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Search of new pharmaceuticals on the basis of darbepoetin in the treatment of ischemic stroke (review of literature) / K.M. Reznikov, N.S. Gorbunova, P.D. Kolesnichenko, A.V. Tverskoy, D.A. Kostina, D.A. Bashkatova, V.A. Nikitina // Research result: pharmacology and clinical pharmacology. – 2017. – Vol. 3, №1 – P. 125-136.

Рус.

UDC 615.036.8 **DOI: 10.18413/2500-235X-2017-3-1-125-136**

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SEARCH OF NEW PHARMACEUTICALS ON THE BASIS OF DARBEPOETIN IN THE TREATMENT OF ISCHEMIC STROKE (REVIEW OF LITERATURE)

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Abstract. The article contains the analysis of medical and biological publications from the global database created by the National Centre for Biology Information (NCBI), an intramural biotechnological division of the US National Library of Medicine. The authors have analyzed publications of the recent ten years. Major results of study of erythropoietins and their recombinant analogues have been generalized and systematized. There has been revealed the significant potential of the preparations of this group to be studied and used. Major advantages and drawbacks of erythropoietins and their recombinant analogues have been described. It has been pointed out that a great variety of erythropoietins and darbepoetins speaks to the fact that there is lack of "universe" erythropoietin meeting all the requirements. Genetically modified erythropoietin having both – pharmacokinetics convenient for clinical application and all properties of the natural analogue – is considered to be the most successful darbepoetin. This property has been resulted from the fact that comparing to standard erythropoietin darbepoetin has bigger molecular weight due to introduction of 2 complementary sites of glycosylation. This, in turn, results in the increase of half-life period and, consequently, decreases application frequency of the preparation that makes it more convenient to use comparing to erythropoietin. Application of erythropoietin and its derivatives in stroke therapy in experimental animal models undoubtedly has positive impact on the reduction of the infarction size and dynamics of recovery of neurological status. Results of the analysis demonstrate that the studied preparations are more effective in the early period following stroke than being applied in the later hours. However, there has been revealed some insufficient knowledge in treatment of brain ischemic lesions and ischemic heart disease.

Key words: erythropoietin, darbepoetin, stroke.

Introduction. Medicine is considered to be actively developing science judging from the dynamics of information accumulation.15% of medical database is renewed every year. This means that in the period less than 7 years practically all medical paradigms are being revised. The fact should be taken into consideration that medical practitioners are being trained for 7-8 years and 2-3 years are necessary to revise, write and publish learning material. Thus, it appears that young health care workers start their practice having knowingly obsolete knowledge and to be updated a young specialist should spend a lot of time studying incoming information. Needless to say, young medical workers have a lot of difficulties learning all new publications appearing during a year. Thus, currently publication of review articles is essential to guide in the actual information flow of periodic.

The objective of this article is to summarize the most interesting experimental models and research results carried out for the last ten years to search new preparations on the basis of darbepoetin for the ischemic stroke therapy.

Materials and methods. To analyze research articles the authors used global database of medical and biological publications in English language created by the National Centre for Biotechnology Information (NCBI), an intramural biotechnological division of the US National Library of Medicine, which houses the most updated and complete articles on the subject of the review. There have been collected 2386 publications regarding erythropoietin in the text database on

biotechnological information (NCBI) for the last ten years, almost a thousand more – regarding its recombinant analogue darbepoetin. This statistical fact alone supports essential medical significance of erythropoietins. Notable interest has been registered in the study of neuroprotective effect of erythropoietin and its analogues for the recent decades [1]. For more suitable analysis the authors of the article have designed a table, where methods and results of the investigational trials are presented concisely and comprehensibly. The Table contains research results of 11 studies demonstrating various models of brain damages, describes in details ways and methods of introduction of the investigated preparations and shows data that prove efficiency of application of erythropoietin, as well as its analogues, in various forms [2].

The advantage of application of erythropoietin and its derivatives in the therapy of brain damages of diverse genesis is considered to be the fact that erythropoietin is a cytokine, which is produced inside the body and its production is genetically determined. A stimulus for erythropoietin synthesis is reported to be decrease of the oxygen content in cells resulting in the formation of hypoxia- inducible factor-1(HIF-1) followed by mRNA and, actually, erythropoietin production [3, 4]. An erythropoietin gene contains a complementary HIF-1a region; binding to it triggers transcription of mRNA erythropoietin. HIF-1 expression is defined in cells just in 30 minutes after hypoxia has started. The major application point of erythropoietin action is granulocytic-monocytic-megakaryocytic-erythrocytic burst and colony forming units that have specific receptors. Erythropoietin is responsible for proliferation, differentiation and inhibition of apoptosis in these cells; decreased apoptosis of bone marrow erythroid progenitor cells being the major effect of erythropoietin in these conditions. Erythropoietin takes an effect through surface receptors that are amounted no more than 1000 per one cell. Receptors to erythropoietin are found in the cells of the nerve tissue, ovaries and testes, uterus, in the vascular smooth muscle cells, cardiomyocytes, endotheliocytes, lung and renal tubules epithelium. These cells are not only able to express erythropoietin receptors; some of them are capable to synthesize erythropoietin itself. Presence of these potencies allows assuming that erythropoietin performs some functions different from hematopoietic function [5]. Due to this fact, a number of investigational trials on animals, which support positive effect of erythropoietin and its analogues on the recovery of the brain functions after damages of various etiologies, have increased. The objective of this article is to review the most interesting experimental models and results of the research studies carried out for the last ten years.

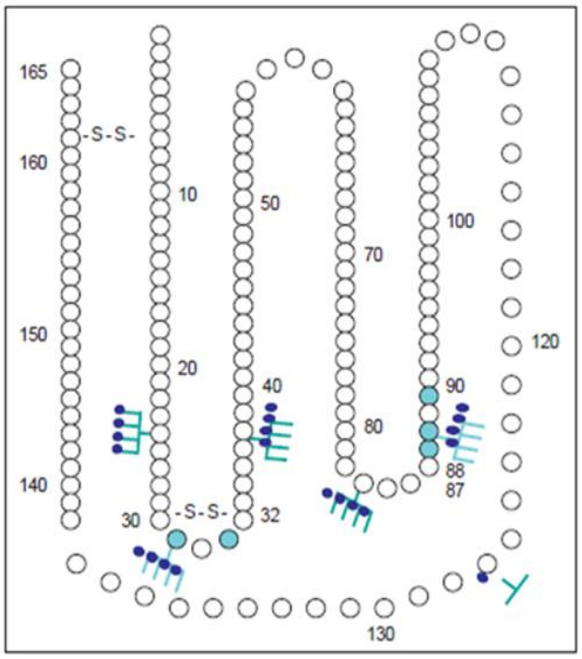


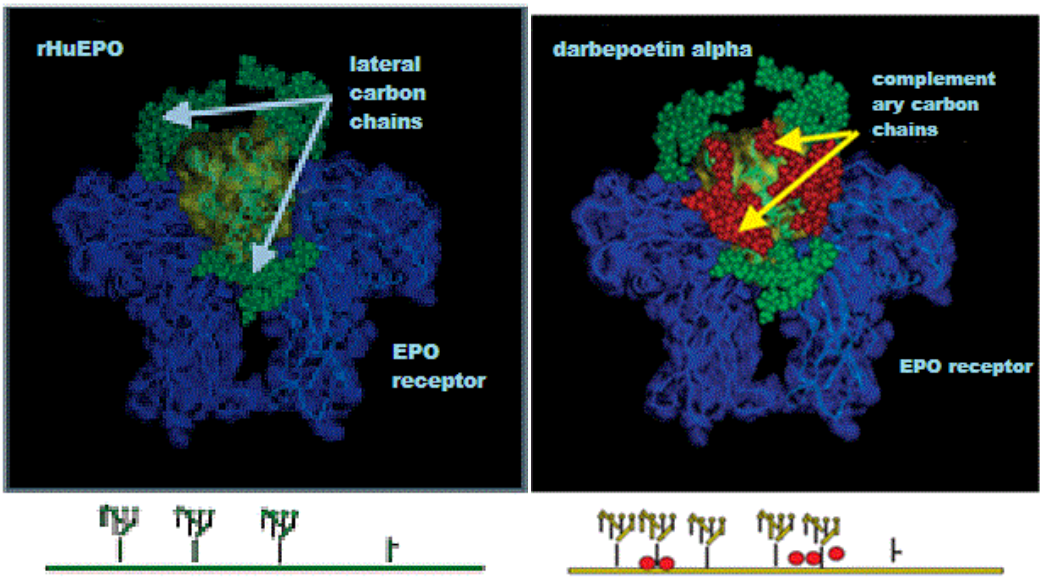
Figure 1. Amino acid sequence of the darbepoetin alpha molecule (replacement of 5 amino acids in polypeptide chain by the method of site-directed mutagenesis allowed creating 5 sites of glycosylation, two of which are in positions 30 and 80). Adapted from Lin F-K et al. Proc Natl Acad Sci USA. 1985; 82: 7580-7584 and Elliot S. et al. Nature Biotechnol. 2003; 21: 414-421.

Erythropoietins of the first generation, recombinant human erythropoietin (rHu-EPO) alpha and beta, were identical to native erythropoietin (EPO) in their chemical structure and represented glycoproteid with molecular weight 30.4 kDa. The structure of an EPO molecule includes a single polypeptide chain comprising 165 aminoacids that is subjected to glycosylation with complementation of 4 complex N-linked hydrocarbon chains (replacement of 3 asparaginic and 1 serine regions); they have several anionic free sialic acid residues determining EPO activity. It appears that not a single homogeneous molecule but a mixture of various isoforms specified on the number of free sialic acid residues is developed. This is associated with the fact that glycosylation of rHu-EPO is a posttranscriptional process and is not under the same strict genetic control as rHu-EPO mRNA translation. N-linked carbon side-chains pre-synthesize with various enzymes and are available for post-translational complementation to polypeptide rHu-EPO. Each isoform has its own bioactivity. Isoform 14 has the most erythropoietic activity. On the other hand, isoforms with the less number of sialic acid residues has more EPO receptor (EPOR) affinity, but a shorter period of circulation. Purified alpha and beta EPO

consist of a mixture of isoforms from 9 to 14. Alpha and beta erythropoietins have a relatively short half-life period that necessitates their introduction into the correction phase as often as 3 times a week, and subcutaneous introduction once a week is allowed only in the maintenance phase of treatment [6, 7].

It is not unexpected that for a long time efforts of clinicians and pharmacologists were aimed at the development of preparations of the new generation with a longer half-life period; that would allow application of more convenient schemes of their introduction (once a week and even once in two weeks). A usual strategy of inhibiting rate of elimination of biomolecules lies in their pegylation, dimerization or synthesis of protein and polypeptide elements [8, 9].

When creating darbepoetin there was applied a novel approach generally aimed at the increase of activity and reduce of clearance including directed re-glycosylation-attachment of 2 complementary N-linked hydrocarbon regions with active sialic residues to a base EPO molecule, so termed "glycoengineering", or site-directed mutagenesis. As a result there was developed a principally new darbepoetin alpha molecule with a weight up to 37.1 kDa having 5 glycosylation regions, and a number of free sialic groups were adjusted to 22. Darbepoetin alpha has less receptor affinity than EPO alpha and beta that is outweighed by the significantly bigger activity and long half-life period. As EPO alpha and beta preparations darbepoetin is produced by ovarian cells of the Chinese hamster subjected to the incorporation of a darbepoetin gene. Amino acid sequence of darbepoetin differs from that of human EPO in 5 positions, the fact that allows attaching complementary hydrocarbon branches to asparaginic residues in positions 30 and 88 without the destruction of total molecule conformation. Thus, darbepoetin differs from EPO by the high content of carbons and sialic residues, higher molecular weight and an increased negative charge [5, 10, 11].



- 3 N-linked hydrocarbon chains
- Up to 14 sialic acid residues
- 30.400 daltons
- Hydrocarbon content up to 40%

- 5 N-linked carbon chains
- up to 22 sialic acid residues
- 37.100 daltons
- hydrocarbon content up to 52

Figure 2. Comparing structures of darbepoetin alpha (on the right) and rHuEPO (on the left) molecules. Arrows show 2 complementary tetra-antenna N-linked carbon chains that led to more erythropoietic activity of darbepoetin due to prolongation of half-life period (explanations are given in the text). Adapted from Sinclair AM, Elliot S, 2005

This provides proliferation, differentiation and survival of cells of erythroid lineage. In spite of the decreased receptor affinity darbepoetin has bigger biological activity and approximately tripled half-life period than EPO alpha [5, 12]. As stated above, darbepoetin has a prolonged period of half-life and inhibited clearance comparing to rHuEPO. Exact mechanisms explaining inhibited clearance of darbepoetin alpha and its metabolism are not completely investigated. Decreased EPO level in blood is described by a double decaying exponential; the first phase of rapid decrease may be conditioned

by binding with endothelial and erythroid cells. When liver, kidneys and bone marrow are considered as possible sites of EPO and darbepoetin degradation, the primary element of its metabolism is erythropoietic tissue through the mechanism of EPO-receptor-inducible uptake. In this case difference between rapid elimination of de-sialylated EPO and inhibited clearance of darbepoetin is caused by the difference in EPO-receptor affinity [13].

Table 1

Comparative characteristic of rHuEPO alpha and beta and darbepoetin alpha (adapted from Deicher R, Horl WH, 2004)

	Epoetin-α	Epoetin-β	Darbepoetin-α
Hydrocarbons content, %	40	40	52
Number of N-linked carbon chains	3	3	5
Half-life period, hours:intravenously, subcutaneously	4-11 19-25,3	8,8-10,4 24	18-25,3 48,8
Bioavailability (subcutaneously), %	30-36	15-50	37
Clearance (intravenously), ml/hour × kg	8,1-8,6	7,9	2,0
Frequency of introduction (number of times a week)	1-3	0,5-3	0,25-1

Table 2

Application of erythropoietin and its analogues in various models of the brain damage.

Author and date of publication	Medicinal product	Reference medicinal product	Model of stroke	Method, dosage and time of introduction of medicinal product	Research results
1	2	3	4	5	6
Ludmila Belayeva; Larissa Khoutorova; Weizhao Zhao; Alexey Vigdorchik; Andrey Belayev; Raul Busto; Ella Magal; Myron D. Ginsberg 2006 [14]	Darbepoetin alpha	Human serum albumin	Right middle cerebral artery occlusion was performed by ligation of this vessel during two hours; the method of intraluminal introduction of poly-L-lysin coated suture was used. Suture material was introduced retrogradely into the right external carotid artery, then into the internal carotid artery and middle cerebral artery at the distance of 20-22 mm from bifurcation.	Darbepoetin alpha dosed 10mkg/ kg was introduced intraperitoneally at the moment of re-perfusion, i.e. in two hours after the onset of middle cerebral artery occlusion. Human serum albumin (0.25%) 10mkg/ kg	Animals were divided into four groups: 1. three-days survival value (darbepoetin alpha n=8) 2. three-days survival value (reference medicinal product n=6) 3. two-weeks survival value (darbepoetin alpha n=8) 4. two-weeks survival value (reference medicinal product n=6) Neurologically significant improvement was in animals receiving reference medicinal products during the first hour after re-perfusion. Histologically areas of cerebral ischemia were significantly less in animals receiving darbepoetin comparing to animals receiving reference medicinal products: • In group 3 (two-weeks survival value) – 28,5±14,1 against 68,0±4,5 mm^3 • In group 1 (three-days survival value) – 28,9±5,3 against 46,4±5,3 mm^3 At that no significant difference in animals between groups were registered.

Table 2 (continued)

1	2	3	4	5	6
Wang Y.; Zhang Z.G.; Rhodes K.; Renzi M.; Zhang R.L.; Kapke A.; Lu M.; Pool C.; Heavner G.; Chopp M. 2007 [15]	Recombinant human erythropoietin	Carbamylated recombinant human erythropoietin	Middle cerebral artery occlusion was performed by vessels embolization.	Recombinant human erythropoietin was introduced in doses 500; 1150 and 5000IU/ kg^2 in 6, 24 and 48 hours after middle cerebral artery occlusion. Carbamylated recombinant human erythropoietin was introduced in doses 50 mkg/ kg^2 in 6, 24 and 48 hours after middle cerebral artery occlusion.	Neurological status of the studied animals was evaluated on the 7th, 14th, 21st and 28th days. Histological examination of the brain was performed in 28 days. Neurological status carbamylated recombinant human erythropoietin was introduced in doses 50 mkg/ kg^2 of animals receiving for 28 days was better comparing to the group of animals receiving recombinant human erythropoietin was introduced in doses 500; 1150 and 5000IU/ kg^2. Histologically the size of ischemia in the cortical area was less in animals receiving recombinant human erythropoietin in doses 500; 1150 and 5000IU/ kg^2 or carbamylated recombinant human erythropoietin in doses 50 mkg/ kg^2 (26% and 30% for recombinant human erythropoietin in doses 500; 1150 and 5000IU/ kg^2 respectively, and 36% for carbamylated recombinant human erythropoietin in doses 50 mkg/ kg^2). recombinant human erythropoietin in a dose 5000IU/ kg^2 significantly decreases ischemia area not only in cortical, but also in sub-cortical layer by 22% and 36% respectively.
Chrystal D Price; Zhongjin Yang; Rachel Karlnoski; Dipak Kumar; Raphael Chaparro; Enric M Gampores 2009 [16]	Asialoerythropoietin	Physiological saline solution	Middle cerebral artery occlusion was performed by vessels embolization.	Rats from the experimental group were introduced asialoerythropoietin in a dose 20 mkg/ kg, 1 mcl/ hour for 24 hours. Rats from the group of comparison were given physiological saline solution in a dose 1 mcl/ hour for 4 days.	The size of cerebral ischemia was assessed by the amount of apoptotic cells and concentration of activated caspase-3 and 9 in the area of penumbra on the 4th day. The size of ischemia significantly decreased in rats of the experimental group comparing to rats from the group of comparison (168±19 mm^3 against 249±28 mm^3), the amount of apoptotic cells and concentration of activated caspase-3 and 9 was also significantly less in the experimental group.
Reitmeir R.; Kilic E.; Kilic U.; Bacigaluppi M.; El Ali A.; Salani G.; Pluchino S.; Gassmann M.; Hermann D.M.	Recombinant human erythropoietin	Physiological saline solution	Middle cerebral artery occlusion was performed by vessels embolization.	The animals of the experimental group were dosed recombinant human erythropoietin through a catheter to the left lateral ventricle of the brain in doses 1 IU/ day and 10 IU/ day; the medication was diluted with 0.9% solution of NaCl in the volume	Functional neurological tests performed on the 3rd, 14th and 42nd days after middle cerebral artery occlusion demonstrated significant improvement of motor abilities and coordination on the 14th and 42nd days after ischemia in the animals receiving 10 IU of recombinant human erythropoietin a day. Immuno-histochemical examination performed on the 14th, 30th and 52nd days after ischemia allowed concluding that a high dose of recombinant human erythropoietin increased neurons "survival value" on the 52nd day of

Table 2 (continued)

1	2	3	4	5	6
2010 [16].				of infusion 0.25 mcL/ hour. The correctness of ischemia modeling was assessed by the Doppler sonography examination.	examination, decreased progressive cerebral atrophy having no affect on the callosal thickness, reduced diffuse astrocytosis and glial scars formation. Decreased level of inflammatory markers (IL-1β, IL-6, leukolysis inhibiting factor, transforming growth factor, tumor necrosis factor, glial fibrillary acid protein) in animals receiving 10 IU of recombinant human erythropoietin per day, which were examined on the 3rd, 14th and 30th day after ischemia, also supported positive impact of high doses of recombinant human erythropoietin.
Marcus Mazur; Robert H.; Miller Shenandoah Robinson 2010 [17]	Erythropoietin	Physiological saline solution	Uterine artery occlusion during 60 min. on the 18th day of embryogenesis (infant rats were born at term, i.e. on the 22nd day of the embryonal development).	Recombinant human erythropoietin was introduced intraperitoneally starting with the 1st day of post-natal period. Animals of group 1 were introduced 500 IU/kg a day. Animals of group 2 were introduced 1000 IU/kg a day for 3 days. Animals of group 3 were introduced 2000 IU/kg a day for 5 days.	Immuno-histological examination was performed on the 2nd, 5th and 9th days after the birth. During 2 weeks after intrauterine ischemia there was increased activity of caspase-3 and increased amount of apoptotic cells in the animals receiving erythropoietin in a dose 1000 IU/kg a day for 3 days. Significantly less amount of immune-positive oligodendrocytes was found in alba of the animals receiving physiological saline solution in contrast to the animals receiving erythropoietin in any dosage. Physiological tests showed that animals receiving 2000 IU/kg a day for 5 days did the tasks better comparing to animals from other groups.
Lella Cherian; J. Clay Goodman; Claudia Robertson 2011 [18]	Darbepoetin alpha	Physiological saline solution	Brain trauma was performed using a craniotome 8 mm in diametre, which was introduced into the right area of the skull above the parietal bone. Then the injury in the form of 8 mm hole was performed in a certain position using a “striking tool” and a stem was introduced through this hole; then 3 mm deformation	Darbepoetin was introduced by 2.5; 5; 10; 25 and 50 mkg/kg subcutaneously in 5 min., 1 hour, 3 hours, 6 hours, 9 hours 12 hours and 24 hours after the trauma.	Histological examination of the size of the brain damage showed significant effect of the darbepoetin therapy in doses 25 and 50 mkg/kg 5 min after brain trauma (8.1±3.1 and 11.1±6 mm^3, respectively), than in animals treated with physiological saline solution (39.1±6.7 mm). There was also significant effect depending on the time of preparation introduction: • When injecting 25 mkg.kg of darbepoetin the size of the damaged area reduced up to 10.5±5 mm^3 when introduced after 5 min after trauma; • When introduced in 1 hour – up to 9.2±3.6 mm^3; • When introduced in 3 hours – up to11.3±2.4 mm^3; • When introduced in 6 hours – up to 14.7±4.4 mm^3

Table 2 (continued)

1	2	3	4	5	6
			of the brain was done using this stem.		The size of the damaged area amounted to 42.9±11mm^3 in the animals that were applied physiological saline solution.
Elizarova O.S.; Balaban'yan V.YU.; SHipulo E.V.; Maksimenko O.O.; Vanchugova L.V.; Litvinova S.A.; Garibova T.L.; Voronina T.A.; Gel'perina S.E. 2012 [19]	Nanosomal form of low co-sialyated recombinant human erythropoietin on the basis of nanoparticles from polylactic-co-glycolic acid stabilized by 1% human serum albumin.	Native low co-sialyated recombinant human erythropoietin (OOO "Proteinovy kontur", Russia)	Localized brain hemorrhage (auto-hemorrhagic left brain stroke) was simulated in the area of internal capsule (capsule interna, coordinates H=5mm, L=3.5 mm, A=2 mm from bregma)	Nanosomal form of low co-sialyated recombinant human erythropoietin and native low co-sialyated recombinant human erythropoietin were injected intravenously in a dose 0.05 mg/kg; prior to injecting nanoparticles preparation was diluted in 1% solution of Pluronic F68. The first introduction was performed in 3-3.5 hours after the operation and recovery of an animal after narcosis. The repeated application was performed on the second and third days after the operation. False-operated rats and rats from the control group with hemorrhagic stroke were introduced physiological saline solution according to the same scheme.	Dynamics of intracerebral post-traumatic hematoma development was studied on the 1st, 3rd and 7th day with death registration. Study of survival dynamics in rats showed that up to the 7th day of observation all false-operated rats survived; in the group of animals with intracerebral post-traumatic hematoma the survival value amounted to 40%. On the background of the repeated 3-days injection of low co-sialyated recombinant human erythropoietin incorporated into nano-particles from polylactic-co-glycolic acid, the survival value in rats was 77.8% till the end of the experiment; this was 40% more than in rats of the control group with intracerebral post-traumatic hematoma. Native low co-sialyated recombinant human erythropoietin did not practically influence the survival value in rats with intracerebral post-traumatic hematoma; this fact might indirectly prove insufficient dose of the introduced preparation for producing therapeutical concentration in the brain and neuroprotective effect.
Alexander M.L.; Hill C.A.; Rosenkrantz T.S.; Fitch R.H. 2012 [1]	Erythropoietin	Physiological saline solution	Ligation of the right common carotid artery for two hours; after that – two hours hypoxia under humidified 8% oxygen and 92% nitrogen.	Animals were divided into 2 groups and 6 sub-groups: Animals of the group 1a were introduced 1000 IU/kg of erythropoietin right after ischemia; animals of the group 1b were introduced 1000 IU/kg of	Statistical analysis of behavioral tests and histological examination was performed using dispersion method. Current study showed that there was practically no therapeutical effect on the introduction of erythropoietin in 60 or 180 min. after ischemia. Assessment of the ventricular pathology revealed the fact that significant swelling of the brain ventricles on the right was registered in the group of animals receiving physiological saline

Table 2 (continued)

1	2	3	4	5	6
				erythropoietin in 60 min. after ischemia; animals of the group 1c were introduced 1000 IU/kg of erythropoietin in 180 min. after ischemia; animals of the groups 2a, b and c were introduced physiological saline solution in volumes and with time intervals equivalent to each of subgroups receiving erythropoietin.	solution, and also in animals that were injected erythropoietin in 180 min. after ischemia comparing to the other groups of animals that did not manifested apparent ventricular pathology. In both – animals receiving erythropoietin in 60 and 180 min. after ischemia and in animals receiving physiological saline solution the amount of apoptotic cells and the size of ischemia histologically significantly increased. There was also histologically proved therapeutical effect of erythropoietin in animals receiving erythropoietin right after ischemia. Thus, the study supported the inefficiency of the delayed introduction of erythropoietin.
Carin Sjolund; John-Kalle Lansberg; Tadeusz Wieloch; Karsten Ruscher; Bertil Romner 2013 [20]	Erythropoietin	Physiological saline solution	Two vessels ten minute ligation with suture materials (right and left carotid arteries)	Animals of the first group were single-dosed 80 IU of erythropoietin multiplied by the volume of distribution (VD; 0.057 ml/g BW), intravenously (Neorecormon Roshe, Switzeland) right after the operation; 160 IU/hour in succeeding 72 hours. Animals of the second group were introduced physiological saline solution according to the same scheme.	Examination of sensor-motor functions and memory tests performed on the 3rd day of the experiment showed that the group of animals receiving erythropoietin did tasks better than the group of animals receiving physiological saline solution. Neuro-protective effect of erythropoietin was also determined by the histological examination of the brain in which neurons of the control animals were specified as big violet cells 30-50 mm in diametre with a large sub-circular nucleus; damaged neurons were specified as red-rosy patches triangle in form with a shrunken dark nucleus. In general histological examination did not reveal significant changes in the brain of the animals receiving erythropoietin as well as in animals receiving physiological saline solution. The results of the experiment demonstrated that treatment with erythropoietin did not affect the amount of apoptotic cells, did not protect from the ischemic damage, but preserved synaptic membrane function; behavioral tests and memory tests proved this.
Sheng-Kai Wu; Ming-Tao Yang; Kai-Hsiang Kang; Houng-Chi Liou;	Recombinant erythropoietin (Merck KGa, Darmstadt,	-	Three vessels ligation with suture materials (right and left carotid arteries, middle cerebral artery)	In the study animals were divided into groups depending on the way of the preparation introduction: Group A (control) – 50 min. ischemia; Group B – 50 min.	Histological picture of the brain showed significant decrease of the brain ischemia size in animals receiving erythropoietin using phonophoresis. Neurological status examination was performed in 24 hours after ischemic reperfusion and it was registered that therapy with the help of phonophoresis significantly improved

Table 2 (continued)

1	2	3	4	5	6
Dai-Hua Lu; Wen-Mei Fu; Win-Li Lin 2014 [21]	Ger-many)	-		ischemia, animals were twice introduced 5000 IU/kg of recom-binant erythropoietin in 5 hours after re-perfusion using phonophoresis; Group C – 50 min. ischemia, animals were single-dosed in-travenously 5000 IU/kg of recombinant erythropoietin in 5 hours after re-perfu-sion; Group D – 50 min. ischemia, animals were single-dosed in-travenously 5000 IU/kg of recombinant erythropoietin in 5 hours after re-perfu-sion using phonophoresis.	neurological functions and reduced neurological assessment scores. Immuno-histochemical staining of the brain performed in 24 hours after ischemia/ reperfusion showed evident neuron death in groups A and C, while in groups B and D phonophoresis had fa-vourable effect on the neurons "survival value'. Thus, the authors concluded that introduction of recombinant erythropoietin using phonophoresis increased penetration of the brain vessels and improved neuroprotective effect of this preparation.
Haiping Zhao; Rongliang Wang; Xiaoning Wu; Jia Liang; Zhifeng Qi; Xiangrong Liu; Lianqui Min; Xunming Ji; Yumin Luo 2015 [21]	Erythro-poietin	-	Occlusion of the middle cerebral artery was per-formed for 2 hours by embolization of vessels followed by 24 hours reperfusion.	Animals of the first group were introduced 800 IU of erythropoietin per kg in the middle cerebral artery in-travascularly. Animals of the second group were introduced 5000 IU of erythropoietin per kg subcutaneously.	Neurobehavioral deficiency and the brain ischemia size was less in the animals receiving 800 IU of erythropoietin per kg in the middle cerebral artery intravas-cularly. Erythropoietin also suppressed expression of stress glucose dependant protein 78 of the endoplasmatic reticulum, activation of the tumor necrosis factor and reduced level of pro-apoptotic caspase-3 in micro-vessels of the brain in these animals. Research results support neuroprotective effect of low doses of erythropoietin (800 IU/kg) when introducing intravascularly in the middle cerebral artery after experimental acute ischemic brain damage.

Results. As the given Table shows, the most popular model of stroke is considered to be two- or three vessels ligation of arteries; simulation of occlusion of uterine arteries was used for the study of erythropoietin effect on the treatment of intra-uterine fetal hypoxia. The attention should be also paid at the interesting and technically complicated method of localized brain hemorrhage (auto-hemorrhagic left brain stroke) in the area of the internal capsule. All the researchers tended to compare low- and high dose effect of erythropoietin and its analogues and to study their effect in various dosages and using various methods of introduction. The most popular method of assessment of research results was histological validation of ischemia and

assessment of neurological status performing various static and dynamic tests. When summarizing all the research results it is evident that neurological symptoms in the animals receiving high doses of erythropoietin and its analogues, especially during the first hours after damage, improved significantly; neurobehavioral deficiency reduced; functions of the limbs and memory functions recovered significantly more rapidly; inefficiency of the delayed introduction of erythropoietin and its derivatives was also registered. The brain ischemia size and the amount of apoptotic cells histologically reduced, concentration levels of inflammatory markers reduced. Analyzing the data obtained the authors may conclude that ways of introduction do not affect the outcome of the studied preparations.

Conclusions. The results of the analysis performed support efficiency of application of erythropoietin in various forms, as well as its genetically-modified analogue – darbepoetin. It has been demonstrated in vitro and in vivo that erythropoietin is considered to be a strong inhibitor of neuron apoptosis induced by ischemia and oxygen deficiency. However, erythropoietin blood-forming activity has unfavourable side effect – increased arterial pressure and risk of blood clot formation – that is, in case of ischemic stroke, strongly counter-indicative even if erythropoietin is applied for a very short period. Related to this fact there are known attempts to develop modified erythropoietin having no blood-forming activity but preserving cytoprotective properties. One of such modified erythropoietin variants is reported to be its de-sialyated form, which has high affinity to classical forms of erythropoietin receptors, but fails to reveal blood-forming activity in vivo due to short half-life period in blood plasma. Another variant of modified erythropoietin represents carbamylated erythropoietin. Protein carbamylation is widely known to be a side-effect of urea application in purification of proteins and as a result of high urea level in the serum. In such cases carbamylation results from urea decomposition into cyanates. Cyanate is responsible for carbamylation of the primary amines of protein in the N-terminal end and amino acid residues of lytic protein subjected to carbamylation. Other amino acid residues possibly subjected to carbamylation are argentine, cysteine, tyrosine, aspartic acid, glutaminic acid, histidine; however, the reaction depends on pH and does not go as rapidly as with the N-terminal end and amino acid residues of lytic protein. Carbamylation of erythropoietin on 7 available lysine residues replaces them on the residues of homocitrullin not involving the profile of glycosylation of the entire molecule. It is demonstrated that carbamylated erythropoietin does not interact with classical erythropoietin receptors but preserves cytoprotective properties. The major advantage of carbamylated erythropoietin comparing to de-sialyated form of erythropoietin lies in the fact that carbamylation in contrast to de-sialyation does not significantly change kinetic profile. Half-life period of carbamylated erythropoietin in the blood plasma, as is has been showed in rats, is the same as of erythropoietin – 3 – 6 hours; this is caused by preservation of sialic acid residues. Darbepoetin is reported to be genetically-modified erythropoietin and has all properties of the natural analogue. Comparing to standard erythropoietin darbepoetin has bigger molecular weight (37.1 kDa and not 30.4 kDa) and maximally possible amount of sialic acid residues (22 against 14 in erythropoietin) due to introduction of 2 complementary sites of glycosylation. This results in the increase of half-life elimination period and, consequently, reduces the application frequency of the preparation [5, 23]. There exists one more form of darbepoetin containing carbamylated groups of all eight amino acid residues of lysine included in a darbepoetin molecule and carbamylated amino acid residue of alanine in the N-terminal end of this protein – carbamylated darbepoetin, which does not affect hemopoetic activity but preserves cytoprotective properties. Carbamylated darbepoetin has more prolonged half-life period comparing to Carbamylated erythropoietin and, consequently, and may be prospective when applying in vivo as a cytoprotective medicinal product in case of disorders resulting in cell death due to hypoxia. There is lack of information in literature about the effect of this substance as a medication with cytoprotective action, and the number of investigational pre-clinical trials is not sufficient; this may become a pre-requisite to new research studies.

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