

INHIBITION OF THE EXPRESSION OF PHYSIOLOGICAL PRIONS WITH ANTISENS-OLIGONUCLEOTIDES

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The aim of the research was to investigate the ability of new complexes as specific single-stranded oligonucleotides (ODN) with dimethylaminomethylmethacrylate (DMAEM) based polymer carriers to inhibit the expression of the physiological prion. It has been established that the introduction of complexes of newly synthesized carriers based on dimethylaminomethyl methacrylate — PEG-DMAEM-MP-27 (magnetic particles) (MP-27), PEG-DMAEM-MP-2 (MP-2), PEG-DMAEM-MP-3 (MP-3) with as ODH into the organism of rats leads to a decrease in the physiological prion content in the tissues of the spleen and small intestine. The influence of complexes of newly synthesized carriers MP-27, MP-2 and MP-3 with as specific single-stranded oligonucleotides ODH on hematological and biochemical blood parameters of Wistar rats was also studied.

Key words: prion infections, antisense-oligonucleotides, polymeric carriers.

Prion infections are caused by a particular pathological form of prion protein, which is present in all mammals. Today, there are no effective methods and means for treating and preventing prion infections [1]. The pathogenesis of prion infections is associated with the synthesis of cellular prion (Pr^{PC}), so that removing this protein from the body may prevent their development [2]. There is a whole arsenal of drugs that can effectively inhibit the biosynthesis of the necessary proteins. Among them are — antisense technology, which results in the use of specific single-stranded oligonucleotides (asODN), which block the mRNA areas and mediate its hydrolysis [3, 4].

The success of using the antisense technology largely depends on the availability of polymer carriers, which is especially important in modern biology, medicine and veterinary medicine. Cationic synthetic and natural polymers are a convenient tool for delivering genetic material to cells, because through electrostatic interactions they effectively bind negatively charged DNA molecules. Thus, polymers screen DNA from the action of hydrolytic ferments, so they continue to stay in the body of genetic constructs and facilitate their penetration into the middle of the cell. In these studies polymers

based on dimethylaminomethylmethacrylate (DMAEM) were used as carriers. They contain tertiary amino groups and significantly increase the efficiency of mammalian transfection of plasmid DNA, however, the molecular mechanisms of this process have not been fully established [5].

The aim of our work was to investigate the ability of new polymer DMAEM based carriers to form conjugates with asODN, to study the efficiency of data transport systems, to analyze their effect on the organism of laboratory rats and to determine the ability to suppress the expression of the physiological prion.

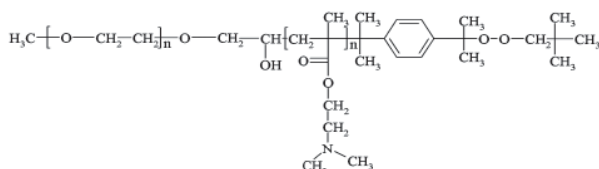
Materials and Methods

For Pr^{PC} gene expression suppressing in studies oligonucleotide sequence 5'-ATGCTTGAGGTTGGTT-3' were used, which are capable of binding to the central portion of the open reading frame mRNA of the cell prions. Antisense oligonucleotides (asODN) were synthesized by AlphaDNA (Canada). As carriers newly asODN synthesized polymers were used based on dimetilaminoetilmetakrilat (DMAEM), namely, PEG-DMAEM-MP-27 (MP-27), PEG-DMAEM-MP-2 (MP-2), PEG-DMAEM-MP-3 (MP-3). Polymers were

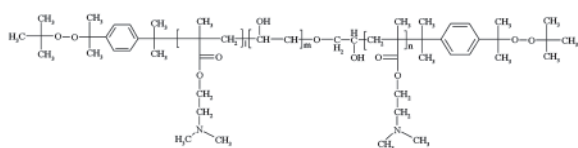
developed at the National University "Lviv Polytechnic" at the Nucleus organic chemistry department.

1. PEG-DMAEM-MP-27 (MP-27) is a resin-like substance of light yellow color. Its linear block is a polyethylene glycol (PEG) copolymer and polydimethylaminomethyl methacrylate (polyDMAEM) with a finite peroxidic moiety. The polymer is soluble in water and in a wide range of pH values (including the physiological pH of the medium), DMSO. The nitrogen content of the polymer is 8%, a molar mass of about 6000 g/mol is calculated. Polymer synthesis was carried out in a medium of DMFA (dimethylformamide) in the presence of a peroxide containing regulator of the polymer chains growth a redox system containing ions of cerium (Ce): $Ce^{4+} + \text{--- PEG-OH}$ was used as the initiator. The polymer was further purified from Ce ions.

Structure:



2. PEG-DMAEM-MP-2 (MP-2) is a brown powdered polymer. In its composition PEG is located in the middle between two blocks of the field DMAEM. Polymer synthesis was carried out in DMF in the presence of environment of peroksid containing growth regulator of the polymer chains (monoperoksin, nmr) a redox system $Ce^{4+} + \text{--- diPEG-OH}$ was used as the initiator. The polymer is further purified from Ce ions.



3. PEG-DMAEM-MP-3 (MP-3) — The general structure is similar to PEG-DMAEM-MP-2, but the polymers were not purified from Ce ions.

The preparation of complexes of asODN and polyDMAEM: 6.6 mg of polyDMAEM was dissolved in 0.01M HCl, the pH of the solution was adjusted to 7.4; 0.5 ml of the prepared polyDMAEM solution was mixed with 0.5 ml of an asODN solution at a concentration of 2 µg/ml. The mixture was incubated for 30 min at room temperature.

The ability of the polymers (polyDMAEM) to bind the oligonucleotides was checked

by electrophoresis in 3% agarose gels. To a solution of asODN with an oligonucleotide concentration of 0.02 mg/ml, 1%, 0.5% and 0.05% of the polymer solution were added. The electrophoresis was conducted in tris-borate buffer with addition of EDTA (89 mm Tris, 89 mm boric acid, 2 mM EDTA) at a constant voltage 2V/cm. The gel was colored with ethidium bromide at the concentration of 2 µg/ml and photographed under ultraviolet light.

Four groups of rats *Rattus norvegicus* var were formed for this research. Alba, Wistar lines: control and three experimental groups, 3 animals each. The animals of the experimental groups were injected into the tail vein with 2 mg/kg of body weight of solutions of the asODN complexes with the MP-2, MP-3 and MP-27 polymers. After 1, 2 and 7 days from the start of the experiment, animals from each group were decapitated under a light Chloroform anesthesia [6].

In the selected blood samples of the animal experimental groups, hematological and biochemical indices were determined for the actions of the asODN complexes with carriers using the Oraphee Mythic-18Vet automatic analyzer (Oragehe, Switzerland).

Histological studies were also carried out to study the effect of the asODN complexes with the polymeric carriers MP-27, MR-2 and MR-3 on the brain structure. To study the toxic effects of the asODH complexes with the polymeric carriers MR-27, MR-3 and MP-2 on the structure of the pre-replicate organs, histological studies of the spleen, small intestine and brain of the white rats were carried out. Samples were taken from pieces of tissue 0.2–0.5 cm thick. Samples were taken from the middle of the ileum, a transverse section of the right side of the spleen and the large hemispheres in the region of the cloak and longitudinal slit of the large brain. The material was fixed in a 10% neutral formalin solution, poured into paraffin, stained with hematoxylin and eosin. Photographs of spleen histograms, a thin section of the intestine and a brain were made with the embedded in a microscope video camera with image recording software Med.Cam.

The immunoblot-detection of the PrPC protein was performed according to the following scheme. The tissue was were lysed in a tenfold volume of lysis buffer pH 7.4 (150 mM NaCl, 1% Triton-X 100, 0.5% Na deoxycholate, 0.1% Na dodecyl sulfate, 50 mM Tris, 0.001% proteinase inhibitor cocktail — Sigma -Aldrich, Germany). The samples were

then centrifuged at 5200 g during 5 minutes at 4 °C. In the finished lysates, the content of the total protein was measured by Lowry method [7]. To equalize the total protein concentrations, samples were diluted with buffer (25 mM Tris-HCl, 150 mM NaCl, 2.5 mM KCl, pH 7.4). Further, the electrophoretic separation of cell lysate proteins was performed in the Lammley system [8] in a 12% polyacrylamide gel. The transfer of proteins from the polyacrylamide gel onto the polyvinyl difluoride membrane was carried out in a Transblot transfer chamber (Bio Rad, USA) [9,10]. At the end of the transfer of proteins, the membrane was incubated for 1 hour at room temperature in 5% skimmed milk (5% skimmed milk powder, 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20). To detect the physiological prion, mouse 6H4 antibodies to the prion protein (Prionics, Switzerland) were used at 1:5000 dilution. With primary antibodies, the membrane was incubated for 12 h at 4 °C. In the next step, the membranes with goat anti-mouse immunoglobulin conjugated with an alkaline phosphatase was conducted (Sigma-Aldrich, Germany) at a dilution of 1:10000. The detection of immune complexes was performed using a chemiluminescent substrate for alkaline phosphatase CDP-Star (Sigma-Aldrich, Germany). The visualization was carried out using an ECL HyperFilm X-ray film (Amersham, USA) and a film development kit (Kodak).

X-ray film with the results of the chemiluminescent detection was scanned. The intensity of the signal was determined using the GelPro 3.1 software. The content of the cellular prion was expressed in conventional units characterizing the integrated optical density of the prion signal in accordance with the control.

Results and Discussion

The conjugation of asODN with newly synthesized polymers is mediated by units of dimethylaminomethylmethacrylate and primary amino groups of aminomethyl methacrylate. When mixing solutions, oligonucleotides condense with cationic polymers, it was found that the formation of complexes was characterized by a decrease in the electrophoretic mobility of oligonucleotides. At the same time, the oligonucleotides connected with polyDMAEM were characterized by lower electrophoretic activity than free oligonucleotides. It was

found that the most effective formation of complexes occurs when mixing 0.5% polymer solutions with a solution of 2 µg/ml asODN.

As a result of the intravenous administration of the test substances to rats, none of the study animals died. Food and water consumption did not change during the experiment. Changes in the nature of animal behavior, intensification of reflexes, and vegetative effects have not been revealed.

The effect of complexes of newly synthesized carriers based on dimethylaminomethyl methacrylate-MP-27, MP-2 and MP-3 with asODN on hematological and biochemical indices of blood of white rats (*Rattus norvegicus* var. Alba, Wistar lines) was studied.

The introduction of complexes of asODN with polymers MP2 and MP-3 leads to a decrease in hemopoiesis and a change in the percentage of leukocytes, in particular, an increase in the monocyte content was noted. The growth of monocytes can be associated with the processes of neutralizing the toxic effect of carriers on the animal organism. Compared with the electrolyte carriers MP-2 and MP-3 complex with the carrier MP-27 have the least effect on blood indices in laboratory rats.

An increase of the urea concentration was noted, which is more likely to affect the functional load on the kidneys after injections with complexes of MP-2 and MP-3 polymers. The indices of the creatinine content were within the limits of physiological fluctuations. With the introduction of complexes of asODN with the carrier of MP-27, no significant changes in the content of creatinine and urea were observed (Fig. 1).

The activity significance of ALT and AST after the introduction of complexes of polymers with asODN also increased (Fig.2). Along with this, already seven days after injections, the activity of aminotransferase was within the limits of the norms, which indicates a functional but short-term load on the liver.

The activity of antioxidant enzymes and the content of lipid peroxidation products in the blood of white rats under the action of complexes of asODN with polymer carriers based on DMAEM fluctuated within physiological norms (Table).

The introduction of complexes of polymers MP-27, MP-2 and MP-3 with asODN into the rats intravenously did not cause changes in the structure of the pre-replicate organs (small intestine, spleen, brain) during the

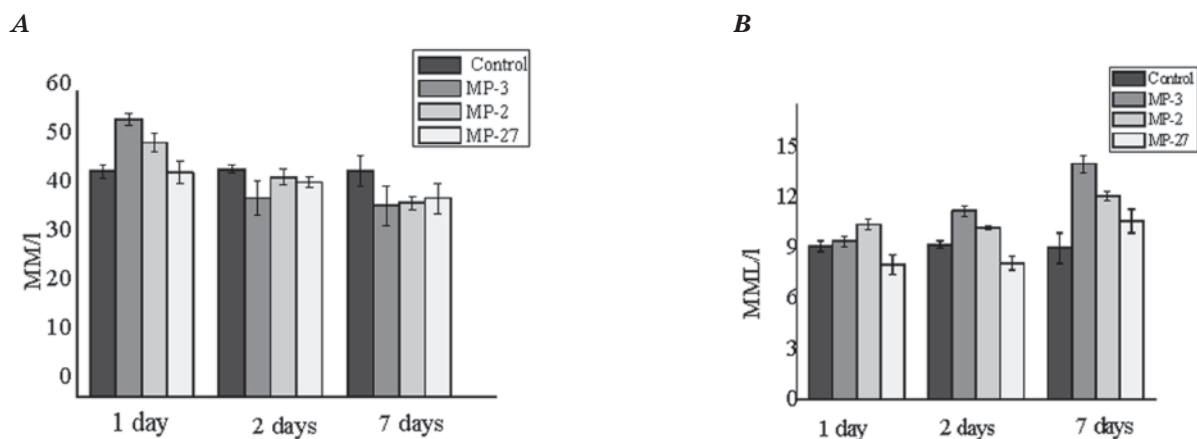


Fig. 1. The indices of the creatinine (A) and urea (B) content in blood of laboratory rats after the introduction of complexes of polymers with asODN
 $M \pm m, n = 5$.

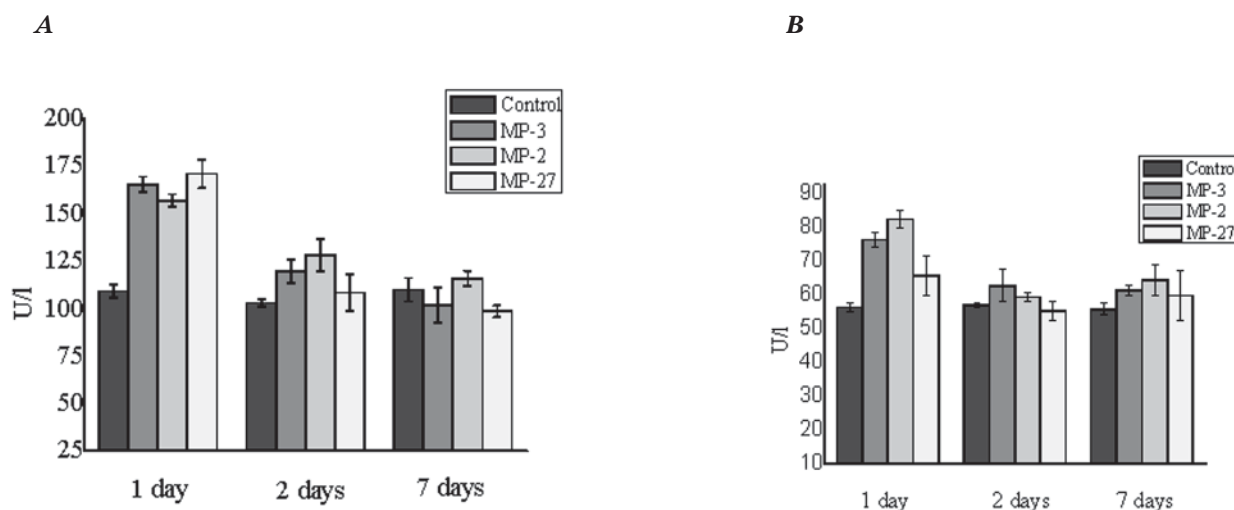


Fig. 2. The activity significance of ALT (A) and AST (B) after the introduction of complexes of polymers with asODN in blood of rats

The antioxidant enzymes and the content of lipid peroxidation products in the blood of white rats under the action of complexes of asODN with polymer carriers

Values	Control group	MP-2+asODN	MP-3+asODN	MP-7+asODN
Catalase, mMol / mg Protein / min	7.42 ± 0.09	6.86 ± 0.41	8.19 ± 0.27	7.71 ± 0.33
GP, nMol / min / Mg protein	50.71 ± 2.11	54.67 ± 1.06	51.86 ± 1.35	53.44 ± 4.31
SOD, mind Unit / mg protein	2.12 ± 0.07	2.06 ± 0.23	1.96 ± 0.46	1.89 ± 0.09
GPL, unit E / ml	6.67 ± 0.33	6.25 ± 0.34	6.44 ± 0.56	6.15 ± 0.16
TBK-active Products, nmol / ml	2.34 ± 0.23	2.77 ± 0.13	2.65 ± 0.24	2.25 ± 0.05

day. On the 7th day after injection of MP-2 and MP-3 polymers with asODN, minor changes in brain histology (decrease in the number of neurons), in the small intestine (destruction of enterocytes, dilatation of the serosa and enlargement of Peyer's plaques) and spleen Follicles, thinning of the capsule and trabeculae, trabecular veins and arteries are slightly differentiated). Complexes of asODN with the carrier of MP-27 on the 7th day after administration into the body did not cause changes in the pre-replicative organs of the rats[11].

Based on the results of Western blot analysis, it was found that the content of PrPC decreased by 46% in the intestines after application of the complexes with the MP-2 carrier 2 days later and 47% 7 days after the administration ($P < 0.05$). However, after the injection of asODN with MP-3, the decrease in total cellular prions was not so rapid (13% and 26%). But it was essential to reduce the content of PrPC in the intestine for the introduction of complexes of asODN with MP-27 polymers. Two days after the administration, the PrPC content decreased by 38%, and after 7 days by 55% compared to the control group (Fig. 3).

The content of the cellular prion in the spleen was reduced by 32% two days after the application of the asODN complexes with the MP-2 carrier. However, after 7 days the effectiveness of these complexes on the content of PrPC fell and fluctuated within

the limits of the control group (Fig. 4). With the introduction of asODN with polymer carrier MP-3, it was noted that the total PrPC content in the spleen decreased by 40% after 2 days and by 48% after 7 days. Analyzing the PrPC content diagram after injections of the asODN complexes with the MP-27 carriers, a decrease in the cellular prion content (by 9% after 2 days and 32% after 7 days) was also noted. Based on the results described in the works [12,13], where the authors described the ability of certain high-molecular polyamines to form complexes with pathological prions, one can assume the existence of a certain affinity between molecules of the physiological prion and polymers based on DMAEM, which may additionally influence the results of the studies.

Analysis of the results after the administration of the asODN complexes with the carriers MP-2 and MP-3 revealed that these polymers did not cause a decrease in the total content of PrPC in rat brain tissues. The reason for this is obviously the presence of the blood-brain barrier (BBB) in the central nervous system, because many water-soluble compounds do not overcome the BBB, which is an important problem in creating effective drugs against CNS diseases. Several excellent results were obtained after the use of asODN in combination with MP-27 polymers, namely: a 28% drop in PrPC content was recorded at 2 days and 34% at 7 days after injection (Fig. 5).

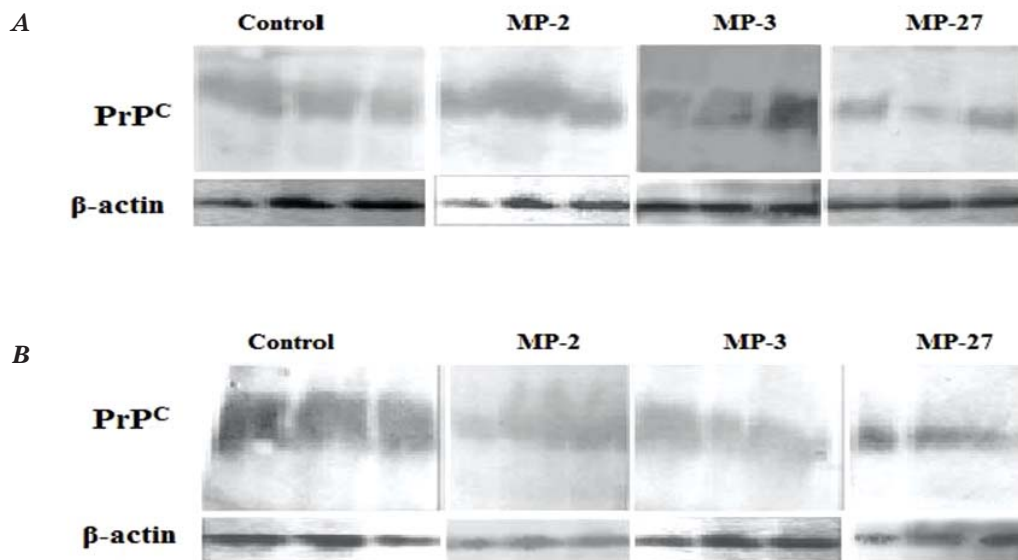


Fig. 3. The content of PrPC in the tissues of the small intestine of rats under the action of complexes of asODN with polymeric carriers based on DMAEM: hereinafter A — two days, B — seven days after the injection; $n = 3$

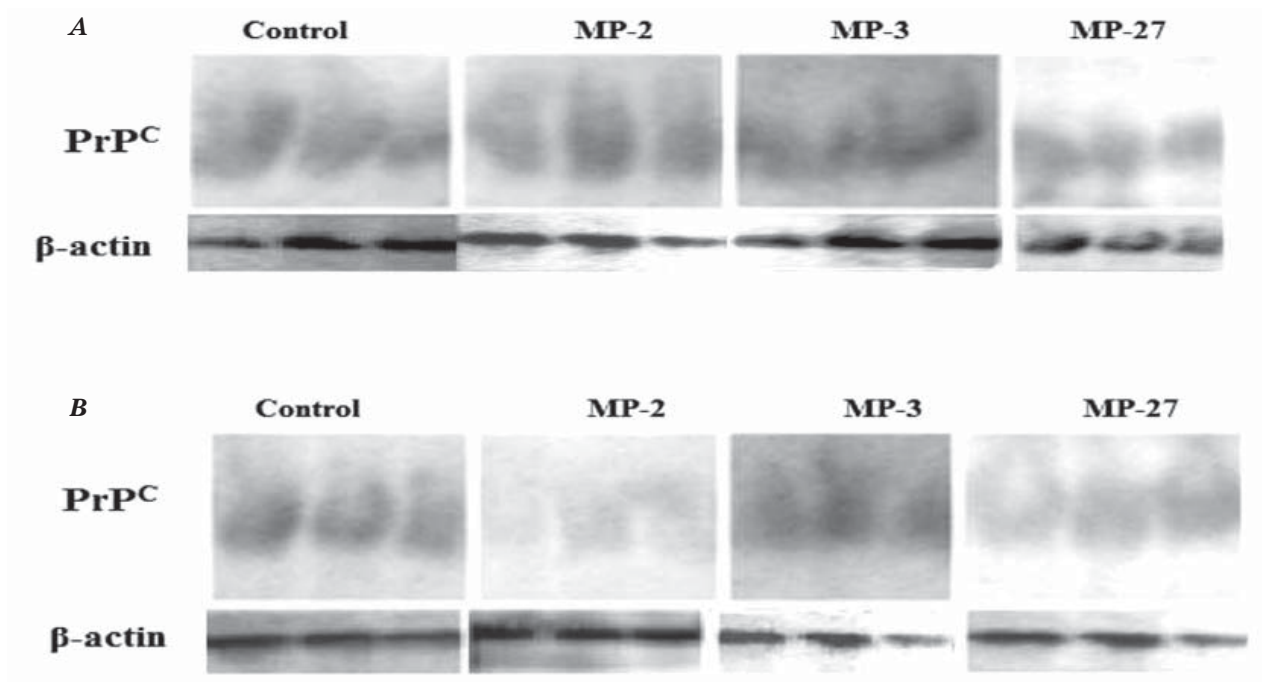


Fig. 4. The content of PrPC in the tissues of rat spleen for the actions of complexes of asODN with polymeric carriers based on DMAEM

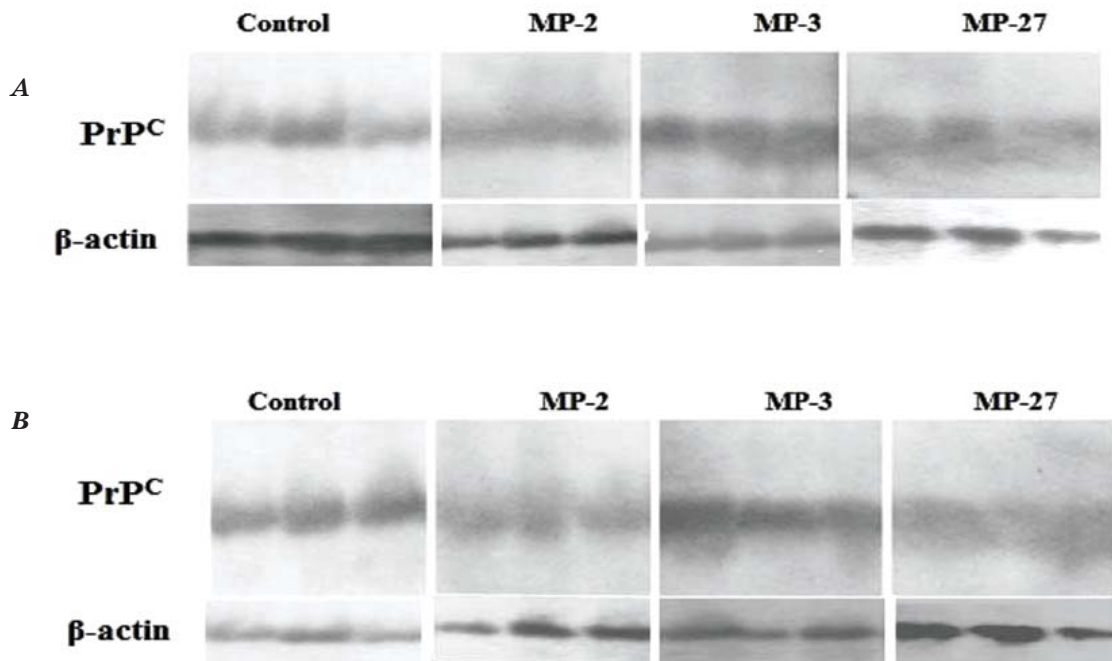


Fig. 5. The content of PrPC in the brain tissues of rats under the action of complexes of asODN with polymeric carriers based on DMAEM

We assume that these results also affect the property of the physiological prion in recircular motions and interaction with some negatively charged compounds. Due to endocytosis, the existing prion molecules are removed from the cell surface, and specific sequences of asODN that penetrate together with the protein into the cell, trigger the mechanism of suppressing the biosynthesis of new prion molecules.

So all newly synthesized polyDMAEM are able to bind and transport oligonucleotides. The introduction of polymers MP-2 and MP-3 leads to a decrease in hemopoiesis and a change in the percentage of leukocytes. Compared with the electrolyte carriers MP-2 and MP-3, the carrier MP-27 causes the least influence on blood indices in laboratory rats.

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**ПРИГНІЧЕННЯ ЕКСПРЕСІЇ
ФІЗІОЛОГІЧНИХ ПРИОНІВ
АНТИСЕНС-ОЛІГОНУКЛЕОТИДАМИ**

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Метою роботи було дослідити здатність нових комплексів специфічних одонитчастих олігонуклеотидів — асОДН — з полімерними носіями на основі диметиламінометилакрилату пригнічувати експресію фізіологічного приона. Встановлено, що введення комплексів новосинтезованих носіїв на основі диметиламінометилметакрилат-PEG-DMAEM-MP-27 (магнітні частинки) (MP-27), PEG-DMAEM-MP-2 (MP-2), PEG-DMAEM-MP-3 (MP-3) з асОДН в організм щурів призводить до зниження вмісту приона у тканинах селезінки і тонкого кишечника. Також було вивчено вплив комплексів нових носіїв на основі диметиламіноетилметакрилату — MP-27, MP-2 та MP-3 з асОДН на гематологічні та біохімічні показники крові щурів Вістар.

Ключові слова: прионні інфекції, антисенс-олігонуклеотиди, полімерні носії.

**УГНЕТЕНИЕ ЭКСПРЕССИИ
ФИЗИОЛОГИЧЕСКИХ ПРИОНОВ
АНТИСЕНС-ОЛИГОНУКЛЕОТИДАМИ**

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Целью работы было исследование способности новых комплексов специфических одонитевых олигонуклеотидов — асОДН — с полимерными носителями на основе диметиламинметилакрилата подавлять экспрессию физиологического приона. Установлено, что введение комплексов вновь синтезированных носителей на основе диметиламинметилметакрилат-PEG-DMAEM-MP-27 (магнитные частички) (MP-27), PEG-DMAEM-MP-2 (MP-2), PEG-DMAEM-MP-3 (MP-3) с асОДН в организм крыс приводит к снижению содержания приона в тканях селезенки и тонкого кишечника. Также было изучено влияние комплексов вновь синтезированных новых носителей на основе диметиламиноэтилметакрилата — MP-27, MP-2 и MP-3 с асОДН на гематологические и биохимические показатели крови крыс Вистар.

Ключевые слова: прионные инфекции, антисенс-олигонуклеотиды, полимерные носители.