцитов улучшало его фармакокинетику путем увеличения периода полувыведения, снижения клиренса препарата и перераспределения в организме с максимальным накоплением в тканях печени и селезёнки – органах, содержащих наибольшее количество клеток ретикулоэндотелиальной системы. Полученные результаты подтверждают гипотезу о том, что тени эритроцитов, наполненные иммунорегуляторными пептидами являются эффективными системами целенаправленной доставки лекарственных средств в печень.

Ключевые слова: целенаправленная доставка, вирусные гепатиты, тени эритроцитов, пегилированный интерферон α– 2b.

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