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Targeted Transport of Low-Sialylated Recombinant Human Erythropoietin by Polymer Nanoparticles through the Blood–Brain Barrier

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Abstract—The research on the nansomal forms s of low-sialylated recombinant human erythropoietin (ls-rhEPO) gave evidence for the efficiency of the nanotechnological approach to targeted delivery of this protein to the brain of experimental animals. Nanosomal ls-rhEPO formulations were found to exhibit neuroprotector activity. The established ability of the nanosomal formulations of ls-rhEPO to enhance BDNF and NGF gene expression in animal brain is to a great extent responsible for the mechanism of the neuroprotector action of this protein.

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INTRODUCTION

Diseases associated with neurodegenerative processes, such as Alzheimer's and Parkinson's diseases, various dementias, traumatic and toxic brain injuries, and strokes are one of the leading reasons of people's disability and death. Even though a lot of drugs for correction of these states are available, the therapy is still not very efficient and not infrequently accompanied by severe impact on body's organs and systems.

The endogenous proteins neurotrophins, a class of peptide growth factors, and, in particular, the hematopoietic growth factor erythropoietin (EPO), attract considerable interest as neuroprotectors. In its chemical nature, human EPO is an acidic glycoprotein. Its protein part consists of 165 amino acids and is covalently bound to heterooligosaccharides. The total content of carbohydrates in the EPO molecule reaches 40–50%. Erythropoietin includes from 4 to 17 sialic acid residues which are responsible for the biological activity and stability of the molecule [1].

In medical practice, recombinant human EPO (rhEPO) is used in the therapy of anemias of different genesis to stimulate erythrocyte growth and differentiation. Moreover, EPO fulfills a broad spectrum of protective functions in the body, for example, it protects brain from injuries. Erythropoietin and its receptor are expressed in the central and peripheral nervous systems and take an active part in the embryonic development of mammals and humans [2]. As shown, the peripheral injection of EPO provides a good protection for the brain tissue injured in different ways due to activation of antiapoptic antigens, triggering of antioxidant and anti-inflammatory mechanisms in neurons and glial and cerebrovascular endothelial cells, as well as due to stimulation of neurogenesis [3, 4]. At the same time, EPO scarcely breaks through the blood–brain barrier, and, therefore, it exhibits useful properties only in doses 20–100 times higher than the therapeutic doses, which poses a high risk of thrombosis [5, 6].

To reduce complications caused by megadoses of rhEPO and also to prolong its effect, rhEPO derivatives with changed pharmacokinetic and pharmacodynamics properties were developed: low-sialylated, asialylated, and carbamylated rhEPO, darbepoietin (DEPO) and carbamylated DEPO, and mimetic peptides [7–9]. However, these derivatives have not found wide application in neurology because of their limited ability to break through the blood–brain barrier.

It is known that nanocarriers for targeting proteins to body's cells and tissues are quite difficult to

develop, because most proteins are unstable under nanosynthesis conditions. Homogenization, as well as contact with organic solvents may lead to denaturation of proteins and, as a result, to loss of their activity.

One of the ways to solve the problem of targeted delivery of pharmacologically active proteins can be provided by their incorporation into biodegradable polymer carriers [10]. We proposed a methodical approach involving EPO adsorption on polymer-based nanoparticles under optimal conditions favoring a high degree of protein binding to nanoparticles. Poly(butyl cyanoacrylate) (PBCA), polylactide (PLA), poly-lactide-co-glycolide (PLGA), albumin, and solid lipid nanoparticles can be used as carriers capable of breaking through the blood–brain barrier [11–15]. Previously rhEPO-loaded poly(butyl cyanoacrylate) nanoparticles [16] and polylactide-co-glycolide micro-particles [17] were shown to act as neuroprotectors. Microcapsulated rhEPO formulations with controlled release were developed [18]. As known, the adsorption capacity of polymer nanoparticles for a drug depends on a whole set of factors responsible for the affinity of the drug to the nanoparticle surface [19]. In particular, quite an important role belongs to an electrostatic drug–surface interaction. This interaction can be a key factor in the case low-syalated rhEPO (ls-rhEPO) which exhibits expressed acidic properties due to the presence of sialic acids in the terminal positions of the carbohydrate chains. Moreover, the binding between EPO molecules and nanoparticle surface can be stabilized by their hydrophobic interactions.

We present here a new nanosomal formulation of ls-rhEPO, an analog of brain erythropoietin (Neuro-EPO), developed on the basis of two polymer carriers: PLGA and PBCA, which transport this protein through the blood–brain barrier and have no impact on the hematopoietic function.

EXPERIMENTAL

Materials. The following materials were used in the work:

– low- and high-molecular homopolymers of lactic acid (Lactel, DURECT, USA);

– copolymers of lactic and glycolic acid with different lactic-to-glycolic unit ratios (Resomer, Boehringer Ingelheim, Germany);

– butyl cyanoacrylate (Sicomet 6000, Sichel-Werke, Germany);

– surfactants (Sigma, USA): poloksamer 188 (Pluronic F-68), polyvinyl alcohol (MW 30–70 kDa), dextran (70 kDa), and human serum albumin (HSA);

– other solvents and reagents from Sigma (USA);

– ls-rhEPO (Protein Contour, Russia).

Methods of synthesis and measurements. *Synthesis of polylactide nanoparticles*. The high-pressure emulsification–solvent evaporation technique described in [20] was used. A solution of the polymer (250 mg) in dichloromethane (5 mL) was added to 25 mL of a 0.5–1% aqueous solution of PVA or HSA, and the mixture was homogenized on an Ultra-Turrax T-25 homogenizer (IKA, Germany) for 2 min at 23600 rpm and then on an EmulsiFlex C-5 homogenizer (Avestin, Canada) for 5 min at 20000–25000 lbf/in^2. Dichloromethane was removed from the resulting emulsion in a vacuum using a Rotavapor R-210 rotary evaporator (Buchi, Germany), a cryoprotectant (mannite, 1% solution) was added to the residue, and the mixture was dried on an Alpha 2–4 LSC freeze drier (Martin Christ, Germany).

Synthesis of poly(butyl cyanoacrylate) nano-particles. The anionic polymerization technique was used. Butyl cyanoacrylate was added to a vigorously stirred 1% solution of dextran in 0.001 N HCl. The mixture was stirred for 1 h, after which it was neutralized with 0.1N NaOH to pH 5.6 and filtered through a porous glass filter (pore size 10 μm) to remove agglomerates formed on polymerization. Mannite was added to the resulting suspension, after which it was freeze-dried.

The size and surface charge (ζ-potential) of nanoparticles were determined using a nanosizer (Malvern Instruments, Great Britain). To remove surfactants, freeze-dried nanoparticles (from a 1-mL volume) were resuspended in 1 mL of distilled water. The suspension was vortexed (VWR, Germany) for 1 min, and nanoparticles were separated on an AvantiJ-30I centrifuge (Beckman Coulter, USA; 30 min, 48384 g, 4–8°C). The supernatant was re-moved, and the nanoparticle precipitate was used for ls-rhEPO sorption. To this end, 1 mL of a solution of the protein (0.60 mg/mL) was added to 10 mg of freeze-dried or washed-off nanoparticles, the mixture was suspended, and the suspension was magnetically stirred (250 rpm) at room temperature for 1 h.

The degree of ls-rhEPO sorption by nanoparticles was determined by UV spectroscopy using a

calibration curve (λ_{max} = 280 nm). A suspension of nanoparticles with the protein was centrifuged (30 min, 48384 g, 4–8°C), and the supernatant was used to measure the free protein concentration (C_{free}). The degree of sorption α was calculated by the formula:

$$\alpha = [(C_{init} - C_{free})/C_{init}]\times 100\%,$$

where C_{init} is the initial protein concentration.

Modeling of post-traumatic brain hematoma. White outbred male rats (200–220 g) were used in biological experiments. Animals were kept in a special room with natural lighting, they had free access to food and water.

The antistroke and neuroprotector properties of the preparations were studied on the model of autohemorrhagic left hemisphere stroke [21, 22] in the internal capsule area. Animals were anesthetized with chloral hydrate (dose 400 mg/kg). False-operated animals were subjected to scalping and craniotomy.

The action of ls-rhEPO sorbed on dextran-stabilized PBCA nanoparticles (ls-rhEPO–PBCA) and on 1% HSA–stabilized PLGA nanoparticles (ls-rhEPO–PLGA). Native EPO (Protein Contour, Russia) was used as reference. The protein and its nanosomal forms were injected intravenously at a dose of 0.05 mg/kg. The first injection was performed 3.0–3.5 h after operation and postanesthetic recovery. Repeated daily injections were performed at the 2nd and 3rd days after operation. The false-operated and control animals with hemorrhagic stroke were saline injected by the same scheme. The dynamics of development of brain hematoma was studied at the 1st, 3rd, and 7th day after animal's death.

Cell morphology was studied on an Olympus BX 51 microscope (Japan) using sections (10 μm) of the sensomotor cortex, which were dewaxed and stained with hematoxylin and eosin. In the quantitative analysis of changes in the brain neocortex of animals, the following morphological parameters were assessed: mean neuronal core and body area, total number of neurons per unit area (1 mm^2), and number of degenerated neurons (cytolysis, karyolysis, or karyopyknosis). The differences between groups were assessed statistically (Student's *t*-test, $p < 0.05$, confidence interval > 95%).

The effect of EPO nanoformulations on hemogenesis was studied by a standard microscopy-based procedure involving scoring of micronucleated reticulocytes [23]. Eight groups of animals (N = 6) were used. The rhEPO–PBCA or rhEPO–PLGA formulation, Eralfon (Sotex PharmFirm, Russia) as reference, and 0.9% saline were injected either intraperitoneally or in the tail vein over the course of 3 days. Two days after the last injection (the time required to reach the highest blood reticulocyte level) blood samples were taken from the tail vein, and smears stained with Brilliant Cresyl Blue were prepared for microscopic examination.

Determination of EPO levels in the brain of experimental animals. Low-sialylated rhEPO and its nanosomals formulations were injected in the tail vein of mice at a dose of 0.5 mg/kg. Forty five minutes after injection, the animals were killed, and their circulatory system was washed with saline to exclude the effect of substances contained in the blood stream on the results of analysis. The brain was removed from the animals *ex tempore* and frozen using dry ice of food quality. The samples were stored at –76°C. Before use they were defrosted on a water bath at 35°C, placed into a freshly prepared cold (4°C) extraction lysis buffer and homogenized in a glass–Teflon homogenizer. After incubation (15 min at 4°C), the homogenates were centrifuged (16000 g at 4°C) for 30 min. Samples of the supernatant were used to determine the protein concentration by immunoenzyme analysis.

Study of the involvement of the brain-derived neurtropic factor (BDNF) and nerve growth factor (NGF) in the mechanism of the neurotopic action of the rhEPO nanoformulations. The BDNF and NGF mRNA levels in rat brain were measured by means of real-time quantitative PCR (M×3000P system, Stratagene) [24]. One hour after intravenous injection of the substances (dose 0.05 mg/kg), rats were decapitated to remove the hippocampus and frontal cortex. The total RNA was isolated by phenol–chloroform extraction using the PeqGold TriFast reagent (PeqLab, Germany). The reverse transcription was performed using a kit from Sileks (Russia). The PCR was performed by a standard protocol using a highly specific dsDNA-binding SYBR Green I stain. The reaction was performed in a system containing the cDNA of the sample, or the reference, or water (negative control), DNTP mixture, $MgCl_2$, solution, tris–HCl buffer (pH 8.8), KCl solution, glycerol, Tween-10 (polysorbate), intercalating SYBR Green I stain, and Taq DNA polymerase (1 unit/μL) with inhibiting antibodies (Sintol, Russia), and sense and antisense primers (Evrogen, Russia). Reaction conditions: start 15 min at 95°C and then 40 cycles including melting (30 s, 95°C), annealing (45 s), and

Table 1. Physicochemical characteristics of ls-rhEPO-loaded nanoparticles

Polymer	Surfactant	Average size, nm	ζ-Potential, mV	Polydispersity	Degree of sorption, %
PLA (ν = 0.34 g/L)	PVA 0.5%	191.9±2.05	–18.9±2.05	0.103±0.02	25.67±2.6
PLA (ν = 0.68 g/L)	PVA 0.5%	203.3±1.2	–23.8±2.34	0.086±0.02	23.4±.0
Resomer 502H	PVA 1%	140.9±0.47	–5.04±0.22	0.101±0.01	69.4±.2
Resomer 502H	HSA 1%	90.09±0.46	–30.1±1.56	0.216±0.01	81.07±8.1
Resomer 752 H	HSA 1%	93.42±0.73	–24.4±2.3	0.196±0.01	60.18±6.0
PBCA	D-70 1%	165.5±1.44	–1.46±0.27	0.107±0.023	77.2±6.0

elongation (45 s, 72°C) with fluorescence measurements at the end of every elongation step.

To confirm specificity of the amplification products, the samples were cooled to 60°C and then heated to 95°C at a rate of 0.03°C/s with continuous fluorescence detection to obtain melting curves. The concentration of the amplification products was determined by the calibration curve with subsequent normalization by *b*-actin.

Statistic treatment of the results was performed using Statistica III software with Student's *t*-test and Fisher's exact test.

RESULTS AND DISCUSSION

The physicochemical parameters of the synthesized polymer nanoparticles, as well as the degrees of ls-rhEPO sorption on them are listed in Table 1. The resulting data give no evidence for a direct correlation between the efficiency of protein sorption and the surface charge of nanoparticles. However, the data in Fig. 1 reveal a clear correlation between sorption efficiency and the structure of the polymer and the type of the stabilizer. Thus, preliminarily separation of PVA from nanoparticles increased the sorption efficiency, whereas washing off HAS much decreased it.

Survival dynamics study showed that by the 7th day of observation all false-operated rats survived, whereas the survival rate in the group of rats with brain hematoma was as low as 40% (Fig. 2). The survival rate on the background of repeated 3-day injection of ls-rhEPO–PLGA was 77.8% by the end of the experiment, which is 40% higher than in the control group with hematoma. In the case of ls-rhEPO–PBCA, 75.5% of rats survived by the end of the experiment, which is 35.5% higher than in the control group with hematoma. The native protein had almost no effect on the survival rate of rats with hematoma, presumably

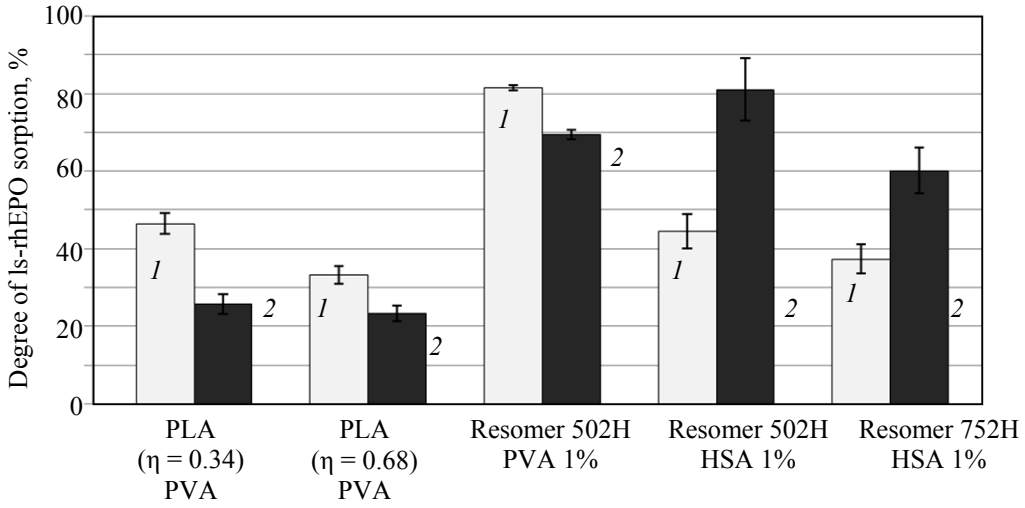

Fig. 1. Effect of the free surfactant in the incubation medium on the degree of ls-rhEPO sorption on different types of nanoparticles. (*1*) Free surfactant was washed off from nanoparticles and (*2*) free surfactant was not washed off from nanoparticles.

implying that the injected dose is too low to reach the therapeutic concentration in the brain and to exert the neuroprotective effect.

Examination of the frontal sections of the sensorimotor neocortex of animals with brain hemorrhage revealed multiple pericellular and perivascular edemas. The most expressed was the perihematomal edema. Reliable evidence was obtained showing that brain hematoma leads to a decrease of neuron density in the brain cortex. Recombinant human EPO sorbed on polymer nanoparticles increased survivability of pyramidal neurons of the brain cortex. An increase in the number of neurons in the ipsilateral hemisphere and a decrease in the neuron death rate in the contralateral hemisphere were established with confidence (Table 2).

As known, ls-rhEPO scarcely affects hemogenesis because of the short plasma half-life of ls-rhEPO [25]. Presumably, nanoparticles are capable of stabilizing the protein molecule in some way, thus favoring hemogenesis and, as a consequence, thrombogenesis, which poses a risk of brain hemorrhages. However, according to the results of blood reticulocyte scoring (Fig. 3), rh-EPO (Eralfon, used for anemia correction) enhances hemogenesis upon intravenous and intraperitoneal injection at a dose of 5000 ME, whereas nanosomal EPO formulations in the equivalent dose caused no statistically significant increase in the number of young blood cells in rats compared to the control group.

Table 2. Effect of ls-rhEPO and its nanosomal formulations on the morphometry parameters of the post-traumatic brain hematoma on brain hemorrhage in rats

Rat group	Volume of the brain damage zone, mm^3	Volume of the brain hemorrhage, mm^3
False-operated animals (control)	0.0	0.0
Brain hemorrhage (control)	6.4±0.07	56.9±0.3
ls-rhEPO injection, 0.05 mg/kg, i/v	5.9±0.06	44.3±1.9
ls-rhEPO−PLGA injection, 0.05 mg/kg, i/v	5.8±0.09	23.4±2.1[a]
ls-rhEPO−PBCA injection, 0.05 mg/kg, i/v	5.4±0.05	22.9±1.1[a]

[a] At $p \le 0.05$ (Fisher's exact test).

The results of analysis of rh-EPO in mouse brain tissue samples taken 45 min after intravenous injection of the tested substances and their combinations showed a statistically significant (3 times, $p < 0.01$) increase of the protein level in the central nervous system of mice given ls-rhEPO-loaded polymer nanoparticles compared to the control group (animals given a 0.9% NaCl solution) (Fig. 4). In the group of animals given ls-rhEPO doped with polysorbate 80 (PS 80), an increased protein level in mouse brain tissues was

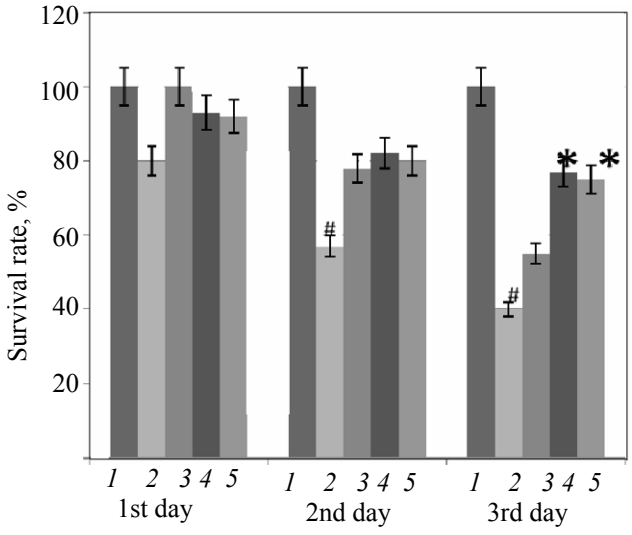

Fig. 2. Effect of ls-rhEPO formulations on the survival rate of rats with post-traumatic brain hematoma: (*1*) control, false-operated animals (FO) ($N = 10$); (*2*) control, rats with rats with post-traumatic brain hematoma (PTBH) ($N = 20$); (*3*) ls-rhEPO, 0.05 mg/kg, i/v ($N = 19$); (*4*) ls-rhEPO−PLGA, 0.05 mg/kg, i/v ($N = 18$), (*5*) ls-rhEPO−PBCA, 0.05 mg/kg, i/v ($N = 18$). (#) Significance of the deviation from FO, $p < 0.05$ (Fisher's exact test); (*) significance of the deviation from the group of rats with PTBH, $p < 0.05$ (Fisher's exact test).

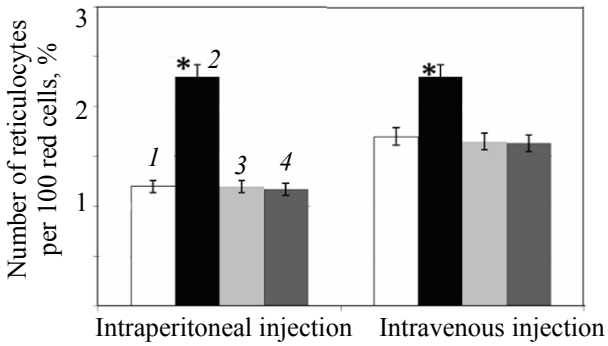

Fig. 3. Results of reticulocyte scoring in rats after injection of EPO formulations. (*) Significance of the deviation from the control group, $p < 0.01$ (Student's *t*-test). (*1*) Saline; (*2*) eralfon, 5000 ME; (*3*) ls-rhEPO−PLGA (0.05 mg/kg); (*4*) ls-rhEPO−PBCA (0.05 mg/kg).

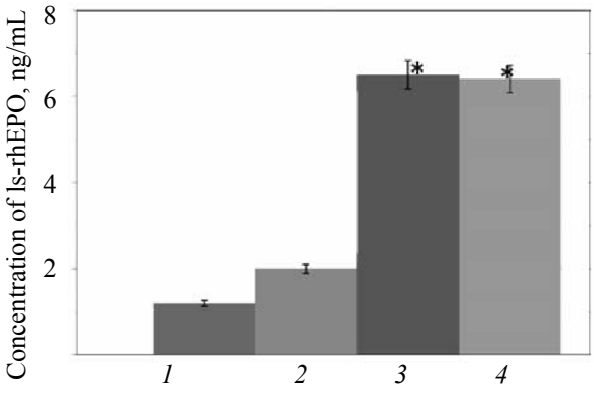

Fig. 4. Concentration of ls-rhEPO in the brain of mice after injection of EPO formulations: (*1*) saline, (*2*) ls-rhEPO–PS-80; (*3*) ls-rhEPO−PBCA; (*4*) ls-rhEPO−PLGA. (*) Statistically significant deviation from the control group, $p < 0.01$ (Student's *t*-test).

revealed but this result could not be considered statistically significant. Thus, the results of our research provide evidence for the ability of polymer nanoparticles to deliver ls-rhEPO through an undamaged blood–brain barrier upon intravenous injection.

According to [26, 27], one of the mechanisms underlying the neuroprotector effect of EPO is activation of neutrophin genes, specifically NGF and BDNF. Our research showed that ls-rhEPO-loaded polymer nanoparticles can break through the blood–brain barrier and act as neuroprotectors. The results of study of the effect of the new rh-EPO formulations on neutrophin gene expression confirmed the "neutrophin" theory of the mechanism of EPO action. Maintaining the viability of neurons is a BDNF dependent process involving glial cells. It can be suggested that the enhancement of BDNF and NGF expression is one the principal molecular mechanisms underlying the therapeutic effect of rhEPO.

In vivo testing of the ability of native ls-rhEPO, as well as ls-rhEPO–PBCA and ls-rhEPO–PLGA to regulate BDNF and NGF gene expression in the frontal cortex and hippocampus of rats showed that the sorbed protein at a dose of 0.05 mg/kg statistically significantly increases the levels of BDNF and NGF mRNAs in the tissue homogenates of the frontal cortex 3.5 and 3.3 times and 1.9 and 1.7 times, respectively (Fig. 5a). According to the results of assessment of BDNF and NGF gene expression in rat hippocampus under the action of ls-rhEPO–PBCA and ls-rhEPO–

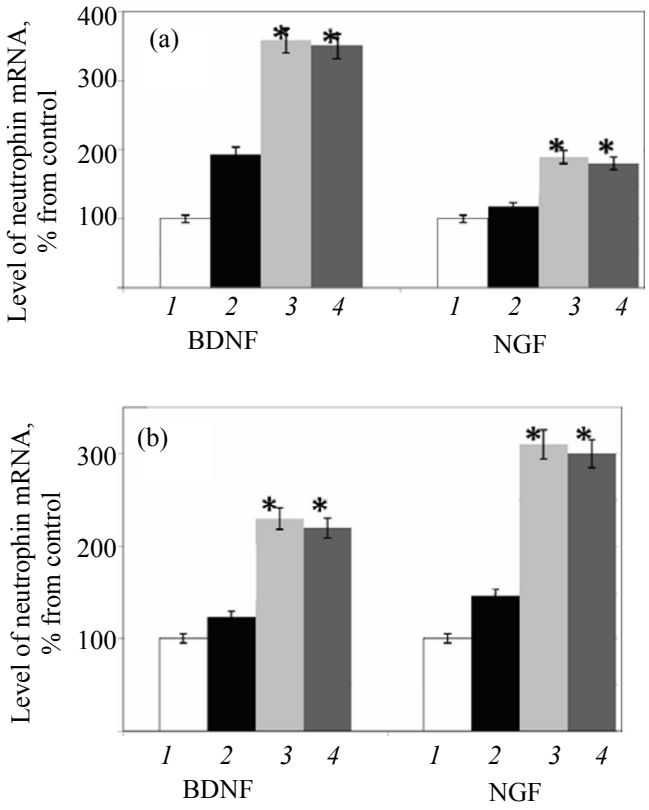

Fig. 5. Effect of ls-rhEPO and its nanosomal formulation on BDNF and NGF gene expression in the (a) frontal cortex of the brain and (b) hippocampus of rats 1 h after intravenous injection: (*1*) saline; (*2*) ls-rhEPO, 0.05 mg/kg; (*3*) ls-rhEPO−PLGA, 0.05 mg/kg; (*4*) ls-rhEPO−PBCA, 0.05 mg/kg. (*) Statistically significant deviation from the group given ls-rhEPO, $p < 0.01$ (Student's *t*-test).

PLGA, the BDNF mRNA level increases 2.3 and 2.1 times compared to control, and the NGF mRNA level increases 3.1 and 3.0 times ($p \leq 0.05$) (Fig. 5b). A considerable increase of BDNF and NGF gene expression in the hippocampus which plays a particular role in neurodegenerative diseases is observed exclusively with nanosomal ls-rhEPO formulations.

CONCLUSIONS

The pharmacological, biochemical, and histological research confirmed the ability of poly(butyl cyanoacrylate) and polylactide-co-glycolide nanoparticles to ensure transport of ls-rhEPO through the blood–brain barrier. However, a final choice of the carrier for ls-rhEPO targeting to the brain can only be made after the safety profile of the protein–nanocarrier complex has been studied in sufficient detail.

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